Journal of Cell Science

Lsp1 partially substitutes for Pil1 function in eisosome assembly under stress conditions

Petra Vesela, Jakub Zahumensky and Jan Malinsky
DOI: 10.1242/jcs.260554

Editor: Jennifer Lippincott-Schwartz

Review timeline
Original submission: 22 August 2022
Editorial decision: 26 September 2022
First revision received: 7 December 2022
Accepted: 22 December 2022

Original submission

First decision letter

MS ID#: JOCES/2022/260554

MS TITLE: Lsp1 complements Pil1 function in eisosome assembly under stress conditions

AUTHORS: Petra Vesela, Jakub Zahumensky, and Jan Malinsky
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.

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 provides new insights into the role of eisosome-associated BAR domain-containing protein, Lsp1 under stress conditions. Previous reports have described the role of Pil1, a paralog of Lsp1, concerning eisosome formation, but little is understood about the physiological role of Lsp1,

which is why lsp1Δ cells do not significantly affect eisosome formation under normal conditions is a reason for this. This study shows that Lsp1 cooperatively contributes to eisosome formation with Seg1 and Nce102 even in the absence of Pil1 due to heat or salt stress, which is an important finding for understanding the role of the eisosome during stress. In addition, by comparing the gene expression profiles of wild-type, pil1Δ, and pil1Δ seg1Δ nce102Δ triple-deficient strains, the authors confirmed that the expression levels of various genes involved in oxidative phosphorylation, tricarboxylic acid cycle, DNA replication, ribosome biogenesis and cell cycle are affected in the triple-deficient strain, whereas the pil1Δ strain does not show significant differences compared to the wild-type strain.

Comments for the author

Major concerns

While the authors present very convincing data on Lsp1-dependent eisosome formation during stress, which has not been clarified before, however, the actual contribution of Lsp1-dependent eisosome formation to stress adaptation during stress seems to be less clear. To discuss the role of Lsp1-dependent eisosome formation in stress adaptation, pil1Δ lsp1Δ cells should also be investigated further in terms of the following comments.

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Furthermore, it would be better to analyze whether Seg1 and Lsp1 co-localize under stress conditions in pil1Δ cells. It is not indicated whether there is a statistically significant difference in Fig. 6B, C. If there is no significant difference, then the expression line 243 "significantly decreased" is not appropriate.

Since Seg1 and Nce102 are required for induction of Lps1-dependent eisosome formation under stress conditions, the authors hypothesize that Lsp1 plays a role in stress adaptation. To directly confirm this idea, the growth of pil1Δ lsp1Δ under a NaCl condition at 37°C should also be added in Fig. 7.

The complete loss of eisosome formation observed at a steady state and under stress conditions in the triple deficient strain, pil1Δ nce102Δ seg1Δ in Fig. 6A is very interesting and leaves no doubt that Lsp1 may play some unknown role under stress conditions. However, I wonder why they did not perform a comparative analysis of gene expression profiles for pil1Δ lsp1Δ. The authors should be explained this.

Additional comments

This manuscript discussed Lsp1-dependent induction of eisosome formation using 1 M NaCl (Fig. 1) and sorbitol (Fig. 4) treatments as the same hyperosmotic stress. However, the results of Fig. S3 indicate that the induction of eisosome formation by Lsp1 under stress conditions does not require a Hog1 kinase-dependent osmotic stress response, and it remains to be discussed whether NaCl-induced stress is because of Na+ or Cl-. The authors should be discussed.

It is interesting that in Fig. 3E, and Fig. S2E, pil1Δ lcb1-100 cells show almost normal eisosome formation as the wild-type cells, but only a little eisosome formation is seen in myriocin-treated cells. The effect of PHS treatment on eisosome formation in pil1Δ lcb1-100 cells should be investigated.

In Fig. 4E, in lcb1-100, a band corresponding to Lsp1-GFP shows faster gel mobility than that in λPPase treated Lsp1-GFP (a dephosphorylated form of Lsp1-GFP). Is this difference in gel mobility due to factors other than phosphorylation?

Minor comments

In Fig. 2, the lack of a concise title at the top of each image makes the data difficult to read. This could be improved by adding a title for each image.

Fig. 4E, F in line 206 should be replaced with Fig. S3A, B.

Fig. 4D, E in line 216 should be replaced with Fig. 4E.

