Journal of Cell Science

Review COMMONS

Septins coordinate cell wall integrity and lipid metabolism in a sphingolipid-dependent process

Alexander Mela and Michelle Momany
DOI: 10.1242/jcs.258336

Editor: James Olzmann

Review timeline
Submission to Review Commons: 9 February 2020
Original submission: 17 December 2020
Accepted: 31 December 2020

Reviewer 1

Evidence, reproducibility and clarity

Septins are highly conserved small GTPase cytoskeletal proteins that function as molecular scaffolds for dynamic cell wall and plasma membrane-remodeling, as well as diffusion barriers restricting movement of membrane and cell wall-associated molecules. Recent work has started to unravel the functional connections between the septins, cell wall integrity MAPK pathway signaling, and lipid metabolism, however most studies have focused on a small sub-set of septin monomers and/or were conducted in primarily yeast-type fungi.

Here the authors show in the filamentous fungus A. nidulans that the core hexamer septins are required for proper coordination of the cell wall integrity pathway, that all septins are involved in lipid metabolism. Especially sphingolipid, but not sterols and phosphoinositides, contributes to the localization and stability of core septins at the plasma membrane.

The experiments are simple and clear, therefore the conclusion is convincing. Fig.8 model, I would like to see the situation of septin mutant.

Significance

Since localization of cell wall synthesis proteins, lipid domains and septins are likely to depend on each other, sometimes difficult to evaluate the effect is direct or indirect. The comprehensive analyses like performed here are helpful to catch the overview in the field.

Reviewer 2

Evidence, reproducibility and clarity

Summary

The study by Mela and Momany describes the function of core septins of A. nidulans and links with the requirement of the cell wall integrity pathway and the sphingolipids which, are required for membrane and cell wall stability. The study is of interest for the fungal genetics community, and the authors have conducted a substantial amount of work in a field they have substantial experience. However, one of the main weaknesses of the manuscript is the assumption whether the CWI pathway controls de septin function of if the core septins control it.

© 2021. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Major comments

In the abstract, the authors claim that double mutant analysis suggested core septins function downstream of the final kinase of the cell wall integrity pathway. However, from the experiments showed, it is difficult to be convinced about that. The authors should make efforts do make it clear in the manuscript and the discussion.

For example:

-Line 25-26 (abstract): "Double mutant analysis suggested core septins function downstream of the final kinase of the cell wall integrity pathway."

-Line 181-182; 219-220 (results) "Double mutant analyses suggest core septins modulate the cell wall integrity pathway downstream of the kinase cascade."

This conclusion is one of the most important of the manuscript. However, this reviewer argues that it cannot be convincingly addressed if at least the phosphorylation ok the MAP kinase MpkA in the septins background is not evaluated under conditions of cell stress and sphingolipid biosynthesis inhibition. The genetic analysis alone maybe not enough to infer if septins control the CWI or the other way around. There may have compensatory effects when the CWI pathway is impaired. For example, most of the septins and mpkA double mutants seems to suppress the defect of the delta mpkA under cell wall stress. The authors should consider this idea.

There is no clear evidences on the manuscript that the core septins AspA, AspB, AspC , and ApsD are epithastic in A. nidulans. Therefore, the authors choice of using different Asp deletion mutants as a proxy for all the septins mutants is questionable. For example, there is no mention of why AspB was chosen for Figure 2 (chitin and ß-1,3-glucan deposition), and AspA was chosen for Figure 3 (chitin synthase localization) since these experiments are correlated. The same is true for Figure S1 where AspB and AspE were used. One can wonder if some of the core septins would have a major impact in the chitin content.

In a related comment about Figure 3, the reallocation of chitin synthases in the absence of septins is very interesting, but consider that all the core septin genes should be tested.
Without a fully functioning cell wall, the formation of septa will be impaired. It makes their results less surprising.

