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The P4-ATPase Drs2 interacts with and stabilizes the multisubunit tethering complex TRAPPIII in yeast

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **10th Oct 2022**

Dear Dr. Gallego,

Thank you for the submission of your research manuscript to EMBO reports. Your manuscript has now been evaluated by three expert referees, whose detailed reports are appended below.

As you will see, the referees acknowledge that the study is well designed, and the findings are novel and important. However, they also raise a number of concerns regarding some experiments and the text, and they provide detailed suggestions for the improvement of the study and the manuscript. All of their concerns should be addressed before your manuscript can be published in EMBO reports.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript. If you have any questions or comments, we can also discuss the revisions in a video chat, if you like.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (January 9th). Please discuss with me the revision progress ahead of this time if you require more time to complete the revisions.

*****
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We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section providing access to data deposited in public databases is missing.

2) Your manuscript contains statistics and error bars based on n=2. Please use scatter plots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.
*****

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1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper unless you opt out of this (please see below for further information).

4) a complete author checklist, which you can download from our author guidelines (<https://www.embopress.org/page/journal/14693178/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).
Specifically, we would kindly ask you to provide public access to the following datasets/data:
- Mass spectrometry (proteomic) data

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below (see also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

8) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the new policy (<https://www.embopress.org/competing-interests>) and update your competing interests statement if necessary. Please name this section 'Disclosure and competing interests statement' and place it after the Acknowledgements section.

9) Figure legends and data quantification:
The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter plots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

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- Please also include scale bars in all microscopy images.

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11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>.

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You can use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

In the manuscript "Drs2 regulates TRAPPIII:...", Pazos et al used the PICT assay to detect possible physical interactions between subunits of MTC and their genetic interactors and found that Drs2 (and its family members) interacts with subunits of multiple MTCs on the Golgi. To explore the physiological significance of such mild physical interactions, the authors focused on the Drs2-Trs85 interaction. The conserved I(S/R)TTK motif in that the N-terminus of Drs2 interacts with Drs85 and facilitates Trs85's function in mobilizing Atg9 vesicles. This study provides data to sort the relationship between Golgi MTCs and the formation and expansion of preautophagosomes, a current research topic. While the quality of the data is pretty high and the writing is clear, some issues made the reading little hard.

Rapamycin inhibits TORC1, which elicits a myriad of downstream reactions. Will this generate a pressure and form new protein-protein interaction? If so, there will be a gap between the capture of interaction (under rapamycin) and the physiological assay of Drs2 and Trs85's functions (23C in normal medium). An explanation of this issue will make the article clearer.
The processing of Ape1 was used in several figures. However, different quantitative measures (% mApe1 in Fig. 2a, processing of Ape1, Ape1 processing rescue...) made it hard for readers to compare the different results.
Drs2 is in autoinhibited. PI4P binds the C-terminus of Drs2 and possibly makes the N-terminus open for binding Trs85 or other partners. I am curious why the authors did not mention the possible role of PI4P in the discussion part.

In addition to the above concerns, there are a few minor issues.
1. The CLEM assay detected Ape1-GFP aggregates in 2% of 660 cells, meaning ~13 Ape1-GFP spots. Does 91.4% mean 12/13 of Ape1-GFP spots? If so, just give the total Ape1-GFP spots analyzed in this assay in the result part.

2. Fig. 3a: Rcy1 is for trafficking toward TGN; the others are for trafficking away from TGN. Including 1 or 2 more genes that work toward TGN and also genetically interact with DRS2 (e. g. Vps13, Vps35) will support the claim that "the biogenesis of the CVT vesicle does not involve the pathways where Drs2 has been reported to participate". The authors detected Drs2-Vps13 interaction in this article. I am curious why they did not include vps13delta in Fig. 3a.

