Osh6 requires Ist2 for localization to the ER-PM contacts and efficient phosphatidylserine transport in budding yeast

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MS TITLE: Osh6 requires Ist2 for localization to the ER-PM contacts and efficient phosphatidylserine transport

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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.

There are some specific cases where further controls are needed and two clear point arising from the reviews. First, the question of whether C2(Lact)-GFP can recognize lyso PS, and second, validation of the functionality of tagged versions of Osh6 and Ist2 is needed. The remaining critiques appear to be largely text changes and clarifications.

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

This manuscript describes an interaction between the lipid transfer protein Osh6/7 and ER-PM contact site protein Ist2, and investigates its impact on PS transport to the plasma membrane. The significance of Osh proteins for lipid transport has been widely appreciated. However, for Osh6 the mechanism of localization to the site of transfer has remained unclear. Data presented in this manuscript allow for the conclusion that Ist2 is required for localization to ER-PM contact sites, and identifies regions in Ist2 and Osh6 that important for this. Furthermore, a clear correlation to PS content and PS transport is shown for cells in which this interaction is disrupted.

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I find that the major claims are supported by the data and therefore support publication in the Journal of Cell Science. However, there are some concerns (listed below) which in my opinion need to be addressed before publication.

As a more general concern, that applies to almost all data presented: Tagged versions of Osh6 and Ist2 are used. Has the functionality of these tagged versions been shown? If not, do results presented in this manuscript offer a way to test for the functionality of tagged versions? If yes, such a control is essential. If no assay is available to test for functionality of tagged versions, this caveat needs to be explicitly stated.

Specific comments
1) The first part of the paper (until Fig. 4) deals with the interaction of Ist2 with Osh6/7. The authors claim that "Osh6 localization depends on its interaction with the cytosolic tail of the ER-PM tether Ist2". In my opinion none of the methods used are sufficient to establish a direct interaction (although a direct interaction is very plausible). To validate such a claim, it would be necessary to show an interaction with the purified proteins/protein fragments. In the absence of such data, the observations are also consistent with an indirect interaction, mediated by another (soluble) protein. Please comment.
2) BiFC shows a lot of diffuse cytoplasmic staining, although Ist2 is membrane-embedded (Fig. 1C, top).
Why is that? This experiment requires additional controls with mutants that do not show an interaction in other assays (e.g. the double-threonine mutant from Fig. 2.).
3) In Figures 2 and 4, bar diagrams are used to quantify Osh6 localization. Since the localization of Osh6 is not an all or nothing event (as clearly visible from the images) it is not clear to me, how the analysis was performed. Numbers in 4B, comparing WT and the RL718-751 mutant are nearly identical, whereas the images show clearly a cytoplasmic pattern for Osh6. I therefore think the analysis is misleading. Also, what does the N=60 refer to? How are error bars generated? Bars are inconsistently labeled.
4) Fig. 4C should also contain a quantification for the RL mutant, so that one can appreciate the maximum difference.
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7) Rescue experiments in Fig. 6D are not performed in a cho1, ist2 double deletion background. Why is that?
8) IPs/Western-Blots in S5A, and 8A are insufficient, both in the way they were performed and in the description. Controls with untagged strain are necessary to show specificity of the detection/pulldown. Is the expression level of GFP-Ist2 too low for the protein to be detected in input samples? Why is there a strong GFP signal in the combination WT-Ist2/AA-Osh6 but not when both proteins are WT. The statement that mutant and WT proteins were expressed at the same level is not supported by Fig. S5A. Why are different temperatures investigated?

9) What does the arrow in S5B indicate?
10) The notion "a motif in the Ist2 tail, conserved in yeasts" is not supported by the data. I recommend either changing this to budding yeasts or use the term "Saccharomycotina". In Fig.3 it is unclear if only the depicted subset or all 58 sequences were used to calculate consensus sequence.

Reviewer 2

Advance summary and potential significance to field

The paper shows how a cytoplasmic lipid transfer protein is localized to a membrane contact site. The underlying mechanism is a protein-protein interaction, rather than the more typical interaction between a protein and a lipid platform. This is new information.

Comments for the author

This is an interesting and generally convincing paper, well-written and clearly explained. It's nice work and the overall conclusion is clear. I have several questions, some going back to the authors' previous work on the role of Osh6/Osh7 in PS transport. It would help the paper if these points were to be clarified.

