JCB

A bipartite sorting signal ensures specificity of retromer complex in membrane protein recycling

Sho Suzuki, Ya-Shan Chuang, Ming Li, Matthew Seaman, and Scott Emr

Corresponding Author(s): Scott Emr, Cornell University

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February 3, 2019

Re: JCB manuscript #201901019

Dr. Scott D Emr
Cornell University
Weill Institute for Cell and Molecular Biology 441 Weill Hall
Ithaca, NY 14853

Dear Scott,

Thank you for submitting your manuscript entitled "A bipartite sorting signal ensures specificity of retromer complex in membrane protein recycling". We apologize again for the delay in providing you with a decision. As noted in our previous correspondence, there was some disagreement among the reviewers about the suitability of the paper for JCB and so we sought further feedback from the reviewers before rendering a decision. In any case, your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that while reviewer#1 is quite enthusiastic about the work, reviewers #2 and 3 both feel that substantially more work is needed before the paper would be suitable for publication in JCB. In particular, these reviewers feel that you will need to provide more conclusive evidence to prove direct binding of Vps26 to the bipartite sorting signals and further validation and/or discussion of the newly identified motifs. Reviewer #3 also feels that you need to determine with Vps26 mutation impacts complex assembly.
Please note that, despite their somewhat more positive inclinations toward the paper, reviewers #1 and #3 have, in the course of our subsequent discussions, indicated that they agree with the points raised by reviewer #2 and would wish to see them addressed as completely as possible in a revised version of the manuscript. We therefore hope that you will be able to address the aforementioned issues, as well as all of the other comments raised by the reviewers, in your revision.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

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Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Ira Mellman, PhD
Senior Editor
Journal of Cell Biology

Tim Spencer, PhD
Deputy Editor
Journal of Cell Biology
ORCiD: 0000-0003-0716-9936

Reviewer #1 (Comments to the Authors (Required)):

The cargo selective complex of retromer (CSC) is comprised of Vps26, Vps29 and Vps35. This complex works together with a SNX-BAR dimer of Vps 5 and Vps17. In this thorough analysis, Emr and colleagues identify a new sorting motif that adds cargo selectivity to retromer cargo recognition. Previously]F/Y/W/]X[L/M/V] was considered to represent the recognition seqeuence, but its presence in non retromer cargoes suggested there was more.

Through extensive mutagenesis combined with localization in yeast cells, the authors identify YSSL and FGEIRL in Vps10 as being important, and sufficient to localize a distinct cargo protein. In Ear1, they identify PPGFEF and INL. They go on to show that CSC is needed for sorting of both cargoes; Vps10 also needed Vps5 and Vps17, but Ear1 only needed Vps5. Both cargoes need the same

complexes as determined from competition experiments. They show that Vps10 and Ear1 engage different binding sites in Vps26 and more specifically than previously thought, due to coincidence detection.

Overall this represents a lot of work that will be of interest to readers of JCB, and the story should be accepted without delay but would benefit from more discussion about other cargoes and how much additional diversity might be anticipated in other cargoes and whether mutation of Vps26 has indicated selective recycling issues for one cargo and not another.

Page 6 bottom-- Rsp5?

Reviewer #2 (Comments to the Authors (Required)):

This paper contains a lot of solid and well presented data, but there are no real mechanistic insights, and the authors have a tendency to gloss over inconsistencies. The study is based on the premise that because the best characterized retromer sorting signal, ΦX[L/M/V] (where Φ is F, Y, or W), can be found in nearly all proteins, retromer cargo proteins are likely to have additional sorting signals. The authors use truncations and alanine scanning mutagenesis to dissect two retromer cargo proteins, Vps10 and Ear1, and they identify two motifs on each that contribute to their recycling away from the vacuole. They also mutate the Vps26 subunit of the retromer complex and show that individual mutations can have different effects on the localization of Vps10 and Ear1. From this they conclude that there are at least three cargo binding sites on Vps26, one of which is shared by Vps10 and Ear1, while the other two sites interact with one but not the other. This is illustrated in their final figure, 5F.

My main reservation is that the authors present no real evidence for a direct interaction between any of their sorting signals and Vps26, so their model is extremely speculative. Moreover, although the paper starts off with the idea that there are other sorting signals in addition to ΦX[L/M/V], in fact none of the motifs they analyse is actually a ΦX[L/M/V]. The Introduction and the first two sentences of the Results make much of this motif, but then the authors go on to examine the YSSL sequence in Vps10, presumably with the assumption that it fits the consensus. Yet there are two residues rather than one residue between the Y and the L, which means not only that the key residues would be spaced further apart than in a ΦX[L/M/V] motif, but also that they would be pointing in different directions. So it is difficult to imagine how a ΦX[L/M/V] motif and a YSSL motif could be binding to the same site. In fact, YSSL looks like a motif for the AP (adaptor protein) complexes. Can the authors rule out the possibility that YSSL binds to AP-1, which also facilitates endosome-to-Golgi retrieval?

Interestingly, a 1995 study from the Emr lab, making use of deletion mutants, pinpointed the sequence FYVF, which does fit the ΦX[L/M/V] consensus, as a key retrieval signal on Vps10. However, the FYVF motif is not discussed, presumably because a subsequent 1996 study from the Stevens lab, which was the first to investigate the YSSL motif, showed by alanine scanning mutagenesis that the FYVF sequence is not strictly necessary for Vps10 retrieval. However, that study, and the present study as well, show that YSSL isn't essential either, so it is not clear to me why they focus so exclusively on the YSSL motif.

A second concern is that, although the authors propose that Vps10 and Ear1 share a binding site on Vps26, the motifs they identify (YSSL plus FGEIRL on Vps10, and INL plus PPGFEF on Ear1) not only don't conform to the ΦX[L/M/V] motif, but also don't appear to share any other consensus

sequences. Thus, it is difficult to imagine how two of these sequences (they don't specify which two) could make use of a common binding site.

It is also not clear why the authors assume that the interactions must be with the Vps26 subunit, rather than with the Vps35 subunit and/or the associated SNX/BAR proteins, all of which have also been implicated in cargo retrieval. Their rationale seems to come from their Vps26 mutagenesis experiments. Initially they mutated I251 and F368, based on the 2016 X-ray crystallography structure of a partial mammalian retromer complex that was co-crystalized with a peptide derived from a cargo protein, which contains a ΦX[L/M/V] motif, YLL. The structure showed that I251 and F368 are in contact with the YLL motif, suggesting that they help to form the cargo binding site. However, in September 2018, a new retromer structure was solved by cryo-EM tomography (PubMed 27889239).The authors do not cite that study, but it is extremely relevant to their own work, not least because it is of a fungal complex. An important difference between fungi (including yeast) and mammals is that in fungi, the SNX/BAR proteins are stably associated with the Vps26-Vps29-Vps35 core. Interestingly, in the fungal structure, the SNX/BAR protein Vps5 occludes the putative cargo-binding pocket in Vps26. Thus, there is a strong possibility that the mutations the authors made in Vps26 prevented the binding of Vps5, and thus that effects on cargo recognition are indirect. The authors go on to make additional mutations in Vps26, but the same caveat applies: these mutations could also affect cargo recognition indirectly.

