Exploring the ATG9A interactome uncovers interaction with VPS13A

Alexander R. van Vliet, Harold B.J. Jefferies, Peter A. Faull, Jessica Chadwick, Fairouz Ibrahim, Mark J. Skehel and Sharon A. Tooze
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Original submission

First decision letter

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AUTHORS: Alexander R. van Vliet, Harold B.J. Jefferies, Peter A. Faull, Fairouz Ibrahim, Mark J. Skehel, and Sharon A. Tooze
ARTICLE TYPE: Short Report

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. in particular, reviewer 2 feels the paper is too preliminary and has suggested ways you could expand on your observations to make the study suitable for publication in JCS. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

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

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

Reviewer 1

Advance summary and potential significance to field

The authors of this study applied a mass-spec approach to identify new interactors of ATG9A, a membrane scrambles that is involved in macroauthophagy. They immunoprecipitated endogenous ATG9A in the presence of two detergents to optimize the detection of specific interactors and identified many proteins involved in lipid homeostasis and transport. ACSL3, VPS13A and VPS13C were among the most significant hits and used to validate the specificity of the approach by co-immunoprecipitation and western blotting. Interestingly, VPS13A/C belong to the same class of lipid channels as ATG2A. The latter cooperates with ATG9A to promote phagophore expansion, while VPS13 is present at various organelle contact sites, including autophagosomes. The function of VPS13 in autophagy remains, however, enigmatic. To characterize the interaction of VPS13A and ATG9A in more detail, four VPS13A fragments comprising different domains of VPS13A, were tested for ATG9A binding. In contrast to ATG2A, which binds ATG9A by its C-terminal domain, the N-terminal Chorein domain and the C-terminal fragment of VPS13A interact with ATG9A. VPS13A is known to be recruited to lipid droplets (LDs) in cells fed with oleic acid. The same was true for its C-terminal fragment. Moreover, ATG9A was recruited to LDs in an VPS13A dependent manner and CLEM experiments showed that LDs were surrounded by vesicles, suggesting that ATG9 vesicles are recruited to LDs by VPS13A. The authors characterized the interaction of the C-terminal fragment of VPS13A with ATG9A in more detail and found that this fragment interacts with the C-terminus of ATG9A.

This study is relevant not only for the autophagy field but also for the broader membrane trafficking community as it discovered new interaction partners of ATG9A. This is an important step to reveal nonautophagic functions of ATG9. The observation that VPS13A binds ATG9 is intriguing because the canonical autophagy interaction partner of ATG9, ATG2, belongs to the same class of lipid channels as VPS13, suggesting that ATG9 couples lipid transfer between membranes with lipid scrambling to equilibrate the lipid composition of both leaflets not only in autophagy, but maybe in other transport processes in cells. While most experiments are convincing and of high quality, the authors should address some concerns before the manuscript can be published. Notably, the CLEM data are not very convincing and need to be repeated. Moreover, the biological relevance of the ATG9-VPS13A interaction should be characterized in more detail by e.g. colocalization studies of full-length proteins and fragments.

Comments for the author

1) Mass spec data were validated by co-IP of ATG9A by RFP-VPS13A, Clover-VPS13C and endogenous ACSL3. Why did the author use different tags for VPS13A and VPS13C? Given, that the interaction of VPS13C with ATG9A appears to be extreamly weak, it might have been better to use the same approach for both isoforms.

2) The authors focused on VPS13A because it was enriched in Triton and LMNG mass-spec datasets. Another argument to focus on VPS13A might have been the apparently stronger interaction of VPS13A and ATG9A in co-IPs. The authors should also consider this as an argument.

3) In co-IP experiments of Flag-ATG9A with GFP-VPS13A fragments, the expression level of the N-terminal fragment 1 is very low, which might be caused by instability or folding defects of that fragment, while fragments 2-4 are strongly expressed. Given that the interaction of fragment 2 with ATG9A is neglectable, the authors could test whether a construct comprising fragments 1 and 2 of VPS13A is more stable to demonstrate binding of the N-terminus of VPS13A to ATG9A. Moreover, the IPs of fragments 2 and 3 were much more efficient than that of fragment 4, but fragment 4 showed strongest binding to ATG9A. Why was the pull-down of fragment 4 so inefficient? It is also not clear how the relative amounts detected in western blots of input and pulldown are related to each other, was the pull-down fraction more concentrated? This would explain why fragment 4 pulls down so much ATG9A.