Reviewer 2

Advance summary and potential significance to field

Vesela et al identify stress conditions (heat stress, salt stress, reduced activity of SPT) under which Lsp1, a previously uncharacterised member of eisosomes that was considered inessential to eisosome assembly,, is sufficient for the formation of a reduced number of eisosome-like structures in the absence of the main organizer, Pil1. They show this using confocal microscopy and freeze-etch electron microscopy. They go on to identify Seg1 and Nce102 as essential factors for the formation of these Lsp1-dependent eisosomes, while they provide some evidence that the phosphorylation of Lsp1 could be important for Lsp1-eisosome assembly. By performing NGS-based comparative transcriptomics of a strain lacking PIL1, SEG1 and NCE102, they identify important differences in gene expression that will allow future analysis of the physiological role of eisosomes. The work is novel and very interesting, the manuscript is very well written and the authors have payed particular attention to keep up with recent and relevant bibliography. Yet, this reviewer feels that more details on the mechanism of the formation of Lsp1-dependent eisosomes would further strengthen the manuscript, since the authors are in a very good position to provide more information about the molecular mechanism and not solely stick to the description of the phenomenon.

Comments for the author

Major comments:

1. In the title, the authors state that Lsp1 “Complements” Pil1 function under stress conditions. To this reviewer’s view, If the function of Lsp1 could complement the function of Pil1, overexpression of Lsp1 would have been able to rescue the reduced number of eisosomes in the absence of Pil1, which is not the case. Moreover,a complementary role of Lsp1 could be claimed if an increase in the number and/or size of MCCs was observed under the described stress conditions, when Pil1 and Lsp1 are simultaneously present. Yet, the results presented do not support a physiological role of Lsp1 in the presence of Pil1. The phenotypes described are minor and only evident in the absence of Pil1. In the presence of Pil1, the absence of Lsp1 seems to have no apparent phenotype. I would, thus, suggest the authors to either provide evidence about / more clearly indicate a physiological role of Lsp1 in the presence of Pil1, or to reduce the tone of their findings, both in the title (for example state that “Pil1-independent partial eisosome assembly by Lsp1 under stress conditions”, and the abstract, e.g. line 21 replace “takes over” with “partially bypasses”. Alternatively, focus could also be set on the formation of the furrow-like invaginations themselves; despite the fact that the number of Lsp1-dependent furrows seems to be reduced to less than 40 % of the wt, the shape of these furrowlike invaginations seems comparable to the wt, Pil1-derived ones.

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3. Regarding Lsp1 phosphorylation in Figure 4E, several inconsistencies exist. First, it is not clearly mentioned which band is supposed to correspond to the non-phosphoyrylated Lsp1. According to the λ-phosphatase treatment, I would say band B. Second, the authors claim that Lsp1 phosphorylation is less pronounced in pil1Δ cells after sorbitol treatment or in the lcb1-100 mutant, while, to this reviewer’s view, it seems to be exactly the opposite. Both conditions, especially the

lcb1-100 mutant, result in an increase in band A and a decrease in band B, which corresponds to the dephosphorylated form of Lsp1 according to phosphatase treatment. I would advise the authors to revise this part of the manuscript, and potentially include some more data, like the ones proposed in my major comment 4.

4. In Lines 209-217 the authors conclude that the remodeling of eisosome remnants into Lsp1-eisosomes is regulated by the phosphorylation of Lsp1. Although quite likely, this has not been formally shown and more mechanistic insight would be much appreciated if possible. Some open questions are the following. Which is/are the kinase(s) that phosphorylate(s) Lsp1, is it Pkh1 and or Pkh2 as previously reported? And is this phosphorylation sufficient for the formation of stress-induced Lsp1 eisosomes? The authors could take the time to answer these questions and make the story more complete. For example, in addition to my comments in major comment 3, they could examine Lsp1 phosphorylation in mutants of the Pkh1/2 kinases. Additionally, they could potentially try to design phospho-mimicking and/or phospho-null mutants of Lsp1 that would show constitutive (even in the absence of stress) or null Lsp1-eisosome stress-induced formation in the absence of Pil1, respectively.

5. Regarding the conclusions in lines 261-271 about Figure 7B: the authors conclude that the effect of deletion of eisosomal genes on the halotolerance of yeast is not only due to missregulation of sphingolipid biosynthesis, since it seems that only deletion of PIL1 affects resistance to myriocin. Yet, the results presented in Figure 7B have only been performed at 28 oC and not at 37 oC, the temperature under which the effect of deletion of eisosomal genes on the halotolerance of yeast is more evident (Figure 7A). Have the authors tried to monitor the sensitivity to Myriocin at 37 oC, in order to be able to better correlate the effect of PIl1 deletion on sphingolipid biosynthesis with that on halotolerance? Moreover, have they tried to see if small concentrations of myriocin, which would reduce the potentially increased biosynthesis of sphingolipids in the pil1Δ strain, could rescue the increased halotolerance of the pil1Δ strain at 37 oC? Such a growth test could reveal whether increased sphingolipid biosynthesis somehow decreases the halotolerance of yeast. Moreover, this halotolerance seems to be rescued in the triple mutant, raising the question of whether the formation of Lsp1 eisosomes is related to this phenotype. Have the authors performed similar growth tests with the pil1Δ lsp1Δ strain?

