Sequestration of the exocytic SNARE Psy1 into multiprotein nodes reinforces polarized morphogenesis in fission yeast

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E20-05-0277
TITLE: Sequestration of the exocytic SNARE Psy1 into multiprotein nodes reinforces polarized morphogenesis in fission yeast

Dear Jaime,

Your manuscript was read by two experts in the field. While the Psy1 localization to nodes is intriguing, both reviewers had major concerns about the interpretations of the later figures and concluding model. I think that both reviewers make excellent points that touch on a number of important issues. While the data for the co-localization of Psy1 at the nodes is an interesting initial observation, the subsequent data suggesting Psy1 sequestration for regulation of exocytosis are not convincing. The reviewers suggest major new experiments to strengthen the proposed function of these Skb1-Psy1 nodes in regulation of exocytosis.

Because of the interesting initial observation and the high technical quality of the work, I encourage you to revise this manuscript with additional experiments to strengthen the conclusions and address the reviewers' concerns thoroughly. Some convincing evidence suggesting a function of Psy1 at the nodes is needed. However, I am aware that the list of substantive experiments needed to make a compelling case is rather open-ended. It is not clear to me where the data might lead. Thus, if you are not able to add significantly to this story at this time, you might consider submitting this to another journal. I would be happy to discuss further.

Additional notes from me:

1. In my view, the most important experiments will be to focus on Psy1 at nodes, and distinguishing whether effects of skb1 mutants are on Psy1 node localization or some other function of Skb1. Is Psy1 concentration increased at the lateral surface if it is not in nodes? Will inappropriate increase or depletion of Psy1 at the lateral regions (not a nodes) sufficient to produce predicted phenotypes?

2. The relevance of the osmotic shock data is not clear, and might be cut. Osmotic shocks can lead to depolarization of many tip proteins.

3. An alternative testable interpretation of Bgs4 FRAP result is that a larger percentage of the Bgs4 is localized at the one cell tip for some reason. Could this reflect a difference in monopolar vs. bipolar growth in the cells assayed? Is there more Bgs4 seen on the sides of cells in skb1D mutants? It is not clear to me how these results strongly support effects on exocytosis: Bgs4 dynamics may be poor proxy for exocytosis, as it is also regulated by endocytic pathways.

Sincerely,

Fred Chang
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Moseley,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you haveopted out of publishing the review history.

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Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

This report entitled "Sequestration of the exocytic SNARE Psy1 into multiprotein nodes reinforces polarized morphogenesis in fission yeast" characterized the localization of the SNARE protein Psy1

in S. pombe. It is found that Psy1 localizes to cortical nodes along the non-growing cell sides and a non-punctate diffuse pattern at growing cell tips. Cortical puncta localization of Psy1 depends on both Slf1 and Skb1 proteins, which this group previously found to be interdependent for cortical node formation. Cells lacking Slf1 or Skb1, and therefore lacking Psy1 at these nodes, display some genetic interactions with proteins involved in exocytosis and polarity. These findings led to a model in which Psy1's function is inhibited by interactions within Skb1/Slf1 cortical nodes, thus spatially restricting exocytosis to cell tips and promoting proper cell polarity.

While the new localization of Psy1 to cortical nodes is intriguing, and the experiments performed rigorously, the results at this stage do not unambiguously support a model of Psy1 sequestration and inhibition. A number of previous findings appear to be relevant to the model that aren't incorporated here, and some of the experimental results appear to be selectively interpreted. In sum, I have some major and minor concerns that I recommend are addressed.

