THE EMBO JOURNAL

Lateral plasma membrane compartmentalization links protein function and turnover

Jon V. Busto, Annegret Elting, Daniel Haas, Felix Spira, Julian Kuhlman, Marco Schäfer-Herte and Roland Wedlich-Söldner

Review timeline:

Submission date:	19 March 2018
Editorial Decision:	9 April 2018
Revision received:	9 May 2018
Editorial Decision:	4 June 2018
Revision received:	8 June 2018
Accepted:	8 June 2018

Editor: Deniz Senyilmaz Tiebe

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.)

1st Editorial Decision 9 April 2018

Thank you for submitting your manuscript entitled 'Lateral plasma membrane compartmentalization links protein function and turnover' for consideration by the EMBO Journal. We have now finally received all three reports on your manuscript, which I am copying below.

As you can see from the comments, all three reviewers express interest in the mechanism that you propose by which plasma membrane compartmentalization provides a regulatory link between function and turnover of plasma membrane proteins. Given these comments I would like to invite you to submit a revised version of your manuscript in which you address the comments of all three referees.

Please feel free to contact me if have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Referee #1:

In this study, Busto et al. use a range of complementary imaging, genetic, and biochemical experiments to examine how the function of a plasma membrane amino acid transporter triggers its internalization. The experiments conducted were systematic and exhaustive, and the conclusions of the present study are interesting and satisfying. I commend the authors on conducting such a thorough study.

While the manuscript in its current form is convincing, it could be greatly improved through edits to the text along with correction of several pervasive shortcomings within the figures. These are some general suggestions that apply repeatedly:

1) The authors make very sparse use of statistics throughout their figures. This is problematic for

two reasons. First and most obviously, it makes it impossible to know whether a difference displayed is significant. Secondly, it makes it hard for the reader to figure out what differences to pay attention to and which ones to write off as noise in the experimental system. Statistics should be applied to all graphs throughout the manuscript (provided a comparison is being made).
2) When possible, showing both channels of a two-color micrograph separately in addition to the merge would help to clarify the data and allow the data presented to be interpreted by colorblind people. In figure 2C, for example, I can hardly tell that Mup1 is becoming less punctate throughout the time course, although I feel certain that I'd be able to see the effect much more clearly were the Mup1 channel shown on its own.
3) Western blots are insufficiently labeled throughout the figures. At a minimum, all tiles of each western blot should be labeled with the primary antibody that was used to probe them. In figure 1c, for example, are both of the top two tiles probed with an anti GFP antibody? Why is there free GFP in the cell, and why does the signal increase with Mup1 degradation? Does it get cleaved off of Mup1 during degradation? Then why is there GFP signal for mutants that fail to degrade Mup1 in figure 1e? I imagine some of this confusion will be cleared up with improved labeling. Molecular weight standard labels wouldn't hurt, either.
4) Information on the length of time arrows is missing for some kymographs.
5) Some of the conclusions in the text seem overstated. For example, the authors do beautiful work in figure 4 to accumulate evidence suggesting that a conformational change in Mup1 triggers its relocalization on the plasma membrane and its internalization. But this conclusion is really based on the speculation that the Mup1 mutants that they have made are locked in alternate conformations, which can't be known without structural biology. The authors should consider addressing these sorts of caveats in the text rather than drawing big conclusions despite the caveats.

