Diversification of CORVET tethers facilitates transport complexity in *Tetrahymena thermophila*

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MS TITLE: Diversification of CORVET tethers facilitates transport complexity in *Tetrahymena thermophila*

AUTHORS: Daniela Sparvoli, Martin Zoltner, Mark Field, and Aaron Turkewitz
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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.

The key issues relate to better validation and presentation of the imaging data in the later figures as well as robust quantification and statistical analysis. Please provide information on the numbers of cells analysed and of biological replicates in each case along with an appropriate statistical test. Many of the comments will likely only require changes to the text and presentation of the figures but some additional experimental work is likely to be needed to address the concerns on localization.

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 this manuscript, Sparvoli et al describe the composition and partially describe the localization of the six CORVET complex variants in Tetrahymena thermophila. CORVET and HOPS are evolutionarily conserved tethering/ Sec1-Munc18 complexes that function in regulating fusion events respectively with early and late endosomes. They share a core of four subunits (Vps11, Vps16, Vps18 and Vps33) and differ by their terminal subunits (Vps8 and Vps3 in CORVET). T. thermophila has lost the genes encoding HOPS-specific terminal subunits, but expresses six different Vps8 paralogues and at least two paralogs of all other subunits (except Vps11). Here, the authors tagged each of the six Vps8 paralogs at their endogenous loci with FLAG or mNeon. They first analyzed immunoprecipitated FLAG-tagged complexes biochemically, and showed by immunoblotting and mass spectrometry that each Vps8 paralog associates with a different combination of Vps8, Vps33, Vps16, Vps18, and Vps3 paralogs (with Vps11 the only shared subunit found in each of the 6 CORVET complexes); a sedimentation assay suggested that at least Vps8a is present in a bona fide hexameric complex. The authors then localized each CORVET complex within cells expressing mNeon-fused Vps8 paralogs by both fixed and live fluorescent microscopy. The data suggest that each Vps8 paralog-containing CORVET complex - including closely related ones that differ only by their Vps8 subunit - have a unique physical and temporal localization, suggesting that the Vps8 paralogs are the major determinants of CORVET complex localization. Consistently, distinct CORVET complexes overlapped to different degrees with endolysosomal Rab proteins.

By defining the composition and documenting distinct localization of the different CORVET complexes in T. thermophila, the authors address an interesting question about the determinants of specificity within CORVET/HOPS complexes for distinct fusion steps. This question has been difficult to address in more complex eukaryotic systems in which seemingly invariant CORVET and HOPS complexes each function in multiple trafficking steps and in which both complexes are essential for life. These studies thus provide a basis for future work that might shed light on functional CORVET binding interactions. The biochemical analyses of the distinct complexes in Figures 1 and 2 are largely well done (although see point #1). However, the localization data are somewhat preliminary and do not fully support the conclusions drawn. With modifications to the text and additional data to strengthen the findings, this work would represent an important contribution to the field.

Comments for the author

Major Concerns:
1. In Figure 1, the co-IPs in panel a are very convincing, but the sedimentation assay lacks essential controls and description. As presented, it is not clear if the HOPS subunits are migrating as expected for the complex or as large aggregates.
a. Which fractions are at the top of the gradient and which are at the bottom? This should be indicated on the figure and in the figure legend.
b. A single protein MW (or s) standard is not sufficient, and the peak fractions for thyroglobulin and another suitable standard (e.g. ferritin at ~440 kDa and IgG or aldolase at ~160 kDa) should be indicated on the figure.
c. What is the predicted MW (or s value) for the complete HOPS complex?
d. Figure 1B should include immunoblotting of lower Mr fractions to support the statement that no monomeric Vps8a-FLAG was detected
e. In Figure 1D and Figure 2A, the axes of the plots need to be defined.

2. The images shown in Figure 3 are neither very informative nor sufficient to support the conclusions drawn; as is, the figure includes only 1 image per cell, no additional markers, and no quantification, making it impossible for the reader to interpret the data.
a. Quantification (e.g. of the size, relative brightness, and number of structures identified) and better labeling of the images must be included. Images of multiple cells can also be added in a

Supplementary figure to support the generality of the patterns. For example, patterns of mNeon-Vps8c look quite different in Fig. 3A, 5A and 7F relative to 5C, 7A and 7B.
b. The text refers to unlabeled structures such as food vacuoles and contractile vacuoles, but there is no labeling in the figure to indicate where these structures are and no basis is provided for their definition for readers unfamiliar with Tetrahymena cell biology. The authors do state that there is a lack of compartmental markers for Tethrahymena, but at minimum can refer to Figures 4 and 7 for more information on some of the complexes, and some basis for interpreting structures as food and contractile vacuoles should be provided.
c. The text also refers to tubular extensions containing Vps8c, but this is not apparent at the magnification shown. Perhaps a magnified inset with arrows and documentation with a video showing tubule movement could be shown. If Vps8c displays tubular extensions not seen in the other Vps8 paralogs, then this should also be quantified for comparison. Note, most membrane tubules are sensitive to fixation, and this is more likely why they are not obvious in fixed cells.
d. The authors state that some orthologues are more mobile than others, but this is not shown (and could be by sequential frames of a video) or quantified.
e. The text also refers to anterior and posterior portions of the cell. This should be labeled for ease of interpretation. Additionally, any anterior or posterior bias should be quantified.
f. The patterns of Vps8b, Vps8e and Vps8f differ substantially in the fixed vs. live cells, and this should be noted and explained.
g. The authors suggest in the text that some of the signal in some samples is due to autofluorescence, but this should be validated by showing similar exposures of wild-type cells and by an indication of relative exposure times/ gain on all samples.
h. Note, the distinct apparent size of the structures observed (e.g. large structures by Vps8c in some images) might simply reflect different concentrations of Vps8 orthologues on these structures - and hence different intensities of fluorescence - rather than an actual size difference of the structure on which they reside. It is also important for the authors to emphasize that the structures observed may be a subdomain and not the entire organelle to which the Vps8 orthologue localizes.

3. The data shown in Figure 4 are intriguing, but also underdeveloped.
a. The data require quantification. What fraction of cells have circularly-arranged Vps8b and e? In what fraction of these positive cells does E. coli localize within the circles? In what fractions of Vps8e- vs. b- enclosed structures are the E. coli intact?
b. If Vps8e and b paralogs localize to early vs late stage food vacuoles, this should be documented by a time-lapse experiment performed after feeding. Considering that the authors have made other cell lines with Vps8e-mNeon and an mCherry-fused Vps paralog, I wonder why a Vps8e-mNeon and Vps8b-mCherry cell line was not made to better visualize the dynamics of their localization to food vacuoles.
c. Whereas Vps8e-mNeon shows clear clustering in one area, Vps8b-mNeon puncta are more widespread throughout the cell. If this is a representative image, then it is not convincing that Vps8b localizes to the anterior part of the cell at a food vacuole. If it is not a representative image, a better image should be chosen.
d. A reading of the text describing these data on the bottom of page 11/ top of page 12 would lead one to believe that all of the Vps8b and e localize to food vacuoles, and that all E. coli label food vacuoles, but neither of these statements is supported by the images in Figures 3 and 4. It should be clearly stated that the majority of Vps8b-mNeon and a substantial fraction of Vps8e-mNeon are present in separate vesicular structures that are nowhere near a food vacuole or other phagosomal structures harboring internalized E. coli.