Referee #2:

Multisubunit Tethering Complexes (MTCs) are essential for vesicle transport by mediating the tethering of the vesicle to the acceptor membrane. Thus far, eight MTCs have been reported in yeast, each of which has been studied separately and the general mechanisms governing the functions of MTCs have remained poorly understood. In this manuscript, the authors systematically searched for binding proteins for MTCs using both genetic interactions and PICT and found that four MTCs functioning at the Golgi (COG, GARP, TRAPPII and TRAPPIII) bind P4-ATPases, a lipid flippase that mediates the movement of PS from the luminal to the cytosolic leaflet. These MTCs are known to be involved in the transport of Atg9 vesicles that are essential for the Cvt pathway, a prototype of selective autophagy. In order to reveal the general mechanism of MTC-P4-ATPase interaction, the authors studied the interaction between TRAPPIII and Drs2, a P4-ATPase, and found that Drs2 regulates the functions of TRAPPIII in Atg9 transport and the Cvt pathway in a manner independent on previously reported functions of Drs2 including the flippase activity. Moreover, the authors showed that Drs2 is essential for the biogenesis of Atg9 vesicles from the reservoirs through a mechanism that requires the I(S/R)TTK motif identified in the N-terminal tail. Importantly, the authors showed that the I(S/R)TTK motif of Drs2 is important for the interaction with not only TRAPPIII, but also other MTCs such as TRAPPII and GARP. Based on the results, the authors proposed that P4-ATPases have a general role in the regulation of the functions of MTCs.
Overall, the experiments are well designed and the obtained data are convincing, and the manuscript is written well and easy to follow. By using various methods that include genetic interactions, PICT, and XL-MS, the authors succeeded in identifying P4-ATPases as a functionally important binding protein for MTCs. The finding that P4-ATPase functions as a general regulator of MTCs is novel and would contribute to understanding the general mechanisms of MTCs functions important for various intracellular events. Although many questions remain unanswered, which include the mechanisms of P4-ATPase in regulating the function of MTCs and the detailed interaction mode between P4-ATPase and MTCs, this work provides many insights into the functions of MTCs and new roles of P4-ATPases and thus deserves publication at EMBO Reports.

Minor point
1) In Figure 5c, it is written in the legend that "Error bars: mean {plus minus} SD, n = 2". However, it is impossible to calculate SD from n=2 data. Perform experiments at least n=3.

Referee #3:

In this manuscript, Pazos et al. conducted a systematic PICT assay combined with a search of reported genetic interactomes, and successfully found the general relationships among MTCs (GARP, TRAPPII, TRAPPIII, and COG) and P4-ATPases (Drs2, Dnf1, Dnf2, and Dnf3 flippases). The authors focused on Drs2, a membrane-embedded flippase on the TGNs and early endosomes, that directly or indirectly interacts with several MTCs, GARP, TRAPPII, and TRAPPIII. The authors showed that Drs2 is involved in selective autophagy (the CVT pathway) as is the TRAPPIII complex (Trs85). Importantly, its function is not as a flippase, but it may be a new function of Drs2 dependent on the I(S/R)TTK motif in the N-terminal tail, which is also conserved in other P4-ATPases Dnf1 and Dnf2. The findings of this study are conceptually important and shed new light on the functional relationships among the MTCs and P4-ATPases. This manuscript is thought to be suitable for consideration for publication in the EMBO reports. Specific comments are as follows:

Major comments:
(1) It is still unknown why the I(S/R)TTK motif of Drs2 is important for the interaction with TRAPPIII. In this context, the authors focused only on Drs2-TRAPPIII and did not examine Drs2-GARP and Drs2-TRAPPII. Also, the authors did not examine the I(S/R)TTK motif conserved in Dnf1 and Dnf2. Comprehensive PICT assays among MTCs (GARP, TRAPPII, and TRAPPIII) and P4-ATPases (Drs2, Dnf1, and Dnf2) should be examined with I(S/R)TTK-motif mutants of Drs2, Dnf1, and Dnf2, to investigate whether the I(S/R)TTK motif is generally involved in the interaction with MTCs.
(Related to this comment, this reviewer could not understand whether the graphic representation shown in Figure S5 are based on the PICT assay or XL-MS. As mentioned in the minor comment (2), representative fluorescence microscopy data of the PICT assay should be shown.)