Minor Points:
1) In figure 1, the authors show that they have varied time and concentration of methionine treatment to see how the rate of Mup1 uptake changes with these variables. I can tell from the methods that 1mM methionine was used for most experiments in the paper, but its not clear to me how long the cells were kept in met(+) media before all of the assays. It would help if this were specified.
2) How was the threshold of network factor determined? By this I mean the dotted line on graphs, above which signals are deemed to be "networks" and below which they are punctate? In figure 5 panel D, the network factor plot is missing the dotted line indicating the threshold. It should be added in for the sake of consistency.
3) Figure 3 and the accompanying text are very difficult to follow. It would help tremendously if the authors stated specifically what all of the genes/pathways manipulated here are and how they are related to each other. It would also help if there was a better justification stated for each of the experiments presented. What question is each experiment trying to answer?
4) The results presented from experiments using myriocin and AureobasidinA would be more convincing if the authors could show that the effects of the drugs are due to their inhibition of sphingolipid synthesis rather than off-target effects. Can the phenotypes by these drugs be rescued through addition of exogenous sphingolipids, such as phytosphingosine?
5) In figure 4, the authors claim that there's less ubiquitination of the W155A Mup1 mutant. If you look at the western blot shown (panel E), it actually looks like there's MORE ubiquitination of that mutant. The authors need to either quantify the reduced ubiquitination of the mutant or remove this claim and explain the hypersensitivity to SeMet differently.
6) The results presented in figure 7 are a little strange. I'd expect there to be no Abp1 spots in the presence of LatB, since Abp1 binds F-actin. Maybe the dose of LatB is too low. Nevertheless, this confusion would be resolved if a different endocytic marker was examined in addition to Abp1; perhaps a component of the coat, which should colocalize with Mup1 patches for longer.

Referee #2:

This manuscript reports an important discovery. Using budding yeast as a model system, the authors carefully characterize the fate of the methionine transporter Mup1 in presence or absence of its substrate methionine. Without its substrate, the majority of Mup1 clusters into distinct plasma membrane domains. A small part of Mup1 is also found in network like strucutres that connect the clusters. These stable domains are called 'Membrane Compartment occupied by Can1' (MCC) and are associated with furrow like invaginations called eisosomes (Pil1 positive structures) Inside

MCCs, Mup1 appears to be protected from endocytosis. The formation of these clusters requires sphingolipids, the tetraspannin Nce102 and TORC2 signaling. Upon addition of methionine, Mup1 exits MCCs and forms a unique network like domain at the cell surface that is described here for the first time. Remarkably, this re-localization only happens when methionine can pass through the transporter into the cell, but it is independent of ubiquitination. Based on careful mutational analysis the authors conclude that substrate passing through Mup1 triggers conformational changes in Mup1 that enable exit from MCC and entry into the network like domain. Thus substrate transport and re-localization into the network like domain are a pre-requisite for ubiquitination and subsequent endocytosis.
A similar study by Bruno Andre using Can1 (an arginine transporter) appeared in pubmed today (PMID: 29559531). However I think that this should no interfere with the publication of this paper. If anything it underlines the importance of this work, because it implies that amino acid transporters regulate their fate - that is endocytosis and degradation - by substrate transport and subsequent changes in lateral plasma membrane compartmentalization (a novel feature of this work). This could be a general concept by which nutrient transporter signal to the endocytic machinery and contribute to the regulation of cellular metabolism.

Major Points:

The experiments are carefully controlled and analyzed and there is generally very little to criticize. I'm not sure that Figure 3 (factor required for Mup1 clustering in MCCs) adds a lot of new information to the paper. All factors described have been involved in the regulation of MCCs.
It will be interesting to identify the factors required for network like domain, but clearly that goes beyond the scope of the present study.

Referee #3:

The manuscript at hand describes the use of the methionine transporter Mup1 of S. cerevisiae as a model for segregation of plasma membrane proteins and their physiological significance. In general, the data provide a certain degree of novelty in that the particular aspect of how residence in specific plasma membrane subdomains affect biological function has been poorly studied, so far. In order to provide an experimental basis for their studies, the authors first confirm and refine the experimental evidence for induction of transporter expression and ubiquitin-dependent degradation of Mup1, followed by an analysis of its lateral membrane distribution at a precision and quantification level not observed before. Finally, relation of these data to transport activity, directly measured or indirectly determined by phenotypic resistance to the toxic analogue selenomethionine, allows the correlation with physiological function. This is substantiated by mutant analyses.
The findings are important not only for experts in the field of yeast membrane composition, but provide insight in the general function of protein distribution in all biological membranes. The manuscript provides a wealth of data and experiments appear to be thoroughly executed and quantified. It is well written, although one may note a certain lack of attention to details, amongst them the reference format, as outlined below.
In summary, I can recommend publication with some minor modifications.