Major concerns

1. In a previous paper from this group, Deng et al., 2014, it was shown that Sbk1 localized exclusively to megadalton cortical nodes, but Slf1 also localized to the cell tip membrane (figure 2B from Deng paper). This was best appreciated in images of Slf1 in the absence of Skb1. Thus, Slf1 is not exclusively localized to cortical nodes, but it is also present at cell tips and septa (Figure 3B from Deng paper). This raises the possibility that Slf1 regulates Psy1 at cell tips and septa, not nodes or in addition to nodes, and could explain why the absence of Sbk1 does not have the same penetrance as the absence of Slf1 in genetic interaction or other experiments. It seems also possible that it is Slf1 (what is the function of this protein?) that influences exocytosis directly. The different localizations and behavior of these two cortical node components should be more explicitly considered in terms of an effect on Psy1 and on exocytosis.
2. A major concern with the paper is that defects due to the loss of Sbk1 and Slf1 nodes are viewed solely through the lens of an effect on Psy1. Given 1)the known interaction of Sbk1 with the polarity kinase Shk1, 2)the complex localization pattern of Slf1, and 3) that the complete composition of Sbk1, Slf1 cortical nodes is unknown, this appears to be unwarranted. I am not convinced that the rescue of a single exocyst mutant by Psy1 overexpression is relevant to a sequestration role of cortical nodes given the function of Psy1. The work needs to go further to support that Psy1 sequestration underlies the genetic or localization data. Possibilities of experiments that will strongly support the sequestration model include sequestering all of Psy1 at tips (GBP-GFP tethering or protein fusion approaches) or making a mutant in Psy1 that fails to localize specifically to nodes. Other possible experiments to bolster the model: If Skb1 or Slf1 are overexpressed (as done in Deng et al 2014), does this result in more Psy1 localized in nodes and less at cell tips, and if so, does this have the same effects? Does overexpression of Psy1 that outruns node components result in ectopic exocytosis?
3. The statement about figure S1B "although we note that a minor portion of Slf1 was displaced from nodes" on page 4 does not match the data presented. Rather, it appears that hexanediol treatment results in a complete loss of Sfl1 from nodes and a redistribution of this protein along the entire plasma membrane. If this is the case, how is it that Slf1 is relocalized by hexanediol treatment but Sbk1 and Psy1 are not given their localization dependencies? Providing max projection in addition to the medial z-slice might be beneficial for interpreting this data.
4. Overall there seems to be a significant difference in the severity of phenotypes for slf1Δ compared to skb1Δ in experiments that examine both (Figure S2, 4C, S3A). Given that lack of either of these proteins results in loss of Psy1 from cortical nodes, it is unclear why there would be any difference between these strains if Psy1 sequestration is the relevant effect. Also, Figures 1B, 3A-B and figure 4D-E should include data for both of deletion strains in order to allow a better comparison between strains.

5. All single channel images should be inverted for clarity.

Minor concerns
1. There are inconsistencies in the the data in figure S2. What is claimed in the text and summarized in the table in S2A does not match all of the data from the spot assays in S2B. For instance, it is stated in the text that there is growth rescue for rho3Δ skb1Δ compared to the single mutant strains, however this is not supported by the spot assay. Rather, rho3Δ slf1Δ has a suppressed growth phenotype. Figure 3A should be amended to reflect the data more accurately.
2. Do the minus signs represent EV controls or no plasmid? Please indicate all these details in materials and methods or figure legend.
3. Please provide either a western blot or microscopy data confirming and characterizing Psy1 overexpression from Figure 3C.
4. Please provide the data of Sec4 localization without the added KCl, as this result is not shown but referenced in the text on page 6 paragraph 3.
5. Please explain the rationale for using the Sec5 marker vs the Sec8 marker in different figure 4 panels.
6. Please provide the p value for WT vs skb1Δ cell width in the figure legend and all data mentioned in text as not being statistically significant.
7. The rational for using osmotic stress in different experiments should be explained in the text.
8. Are there any genetic interactions between Slf1, Skb1 and Rga4/6?
9. In figure 1A figure legend "co-immunoprecipitation" and "coimmunoprecipitation" are both used
10. Were cell width measurements made with DIC images as shown in the figure or with Blankophor stained cells as indicated in Materials and Methods?

Figure design
1. As in figure 1B, a blow up of the GFP-Psy1 LifeAct-mCh is needed to be able to determine if the proteins do or do not co-localize as stated in the figure legend.
2. Label is missing for y axis in figure 1D.
3. Figure 2A and 2B are missing scale bars.
4. What is the overall elapsed time in right panel of 2B?
5. Figure 2B, it is difficult to see the dynamic exchange at the cell tips, perhaps only showing one cell tip but enlarging the image would be helpful.
6. Figure 4D µM is used instead of µm.
7. Figure 4D there is no scale bar.

Reviewer #2 (Remarks to the Author):

This manuscript by Miller, Moseley and colleagues presents an interesting observation on the plasma membrane t-SNARE Psy1 in fission yeast, which forms large clusters at the plasma membrane in the non-growing regions of the cell. These clusters are organized by the proteins Skb1 and Slf1, whose deletions the authors use to probe the consequence of loss of Psy1 clusters. They find that these cells exhibit genetic interactions with exocyst and formin mutants, and that exocyst markers ectopically localize to cell sides and cells acquire more round shapes when these mutants are sensitized either by salt stress or by further mutation of Cdc42 GTPases that prevent Cdc42 activity at cell sides. This leads them to conclude that Skb1-Slf1 nodes act to sequester Psy1 at cell sides to prevent exocytosis at these locations, and that this pathway acts in parallel to

Cdc42 inhibition to restrict growth to cell tips.