4. Figure 5 suffers from similar concerns as Figure 3, particularly given the varying distribution of Vps8e-mCherry.
a. It is difficult to draw conclusions from the microscopy images at this magnification. I suggest adding a magnified insert with arrows to point out puncta of interest. In the unmagnified image, the area of magnification should be shown with a white box.
b. Since the actual structures to which Vps8 orthologs localize are not detected, how confident are the authors that the pairs shown in panel C, top and bottom (a vs. e and a vs. c) are actually on different structures rather than distinct domains on the same structure?

5. Overall note in interpreting Manders' coefficients - the numbers do not indicate % localization, as inferred in the text. These are relative correlation coefficients and are not absolute measures of colocalization. A good example of this is in Figure 7E, in which the Manders' coefficients indicate a

very high correlation for Vps8a and Rab4b. However, inspection of the image shows that at least half (if not more) of the Vps8a-containing spots lack Rab4b. An object-based colocalization method would thus provide a value closer to 40-50%. Please amend the text to reflect this reality.

Minor concerns:

6. For all existing quantification: statistical analyses are missing and need to be included.

7. In Figure 1A, are the differences in the expression of the endogenously-tagged subunits consistent with what has previously been observed? For example, there is a very low level of Vps33a relative to Vps33b, and of Vps18d and Vps18a compared to Vps18b and Vps18c.

8. In Suppl. Fig. 1B, although the data are nice, the band labeling is very confusing. Would it be possible to label them as 8, 18, 16, 11, 3 and 33 instead of 1-6? Also in this figure, what are the other bands? Were other protein constituents of these complexes appear in the mass spec?

9. In Supp. Fig. 2A, the lower Mr bands (~50, ~34, and ~25 kDa) should be indicated as background bands and explained as such (based on their presence in anti-myc IPs from WT cells) in the text of either the results or, minimally, the figure legend. What is the basis for the statement on page 10 that "proteolytic cleavage may occur during immunoprecipitation, rather than within live cells"?

10. In the text describing the cells expressing the mNeon-tagged Vps8 orthologues on page 10, the authors state that cells expressing tagged Vps8c and 8d had no phenotype. Was this also true of cells expressing Vps8b, e and f?

11. In Figure 4:
a. A white box should be added in the unmagnified image around the area that is magnified in the inset.
b. The bottom panel needs to have magnified insets to be consistent with the top panel.

12. In Supplementary Figure 4A, the fluorescence intensity is too weak to draw any conclusions.

13. In Figure 6A, if the Vps11 is found in each of the distinct CORVET complexes, then all Vps3a-GFP should colocalize with Vps11-mCherry but not all Vps11-mCherry should colocalize with Vps3a-GFP. Considering that these are tagged at their endogenous loci, shouldn't there be Vps11-mCherry puncta that do not colocalize with Vps3a-GFP?

14. Some of the quantification methods used in the current manuscript are confusing. For example, in the Methods section on Colocalization Analyses, the authors write: "To measure the colocalization between Vps8c-mNeon and Vps8c-mCherry, 14 cells were analyzed and the coefficients were derived from 261 non-overlapping images." Where did the 261 images come from?

15. In the discussion, the authors might consider noting that the association of Vps8a with Rab4b-containing endosomal domains and its function in cargo delivery to the mucocyst is consistent with the role of early endosome-derived tubules in cargo delivery to melanosomes in melanocytes (e.g. see Delevoye et al., 2009 JCB, Dennis et al., 2015 JCB and Delevoye et al., 2016 Curr. Biol.), suggesting conservation in LRO biogenesis pathways. Also in this respect, C. elegans expresses two distinct subunit pairs for CCZ-1 - a MON1 orthologue and another (GLO3) that was hard to identify as a MON1 paralogue by sequence identification (see Morris et al., 2018, PLoS Genet.); thus, the failure to identify a clear RAB7 GEF subunit (CCZ-1) orthologue in T. thermophila has some precedent in C. elegans.

Reviewer 2

Advance summary and potential significance to field

This study represents a detailed biochemical characterization of the CORVET complexes in Tetrahymena. Together with the HOPS complex, these tether complexes mediate endosome maturation via their specific interaction with Rab GTPases. Though the current study lacks

functional characterization of the distinct CORVET complexes, their distinct intracellular localization using the defining Vps8 subunits does support functional differences.

Comments for the author

1. Last sentence in background states that "…Vps18 plays a key role in shaping the specificity of complex assembly". There is however, no evidence provided for the function of Vps18 in the manuscript. I suppose this is a typo.

2. The background is rather long. Think it could be shortened to make the research question clearer. However, it is not clear if the loss of HOPS is Tetrahymena-specific or also found in other ciliates.

3. I found it puzzling that Vps8c-mNeon and Vps8c-mCherry do not colocalize completely (Fig. 5A). Does this imply that different tags may have different effects on Vps8c localization and/or dynamics? This would make the rest of Fig 5 and even Fig 6 difficult to interpret. Other than mNeon and mCherry, it would be important to use other tags for these co-localization studies. A non-fluorescent tag may be desirable in this case, to rule out any possible FRET effects. This is particularly relevant in Fig 7 when potential interacting proteins are tagged respectively with mNeon and mCherry. In a recent study (https://www.biorxiv.org/content/10.1101/694190v2.full), mCherry was found to be an efficient FRET acceptor for mNeonGreen.

First revision

Author response to reviewers' comments

We sincerely thank the reviewers for their careful reading of the manuscript, and hope our resubmission addresses their concerns.

Reviewer 1
Major Concerns:
1. In Figure 1, the co-IPs in panel a are very convincing, but the sedimentation assay lacks essential controls and description. As presented, it is not clear if the HOPS subunits are migrating as expected for the complex or as large aggregates.
a. Which fractions are at the top of the gradient and which are at the bottom? This should be indicated on the figure and in the figure legend.

Thank you, the suggested changes have been made, adding to the legend in figure 1 as follow: "250µl fractions were collected from top to bottom, of which 25µl aliquots were subjected to SDS-PAGE (4-20% gel). Fractions 17-29 (top-bottom) are shown"

b. A single protein MW (or s) standard is not sufficient, and the peak fractions for thyroglobulin and another suitable standard (e.g. ferritin at ~440 kDa and IgG or aldolase at ~160 kDa) should be indicated on the figure.

In response, we are now showing data on the sedimentation of thyroglobulin, alcohol dehydrogenase and bovine serum albumin (BSA), which we had analyzed in parallel with the CORVET complexes. Coomassie blue-stained glycerol gradient fractions are shown in Supplementary figure 1, and we indicate in bold the peak fraction for each standard. In the legend of figure 1 we changed the text to "Thyroglobulin, sedimented in parallel as a size standard, appeared in fractions 24-29 (top-bottom), with a peak in fraction F26 (arrow at the top). 8A-CC sediments more slowly than expected for a 727kDa complex." In the legend of supplementary figure 1 we have added "A) Coomassie Blue staining of sedimentation standards for the experiment shown in figure 1C. The peak fractions for thyroglobulin (660 kDa), yeast alcohol dehydrogenase (150 kDa), and bovine serum albumin (BSA, 66 kDa), are F26, F12 and F7, respectively." We added the following sentence in the corresponding Results section:

"Thyroglobulin, yeast alcohol dehydrogenase and bovine serum albumin were sedimented in parallel to provide size standards (Fig. S1A)."

c. What is the predicted MW (or s value) for the complete HOPS complex?