Why was chitin synthase B chosen to be analyzed in terms of reallocation? How many chitin synthases are in the A. nidulans genome. This rationale should be explained in the manuscript.

Figure 3 and Figure 4. The authors should make efforts to quantify the phonotypes they claim. They are overall very subtle, especially for Figure 3. Also, a decrease of fluorescence is a tricky observation that should be better reported by quantification.

Again, in Figures 5, 6, and 7, it is clear that the different septins respond differently when ergosterol or sphingolipids synthesis is impaired. It also raises the question again if there are differences in the role of septin genes. Can the authors use previous information about differences in septin function to improve the model (Figure 8)

For the above-discussed reasons, the conclusion on lines 384-388 (discussion) is not completely supported by the experiments shown in the manuscript. The authors need to make a better structured and more straightforward story emphasizing the stronger points and reducing descriptions of more speculative points.
Minor comments
Overall the figure caption could be shortened. They are too descriptive and contain details that are easily inferred for the images and from the materials and methods.

The authors made every effort to cove the precedent literature, but the manuscript has 115 references. The authors should evaluate if all the cited literature is extremely relevant. The manuscript would benefit for that conciseness.

© 2021. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Line 124, 493: Replace 10^7, 10^4 to 107, 104, etc

The use of fludioxonil as a probe to detect cell wall impairment is perhaps out of context. This drug responds primarily to the HOG pathway and also respond to oxidative damage. So, these results could be suppressed.

Line 140: "exposure" would be more appropriate than architecture. Please also consider that the difference in the cell wall reported in Figure S1 are very subtle. Are they relevant?

Line 144: explain briefly what it is about and why it was chosen instead of the total detection of chitin sugar monomers.
Line 538: Cell wall extraction section. Is this a new method? There is no supporting literature.

The results described on lines 232-257 are marginal to the study and are not exploited by the authors to address the central question of the manuscript, which is the role of the CWI pathway, septins, and sphingolipids. This section could be suppressed or very briefly mentioned in the preceding section.

Significance

The topic of the manuscript is highly relevant to the fungal biology field and employs a very important genetic model. The cooperation of signaling pathways in mains aspects of fungal physiology is the main significant contribution of this manuscript.

Reviewer 3

Evidence, reproducibility and clarity

Summary:

In this work the authors use genetic analysis in Aspergillus nidulans to identify phenotypes of septin mutants that point to roles for septins in coordinating the cell wall integrity pathway with lipid metabolism in a manner involving sphingolipids. Most of the major conclusions derive from monitoring the effects of combined genetic or chemical manipulations that target specific components of the pathways of interest. Additionally, the authors monitor the subcellular localization of septins, cell-wall modifying enzymes, and components of the cell wall itself.

Major comments:

The key conclusions are convincing, with the unavoidable caveat that null mutations of this sort and chemical inhibitors of these kinds could have unanticipated effects, such as upregulation of unexpected pathways or other compensatory alterations. The authors qualify their conclusions appropriately in this regard.

The methods are explained very clearly and the data are presented appropriately. In some cases results are shown as representative images illustrating altered localization of a protein or a cell wall component. The changes observed in the experimental conditions are fairly obvious, but some quantification would not be difficult and would likely make the results even more obvious. For example, the Calcofluor White staining patterns might be nicely quantified by linescans along the hyphal length, and the same is true for AspB-GFP localization upon addition of drugs.

I could imagine one simple experiment that might generate interesting and relevant results, but by no means would this be a critical experiment for this study. In yeast, exposure to Calcofluor triggers increased chitin deposition in the wall. It would be interesting to know how Calcofluor staining looks in WT or septin-mutant cells that have been growing the presence of Calcofluor for some time, particularly with regard to the localization of chitin deposition in these cells. Such experiments could help connect the idea of septins as sensors of membrane lipid status and also effectors of CWI signaling.

© 2021. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Minor comments:

- Body text refers to Figure 1A and 1B but the figure itself does not have panels labeled A or B.
- Line 885: "S3" is missing from the beginning of the title of the figure.