Minor comments:

1. Lines16-17. The way the sentences are structured indicate that members of the Pil1 family organize eisosomes also in alge, something that has not been shown. The authors could modify the second sentence as follows: “In fungi, they are organized...”

2. L137-138. In order to show that the addition of exogenous PHS has a effect to Lsp1-derived eisosomes that is comparable to heat stress, a direct statistical comparison should be shown in quantifications of Figure 3. To this reviewer’s view, the effect of PHS seems less pronounced than that of heat stress.

3. L193. Similarly, the authors could include a heat-stress control in the graphs and statistical analyses of figure 4, for directly comparing the effect of HOG or CFW activation with the effects mentioned in line 193.

4. In line 206, Figures 4E and 4F refer to results that are shown in Figure S3.

5. Line 236. In the pil1Δ lsp1Δ strain discernible Seg1-GFP foci are always present, as the authors also acknowledge in lines 231-232. So, I would suggest to rephrase the results about the double mutant.

6. Line 255. Correct “homologous” to “paralogous”.

First revision

Author response to reviewers' comments

Reviewer 1

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This manuscript provides new insights into the role of eisosome-associated BAR domain-containing protein, Lsp1 under stress conditions. Previous reports have described the role of Pil1, a paralog of Lsp1, concerning eisosome formation, but little is understood about the physiological role of Lsp1, which is why lsp1Δ cells do not significantly affect eisosome formation under normal conditions is a reason for this. This study shows that Lsp1 cooperatively contributes to eisosome formation with Seg1 and Nce102 even in the absence of Pil1 due to heat or salt stress, which is an important finding for understanding the role of the eisosome during stress. In addition, by comparing the gene expression profiles of wild- type, pil1Δ, and pil1Δ seg1Δ nce102Δ triple-deficient strains, the authors confirmed that the expression levels of various genes involved in oxidative phosphorylation, tricarboxylic acid cycle, DNA replication, ribosome biogenesis, and cell cycle are affected in the triple-deficient strain, whereas the pil1Δ strain does not show significant differences compared to the wild-type strain.

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The authors speculate that the formation of eisosomes during stress is dependent on Lsp1, and indeed, the complete absence of plasma membrane foci visualized by Seg1-GFP in the pil1Δ lsp1Δ cells in Fig. 5 is very convincing. However, freeze-fracture electron microscopy images should be used to determine if there is a complete loss of eisosome formation in pil1Δ lsp1Δ cells as shown in Fig. 2. Furthermore, it would be better to analyze whether Seg1 and Lsp1 co-localize under stress conditions in pil1Δ cells. It is not indicated whether there is a statistically significant difference in Fig. 6B, C. If there is no significant difference, then the expression line 243 "significantly decreased" is not appropriate.

The *lsp1Δpil1Δ* cells do not form eisosomes. However, their plasma membrane is not completely flat, and like the *pil1Δ* mutant cells, they form eisosome remnants. To make this clearer we have included respective images with adjusted contrast in Fig. 5A and indicated the position of eisosome remnants with arrowheads directly in the images. We have also adjusted the text of the Results section to make this clear to the reader. In transmission electron microscopy, these remnants (Fig. R1) contain convoluted plasma membrane material and do not differ at first glance from those described in *pil1Δ* strain (e.g., Supplementary Figure in the study of Stradalova et al., JCS 2009). Such structures are extremely prone to breakage during the procedure of freeze-fracture and pictures of their intact surface are therefore quite rare. For all these reasons, we do not see a clear benefit of the eventual inclusion of the freeze-fractured detail of eisosome remnant in *lsp1Δpil1Δ* mutant to the Results part.

We have analyzed the relative localization of Seg1 and Lsp1 and found that they colocalize under control conditions, as well as under both heat and salt stress, as expected. As Fig. 5 is focused on salt stress, we added the colocalization under salt stress as Fig. 5E.

We have changed the text in 243 to not include the word “significantly”.

Since Seg1 and Nce102 are required for induction of Lps1-dependent eisosome formation under stress conditions, the authors hypothesize that Lsp1 plays a role in stress adaptation. To directly confirm this idea, the growth of pil1Δlsp1Δ under a NaCl condition at 37˚C should also be added in Fig. 7.

We have included the growth analysis of *lsp1*Δ and *pil1*Δ*lsp1*Δ mutants under both salt stress and myriocin treatment, at both 28˚C and 37˚C, as requested. We adjusted the Results section accordingly. As documented by the added data, the phenotypes of the strains lacking the *LSP1* gene do not differ from their respective parent strains under respective conditions.

