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Visual detection of binary, ternary and quaternary protein interactions in fission yeast using a Pil1 co-tethering assay

Zhong-Qiu Yu, Xiao-Man Liu, Dan Zhao, Dan-Dan Xu and Li-Lin Du
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MS TITLE: Visual detection of binary, ternary, and quaternary protein-protein interactions in fission yeast by Pil1 co-tethering assay

AUTHORS: Zhong-Qiu Yu, Xiao-Man Liu, Dan Zhao, Dan-Dan Xu, and Li-Lin Du
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

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

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

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

Reviewer 1

Advance summary and potential significance to field

In this paper, a clever scheme of protein misdirection is applied to identify interactions between components of multi-protein autophagy complexes in the fission yeast Schizosaccharomyces pombe, and finer mapping of interaction domains. Although the conceptually similar strategy of fusing a protein of interest to a SPB targeting domain has been used previously for the same purpose, this new strategy of mis-directing proteins to eisosomes at the plasma membrane via fusion to Pil1 may be an advantage for dissecting certain membrane-interacting proteins or complexes. The authors validate their strategy on known interacting proteins and then extend it to map interacting domains and to identify previously unknown protein interactions.

Overall, this new strategy seems that it will be very useful for the yeast community and already new insights into autophagy have been discovered. Also, the paper is well-written, and the data clear and convincing. I do have some recommendations about improving the presentation.

Comments for the author

Major points:
1. A strategy of quantifying co-localization was developed that can be used to predict a direct protein-protein interaction. However, it should not be used as a proxy for the "strength" of the interaction nor positive validation of a direct interaction but rather only as a guide or prediction. This of course has to do with the use of tags, a caveat acknowledged by the authors in their discussion. Therefore, the text should be carefully re-edited to avoid statements about interaction strength based on this assay (e.g. four pairs of strong protein-protein interactions should be "four pairs of strong protein co-localizations"). "The introduction of PCC to evaluate the degree of colocalization between bait and prey proteins allows the strengths of the interactions to be measured in a quantitative manner" should be re-worded to avoid "strengths of the interactions", etc.

2. It is reported that the co-localization strategy can be adopted for TM-containing proteins and Atg9 and Ctl1 were used as example. Please indicate in the paragraph beginning line 152 where Atg9 and Ctl1 ordinarily localize. Also, given that Pil1 is not a TM-containing protein can it be clarified how TM proteins fused to Pil1 are processed and whether they are actually integrated into the membrane and if so, what membrane?

3. I recommend citing in the introduction the conceptually similar scheme used previously in fission yeast for misdirecting proteins to the SPB to demonstrate interactions between proteins. It would also be worthwhile to introduce the conceptually similar scheme of using GBP-GFP tags to misdirect proteins and demonstrate interactions and dependencies thereof.

Minor:
1. Please change the color scheme that disadvantages red-green color-blind individuals. I recommend a magenta-green false color scheme in which co-localization is white. This allows the maximum number of individuals to appreciate the images. While this is a lot of work for this paper, at least the main figures should be changed.

2. line 307; "used to detect ternary interactions". What the assay did in this case is to demonstrate an indirect, not a ternary, interaction in a trimeric complex. An example of a bonafide ternary interaction will come later in the paper.

3. The fusion proteins were assembled into plasmids and integrated into the genome. However, the description of the experimental scheme in lines 100-108 of the text does not mention this critical step of plasmid integration. Please describe plasmid integration in this first paragraph that so it is clear that the scheme is not using two extra-chromosomal plasmids.

4. It was observed, as with the GBP-GFP system, that proteins can be pulled in the opposite direction than intended – here, it seems that the majority of Pil1 was pulled into the nucleus by a

certain fusion protein interaction. Therefore, this co-localization strategy may not always be effective and this caveat should also be acknowledged in the limitations section.

5. The results summarized in Figure 4D and E suggest a direct interaction between Atg1 and Atg17 that was not apparently previously known. However, from the scheme, it seems that this might be bridged by Atg13. Has this interaction been tested in the absence of Atg13 like the interaction between Atg11 and Atg13 was tested in the absence of Atg1?

6. It isn't clear what motivated the experiment described in lines 313-317 and given the negative results this section could be removed.

Editorial:
1. Line 125-126. "totally abolished". Either something is abolished or it is not. Please delete "totally".
2. Line 394: complex seems vary among different species. Please add "to" between "seems" and "vary".
3. Line 187: to exhaustively examined the… Should be examine rather than examined.
4. The y-axis in Figure 5G needs a label.