(2) It is important to investigate trs85-delta (single deletion) and drs2-delta trs85-delta (double deletion) cells in Figure 6a to clarify the relationship between Drs2 and TRAPPIII.

(3) TRAPPIII is also involved in starvation-induced autophagy. Thus, starvation-induced autophagy should be examined (in Figure 4d).

(4) The western blot shown in Figure 3c appears to have been processed inappropriately.

Minor comments:

(1) Since Drs2 is a membrane protein, it is difficult to understand what membranous structure is co-localized with Trs85/Trs130-FRB in Figure 1c. It is better to investigate the TGN and early endosome markers in the same experimental conditions in Figure 1c. Alternatively, it is better to discuss this issue in the Discussion part.

(2) Representative fluorescence microscopy data of the PICT assay should be shown in Figures 5c and 6c.

(3) There is only one label for two bars in the bar graphs in Figures 6b and 6c.

We thank the reviewers for their positive and constructive comments concerning the manuscript. We have now considered all the comments, which we believe have considerably improved the manuscript.

Following we answer (in blue) all the referee's comments on a point-by-point basis:

Referee #1:

In the manuscript "Drs2 regulates TRAPPIII:...", Pazos et al used the PICT assay to detect possible physical interactions between subunits of MTC and their genetic interactors and found that Drs2 (and its family members) interacts with subunits of multiple MTCs on the Golgi. To explore the physiological significance of such mild physical interactions, the authors focused on the Drs2-Trs85 interaction. The conserved I(S/R)TTK motif in that the N-terminus of Drs2 interacts with Drs85 and facilitates Trs85's function in mobilizing Atg9 vesicles. This study provides data to sort the relationship between Golgi MTCs and the formation and expansion of preautophagosomes, a current research topic. While the quality of the data is pretty high and the writing is clear, some issues made the reading little hard.

Rapamycin inhibits TORC1, which elicits a myriad of downstream reactions. Will this generate a pressure and form new protein-protein interaction? If so, there will be a gap between the capture of interaction (under rapamycin) and the physiological assay of Drs2 and Trs85's functions (23C in normal medium). An explanation of this issue will make the article clearer.

We agree that this is key for interpreting the data and such a relevant point should be more clearly exposed to the reader. All experiments with rapamycin (PICT assays) were done on yeast strains that express mutated *tor1-1.* This mutant form of Tor1 is not inhibited by the rapamycin-FKBP complex. This is well reported in the literature and we had an explanatory sentence in the Material and Methods section, under the "Protein interactions from Imaging Complexes after Translocation (PICT)" caption. We now have added an explanatory sentence in the results section as follows: "In our strains rapamycin has no effect on endogenous TORC1 as this has been mutated to the rapamycin-insensitive form *tor1-1* (see Materials and Methods)."

The processing of Ape1 was used in several figures. However, different quantitative measures (% mApe1 in Fig. 2a, processing of Ape1, Ape1 processing rescue...) made it hard for readers to compare the different results.

We apologize for this heterogeneity in the way we exposed our results. We agree with this referee, and we have modified the Ape1 processing assays to express the measurements as

the % of mApe1 from total Ape1 detected in the WB. Results are normalized to the relevant strain. This is also explained in Material and Methods.

Drs2 is in autoinhibited. PI4P binds the C-terminus of Drs2 and possibly makes the N-terminus open for binding Trs85 or other partners. I am curious why the authors did not mention the possible role of PI4P in the discussion part.

We acknowledge the role of PI4P in regulating Drs2 and we have added this Referee's suggestion, together with other proteins that bind Drs2, in the discussion.