The proposed model of t-SNARE sequestration to help define sites of active growth is interesting and novel and I like it very much. However, I am not yet convinced that the data lends enough support to the model and I feel it is strongly over-interpreted. There are a number of points that need to be addressed, listed below.

1. The major issue is that the authors use the skb1Δ as a proxy for delocalization of Psy1 from lateral nodes, but do not convince that the phenotypes are caused by this delocalization rather than another role of Skb1. The fact that skb1Δ is used throughout the paper rather than slf1Δ is problematic. Given both deletions have the same effect on Psy1 distribution, but skb1Δ has stronger phenotypes, the simplest interpretation is that Skb1 has additional Psy1 distribution-unrelated functions. Thus, the slf1Δ phenotype is likely to more closely represent the phenotype of Psy1 absence from nodes. Using the skb1Δ phenotype is exaggerating the importance of this localization change and is thus misleading. It would be important to perform all experiments with slf1Δ and present them in the main figures, keeping those with skb1Δ for supplementary figures. If the genetic interactions, localization of exocyst to cell side and increase of cell roundness in combination with rga4Δ rga6Δ also occur in slf1Δ, even if the phenotypes are weak, I'll be more convinced that the sequestration proposed by the authors contributes to cell shape regulation.

2. Even with the use of slf1Δ rather than skb1Δ, the support for the model will remain a correlation. To lend strong support to the proposed conclusion, the authors would need to test the phenotype of specific mutations in Psy1 node localization (and/or test the effect of re-establishing this localization in the mutant backgrounds). While this is far from being a simple task and likely beyond the present study, there are steps the authors can take to help support their model. For instance, they could test whether psy1 mutants reverse the septation phenotypes of double mutant cells, as a way to probe whether the skb1Δ/slf1Δ phenotypes are due to changes in Psy1.

3. The idea of sequestration is that clustering takes Psy1 away from its diffuse membrane localization at non-growing cell regions. However, from the data presented, it is not clear whether the diffuse distribution of Psy1 described for growing tips is absent from cluster-containing regions. In the projection images of Psy1 in Fig 1, there seems to be a continuous line of signal all around the cell in addition to the punctate pattern at non-growing regions. This is an important question, as the interpretation of all results hinges on Psy1 in nodes reducing the amount of free Psy1. Could the authors quantify the relative levels of Psy1 outside nodes in non-growing vs growing cell regions and in non-growing cell regions in WT vs skb1Δ/slf1Δ? Is this level low at non-growing regions and increased in the mutants as implied by the interpretation? To test the idea of sequestration, it would also be interesting to perform FRAP experiments of GFP-Psy1 at cell tips to complement the analysis at clusters, to test whether the diffuse distribution of Psy1 represents a more dynamic population, as predicted.

4. More careful wording needs to be used to express indirect interpretations of results and avoid over-interpretation. In some case, the thinking needs to be clarified. For instance:

a. To my knowledge the role of Psy1 in exocytosis and growth at cell tips has not be characterized. The authors should acknowledge this with appropriate phrasing in their text. For instance, the statement "Psy1 is necessary for exocytosis and growth at cell tips" should be re-written as "Psy1 is predicted to be necessary for exocytosis and growth at cell tips" or similar.
b. The suppression of sec3-2 growth defect by overexpression of Psy1 is not particularly surprising: this is the suppression of an exocyst mutant by overexpression of a t-SNARE - a rather expected

finding. Similar suppression was previously observed of psy1 mutant growth defects by overexpression of v-SNARE proteins (Maeda et al, 2009). Interpreting this as a specific function linked to Psy1 localization to nodes or in free form is a vast over-interpretation.
c. I am not sure I follow the interpretation of the Bgs4 FRAP at cell tips in skb1Δ vs WT. A reduction in the mobile fraction means that there is a larger proportion of Bgs4 that is immobile *at cell tips*. How could this be explained by a re-direction to ectopic sites? If Bgs4 is generally re-directed to ectopic sites in skb1Δ, one would expect a lower steady-state level of this protein at cell tips. Is this the case? It is more difficult to explain a change in the ratio of mobile to immobile pools. Is the thinking that the amount of mobile (trafficking) Bgs4 is reduced but compensated for by an increase immobile (PM/cell-wall anchored) protein? This should be clarified.