We have now added this information to the figure legends and to the Results. For the former: supplementary figure 1 now includes the following legend: "B) Size of 8A-CC inferred by sedimentation. The molecular weights of standards were plotted as a function of the corresponding peak fractions. Based on these positions, the position of 8A-CC corresponds to approximately 550 kDa."
8A-CC thus migrates as significantly smaller than predicted, although it is clear from the analysis that all six subunits are present in the peak fraction. We omitted this information from the previous submission because we cannot tell whether this anomalous behavior is specific to the Tetrahymena complex. HOPS and CORVET in other organisms have been analyzed by gradient centrifugation in the published literature, but in those cases sedimentation standards were not shown, as far as we are aware; in no case have we been able to find published data on the size of a HOPS or CORVET complex as inferred by its S-value. To clarify this, we have added the following sentence in the Results section: "Although the predicted size for 8A-CC is 727 kDa, and all six subunits are visible by silver staining, the size of the peak glycerol gradient fraction corresponds to ~550 kDa (Fig. S1B). Whether such anomalous sedimentation for CORVET (or HOPS) is specific to the complex from Tetrahymena remains to be determined."

d. Figure 1B should include immunoblotting of lower Mr fractions to support the statement that no monomeric Vps8a-FLAG was detected

We have now added the requested data to supplementary figure 1, with a note added to the Figure 1 legend: "Fractions 4-16 (top-bottom) were similarly analyzed by SDS-PAGE and western blotting (see Fig. S1C)." The supplementary figure 1 data are now described as follows: "C) Glycerol gradient fractions 4-16 (top-bottom) from the sedimentation analysis of 8A-CC shown in figure 1B, C. Vp8a-FLAG was detected by Western blot with anti-FLAG antibodies. "INPUT" corresponds to 1% of the total eluate. No monomeric Vps8a-FLAG was detected."

e. In Figure 1D and Figure 2A, the axes of the plots need to be defined.

We changed the names of the Y and X axis in the volcano plots to "-log10 p-value" and "t-test difference" and we added the following text to the figure legends in Figure 1D and Figure 2A: "To generate the volcano plot, the -log10 t-test p-value was plotted versus the t-test difference (difference between means). The cut-off curve is based on the false discovery rate and the artificial factor s0, controlling the relative importance of the t-test p-value and difference between means."

2. The images shown in Figure 3 are neither very informative nor sufficient to support the conclusions drawn; as is, the figure includes only 1 image per cell, no additional markers, and no quantification, making it impossible for the reader to interpret the data.
a. Quantification (e.g. of the size, relative brightness, and number of structures identified) and better labeling of the images must be included. Images of multiple cells can also be added in a Supplementary figure to support the generality of the patterns. For example, patterns of mNeon-Vps8c look quite different in Fig. 3A, 5A and 7F relative to 5C, 7A and 7B.

Thank you for these suggestions. We have added new data, which we believe address these issues. First, we have added multiple images of fixed cells for each paralog, in supplementary figure 3D. To the figure 3 legend we have added the following: "For additional images see Fig. S3D." The Figure S3D legend reads: "D) Confocal sections of fixed cells expressing mNeon-tagged Vps8 paralogs with paired DIC images, in addition to those shown in figure 3B. Arrowheads indicate the Vps8b-labeled phagosomes/phagolysosomes; asterisks indicate localization of Vps8d at contractile vacuoles; white arrows indicate Vps8e vesicles at the cell anterior; black arrows indicate the oral apparatus (OA). Scale bars, 10 μm." We have added to Results: "Additional images of fixed cells showing the localization of Vps8 paralogs are in figure S3D."
We have also provided individual videos of live cells expressing each of the six mNeon-tagged Vps8 paralogs. We quantified the size, number and brightness of the vesicular structures observed in

Vps8a, Vps8b, Vps8c, Vps8e and Vps8f, and the quantification is reported in figure 3 D, E, F. We added the following to the legend of Figure 3: "D) Estimation of the number of Vps8-labeled particles with size between 0.1 and 2 μm2, in cells individually expressing mNeon-tagged Vps8a, Vps8b, Vps8c, Vps8e and Vps8f. For all paralogs, most particles are 0.1<>0.5 μm2, with fewer particles in the 0.5<>1 μm2 and 1<>2 μm2 size classes. The analysis was performed on 143/155/144/148/143 non-overlapping optical sections for Vps8a, Vps8b, Vps8c, Vps8e and Vps8f, respectively. p-values (p) for each combination of Vps8 paralogs in each size class, were determined by two-tailed t-test. Error bars represent standard deviations. E) Analysis of the number and size of the Vps8-labeled particles observed in D, limited to those belonging to the 0.1<>0.5 μm2 size class. The size distributions of fluorescent puncta for Vps8a, Vps8c and Vps8f were distinct from one another, while those for Vps8b and Vps8e were more similar to one another. Measurements were performed as in D. F) Analysis of the relative brightness of the Vps8-labeled particles analyzed in D and E, by measuring the integrated density (RFU, relative fluorescence units) for each size class. For each paralog, particle brightness increases with size. Among the smallest size class, Vps8a-labeled particles appear brightest, while Vps8C-labeled particles are brightest in the 0.5-2 μm2 class, which correspond to the larger structures observed in A and B. Vps8b particles are the least bright in all size classes. The analysis was performed as in D." We have added the following text to the Results section: "Overall, this microscopy revealed non-identical patterns of fluorescent puncta for five of six paralogs, and this was consistent with measured differences between paralogs in the number, size and relative brightness of puncta (Fig. 3D, E and F)."
The reviewer feels that the pattern of Vps8c puncta is confusingly variable in different figures. An important detail may have been overlooked, namely that figure 3A shows single focal planes for each cell, while the other figures mentioned by the reviewer feature Z-projections. We agree that the Vps8c structures are polymorphic and say in the text: "The most closely related paralog, Vps8c, also localizes to cytoplasmic puncta, but these are larger and more irregular. In addition, the Vps8c-labeled structures frequently show dynamic tubular extensions (Fig. 3A, B, third panels; see also video "Vps8c-mNeon"), which are absent or less apparent for the other Vps8 paralog-labeled structures. At least one such tubulovesicular structure was seen in every cell, and they are more frequently located toward the posterior." In some images the posterior accumulation of vesicles is more evident than others. To address this issue, we have now quantified Vps8c posterior distribution and the results are reported in supplementary figure 3B with the following description: "B) Vps8c puncta are concentrated toward the cell posterior. 30 z-stacks of randomly-chosen cells expressing fluorescently-tagged Vps8c were scored for the absence or presence of concentrated Vps8c signal in the posterior half of the cell. The oral apparatus (OA) was first used to unambiguously define the anterior, and z-projections were then generated to visualize the entire pool of fluorescent puncta within each cell. As shown in the table, ~80% of cells showed distinct posterior accumulation. N/A indicates cells in which the oral apparatus could not be visualized, and such cells were not counted."

b. The text refers to unlabeled structures such as food vacuoles and contractile vacuoles, but there is no labeling in the figure to indicate where these structures are and no basis is provided for their definition for readers unfamiliar with Tetrahymena cell biology. The authors do state that there is a lack of compartmental markers for Tetrahymena, but at minimum can refer to Figures 4 and 7 for more information on some of the complexes, and some basis for interpreting structures as food and contractile vacuoles should be provided.