I have only one major concern regarding the interpretation of mutant analyses. The authors introduce quite drastic amino acid substitutions in the TMD and the tail of Mup1, such as G78N or aromatic amino acids for alanines, without providing data on the 3D structure of the resulting variants. While gathering of such data may be clearly beyond the scope of this manuscript, one has to bear in mind that such alterations may affect much more than the intended substrate binding or the "plug" function the authors intend to attack. In fact, most of the data on relocalization of the mutant Mup1 proteins could also be explained by more drastic effects than those intended. I would therefore recommend to rephrase the respective parts of the manuscript to insert a little more caution on the conclusions drawn.
One minor point is the removal of prospective phosphorylation sites (bottom of page 11). This frequently results in the use of alternative residues being phosphorylated in the same domain. I wonder whether the authors considered to use mass-spec analysis to clarify this point?

Some recommendations for minor corrections:
p1, l12: Leioa is Basque dialect for the Spanish city of Lejona and does not appear as such in

Google maps
p2, l6: I would suggest to substitute "biochemical function" for "physiological function", since the authors investigated the role of the transporter and its distribution in vivo, not in an isolated biochemical setting
p2, l8: I would suggest to add "Clustering in eisosomes requires sphingolipids ..." to leave this point clear in the abstract, since one novel finding is the abundance of Mup1 in eisosomes in the absence of its substrate.
p2, l9: "tetraspanner protein Nce102" and "signaling through the TOR2 complex" may be more clear to the unprepared reader
p4, l28: "Yeast cells are constantly ..."
p4, l30: "must therefore be dynamically modulated."
p8, l126: "with aureobasidin A ..."
p8, l135: The authors frequently use abbreviations, here Rap for rapamycin, which with the few places this word appears in the text is more confusing then helpful. I would suggest to stick to the full name. The same holds true for methione instead of "Met" and sphingolipids instead of "SLs". Yeast nomenclature per se is confusing enough, without adding another level.
p9, l161: "we deleted NCE102". You can either delete the wild-type gene or work with an nce102 deletion, but you cannot delete the deletion allele.
p10, l184: "with two inverted repeats of five transmembrane domains"
p11, l218: "both N- and C-termini did not ...."
p12, l244: I suggest to remove the sentence starting with "Moreover, addition of ...". Clearly, the authors showed that the mutant does not reside in eisosomes in the first place, a localization influenced by substrate addition. Thus, a change of pattern after its addition is not expected.
Also, the claim of this being consistent with a closed conformation is somewhat misleading, since it is also consistent with a complete change of conformation affecting distribution, not necessarily meaning that it has to be a closed conformation (see comment above on interpretation of mutant analyses).
p13, l269: "... reduced its biochemical activity": What is really observed is an increase in resistance towards selenomethionine, which presumably/most likely reflects a decrease in transport activity. Please rephrase.
p18, l376: "depletion of Pil1". What is depleted is the protein, not the deletion allele of the gene.
p18, l390: Our data indicate ...
p19, l413: "In keeping ..." may be substituted by "In accordance with/Consistent with"
p20, l437: "First time" claims are not allowed in several scientific journals. Especially in this case, the authors themselves previously demonstrated a functional relationship of Can1 to its localization in the PM. Thus, although this is more convincingly shown in the current manuscript, it is not the first time.
p20, l443: "... and cell types."
p21, l448: Mating type in yeast is spelled with MAT in all capital letters and in italics
p21, l452: kanMX should be written in italics
p24, l535: please check spelling with capital letters (glycerol, bromophenol blue)
p24, l538: is the PAA gel really prepared with 13.3% glycerol?
p25, l548: "after start of the reaction". I assume that radioactive methionine has been added in combination with "cold" methionine? - Please specify assay conditions.
p26, l575: 100% ethanol does not exist, best I could find is >99.8%
p27, l600: please give sequence of oligonucleotides used for constructions, or at least details on the exact points of fusion, the nature of linker peptides inserted etc.. Since this is frequently crucial for biological function, such details are not trivial and should be provided.