"…In consonance, a former study showed that Drs2 is not required for the processing of Ape1 at the stationary phase (Wang *et al*, 2017 PMID 28627726), confirming that Drs2 function is controlled according to different cellular stimuli. Indeed, Drs2 activity is regulated by multiple interactions. The small GTPase Arl1 binds within the N-terminal unstructured region of Drs2 and serves to stimulate Drs2 flippase activity (Tsai et al 2013 PMID 23345439). Drs2 is autoinhibited by its C-terminal tail and interactions with PI4P and Gea2 in the C-terminal region, along with Arl1 at the N-terminus, serve to displace the autoinhibitory domain and activate Drs2 (Natarajan et al 2004 PMID 15249668, Chantalat et al 2004 PMID 14734650, Zhou et al 2013 PMID 24045945, Timcenko et al 2019 PMID 31243363). Investigating the relevance of these interactions in opening up the I(S/R)TTK motif for binding to Trs85 might shed light on the mechanism that control Drs2 role in the CVT."

In addition to the above concerns, there are a few minor issues.
1. The CLEM assay detected Ape1-GFP aggregates in 2% of 660 cells, meaning ~13 Ape1-GFP spots. Does 91.4% mean 12/13 of Ape1-GFP spots? If so, just give the total Ape1-GFP spots analyzed in this assay in the result part.

We apologize. As pointed by this Referee, the Material and Methods section explainig the CLEM analysis was ambiguous. We first estimated the fraction of cells that presented Ape1-GFP aggregates in our images by analyzing 660 cells (from 5 sections). We could detect Ape1-GFP aggregates in 2% of these cells. However, in total, for the CLEM experiments we analyzed at higher magnification 58 Ape1-GFP aggregates found in 52 of sections. We have modified the relevant text in the Material and Methods section to clarify the protocol used.

2. Fig. 3a: Rcy1 is for trafficking toward TGN; the others are for trafficking away from TGN. Including 1 or 2 more genes that work toward TGN and also genetically interact with DRS2 (e. g. Vps13, Vps35) will support the claim that "the biogenesis of the CVT vesicle does not involve the pathways where Drs2 has been reported to participate". The authors detected Drs2-Vps13 interaction in this article. I am curious why they did not include vps13delta in Fig. 3a.

The reason why we did not include Vps13 deletion in Fig 3A is because we couldn't find any literature where Drs2 was reported to work together Vps13 (or Vps35). Instead, reported negative genetic interactions suggest that Vps13 (and Vps35) controls pathways where Drs2 is not involved. Indeed, it was already shown that Vps35 and Drs2 control different (yet parallel) vesicle transport pathways from endosomes to the Golgi (Best et al 2020, PMID

32074001). Interestingly, Vps13 is known to regulate the P4-ATPase Neo1 (Dalton et al 2017 PMID 28404745), but no evidence was found to control Drs2 (or Dnf1/Dnf2). Thus we do not have an answer on which might be the role of the interaction between Drs2 and Vps13 found in our XL-MS analysis, but in any case, this is not a pathway where Drs2 has already been reported to participate. We agree that it would be very interesting to investigate the potential interplay between these two proteins, but we believe that this will not benefit the present manuscript which is centered to study the interplay between P4-ATPases and MTCs.

Referee #2:

Multisubunit Tethering Complexes (MTCs) are essential for vesicle transport by mediating the tethering of the vesicle to the acceptor membrane. Thus far, eight MTCs have been reported in yeast, each of which has been studied separately and the general mechanisms governing the functions of MTCs have remained poorly understood. In this manuscript, the authors systematically searched for binding proteins for MTCs using both genetic interactions and PICT and found that four MTCs functioning at the Golgi (COG, GARP, TRAPPII and TRAPPIII) bind P4-ATPases, a lipid flippase that mediates the movement of PS from the luminal to the cytosolic leaflet. These MTCs are known to be involved in the transport of Atg9 vesicles that are essential for the Cvt pathway, a prototype of selective autophagy. In order to reveal the general mechanism of MTC-P4-ATPase interaction, the authors studied the interaction between TRAPPIII and Drs2, a P4-ATPase, and found that Drs2 regulates the functions of TRAPPIII in Atg9 transport and the Cvt pathway in a manner independent on previously reported functions of Drs2 including the flippase activity. Moreover, the authors showed that Drs2 is essential for the biogenesis of Atg9 vesicles from the reservoirs through a mechanism that requires the I(S/R)TTK motif identified in the N-terminal tail. Importantly, the authors showed that the I(S/R)TTK motif of Drs2 is important for the interaction with not only TRAPPIII, but also other MTCs such as TRAPPII and GARP. Based on the results, the authors proposed that P4-ATPases have a general role in the regulation of the functions of MTCs.
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Minor point
1) In Figure 5c, it is written in the legend that "Error bars: mean {plus minus} SD, n = 2". However, it is impossible to calculate SD from n=2 data. Perform experiments at least n=3.