4) To correlate the pull-down efficiency of the VSP13A fragments with that of VPS13A full-length, the latter should be included in the experiment and a blot containing FL and fragments should be presented and the interaction with ATG9A quantified.

5) Why did the authors only analyze colocalization of the C-terminal fragment 4 with ATG9A in cells? Even if the N-terminus of VPS13A is not recruited to LDs, it should be present at other organelles allowing to study its colocalization with ATG9A. A mainly cytoplasmic localization of

fragment 1 would indicate that the fragment is instable or misfolded and in this case a construct comprising fragments 1 and 2 should be tested (as suggested for co-IPs, see point 3).

6) The authors detected vesicles surrounding LDs in electron micrographs from CLEM experiments, but the presented images are not very clear/convincing. The ATG9 fluorescence is very diffuse and the colocalization with LDs might just reflect cytoplasmic localization of ATG9 in the crowed LD cluster. The correlation of ATG9 fluorescence with vesicles and its specific recruitment to LD should be shown by better CLEM images. Moreover, mitochondria appear to be damaged (characteristic white inclusions), indicating that the cells are very sick. This might be a consequence of feeding with oleic acid. Given that VSP13A is found on ER-mitochondria contact sites, the authors should study colocalization of both proteins under physiologic conditions. This will be relevant because both, mitochondria and ER are in close vicinity to phagophores.

7) Why does ATG9 FL does not pull down VPS13A fragment 4 in Fig 3B? The ATG9 FL pull-down in Fig. 3C is very inefficient and the experiment should be repeated. The low expression of ATG9 delta C indicates folding or stability problems and this should be critically discussed as a limitation of the study. Given the low expression of the fragment and its extremely low abundance, no conclusions can be drawn regarding its biding to VPS13A.

8) Previous studies (PMID: 30993752) revealed that a chimera in which the N-terminus of ATG2A was replaced by that of VPS13A complements the autophagy defect in ATG2A KO cells. This suggests that the N-termini of VPS13A and ATG2A have similar functions. The authors could perform similar experiments by generating ATG2 chimera that contain the C-terminus of VSP13A to reveal more insights into the functional conservation of the C-terminal domain.

Reviewer 2

Advance summary and potential significance to field

The manuscript by van Vliet et al. describes a novel interaction of ATG9A with the lipid transfer protein VPS13A. The interaction was detected in a mass spectrometry analysis of novel binding proteins for ATG9A. In agreement, VPS13A can recruit ATG9A positive structures, possibly vesicles, to lipid droplets, where VPS13A localizes. The identified interaction of both proteins is distinct from the previously described interaction of ATG9A with ATG2A.

Comments for the author

This is an overall interesting, yet preliminary manuscript. While the interaction of VPS13A with ATG9A is certainly exciting, the paper does not demonstrate its functional relevance in any follow up assays. Neither interfaces between the proteins are mapped, nor distinct mutants are shown, nor do the authors analyze the role of the complex of VPS13A and ATG9A in – as it seems here - lipid droplet biogenesis or lipid transfer. The authors provide a very attractive starting point of a study, but the advances are in my view not yet sufficient for publication.

First revision

Author response to reviewers' comments

Reviewer 1 Comments for the Author:

1) Mass spec data were validated by co-IP of ATG9A by RFP-VPS13A, Clover-VPS13C and endogenous ACSL3. Why did the author use different tags for VPS13A and VPS13C? Given, that the interaction of VPS13C with ATG9A appears to be extremely weak, it might have been better to use the same approach for both isoforms.

The reviewer makes a good point. The current approach was done because cloning VPS13 constructs proved challenging. We tried to replace these tags with other fluorescent proteins, but the very low success rate and time-consuming nature to troubleshoot made us opt to keep the original tags, which were obtained from addgene (plasmid # #118758 and #118760).

2)The authors focused on VPS13A because it was enriched in Triton and LMNG mass-spec datasets. Another argument to focus on VPS13A might have been the apparently stronger interaction of VPS13A and ATG9A in co-IPs. The authors should also consider this as an argument.

This is a very good point, and this line of reasoning has been added to the text on page 4, line 25-29.