1. Does C2(Lact)-GFP recognize lyso PS? If it does, then how can it be ruled out that the peripheral signal does not simply arise from the flip of lyso-PS to the cyto face of the PM instead of (or in addition to) the model proposed - which is transport to the ER (how is this achieved??), acylation, then Osh6/Osh7-mediated anterograde transport to the PM. I realize that this is the model presented in a previous paper, but it remains unclear as to exactly what is going on.

2. The accumulation of PS in the ER is hard to conclude - for example, the graphs in Fig 6B show that peripheral staining is reduced and internal signal is increased. Is the C2(Lact)-GFP probe actually associated with the ER or simply diffusely present in the cytoplasm? If it cannot be conclusively shown that the probe is at the ER, then several statements (e.g. p 7, main para 2, line 7) stating that 'PS accumulated at the ER' should be rephrased.

3. Related to points 1 and 2, the steady state pool of PS is proposed to be mainly in the ER lumen (with the exception of a recent paper that indicates otherwise - the authors do not cite this paper) - how does this impact the authors' assay? Obviously the C2(Lact)-GFP probe would not recognize PS in the ER lumen, but as PS is constitutively scrambled, it may end up trapping PS on the cytoplasmic face of the ER.

4. The change in the amount of peripheral PS seen in only one out of three experiments (Fig S3). This is not really the impression given in the main text (p7, main para 1, line 5).

5. There is only one specific data set pertaining to Osh7 as far as I could see (Fig 2C, yeast 2-hybrid). There are no other explicit tests of Osh7's interaction with Ist2. yet, Osh7 is lumped in together with Osh6 in several places, starting at the bottom of p7. I guess that this is an extrapolation that the authors want to make, but as the dataset for Osh7 is less complete/rigorous, perhaps they should qualify their conclusion a little bit.

6. Figure 1A is barely convincing. I realize that the work with Ist2 speaks for itself, but this is hardly the candidate that jumps out when looking at the silver-stained gel! Is the identity of the strong band (comparable intensity to Osh6-TAP) ~55 kDa known?

7. Methods: was the mass spec analysis done in replicate? how was the 'high pressure lysis' of the yeast cells achieved? 'Fluorescent Microscopy' on p13, should be Fluorescence Microscopy.

8. Fig 1C - the labels are switched in the figure legend vs the figure itself. Osh6-VC should be Osh6-VN, or vice versa. A control for BiFC is also missing - BiFC is not very reliable and requires a negative control to show that the seen interaction is not unspecific.

9. Page 10, last line. The Bushell 2019 paper is not really relevant to the discussion and I think that the paper comes to the wrong conclusion. TMEM16K is a very poor scramblase; it is also Ca-activated. The ER has constitutive phospholipid scramblase activity, so it is hard to see how Ist2 and Osh6 could be functionally coupled in this regard.

First revision

Author response to reviewers' comments

D'Ambrosio et al., Point-by-point response to reviewers' comments:

Reviewer 1 Advance Summary and Potential Significance to Field:
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We thank the reviewer for his overall positive comments on our work. We agree that the question of functionality of tagged proteins is important, in particular for Osh6.
Tagging Osh6:
Results presented in this manuscript show that Osh6-mCherry transports PS in our transport assay, as does endogenous untagged Osh6. Moreover, we showed previously, in Moser von Filseck, 2015, and Lipp, 2019, that there was a good agreement between the behavior of various Osh6-mCherry mutants in cellular PS transport assays and the results of the in vitro PS transport assays with purified untagged Osh6. We have also demonstrated the functionality of the C-ter tagged Osh6-mCherry construct in previous work, most directly in Lipp 2019, where we have shown that Osh6-mCherry rescues the growth of the *osh1Δ-7Δ osh4-ts* at the non- permissive temperature (as does untagged Osh6 by Quon et al., PLOS Biol 2018 and Smindak et al, J Cell Sci 2017). Nevertheless, we cannot exclude the possibility that a tag would affect the behavior of Osh6 in the contact site in a subtle manner. Due to the lack of a growth phenotype for *osh6Δ osh7Δ* cells, it is difficult to test for such effects.