Reviewer 2

Advance summary and potential significance to field

In this paper, Du and colleagues describe a technique to study protein-protein interactions in fission yeast cells. The authors tether one mCherry-tagged "bait" protein to eisosomes, which appear as prominent filaments at the plasma membrane. The second "prey" protein is tethered to GFP, and colocalization is detected by fluorescence microscopy and calculation of PCC. The authors use this novel assay to characterize protein-protein interactions amongst autophagy proteins and PI3K complexes. In some cases the assay is extended to include expression of a third protein that "bridges" the bait and prey, therefore demonstrating an indirect physical interaction between bait and prey. Overall, the paper is very solid with appropriate controls and rigorous data analysis. The results confirm previously known interactions and also demonstrate novel protein-protein interactions. I found that Discussion section to be well balanced on the strengths and limitations of the new approach. Given the potential utility of this new method, I would have liked to see it used to show protein-protein interaction for non-pombe proteins. As presented, it will be very useful for fission yeast researchers but definitely has the potential for use with testing interactions between proteins from human, fly, worm, budding yeast, etc. It would be nice to see a demonstration of this potential to gain further impact for the work. However, the paper as presented is a clear step forward and a very nice paper, so this suggestion need not be required for publication of the work.

Comments for the author

Specific comments:
1. The method relies on recruitment of a prey protein to eisosomes, dependent on protein-protein interaction between bait and prey. It should be noted that the bait-prey interaction sometimes recruits the prey protein away from eisosomes, resulting in colocalization elsewhere in the cells. The PCC is still strong, but at that point it is not an eisosome co-tethering assay. Some examples are found in Figs 3 and 4 (e.g. 4B for Atg17-Atg13 and Atg17-Atg17). The authors should include this point in the discussion section as a limitation that depends on the strength of prey protein localization signals.

2. Colocalization in Figure 8C is quite limited and should be presented accordingly in the results section. A strength of the PCC approach is the calculation of a quantitative metric for the interaction. In this case, it would fall into a "weak colocalization" category.

3. Pil1 binds to membrane lipids and the new assay relies on colocalization, not necessarily protein-protein interaction. Therefore, the authors should note (in the Discussion, perhaps?) the possibility that the prey protein could colocalize with eisosomes due to interactions with membrane lipids. Eisosomes appear to generate membrane lipid microenvironments, and recruitment of prey

proteins to this microenvironment would lead to colocalization of bait and prey without protein-protein interactions.

4. The following suggestion is not necessary for publication of this manuscript in my opinion. However, I would like to point out that the authors could use this assay to have 2 differently tagged "prey" proteins compete for interaction with a single "bait" protein. The prey proteins (e.g. Atg14 and Vps38) could be differently tagged with GFP and CFP, and a bait protein (e.g. Atg6) could be tagged with mCherry-Pil1. The resulting PCCs would indicate whether Atg14 or Vps38 outcompetes the other for binding to Atg6 within the cellular environment, dependent on expression level of the 2 prey proteins.

Reviewer 3

Advance summary and potential significance to field

This manuscript from Yu et al presents a useful method to simply assess protein-protein interactions in fission yeast cells. The method relies on the concentration of a bait protein at Pil1-marked eisosomes, plasma membrane invaginations that show a distinctive localization easily identified by light microscopy, and assess the extent of recruitment of prey proteins tagged in a different colour. The assay seems robust, is generally well presented and is likely to be useful to the field given its ease of application. I have however several comments that should be addressed to strengthen the study and make the method easily transferable to any lab.

Comments for the author

The method is very convincing for all the cytosolic proteins tested, but I am less convinced about the nuclear and especially transmembrane proteins shown:

- For nuclear proteins, the system would be strongly improved by introducing an NES signal in the tagging plasmids. The example shown with Lig4 and Xrc4 shows it can work, but the result will be strongly affected by the strength of the NLS localization signal of one or the other binding partners. In fact, even in the example shown, the assay looks more like a Pil1 nuclear recruitment than the opposite. The authors solve the problem by deleting the NLS. However introducing a NES systematically in the plasmids would be an easier way, especially for proteins for which the NLS has not been characterized.

- I am not sure how the assay works for transmembrane proteins. What is the physiological localization of the two TM proteins tested, Atg9 and Ctl1? Are these plasma membrane-localized? I am surprised that they should both localize to eisosomes. Are the fusion proteins membrane-inserted? To claim generally that the assay works for membrane proteins, more evidence should be provided that Pil1 tethering does not influence membrane insertion, and also show this for at least a second pair of TM domain-containing interactors.