We thank the reviewer for raising the point that interpreting our figures could be difficult for readers unfamiliar with Tetrahymena. To address this, we have now added a cartoon showing a Tetrahymena cell with the specific structures mentioned in the paper, and labeled the anterior and posterior. The cartoon is in figure 3C. We added labeling on the images in figures 3, 4, 5, 6, 7, S3 and S5, to specify structures relevant for the localization of particular Vps8 paralogs, and modified the figure 3 legend accordingly. We added a description of the cartoon in the legend of figure 3: "C) Tetrahymena cell cartoon. The phagocytic pathway is shown in the cell's upper left to right. Food particles (red rectangle) are taken up at the anterior oral apparatus (OA) into food vacuoles/phagosomes (P), from whence they move in a posterior direction and eventually egest undigested material at the cytoproct (CP). Also in the posterior is a water-pumping compartment, the contractile vacuole (CV). Other structures shown are the polyploid vegetative macronucleus (M), and diploid germline micronucleus (m). Cell length is 50 μM." In the main text we have added

the following: "Morphological studies suggest at least four distinct pathways for uptake in Tetrahymena including clathrin-mediated endocytosis (Elde et al., 2005; Nilsson and Van Deurs, 1983). Phagocytosis begins with phagosome formation at an anterior portal called the oral apparatus, followed by phagolysosome maturation via fusion with multiple classes of endosomes (Jacobs et al., 2006; Nilsson, 1979; Plattner, 2010) that deliver hydrolytic enzymes required to digest the phagolysosome contents. Maturing phagolysosomes are eventually transported to the posterior of the cell where they release undigested material via exocytosis."

c. The text also refers to tubular extensions containing Vps8c, but this is not apparent at the magnification shown. Perhaps a magnified inset with arrows and documentation with a video showing tubule movement could be shown. If Vps8c displays tubular extensions not seen in the other Vps8 paralogs, then this should also be quantified for comparison. Note, most membrane tubules are sensitive to fixation, and this is more likely why they are not obvious in fixed cells.

We have added videos of cells expressing each of the Vps8 paralogs to make the differences more apparent. We provide the full video from whence the sequential frames shown in figure S6A and B were taken, highlighting the regions of the cell where tubule outgrowth and fission appear. Other similar events can be detected. We wish to clarify that we do not exclude the possibility that other paralogs, for example Vps8a, also label tubulovesicular structures, but any such structures were less evident than for Vps8c. We thus modified the text as follows (also cited above): "The most closely related paralog, Vps8c, also localizes to cytoplasmic puncta, but these are larger and more irregular. In addition, the Vps8c-labeled structures frequently show dynamic tubular extensions (Fig. 3A, B, third panels; see also video "Vps8c-mNeon), which are absent or less apparent for the other Vps8 paralog-labeled structures".

d. The authors state that some orthologues are more mobile than others, but this is not shown (and could be by sequential frames of a video) or quantified.

We have removed any mention of vesicle mobility and leave it to interested readers to draw their own conclusions from the videos.

e. The text also refers to anterior and posterior portions of the cell. This should be labeled for ease of interpretation. Additionally, any anterior or posterior bias should be quantified.

Addressed above, and below for Vps8b vs Vps8e

f. The patterns of Vps8b, Vps8e and Vps8f differ substantially in the fixed vs. live cells, and this should be noted and explained.

On re-reading, we agree that the explanation given in the original text is not sufficient.
As detailed in the text, different conditions were found to be optimal for collecting images in live vs fixed cells. Fixed cells came from growing cultures, while live cells were semi-starved. We used different conditions because live imaging of these highly mobile cells imposes some constraints. The differences appeared to have the greatest impact for the patterns of Vps8b, Vps8e and Vps8f, and can be rationalized based on the likely role of these paralogs in phagosome formation and/or maturation (Sparvoli et al., 2018). Fixed cells, which come from growing cultures, have a large number of food vacuoles. In contrast, for live imaging, the cells needed to be immobilized in agarose, and pre-incubated in a starvation-like medium to reduce autofluorescence. Both the semi-starvation and the agarose suppress food vacuole formation. A full understanding of the differences between growing vs our starved conditions will require additional markers and is beyond the scope of the present paper.

To clarify the differences for readers, we have added the following to the Results section: "Differences observed in the distributions of Vps8b, Vps8e and Vps8f in live versus fixed cells might be related to the fact that fixed cells were obtained from growing cultures, while optimal live cell imaging was achieved using partially starved cultures. Tetrahymena undergo rapid physiological changes when shifted from growth medium to non-nutrient medium, which might be particularly relevant for the Vps8b, 8e and 8f complexes that are likely to be associated with phagosome formation and maturation (Sparvoli et al., 2018)."

g. The authors suggest in the text that some of the signal in some samples is due to autofluorescence, but this should be validated by showing similar exposures of wild-type cells and by an indication of relative exposure times/ gain on all samples.

We show images of live and fixed wildtype cells in figure 3. Exposure times were equivalent for all samples, and are provided in Materials & Methods. We changed the corresponding text in the Results section as follows: "For images of live cells, the increased contrast that was used to enhance those paralogs with weak signals also enhanced the background fluorescence. This included auto-fluorescence within food vacuoles, also seen in cells not expressing any fluorescent proteins (Fig. 3A, B, seventh panels)."

h. Note, the distinct apparent size of the structures observed (e.g. large structures by Vps8c in some images) might simply reflect different concentrations of Vps8 orthologues on these structures - and hence different intensities of fluorescence - rather than an actual size difference of the structure on which they reside. It is also important for the authors to emphasize that the structures observed may be a subdomain and not the entire organelle to which the Vps8 orthologue localizes.

Thank you. To clarify this for readers, we added the following sentences to the Results section, borrowing the reviewer's language: "With regard to the apparent sizes of the structures associated with the individual paralogs, it is important to note that the differences might reflect variation in the concentration of Vps8 proteins on their target membranes - and hence different intensities of fluorescence - rather than actual size differences between the structures on which they reside.", and to the Discussion: "While detailed functional analysis has yet to be achieved, our localization data strongly suggest that the complexes diversified to associate with distinct compartments or subdomains of the same compartment."

3. The data shown in Figure 4 are intriguing, but also underdeveloped.
a. The data require quantification. What fraction of cells have circularly-arranged Vps8b and e? In what fraction of these positive cells does E. coli localize within the circles? In what fractions of Vps8e- vs. b- enclosed structures are the E. coli intact?
b. If Vps8e and b paralogs localize to early vs late stage food vacuoles, this should be documented by a time-lapse experiment performed after feeding. Considering that the authors have made other cell lines with Vps8e-mNeon and an mCherry-fused Vps paralog, I wonder why a Vps8e-mNeon and Vps8b-mCherry cell line was not made to better visualize the dynamics of their localization to food vacuoles.
c. Whereas Vps8e-mNeon shows clear clustering in one area, Vps8b-mNeon puncta are more widespread throughout the cell. If this is a representative image, then it is not convincing that Vps8b localizes to the anterior part of the cell at a food vacuole. If it is not a representative image, a better image should be chosen.