Minor comments:

In the genetic interaction analysis (Fig S2), I am confused by why slf1Δ generally appears to cause less pronounced phenotypes, except in the case of rho3Δ, where it is showing an impressive suppression.

What is the effect of skb1Δ/slf1Δ on the septation index of for3Δ?

The transition to use of 0.5M KCl is rather abrupt, and this is not commented upon in Fig S2.

Why is Sec5-GFP first used as exocyst marker, and then Sec8-mNG? Are these two markers co-localizing in all conditions?

The presence of Sec8 at sides and rounder shape of skb1Δ rga4Δ rga6Δ mutants compared with rga4Δ rga6Δ mutants is in line with the model proposed by the authors that exocytosis on cell sides leads to rounder cell shape. However, it does not exclude the alternative view that the exocytosis on cell sides is a consequence of the rounder shape. This is a pervasive chicken-or-egg problem in cell morphogenesis. Because cell dimensions overlap at the population level, one way to address the problem would be to restrict the quantification of Sec8 localization to cells of equivalent width in the two genotypes.

Additional notes from the Editor:

1. In my view, the most important experiments will be to focus on Psy1 at nodes, and distinguishing whether effects of skb1 mutants are on Psy1 node localization or some other function of Skb1. Is Psy1 concentration increased at the lateral surface if it is not in nodes? Will inappropriate increase or depletion of Psy1 at the lateral regions (not a nodes) sufficient to produce predicted phenotypes?

AUTHOR RESPONSE: We have added new data quantifying the Psy1 concentration and dynamics at cell sides when it is present in nodes compared to when it is diffused and not in nodes (Figure 2 & 3). These experiments show that *skb1*Δ and *slf1*Δ mutants have increased diffuse Psy1 at cell sides. In addition, Psy1 dynamics at cell sides are much faster in *skb1*Δ and *slf1*Δ mutants, similar to the dynamics of Psy1 at cell tips in wild type cells. We also performed a structure-function analysis of Psy1 and used the results to generate a panel of point mutants, including the new psy1-Ha-m1 mutant that no longer localizes to nodes. In addition to losing node localization, this mutant exhibits ectopic exocytosis at cell sides and increased cell width (Figure 7 & 8), consistent with our model that Psy1 sequestration into Skb1-Slf1 nodes at cell sides promotes polarized morphogenesis (Figure 9). We feel that this new mutant directly addresses concerns regarding the functional role of Psy1 localization to nodes.

2. The relevance of the osmotic shock data is not clear, and might be cut. Osmotic shocks can lead to depolarization of many tip proteins.

AUTHOR RESPONSE: We removed the osmotic shock data as suggested.

3. An alternative testable interpretation of Bgs4 FRAP result is that a larger percentage of the Bgs4 is localized at the one cell tip for some reason. Could this reflect a difference in monopolar vs. bipolar growth in the cells assayed? Is there more Bgs4 seen on the sides of cells in skb1D mutants? It is not clear to me how these results strongly support effects on exocytosis: Bgs4 dynamics may be poor proxy for exocytosis, as it is also regulated by endocytic pathways.

AUTHOR RESPONSE: We have modified our interpretation of the Bgs4 data based on the helpful suggestions here and from Reviewer 2 (See page 5 of revised MS).

Reviewer #1 (Remarks to the Author):

This report entitled "Sequestration of the exocytic SNARE Psy1 into multiprotein nodes reinforces polarized morphogenesis in fission yeast" characterized the localization of the SNARE protein Psy1 in S. pombe. It is found that Psy1 localizes to cortical nodes along the non-growing cell sides and a non-punctate diffuse pattern at growing cell tips. Cortical puncta localization of Psy1 depends on both Slf1 and Skb1 proteins, which this group previously found to be interdependent for cortical node formation. Cells lacking Slf1 or Skb1, and therefore lacking Psy1 at these nodes, display some genetic interactions with proteins involved in exocytosis and polarity. These findings

led to a model in which Psy1's function is inhibited by interactions within Skb1/Slf1 cortical nodes, thus spatially restricting exocytosis to cell tips and promoting proper cell polarity.

While the new localization of Psy1 to cortical nodes is intriguing, and the experiments performed rigorously, the results at this stage do not unambiguously support a model of Psy1 sequestration and inhibition. A number of previous findings appear to be relevant to the model that aren't incorporated here, and some of the experimental results appear to be selectively interpreted. In sum, I have some major and minor concerns that I recommend are addressed.