The statement that the PCC measure provides a quantitative measure of the strength of protein-protein interaction should be tempered down. The parallel to the interaction surface predicted from structural work is suggestive that the PCC measure is indicative of the strength of the interaction, but the evidence is not sufficient to make this claim. This would require careful in vitro measurements of binding constants for all interaction and testing of correlation with the PCC values. I suspect that the PCC value may be also influenced by other parameters in vivo.

I suggest that the text includes a section explaining how overexpression of the proteins from the nmt41 promoter contributes to the assay and how nmt41 vs nmt1 promoters were chosen (in some cases, the strong nmt1 promoter was used, but this is not clearly stated nor explained why). The authors state in the discussion that the assay cannot distinguish whether interaction is direct or indirect, which I completely agree with. However, although not explicitely stated, they also show in their ternary/quaternary complex investigation that the endogenous protein does not suffice to bridge the test proteins. This is likely due to the substantially lower endogenous expression levels, which do not produce sufficient protein to bridge the quantity of protein expressed under the nmt41 promoter.

Importantly, as this is a method paper, it should include a figure presenting the tools, i-e the plasmids that allow expression and tagging of proteins in the assay, showing polylinkers and method of genome integration: in particular "empty plasmids" pDUAL-41nmt1p-Pil1-mCherry, pDUAL-

41nmt1p-GFP (or YFP), pHIS3H-41nmt1p-CFP, pHIS3H-41nmt1p-13Myc... It would make it easier for readers to plan use of the tools for their own experiments. These plasmids (and at least a set of positive interactors that others can use as positive controls) should also be made available through deposition on resource centres (for instance NBRP and/or Addgene). It was also not clear to me how the authors integrated 4 different vectors in their quaternary complex tests.

Minor comments:
In Fig 2G, why are you using Atg8(1-115) rather than full-length Atg8?
Is the Atg1-Atg17 interaction independent of atg13? This would be worth checking given the Atg1-Atg13 and Atg13-Atg17 interactions.
It is not entirely clear how the authors focused on the 10 aa residues between 153 and 161 in Atg38. Is that a particularly conserved region? Perhaps the authors could specify.
The Pho8Δ60 assay could be explained with slightly more detail to help the non-specialist understand it.

First revision

Author response to reviewers' comments

Response to reviewers

Reviewer 1

Advance Summary and Potential Significance to Field:

In this paper, a clever scheme of protein misdirection is applied to identify interactions between components of multi-protein autophagy complexes in the fission yeast, Schizosaccharomyces pombe, and finer mapping of interaction domains. Although the conceptually similar strategy of fusing a protein of interest to a SPB targeting domain has been used previously for the same purpose, this new strategy of mis-directing proteins to eisosomes at the plasma membrane via fusion to Pil1 may be an advantage for dissecting certain membrane-interacting proteins or complexes. The authors validate their strategy on known interacting proteins and then extend it to map interacting domains and to identify previously unknown protein interactions. Overall, this new strategy seems that it will be very useful for the yeast community and already new insights into autophagy have been discovered. Also, the paper is well-written, and the data clear and convincing. I do have some recommendations about improving the presentation.

Reviewer 1

Comments for the Author:

Major points:

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Response: We thank the reviewer for raising this point and we agree with the reviewer. In the revised manuscript, we avoided using the strength of colocalizations to indicate the strength of

protein-protein interactions. For the two examples the reviewer mentioned, we changed “four pairs of strong protein-protein interactions” to “four pairs of protein-protein interactions”, and changed “The introduction of PCC to evaluate the degree of colocalization between bait and prey proteins allows the strengths of the interactions to be measured in a quantitative manner” to “The introduction of PCC to evaluate the degree of colocalization between bait and prey proteins is helpful in judging whether point mutations, the presence/absence of other proteins, or environmental changes influence the interactions”.

2. *It is reported that the co-localization strategy can be adopted for TM-containing proteins and Atg9 and Ctl1 were used as example. Please indicate in the paragraph beginning line 152 where Atg9 and Ctl1 ordinarily localize. Also, given that Pil1 is not a TM-containing protein, can it be clarified how TM proteins fused to Pil1 are processed and whether they are actually integrated into the membrane and if so, what membrane?*
Response: Under nutrient-rich conditions, both Atg9 and Ctl1 mainly localize to the Golgi. We agree with the reviewer that it is uncertain whether transmembrane proteins fused to Pil1 are actually inserted into the membrane. In addition, if Pil1-fused Ctl1 can still localize to the Golgi, it may tether the Golgi to the eisosome filaments and cause not only Atg9 but all Golgi proteins to colocalize to the eisosome filaments. Because of these uncertainties, we have decided to remove the results on Atg9 and Ctl1 and no longer make the claim that the Pil1 co-tethering assay can be applied to transmembrane proteins.