Reviewer Identity: This is Michael McMurray, PhD, Associate Professor of Cell and Developmental Biology, University of Colorado Anschutz Medical Campus

Significance

This is an important conceptual advance in our understanding of septin function because previous work in fungal septins mostly points toward them being important in directing or restricting the localization of other proteins that modify the cell wall or plasma membrane. This new work suggests that septins can play a sensing role, as well. As a fungal (budding yeast) septin researcher myself, I think that other fungal septin researchers would be very interested in these results, and I also think the broader septin community would appreciate it. Additionally, those studying fungal cell wall and plasma membrane biogenesis and coordination, including the Cell Wall Integrity Pathway, will be interested.

REFEREES CROSS COMMENTING

After reading Reviewer #1's comments, I agree that it would be appropriate to modify the wording of the authors' conclusions about where the septins lie in the CWI pathway (upstream or downstream). While they do mention that there may be other ways to interpret their results, a reader would have to search for the mention of these caveats and if the reader did not, then the strong conclusion statements might be taken as fact.
On the other hand, I don't think additional experiments looking at deletions of the other core septins will be worthwhile. I think that there is sufficient evidence to suspect that any single core septin deletion mutant will behave similar to another, and therefore that any one can be taken as representative. While it's possible that the authors might find something informative by looking at other mutants, I personally find the likelihood too low to justify additional experimentation along those lines.

Author response to reviewers' comments

Response to Reviewers **(Review Commons Refereed Preprint #RC-2020-00180)**
"Septins coordinate cell wall integrity and lipid metabolism in a sphingolipid-dependent process"

We sincerely appreciate the reviewers' care in evaluating our work and their insightful comments. We have made many improvements to the manuscript in response to the reviews as described below. We also apologize for the delay in responding. The many changes and extra duties associated with COVID-19 made what should have been a relatively quick process very slow.

Sincerely,
Michelle Momany (and Alex Mela)

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Septins are highly conserved small GTPase cytoskeletal proteins that function as molecular scaffolds for dynamic cell wall and plasma membrane-remodeling, as well as diffusion barriers restricting movement of membrane and cell wall-associated molecules. Recent work has started to unravel the functional connections between the septins, cell wall integrity MAPK pathway signaling,

© 2021. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

and lipid metabolism, however most studies have focused on a small sub-set of septin monomers and/or were conducted in primarily yeast-type fungi.

Here the authors show in the filamentous fungus A. nidulans that the core hexamer septins are required for proper coordination of the cell wall integrity pathway, that all septins are involved in lipid metabolism. Especially sphingolipid, but not sterols and phosphoinositides, contributes to the localization and stability of core septins at the plasma membrane.

The experiments are simple and clear, therefore the conclusion is convincing. Fig.8 model, I would like to see the situation of septin mutant.

We thank the reviewer for the positive comments. In response to the request from this reviewer and a similar one from reviewer 2 for more on the effect of the loss of individual septins, we added text clarifying the roles of core hexamer, core octamer and noncore septins throughout the manuscript including in the legend to Fig 8 (li 439-444) and the discussion (li 388-402). Please see responses to reviewer 2 comments for more detail.

Reviewer #1 (Significance (Required)):

Since localization of cell wall synthesis proteins, lipid domains and septins are likely to depend on each other, sometimes difficult to evaluate the effect is direct or indirect. The comprehensive analyses like performed here are helpful to catch the overview in the field.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Summary

The study by Mela and Momany describes the function of core septins of A. nidulans and links with the requirement of the cell wall integrity pathway and the sphingolipids which, are required for membrane and cell wall stability. The study is of interest for the fungal genetics community, and the authors have conducted a substantial amount of work in a field they have substantial experience. However, one of the main weaknesses of the manuscript is the assumption whether the CWI pathway controls de septin function of if the core septins control it.