We apologize for this. We have repeated this experiment in three independent biological

replicates and updated Fig 5C and corresponding figure legend.

Referee #3:

In this manuscript, Pazos et al. conducted a systematic PICT assay combined with a search of reported genetic interactomes, and successfully found the general relationships among MTCs (GARP, TRAPPII, TRAPPIII, and COG) and P4-ATPases (Drs2, Dnf1, Dnf2, and Dnf3 flippases). The authors focused on Drs2, a membrane-embedded flippase on the TGNs and early endosomes, that directly or indirectly interacts with several MTCs, GARP, TRAPPII, and TRAPPIII. The authors showed that Drs2 is involved in selective autophagy (the CVT pathway) as is the TRAPPIII complex (Trs85). Importantly, its function is not as a flippase, but it may be a new function of Drs2 dependent on the I(S/R)TTK motif in the N-terminal tail, which is also conserved in other P4-ATPases Dnf1 and Dnf2. The findings of this study are conceptually important and shed new light on the functional relationships among the MTCs and P4-ATPases. This manuscript is thought to be suitable for consideration for publication in the EMBO reports. Specific comments are as follows:

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We agree with this Referee that it is fundamental to tackle the general relevance of the I(S/R)TTK motif. As suggested by this Referee, we embarked on a systematic PICT assay to test if the P4-ATPases I(S/R)TTK motif is generally involved in the interaction with MTCs. The experiments were designed to complement the PICT assay and the XL-MS experiment where we already confirmed the role of Drs2 I(S/R)TTK motif in binding GARP, TRAPPII and TRAPPIII (Fig 5B, 5C and Fig EV 4B).

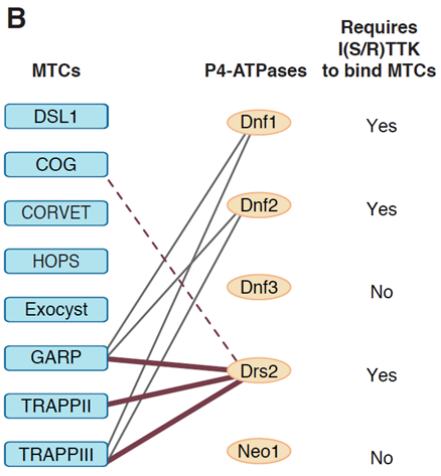
For coherence with former Drs2 experiments (both PICT and XL-MS), the assay was initially conceived using *Δdrs2* cells transformed with centromeric plasmids that express GFP fusions

of Drs2 (wild-type and mutant) under the DRS2 promoter. COG was excluded because XL-MS indicated that its binding is likely to be indirect. The "PICT+ centromeric plasmid" approach successfully confirmed the XL-MS data showing the essential role of the Drs2 I(S/R)TTK motif to bind TRAPPII and TRAPPIII. However, this approach did not recapitulate the interaction between Drs2-GFP and GARP that we had seen by XL-MS (Fig 5B) and PICT on cells expressing endogenous Drs2-GFP (Fig 1D). This is likely to reflect the sensitivity limit of the assay when using heterologous expression of Drs2-GFP. As the contribution of the I(S/R)TTK motif in the interaction between Drs2 and GARP had already been proved by a more stringent XL-MS (a technique that informs about binding and proximity), we think that using the "PICT+centromeric plasmid" approach to test Drs2-GARP interaction is not necessary for the conclusions of this manuscript.