3. *I recommend citing in the introduction the conceptually similar scheme used previously in fission yeast for misdirecting proteins to the SPB to demonstrate interactions between proteins. It would also be worthwhile to introduce the conceptually similar scheme of using GBP-GFP tags to misdirect proteins and demonstrate interactions and dependencies thereof.*
Response: We have searched the literature and consulted with a fission yeast colleague studying the SPB. We were not able to find any publications using the strategy of misdirecting proteins to the SPB in fission yeast to demonstrate interactions between proteins. We found two papers in which the authors used the C-terminal region of Ppc89 as a SPB-targeting domain to substitute for the SPB-targeting region of Sid4 (Rosenberg et al. 2006 PMID: 16775007 and Johnson and Gould 2010 PMID: 21131906), but the authors did not use SPB targeting for the purpose of detecting protein-protein interactions. We may have missed the publication that the reviewer had in mind and will be happy to add the citation if the reviewer can kindly inform us which paper we should cite. For the GBP-GFP approach, we are aware of a number of papers in which the GBP-GFP interaction was used to tether proteins to the SPB in fission yeast (Grallert et al. 2013 PMID: 23222840; Masuda and Toda 2016 PMID: 27053664; Chen et al. 2017 PMID: 28082423; Jones et al. 2018 PMID: 29975113), but none of these papers used the GBP-GFP approach for the purpose of examining protein-protein interactions. Again, we may have missed the relevant papers and will be happy to cite them if the reviewer can let us know which papers have used the GBP-GFP approach for the purpose of detecting protein-protein interactions.

Minor:

1. *Please change the color scheme that disadvantages red-green color-blind individuals. I recommend a magenta-green false color scheme in which co-localization is white. This allows the maximum number of individuals to appreciate the images. While this is a lot of work for this paper, at least the main figures should be changed.*
Response: We thank the reviewer for this suggestion. As suggested by the reviewer, we have changed all the “red-green” combination to the color blind friendly “magenta- green” combination, and all the “red-green-blue” combination to the color blind friendly “magenta-green-blue” combination both in the main figures and in the supplementary figures.

2. *line 307; “used to detect ternary interactions”. What the assay did in this case is to demonstrate an indirect, not a ternary, interaction in a trimeric complex. An example of a bona fide ternary interaction will come later in the paper.*
Response: We agree with the reviewer that in the Vps15-Vps34-Atg38 interaction, Vps34 bridges an indirect interaction between Vps15 and Atg38. Such a situation of one protein bridging two other proteins in a trimeric complex is often called a ternary interaction in the literature. We list four example publications below, one of which is a review article on methods for detecting protein-protein interactions.

Datta, P., Dasgupta, A., Singh, A.K., Mukherjee, P., Kundu, M., and Basu, J. (2006). Interaction between FtsW and penicillin-binding protein 3 (PBP3) directs PBP3 to mid- cell, controls cell septation and mediates the formation of a trimeric complex involving FtsZ, FtsW and PBP3 in mycobacteria. Mol. Microbiol. *62*, 1655–1673.

Houtman, J.C.D., Brown, P.H., Bowden, B., Yamaguchi, H., Appella, E., Samelson, L.E., and Schuck, P. (2007). Studying multisite binary and ternary protein interactions by global analysis of isothermal titration calorimetry data in SEDPHAT: application to adaptor protein complexes in cell signaling. Protein Sci. Publ. Protein Soc. *16*, 30–42.

Lee, H.-C., Portnoff, A.D., Rocco, M.A., and DeLisa, M.P. (2014). An engineered genetic selection for ternary protein complexes inspired by a natural three-component hitchhiker mechanism. Sci. Rep. *4*, 7570.

Xing, S., Wallmeroth, N., Berendzen, K.W., and Grefen, C. (2016). Techniques for the Analysis of Protein-Protein Interactions in Vivo. Plant Physiol. *171*, 727–758.

3. The fusion proteins were assembled into plasmids and integrated into the genome. However, the description of the experimental scheme in lines 100-108 of the text does not mention this critical step of plasmid integration. Please describe plasmid integration in this first paragraph that so it is clear that the scheme is not using two extra-chromosomal plasmids.
Response: As suggested by the reviewer, in the first paragraph of the Results section of the revised manuscript, we emphasized that the plasmids were integrated into the genome.