The authors end by concluding that retromer cargo proteins have bipartite recycling signals, making their recognition more specific. This is a sensible conclusion, but it is not a new one: over 20 years ago, Cooper and Stevens proposed that Vps10 has more than one retrieval signal (PubMed 8636229). They then point out that the AP-1 and AP-2 complexes can recognize both YXXΦ motifs and [D/E]XXXL[L/I] motifs, and they suggest that bipartite signal recognition may be a general mechanism in membrane trafficking. However, this is not really a good comparison, because YXXΦ motifs and [D/E]XXXL[L/I] motifs can act in isolation (PubMed 20214754 and 28003333). Moreover, YXXΦ sequences are almost as common as ΦX[L/M/V] sequences, but for them to act as sorting signals, they need to be within an unstructured cytoplasmic domain of a transmembrane protein, and this is a lot less common. In addition, although coincidence detection is certainly important in membrane trafficking pathways, the coincidences do not have to be within the cytoplasmic domain of a single membrane protein. Interactions with phosphoinositides and small GTPases are important for getting the trafficking machinery onto the right membrane in the first place, where it can then find its cargo, and this applies equally well to retromer and to AP complexes.

In summary, the authors have generated some useful data, and future studies will no doubt show how the sequences they have identified actually function. But the present study is more of a collection of observations than an insight into actual mechanisms.

If the authors are invited to submit a revised version, these are the most important points to be addressed:

1. Clarify what is and what isn't supposed to be a canonical ΦX[L/M/V].

2. Take the new cryo-EM structure into account.

3. Test by immunoprecipitation whether any of their Vps26 mutations affect assembly of the complex.

4. Provide evidence for a direct interaction between Vps26 and sorting signals.

Reviewer #3 (Comments to the Authors (Required)):

Review Suzuki et al., 2019, JCB

In the present study, Suzuki et al. investigate how the yeast hydrolase transporter, the Vps10 receptor, is recognized and retrogradely transported through the retromer complex. Using extensive mutagenesis and an imaging based sorting assay, they thoroughly dissect the sorting requirements for VPS10 (and Ear1) to conclude that retromer requires a complex, bipartite sequence to retrieve these receptors from the vacuolar pathway.

The mutagenesis and sorting data are clean, very convincing and leave little doubt about the identified sorting signal. Given that recent data on the mammalian retromer and the Vps10p equivalent CI-MPR has caused considerable confusion in the field, the study is also timely and of importance for the field. The report format is appropriate as the authors report a single finding with considerable impact.

The only weakness of the study is the use of a single assay to identify and verify the sorting requirements. All the results are based on mutagenesis of either Vps10 or VPS26 and imaging based analysis of retrograde sorting. Given that the retromer associated SNX-BAR proteins have recently been shown to directly bind CI-MPR (Kvainickas et al., 2017/ Simonetti et al, 2017) it would be great if the authors could use a biochemical approach to verify that the Vps10 tail indeed binds to the core retromer trimer and not to the Vps5/Vps17 subunit. At present, the data do not fully exclude that Vps10 binds to Vps5 or Vps17 as the mutagenesis of VPS26 could also disrupt retromer function in an unspecific way. A binding assay between purified Vps10 tail and retromer components could then also be used to test whether the sorting motif within Vps10 that was identified through mutagenesis and imaging indeed mediates the binding. At least for the mammalian proteins, the VPS29/VPS35/VPS26 and the SNX-BAR subcomplexes can be individually expressed and purified. In my opinion, it would really strengthen the study if the authors showed some form of binding assay with the wildtype and mutant Vps10 tail with the individual retromer subcomplexes. This could be a GST pulldown with recombinant and purified proteins or maybe even co-IPs from mammalian HEK293 cells if the proteins express poorly in bacteria.

Some additional minor points:
In the introduction, the authors state that the Parkinson associated VPS35-D620N mutant causes lysosomal dysfunction. I am not sure whether this has been conclusively shown. The data from the D620N knock-in mouse (Ishizu et al., 2016) strongly suggests that the D620N mutant is an extremely subtle loss of function mutant even in D620N homozygous mice. It is unlikely to cause significant lysosomal dysfunction as it retains most cargo sorting functions.

At the end of the results section, the authors state that SNX3 is recruited to the endosomal membrane via binding to VPS26. Given that SNX3 has intrinsic lipid binding capabilities through its PX domain and has been shown to mediate retromer recruitment together with RAB7-GTP (Harrison et al., 2014, PNAS), this is really surprising. The authors cite Lucas et al. in this context, but that study also only shows the recruitment of retromer via SNX3, not the other way around?

Reviewer #1 (Comments to the Authors (Required)):

The cargo selective complex of retromer (CSC) is comprised of Vps26, Vps29 and Vps35. This complex works together with a SNX-BAR dimer of Vps5 and Vps17. In this thorough analysis, Emr and colleagues identify a new sorting motif that adds cargo selectivity to retromer cargo recognition. Previously]F/Y/W/]X[L/M/V] was considered to represent the recognition segeuence, but its presence in non retromer cargoes suggested there was more.

Through extensive mutagenesis combined with localization in yeast cells, the authors identify YSSL and FGEIRL in Vps10 as being important, and sufficient to localize a distinct cargo protein. In Ear1, they identify PPGFEF and INL. They go on to show that CSC is needed for sorting of both cargoes; Vps10 also needed Vps5 and Vps17, but Ear1 only needed Vps5. Both cargoes need the same complexes as determined from competition experiments. They show that Vps10 and Ear1 engage different binding sites in Vps26 and more specifically than previously thought, due to coincidence detection.

Overall this represents a lot of work that will be of interest to readers of JCB, and the story should be accepted without delay but would benefit from more discussion about other cargoes and how much additional diversity might be anticipated in other cargoes and whether mutation of Vps26 has indicated selective recycling issues for one cargo and not another.

Thank you! We also tested Kex2 localization in *vps26* mutants (Fig. Rev. 1). Kex2-GFP was mislocalized in I251E/F368E or L285E, but not in F334E, suggesting that I251/F368 recognized at least Ear1 and Kex2. Because of the space restriction for the report format, we decided not to include this data in the revised manuscript.