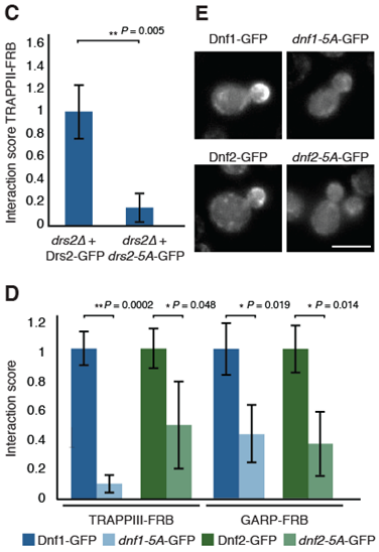
We also tried the "PICT+ centromeric plasmid" approach to assess the role of the I(S/R)TTK motif in the interaction between Dnf1/2 and MTCs. Unfortunately, a few weeks ago, the company Genscript informed us of an issue while cloning the constructs that delayed the experiment significantly. As alternative, we used CRISPR/Cas9 to generate cells that express GFP fusions of Dnf1/2 with their I(S/R)TTK motif mutated to five alanines in otherwise the exact same strains used to test the network of interactions between their wild-type counterparts and MTCs (Fig 1D). Gene editing rules-out possible artifacts arising from heterologous expression. Using PICT+CRISPR/Cas9, we measured the impact that the 5A mutation has on the interaction between Dnf1/2 and MTCs (GARP and TRAPPIII, the two MTCs bound by Dnf1 and Dnf2). The interaction of *dnf1-5A*-GFP and *dnf2-5A*-GFP with MTCs decreased 50% to 90% when compared to the binding of wild-type P4-ATPases. Interestingly, both *dnf1-5A*-GFP and *dnf2-5A*-GFP present a defect in polarization as expected for the lack of binding to GARP (Shirahama-Noda et al, 2013 PMID 22177957).

All these results are clearly explained in the results section under the caption "Drs2 I(S/R)TTK mediates proximal interactions with MTCs".

These results are summarized now on Fig 4B



And shown in detail in Fig EV 4



(Related to this comment, this reviewer could not understand whether the graphic representation shown in Figure S5 are based on the PICT assay or XL-MS. As mentioned in the minor comment (2), representative fluorescence microscopy data of the PICT assay should be shown.)

We apologize and we acknowledge the difficulty to understand former Fig S5. We have corrected the corresponding figure legend (now Fig EV 4B) as follows to specify that this is data derived from XL-MS analysis:

"B Graphic representation of the 26 proteins whose interaction with Drs2 is diminished by the mutation of the I(S/R)TTK motif in XL-MS experiments. Binding to Atg9 as reported in SGD (Data ref: Saccharomyces Genome Database, 2021). BFDR, Bayesian False Discovery Rate."

(2) It is important to investigate trs85-delta (single deletion) and drs2-delta trs85-delta (double deletion) cells in Figure 6a to clarify the relationship between Drs2 and TRAPPIII.

We agree with this Referee and we tried to generate these strains with no success. Drs2 and Trs85 have a negative genetic interaction that is likely to prevent the construction of viable double mutant.

(3) TRAPPIII is also involved in starvation-induced autophagy. Thus, starvation-induced autophagy should be examined (in Figure 4d).

We agreed with the Referee and have performed the suggested experiment. Under starvation conditions Trs85 is dispensable for the delivery of Atg9 to the autophagosome

and for the subsequent processing of Ape1, because the pathway controlled by the retromer and GARP can bypass the loss of TRAPPIII (Shirahama-Noda et al, 2013 PMID 23986483). Accordingly, starving cells lacking Trs85 show a small 22% decrease in Ape1 maturation, similar to that of cells lacking Drs2 (27%). This observation further supports a coordinated role between Trs85 and Drs2. We now show these results in Fig EV 2E .