The complete loss of eisosome formation observed at a steady state and under stress conditions in the triple deficient strain, pil1Δnce102Δseg1Δ in Fig. 6A is very interesting and leaves no doubt that Lsp1 may play some unknown role under stress conditions. However, I wonder why they did not perform a comparative analysis of gene expression profiles for pil1Δlsp1Δ. The authors should be explained this.

As we wrote in the Results part: "Our data indicate that the newly constructed eisosome-less *seg1*Δ*nce102*Δ*pil1*Δ strain has growth phenotypes distinct from both the wild type and the *pil1*Δ strain." This was the reason why only these three strains were compared in our analysis. In our growth assays, we did not find a difference in the growth of *lsp1*Δ*pil1*Δ and *pil1*Δ strains under any tested condition, just as there was no difference between *lsp1*Δ and the wild type. In addition, as evidenced above, eisosome remnants form in the *lsp1*Δ*pil1*Δ strain, which is not the case in the *seg1*Δ*nce102*Δ*pil1*Δ strain. We, therefore, did not expect any interesting result to be obtained from whole transcriptome analysis of any of the two strains lacking *LSP1*.

Additional comments

This manuscript discussed Lsp1-dependent induction of eisosome formation using 1 M NaCl (Fig. 1) and sorbitol (Fig. 4) treatments as the same hyperosmotic stress. However, the results of Fig. S3 indicate that the induction of eisosome formation by Lsp1 under stress conditions does not require a Hog1 kinase-dependent osmotic stress response, and it remains to be discussed whether NaCl-induced stress is because of Na+ or Cl-. The authors should be discussed.

We agree with the Reviewer that NaCl-induced cellular response represents quite a complex process combining responses to hyperosmotic stress and the stress evoked by specific ions. As documented by the data in the manuscript, we see the same effect following NaCl and sorbitol treatment, suggesting that the observed changes are not due to cationic/anionic (sodium/chlorine) stress, but rather osmotic stress. As we admit in the manuscript, for the time being, we did not identify the trigger at the molecular level leading to Lsp1-eisosome formation. We cannot even say whether it is a specific protein factor taking part here, or whether it is a direct reaction to the changes in the local lipid composition of the membrane, or in membrane tension, to name a few possibilities. At the present time, we can only speculate. Concerning the role of Hog1 pathway, we can only repeat the sentence already written in the Results: "If HOG pathway takes part in the process, then the step triggering eisosome formation lies upstream of Hog1."

It is interesting that in Fig. 3E, and Fig. S2E, pil1Δ lcb1-100 cells show almost normal eisosome formation as the wild-type cells, but only a little eisosome formation is seen in myriocin-treated cells. The effect of PHS treatment on eisosome formation in pil1Δ lcb1-100 cells should be investigated.

The difference between the *lcb1-100* and myriocin-treated wild type is that the former has chronically low sphingolipid levels (but not zero) and is able to compensate. The myriocin-treated cultures, on the other hand, have completely stopped sphingolipid biosynthesis, and continue to divide only until the preexisting sphingolipids are able to support the growth of the new bud, roughly for 5 hours (Sun et al., 2000; doi: 10.1128/MCB.20.12.4411-4419.2000).

As suggested, we investigated the effect of PHS on the Lsp1 eisosomes in the *pil1*Δ *lcb1-100* strain. Specifically, we either exposed exponentially growing cultures to 20 μM and 50 μM PHS for 2 hours or cultivated them in the presence of PHS (1, 3, 10, 20, 30, 50 μM; added as a spike at time t=0)

for 6 or 24 hours. We found that the density of eisosomes in the plasma membrane decreased in a dose- dependent manner. The effect was not extensive and was more pronounced when 10 µM PHS was added to the preculture. PHS had a growth inhibitory effect, as evidenced by decreased OD_{600} compared to the non-treated control, increased mean cell size, and a decreased incidence of buds (especially apparent in the 6-hour cultures). These results indicated a negative effect of PHS on the cell cycle. For these reasons, unambiguous interpretation of these data is difficult.

In Fig. 4E, in lcb1-100, a band corresponding to Lsp1-GFP shows faster gel mobility than that in λPPase treated Lsp1-GFP (a dephosphorylated form of Lsp1-GFP). Is this difference in gel mobility due to factors other than phosphorylation?

We believe that the faster gel mobility of this band was due to its significantly higher load. This issue originated partially from the inherently high difference in the Lsp1 protein amount between *pil1* and *pil1lcb1-100* samples and partially because it is not possible to use either Pierce BCA or Bradford assay with the specific lysis buffers (due to the presence of DTT and Triton) used for the analysis of protein phosphorylation. We repeated the experiment using a NanoDrop ND-1000 Spectrophotometer, which we were able to borrow temporarily, to directly measure the protein amount in our samples. Correct loading completely abolished the faster migration of the *lcb1-100* sample (Fig. R2). However, corresponding to the change in Lsp1-GFP intensity in Fig. 3E, the detected Lsp1-GFP amount in the sample was very low compared to those detected in other lanes, at a level comparable to the background. This made the analysis of the *lcb1-100* band intensities unreliable. For this reason, we decided to omit the RH1800 cells from the Lsp1 phosphorylation analysis in the revised version of the manuscript.