Major concerns

1. In a previous paper from this group, Deng et al., 2014, it was shown that Sbk1 localized exclusively to megadalton cortical nodes, but Slf1 also localized to the cell tip membrane (figure 2B from Deng paper). This was best appreciated in images of Slf1 in the absence of Skb1. Thus, Slf1 is not exclusively localized to cortical nodes, but it is also present at cell tips and septa (Figure 3B from Deng paper). This raises the possibility that Slf1 regulates Psy1 at cell tips and septa, not nodes or in addition to nodes, and could explain why the absence of Sbk1 does not have the same penetrance as the absence of Slf1 in genetic interaction or other experiments. It seems also possible that it is Slf1 (what is the function of this protein?) that influences exocytosis directly. The different localizations and behavior of these two cortical node components should be more explicitly considered in terms of an effect on Psy1 and on exocytosis.

AUTHOR RESPONSE: We have now added data with *slf1*Δ cells to compare to *skb1*Δ cells in Figures 2-6 in the revised MS. We also explicitly state in the Results and Discussion that there are differences in the severity of *skb1*Δ and *slf1*Δ mutations, "likely reflecting differences in the functions and protein-protein interactions for Skb1 and Slf1."

2. A major concern with the paper is that defects due to the loss of Sbk1 and Slf1 nodes are viewed solely through the lens of an effect on Psy1. Given 1)the known interaction of Sbk1 with the polarity kinase Shk1, 2)the complex localization pattern of Slf1, and 3) that the complete composition of Sbk1, Slf1 cortical nodes is unknown, this appears to be unwarranted. I am not convinced that the rescue of a single exocyst mutant by Psy1 overexpression is relevant to a sequestration role of cortical nodes given the function of Psy1. The work needs to go further to support that Psy1 sequestration underlies the genetic or localization data. Possibilities of experiments that will strongly support the sequestration model include sequestering all of Psy1 at tips (GBP-GFP tethering or protein fusion approaches) or making a mutant in Psy1 that fails to localize specifically to nodes. Other possible experiments to bolster the model: If Skb1 or Slf1 are overexpressed (as done in Deng et al 2014), does this result in more Psy1 localized in nodes and less at cell tips, and if so, does this have the same effects? Does overexpression of Psy1 that outruns node components result in ectopic exocytosis?

AUTHOR RESPONSE: We thank the reviewer for these suggestions. We generated a psy1 mutant that fails to localize to nodes and found that it has defects in exocyst localization and has increased cell width (Figure 7 & 8), similar to defects observed with the loss of Skb1 and Slf1 nodes.

3. The statement about figure S1B "although we note that a minor portion of Slf1 was displaced from nodes" on page 4 does not match the data presented. Rather, it appears that hexanediol treatment results in a complete loss of Sfl1 from nodes and a redistribution of this protein along the entire plasma membrane. If this is the case, how is it that Slf1 is relocalized by hexanediol treatment but Sbk1 and Psy1 are not given their localization dependencies? Providing max projection in addition to the medial z-slice might be beneficial for interpreting this data.

AUTHOR RESPONSE: We apologize for any confusion from the figure in our initial submission. In the revised manuscript, we provide maximum projection images of the top half of cells as suggested (Figure S1B). These images show that both Psy1 and Slf1 remain at nodes after 1,6-hexanediol treatment.

4. Overall there seems to be a significant difference in the severity of phenotypes for slf1Δ compared to skb1Δ in experiments that examine both (Figure S2, 4C, S3A). Given that lack of either of these proteins results in loss of Psy1 from cortical nodes, it is unclear why there would be any difference between these strains if Psy1 sequestration is the relevant effect. Also, Figures 1B, 3A-B and figure 4D-E should include data for both of deletion strains in order to allow a better comparison between strains.

AUTHOR RESPONSE: We agree with the reviewer on this point. In the revised manuscript, we have added data for *slf1*Δ cells in Figures 2-6 so that any differences between the two mutants are clearly shown and stated. We have also added analysis of the new psy1-Ha-m1 mutant, which does not localize to nodes. All three strains (*skb1*Δ, *slf1*Δ, and psy1-Ha-m1) show similar defects although the severity is slightly different in some cases. We acknowledge these differences in the Discussion section by stating: “We note that the severity of phenotypes varies among these three mutants, likely reflecting differences in the functions and protein-protein interactions for Skb1 and Slf1. In addition, we do not exclude the possibility that the *psy1-Ha-m1* mutation alters the activity of Psy1.”

5. All single channel images should be inverted for clarity.

AUTHOR RESPONSE: Done.