Tagging Ist2: We have strong evidence, presented in this manuscript, that N-ter tag on Ist2 does not influence the functionality of the Ist2 tail, i.e. its function in Osh6-mediated PS transport. First, both N- and C-ter tagged Ist2 fusions localize to cortical ER (Fig. 1, Fig. 4). Second, Osh6-mCherry/GFP localizes to contact sites in strains expressing endogenous untagged Ist2 or tagged Ist2 (Fig.1). Most importantly, Osh6-mediated PS transport proceeds in a strain with endogenous untagged Ist2, as well as when N-ter tagged Ist2 is expressed as the sole Ist2 copy. We have tried to test the functionality of GFP-Ist2 using increased salt tolerance of *ist2Δ* cells.
However, this phenotype is weak and can only be observed on rich media, precluding the testing of our plasmid- borne GFP-Ist2 (see Figure i below).

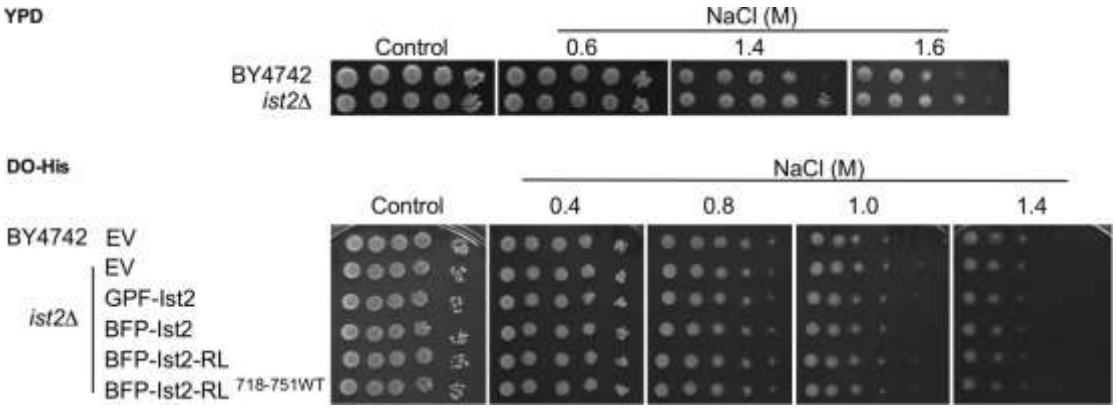

Figure i. Upper panel: Growth of *ist2Δ* vs WT (BY4742) cells on YPD media with increasing conc. of NaCl. 10-fold serial dilutions are plated, plates were incubated 3 days at 30C. Note the small improvement in growth of ist2Δ cells, in particular on plate with 1.6 M NaCl. Bottom panel: growth of *ist2Δ* bearing plasmids as indicated, vs WT, on minimal media (DO-His) with increasing conc of NaCl. EV indicated 'empty vector'. Plates shown were incubated 3 days at 30C. The same result (i.e. no difference in growth) was obtained after incubation at lower or higher temperatures or after a longer incubation period.

Specific comments

1) *The first part of the paper (until Fig. 4) deals with the interaction of Ist2 with Osh6/7. The authors claim that "Osh6 localization depends on its interaction with the cytosolic tail of the ER-PM tether Ist2". In my opinion none of the methods used are sufficient to establish a direct interaction (although a direct interaction is very plausible). To validate such a claim, it would be necessary to show an interaction with the purified proteins/protein fragments. In the absence of such data, the observations are also consistent with an indirect interaction, mediated by another (soluble) protein. Please comment.*

We agree that this is a possibility. We have revised the text, which now mentions that the interaction between Ist2 and Osh6 could also be mediated by an intermediate adaptor protein – this is explicitly stated in the Results section, pg. 8, and we have likewise revised the text in other places to say that Osh6 and Ist2 *may* directly interact.

2) *BiFC shows a lot of diffuse cytoplasmic staining, although Ist2 is membrane-embedded (Fig. 1C, top). Why is that? This experiment requires additional controls with mutants that do not show an interaction in other assays (e.g. the double-threonine mutant from Fig. 2.).*

The diffuse cytoplasmic staining was due to the images being taken with a widefield microscope. We have repeated the imaging in z-section using spinning disc microscope and have revised the figure using these new images. Fluorescence complementation is now largely restricted to the cortical ER. We have also included an additional control using Ist2Δ590-VC, where the cytosolic tail is missing. No fluorescence complementation with Osh6-VN can be observed.