We agree that while our data clearly indicate interactions between the septins and the CWI pathway, which component controls the other is not clear. We have modified the text to address this concern in several places as detailed in responses to the reviewer's specific comments below.

Major comments

In the abstract, the authors claim that double mutant analysis suggested core septins function downstream of the final kinase of the cell wall integrity pathway. However, from the experiments showed, it is difficult to be convinced about that. The authors should make efforts do make it clear in the manuscript and the discussion.

For example:

-Line 25-26 (abstract): "Double mutant analysis suggested core septins function downstream of the final kinase of the cell wall integrity pathway."

We agree that while the double mutant analysis shows interaction of septins with the CWI pathway, the evidence for them being downstream is not strong. We have revised the abstract as follows:

Li29-30: Double mutant analysis with *ΔmpkA* suggested core septins interact with the cell wall integrity pathway."

-Line 181-182; 219-220 (results) "Double mutant analyses suggest core septins modulate the cell

© 2021. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

wall integrity pathway downstream of the kinase cascade."

This conclusion is one of the most important of the manuscript. However, this reviewer argues that it cannot be convincingly addressed if at least the phosphorylation ok the MAP kinase MpkA in the septins background is not evaluated under conditions of cell stress and sphingolipid biosynthesis inhibition. The genetic analysis alone maybe not enough to infer if septins control the CWI or the other way around.

There may have compensatory effects when the CWI pathway is impaired. For example, most of the septins and mpkA double mutants seems to suppress the defect of the delta mpkA under cell wall stress. The authors should consider this idea.

Although we discuss the epistasis experiments as one possible interpretation, we agree the genetic analysis is not enough to definitively show that the septins are upstream of the CWI pathway or the other way around. The suppression of cell wall defects by deletion of septins in a mpkA null mutant background under cell wall stress suggests a bypass of the CWI pathway for remediation of the cell wall or some other alternate regulatory node. One possible interpretation of these data could be that by inactivation of normal CWI integrity function through deletion of the final kinase, in addition to deletion of septins (possibly acting as negative regulators of CWI components), there may be a parallel node by which cell wall remediation could still occur.

Wording throughout the abstract, results, and discussion has been modified accordingly.
Li 29-30: Double mutant analysis with *ΔmpkA* suggested core septins interact with the cell wall integrity pathway.

Li 208-209: ***Double mutant analyses suggest the core septin aspB^{cdc3} modulates the cell wall integrity pathway in the ΔmpkA background under cell wall stress.***

Li 221-225: When challenged with low concentrations of CASP and CFW, the *ΔaspB*^{cdc3}*ΔmpkA*^{slt2} and *ΔaspE ΔmpkA*^{slt2} mutants were more sensitive than *ΔaspB*^{cdc3} and *ΔaspE* single mutants, but suppressed the colony growth defects of *ΔmpkA*^{slt2} . The novel phenotype of the double mutants shows that septins are involved in cell wall integrity and raises the possibility that they act in a bypass or parallel node for remediation of cell wall defects (Fig 4)**.**

Li 227-228: **Fig 4. Double mutant analyses suggest core septins modulate the cell wall integrity pathway.**

Li 464-468: Double mutant analyses between septins and CWI pathway kinases also support a role for core septins in maintaining cell wall integrity under stress (Fig 4). Suppression of cell wall defects under cell wall stress by deletion of septins in an *ΔmpkA*^{slt2} background suggests a parallel node by which septins negatively regulate cell wall integrity pathway sensors or kinases could exist.

There is no clear evidences on the manuscript that the core septins AspA, AspB, AspC, and ApsD are epithastic in A. nidulans. Therefore, the authors choice of using different Asp deletion mutants as a proxy for all the septins mutants is questionable. For example, there is no mention of why AspB was chosen for Figure 2 (chitin and β-1,3-glucan deposition), and AspA was chosen for Figure 3 (chitin synthase localization) since these experiments are correlated. The same is true for Figure S1 where AspB and AspE were used. One can wonder if some of the core septins would have a major impact in the chitin content.