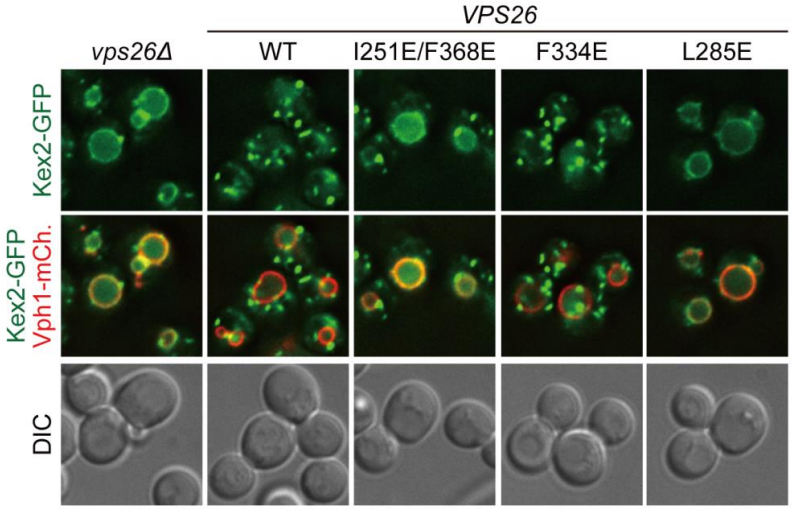

Fig. Rev. 1

Page 6 bottom-- Rsp5?

Thanks for pointing this out. We have corrected it in the manuscript to avoid confusion (Page 6, paragraph 1, lane 16).

Reviewer #2 (Comments to the Authors (Required)):

This paper contains a lot of solid and well presented data, but there are no real mechanistic insights, and the authors have a tendency to gloss over inconsistencies. The study is based on the premise that because the best characterized retromer sorting signal, ΦX[L/M/V] (where Φ is F, Y, or W), can be found in nearly all proteins, retromer cargo proteins are likely to have additional sorting signals. The authors use truncations and alanine scanning mutagenesis to dissect two retromer cargo proteins, Vps10 and Ear1, and they identify two motifs on each that contribute to their recycling away from the vacuole. They also mutate the Vps26 subunit of the retromer complex and show that individual mutations can have different effects on the localization of Vps10 and Ear1. From this they conclude that there are at least three cargo binding sites on Vps26, one of which is shared by Vps10 and Ear1, while the other two sites interact with one but not the other. This is illustrated in their final figure, 5F.

My main reservation is that the authors present no real evidence for a direct interaction between any of their sorting signals and Vps26, so their model is extremely speculative.

In the revised manuscript, we have included the biochemical evidence for the cargo binding of retromer (Fig. 2E, 4E, 4F, 5E, 5F, S2E, and S2F were added in the revised manuscript). We believe these new data strengthen our conclusion.

Moreover, although the paper starts off with the idea that there are other sorting signals in addition to ΦX[L/M/V], in fact none of the motifs they analyse is actually a ΦX[L/M/V].

Thanks for pointing this out. In the initial manuscript, we used ΦX[L/M/V] as a consensus motif defined by the mammalian retromer cargos (Cullen and Steinberg., 2018). However, when we listed all identified yeast recycling sequences (Fig. S1A was added in the revised version), we realized that that 4 out of 8 identified recycling signals such as YSSL of Vps10, FQFND of Ste13, YEF of Kex2, and WKY of Stv1 do not follow this rule. It could be that yeast recycling signals and mammalian recycling signals are different. The only common feature of yeast recycling signals is the presence of hydrophobic residues. In the revised manuscript, we addressed this in the text (Page 3, paragraph 3, line 3).

The Introduction and the first two sentences of the Results make much of this motif, but then the authors go on to examine the YSSL sequence in Vps10, presumably with the assumption that it fits the consensus. Yet there are two residues rather than one residue between the Y and the L, which means not only that the key residues would be spaced further apart than in a ΦX[L/M/V] motif, but also that they would be pointing in different directions. So it is difficult to imagine how a ΦX[L/M/V] motif and a YSSL motif could be binding to the same site.

Our study proposes that the retromer has multiple binding sites. Currently, we don't have any evidence regarding which binding site binds to which sequence. However, how the different recycling sequences bind to the retromer complex will be the focus of our investigation in the future.

In fact, YSSL looks like a motif for the AP (adaptor protein) complexes. Can the authors rule out the possibility that YSSL binds to AP-1, which also facilitates endosome-to-Golgi retrieval?

We tested Vps10-GFP localization in AP-1 mutants (*apl2*Δ cells) (Fig. Rev. 2). However, it still localized on punctate structures similar to WT cells, suggesting that AP-1 does not facilitate Vps10 recycling. Also, consistent with our interpretation, a recent study reports that AP-1 is required for intra-Golgi recycling, not endosome-to-Golgi retrieval (Casler et al., JCB 2019).

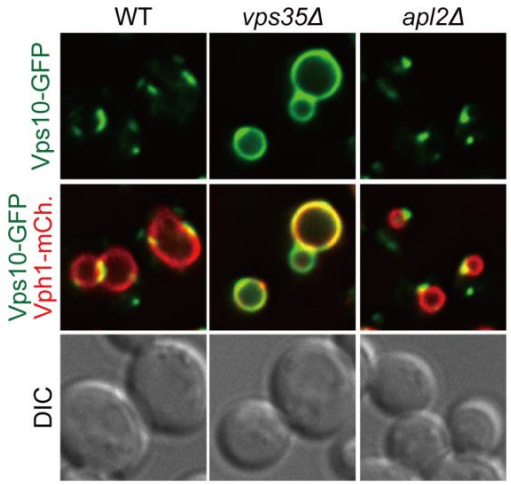

Fig. Rev. 2

Interestingly, a 1995 study from the Emr lab, making use of deletion mutants, pinpointed the sequence FYVF, which does fit the ΦX[L/M/V] consensus, as a key retrieval signal on Vps10. However, the FYVF motif is not discussed, presumably because a subsequent 1996 study from the Stevens lab, which was the first to investigate the YSSL motif, showed by alanine scanning mutagenesis that the FYVF sequence is not strictly necessary for Vps10 retrieval.

Consistent with a 1996 study from the Stevens lab, both 1447-1456A and 1457-1466A mutants which include the sequence 1456-FYVF-1459 did not exhibite striking phenotype (Fig. 1F). Accordingly, we revised the text in the manuscript (Page 4, paragraph 1, line 7).

However, that study, and the present study as well, show that YSSL isn't essential either, so it is not clear to me why they focus so exclusively on the YSSL motif.