Minor comments

In Fig. 2, the lack of a concise title at the top of each image makes the data difficult to read. This could be improved by adding a title for each image.

We added descriptions to each image as requested. We also modified the Figure legend to make it clearer and prevent any confusion for the reader.

Fig. 4E, F in line 206 should be replaced with Fig. S3A, B.

Thank you, we have corrected this mistake.

Fig. 4D, E in line 216 should be replaced with Fig. 4E.

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Reviewer 2

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Vesela et al identify stress conditions (heat stress, salt stress, reduced activity of SPT) under which Lsp1, a previously uncharacterised member of eisosomes that was considered inessential to eisosome assembly, is sufficient for the formation of a reduced number of eisosome-like structures in the absence of the main organizer, Pil1. They show this using confocal microscopy and freeze-etch electron microscopy. They go on to identify Seg1 and Nce102 as essential factors for the formation of these Lsp1-dependent eisosomes, while they provide some evidence that the phosphorylation of Lsp1 could be important for Lsp1-eisosome assembly. By performing NGS-based comparative transcriptomics of a strain lacking PIL1, SEG1 and NCE102, they identify important differences in gene expression that will allow future analysis of the physiological role of eisosomes. The work is novel and very interesting, the manuscript is very well written and the authors have payed particular attention to keep up with recent and relevant bibliography. Yet, this reviewer feels that more details on the mechanism of the formation of Lsp1-dependent eisosomes would further strengthen the manuscript, since the authors are in a very good position to provide more information about the molecular mechanism and not solely stick to the description of the phenomenon.

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The reviewer is correct. Our choosing the word "complement" was inappropriate. We have changed the phrasing to "partially substitute" to make the claims correspond better to the presented data.

2. Results in Figure 2 are most convincing. Yet, the authors have not formally shown that these invaginations formed under salt stress require Lsp1. The same experiment with the pil1Δ lsp1Δ strain could be included to strengthen the conclusions of the authors.

The idea of the requirement of Pil1-like proteins for plasma membrane furrows formation is not new and has been repeatedly verified in many cell-walled organisms by different research groups worldwide. *S. cerevisiae* contains only two proteins of the Pil1 family, Pil1 and Lsp1. We show in Fig. 5 that in the *lsp1Δpil1Δ* mutant, Seg1-GFP does not localize to clearly defined plasma membrane foci, only large eisosome remnants of irregular shape, in both the absence and presence of stress. In our opinion, this clearly indicates that Lsp1 is required for the formation of furrows under salt stress.

3. Regarding Lsp1 phosphorylation in Figure 4E, several inconsistencies exist. First, it is not clearly mentioned which band is supposed to correspond to the non-phosphoyrylated Lsp1. According to the λ-phosphatase treatment, I would say band B. Second, the authors claim that Lsp1 phosphorylation is less pronounced in pil1Δ cells after sorbitol treatment or in the lcb1-100 mutant, while, to this reviewer's view, it seems to be exactly the opposite. Both conditions, especially the lcb1-100 mutant, result in an increase in band A and a decrease in band B, which corresponds to the dephosphorylated form of Lsp1 according to phosphatase treatment. I would advise the authors to revise this part of the manuscript, and potentially include some more data, like the ones proposed in my major comment 4.

As we stated at the end of the legend for Fig. 4, "A, B, C denote non-phosphorylated and two phosphorylated bands, respectively; annotation based on previous reports (Luo et al, 2008)". The confusing irregularity in the original image was caused by the fact that one of the wells was overloaded, and proteins in different lanes did not migrate at the same rate. In the revised version of the manuscript, we show the result of another biological replicate of this experiment, in which we prevented the overloading. Now it should be clear that the lowest band corresponds to the non- phosphorylated protein. To facilitate the comparison, we also added density profiles into Fig. 4E. Unfortunately, the detected Lsp1-GFP amount in the *lcb1-100 pil1Δ* sample was systematically significantly lower compared to the *LCB1* sample (in accordance with the change in the overall intensity of Lsp1-GFP fluorescence in Fig. 3E), and the signal was comparable to the background. This made the analysis of the band intensities unreliable (Fig. R2). For this reason, we decided to omit the RH1800 cells from the Lsp1 phosphorylation analysis in the revised version of the manuscript.