3)In co-IP experiments of Flag-ATG9A with GFP-VPS13A fragments, the expression level of the N-terminal fragment 1 is very low, which might be caused by instability or folding defects of that fragment, while fragments 2-4 are strongly expressed. Given that the interaction of fragment 2 with ATG9A is neglectable, the authors could test whether a construct comprising fragments 1 and 2 of VPS13A is more stable to demonstrate binding of the N-terminus of VPS13A to ATG9A.

We thank the reviewer for this creative suggestion. We took up the suggestion and cloned a construct encompassing fragment 1 and fragment 2 of VPS13A. However, this construct did not improve expression levels compared either to fragment 1 alone or relative to fragment 2, and we therefore did not continue with it. An example western blot is shown below where we compare both mCherry tagged fragment 1+2 (left) or GFP tagged fragment 1+2 (right) to the respectively tagged fragment 1 or 2.

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

Moreover, the IPs of fragments 2 and 3 were much more efficient than that of fragment 4, but fragment 4 showed strongest binding to ATG9A. Why was the pull-down of fragment 4 so inefficient? It is also not clear how the relative amounts detected in western blots of input and pulldown are related to each other, was the pull-down fraction more concentrated? This would explain why fragment 4 pulls down so much ATG9A.

In order to address this point, we have adjusted the input signals from this blot to show the same exposure as the IP signals. To avoid confusion, we have done the same for the new western blots in the paper as well. We think this illustrates that the IP for all fragments of VPS13A was efficient. We recognize that the pull-down of fragments 2 and 3 may be more efficient than for fragment 4, but for this reason we normalize the ATG9A signal to the IP'd VPS13A fragment signal, to control for differences in IP efficiency. In addition, in order to clarify further the relation between input and IP we state for each IP blot how much % of lysate was loaded as an input in the figure legends.

4)To correlate the pull-down efficiency of the VSP13A fragments with that of VPS13A full-length, the latter should be included in the experiment and a blot containing FL and fragments should be presented and the interaction with ATG9A quantified.

We appreciate this comment and performed the experiment as suggested by the reviewer. However, we struggled with the discrepancy between the low expression levels of full length VPS13A and the high expression levels of the VPS13A fragments. The IP we show of full length VPS13A in figure 1 was performed using LMNG as a detergent, which stabilized the interaction. The fragment IP's in figure 2 were done with Triton X-100 which in the case of the fragment IPs gave a more consistent result. IP with full length VPS13A and ATG9A using Triton X-100 is relatively inefficient, exacerbating the problem. We have thus chosen to maintain the current figure panel. Our aim with the pull down of the four fragments was to identify which fragment exhibited the most ATG9A binding. Our interpretation of this blot is therefore not reliant on the presence of the full-length VPS13A, as we are not making any claims regarding its relative efficiency compared to the full length. We hope that this explanation is acceptable to the reviewer.

5)Why did the authors only analyze colocalization of the C-terminal fragment 4 with ATG9A in cells? Even if the N-terminus of VPS13A is not recruited to LDs, it should be present at other organelles allowing to study its colocalization with ATG9A. A mainly cytoplasmic localization of fragment 1

would indicate that the fragment is instable or misfolded and in this case a construct comprising fragments 1 and 2 should be tested (as suggested for co-IPs, see point 3).

As has been reported before (Kumar et al. among others), VPS13A is already known to bind to the ER through its N-terminus using a FFAT motif to bind to VAPB. In addition to this, the C-terminus of VPS13A shows high homology with the C-terminus of ATG2A, which is the main binding site for ATG9A in ATG2A. Having said that, we did initially try to repeat the same assay for fragment 1 of VPS13A, but expression of this construct was too low to allow meaningful interpretation using fluorescence microscopy. In addition, we wanted to mainly focus on the C-terminal fragment of VPS13A.
As stated above, the expression of a construct comprising fragment 1 and 2 did not improve expression unfortunately and so was not used going forward.

6)The authors detected vesicles surrounding LDs in electron micrographs from CLEM experiments, but the presented images are not very clear/convincing. The ATG9 fluorescence is very diffuse and the colocalization with LDs might just reflect cytoplasmic localization of ATG9 in the crowed LD cluster.

As stated by the reviewer, there is diffuse cytosolic ATG9A fluorescence in these cells. However, our quantification analysed average fluorescence intensity at the site of lipid droplets versus total cell and this shows a significant increase when expressing VPS13A fragment 4. This shows that the cytosolic pool of ATG9A vesicles is recruited to lipid droplets more when expressing VPS13A fragment 4 than in control.