p29: The list of references is a complete disaster regarding its format. Only in some cases are journal abbreviations maintained. Mostly full journal names are given, but only with the first letter in capitals. Titles are given sometimes with, sometimes without capital letters. Obviously, none of the authors cared to have a look at the reference list prior to submission.
p33: In the legend of Fig. 4B, please give the conditions of drop dilution assays, i.e. these are 2-fold or 10-fold dilutions and from top to bottom (since in other figures its from left to right)?
More importantly, at first sight drop dilution assays give the impression that they were all done on the same plate in one experiment within each figure. However, close inspection reveals different shades of darkness of the background in some lanes, indicating that these pictures are composites, e.g. in the last lane of Fig. 4B or middle lane of Fig. 5D. Thus, lanes have to be clearly separated and the figure legends should state, if results were compiled from different assays.

p35: Legend of Fig. 8 should state that green arrows indicate activation, red lines with dots inhibition of the indicated molecular targets. Commonly, the latter is indicated by lines with bars, rather than dots, which the authors may want to consider.

1st Revision - authors' response 9 May 2018

Referee #1

In this study, Busto et al. use a range of complementary imaging, genetic, and biochemical experiments to examine how the function of a plasma membrane amino acid transporter triggers its internalization. The experiments conducted were systematic and exhaustive, and the conclusions of the present study are interesting and satisfying. I commend the authors on conducting such a thorough study.

While the manuscript in its current form is convincing, it could be greatly improved through edits to the text along with correction of several pervasive shortcomings within the figures. These are some general suggestions that apply repeatedly:

1) The authors make very sparse use of statistics throughout their figures. This is problematic for two reasons. First and most obviously, it makes it impossible to know whether a difference displayed is significant. Secondly, it makes it hard for the reader to figure out what differences to pay attention to and which ones to write off as noise in the experimental system. Statistics should be applied to all graphs throughout the manuscript (provided a comparison is being made).

We have now added statistical tests for all relevant comparison s (t-test for paired, ANOVA for multiple comparisons using either Bonferroni or Dunnett post hoc tests). To avoid overloading the graph-panels we have added the relevant p-values to a supplementary table S3 and additionally provide simple bars to the panels indicating significant differences (green) vs. non-significant (red).

2) When possible, showing both channels of a two-color micrograph separately in addition to the merge would help to clarify the data and allow the data presented to be interpreted by colorblind people. In figure 2C, for example, I can hardly tell that Mup1 is becoming less punctate throughout the time course, although I feel certain that I'd be able to see the effect much more clearly were the Mup1 channel shown on its own.

We agree that the effect in figure 2C is not easy to see. We have now added single color images for this panel. In most cases with patch vs network localization the differences in colocalization are very clear in the chosen examples. For most instances we also provide additional single channel GFP images for the network factor analysis.

3) Western blots are insufficiently labeled throughout the figures. At a minimum, all tiles of each western blot should be labeled with the primary antibody that was used to probe them. In figure 1C, for example, are both of the top two tiles probed with an anti GFP antibody? Why is there free GFP in the cell, and why does the signal increase with Mup1 degradation? Does it get cleaved off of Mup1 during degradation? Then why is there GFP signal for mutants that fail to degrade Mup1 in figure 1E? I imagine some of this confusion will be cleared up with improved labeling. Molecular weight standard labels wouldn't hurt, either.

We have now added labels for primary antibodies and the molecular weight markers to make the indicated points clearer. GFP does indeed get cleaved from Mup1during degradation. While strongly increased upon addition of Met (Fig. 1C, time 15-60) this degradation also happens at background levels during sample preparation (Fig. 1C, time 0), and this background is also not affected in mutants that are no longer ubiquitinated (Fig. 1E). Interestingly, the ΔN mutant shows no free GFP band, indicating that this mutant is less prone to degradation. As we currently don´t understand the reason for this, we have not added any discussion of this point to the manuscript.

4) Information on the length of time arrows is missing for somekymographs.
This has been corrected.

5) Some of the conclusions in the text seem overstated. For example, the authors do beautiful work in figure 4 to accumulate evidence suggesting that a conformational change in Mup1 triggers its relocalization on the plasma membrane and its internalization. But this conclusion is really based on the speculation that the Mup1 mutants that they have made are locked in alternate conformations, which can't be known without structural biology. The authors should consider addressing these sorts of caveats in the text rather than drawing big conclusions despite the caveats.