3) *In Figures 2 and 4, bar diagrams are used to quantify Osh6 localization. Since the localization of Osh6 is not an all or nothing event (as clearly visible from the images) it is not clear to me, how the analysis was performed. Numbers in 4B, comparing WT and the RL718-751 mutant are nearly identical, whereas the images show clearly a cytoplasmic pattern for Osh6. I therefore think the analysis is misleading. Also, what does the N=60 refer to? How are error bars generated? Bars are inconsistently labeled.*

The analysis was performed manually, by counting the cells in which some enrichment of cortical Osh6 fluorescence could be observed, versus cells where Osh6 signal is uniformly cytosolic. This explanation has been added to the Methods section.
We have clarified in the figure legends what n refers to, and how error bars were generated. We have removed the labels on some bars.

4) *Fig. 4C should also contain a quantification for the RL mutant, so that one can appreciate the maximum difference.*

We have included the quantification for the Ist2-RL mutant in Fig. 4C.

5) *The lipidomics data is very clear. However, this seems not to be reflected in Figure S3. The statement to S3, "we usually observed a decrease in the level of C2lact-GFP at the plasma membrane …", is not supported by the data. Please comment if there is a discrepancy between lipidomics data and the PS- Sensor . Statistical analysis is missing.*

Statistical analysis of the data presented in Fig. S3 (now Fig. S4) is now included, and we have revised the description of the data presented in Fig. S4 in the Results section to improve its clarity. No, there is no discrepancy between lipidomics data and the PS sensor because these two experiments do not measure the same thing: total level of PS in the first case, subcellular distribution of a PS sensor in the second case. Lipidomics data show a very consistent and significant decrease in total cellular PS levels in osh6 osh7 and ist2 mutant cells. In the case of the PS probe, the trend is the same but we observe significant variability between the results of independent experiments, as illustrated in the corresponding Fig. S4. We could not determine the source of variability in C2Lact-GFP distribution assay. One important thing to note here is that fluorescent protein probes for lipids do not necessarily have a linear response and have been shown to be affected by other membrane parameters.

6) Fig.6 and Fig 8 are insufficiently explained: What does Arg-CMAC mean? Why is this method used here? Why is the dynamic range in Fig.8C (0.2 to 1.0, for WT) different from data in Fig. 6B, and D (0.1 to 0.4/0.5). Please explain.

PS transport assays to follow Osh6 function in cells have been used by us and other in previous studies (Maeda et al, Nature 2013, Moser et al., Science 2015, Lipp et al., 2019). Despite refinements of our protocol, this assay remains technically as well as time-demanding and the experiments presented in the manuscript were performed over a longer period.
Variability in the dynamic range between some experiments (but not all) is due to two things:
- some changes in the power of the laser on our spinning disc microscope
- variability in lyso-PS preparation, which remains difficult to control, thus despite our very careful work, we cannot completely control the effective concentration of lyso-PS that we add to the medium. This is explained in our Methods paper, D'Ambrosio et al., 2019.

Because of this variability, we in most cases simultaneously monitored two strains at the same time, one of them serving as an internal control. To distinguish between the two strains, one of them is stained with the vacuolar dye CMAC just prior to imaging. We added more explanation about CMAC staining to the Methods, and additional clarification to figure legends in Fig. 6 & 8. We noticed that we sometimes used Arg-CMAC and sometimes CMAC; we have corrected this.

7) *Rescue experiments in Fig. 6D are not performed in a cho1, ist2 double deletion background. Why is that?*

We were not able to construct a *cho1Δ ist2Δ* double mutant strain, despite a lot of trying. We suspect that the two mutations are synthetic lethal, or at least that they have a very strong synthetic interaction. However, we have not been able to confirm this, because *cho1Δ* cells do not mate and they are also sensitive to 5-FOA. Without a confirmation, we prefer to not make any statements about the synthetic interaction in the text.

8) *IPs/Western-Blots in S5A, and 8A are insufficient, both in the way they were performed and in the description. Controls with untagged strain are necessary to show specificity of the detection/pulldown.*

We have added additional description of these experiments in the Methods section and in the figure legends. We regret that the experiments shown in Fig. 8A and Fig. S5A (now S6A) lack controls with untagged Osh6 and untagged Ist2 to show the specificity of detection with anti-GFP and anti-mCherry antibodies. We were in the middle of repeating the experiments in S5A and 8A

that would include the untagged controls on the same membrane, when our lab was shut down because of the covid-19 pandemic. However, we hope that the reviewer will agree that our existing experiments are sufficient for the reasons explained below and illustrated with additional figures.