We agree with the reviewer that not all four core septins are equivalent. Previously published work from our lab shows that AspA^{Cdc11}, AspB^{Cdc3}, AspC^{Cdc12}, and AspD^{Cdc10} form octamers and that AspA^{Cdc11}, AspB^{Cdc3}, and AspC^{Cdc12} form hexamers, that both of these heteropolymers co-exist, and that the noncore septin AspE is not part of either core heteropolymer, though it appears to influence them possibly through brief interactions (Lindsay et al., 2010; Hernandez-Rodriguez et al., 2012; Hernandez-Rodriguez et al., 2014). This previous work also clearly shows that strains in which the hexameric septins have been deleted (*ΔaspA*, *ΔaspB*, and *ΔaspC)* have very similar phenotypes while strains in which the octamer-exclusive septin has

© 2021. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

been deleted (*ΔaspD*) have different phenotypes.

In our attempt to simplify the current manuscript we discussed the four core septins as a group. In retrospect this caused us to miss important distinctions on the roles of hexamer vs octamer septins and we are grateful to the reviewer for pointing this out. We have modified language throughout the revised manuscript to specify whether results and interpretations apply to core hexamer septins, core octamer septins, the noncore septin, or individual septins. This more detailed analysis has given us several new ideas to test in future work.

While we cannot exclude the possibility that interesting results might be produced by analyzing null alleles of each individual septin gene for all experiments, we agree with the cross-reference by Reviewer #3 that there is a very low likelihood that we would see different results by analyzing all individual septins within each subgroup (hexamer, octamer or noncore).

To the reviewer's questions on choice of septins for Fig 2, Fig 3, and Fig S1:

***ΔaspA*, *ΔaspB*, and *ΔaspC* showed similar sensitivity to cell wall-disturbing agents in the plate-based assays in Fig 1 and are all part of the core hexamer. We have modified text including the figure legends to make it clear which septins were used in the experiments and which group they belong to.**

In a related comment about Figure 3, the reallocation of chitin synthases in the absence of septins is very interesting, but consider that all the core septin genes should be tested. Without a fully functioning cell wall, the formation of septa will be impaired. It makes their results less surprising.

In the case of Fig 3, we were unable to recover ChsB-GFP in the *ΔaspB* or *ΔaspC* backgrounds but were able to recover it in the *ΔaspA* background. We have clarified as follows:

Li184-187: To determine the localization of synthases, a chitin synthase B-GFP (*chsB-GFP*) strain was crossed with strains in which core hexamer septins were deleted. After repeated attempts, the only successful cross was with core hexamer deletion strain *ΔaspA^{cdc11}*.

Figure 3, Panels A and B, chitin was also labeled by Calcofluor White which clearly shows that the formation of septa was not impaired even in the septin null mutant background (this is in agreement with previous work form our lab which shows that septa still forms in individual septin null mutatns). The results showed that unlike WT cells, chitin synthase is not only absent in most branch tips in the septin null mutant background, but seems to be limited primarily to longer (presumably actively growing/non-aborted) branches; these findings were surprising to us, considering other major cell wall synthesis events, such as targeting of cell wall synthases to septa during septation appeared to be unimpaired (based on the presence of fully-developed, chitin-labeled septa).

The labeling of septa by calcofluor is now noted in the legend to Figure 3 as follows:

Li 201: Calcofluor White labeling shows the presence of the polymer chitin at septa, main hyphal tips, branches, and …

Why was chitin synthase B chosen to be analyzed in terms of reallocation? How many chitin synthases are in the A. nidulans genome. This rationale should be explained in the manuscript.