We thank the reviewer for this comment. We absolutely agree that our current conclusions are based on cumulative, but ultimately correlative results. We lack the ultimate proof from actual structural analysis. We have now tried to make this distinction clear in the results and discussion sections (line 228-231, 266-269, 436-439). Also we now reference the work by Bruno André that describes similar results for Can1, where the conformational states are additionally backed up by previous molecular dynamics simulations.

Minor Points:
1) In figure 1, the authors show that they have varied time and concentration of methionine treatment to see how the rate of Mup1 uptake changes with these variables. I can tell from the methods that 1mM methionine was used for most experiments in the paper, but its not clear to me how long the cells were kept in met(+) media before all of the assays. It would help if this were specified.

In all shown experiments cells were kept in Met deficient medium for 2.5 h to reach maximal levels of Mup1 at the PM but avoid interference from beginning degradation at later time points. For TIRFM imaging of Mup1 patterns at the PM we observed cells 30 min after addition of Met. For equatorial imaging of Mup1 internalization we observed cells 60 min after addition of Met. We have added this information to the methods section (line 517-519).

2) How was the threshold of network factor determined? By this I mean the dotted line on graphs, above which signals are deemed to be "networks" and below whichthey are punctate? In figure 5 panel D, the network factor plot is missing the dotted line indicating the threshold. It should be added in for the sake of consistency.

From our previous study (Spira et al, NCB 2012) and our current results we accumulated many examples of proteins that exhibit network-like distributions. The threshold was set to reflect our qualitative evaluations and is described in the methods section (lines 542-544). We have now added the dotted line to Figure 6D.

3) Figure 3 and the accompanying text are very difficult to follow. It would help tremendously if the authors stated specifically what all of the genes/pathways manipulated here are and how they are related to each other. It would also help if there was a better justification stated for each of the experiments presented. What question is each experiment trying to answer?

We have rewritten this part of the results to make the rational e behind each experiment clearer. We have now added a schematic to indicate the pathway and drug activities for sphingolipid biosynthesis in Figure EV2A. In addition, we have included description of active/inactive TORC1/2 in the relevant panels to better explain the complicated genetic backgrounds. Finally, we have added bars to show statistically significant or non-significant values where relevant to the main text (see also major point 1). Finally, the new data on rescue of Myr effects by addition of phytosphingosine also help to better follow this section of thechapter (Figure EV2A, see also minor point 4).

4) The results presented from experiments using myriocin and AureobasidinA would be more convincing if the authors could show that the effects of the drugs are due to their inhibition of sphingolipid synthesis rather than off-target effects. Can the phenotypes by these drugs be rescued through addition of exogenous sphingolipids, such as phytosphingosine?

We have performed the requested rescue experiments. Indeed, add ition of phytosphingosine to cells led to a rescue of the observed Myr effects on Slm1 and Mup1 distribution (network factor and Pearson Mean). These results are now included in Figure EV2 A (lines 140-144). We now also

include the reference to the publication by the André lab (see comment by reviewer 2) that shows similar results for the Arginine permease Can1 (lines 144-146).

5) In figure 4, the authors claim that there's less ubiquitination of the W155A Mup1 mutant. If you look at the western blot shown (panel E), it actually looks like there's MORE ubiquitination of that mutant. The authors need to either quantify the reduced ubiquitination of the mutant or remove this claim and explain the hypersensitivity to SeMet differently.

We have now removed this claim. In fact, the ubiquitination pattern seen for Mup1 is fairly complex. A lower band can even be seen in the 2KR mutant (Figure 4E), indicating that it reflects modifications that are not critical for endocytic uptake. In contrast, several higher weight bands are absent or reduced in all internalization mutants including the W155A mutant. The main point regarding the hypersensitivity is the reduction of internalization for W155A, which increases the number of transporters available for SeMet uptake. The relevantsentences (lines 214-219) were changed to make this point clearer.

6) The results presented in figure 7 are a little strange. I'd expect there to be no Abp1 spots in the presence of LatB, since Abp1 binds F-actin. Maybe the dose of LatB is too low. Nevertheless, this confusion would be resolved if a different endocytic marker wasexamined in addition to Abp1; perhaps a component of the coat, which should colocalize with Mup1 patches for longer.