(4) The western blot shown in Figure 3c appears to have been processed inappropriately.

We apologize for this and we would like to thank the Referee to spot such a mistake. We have fixed the figure 3C accordingly.

Minor comments:

(1) Since Drs2 is a membrane protein, it is difficult to understand what membranous structure is co-localized with Trs85/Trs130-FRB in Figure 1c. It is better to investigate the TGN and early endosome markers in the same experimental conditions in Figure 1c. Alternatively, it is better to discuss this issue in the Discussion part.

We agree with this Referee, we have not considered enough the location of the Drs2 that interacts with Trs85. Unfortunately, the PICT experiments cannot resolve this question as anchoring of Trs85-FRB might accumulate a variety of binding proteins, likely from different compartments. Thus, the recruitment of a marker could not be unambiguously assigned to Drs2. We have added the following text to the discussion section:

"…TRAPPIII is a MTC that controls the transport of Atg9 between reservoirs at the endocytic structures and the Golgi. TRAPPIII is essential so that a small fraction of Atg9 can eventually be delivered to the PAS for the formation of the CVT vesicle. Drs2 is a lipid flippase that circulates between early endosomes and the TGN. As we could not label Drs2 from different compartments, the PICT technique did not allow us to resolve which subpopulation of Drs2 interacts with TRAPPIII. Nevertheless, given the role of TRAPPIII in transporting Atg9 from endosomes to Golgi (Shirahama-Noda et al, 2013 PMID 23986483), it is plausible that Drs2 and TRAPPIII interact on endosomal structures that serve as Atg9 reservoir."

(2) Representative fluorescence microscopy data of the PICT assay should be shown in Figures 5c and 6c.

We agree with this Referee and we have added illustrative images for these experiments.

(3) There is only one label for two bars in the bar graphs in Figures 6b and 6c.

We apologize for this mistake. We have now fixed this in the corresponding figures.

Dear Oriol,

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of comments from the three referees that were asked to re-evaluate your study. All referees find that their concerns were successfully addressed, and they now recommend publication. There are only 2 typos that referee #1 identified (please see the comments appended at the end of this email), which should be corrected in the final version of the manuscript.

From the editorial side, there are also a few things that we need from you before we can proceed with the acceptance of your manuscript:

- Please note that all deposited data (mass spectrometry/proteomic data) must be publicly available at the time of publication.

- The author contributions statement should be removed from the manuscript file. Instead, we now use CRediT to specify the contributions of each author in the journal submission system. Please use the free text box to provide more detailed descriptions. See also guide to authors:
<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>.

- The top section of the author checklist (with author and manuscript information) should be completed.

- Figure callouts for Fig. EV2E are missing, please make sure that all figure panels are called out in your revised manuscript.

- Tables EV1 to EV4 should be renamed Dataset EV1 to EV4. Please make sure that all callouts are revised accordingly.

- Table EV5 should be renamed Table EV1. Please make sure that all callouts are revised accordingly.

- The names in the movie files need correcting to "Movie EV#". A single ZIP file should be submitted for each movie, containing the movie file and a separate plain text README file with the movie legend.

- We request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator has contacted you to discuss which figure panels we need source data for and has provided you with helpful tips on how to organize and upload the files. We noted that you have not uploaded your source data yet. Please do so while submitting your revised manuscript. For your convenience, I will send you the original message of our source data curator again.

- Your Figure legends have been inspected by our data editors for completeness and accuracy. Please see the required changes in the attached Word file and address all comments in your revised manuscript (with tracked changes).

- I took the liberty to suggest a few improvements in the title, the abstract, the short summary and the bullet points (below). Please review them and let me know if you agree with the changes, or feel free to improve them further (in your revised manuscript). Please note that the title should be short and as informative and accurate as possible, while the abstract should be a single paragraph describing all key novel findings of the study, written in present tense, and it should not exceed 175 words.