Indeed, Cooper and Stevens showed YSSL is not essential. However, they observed 36% of CPY is missorted in Y1492A mutants (described as $Y_{77}A$ in Figure 8A in Cooper and Stevens., 1996). From this data, they concluded "a tyrosine-based signal ($YSSL_{80}$) within the cytosolic domain enables vps10p to cycle between the late-Golgi and prevacuolar/endosomal compartments" (Cooper and Stevens., 1996). Based on this data and our study, we believe the YSSL motif is important for recycling.

A second concern is that, although the authors propose that Vps10 and Ear1 share a binding site on Vps26, the motifs they identify (YSSL plus FGEIRL on Vps10, and INL plus PPGFEF on Ear1) not only don't conform to the ΦX[L/M/V] motif, but also don't appear to share any other consensus sequences. Thus, it is difficult to imagine how two of these sequences (they don't specify which two) could make use of a common binding site.

As mentioned above, yeast recycling motifs do not share any clear consensus motif (Fig. S1A in the revised manuscript). Determination of the genuine consensus motif for retromer binding is an important question in the field. However, to answer this, structural analysis will ultimately be required. We feel this is beyond the scope of the current manuscript. We hope that we will be able to answer this in future studies.

It is also not clear why the authors assume that the interactions must be with the Vps26 subunit, rather than with the Vps35 subunit and/or the associated SNX/BAR proteins, all of which have also been implicated in cargo retrieval. Their rationale seems to come from their Vps26 mutagenesis experiments.

Thanks for pointing this out. In the revised manuscript, we examined cargo binding to the retromer in each retromer subunit mutant (Fig. 4E, 4F, S2E, and S2F were added in the revised manuscript) and show that cargo binding of retromer requires both Vps26 and Vps35. There were two reasons to investigate cargo-binding by Vps26, firstly it is an arrestin-type protein and therefore a good candidate for cargo-binding by analogy with other arrestin proteins and secondly the crystal structure of cargo-bound to Vps26 has been reported (Lucas et al., 2016), thus enabling predictions based on the structural data. How Vps35 interacts with the cargos will be the focus of future studies.

Initially they mutated I251 and F368, based on the 2016 X-ray crystallography structure of a partial mammalian retromer complex that was co-crystalized with a peptide derived from a cargo protein, which contains a ΦX[L/M/V] motif, YLL. The structure showed that I251 and F368 are in contact with the YLL motif, suggesting that they help to form the cargo binding site. However, in September 2018, a new retromer structure was solved by cryo-EM tomography (PubMed 27889239).The authors do not cite that study, but it is extremely relevant to their own work, not least because it is of a fungal complex. An important difference between fungi (including yeast) and mammals is that in fungi, the SNX/BAR proteins are stably associated with the Vps26-Vps29-Vps35 core. Interestingly, in the fungal structure, the SNX/BAR protein Vps5 occludes the putative cargo-binding pocket in Vps26. Thus, there is a strong possibility that the mutations the authors made in Vps26 prevented the binding of Vps5, and thus that effects on cargo recognition are indirect. The authors go on to make additional mutations in Vps26, but the same caveat applies: these mutations could also affect cargo recognition indirectly.

As suggested by the reviewer, we performed CoIP analysis and confirmed that the Vps5-Vps26 interaction was unaffected in the *vps26* mutants (Fig. 5E was added to the revised version.). We have also discussed the differences with the Cryo-EM structure in the text.

The authors end by concluding that retromer cargo proteins have bipartite recycling signals, making their recognition more specific. This is a sensible conclusion, but it is not a new one: over 20 years ago, Cooper and Stevens proposed that Vps10 has more than one retrieval signal (PubMed 8636229).

Cooper and Stevens showed that $Y_{77}A/F_{106}A$ mutants exhibited a stronger CPY missorting phenotype than $Y_{77}A$ ($Y_{77}A/F_{106}A$: 49%, $Y_{77}A$: 36%). From this result, they concluded "Y_{77} and F_{106} signal plays a major role in the membrane trafficking of Vps10p yet other residues are likely to contribute". We directly examined Vps10-GFP localization of these mutants. We found that $Y_{77}A$ (Y1492A) mutants showed a partial recycling defect (Fig. Rev. 3). However, we could not see a clear difference between $Y_{77}A$ (Y1492A) and $Y_{77}A/F_{106}A$ (Y1492A/F1521A) mutants.

In our study, we found that 1428-FGEIRL-1433 and 1492-YSSL-1495 motifs of Vps10 are required for retromer recognition. Importantly, when we mutated both motifs (FGEIRL>AAAAAA and YSSL>AAAA), this double mutant did not show any detectable punctate structures (Fig. 2C), suggesting that these two motifs are major recycling motifs for Vps10 (Fig. 2C and Fig. 2D). Also, the other retromer cargo, Ear1, also has two discontinuous motifs, 453-PPGFEF-458 and 473-INL-475, sufficient for its recycling mediated by the retromer complex.

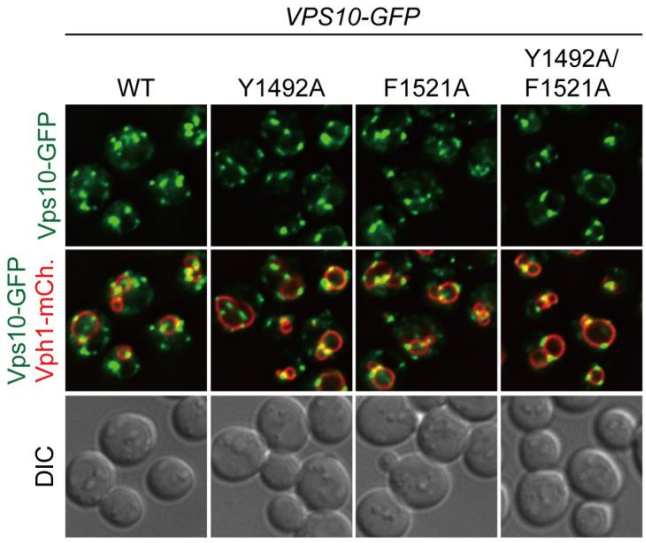

Fig. Rev. 3

They then point out that the AP-1 and AP-2 complexes can recognize both YXXΦ motifs and [D/E]XXXL[L/I] motifs, and they suggest that bipartite signal recognition may be a general mechanism in membrane trafficking. However, this is not really a good comparison, because YXXΦ motifs and [D/E]XXXL[L/I] motifs can act in isolation (PubMed 20214754 and 28003333). Moreover, YXXΦ sequences are almost as common as ΦX[L/M/V] sequences, but for them to act as sorting signals, they need to be within an unstructured cytoplasmic domain of a transmembrane protein, and this is a lot less common. In addition, although coincidence detection is certainly important in membrane trafficking pathways, the coincidences do not have to be within the cytoplasmic domain of a single membrane protein. Interactions with phosphoinositides and small GTPases are important for getting the trafficking machinery onto the right membrane in the first place, where it can then find its cargo, and this applies equally well to retromer and to AP complexes.