We thank the reviewer for this comment. It has actually been reported that treatment of yeast cells for short periods or with lower doses of Latrunculin leads to a cortical stabilization of endocytic patches that retain typical markers (Kaksonen, Cell 2003). The 100 µM of LatB (weaker effects than LatA) we used were titrated in order to obtain exactly this effect. In response to the reviewer we have also used Ede1 as marker for early endocytic events in our colocalization studies. However, the patterns obtained with this marker were complex, not only reflected sites of endocytic internalization and were difficult to interpret. We have therefore decided not to include those results. We now explain that brief treatment with LatB at the used concentration only stops actin dynamics but does not disrupt actin patches. We added a reference (Kaksonen, Cell 2003) to support this approach (lines 337-338).

Referee #2:

This manuscript reports an important discovery. Using budding yeast as a model system, the authors carefully characterize the fate of the methionine transporter Mup1 in presence or absence of its substrate methionine. Without its substrate, the majority of Mup1 clusters into distinct plasma membrane domains. A small part of Mup1 is also found in network like structures that connect the clusters. These stable domains are called 'Membrane Compartment occupied by Can1' (MCC) and are associated with furrow like invaginations called eisosomes (Pil1 positive structures) Inside MCCs, Mup1 appears to be protected from endocytosis. The format ion of these clusters requires sphingolipids, the tetraspanner Nce102 and TORC2 signaling. Upon addition of methionine, Mup1 exits MCCs and forms a unique network like domain at the cell surface that is described here for the first time. Remarkably, this re-localization only happens when methionine can pass through the transporter into the cell, but it is independent of ubiquitination. Based on careful mutational analysis the authors conclude that substrate passing through Mup1 triggers conformational changes in Mup1 that enable exit from MCC and entry into the network like domain. Thus substrate transport and re-localization into the network like domain are a pre-requisite for ubiquitination and subsequent endocytosis.

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Major Points:
The experiments are carefully controlled and analyzed and thereis generally very little to criticize.

I'm not sure that Figure 3 (factor required for Mup1 clustering in MCCs) adds a lot of new information to the paper. All factors described have been involved in the regulation of MCCs. It will be interesting to identify the factors required for network like domain, but clearly that goes beyond the scope of the present study.

We thank the reviewer for the positive and encouraging remarks. We are aware of the recent publication by the André lab and have now included references to this work in the introduction, results and discussion sections (see points by reviewer 1). We agree that the involvement of sphingolipids, TORC2 signaling and Nce102 in MCC or eisosome integrity has been reported before. However, several of our findings are relevant. In particular the effects of TORC2 inhibition on clustering of various MCC components (Slm1, Nce102) have not been previously reported. We also demonstrate that growth conditions used in our experiments (-Met) can modulate the patterns of Mup1 distribution. Importantly, no previous data has been obtained on the effects of the identified factors on Mup1. Finally, we report that deletion of the Avo3 C-terminus seems to have effects on the degree of Mup1 clustering within MCC patches (Figure 3C). This finding might serve as a useful caution for future studies using this mutant in the study of TORC2 signaling. We therefore decided to retain the information of this chapter for the manuscript.

Referee #3:

The manuscript at hand describes the use of the methionine transporter Mup1 of S. cerevisiae as a model for segregation of plasma membrane proteins and their physiological significance. In general, the data provide a certain degree of novelty in that the particular aspect of how residence in specific plasma membrane subdomains affect biological function has been poorly studied, so far. In order to provide an experimental basis for their studies, the authors first confirm and refine the experimental evidence for induction of transporter expression and ubiquitin-dependent degradation of Mup1, followed by an analysis of its lateral membrane distribution at a precision and quantification level not observed before. Finally, relation of these data to transport activity, directly measured or indirectly determined by phenotypic resistance to the toxic analogue selenomethionine, allows the correlation with physiological function. This is substantiated by mutant analyses.

The findings are important not only for experts in the field of yeast membrane composition, but provide insight in the general function of protein distribution in all biological membranes. The manuscript provides a wealth of data and experiments appear to be thoroughly executed and quantified. It is well written, although one may note a certain lack of attention to details, amongst them the reference format, as outlined below.