Title:
The P4-ATPase Drs2 interacts with and stabilizes the multisubunit tethering complex TRAPPIII in yeast

Abstract:
Multisubunit Tethering Complexes (MTCs) are a set of conserved protein complexes that tether vesicles at the acceptor membrane. Interactions with other components of the trafficking machinery regulate MTCs through mechanisms that are partially understood. Here we systematically investigate the interactome that regulates MTCs. We report that P4-ATPases, a family of lipid flippases, interact with MTCs that participate in the anterograde and retrograde transport at the Golgi, such as TRAPPIII. We use the P4-ATPase Drs2 as a paradigm to investigate the mechanism and biological relevance of this interplay during transport of Atg9 vesicles. Binding of Trs85, the sole specific subunit of TRAPPIII, to the N-terminal tail of Drs2 stabilizes TRAPPIII on membranes loaded with Atg9 and is required for Atg9 delivery during selective autophagy, a role that is independent of P4-ATPase canonical functions. This mechanism requires a conserved I(S/R)TTK motif that also mediates the interaction of the P4-ATPases Dnf1 and Dnf2 with MTCs, suggesting a broader role of P4-ATPases in MTC regulation.

Short summary:
Binding of Trs85 to the I(S/R)TTK motif in the N-terminal tail of Drs2 stabilizes TRAPPIII on membranes loaded with Atg9. This is required for Atg9 delivery to the pre-autophagosomal structure during Cytoplasm-to-vacuole targeting.

Bullet points:
- P4-ATPases, a family of lipid flippases, interact with Multisubunit Tethering Complexes (MTCs) in yeast.

- Binding of MTC subunits to P4-ATPases requires the N-terminal I(S/R)TTK motif of the flippases.
- The P4-ATPase Drs2 stabilizes TRAPPIII on Atg9-loaded compartments to facilitate transport of Atg9 vesicles.
- The regulatory role of Drs2 is critical for Cytoplasm-to-vacuole targeting at low temperatures and is independent of reported functions of this lipid flippase.

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We would also welcome the submission of cover suggestions or motifs to be used by our Graphics Illustrator in designing a cover.

We look forward to seeing a final version of your manuscript as soon as possible.

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

The revised edition addressed all my concerns.

During my reading, I caught 2 typos:
1. People use 'Cvt' instead of 'CVT'
2. -2 line of abs: Dfn1 should be Dnf1

Referee #2:

The authors have addressed my concern.

Referee #3:

I have reviewed the revised manuscript and the point-by-point response to my previous comments. All my concerns have been appropriately addressed and the added data strengthens the authors' new findings showing general relationships between P4-ATPases and MTCs. I recommend publication of this work in EMBO Reports.

We thank the referees for their positive and constructive comments concerning the manuscript. We have now considered all the comments.

Following we answer (in blue) all the referee's comments on a point-by-point basis:

Referee #1:

The revised edition addressed all my concerns.

During my reading, I caught 2 typos:
1. People use 'Cvt' instead of 'CVT'
2. -2 line of abs: Dfn1 should be Dnf1

Thank you very much for the correction, we have fixed both typos. We appreciate all the constructive comments given.

Referee #2:

The authors have addressed my concern.

Thank you very much for the positive comment and all the feedback given.

Referee #3:

I have reviewed the revised manuscript and the point-by-point response to my previous comments. All my concerns have been appropriately addressed and the added data strengthens the authors' new findings showing general relationships between P4-ATPases and MTCs. I recommend publication of this work in EMBO Reports.

Thank you very much for the positive comment and all the feedback given.

Oriol Gallego
UPF
Department of Experimental Science and Health
Doctor Aiguader 88
Barcelona, Barcelona 08003
Spain

Dear Oriol,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

****

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- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
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- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

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- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
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- a statement of how many times the experiment shown was independently replicated in the laboratory.
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  - definition of error bars as s.d. or s.e.m.

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