4. It was observed, as with the GBP-GFP system, that proteins can be pulled in the opposite direction than intended – here, it seems that the majority of Pil1 was pulled into the nucleus by a certain fusion protein interaction. Therefore, this co-localization strategy may not always be effective and this caveat should also be acknowledged in the limitations section.
Response: We thank the reviewer for pointing out this issue. We added this caveat in the limitations section in the revised manuscript.

5. The results summarized in Figure 4D and E suggest a direct interaction between Atg1 and Atg17 that was not apparently previously known. However, from the scheme, it seems that this might be bridged by Atg13. Has this interaction been tested in the absence of Atg13 like the interaction between Atg11 and Atg13 was tested in the absence of Atg1?
Response: During the revision, we have performed additional experiments to examine whether the absence of Atg13 had an influence on the interaction between Atg1 and Atg17 and found that Atg13 is not required for this interaction. We added the results in Fig. S3A, B, C, and D of the revised manuscript.

6. It isn't clear what motivated the experiment described in lines 313-317, and given the negative results this section could be removed.
Response: We presented the results to show that ectopic expression of Vps34 specifically bridge the interactions between Vps15 and Atg38, but not other subunits of the two PtdIns3K complexes. We explained the motivation of the experiment in the revised manuscript.

Editorial:

1. Line 125-126. "totally abolished". Either something is abolished or it is not. Please delete "totally".
Response: We deleted "totally" as suggested by the reviewer.

2. Line 394: complex seems vary among different species. Please add "to" between "seems" and "vary".
Response: We added "to" between "seems" and "vary" as suggested by the reviewer.

3. Line 187: to exhaustively examined the… Should be examine rather than examined
Response: We changed "examined" to "examine".

4. The y-axis in Figure 5G needs a label.
Response: We added "Pho8Δ60 activity" as the label to the y-axis in Fig. 5G in the revised manuscript.

Reviewer 2

Advance Summary and Potential Significance to Field:

In this paper, Du and colleagues describe a technique to study protein-protein interactions in fission yeast cells. The authors tether one mCherry-tagged "bait" protein to eisosomes, which appear as prominent filaments at the plasma membrane. The second "prey" protein is tethered to GFP, and colocalization is detected by fluorescence microscopy and calculation of PCC. The authors use this novel assay to characterize protein-protein interactions amongst autophagy proteins and PI3K complexes. In some cases, the assay is extended to include expression of a third protein that "bridges" the bait and prey, therefore demonstrating an indirect physical interaction between bait and prey. Overall, the paper is very solid with appropriate controls and rigorous data analysis. The results confirm previously known interactions and also demonstrate novel protein-protein interactions. I found that Discussion section to be well balanced on the strengths and limitations of the new approach. Given the potential utility of this new method, I would have liked to see it used to show protein-protein interaction for non-pombe proteins. As presented, it will be very useful for fission yeast researchers but definitely has the potential for use with testing interactions between proteins from human, fly, worm, budding yeast, etc. It would be nice to see a demonstration of this potential to gain further impact for the work. However, the paper as presented is a clear step forward and a very nice paper, so this suggestion need not be required for publication of the work.
Response: We thank the reviewer for the suggestions. We agree that it would be useful to demonstrate that this assay can be used to examine the interactions between non-pombe proteins. However, because the first author of this paper is leaving my lab soon, we decided to not perform such additional experiments.

Reviewer 2

Comments for the Author:

Specific comments:

1. The method relies on recruitment of a prey protein to eisosomes, dependent on protein-protein interaction between bait and prey. It should be noted that the bait-prey interaction sometimes recruits the prey protein away from eisosomes, resulting in colocalization elsewhere in the cells. The PCC is still strong, but at that point it is not an eisosome co-tethering assay. Some examples are found in Figs 3 and 4 (e.g. 4B for Atg17-Atg13 and Atg17-Atg17). The authors should include this point in the discussion section as a limitation that depends on the strength of prey protein localization signals.
Response: We thank the reviewer for raising this issue. We agree that in some instances bait-prey interactions result in colocalizations not only on eisosomes but also on some other subcellular localizations. In the revised manuscript, we added this point in the Discussion section as a limitation of the Pil1 co-tethering assay.

2. Colocalization in Figure 8C is quite limited and should be presented accordingly in the results section. A strength of the PCC approach is the calculation of a quantitative metric for the interaction. In this case, it would fall into a "weak colocalization" category.
Response: As the reviewer suggested, we have revised the wording and described the colocalization shown in Fig. 8C as "weak but noticeable".