In summary, I can recommend publication with some minor modifications.
I have only one major concern regarding the interpretation of mutant analyses. The authors introduce quite drastic amino acid substitutions in the TMD and the tail of Mup1, such as G78N or aromatic amino acids for alanines, without providing data on the 3D structure of the resulting variants. While gathering of such data may be clearly beyond the scope of this manuscript, one has to bear in mind that such alterations may affect much more than the intended substrate binding or the "plug" function the authors intend to attack. In fact, most of the data on relocalization of the mutant Mup1 proteins could also be explained by more drastic effects than those intended. I would therefore recommend to rephrase the respective parts of the manuscript to insert a little more caution on the conclusions drawn.

We absolutely agree that the mutational data we gathered on Mup1 is correlative and not backed up by hard structural data. Regarding the potential effects on general structure of Mup1 we can at least clearly state that overall expression levels, folding and PM delivery of Mup1 were not visibly altered by the mutations. This does not rule out additional effects on Mup1 integrity but would at least make the consequences specific to the alterations in lateral distribution and posttranslational modifications that we observed. For the strong G78N mutation we found that expression, PM delivery and lateral segregation into MCC clusters was indistinguishable from the wildtype protein, further supporting our assumption that no gross changes in protein folding or structure were introduced. We added a statement regarding the conclusionsfrom structural mutants to make clear that all results are based on homology models (lines 2278-231).

One minor point is the removal of prospective phosphorylation sites (bottom of page 11). This frequently results in the use of alternative residues being phosphorylated in the same domain. I wonder whether the authors considered to use mass-spec analysis to clarify this point?

Our structure-function analysis of the Mup1 C-terminus showed that we could remove all residues up to the FWRV motif without affecting lateral relocation of Mup1 (Figure EV4). The only remaining Serine residue of the C-terminus (S525) is located within the proposed C-plug (not accessible) and is not predicted to undergo phosphorylation.

Some recommendations for minor corrections:
p1, l12: Leioa is Basque dialect for the Spanish city of Lejona and does not appear as such in Google maps.

Leioa is the official name of the municipality since 1979. Unfortunately many websites still use outdated place name databases.

p2, l6: I would suggest to substitute "biochemical function" for "physiological function", since the authors investigated the role of the transporter and its distribution in vivo, not in an isolated biochemical setting

done

p2, l8: I would suggest to add "Clustering in eisosomes require s sphingolipids ..." to leave this point clear in the abstract, since one novel finding is the abundance of Mup1 in eisosomes in the absence of its substrate.

We now added the term “clustering” in the abstractbut did not choose to include the name of the domain in the abstract as we did not want to focus attention ona particular yeast-specific nomenclature at this point.

p2, l9: "tetraspanner protein Nce102" and "signaling through the TOR2 complex" may be more clear to the unprepared reader

done

p4, l28: "Yeast cells are constantly ..."

done

p4, l30: "must therefore be dynamically modulated."

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p8, l126: "with aureobasidin A ..."

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p8, l135: The authors frequently use abbreviations, here Rap for rapamycin, which with the few places this word appears in the text is more confusing then helpful. I would suggest to stick to the full name. The same holds true for methionine instead of "Met" and sphingolipids instead of "SLs". Yeast nomenclature per se is confusing enough, without adding another level.

We removed the Rap abbreviation from the main text but kept it in the figure legends due to space restrictions (Rap explained in the legends for Figures 3 and EV2). Met and SL is used many times throughout the manuscript (>10) and we prefer to keep the abbreviation for those terms. Myr and AbA are commonly used abbreviations and appear many times (7 and 11x) throughout the main text. We therefore also kept those two abbreviations.

p9, l161: "we deleted NCE102". You can either delete the wild-type gene or work with an nce102 deletion, but you cannot delete the deletion allele.

This was corrected throughout

p10, l184: "with two inverted repeats of five transmembrane dom ains"

done

p11, l218: "both N- and C-termini did not ...."

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p12, l244: I suggest to remove the sentence starting with "More over, addition of …". Clearly, the authors showed that the mutant does not reside in eisosomes in the first place, a localization influenced by substrate addition. Thus, a change of pattern after its addition is not expected. Also, the claim of this being consistent with a closed conformation is somewhat misleading, since it is also consistent with a complete change of conformation affecting distribution, not necessarily meaning that it has to be a closed conformation (see comment above on interpretation of mutant analyses).