We know that YXXΦ motifs and [D/E]XXXL[L/I] motifs can act in isolation. However, there are examples of membrane proteins containing both signals (Kozik et al., 2010) and the conformational change induced in AP2 when it binds D/ExxLL motifs could facilitate further binding to YxxL. To avoid confusion, we have deleted this discussion.

In summary, the authors have generated some useful data, and future studies will no doubt show how the sequences they have identified actually function. But the present study is more of a collection of observations than an insight into actual mechanisms.

If the authors are invited to submit a revised version, these are the most important points to be addressed:

1. Clarify what is and what isn't supposed to be a canonical ΦX[L/M/V].

As mention earlier, we used ΦX[L/M/V] as a consensus motif defined by the mammalian retromer cargos (Cullen and Steinberg, 2018). However, we realized that 4 out of 8 identified recycling signals in yeast such as YSSL of Vps10, FQFND of Ste13, YEF of Kex2, and WKY of Stv1 do not follow this rule (Fig. S1A was added in the revised version). Hence, the yeast recycling sequence does not follow the ΦX[L/M/V] consensus. Defining the consensus sequence will be the focus of our future investigations.

2. Take the new cryo-EM structure into account.

Collins lab with others recently solved the Cryo-EM structure of *C. thermophilum* Vps5-Vps5-Vps26-Vps29-Vps35 complex (Kovtun et al., 2018). However, we believe that this Cryo-EM structure and the yeast retromer structure are different for the following reasons. In the Cryo-EM structure, the CSC (Vps26-Vps29-Vps35 complex) and the SNX-BAR dimers (Vps5-Vps17 complex) form a complex through the Vps5-Vps26 interaction, which is a sole contact between CSC and SNX-BAR dimers. Vps29 and Vps35 do not interact with the SNX-BAR dimer, which suggests that Vps29 and Vps35 require Vps26 to interact with the SNX-BAR dimer. However, it has been reported that SNX-BAR (Vps5) still interacts with Vps29 and Vps35 in vps26Δ cells (Reddy and Seaman, 2001; Seaman and Williams, 2002). Also, in the Cryo-EM structure, Vps26 directly interacts with SNX-BAR (Vps5), which would mean that Vps26 does not require Vps29 or Vps35 for the interaction with SNX-BAR (Vps5). However, Collins himself previously reported that Vps26 requires Vps29 to interact with SNX-BAR (Vps17) (Collins et al., 2005). Also, the Seaman lab has reported that Vps26 fails to interact with SNX-BAR (Vps5) in vps29Δ (Reddy and Seaman, 2001; Seaman and Williams., 2002). Importantly, in the Cryo-EM structure (Kovtun et al., 2018), this L252 residue of Vps29, which is required for binding to the SNX-BAR dimer (Collins et al., 2005), does not face towards any of other retromer subunit including SNX-BAR dimer (Fig. Rev. 4A and 4B). Unfortunately, Kovtun et al. did not cite these previous observations in their Cryo-EM study (Kovtun et al., 2018). Also, they did not confirm whether Vps5 directly interacts with Vps26 in vivo (Kovtun et al., 2018). We examined the retromer complex formation in the revised manuscript to ask if the model suggested by the Cryo-EM structure fits with the IP results (Fig Rev.5; IP results were added to the revised version as Fig. 4B). We tagged *VPS5* and *VPS17* at their endogenous locus with FLAG and HA epitopes, respectively. We confirmed that Vps10 is recycled in this strain, implying that Vps5-FLAG and Vps17-HA are functional. When we immunoprecipitated Vps5-FLAG from yeast cell lysates, Vps17-HA, Vps26, Vps29, and Vps35 co-precipitated. Strikingly, Vps17-HA, Vps29, and Vps35 still co-precipitated with Vps5-FLAG in vps26Δ cells. On the other hand, the association of Vps26 with Vps5-FLAG was abolished in vps29Δ or vps35Δ cells. These results strongly suggest that the CSC interacts with the SNX-BAR dimer through Vps29 and Vps35, and not through Vps26. This conclusion is consistent with the previously published results by Seaman's lab (Reddy and Seaman.,

2001; Seaman and Williams., 2002) and Collins's lab (Collins et al., 2005), but inconsistent with the Cryo-EM structure (Kovtun et al., 2018). Based on this finding, we conclude that complex formation of yeast retromer and C. thermophilum Vps5-Vps5-Vps26-Vps29-Vps35 are probably different. Hence, we were unable to take the Cryo-EM data into account in our models. It is interesting to note also that a recent study from the Jackson Lab has indicated that retromer can form more diverse structures than the Kovtun et al. study indicated and the angle of the CSC related to the SNX-BAR dimer is more variable hinting at a dynamic and/or flexible arrangement of the retromer subcomplexes (Kendal et al., 2019 -https://doi.org/10.1101/639575)

The recent Cryo-EM structure is also inconsistent with other previous observations. The retrieval of several cargos (i.e. Kex2, Ste13, Pep12, Ear1 etc.) is known to require Snx3 (Fig. Rev. 6). Snx3 interacts with retromer through Vps26 (Lucas et al., 2016). However, this Snx3 binding site on Vps26 is used for Vps5 binding (Kovtun et al., Nature 2018). Also, the DMT1-II (cargo) binding site determined by crystal structure and also confirmed by in vivo experiments is used for Vps5 binding (Kovtun et al., 2018). These inconsistencies support our interpretation that yeast retromer and the Cryo-EM structure are different.

Several possibilities may explain why assembly of the C. thermophilum Vps5-Vps5-Vps26-Vps29-Vps35 complex and the yeast retromer are different. It could be because components of the Cryo-EM structure and yeast retromer complex are different. Yeast retromer complex consists of Vps5, Vps17, Vps26, Vps29, and Vps35. All of them have a 1:1:1:1:1 stoichiometry. On the other hand, Kovtun et al. used two Vps5, Vps26, Vps29, and Vps35. They did not use Vps17 and in essence therefore did not report the structure of 'retromer'. In yeast, Vps5 makes a heterodimer with Vps17 (Horazdovsky et al., 1997; Seaman et al., 1998). However, Kovtun et al. claimed (without evidence) "Vps17 is likely to be structurally homologous to Vps5, in which case a heterodimeric array of Vps5 and Vps17 would form through equivalent contacts". However, vps17Δ cells exhibit a strong CPY missorting phenotype like vps5Δ cells (Horazdovsky et al., 1997). Also, we confirmed that Vps5 is unable to interact with Vps5 even in the absence of Vps17 (Fig. Rev. 7). These facts strongly suggest that the Vps5-Vps5 interaction and Vps5-Vps17 interaction are different. To answer this question, solving the retromer structure consisting of Vps5, Vps17, Vps26, Vps29, and Vps35 is essential.