3. Pil1 binds to membrane lipids and the new assay relies on colocalization, not necessarily protein-protein interaction. Therefore, the authors should note (in the Discussion, perhaps?) the possibility that the prey protein could colocalize with eisosomes due to interactions with membrane lipids. Eisosomes appear to generate membrane lipid microenvironments, and

recruitment of prey proteins to this microenvironment would lead to colocalization of bait and prey without protein-protein interactions.
Response: We thank the reviewer for raising this possibility. In the Discussion section of revised manuscript, we have added this limitation of the assay.

4. The following suggestion is not necessary for publication of this manuscript in my opinion. However, I would like to point out that the authors could use this assay to have 2 differently tagged "prey" proteins compete for interaction with a single "bait" protein. The prey proteins (e.g. Atg14 and Vps38) could be differently tagged with GFP and CFP, and a bait protein (e.g. Atg6) could be tagged with mCherry-Pil1. The resulting PCCs would indicate whether Atg14 or Vps38 outcompetes the other for binding to Atg6 within the cellular environment, dependent on expression level of the 2 prey proteins.
Response: During the revision, we have performed the experiments proposed by the reviewer. As predicted by the reviewer, when Vps38 was strongly overexpressed, the colocalization between Atg6 and Atg14 was obviously reduced. Similarly, the colocalization between Atg6 and Vps38 was reduced when Atg14 was strongly overexpressed. We presented the results in Fig. S6C, D, E, and F of the revised manuscript.

Reviewer 3

Advance Summary and Potential Significance to Field:

This manuscript from Yu et al presents a useful method to simply assess protein- protein interactions in fission yeast cells. The method relies on the concentration of a bait protein at Pil1-marked eisosomes, plasma membrane invaginations that show a distinctive localization easily identified by light microscopy, and assess the extent of recruitment of prey proteins tagged in a different colour. The assay seems robust, is generally well presented and is likely to be useful to the field given its ease of application. I have however several comments that should be addressed to strengthen the study and make the method easily transferable to any lab.

Reviewer 3

Comments for the Author:

The method is very convincing for all the cytosolic proteins tested, but I am less convinced about the nuclear and especially transmembrane proteins shown:
For nuclear proteins, the system would be strongly improved by introducing an NES signal in the tagging plasmids. The example shown with Lig4 and Xrc4 shows it can work, but the result will be strongly affected by the strength of the NLS localization signal of one or the other binding partners. In fact, even in the example shown, the assay looks more like a Pil1 nuclear recruitment than the opposite. The authors solve the problem by deleting the NLS. However introducing a NES systematically in the plasmids would be an easier way, especially for proteins for which the NLS has not been characterized.
Response: We agree with the reviewer that introducing an NES signal in the tagging plasmids would improve the applicability of the Pil1 co-tethering assay system for nuclear proteins. In the revised manuscript, we have emphasized that the Pil1 co- tethering assay in its current format may not be suitable for all nuclear proteins and recommended that researchers who intend to use this assay to study nuclear proteins introduce an NES signal into the plasmids.

I am not sure how the assay works for transmembrane proteins. What is the physiological localization of the two TM proteins tested, Atg9 and Ctl1? Are these plasma membrane-localized? I am surprised that they should both localize to eisosomes. Are the fusion proteins membrane-inserted? To claim generally that the assay works for membrane proteins, more evidence should be provided that Pil1 tethering does not influence membrane insertion, and also show this for at least a second pair of TM domain-containing interactors.
Response: Under nutrient-rich conditions, Atg9 and Ctl1 mainly localize to the Golgi. We cannot rule out the possibility that membrane insertion of Atg9 and Ctl1 was affected in our assay. In addition, it is possible that when we fused Pil1 to the N- terminus of Ctl1, which is predicted to

face the cytosol, the fusion protein still localizes to the Golgi and tethers the Golgi to the eisosome filaments. If this is true, we cannot rule out the possibility that all Golgi-localized proteins, not just Atg9, exhibit co- localization to the eisosome filaments under such a condition. Because of these possibilities, we decided to remove the results on Atg9 and Ctl1 from the manuscript and no longer make the claim that the Pil1 co-tethering assay can be applied to transmembrane proteins.