The correlation of ATG9 fluorescence with vesicles and its specific recruitment to LD should be shown by better CLEM images. Moreover, mitochondria appear to be damaged (characteristic white inclusions), indicating that the cells are very sick. This might be a consequence of feeding with oleic acid. Given that VSP13A is found on ER-mitochondria contact sites, the authors should study colocalization of both proteins under physiologic conditions. This will be relevant because both, mitochondria and ER are in close vicinity to phagophores.

We thank the reviewer for this important comment and spent a significant amount of time addressing this point. We redid the entire EM experiment and tried to optimize fixation and embedding. We hope the reviewer can appreciate that cellular structures and organelles like mitochondria look better preserved. We have also further optimized the CLEM correlation by using the EC_Clem plugin for the ICY software in order to overlay the fluorescence signal better with vesicular and tubular membranes making contact with the limiting membrane of the lipid droplets. These new panels are in Figure 3A.

7)a) Why does ATG9 FL does not pull down VPS13A fragment 4 in Fig 3B?
b) The ATG9 FL pull-down in Fig. 3C is very inefficient and the experiment should be repeated. c) The low expression of ATG9 delta C indicates folding or stability problems and this should be critically discussed as a limitation of the study. Given the low expression of the fragment and its extremely low abundance, no conclusions can be drawn regarding its biding to VPS13A.

These are very good points and we have addressed these comments together. After reading these comments we optimized transfection levels again from scratch and tried to ensure pull down levels between constructs was similar. We redid all these experiments with the new parameters. The new blots are now in figure 1F and supplemental figure 1B. Expression levels of all constructs are now more equal and more importantly, the pull down of the different constructs is now similar. We rearranged figure 2 and 3, and because of the order change, the original blot in figure 2B no longer fitted with the narrative and so we moved this to a new figure S1.

8)Previous studies (PMID: 30993752) revealed that a chimera in which the N-terminus of ATG2A was replaced by that of VPS13A complements the autophagy defect in ATG2A KO cells. This suggests that the N-termini of VPS13A and ATG2A have similar functions. The authors could perform similar experiments by generating ATG2 chimera that contain the C-terminus of VSP13A to reveal more insights into the functional conservation of the C-terminal domain.

We thank the reviewer for this excellent comment. We have done as suggested and replaced the entire C-terminus of ATG2A (CLR + ATG_C) domain with the C-terminus of VPS13A (ATG_C and PH domains included). We then made new stable cells expressing the ATG2A-VPS13A chimera construct in an ATG2AB DKO background. To our surprise, this construct was not able to rescue autophagic flux. We confirmed that the construct retained ATG9A binding (Figure 3B-D). We have written about these experiments on page 6, lines 20-32, and discuss the new data on page 7, lines 1-9. We are very excited by these results and hypothesize that they could inform us on why VPS13A is not essential for autophagy and could hint at its role in autophagosome formation, possibly as a tether. We do realize that there is a chance that lipid transfer could be affected by this change, and we discuss this possibility in the limitations of the study.

Reviewer 2 Comments for the Author:
This is an overall interesting, yet preliminary manuscript. While the interaction of VPS13A with ATG9A is certainly exciting, the paper does not demonstrate its functional relevance in any follow up assays. Neither interfaces between the proteins are mapped, nor distinct mutants are shown, nor do the authors analyze the role of the complex of VPS13A and ATG9A in – as it seems here - lipid droplet biogenesis or lipid transfer. The authors provide a very attractive starting point of a study, but the advances are in my view not yet sufficient for publication.

We thank the reviewer for their enthusiasm about our discovery. We recognize their criticisms but feel that experiments to address the functional relevance would require substantial additional work and would take this study beyond the scope of a short report. In addition, the paper reports more than just an interaction between ATG9A-VPS13A, as we also provide a comparative interactomics analysis of endogenous ATG9A, which contains many new and interesting hits and can be used as a tool for the community. A table with all hits is included in the manuscript.
Having said this, we have tried to address some of the reviewer's comments. We have added additional data where we describe the putative ATG9A binding site in the C-terminus of VPS13A (Figure 2C, D, discussed in text on page 5, lines 21-32, and on page 7, lines 1-2). In addition to this, we investigate whether the C-termini of ATG2A and VPS13A could have similar functions by making an ATG2A-VPS13A chimera where we replace the CLR and ATG_C domains of ATG2A with the ATG_C and PH domains of VPS13A (Figure 3B-D). To our surprise, the Chimera is unable to rescue autophagic flux, indicating unique properties of the ATG2A C-terminal domain. We hope these additional experiments and insights will go some way as to address the reviewer's point.