Because the reviewer is frustrated by our analysis and comparison of Vps8b and Vps8e, we have re-analyzed these cells by taking a different approach. First, we did a careful time-course of protein localization after bacterial feeding, to determine the optimal conditions for co-localization. With the optimized conditions, we then individually co-localized Vps8b and Vps8e with internalized dsRed-E. coli. However, because it is not always straight-forward to distinguish intact from degraded bacteria, and very difficult to quantify, we took a different approach. To distinguish between younger vs older phagosomes, we exploited the fact that phagosomes form at the cell anterior, and are then translocated to the posterior, as described above and shown in new Fig 3C. Thus we measured the distance, for each cell, from the cell anterior to the structure labeled by Vps8b or Vps8e. The data, shown in Fig. 4 and including quantitation, show unambiguously that Vps8e shows biased association with anterior phagosomes, while Vps8b is associated with more posterior phagosomes.
We have removed all the previous data and text relating to localization of Vps8e and Vps8b, because our new analysis is much more straight-forward for demonstrating that the two CORVET complexes are associated with different populations of phagosomes. We thank the reviewer for prompting these changes. The new description of the analysis is as follows:

Tetrahymena are avid bacterivores and rapidly concentrate bacteria via ciliary beating at the anterior-positioned oral apparatus. From the base of the anterior oral apparatus bacteria are taken up by phagocytosis into newly-formed phagosomes called food vacuoles. The food vacuoles

then mature as they move posteriorly, and as their contents are digested (Nilsson, 1979). Based on their fluorescence patterns, a fraction of Vps8b-mNeon and Vps8e-mNeon puncta appeared to localize to food vacuoles. To confirm this, we labeled food vacuoles by incubating Tetrahymena with dsRed-expressing E. coli. Note that not all food vacuoles will be labeled under these conditions, since phagocytosis can also occur without bacterial ingestion. Both Vps8b-mNeon and Vps8e-mNeon were concentrated in ~60% of the cells around clearly labeled food vacuoles (Fig 4A), while in the remaining cells the fluorescent puncta were dispersed. To ask whether Vps8b and 8e were associated with food vacuoles at different stages of maturation, we took advantage of the fact that vacuoles form in the cell anterior, and move posteriorly as they mature. For each cell in which we detected a food vacuole associated with Vps8b or 8e, we measured the distance of that vacuole from that cell's anterior end as well as the cell length, and calculated an index to report the relative vacuole position (Fig 4B). Notably, Vps8e-labeled phagosomes were positioned closer to the anterior end, while those labeled by Vps8b were positioned more posteriorly (Fig. 4C). Representative images of cells used for the quantification are shown in figure S4A-B. These results strongly suggest a role for 8E-CC and 8B-CC at successive stages in the formation and maturation of food vacuoles. Our analysis was limited to fixed cells, because the semi-starvation conditions that favor live cell imaging inhibit food vacuole formation. We found that Vps8b-associated food vacuoles, but not those associated with Vps8e, could still be found in cells starved for several hours, consistent with the idea that Vps8b associates with old food vacuoles (not shown).

Figure 4 legend:
Biased posterior vs. anterior localization of Vps8b vs. Vps8e to bacteria-containing phagosomes. A) Cells expressing Vps8b-mNeon or Vps8e-mNeon were fed with E. coli expressing dsRed and then fixed, as described in Materials and Methods. The patterns of both Vps8b and Vps8e are similar to those seen in live cells (Fig 3). Scale bars, 10 μm. B) and C) Calculating a position index for Vps8b- and Vps8e-labeled phagosomes. The position index corresponds to the distance of a vacuole to the anterior tip of the cell divided by the cell length. The plot of the position indices for Vps8b- and Vps8e-labeled phagosomes reveals clear posterior vs anterior biases, respectively.

Additional images of cells expressing Vps8b and Vps8e have been added to supplementary figures 3 and 4.

d. A reading of the text describing these data on the bottom of page 11/ top of page 12 would lead one to believe that all of the Vps8b and e localize to food vacuoles, and that all E. coli label food vacuoles, but neither of these statements is supported by the images in Figures 3 and 4. It should be clearly stated that the majority of Vps8b-mNeon and a substantial fraction of Vps8e-mNeon are present in separate vesicular structures that are nowhere near a food vacuole or other phagosomal structures harboring internalized E. coli.

This point has now been clarified in the new text provided above, explaining that not all food vacuoles are labeled with E. coli.

We have also added some clarification to the description of Vps8b and Vps8e localization in live cells:

"Vps8b-mNeon fluorescence appears in both live and fixed cells as a tightly-spaced array of puncta at the periphery of large circular structures, whose size and shape are sufficient to mark them as probable food vacuoles (Fig. 3A, B, second panels; video "Vps8b-mNeon"), since there are no other similarly-sized organelles in Tetrahymena. In addition, a substantial fraction of Vps8b localizes to isolated small cytoplasmic puncta visible in fixed cells, and, to a lesser extent, in favorable focal planes of live cells (Fig. S3C)".

"Vps8e-mNeon fluorescence in live cells appears in numerous small puncta throughout the cell cytoplasm (Fig. 3A fifth panel; video "Vps8e-mNeon"). However, in some fixed cells, puncta were concentrated around a single circular structure near the cell anterior, close to the oral apparatus where food vacuoles are formed (Fig 3B, fifth panel)."

4. Figure 5 suffers from similar concerns as Figure 3, particularly given the varying distribution of Vps8e-mCherry.

a. It is difficult to draw conclusions from the microscopy images at this magnification. I suggest adding a magnified insert with arrows to point out puncta of interest. In the unmagnified image, the area of magnification should be shown with a white box.

We have made the requested changes to figure 5 and we also provided an additional image for Vps8e-mCherry in supplementary figure 5A, to confirm the consistent pattern at the oral apparatus. We added the following legend for the new panel in figure S5A: "A) Confocal section of a fixed cell expressing Vps8e-mCherry, with paired DIC images. The white arrow indicates vesicles at the cell anterior, similar to those observed with the mNeon-tagged version. Scale bar, 10 μm." We added the following to the Results: "In our negative controls, we found the expected limited overlap between co-expressed Vps8e-mCherry, which was concentrated near the cell anterior (see magnified insets in figure 5C' and C"; Fig. S5A), and either Vps8a-mNeon or Vps8c-mNeon"

b. Since the actual structures to which Vps8 orthologs localize are not detected, how confident are the authors that the pairs shown in panel C, top and bottom (a vs. e and a vs. c) are actually on different structures rather than distinct domains on the same structure?

We have added the possibility of subdomains to the Discussion, as mentioned above.

5. Overall note in interpreting Manders' coefficients - the numbers do not indicate % localization, as inferred in the text. These are relative correlation coefficients and are not absolute measures of colocalization. A good example of this is in Figure 7E, in which the Manders' coefficients indicate a very high correlation for Vps8a and Rab4b. However, inspection of the image shows that at least half (if not more) of the Vps8a-containing spots lack Rab4b. An object-based colocalization method would thus provide a value closer to 40-50%. Please amend the text to reflect this reality.

As we understand it, there is no single standard method to measure co-localization between different fluorescent signals; see for example https://jcs.biologists.org/content/joces/131/3/jcs211847.full.pdf. The Pearson's coefficient, another popular parameter for measuring co-localization, is a correlation coefficient, but it is heavily influenced by signal-to-noise ratio (SNR). Mander's coefficient on the other hand is relatively insensitive to SNR and it reports co-occurrence (Ref: Dunn: A practical guide to evaluating colocalization in biological microscopy). It determines the overlap of two images while taking into account pixel intensity values in the areas that contain both signals of interest. Given these considerations, we would prefer not to revisit our the approaches used to quantity co-localization. We have altered the description of the Mander's coefficient in Materials and Methods (Colocalization analysis), replacing in two places the phrase "measure the extent of overlap between" with "determine the correlations between". We have added an additional Vps8a-rab4b image, in which co-localization between the two signals is more easily seen, consistent with the quantitation reported in figure 7G.

Minor concerns:
6. For all existing quantification: statistical analyses are missing and need to be included.