The statement that the PCC measure provides a quantitative measure of the strength of protein-protein interaction should be tempered down. The parallel to the interaction surface predicted from structural work is suggestive that the PCC measure is indicative of the strength of the interaction, but the evidence is not sufficient to make this claim. This would require careful in vitro measurements of binding constants for all interaction and testing of correlation with the PCC values. I suspect that the PCC value may be also influenced by other parameters in vivo.
Response: We thank the reviewer for raising this issue. We agree that PCC values cannot be reliably used to compare the strength of the protein-protein interactions between different protein pairs. In the revised manuscript, we avoided directly using the PCC value or the degree of colocalization to indicate the strength of protein-protein interactions. However, we noted in the discussion section that it is still meaningful to use PCC to indicate the relative strength of the interactions when judging whether point mutations, the presence/absence of other proteins, or environmental changes influence the interaction of a particular pair of bait and prey proteins.

I suggest that the text includes a section explaining how overexpression of the proteins from the nmt41 promoter contributes to the assay and how nmt41 vs nmt1 promoters were chosen (in some cases, the strong nmt1 promoter was used, but this is not clearly stated nor explained why). The authors state in the discussion that the assay cannot distinguish whether interaction is direct or indirect, which I completely agree with.
However, although not explicitly stated, they also show in their ternary/quaternary complex investigation that the endogenous protein does not suffice to bridge the test proteins. This is likely due to the substantially lower endogenous expression levels, which do not produce sufficient protein to bridge the quantity of protein expressed under the nmt41 promoter.
Response: We stated in the Materials and Methods section that choosing the *41nmt1* promoter to ectopically express bait proteins is because this promoter is strong enough to generate robust fluorescence signal but not too strong to cause abnormal cell morphology and reduced growth rates that can result from strong overexpression of Pil1. We also used the *41nmt1* promoter to express prey proteins in most cases, so that the expression levels of prey proteins can match those of bait proteins. The only exception is the prey protein Atg9-YFP in Fig. 3B, for which we used the stronger *nmt1* promoter. We did not intentionally choose the *nmt1* promoter over the *41nmt1* promoter for expressing Atg9-YFP. It was a decision out of convenience because we already had a pDUAL-*nmt1p*-Atg9-YFP plasmid. For reasons given earlier, we have decided to remove the experimental data on Atg9 and Ctl1 from the manuscript. In the data shown in the revised manuscript, all bait and prey proteins were expressed from the *41nmt1* promoter.
As for the ternary/quaternary interactions in the two PI3K complexes, we agree with the reviewer's explanation that the insufficiency of endogenous proteins to bridge the interactions is due to endogenous expression levels being substantially lower than the expression levels under the control of the *41nmt1* promoter. We have added this explanation in the revised manuscript.

Importantly, as this is a method paper, it should include a figure presenting the tools, i-e the plasmids that allow expression and tagging of proteins in the assay, showing polylinkers and method of genome integration: in particular "empty plasmids" pDUAL- 41nmt1p-Pil1-mCherry, pDUAL-41nmt1p-GFP (or YFP), pHIS3H-41nmt1p-CFP, pHIS3H-41nmt1p-13Myc... It would make it easier for readers to plan use of the tools for their own experiments. These plasmids (and at least a set of positive interactors that others can use as positive controls) should also be made available through deposition on resource centres (for instance NBRP and/or Addgene). It was also not clear to me how the authors integrated 4 different vectors in their quaternary complex tests.
Response: We thank the reviewer for these suggestions.
We have added a figure panel (Fig. 1C in the revised manuscript) showing information about the plasmids and methods of integration.
We have deposited the following 9 plasmids in Addgene.
Basic plasmids:
pDUAL-*41nmt1p*-Pil1-mCherry (Addgene ID 174012)
pDUAL-*41nmt1p*-GFP (Addgene ID 174013)

pHIS3H-*41nmt1p*-CFP-Atg6 (Addgene ID 174014) (we have no empty plasmid containing CFP, but the atg6 coding sequence in this plasmid can be replaced by any other sequence by digesting this plasmid using BamHI and AscI and inserting another DNA fragment)
pHIS3H-*41nmt1p*-13Myc-Vps15 (Addgene ID 174015) (we have no empty plasmid containing 13Myc, but the vps15 coding sequence in this plasmid can be replaced by any other sequence by digesting this plasmid using AscI and inserting another DNA fragment)

Positive controls:

pDUAL-*41nmt1p*-Pil1-mCherry-Atg38(161-190) (Addgene ID 174016)
pDUAL-*41nmt1p*-GFP-Atg8 (Addgene ID 174017)
pDUAL-*41nmt1p*-Pil1-mCherry-Atg14 (Addgene ID 174018)
pDUAL-*41nmt1p*-Pil1-mCherry-Vps38 (Addgene ID 174019)
pDUAL-*41nmt1p*-GFP-Atg6 (Addgene ID 174020)