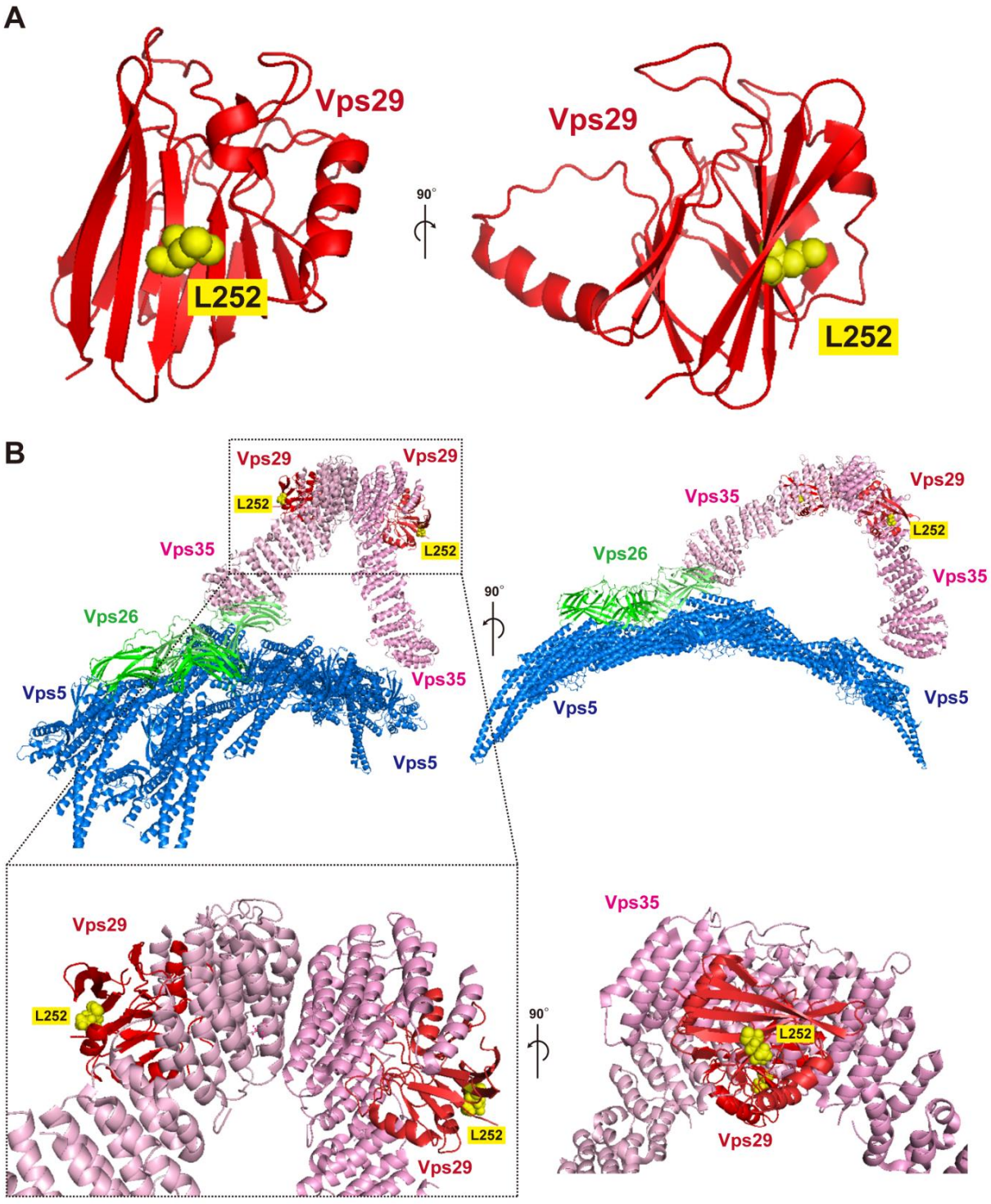
A
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Fig. Rev. 4