All bands shown in the Western blots in Fig. S5A (now S6A) and 8A are in fact specific for Ist2-GFP and Osh6- mCherry. This is demonstrated in two additional experiments, shown in Figure ii and iii below. We also note that the 3 bands that we consistently observe in Osh6-mCherry input fractions are in agreement with what Maeda et al. have observed during the purification of Osh6-TAP (see Suppl. Fig. 2 in Maeda et al., Science 2013). We currently do not know why Osh6-mCherry runs as 3 bands on SDS-PAGE, and why only 2 of these bands co- purify with Ist2. We have added some additional comment on this below and in figure legends, and the Osh6- mCherry and GFP-Ist2 specific bands are marked with arrows in Fig. 8A and Fig. S6A of our manuscript.

In the case of GFP-Ist2, we observed a single band in all input fractions, migrating somewhat faster than at the predicted size of 135 kDa. This can be appreciated in Fig. ii, by comparing the migration of full-length GFP-Ist2 with that of C-ter truncated GFP-Ist2. In the bound fractions, where GFP-Ist2 is highly concentrated, we observed a double band in many experiments, likely due to proteolytic degradation. A much more slowly migrating band also appears (see Fig. i), which is likely due to protein aggregation.

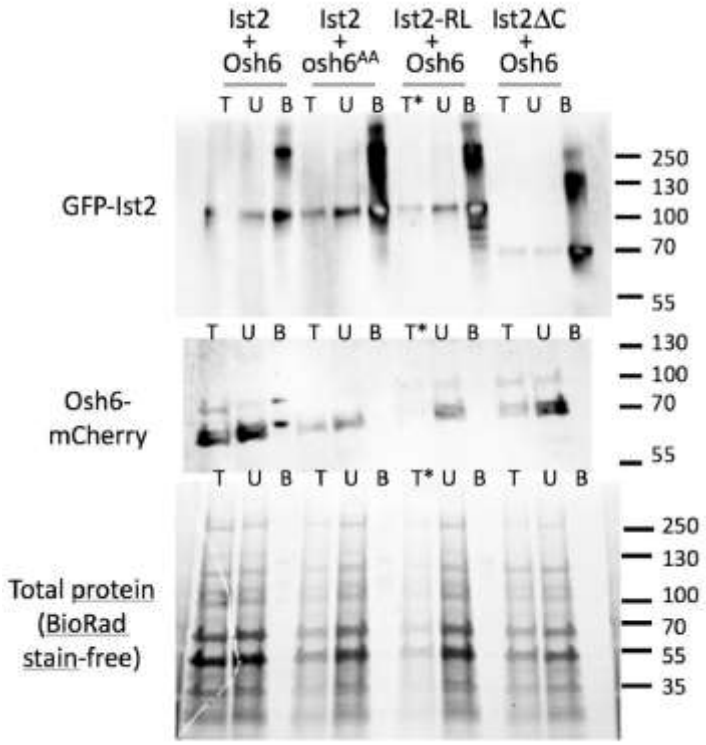

Figure ii. Co-IP of Osh6-mCherry or Osh6[AA]-mCherry with GFP-Ist2, GFP-Ist2-RL (randomized linker), or GFP- Ist2ΔC (protein truncated after TM region, lacking the whole cytosolic tail). Note the faster migration of GFP- Ist2ΔC, whose predicted size is ~95 kDa (40 kDa shorter than GFP-Ist2). T: Total lysate before IP (0.3 OD equivalents loaded on gel); U: Unbound fraction after incubation with anti-GFP beads (0.3 OD equivalents); B: Bound fraction eluted from anti-GFP beads (this fraction is ~35x concentrated compared to T; 1.5 OD equivalents loaded for anti-GFP WB, 10 OD equivalents for anti-mCherry WB). Note that some total fractions were incompletely precipitated, in particular T*.

Regarding Osh6-mCherry, we consistently observed 3 specific bands in the total fractions, only 2 of which interact with GFP-Ist2. The band running at >100 kDa is likely an aggregate. The two bands at ~80 kDa, in agreement with the predicted size of Osh6-mCherry (52 kDa + 29 kDa) may differ in post-translational modifications; however, we were not able to see a mobility shift after treatment

with different phosphatases. The lower band may also arise from some proteolytic degradation of Osh6. We tested whether this might be degradation of the 35 N- ter aa of Osh6, which are disordered, but this does not seem to be the case (see Fig. iii below). This will be the subject of future investigations.