4. In Lines 209-217 the authors conclude that the remodeling of eisosome remnants into Lsp1-eisosomes is regulated by the phosphorylation of Lsp1. Although quite likely, this has not been formally shown and more mechanistic insight would be much appreciated if possible. Some open questions are the following. Which is/are the kinase(s) that phosphorylate(s) Lsp1, is it Pkh1 and or Pkh2 as previously reported? And is this phosphorylation sufficient for the formation of stress-induced Lsp1 eisosomes? The authors could take the time to answer these questions and make the story more complete. For example, in addition to my comments in major comment 3, they could examine Lsp1 phosphorylation in mutants of the Pkh1/2 kinases. Additionally, they could potentially try to design phospho-mimicking and/or phospho-null mutants of Lsp1 that would show constitutive (even in the absence of stress) or null Lsp1-eisosome stress-induced formation in the absence of Pil1, respectively.

The identity of both specific Lsp1 phosphorylation sites and of a kinase responsible is indeed an intriguing issue, which we are currently pursuing further. However, it is a matter of considerable complexity and therefore beyond the scope of the current manuscript. For instance, the Pkh kinases are involved in the regulation of multiple pathways, such as sphingolipid biosynthesis (via Ypk kinases), cell wall integrity pathway (via Pkc1) and TORC1-related signaling (via Sch9). Eliminating the Pkh activity (by deletion of respective genes or use of the thermosensitive *pkh1-ts* allele) will necessarily affect multiple pathways, including those involved in the regulation of plasma membrane lipids and cell wall material. This will cause a change in the composition of these structures, and in turn, affect the propensity of Lsp1 to bind the plasma membrane. In addition, the individual pathways downstream of Pkh contain additional kinases. To pinpoint which of the possible kinases is indeed involved in Lsp1 phosphorylation is not completely straightforward. Besides Pkh, Ypk, Pkc1 and Sch9 kinases, there are additional candidate kinases that can play a role in the phosphorylation of Lsp1 under the examined stress conditions (http://netphorest.science/showPrediction.shtml?id=21592860970360939547597302145402681338 8). Furthermore, since there have been at least six Lsp1 phosphorylation sites reported in the literature, and more predicted (see above link), the analysis of all phosphor-mimicking and non-phosphorylatable mutants (either single site or in combination) is quite time-consuming. And, while there is a lot of information on phosphorylation sites in both Pil1 and Lsp1, a clear consensus about the effect of the phosphorylation of individual sites (or their combination) on the assembly of eisosomes is missing. We are of the opinion that the main message of our current manuscript should stay in the frame of its title: "Yes, there is a specific function for Lsp1 in the yeast *S. cerevisiae*, and this is pronounced during the stress response." Since the original Nature paper of Walther et al. (2006), which described the subcellular localization of both Pil1 and Lsp1, this information is completely new and, in our opinion, in itself also important for understanding the structure and function of the eisosome.

5. Regarding the conclusions in lines 261-271 about Figure 7B: the authors conclude that the effect of deletion of eisosomal genes on the halotolerance of yeast is not only due to missregulation of sphingolipid biosynthesis, since it seems that only deletion of PIL1 affects resistance to myriocin. Yet, the results presented in Figure 7B have only been performed at 28 oC and not at 37 oC, the temperature under which the effect of deletion of eisosomal genes on the halotolerance of yeast is more evident (Figure 7A). Have the authors tried to monitor the sensitivity to Myriocin at 37 oC, in order to be able to better correlate the effect of Pil1 deletion on sphingolipid biosynthesis with that on halotolerance? Moreover, have they tried to see if small concentrations of myriocin, which would reduce the potentially increased biosynthesis of sphingolipids in the pil1Δ strain, could rescue the increased halotolerance of the pil1Δ strain at 37 oC? Such a growth test could reveal whether increased sphingolipid biosynthesis somehow decreases the halotolerance of yeast. Moreover, this halotolerance seems to be rescued in the triple mutant, raising the question of whether the formation of Lsp1 eisosomes is related to this phenotype. Have the authors performed similar growth tests with the pil1Δ lsp1Δ strain?

Yes, we have indeed analyzed the growth of the mutants exposed to myriocin also at 37°C and found that the effects of increased cultivation temperature and sphingolipid synthesis inhibition are of the same character, and additive. This we expected, since increased temperature results in increased fluidity of the membrane, which is generally compensated by de-novo sphingolipid synthesis. Both sphingolipid inhibition and increase of cultivation temperature, therefore, represent conditions with increased sphingolipid demand. We have added respective images to Fig. 7 and adjusted the corresponding text in the Results section accordingly.

We did not combine myriocin and NaCl at either 28°C or 37°C.

We have included the growth analysis of *lsp1*Δ and *pil1*Δ*lsp1*Δ mutants under both salt stress and myriocin treatment, at both 28°C and 37°C, and adjusted the Results section accordingly. The phenotype of the *lsp1*Δ strains does not differ from their respective parent strains.