We have calculated the p-value (p) for each coefficient by two-tailed t-tests and report the results in the corresponding figure legends.

7. In Figure 1A, are the differences in the expression of the endogenously-tagged subunits consistent with what has previously been observed? For example, there is a very low level of Vps33a relative to Vps33b, and of Vps18d and Vps18a compared to Vps18b and Vps18c.

Since Vps33a and Vps18a are each present in just 1 of the 6 complexes, it is likely that both subunits are less abundant than Vps33b (present in 5 complexes) or other Vps18 paralogs. The differences between the abundance of some paralogs could be due to their additional functions unrelated to the cognate CORVET complex, e.g., we have preliminary data suggesting that a pool of Vps8d may not be complex-associated, which we mention in the Discussion. However, another factor is that the solubilization conditions used, which we optimized for Vps8a, may be more or less efficient for other complexes. We feel this discussion is too speculative to be a useful addition to the manuscript.

8. In Suppl. Fig. 1B, although the data are nice, the band labeling is very confusing. Would it be possible to label them as 8, 18, 16, 11, 3 and 33 instead of 1-6? Also in this figure, what are the other bands? Were other protein constituents of these complexes appear in the mass spec?

We have changed the labeling in Suppl. Fig. 1B (now renamed Fig. S2B) following the reviewer's request. The additional bands, common to all samples, are non-specific proteins which co-elute with the complexes and which are also present in unrelated pulldowns. Thus, we added the following text to the legend: "The additional bands common to all samples are non-specific bands captured by the FLAG-beads." No additional protein constituents of the complexes appeared in the mass spectrometry data with statistical significance.

9. In Supp. Fig. 2A, the lower Mr bands (~50, ~34, and ~25 kDa) should be indicated as background bands and explained as such (based on their presence in anti-myc IPs from WT cells) in the text of either the results or, minimally, the figure legend. What is the basis for the statement on page 10 that "proteolytic cleavage may occur during immunoprecipitation, rather than within live cells"?

We added to the legend of supplementary figure 2A (now renamed figure S3A) the following sentence: "The three lower bands, common to all samples, are cross-reactive bands."
In the statement on page 10, we are simply acknowledging that we cannot rule out the possibility that artefactual proteolysis occurs in the cell lysate during the pulldown. Unfortunately, the level of the endogenous protein is too low to be detected directly in whole cell lysates, e.g, following TCA precipitation, so we cannot determine with certainty if the tag is removed in vivo or during the IP procedure. To clarify this in the text, we have added the following:

"The weak fluorescence for Vps8e correlates with its apparent partial proteolytic cleavage (Fig. S3A, sixth lane), although we cannot rule out the possibility that proteolytic cleavage occurred during immunoprecipitation, notwithstanding the addition of protease inhibitors during cell lysis. "

10. In the text describing the cells expressing the mNeon-tagged Vps8 orthologues on page 10, the authors state that cells expressing tagged Vps8c and 8d had no phenotype. Was this also true of cells expressing Vps8b, e and f?

Cells expressing tagged Vps8b, 8e and 8f showed no detectable growth defects, which was also true for the respective knockouts (Sparvoli et al., 2018). We have now added this to the Results: "Since the cells relying on mNeon-tagged Vps8c and 8d had no detectible growth phenotypes, which was also true for those expressing mNeon-tagged Vps8b, 8e and 8f…"

11. In Figure 4:
a. A white box should be added in the unmagnified image around the area that is magnified in the inset.
b. The bottom panel needs to have magnified insets to be consistent with the top panel.

Thank you, these changes have been made.

12. In Supplementary Figure 4A, the fluorescence intensity is too weak to draw any conclusions.

We have now provided the original video from which we selected the magnified frames shown in this figure (now renamed figure S6A), to show the outgrowth and fission events described in the picture.

13. In Figure 6A, if the Vps11 is found in each of the distinct CORVET complexes, then all Vps3a-GFP should colocalize with Vps11-mCherry but not all Vps11-mCherry should colocalize with Vps3a-GFP. Considering that these are tagged at their endogenous loci, shouldn't there be Vps11-mCherry puncta that do not colocalize with Vps3a-GFP?

We agree with the reviewer's point. There are indeed such puncta, and we have slightly increased the brightness and contrast of the cell images to make this more evident. This was necessary because our mCherry-labeled proteins are less bright than those labeled with GFP or GFP variants, and this difference is exacerbated when the tagged protein is of low abundance.

14. Some of the quantification methods used in the current manuscript are confusing. For example, in the Methods section on Colocalization Analyses, the authors write: “To measure the colocalization between Vps8c-mNeon and Vps8cmCherry, 14 cells were analyzed and the coefficients were derived from 261 non-overlapping images.” Where did the 261 images come from?

For 14 cells we collected 14 Z-stacks with an average of 18 focal planes per stack, from which we quantitatively analyzed a total of 261 non-overlapping focal planes.
To avoid any confusion, we have changed the text of the “colocalization analysis” paragraph in the M&M, denoting the number of independent Images analyzed.

15. In the discussion, the authors might consider noting that the association of Vps8a with Rab4b-containing endosomal domains and its function in cargo delivery to the mucocyst is consistent with the role of early endosome-derived tubules in cargo delivery to melanosomes in melanocytes (e.g. see Delevoye et al., 2009 JCB, Dennis et al., 2015 JCB and Delevoye et al., 2016 Curr. Biol.), suggesting conservation in LRO biogenesis pathways. Also in this respect, C. elegans expresses two distinct subunit pairs for CCZ-1 - a MON1 orthologue and another (GLO3) that was hard to identify as a MON1 paralogue by sequence identification (see Morris et al., 2018, PLoS Genet.); thus, the failure to identify a clear RAB7 GEF subunit (CCZ-1) orthologue in T. thermophila has some precedent in C. elegans.

We thank the reviewer for these valuable references, which we have added to the discussion section:

“Interestingly, Vps8 in Hela cells similarly associates with Rab4, as part of a sub-complex that provides a function distinct from holo-CORVET (Jonker et al., 2018). Moreover, the association of Vps8a with Rab4b-containing endosomal domains, and its function in cargo delivery to mucocysts is consistent with the role of early endosome-derived tubules in cargo delivery to melanosomes (Delevoye et al., 2009 JCB; Delevoye et al., 2016; Dennis et al., 2015), suggesting potential conservation in LRO biogenesis pathways.”
“Interestingly, while Tetrahymena has an unambiguous Mon1 homolog, there is no convincing ortholog for Ccz1 (unpublished), similar to what has been reported in C. elegans (Morris et al., 2018).”

Reviewer 2 Advance summary and potential significance to field...
This study represents a detailed biochemical characterization of the CORVET complexes in Tetrahymena. Together with the HOPS complex, these tether complexes mediate endosome maturation via their specific interaction with Rab GTPases. Though the current study lacks functional characterization of the distinct CORVET complexes, their distinct intracellular localization using the defining Vps8 subunits does support functional differences.
Reviewer 2 Comments for the author...
1. Last sentence in background states that “…Vps18 plays a key role in shaping the specificity of complex assembly”. There is however, no evidence provided for the function of Vps18 in the manuscript. I suppose this is a typo.

The evidence in the manuscript comes from the pattern of association of Vps18 paralogs with specific paralogs of other subunits, and is described in the paragraph beginning “The pattern of subunit variation between complexes suggests that the core Vps18 subunit may determine which Vps8 paralog is included.” However, the reviewer makes the point that our statement in the background might lead some readers to think we will present functional analysis. Thus we have removed that statement from the background.