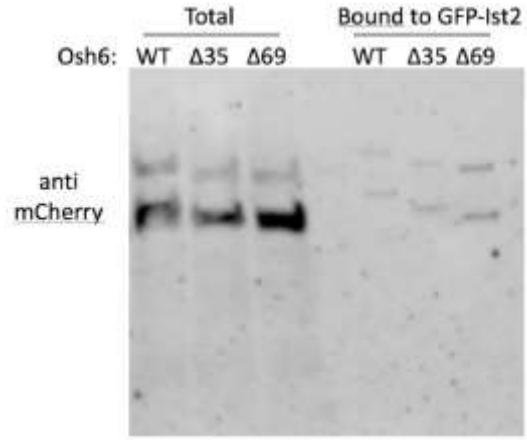

Figure iii. Co-IP od Osh6-mCherry or osh6Δ35-mCherry (missing 35 N-ter aa), or osh6Δ69-mCherry (missing 69 N-ter aa, which form the lid; see Lipp et al, 2019) with GFP-Ist2. Only anti-mCherry WB is shown. Note the shift in mobility of the truncated Osh6 mutants, which still interact with Ist2, in agreement with the BiFC data shown in Fig. S5A.

Finally, we would like to note that we have used the most stringent controls possible for the specifity of the co-IP: We have never observed Osh6-mCherry co-purifying with GFP-Ist2-RL, which differs from GFP-Ist2 only in that the cytosolic tail, containing the Osh6-binding site, is randomized. Likewise, we have never observed any co- purification of the Osh6-AA mutant, which differs only in two aa from Osh6-WT, with GFP-Ist2.

Is the expression level of GFP-Ist2 too low for the protein to be detected in input samples? Why is there a strong GFP signal in the combination WT-Ist2/AA-Osh6 but not when both proteins are WT. The statement that mutant and WT proteins were expressed at the same level is not supported by Fig. S5A.

Ist2 is a transmembrane protein with 8 TM and solubilization of such proteins after precipitation can be an issue. We made a number of adjustments to the precipitation protocol that we used to improve reproducibility of our Western blots. Still, there is some variability in the amount of precipitated protein, which is fairly demonstrated in Fig. S5A. We have corrected the text in Results section, pg. 8, to say that the two proteins were expressed at a *similar* level.
GFP-Ist2 is detected in all input samples and signal intensities are very similar. We have added arrows to the gel to indicate that the bands in input samples are specific and belong to Ist2. However, in bound fractions, where GFP-Ist2 is highly concentrated, there is a shift in the size of the GFP-Ist2 band. This may be due to some degradation or dephosphorylation, and possibly also to changes in protein mobility due to aggregation on the beads. Despite a high concentration of protease and phosphatase inhibitors throughout our purification procedure, we could not prevent the mobility shift in most experiments (note that in the experiment shown in Fig. 1, the degradation was less pronounced). We do not know why there is a stronger GFP signal WT-Ist2/AA-Osh6 bound sample, given that input GFP-Ist2 signals are similar. However, this would, if anything lead to a higher amount of AA-Osh6 bound; therefore, it does not interfere with our conclusion that AA-Osh6 mutant binds less strongly to GFP-Ist2 than WT.

Why are different temperatures investigated?

Based on the position of residues D141 and L142 in Osh6 structure, we worried that mutation of this residues (in particular L142) might be making the protein thermally unstable. This is why we checked protein levels at 37C as well as with 30C (our standard growth temperature) and saw that thermal stability was not a problem. We have added a comment on this to the Results section.

9) *What does the arrow in S5B indicate?*

We added explanation in figure legend of what is now Fig. S6B.

10) *The notion "a motif in the Ist2 tail, conserved in yeasts" is not supported by the data. I recommend either changing this to budding yeasts or use the term "Saccharomycotina". In Fig.3 it is unclear if only the depicted subset or all 58 sequences were used to calculate consensus sequence.*

Consensus sequence and the conservation score shown in Figure 3 was calculated from a subset of 34 sequences extracted, but not re-aligned, from the MAFFT alignment that served to construct the tree, i.e. 68 sequences from 62 species (see Methods – the exact number of species and sequences has been corrected). One duplicated paralog from *Kazachstania saulgeensis* was deleted; we presume that the Osh6-binding site in the second copy was lost after gene duplication. We have added an additional supplemental figure (Figure S7) that shows all sequences used in the calculation of the conservation score.
The subset of 34 sequences corresponds to a monophyletic clade that includes Saccharomycodacae and Saccharomycetacea. This monophyletic clade (purple on the tree) has been described by Shen et al. (2018), but it has no name. Remarkably, in this unnamed clade, all species (retrieved in database) have a unique TMEM16 with a long C-ter tail containing PM-recognition patch and one conserved motif in the middle found to be implied in the interaction between Ist2p and Osh6p.