We have added the following:

Lines 173-182: *A. nidulans* contains six genes for chitin synthases: *chsA*, *chsB*, *chsC*, *chsD*, *csmA*, and *csmB*. Chitin synthase B localizes to sites of polarized growth in hyphal tips, as well as developing septa in vegetative hyphae and conidiophores, a pattern very similar to septin localization. Deletion of chitin synthase B shows severe defects in most filamentous fungi analyzed thus far, and repression of the chitin synthase b gene expression in *chsA*, *chsC*, and *chsD* double mutants exacerbated growth defects from a number of developmental states observed in each single mutant, suggesting it plays a major role in chitin synthesis at most growth stages (Fukuda et al., 2009). For these reasons, we chose chitin synthase B as a

© 2021. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

candidate to observe in septin mutant background for possible defects in localization.

Figure 3 and Figure 4. The authors should make efforts to quantify the phonotypes they claim. They are overall very subtle, especially for Figure 3. Also, a decrease of fluorescence is a tricky observation that should be better reported by quantification.

Line scans of aniline blue and CFW label were conducted and added as Fig S1. Quantitation was performed and added as Fig S3. See author's response to Reviewer #3 below for details.

Again, in Figures 5, 6, and 7, it is clear that the different septins respond differently when ergosterol or sphingolipids synthesis is impaired. It also raises the question again if there are differences in the role of septin genes. Can the authors use previous information about differences in septin function to improve the model (Figure 8)

As described above, we have modified the manuscript throughout to clarify which phenotypes are seen for core hexamer, core octamer, and noncore septin deletions. As the reviewer notes, these are especially relevant for the sphingolipid-disrupting agents. Our model includes interaction of septins with sterol rich domains that contain both sphingolipids and ergosterol. Because it is not yet clear how subgroups of septins interact with each other and are organized at SRDs, we show all core septins in our model without distinguishing hexamers and octamers in the drawing, but we have now added text to clarify roles and outstanding questions.

The changes are summarized in the abstract as follows:

Li 37-40: Our data suggest that the core hexamer and octamer septins are involved in cell wall integrity signaling with the noncore septin playing a minor role; that all five septins are involved in monitoring ergosterol metabolism; that the hexamer septins are required for sphingolipid metabolism; and that septins require sphingolipids to coordinate the cell wall integrity response.

The clarifications are reflected in the Figure 8 legend (and associated sections of the discussion) as follows:

Li 436-441: As described in the text, our data suggest that all five septins are involved in cell wall and membrane integrity coordination. The core septins that participate in hexamers appear to be most important for sphingolipid metabolism while all septins appear to be involved in ergosterol metabolism and cell wall integrity. Because SRDs contain both sphingolipids and ergosterol and because it is not yet clear how subgroups of septins interact with each other at SRDs, we show all core septins in our model without distinguishing hexamers and octamers.

For the above-discussed reasons, the conclusion on lines 384-388 (discussion) is not completely supported by the experiments shown in the manuscript. The authors need to make a better structured and more straightforward story emphasizing the stronger points and reducing descriptions of more speculative points.

As discussed above, we have made changes throughout the manuscript to clarify which subgroups of septins are involved in which process and to refine our conclusions accordingly. The beginning of the discussion section has been changed as follows:

Li 384-399: Our data show that *A. nidulans* septins play roles in both plasma membrane and cell wall integrity and that distinct subgroups of septins carry out these roles. Previous work has shown that the five septins of *A. nidulans* septins form hexamers (AspA^{Cdc11}, AspB^{Cdc3}, and AspC^{Cdc12}) and octamers (AspA^{Cdc11}, AspB^{Cdc3}, AspC^{Cdc12}, and AspD^{Cdc10}) and that the noncore septin AspE does not appear to be a stable member of a heteropolymer (20). The current work suggests that though all septins are involved in coordinating cell wall and membrane integrity, the roles of hexamers, octamers, and the noncore septin are somewhat different. Core hexamer septins appear to be most important for sphingolipid metabolism, all five septins appear to be involved in ergosterol metabolism, and core septins are most important for cell wall integrity pathway with the noncore septin possibly playing a minor role. As summarized in Figure 8 and discussed in more detail below, our previous and current data are consistent with