Minor concerns
1. There are inconsistencies in the the data in figure S2. What is claimed in the text and summarized in the table in S2A does not match all of the data from the spot assays in S2B. For instance, it is stated in the text that there is growth rescue for rho3Δ skb1Δ compared to the single mutant strains, however this is not supported by the spot assay. Rather, rho3Δ slf1Δ has a suppressed growth phenotype. Figure 3A should be amended to reflect the data more accurately.

AUTHOR RESPONSE: We changed the text on page 5 to point out the differences of *rho3*Δ growth suppression with *skb1*Δ vs *slf1*Δ more clearly.

2. Do the minus signs represent EV controls or no plasmid? Please indicate all these details in materials and methods or figure legend.

AUTHOR RESPONSE: We included a key below the table in revised Figure S2A

3. Please provide either a western blot or microscopy data confirming and characterizing Psy1 overexpression from Figure 3C.

AUTHOR RESPONSE: We have removed the *psy1* overexpression data based on comments from Reviewer 2.

4. Please provide the data of Sec4 localization without the added KCl, as this result is not shown but referenced in the text on page 6 paragraph 3.

AUTHOR RESPONSE: We have removed Sec4 localization data based on the helpful suggestion of the editor.

5. Please explain the rationale for using the Sec5 marker vs the Sec8 marker in different figure 4 panels.

AUTHOR RESPONSE: For clarity and consistency, we have only used Sec8 as a marker for exocytosis in Figures 6 and 8 of the revised manuscript.

6. Please provide the p value for WT vs skb1Δ cell width in the figure legend and all data mentioned in text as not being statistically significant.

AUTHOR RESPONSE: We have added new statistical analysis for these mutants. For comparisons that are not statistically significant, we now denote "ns" in the figure, which is defined as $p > 0.05$ in the figure legend (e.g. Figure 5D, 6A, and 8B). For the specific example requested by the reviewer, the p value for comparing WT vs *skb1*Δ cell width is 0.3897.

7. The rational for using osmotic stress in different experiments should be explained in the text.

AUTHOR RESPONSE: We have removed the osmotic stress data based on the suggestion of the editor.

8. Are there any genetic interactions between Slf1, Skb1 and Rga4/6?

AUTHOR RESPONSE: We observed genetic interactions in cell morphology but not on viability or growth rate. More specifically, *rga4*Δ *rga6*Δ modifies cell shape without affecting growth rate in triple mutants with *skb1*Δ or *slf1*Δ.

9. In figure 1A figure legend "co-immunoprecipitation" and "coimmunoprecipitation" are both used

AUTHOR RESPONSE: We have fixed this error in revised Figure 1C legend.

10. Were cell width measurements made with DIC images as shown in the figure or with Blankophor stained cells as indicated in Materials and Methods?

AUTHOR RESPONSE: We measured cell width using images of blankophor stained cells as indicated in the materials and methods. The DIC images are provided for visual examples of overall morphology.

Figure design
1. As in figure 1B, a blow up of the GFP-Psy1 LifeAct-mCh is needed to be able to determine if the proteins do or do not co-localize as stated in the figure legend.

AUTHOR RESPONSE: Thank you for the suggestion. We have updated Figure 1A accordingly.

2. Label is missing for y axis in figure 1D.

AUTHOR RESPONSE: Thank you for pointing this out. See revised Figure 1D.

3. Figure 2A and 2B are missing scale bars.

AUTHOR RESPONSE: These images have been replaced in the revised MS.

4. What is the overall elapsed time in right panel of 2B?

AUTHOR RESPONSE: We added the elapsed time to the revised Figure 3C legend.

5. Figure 2B, it is difficult to see the dynamic exchange at the cell tips, perhaps only showing one cell tip but enlarging the image would be helpful.

AUTHOR RESPONSE: We revised this figure (3C) by including only one tip and inverting the look-up table. We hope that the dynamics of Psy1 at the tip versus the side is more clearly presented now.

6. Figure 4D µM is used instead of µm.

AUTHOR RESPONSE: We have fixed this error in revised Figure 5C.

7. Figure 4D there is no scale bar.

AUTHOR RESPONSE: A scale bar has been added to this figure (now Figure 5C).

Reviewer #2 (Remarks to the Author):

This manuscript by Miller, Moseley and colleagues presents an interesting observation on the plasma membrane t-SNARE Psy1 in fission yeast, which forms large clusters at the plasma membrane in the non-growing regions of the cell. These clusters are organized by the proteins Skb1 and Slf1, whose deletions the authors use to probe the consequence of loss of Psy1 clusters. They find that these cells exhibit genetic interactions with exocyst and formin mutants, and that exocyst markers ectopically localize to cell sides and cells acquire more round shapes when these mutants are sensitized either by salt stress or by further mutation of Cdc42 GTPases that prevent Cdc42 activity at cell sides. This leads them to conclude that Skb1-Slf1 nodes act to sequester Psy1 at cell sides to prevent exocytosis at these locations, and that this pathway acts in parallel to Cdc42 inhibition to restrict growth to cell tips.