Second decision letter

MS ID#: JOCES/2023/261081

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AUTHORS: Alexander R. van Vliet, Harold B.J. Jefferies, Peter A. Faull, Jessica Chadwick, Fairouz Ibrahim, Mark J. Skehel, and Sharon A. Tooze
ARTICLE TYPE: Short Report

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As you will see, the reviewers gave favourable reports but reviewer #2 has asked for some minor improvements to the font sizes, layout and description of some of the figures. Please make these changes and then return your paper to us.

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

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box.

Reviewer 1

Advance summary and potential significance to field

This study identified Vps13A as a new interactor of ATG9, revealed the binding sites and reported that the interaction leads to the recruitment of ATG9 vesicle to lipid droplets. Although some elements of the study and the biological function of the identified interaction are still preliminary, the study will promote the discovery of new functions of ATG9 in lipid droplet biology and autophagy.

Comments for the author

The authors addressed all my concerns by performing additional experiments or by explaining the reason why this was not possible. Notably, the authors repeated many experiments to improve data quality. I recommend publication of the manuscript in its present form.

Reviewer 2

Advance summary and potential significance to field

The authors provide evidence that Atg9 interacts with Vps13, another lipid transfer protein involved in interorganellar lipid flux. Using mass spectrometry and pull-down analyses, they confirm that the interaction is direct.

Comments for the author

The authors addressed some of my points by now providing in addition a chimera of Atg2A with Vps13, which restores the interaction, yet does not functionally complement Atg2A. This is a start, yet also casts a little doubt on the meaning of the observed interaction. However, given that the Vps13 function in Autophagy has been suggested elsewhere, it is worth reporting the overall finding.

I have some general suggestions. The figures in the manuscript are not very homogeneous regarding the use of fonts, which differ a lot in sizes and are thus very difficult to read. This applies in particular to Figure 1A and B, where the identified proteins are poorly readable. Also the labeling of the axes is very tiny in many figures, and the font differs between subfigures (e.g. Figure 1E, bottom). In Figure 3, the chimera is difficult to understand from the text vs. the 3D representation. A schematic representation of their construct in addition to the model would be helpful.

Second revision

Author response to reviewers' comments

Response to Reviewers
Reviewer 1 Advance Summary and Potential Significance to Field:
This study identified Vps13A as a new interactor of ATG9, revealed the binding sites and reported that the interaction leads to the recruitment of ATG9 vesicle to lipid droplets. Although some elements of the study and the biological function of the identified interaction are still preliminary, the study will promote the discovery of new functions of ATG9 in lipid droplet biology and autophagy.

Reviewer 1 Comments for the Author:

The authors addressed all my concerns by performing additional experiments or by explaining the reason why this was not possible. Notably, the authors repeated many experiments to improve data quality. I recommend publication of the manuscript in its present form.

Our Response: We would like to thank the reviewer again for their helpful suggestions, and are very happy that the reviewer appreciates our revised manuscript.

*****

Reviewer 2 Comments for the Author:
The authors addressed some of my points by now providing in addition a chimera of Atg2A with Vps13, which restores the interaction, yet does not functionally complement Atg2A. This is a start, yet also casts a little doubt on the meaning of the observed interaction. However, given that the Vps13 function in Autophagy has been suggested elsewhere, it is worth reporting the overall finding.

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Our Response: We appreciate the reviewer taking the time to go over the figures in detail and making suggestions on how to improve them. We have taken these comments to heart and have tried to address all of them. Across all figures we have harmonized the font + increased the text size, especially for the graphs and the protein annotations. We have added a new scheme in Figure 3B in addition to an additional explanation in the main text (line 6 lines 22-27) and in the figure legend (page 19 lines 31-33 and page 20 line 1-4).
In addition to these improvements, we have adopted the JCS guidelines for formatting of bar graphs and adapted the graphs in Figure 2B and D, and Figure 3A and D.

Third decision letter

MS ID#: JOCES/2023/261081

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AUTHORS: Alexander R. van Vliet, Harold B.J. Jefferies, Peter A. Faull, Jessica Chadwick, Fairouz Ibrahim, Mark J. Skehel, and Sharon A. Tooze
ARTICLE TYPE: Short Report

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