© 2021. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

a model in which: (A) All five septins assemble at sites of membrane and cell wall remodeling in a sphingolipid-dependent process; (B) All five septins recruit and/or scaffold ergosterol and the core hexamer septins recruit and/or scaffold sphingolipids and associated sensors at these sites, triggering changes in lipid metabolism; and (C) The core septins recruit and/or scaffold cell wall integrity machinery to the proper locations and trigger changes in cell wall synthesis. The noncore septin might play a minor role in this process.

Minor comments
Overall the figure caption could be shortened. They are too descriptive and contain details that are easily inferred for the images and from the materials and methods.

Legends to the following figures have been streamlined by removing portions that belong in the methods: Figure 2, Fig 3, and Fig 6

The authors made every effort to cove the precedent literature, but the manuscript has 115 references. The authors should evaluate if all the cited literature is extremely relevant. The manuscript would benefit for that conciseness.

Because this manuscript addresses septins, ergosterol, sphingolipids, cell wall integrity, and multiple different pathways, there is a lot of literature underlying our approaches. Our strong preference is to cite primary literature, however we can shorten our reference list by relying on reviews if requested by the journal.

Line 124, 493: Replace 10ˆ7, 10ˆ4 to 107, 104, etc

"10^7" and all other scientific notation was altered to replace carrots "^7" with superscripts^{7}" throughout.

The use of fludioxonil as a probe to detect cell wall impairment is perhaps out of context. This drug responds primarily to the HOG pathway and also respond to oxidative damage. So, these results could be suppressed.

Previous work by Kojima et al., 2006 showed that in addition to the HOG pathway, cell wall integrity is required for resistance to fludioxonil treatment. *C. neoformans* cell wall integrity mutants *bck1*, *mkk1*, and *mpk1* (*Aspergillus nidulans bckA*, *mkkA*, and *mpkA* homologues) all exhibit hypersensitivity to fludioxonil, and this was shown to be remediated by the addition of osmotic stabilizers, suggesting cell wall impairment was involved in the growth defect produced by this treatment. Although this drug seems to act primarily through the HOG pathway, the CWI and HOG pathways have been shown to antagonize/negatively regulate one another through a parallel pathway (SVG pathway in yeast) (Lee and Elion, 1999). It has been hypothesized that internal accumulation of glycerol by constitutive activation of the HOG pathway causes decreased cell wall integrity. Due to the apparent cross-pathway control between the HOG and CWI pathways, as well as the high level of conservation of these pathway components in filamentous fungi, we thought this treatment was rightfully dual-purposed to investigate both cell wall impairment in the septin mutants and any possible involvement of the HOG pathway. This seems to be would a reasonable drug treatment to look at cell wall impairment that is not likely to be redundant with the modes of action observed in the other Figure 1 treatments (e.g. CFW, Congo Red, and Caspofungin).

The text clarifies this point as follows: li 110-112: Fludioxonil (FLU), a phenylpyrrol fungicide that antagonizes the group III histidine kinase in the osmosensing pathway and consequently affects cell wall integrity pathway signaling (Fig 1**)(58-67).**

Line 140: "exposure" would be more appropriate than architecture. Please also consider that the difference in the cell wall reported in Figure S1 are very subtle. Are they relevant?
The differences in the cell wall content reported in Figure S1 (Figure S2 in the revised manuscript) showed that the peak for 4-Glc was almost identical in WT and *aspB* null mutant, however the overall ratio of peaks switched, where 4-GlcNac content exceeded the 4-Glc content in the mutant compared to WT. By comparison, this was not the case with the septin aspE null mutant. Although this could be considered a 'subtle' change in chitin content, we