The proposed model of t-SNARE sequestration to help define sites of active growth is interesting and novel and I like it very much. However, I am not yet convinced that the data lends enough support to the model and I feel it is strongly over-interpreted. There are a number of points that need to be addressed, listed below.

1. The major issue is that the authors use the skb1Δ as a proxy for delocalization of Psy1 from lateral nodes, but do not convince that the phenotypes are caused by this delocalization rather than another role of Skb1. The fact that skb1Δ is used throughout the paper rather than slf1Δ is problematic. Given both deletions have the same effect on Psy1 distribution, but skb1Δ has stronger phenotypes, the simplest interpretation is that Skb1 has additional Psy1 distribution-unrelated functions. Thus, the slf1Δ phenotype is likely to more closely represent the phenotype of Psy1 absence from nodes. Using the skb1Δ phenotype is exaggerating the importance of this localization change and is thus misleading. It would be important to perform all experiments with slf1Δ and present them in the main figures, keeping those with skb1Δ for supplementary figures. If the genetic interactions, localization of exocyst to cell side and increase of cell roundness in combination with rga4Δ rga6Δ also occur in slf1Δ, even if the phenotypes are weak, I'll be more convinced that the sequestration proposed by the authors contributes to cell shape regulation.

AUTHOR RESPONSE: We agree with this helpful comment. In the revised manuscript, we have added data with *slf1*Δ cells to compare to *skb1*Δ cells in all relevant figures.

2. Even with the use of slf1Δ rather than skb1Δ, the support for the model will remain a correlation. To lend strong support to the proposed conclusion, the authors would need to test the phenotype of specific mutations in Psy1 node localization (and/or test the effect of re-establishing this localization in the mutant backgrounds). While this is far from being a simple task and likely beyond the present study, there are steps the authors can take to help support their model. For instance, they could test whether psy1 mutants reverse the septation phenotypes of double mutant cells, as a way to probe whether the skb1Δ/slf1Δ phenotypes are due to changes in Psy1.

AUTHOR RESPONSE: We generated a psy1 mutant that does not localize to nodes and found that it has defects in exocyst localization and has increased cell width (Figure 7 & 8), similar to the phenotypes observed from the loss of Skb1 and Slf1.

3. The idea of sequestration is that clustering takes Psy1 away from its diffuse membrane localization at non-growing cell regions. However, from the data presented, it is not clear whether the diffuse distribution of Psy1 described for growing tips is absent from cluster-containing regions. In the projection images of Psy1 in Fig 1, there seems to be a continuous line of signal all around the cell in addition to the punctate pattern at non-growing regions. This is an important question, as the interpretation of all results hinges on Psy1 in nodes reducing the amount of free Psy1. Could the authors quantify the relative levels of Psy1 outside nodes in non-growing vs growing cell regions and in non-growing cell regions in WT vs skb1Δ/slf1Δ? Is this level low at non-growing regions and increased in the mutants as implied by the interpretation? To test the idea of sequestration, it would also be interesting to perform FRAP experiments of GFP-Psy1 at cell tips to complement the analysis at clusters, to test whether the diffuse distribution of Psy1 represents a more dynamic population, as predicted.

AUTHOR RESPONSE: We thank the reviewer for these helpful suggestions. We have added additional data quantifying Psy1 concentration and dynamics at cell sides when it is present in nodes compared to when it is diffuse and not in nodes (Figure 2 & 3). We show that *skb1*Δ and *slf1*Δ mutants have increased diffuse Psy1 at cell sides. Also, the diffuse Psy1 at the sides of *skb1*Δ (or *slf1*Δ) cells is very dynamic, similar to Psy1 at growing cell tips.

4. More careful wording needs to be used to express indirect interpretations of results and avoid over-interpretation. In some case, the thinking needs to be clarified. For instance:

a. To my knowledge the role of Psy1 in exocytosis and growth at cell tips has not be characterized. The authors should acknowledge this with appropriate phrasing in their text. For instance, the statement "Psy1 is necessary for exocytosis and growth at cell tips" should be re-written as "Psy1 is predicted to be necessary for exocytosis and growth at cell tips" or similar.