2. The background is rather long. Think it could be shortened to make the research question clearer. However, it is not clear if the loss of HOPS is Tetrahymena-specific or also found in other ciliates.

We previously showed that HOPS was lost in ciliates belonging to the Oligohymenophorea lineage, including Paramecium caudatum and Ichthyophthirius multifiliis (Sparvoli et al.,2018). We have clarified this in the introduction to address the reviewer’s concern.

With regard to shortening the introduction, we have removed ~200 words from the introduction. Beyond this, we do not see additional material we can remove without compromising the accessibility of the manuscript to a broad readership.

3. I found it puzzling that Vps8c-mNeon and Vps8c-mCherry do not colocalize completely (Fig. 5A). Does this imply that different tags may have different effects on Vps8c localization and/or dynamics? This would make the rest of Fig 5 and even Fig 6 difficult to interpret. Other than mNeon and mCherry, it would be important to use other tags for these co-localization studies. A non-fluorescent tag may be desirable in this case, to rule out any possible FRET effects. This is particularly relevant in Fig 7 when potential interacting proteins are tagged respectively with mNeon and mCherry. In a recent study (https://www.biorxiv.org/content/10.1101/694190v2.full), mCherry was found to be an efficient FRET acceptor for mNeonGreen.

We did not expect to see 100% colocalization between the two different tagged versions, based on similar experiments in the literature that were used to establish baselines for comparing localization of two different proteins. In the specific case of Vps8c, the number of complexes present on a single structure (e.g., vesicle) may be small. Based on preliminary data, we currently estimate that there may be 12-20 copies of Vps11 per vesicle. With such small numbers, it is not surprising that some vesicles will have too little of either the mCherry or mNeon-tagged copy to be detected.
We agree with the reviewer that one cannot completely eliminate the possibility that one or both tags perturb protein function. However, any such perturbation must be subtle, based on the absence of detectible phenotypes in cells expressing these proteins. Because we think these considerations do not compromise our fundamental conclusions, and because of the time that would be required to generate cell lines expressing differently-tagged proteins as gene replacements, we would strongly prefer to rely on our current data for the present manuscript.

Because the additions to the manuscript requested above increased the content above the word limit, we have removed ~700 words from the manuscript in a variety of places.

Second decision letter

MS ID#: JOCES/2019/238659

MS TITLE: Diversification of CORVET tethers facilitates transport complexity in *Tetrahymena thermophila*

AUTHORS: Daniela Sparvoli, Martin Zoltner, Chao-Yin Cheng, Mark Field, and Aaron Turkewitz
ARTICLE TYPE: Research Article

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 gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

Please could you address explicitly the question of the potential for FRET between your probes, at least in the text if not through new experiments.

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

By defining the composition and documenting distinct localization of the different CORVET complexes in T. thermophila, the authors address an interesting question about the determinants of specificity within CORVET/HOPS complexes for distinct fusion steps. This question has been difficult to address in more complex eukaryotic systems in which seemingly invariant CORVET and HOPS complexes each function in multiple trafficking steps and in which both complexes are essential for life. These studies thus provide a basis for future work that might shed light on functional CORVET binding interactions. They also begin to document Rab interaction specificities for some of the distinct CORVET Vps8 subunits and a role for two of the Vps8 subunits in different stages of phagosome maturation.

Comments for the author

The manuscript by Sparvoli et al has been extensively revised and is vastly improved over the original version in nearly all aspects. The data are better presented and described, methods are better described, quantification has been added, and heterogeneity among data are more clearly delineated and explained. The addition of Figure 4 is quite nice in showing successive roles for Vps8e and b with maturing food vacuoles. This manuscript now provides an important, interesting, and rigorous contribution to the field.

I have only a few minor concerns/suggestions regarding the revisions that can all be addressed by changes to the text and or altered labeling of graphs.

Most critical:

1. In Figure 3D-F, it is not clear what the number of particles refers to - it does not look like this is per cell, is it? If this is over the entire group of images analyzed, then perhaps it would be best to convert the "number" to "percentage" of total structures analyzed (as the number itself would be meaningless). Alternatively, a number per cell can be calculated, or if this number is per cell, it should be indicated as such.

Minor:

2. In the Introduction page 4 line 5, the authors note that VPS33B and VPS16B are part of the CHEVI complex; data in Ambrosio and Di Pietro (2019; Blood Adv. 3: 2617-2626) essentially prove (in this reviewer's opinion) that VPS33B and VPS16B are the sole subunits of CHEVI. Also, CHEVI is associated with the biogenesis of alpha granules and lamellar bodies, not with the organelles themselves; this is supported by studies in Ambrosio and Di Pietro 2019 and suggested by biochemical interaction studies in Gruber et al 2017 (J. Invest. Dermatol. 137: 845).

3. In describing the results of the sedimentation analyses on page 6, it is not quite correct to refer to the sedimentation of the complex as "anomolous". Sedimentation is not only a property of the mass, but also of the density (partial specific volume). Deviation from the migration expected for a globular protein of 730 kDa is likely indicative of either an elongated shape, as expected from the negative stain architecture of HOPS (Bröcker et al, 2012, PNAS 109: 1991; Chou et al., 2016, Nat. Struct. Mol. Biol. 23: 761), or a low density of the protein complex due to other properties. I think the important point to note here is that the data on the sedimentation of Vps8a-cc from cells are consistent with that of a large complex - and now much more nicely documented than in the original manuscript - and reservations about migration differences for a globular protein of its predicted mass should be minimized.

4. In the legend to Suppl. Fig. 2, 3rd line from bottom, "note species migrating close to band 5" - it is not clear what "band 5" is. It would be more clear to denote this as "the Vps3 band".

5. The movies are really cool (particularly of Vps8b-mNeon in Movie 3 and Vps8d-mNeon in Movie 4 - these would be great movies for undergraduates!). If it would not be too difficult, it would be helpful for readers if the name of the VPS8 orthologue were included at least in the first frame of each of the movies.

6. The cartoon in Fig. 4B is a little bit opaque. What is the structure at the top right? Does this structure help to define the anterior end of the organism, and does it correlate with the darkened region of the cell shown in panel A top? If not, how is the anterior end of the organism defined?

7. The authors acknowledge the previously raised concern about the Mander's coefficient as a measure of relative colocalization, but the wording in the text on page 12 regarding "90% colocalization" or "50% colocalization" (describing results in Figure 7) still needs to be changed. As the authors acknowledge and as they cite in the classic article by Dunn and colleagues, Mander's (like Pearson's) is a relative measure of correlation between two fluorophores and not an absolute value of colocalization. It is a fine measure to use, and the data are very convincing that Rab7 and Rab22a colocalize to a great extent with Vps8c but not Vps8a, but the numbers obtained cannot be interpreted in absolute terms of colocalization. I would also argue that the statement in the last paragraph of the Results on page 13 - "8C-CC shows significant co-localization with early endosomal Rab22a, and almost complete co-localization with late endosomal Rab7" is impossible and at odds with the images. It would be better to leave this statement more qualitative like "strong co-localization with Rab7 and modest overlap with Rab22a".

Reviewer 2

Advance summary and potential significance to field

I agree with the authors that mNeon and mCherry tags may not affect the function or localization of the proteins. But I am afraid that the potential FRET between mNeon and mCherry was not sufficiently considered.