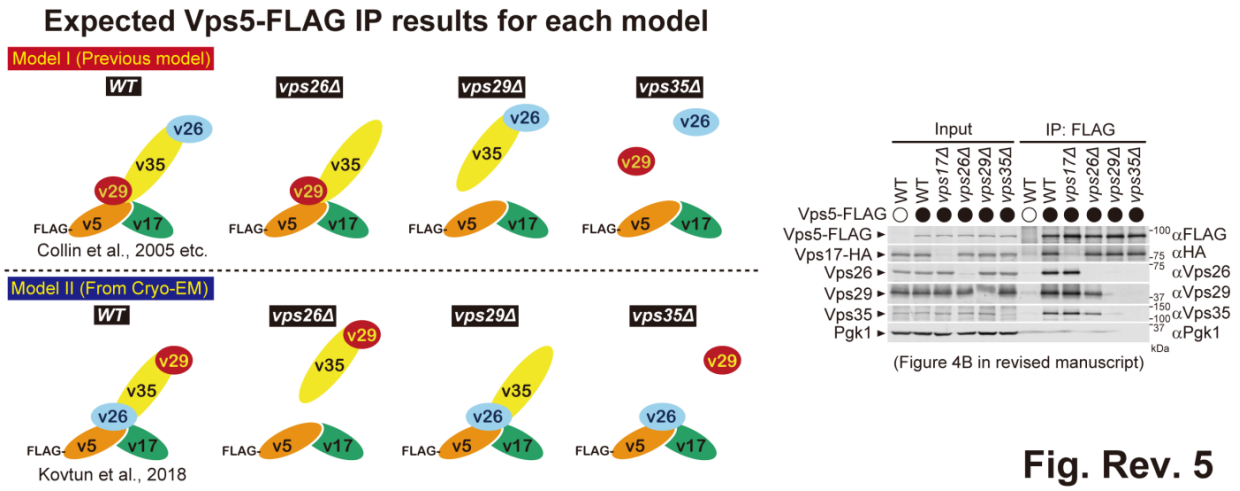

Fig. Rev. 5

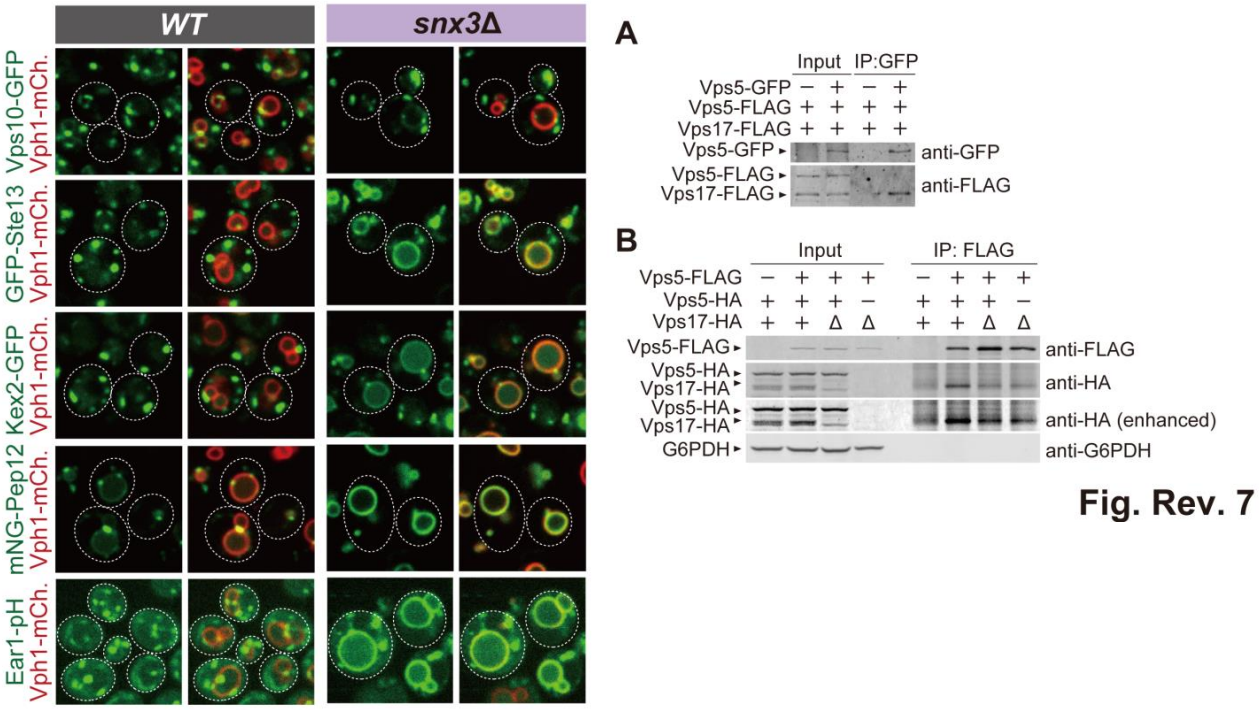

Fig. Rev. 6

Fig. Rev. 7

3. Test by immunoprecipitation whether any of their Vps26 mutations affect assembly of the complex.

As mention above, we examined the retromer complex formation in our mutants and confirmed that our mutations do not affect it (Fig. 5E was added to the revised version.).

4. Provide evidence for a direct interaction between Vps26 and sorting signals.

To investigate this interaction, we examined Vps10 binding to the retromer complex by CoIP analysis (Fig. 2E was added in the revised manuscript). Wild-type Vps10-GFP was co-precipitated with Vps26-FLAG, but a Vps10 sorting signal mutant did not, suggesting that the sorting signal is required for the interaction with retromer. To determine whether this binding is mediated by Vps26, we tested this interaction in retromer subunit mutants (Fig. 4E, 4F, S2E, and S2F were added in the revised manuscript) and found that Vps26 is essential for cargo binding. Furthermore, we examined cargo binding of retromer in a Vps26(F334E) mutant. In this mutants, retromer is assembled normally (Fig. 5E was added in the revised manuscript), but cargo binding is impaired (Fig. 5F was added in the revised manuscript). Collectively, we propose that Vps26 directly interacts with the sorting signals.

Reviewer #3 (Comments to the Authors (Required)):

Review Suzuki et al., 2019, JCB

In the present study, Suzuki et al. investigate how the yeast hydrolase transporter, the Vps10 receptor, is recognized and retrogradely transported through the retromer complex. Using extensive mutagenesis and an imaging based sorting assay, they thoroughly dissect the sorting requirements for VPS10 (and Ear1) to conclude that retromer requires a complex, bipartite sequence to retrieve these receptors from the vacuolar pathway.

The mutagenesis and sorting data are clean, very convincing and leave little doubt about the identified sorting signal. Given that recent data on the mammalian retromer and the Vps10p equivalent CI-MPR has caused considerable confusion in the field, the study is also timely and of importance for the field. The report format is appropriate as the authors report a single finding with considerable impact.

Thank you!

The only weakness of the study is the use of a single assay to identify and verify the sorting requirements. All the results are based on mutagenesis of either Vps10 or VPS26 and imaging based analysis of retrograde sorting. Given that the retromer associated SNX-BAR proteins have recently been shown to directly bind CI-MPR (Kvainickas et al., 2017/ Simonetti et al, 2017) it would be great if the authors could use a biochemical approach to verify that the Vps10 tail indeed binds to the core retromer trimer and not to the Vps5/Vps17 subunit. At present, the data do not fully exclude that Vps10 binds to Vps5 or Vps17 as the mutagenesis of VPS26 could also disrupt retromer function in an unspecific way. A binding assay between

purified Vps10 tail and retromer components could then also be used to test whether the sorting motif within Vps10 that was identified through mutagenesis and imaging indeed mediates the binding. At least for the mammalian proteins, the VPS29/VPS35/VPS26 and the SNX-BAR subcomplexes can be individually expressed and purified. In my opinion, it would really strengthen the study if the authors showed some form of binding assay with the wildtype and mutant Vps10 tail with the individual retromer subcomplexes. This could be a GST pulldown with recombinant and purified proteins or maybe even co-IPs from mammalian HEK293 cells if the proteins express poorly in bacteria.

As suggested by the reviewer, we first tried a GST pulldown assay by using recombinant proteins. However, we could not detect a Vps26-Vps10^{tail} interaction (Fig. Rev. 8). In this assay, we used GST-Chs3^{tail} as a control, because the Burd lab successfully detected the Vps26-Chs3 interaction by this vitro binding assay (Cui et al., 2017). However, although we used the same construct (GST-Chs3^{tail} construct from Fromme lab used in Cui et al., 2017), we could not detect the Vps26-Chs3 interaction. Hence, we tried to examine the cargo binding of retromer by in vivo CoIP analysis. We expressed Vps10-GFP and Vps26-FLAG, and performed immunoprecipitation experiments. Vps10-GFP efficiently co-immunoprecipitated Vps26-FLAG (Fig. 2E added to the revised manuscript). However, sorting signal mutants (1428-1433A/1492-1495A double mutants) did not co-precipitate with Vps26-FLAG. Furthermore, cargo binding of retromer was observed in vps5Δ, vps17Δ, or vps29Δ, but it was not detected in vps26Δ or vps35Δ (Fig. 4E and 4F were added in the revised manuscript). These results suggest that the Vps10 sorting signal is required for the association with the retromer complex and that this association requires the CSC (Vps26 and Vps35), not the SNX-BAR dimer (Vps5 and Vps17).