AUTHOR RESPONSE: We revised the text on page 4 bottom paragraph as suggested.

b. The suppression of sec3-2 growth defect by overexpression of Psy1 is not particularly surprising: this is the suppression of an exocyst mutant by overexpression of a t-SNARE - a rather expected finding. Similar suppression was previously observed of psy1 mutant growth defects by overexpression of v-SNARE proteins (Maeda et al, 2009). Interpreting this as a specific function linked to Psy1 localization to nodes or in free form is a vast over-interpretation.

AUTHOR RESPONSE: We appreciate this comment and agree with the reviewer. We have removed this result from the revised manuscript.

*c. I am not sure I follow the interpretation of the Bgs4 FRAP at cell tips in skb1Δ vs WT. A reduction in the mobile fraction means that there is a larger proportion of Bgs4 that is immobile *at cell tips*. How could this be explained by a re-direction to ectopic sites? If Bgs4 is generally re-directed to ectopic sites in skb1Δ, one would expect a lower steady-state level of this protein at cell tips. Is this the case? It is more difficult to explain a change in the ratio of mobile to immobile pools. Is the thinking that the amount of mobile (trafficking) Bgs4 is reduced but compensated for by an increase immobile (PM/cell-wall anchored) protein? This should be clarified.*

AUTHOR RESPONSE: We have modified our interpretation of the Bgs4 data based on these helpful suggestions (See page 5 of revised manuscript).

Minor comments:

1. In the genetic interaction analysis (Fig S2), I am confused by why slf1Δ generally appears to cause less pronounced phenotypes, except in the case of rho3Δ, where it is showing an impressive suppression.

AUTHOR RESPONSE: Our previous work showed that *skb1*Δ and *slf1*Δ do not have identical phenotypes (Deng *et al.*, MBoC, 2014), consistent with some of the differences observed in our current study. Regarding the specific example of *rho3*Δ, future work would be needed to understand why *slf1*Δ provides such a striking suppression in this case. More generally, we have added the following statement in the Discussion: "We note that the severity of phenotypes varies among these three mutants, likely reflecting differences in the functions and protein-protein interactions for Skb1 and Slf1."

2. What is the effect of skb1Δ/slf1Δ on the septation index of for3Δ?

AUTHOR RESPONSE: As shown in the revised Figure 4A, both *skb1*Δ and *slf1*Δ cause a very minor increase in the septation index of *for3*Δ cells.

3. The transition to use of 0.5M KCl is rather abrupt, and this is not commented upon in Fig S2.

AUTHOR RESPONSE: We have removed the osmotic stress experiments in the revised manuscript.

4. Why is Sec5-GFP first used as exocyst marker, and then Sec8-mNG? Are these two markers co-localizing in all conditions?

AUTHOR RESPONSE: To increase consistency in the revised manuscript, we shifted to using only Sec8 as an exocyst marker (see Figures 6 and 8).

5. The presence of Sec8 at sides and rounder shape of skb1Δ rga4Δ rga6Δ mutants compared with rga4Δ rga6Δ mutants is in line with the model proposed by the authors that exocytosis on cell sides leads to rounder cell shape. However, it does not exclude the alternative view that the exocytosis on cell sides is a consequence of the rounder shape. This is a pervasive chicken-or-egg problem in cell morphogenesis. Because cell dimensions overlap at the population level, one way to address the problem would be to restrict the quantification of Sec8 localization to cells of equivalent width in the two genotypes.

AUTHOR RESPONSE: We understand the reviewer's concern regarding causality of these two phenotypes. In the revised manuscript, we have changed the wording in several places to state that we observe ectopic exocytosis and increased cell width, without stating that one causes the other. For example, our revised abstract states: "Mutations that prevent node assembly or inhibit Psy1 localization to nodes lead to aberrant exocytosis at cell sides and increased cell width." Similar changes have been made in the Results section (*e.g.* "Defects in spatial control of exocytosis should be accompanied by morphological consequences in cell shape.").

RE: Manuscript #E20-05-0277R
TITLE: "Sequestration of the exocytic SNARE Psy1 into multiprotein nodes reinforces polarized morphogenesis in fission yeast"

Dear Jamie, Kristi, Joseph and Noelle,

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

I looked at your revised manuscript myself without going back the reviewers. I think you did an excellent job of addressing the concerns of the reviewers, especially with the addition of the new psy1 mutant. I appreciate the quality of the data and the careful wording used in some of the interpretations. Congratulations on this interesting work!

Sincerely,

Fred

Fred Chang
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Moseley:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

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