Minor comments:

1. Lines16-17. The way the sentences are structured indicate that members of the Pil1 family organize eisosomes also in alge, something that has not been shown. The authors could modify the second sentence as follows: "In fungi, they are organized..."

As the Reviewer pointed out, there is no evidence so far that Pil1 family proteins organize eisosomes in algae. Or, to cite an earlier study of Goodenough's group: …there is no evidence for a "universal" set of eisosome-associated *BAR* proteins in eukaryotes" (Lee et al., 2015). We have adjusted the phras- ing in the Abstract accordingly.

2. L137-138. In order to show that the addition of exogenous PHS has a effect to Lsp1-derived eisosomes that is comparable to heat stress, a direct statistical comparison should be shown in quantifications of Figure 3. To this reviewer's view, the effect of PHS seems less pronounced than that of heat stress.

We have added heat stress data to the quantification of the eisosome number in Fig. 3. We performed an unpaired *t*-test, which did not reveal any significant difference between the two treatments – heat stress and PHS addition.

3. L193. Similarly, the authors could include a heat-stress control in the graphs and statistical analyses of figure 4, for directly comparing the effect of HOG or CFW activation with the effects mentioned in line 193.

We assume that in this case, the Reviewer meant salt stress, as that is what is described in the respective paragraph. We hence added salt stress to the quantification of the eisosome number in Fig. 4. We performed an unpaired *t*-test, which did not reveal a significant difference between hyperosmotic stress induced by sorbitol and salt stress.

4. In line 206, Figures 4E and 4F refer to results that are shown in Figure S3.

Thank you, we have corrected this mistake.

5. Line 236. In the pil1Δ lsp1Δ strain discernible Seg1-GFP foci are always present, as the authors also acknowledge in lines 231-232. So, I would suggest to rephrase the results about the double mutant.

The reviewer is correct. We have adjusted the respective part of the Results.

6. Line 255. Correct "homologous" to "paralogous".

We changed as suggested.