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We agree with the reviewer that is in not completely clear what happens during the assay. However, previous work by us and others (Maeda 2013, Moser 2015, Lipp 2019) is robust and the results are consistent with results obtained in vitro for various Osh6 mutants. Furthermore, Spira et al., Nat Cell Biology 2012, demonstrated that lysoPS addition to cho1¤ cells largely corrects the organization of PM-localized proteins to the WT situation. We added a reference to Spira et al. to the Results section.

We have addressed this question extensively in a previous Methods paper: Ambrosio et al., 2019. In brief, yes, C2Lact-GFP does recognize lyso-PS. How can we then distinguish between the exogenously-added lyso-PS in the PM and the lyso-PS-generated PS that has been transported to the PM from the ER by Osh6? As we explain in Ambrosio et al., the assay only works when lyso-PS concentration in the medium is low. In this case, lyso-PS is taken up by the cells and we can observe a small and transient increase in Lact-C2 PM fluorescence, which then returns to basal level. A lag phase ensues, during which Lact-C2 remains cytosolic and lyso-PS is accumulating at the ER and being acylated into PS by ER-localized acyl-transferases (probably primarily Ale1). A threshold level of PS at the ER needs to be achieved before PS transport to the PM can be observed, and it is thanks to this threshold and the fact that lyso-PS does not accumulate at the PM that this assay works.

2. The accumulation of PS in the ER is hard to conclude - for example, the graphs in Fig 6B show that peripheral staining is reduced and internal signal is increased. Is the C2(Lact)-GFP probe actually associated with the ER or simply diffusely present in the cytoplasm? If it cannot be conclusively shown that the probe is at the ER, then several statements (e.g. p 7, main para 2, line 7) stating that 'PS accumulated at the ER' should be rephrased.

The ER has a complex geometry as its surface is at least 10 times larger than that of the PM (see e.g. Cell Biology by the Numbers; http://book.bionumbers.org/how-big-is-the-endoplasmic-reticulum-of-cells/). These two properties imply that PS accumulation at the ER cannot give an intense and well localized signal in contrast to the signal at the PM. Maeda et al., 2013 (Suppl fig 8), already showed that C2Lact-GFP in *cho1 osh6 osh7* cells colocalizes with mCherry-HDEL after lyso-PS addition, even if the intensities of signals are not the same.

3. Related to points 1 and 2, the steady state pool of PS is proposed to be mainly in the ER lumen (with the exception of a recent paper that indicates otherwise - the authors do not cite this paper) - how does this impact the authors' assay? Obviously the C2(Lact)-GFP probe would not recognize PS in the ER lumen, but as PS is constitutively scrambled, it may end up trapping PS on the cytoplasmic face of the ER.

Our assay relies on the use of *cho1¤* cells, which lack the PS synthase Cho1 and therefore have no endogenous PS. PS is synthesized via acylation of lyso-PS. The active site of acyl transferases is believed to be cytosolic, therefore we expect that in this case, as in WT cells, PS is produced in the cytosolic leaflet of the ER membrane. However, we have no information on the distribution of PS within the ER in this case. We agree that this is a very interesting question, but beyond the scope of this study.
The study of Tsuji et al 2019 is technically impressive, but their results are in striking contrast with what was reported previously regarding PS asymmetry within membranes, ER in particular, at steady state. We hope that additional studies will resolve this controversy; our data does not address it, which is why we did not include this reference initially. However, we completely agree with the reviewer that these questions are highly relevant for understanding how Osh6 and Ist2 cooperate. We have added a sentence in the Discussion and we now include a ref to Tsuji 2019.

4. The change in the amount of peripheral PS seen in only one out of three experiments (Fig S3). This is not really the impression given in the main text (p7, main para 1, line 5).

We have added statistical analysis to Fig S3 (now Fig S4), which shows that the differences between WT cells and *osh6Δ osh7Δ* or *ist2Δ* cells are statistically significant in most cases. However, we completely agree that there is a variability in the steady-state distribution of C2Lact-GFP between individual experiments, which is why we show results of all our experiments. We have revised the text on p7 to further emphasize this point.