Comments for the author

FRET could potentially affect the colocalization analyses and results. For example, if FRET occurs between mNeon and mCherry (depending on the proteins each reporter is fused to), mNeon fluorescence could be greatly diminished due to FRET resulting in underestimation of colocalization. I understand that making new cell lines will be time-consuming, but a control for FRET or the lack of would be helpful.

Second revision

Author response to reviewers' comments

Most critical:
1. In Figure 3D-F, it is not clear what the number of particles refers to - it does not look like this is per cell, is it? If this is over the entire group of images analyzed, then perhaps it would be best to convert the "number" to "percentage" of total structures analyzed (as the number itself would be meaningless). Alternatively, a number per cell can be calculated, or if this number is per cell, it should be indicated as such.

The reviewer is correct, the number of particles refers to “number of particles per cell”, reported as Mean value. Figure 3D-E has been modified accordingly. The text in figure legend 3 has been updated to add:

D) …. The reported values represent average number of particles per cell.

Minor:
2. In the Introduction page 4 line 5, the authors note that VPS33B and VPS16B are part of the CHEVI complex; data in Ambrosio and Di Pietro (2019; Blood Adv. 3: 2617-2626) essentially prove (in this reviewer's opinion) that VPS33B and VPS16B are the sole subunits of CHEVI. Also, CHEVI is associated with the biogenesis of alpha granules and lamellar bodies, not with the organelles themselves; this is supported by studies in Ambrosio and Di Pietro 2019 and suggested by biochemical interaction studies in Gruber et al 2017 (J. Invest. Dermatol. 137: 845).

We have changed the wording to "CHEVI functions during biogenesis of …."
We have changed the wording to say "….Vsp16B and Vps33B form a distinct complex….." but the constraint of word limits for the manuscript prevent us from adding more description and references for CHEVI complex.

3. In describing the results of the sedimentation analyses on page 6, it is not quite correct to refer to the sedimentation of the complex as "anomolous". Sedimentation is not only a property of the mass, but also of the density (partial specific volume). Deviation from the migration expected for a globular protein of 730 kDa is likely indicative of either an elongated shape, as expected from the negative stain architecture of HOPS (Bröcker et al, 2012, PNAS 109: 1991; Chou et al., 2016, Nat. Struct. Mol. Biol. 23: 761), or a low density of the protein complex due to other properties. I think the important point to note here is that the data on the sedimentation of Vps8a-cc from cells are consistent with that of a large complex - and now much more nicely documented than in the original manuscript - and reservations about migration differences for a globular protein of its predicted mass should be minimized.

We have removed the phrase that included the word "anomalous"

4. In the legend to Suppl. Fig. 2, 3rd line from bottom, "note species migrating close to band 5" - it is not clear what "band 5" is. It would be more clear to denote this as "the Vps3 band".

We thank the reviewer for noticing this mistake. The band 5 belongs to the previous labeling of supplementary figure 2, which we changed accordingly to the reviewer's request but without replacing the number in the figure legend. The supplementary figure 2B legend has now been updated as follows:

B) … All six CORVET subunits were detected for Vps8c, Vps8d, Vps8e, and Vps8f. In the Vps8b-FLAG eluate, the Vps18 subunit (Vps18b) was not clearly detected at the expected size, but may migrate anomalously (note species migrating close to the Vps3 band, unique to this sample).

5. The movies are really cool (particularly of Vps8b-mNeon in Movie 3 and Vps8d-mNeon in Movie 4 - these would be great movies for undergraduates!). If it would not be too difficult, it would be helpful for readers if the name of the VPS8 orthologue were included at least in the first frame of each of the movies.

These additions have been made.

6. The cartoon in Fig. 4B is a little bit opaque. What is the structure at the top right? Does this structure help to define the anterior end of the organism, and does it correlate with the darkened region of the cell shown in panel A top? If not, how is the anterior end of the organism defined?

The structure at the top right represents the oral apparatus (OA) and it has been used to define the anterior portion of the cell in this experiment. We have now labeled the "OA" structure in figure 4B. We also updated the figure legend accordingly:

"B) and C) Calculating a position index for Vps8b- and Vps8e-labeled phagosomes. The position index corresponds to the distance of a vacuole to the tip of the cell divided by the cell length. The oral apparatus (OA) defines the anterior of the cell. The plot for Vps8b- and Vps8e-labeled phagosomes reveals clear posterior vs anterior biases, respectively."

7. The authors acknowledge the previously raised concern about the Mander's coefficient as a measure of relative colocalization, but the wording in the text on page 12 regarding "90% colocalization" or "50% colocalization" (describing results in Figure 7) still needs to be changed. As the authors acknowledge and as they cite in the classic article by Dunn and colleagues, Mander's (like Pearson's) is a relative measure of correlation between two fluorophores and not an absolute value of colocalization. It is a fine measure to use, and the data are very convincing that Rab7 and Rab22a colocalize to a great extent with Vps8c but not Vps8a, but the numbers obtained cannot be interpreted in absolute terms of colocalization. I would also argue that the statement in the last paragraph of the Results on page 13 - "8C-CC shows significant co-localization with early endosomal Rab22a, and almost complete co-localization with late endosomal Rab7" is impossible and at odds with the images. It would be better to leave this statement more qualitative like "strong colocalization with Rab7 and modest overlap with Rab22a".

We have modified the text as suggested:
Page 12: "We previously reported the surprising finding that Vps8a shows negligible co-localization with Rab22a but substantial overlap with Rab7(Sparvoli et al., 2018). To determine if this was also the case for Vps8c, we expressed N-terminal mCherry-tagged Rab7 (Fig. 7A) and Rab22a (Fig. 7B) in Vps8c-mNeon expressing cells, and measured the extent of overlap. Vps8c co-localized more strongly with both Rabs than Vps8a (Fig. 7C, D), and in particular overlapped extensively with Rab7 (Fig. 7C)."

Page 13: "8C-CC shows mild co-localization with early endosomal Rab22a, and strong co-localization with late endosomal Rab7."

Reviewer 2
Advance summary and potential significance to field... I agree with the authors that mNeon and mCherry tags may not affect the function or localization of the proteins. But I am afraid that the potential FRET between mNeon and mCherry was not sufficiently considered.

Reviewer 2 Comments for the author...
FRET could potentially affect the colocalization analyses and results. For example, if FRET occurs between mNeon and mCherry (depending on the proteins each reporter is fused to), mNeon fluorescence could be greatly diminished due to FRET, resulting in underestimation of colocalization. I understand that making new cell lines will be time-consuming, but a control for FRET or the lack of would be helpful.

Thank you for your consideration. We have studied the manuscript suggested by the reviewer, and have also discussed this matter with colleagues who regularly use paired fluorophores for quantitative localization studies. As we are measuring the green and red signals separately, we would not be detecting FRET signals in our experiments. If the red and green fluorophores are in very close contact, there is a possibility that the green signal would be slightly diminished. This would be a small effect, and our analysis in Fig 7 does not depend on the intensity of signals, as long as they are detectible. For those reasons, we believe it is unlikely that any FRET, if it occurs, would qualitatively affect our conclusions regarding overlap between signals. We have added the following to the Fig 7 legend:

In cases of strong overlap, FRET between mNeon and mCherry could reduce green emission.

Third decision letter

MS ID#: JOCES/2019/238659

MS TITLE: Diversification of CORVET tethers facilitates transport complexity in *Tetrahymena thermophila*

AUTHORS: Daniela Sparvoli, Martin Zoltner, Chao-Yin Cheng, Mark Field, and Aaron Turkewitz
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.