Figures for Reviewers

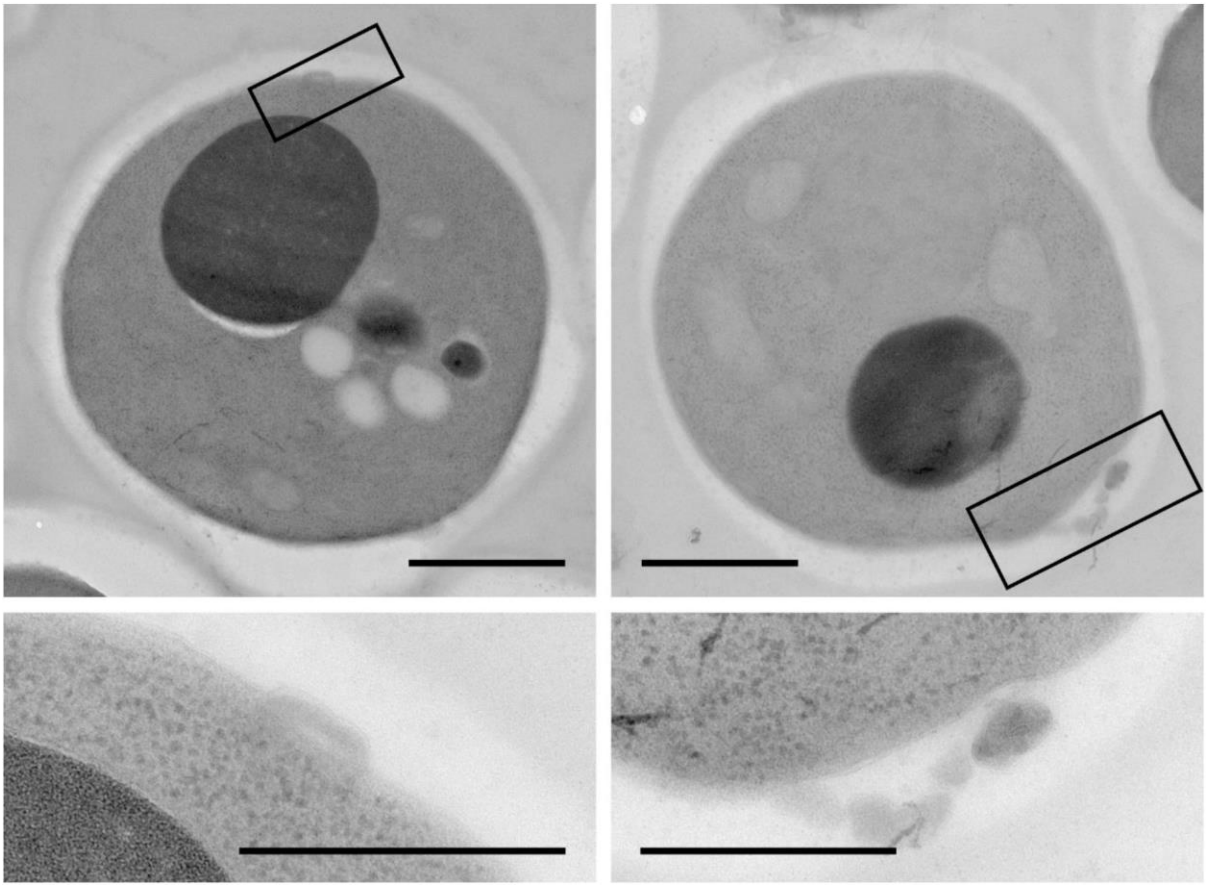

Fig. R1 Eisosome remnants in *pil1Δlsp1Δ* yeast. Two examples of *lsp1Δpil1Δ* cells with sectioned eisosome remnant on their surface (upper row) and respective details of these remnants (lower row, corresponding to black frames in upper row images), as revealed on a single transversal thin section using transmission electron microscopy. The sample was prepared as follows: Living yeast cells (overnight culture in YPD medium) were concentrated by suction filtration onto a filter and this was then placed onto a YPD agar plate. The yeast paste was scraped from the filter, put on a flat specimen carrier (Leica, 1.2 mm cavity diameter) and quickly frozen in a Leica EM PACT high-pressure freezer. Frozen samples in the carriers were transferred under liquid nitrogen to freeze substitution medium in cryovials and placed in a Leica AFS machine. Cells were freeze-substituted at -90°C for 3 days. Thereafter, the temperature was elevated to -50°C (5°C per hour) and samples were kept at this temperature for about 12 hours. After this period, the specimens were washed six times with fresh pre-cooled acetone at -50°C and then infiltrated with Lowicryl HM20. For the best structure preservation, the FS medium consisted of 3% glutaraldehyde (10% acetone stock; EMS), 0.1% uranyl acetate (20% methanolic stock; Polysciences) and 2% H_2O in acetone (glass distilled; EMS). Following the acetone wash at -50°C, the samples were infiltrated subsequently in 3:1, 1:1, 1:3 (v:v) acetone:HM20 mixtures for 4 hours each at -50°C; then incubated in 100% HM20 at -50°C for 2 hours and finally placed in fresh resin and polymerized with UV at -40°C for 48 hours, and at 20°C for ~3 days. Ultrathin sections (65 nm) were cut with Ultracut S ultramicrotome equipped with a diamond knife (35°; Diatome) and placed on copper formvar-coated grids. Sections were contrasted with a saturated aqueous solution of uranyl acetate for 1 h, washed, air-dried and examined in an FEI Morgagni 268(D) transmission electron microscope at 80 kV. Bars: 1 µm (upper row), 0.5 µm (lower).

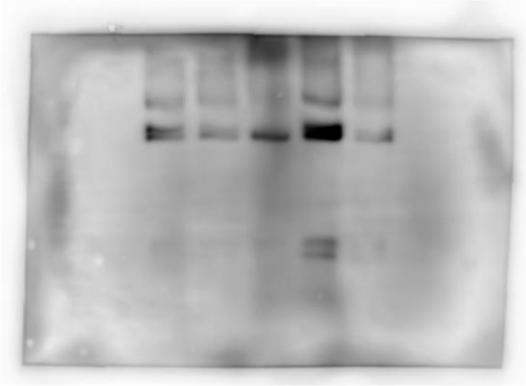

Fig. R2 Western blot analysis of Lsp1-GFP phosphorylation change in response to a decrease in SPT activity. We detected Lsp1-GFP in cell protein extracts prepared from BY4742-derived *LSP1-GFP* expressing *pil1*Δ cells grown exponentially for 6 hours and treated with either nothing (Lanes 1, 3) or with 1M sorbitol (Lane 2) for 25 minutes, and from RH1800-derived *LSP1-GFP* expressing *pil1*Δ cells carrying either native *LCB1* or the hypoactive *lcb1-100* mutant allele (Lanes 4, 5, respectively). To verify that the multiple detection bands originate in phosphorylation, the sample was treated with λ phosphatase (λPPase, Lane 3).

Second decision letter

MS ID#: JOCES/2022/260554

MS TITLE: Lsp1 partially substitutes Pil1 function in eisosome assembly under stress conditions

AUTHORS: Petra Vesela, Jakub Zahumensky, and Jan Malinsky
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 2

Advance summary and potential significance to field

Results provided are novel and important to the field (as stated in this reviewer's evaluation of the first version of the manuscript), and with the current modifications fully support the conclusions drawn.

Comments for the author

The authors have successfully addressed all my comments, either by additional experiments or via well-justified argumentation.