To directly assess whether the SNX-BAR dimer interacts with cargo, we also examined the cargo binding of the SNX-BAR dimer in vps29Δ cells, because in this mutant, the SNX-BAR dimer is formed, but cannot interact with the CSC (Fig. 4B was added in the revised manuscript). In WT cells, SNX-BAR dimer (Vps5-FLAG) was able to interact with cargos (Vps10-GFP) (Fig. S2E and S2F was added in the revised manuscript). However, the Vps5-Vps10 interaction was abolished in vps29Δ cells. This data also strengthen our conclusion that the cargo is recognized by the CSC, not the SNX-BAR dimer.

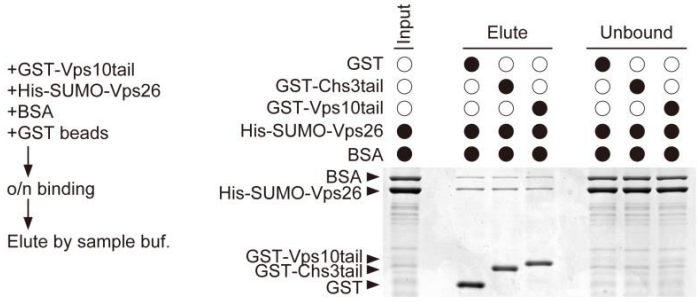

Fig. Rev. 8

Some additional minor points:

In the introduction, the authors state that the Parkinson associated VPS35-D620N mutant causes lysosomal dysfunction. I am not sure whether this has been conclusively shown. The data from the D620N knock-in mouse (Ishizu et al., 2016) strongly suggests that the D620N mutant is an extremely subtle loss of function mutant even in D620N homozygous mice. It is unlikely to cause significant lysosomal dysfunction as it retains most cargo sorting functions.

Thanks for pointing this out. Indeed, Ishizu et al. did not observe any lysosomal dysfunction. However, Follett et al. and McGough et al. observed mislocalization of CatD or M6PR, respectively (Follett et al., 2013; McGough et al., 2014). We revised the manuscript to reflect this (Page 2, paragraph 2, line 13).

At the end of the results section, the authors state that SNX3 is recruited to the endosomal membrane via binding to VPS26. Given that SNX3 has intrinsic lipid binding capabilities through its PX domain and has been shown to mediate retromer recruitment together with RAB7-GTP (Harrison et al., 2014, PNAS), this is really surprising. The authors cite Lucas et al. in this context, but that study also only shows the recruitment of retromer via SNX3, not the other way around?

Thank you for pointing this out. Mammalian SNX3 is recruited to the endosomal membrane in a PI3P dependent manner, allowing retromer recruitment on the endosome (Lucas et al., 2016). However, yeast Snx3 is also recruited to the endosomal membrane in a PI3P dependent manner (Strochlic et al., 2007), but this PI3P binding is not sufficient. The endosomal localization of Snx3 requires at least Vps26 (Fig. Rev. 9A). On the other hand, Vps26 localization to the endosome does not require Snx3 (Fig. Rev. 9B). Thus, yeast Snx3 requires both lipid binding and Vps26 binding for its recruitment to the endosome, but the endosomal localization of retromer does not require Snx3. This conclusion is consistent with the result that the retromer complex can recycle Vps10 even in snx3Δ cells (Fig. Rev 6). Collectively, the yeast retromer complex (Vps5-Vps17-Vps26-Vps29-Vps35 complex) is recruited onto the endosomal membrane in a Snx3 independent manner, whereas Snx3 is recruited onto the endosomal membrane through both Vps26 and PI3P binding.

In the original manuscript, we had examined Snx3-GFP localization to evaluate retromer complex formation in vps26 mutants. However, we have now directly assessed retromer complex formation by CoIP experiments (Fig. 5E was added to the revised manuscript), and have included this in the revised manuscript.

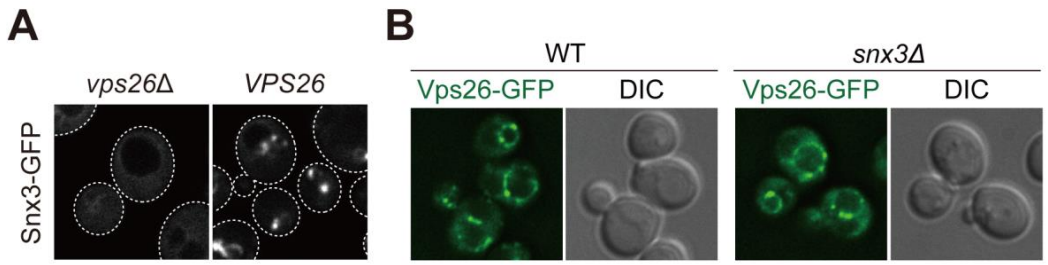

Fig. Rev. 9

June 24, 2019

RE: JCB Manuscript #201901019R

Dr. Scott D Emr
Cornell University
Weill Institute for Cell and Molecular Biology 441 Weill Hall
Ithaca, NY 14853

Dear Scott:

Thank you for submitting your revised manuscript entitled "A bipartite sorting signal ensures specificity of retromer complex in membrane protein recycling". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

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2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.) - in your current manuscript you only say "at least 30 cells were classified and the data from three independent experiments were used for statistical analysis" but do not indicate what statistical tests were used or their parameters. Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts.

5) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

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a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

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9) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

10) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Ira Mellman, Ph.D.
Editor
The Journal of Cell Biology

Tim Spencer, PhD
Deputy Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have responded thoughtfully to the reviewer comments and the manuscript should be accepted for publication. Thanks!

Reviewer #2 (Comments to the Authors (Required)):

The authors have done an excellent job of addressing my comments and those of the other

reviewers. They have included new experiments that greatly strengthen the paper. Although it would have been nice to see more about molecular mechanisms, the authors' conclusions are now backed by solid data. This paper will make a valuable contribution to our understanding of protein sorting in the endocytic pathway.

Reviewer #3 (Comments to the Authors (Required)):

The authors have performed significant additional experimentation which has addressed most of my main concerns. I am therefore supportive of publication of the revised manuscript.