5. There is only one specific data set pertaining to Osh7 as far as I could see (Fig 2C, yeast 2-hybrid). There are no other explicit tests of Osh7's interaction with Ist2. yet, Osh7 is lumped in together with Osh6 in several places, starting at the bottom of p7. I guess that this is an extrapolation that the authors want to make, but as the dataset for Osh7 is less complete/rigorous, perhaps they should qualify their conclusion a little bit.

We have added additional data to Fig S2, showing fluorescence complementation between Osh7-VN and Ist2-VC in the BiFC assay at the cortical ER. Furthermore, it has been shown by Schulz et al., 2009, that Osh6-GFP and Osh7-GFP display a very similar localization at the cortical ER. Other data that we show in the manuscript also strongly suggest that like Osh6, Osh7 localizes to the cortical ER via Ist2: conservation of the two sequences (70% identity), in particular of residues D141 and L142, involved in the interaction with Ist2 in Osh6 (Fig. 7C and 7D and associated text), and the lack of PS transport in cells with mutations in Ist2-tail, which contain WT copies of *OSH6* and *OSH7* (Fig. 6A,B). However, as the reviewer points out, the majority of our experiments follow Ohs6 and not Osh7. We have qualified our conclusion about Ohs7 in the Discussion accordingly.

6. Figure 1A is barely convincing. I realize that the work with Ist2 speaks for itself, but this is hardly the candidate that jumps out when looking at the silver-stained gel! Is the identity of the strong band (comparable intensity to Osh6-TAP) ~55 kDa known?

Indeed, there are many bands in the sliver-stained gel that are intriguing! However, I hope the reviewer will understand that when we saw “Ist2” in the list of names, we immediately focused on it given its known localization at ER-PM contact site. In other words, mass spectrometry was the preliminary experiment that led us to explore the possible interaction between Osh6 and Ist2. We have now moved this figure to Suppl. Fig1. We used other experiments, as presented in the paper, to confirm this interaction.

7. Methods: was the mass spec analysis done in replicate? how was the 'high pressure lysis' of the yeast cells achieved? 'Fluorescent Microscopy' on p13, should be Fluorescence Microscopy.

The mass spec analysis was not done in replicate (see above). It will be important to repeat this experiment under different conditions and using different constructs to identify other possible partners of Osh6. This will be (we hope!) the object of future work – ours or somebody else’s, which is why we think it is important to publish the full data set of this experiment and the exact experimental conditions. We have added additional clarification for how the cell lysis was achieved. We have corrected the typo on p13.

8. Fig 1C - the labels are switched in the figure legend vs the figure itself. Osh6-VC should be Osh6-VN, or vice versa. A control for BiFC is also missing - BiFC is not very reliable and requires a negative control to show that the seen interaction is not unspecific.

We thank the reviewer for pointing out this typo, which we have now corrected. We have also included a control with truncated Ist2 (missing the whole cytosolic tail), tagged with VC, which does not show any complementation with Osh6-VN.

9. Page 10, last line. The Bushell 2019 paper is not really relevant to the discussion and I think that the paper comes to the wrong conclusion. TMEM16K is a very poor scramblase; it is also Ca-activated. The ER has constitutive phospholipid scramblase activity, so it is hard to see how Ist2 and Osh6 could be functionally coupled in this regard.

We have revised the final paragraph of the discussion to not overstate the possibility that TMEM16K functions as a scramblase. However, we still think that it is important to cite the Bushell 2019 study, as we find this to be the most convincing demonstration of a mammalian TMEM16 protein localizing to the ER. We now include an additional reference, Jha 2019, which suggests that TMEM16H, which is most closely related to TMEM16K, may also reside with its TM part in the ER. As the reviewer points out, it is currently not known what is the function of the TM domain of Ist2; however, its homology with the TMEM16 proteins (which is highest for TMEM16H and K for the mammalian proteins) is impossible to disregard. Future work will be required to make conclusions beyond this one.

Second decision letter

MS ID#: JOCES/2020/243733

MS TITLE: Osh6 requires Ist2 for localization to the ER-PM contacts and efficient phosphatidylserine transport

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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. Thanks you for the careful and extensive revisions to your work. I did not on this occasion feel the need to return it to the reviewers.