We removed those sentences and added a reference to the recent publication by Gournas et al on Can1.

p13, l269: "... reduced its biochemical activity": What is really observed is an increase in resistance towards selenomethionine, which presumably/most likely reflectsa decrease in transport activity. Please rephrase.

The figure this refers to (now Figure 6D) shows both, increase resistance to SeMet and decreased uptake of radioactive Met. We change the term to "reduced its Met uptake activity"

.
p18, l376: "depletion of Pil1". What is depleted is the protein , not the deletion allele of the gene.

Changed throughout

p18, l390: Our data indicate ...

done

p19, l413: "In keeping ..." may be substituted by "In accordance with/Consistent with"

done

p20, l437: "First time" claims are not allowed in several scientific journals. Especially in this case, the authors themselves previously demonstrated a functional relationship of Can1 to its localization in the PM. Thus, although this is more convincingly shown in the current manuscript, it is not the first time.

We removed this claim

p20, l443: "... and cell types."

done

p21, l448: Mating type in yeast is spelled with MAT in all capital letters and in italics

done

p21, l452: kanMX should be written in italics

done

p24, l535: please check spelling with capital letters (glycerol, bromophenol blue)

done

p24, l538: is the PAA gel really prepared with 13.3% glycerol?

This is indeed correct and was used to better separate ubiquitinated protein samples. We added a sentence to the methods section to make this point clear.

p25, l548: "after start of the reaction". I assume that radioactive methionine has been added in combination with "cold" methionine? - Please specify assay conditions.

This is clarified now – there was no cold Met added.

p26, l575: 100% ethanol does not exist, best I could find is >99.8%

This was now changed to “absolute ethanol”

p27, l600: please give sequence of oligonucleotides used for constructions, or at least details on the exact points of fusion, the nature of linker peptides inserted etc.. Since this is frequently crucial for biological function, such details are not trivial and should be provided.

We now provide this information in supplementary table S1.

p29: The list of references is a complete disaster regarding its format. Only in some cases are journal abbreviations maintained. Mostly full journal names are given, but only with the first letter in capitals. Titles are given sometimes with, sometimes without capital letters. Obviously, none of the authors cared to have a look at the reference list prior to submission.

The format has now been adapted to EMBO style.

p33: In the legend of Fig. 4B, please give the conditions of drop dilution assays, i.e. these are 2-fold or 10-fold dilutions and from top to bottom (since in other figures its from left to right)? More importantly, at first sight drop dilution assays give the impression that they were all done on the same plate in one experiment within each figure. However, close inspection reveals different shades of darkness of the background in some lanes, indicating that these pictures are composites, e.g. in the last lane of Fig. 4B or middle lane of Fig. 5D. Thus, lanes have to be clearly separated and the figure legends should state, if results were compiled from different assays.

The legends were adapted as requested (all are 5fold dilution series). Lanes are always taken from the same growth assay. Shading differences are due to control lanes that were removed to simplify the results. We now added separator lines to clearly indicate removed lanes and mention those in the figure legends.

p35: Legend of Fig. 8 should state that green arrows indicate activation, red lines with dots inhibition of the indicated molecular targets. Commonly, the latter is indicated by lines with bars, rather than dots, which the authors may want to consider.

done

2nd Editorial Decision 4 June 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees whose comments are shown below.

As you will see they both find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can officially accept the manuscript there are a

few editorial issues concerning text and figures that I need you to address.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your revision.

Referee #1:

The authors have done an excellent job addressing the concerns of the original review. I recommend publication without further revision.

Referee #3:

All comments of myself and the other Referees on the first Version of the manuscript were addressed and I am O.K. with the revised Version.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Roland Wedlich-Söldner
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2018-99473

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	all data obtained with biological replicates and large yeast cell numbers (n), automated image analysis routines reduce possibilities of subjective bias in analysis
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	no assumption of gaussian distribution for statistical tests (see methods) but all data sets generally close to normally distributed
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	done
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	done
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No