Here, we use the example of a strain used in the experiments shown in Fig. 8A to explain how we constructed strains with four integrated plasmids. One pDUAL-based plasmid expressing GFP-Atg14 was linearized with NotI digestion and integrated at the *leu1* locus (selecting for Leu+), another pDUAL-based plasmid expressing Pil1- mCherry-Vps34 was linearized with MluI digestion and integrated at the *ars1* replication origin region (selecting for Ura+), the third plasmid, which is a pHIS3H- based plasmids expressing CFP-Atg6, was linearized with NotI digestion and integrated at the *his3* locus (selecting for hygromycin resistance), and the fourth plasmid expressing 13Myc-Vps15, which is also based on pHIS3H, was linearized with SalI and integrated at the *vps15* locus (selecting for hygromycin resistance). Because the third plasmid and the fourth plasmid have the same hygromycin resistance marker, they were first integrated into separate strains and then brought into the same strain by crossing.

In Fig 2G, why are you using Atg8(1-115) rather than full-length Atg8?
Response: During nitrogen starvation, wild-type Atg8 is conjugated to phosphatidylethanolamine (PE) through its glycine 116 residue in a process called lipidation. Atg8(1-115) cannot be lipidated, and thus using Atg18(1-115) as bait can rule out the involvement of lipidation. However, because the Pil1 co-tethering assays shown in this manuscript were all conducted under nutrient-rich conditions where wild- type Atg8 is not lipidated, using wild-type or Atg8(1-115) as bait should not make any differences. We have added explanations on the use of Atg18(1-115) in the revised manuscript.

Is the Atg1-Atg17 interaction independent of atg13? This would be worth checking given the Atg1-Atg13 and Atg13-Atg17 interactions.
Response: We thank the reviewer for raising this question. During the revision, we have performed additional experiments to address this question. We examined whether the absence of Atg13 influences the Atg1-Atg17 interaction and found that this interaction is independent of Atg13. We added the results in Fig. S3A, B, C, and D of the revised manuscript.

It is not entirely clear how the authors focused on the 10 aa residues between 153 and 161 in Atg38. Is that a particularly conserved region? Perhaps the authors could specify.
Response: There are two short linear segments in Atg38 that exhibit strong sequence conservation among four fission yeast species. One is a 13-amino-acid sequence between residues 173 and 185, which encompasses the AIM and is responsible for interacting with Atg8. The other is the 10-amino-acid sequence between 152 and 161. We showed a sequence alignment of this region in Fig. 5D of this manuscript. The sequence conservation suggests functional importance. We hypothesized that the Vps34-Atg38 interaction is functionally important, and therefore focused our attention on this conserved segment.

The Pho8Δ60 assay could be explained with slightly more detail to help the non- specialist understand it.
Response: We have added more details about this assay in the revised manuscript.

Second decision letter

MS ID#: JOCES/2021/258774

MS TITLE: Visual detection of binary, ternary, and quaternary protein interactions in fission yeast by Pil1 co-tethering assay

AUTHORS: Zhong-Qiu Yu, Xiao-Man Liu, Dan Zhao, Dan-Dan Xu, and Li-Lin Du
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

The authors have satisfactorily addressed all the points I raised. This is a very nice paper and the strategy will be a very useful for the community.

Just for the authors' information, for the conceptually similarly SPB strategy I apologize for not providing a relevant citation. It is indeed buried in papers and I also can find only one right now. The system I am familiar with was developed by the Sirotkin lab and described in PMID: 31391237

Also, I stand corrected about the GFP-GBP approach! I was thinking of Dr. Quan-Wen Jin's and Dr. Mohan Balasubramanian's papers actually (citations below) but the authors are completely correct that this was used for moving proteins around, not testing for interactions:

- PMID: 25176634 DOI: 10.1016/j.cub.2014.07.074
- PMID: 28082423

Comments for the author

I have no further suggestions for the authors's consideration.

Reviewer 2

Advance summary and potential significance to field

The paper presents a new microscopy-based assay for protein-protein interactions of fusion proteins in fission yeast. The assay should be useful for researchers testing protein-protein interactions from any cell type or organism.

Comments for the author

The authors have revised the text and added new data to address previous concerns from all reviewers including me. I have no remaining concerns and support publication of this excellent study.

Reviewer 3

Advance summary and potential significance to field

This is a very nice new method addition.

Comments for the author

The authors have adequately addressed my comments.