© 2021. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

believe this was an important unbiased analysis of the cell wall polysaccharide content and addressed some of the cell wall sensitivity phenotypes we observed, not only between WT and the septin mutants, but also between the septin null mutants which showed sensitivity to cell wall disturbing agents (i.e. *aspA*, *aspB*, and *aspC*) vs. those that did not show significant sensitivity (e.g. *aspE*). For these reasons we believe this warranted at the very least a supplemental figure for these data.
Though our idea of cell wall architecture includes changes in polymer exposure, as pointed out by the reviewer, others might use the phrase to mean only content changes. To avoid this misunderstanding, we have replaced the word "architecture" with "organization" in Li 147-148: These data show that cell wall organization is altered in *ΔaspB* ^{cdc3} and raise the possibility that it might be altered in other core hexamer septin null mutants as well.

Line 144: explain briefly what it is about and why it was chosen instead of the total detection of chitin sugar monomers.

Line 538: Cell wall extraction section. Is this a new method? There is no supporting literature.
We chose this method because it provides an analysis of all cell wall polysaccharide components and associated linkages. Detection of chitin sugar monomers would have also been a reasonable analysis if this were the only component of the cell wall we were investigating initially. The results showed differences in cell wall chitin content, so these were the data we presented.

This was addressed on lines 574-576: "Cell walls were isolated from a protocol based on (Bull, 1970); cell wall extraction and lyophilization were conducted as previously described in (Guest and Momany, 2000) with slight modifications listed in full procedure below."

The results described on lines 232-257 are marginal to the study and are not exploited by the authors to address the central question of the manuscript, which is the role of the CWI pathway, septins, and sphingolipids. This section could be suppressed or very briefly mentioned in the preceding section.

The authors concur that these data did not show any additional involvement of septins in the Calcineurin and cAMP-PKA pathways, and the relevance of the TOR signaling pathway connection is still quite unclear. For this reason, these data were added as a supplemental figure. On the other hand, there are a number of important signaling pathways which have been shown to affect the Cell Wall Integrity pathway directly and indirectly (these three pathways in particular), which is part of the central question of the manuscript. Considering such extensive 'cross-talk' between pathways (references produced on Line 65) in filamentous fungi, we felt it necessary to inspect possible involvement of these pathways in septin function via plate-based assays and feel that this s most clearly communicated as its own brief section in the text.

Reviewer #2 (Significance (Required)):

The topic of the manuscript is highly relevant to the fungal biology field and employs a very important genetic model. The cooperation of signaling pathways in mains aspects of fungal physiology is the main significant contribution of this manuscript.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:

In this work the authors use genetic analysis in Aspergillus nidulans to identify phenotypes of septin mutants that point to roles for septins in coordinating the cell wall integrity pathway with lipid metabolism in a manner involving sphingolipids. Most of the major conclusions derive from monitoring the effects of combined genetic or chemical manipulations that target specific components of the pathways of interest. Additionally, the authors monitor the subcellular localization of septins, cell-wall modifying enzymes, and components of the cell wall itself.

© 2021. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Major comments:

The key conclusions are convincing, with the unavoidable caveat that null mutations of this sort and chemical inhibitors of these kinds could have unanticipated effects, such as upregulation of unexpected pathways or other compensatory alterations. The authors qualify their conclusions appropriately in this regard. The methods are explained very clearly and the data are presented appropriately. In some cases results are shown as representative images illustrating altered localization of a protein or a cell wall component. The changes observed in the experimental conditions are fairly obvious, but some quantification would not be difficult and would likely make the results even more obvious. For example, the Calcofluor White staining patterns might be nicely quantified by linescans along the hyphal length, and the same is true for AspB-GFP localization upon addition of drugs.

We thank the reviewer for the positive comments and have made the suggested changes as follows:

Line scans of aniline blue and CFW label were conducted and added as Fig S1. Text has beend modified accordingly (Li 140-147).

Quantification of Chitin synthase-GFP localization and CFW staining and statistical analysis have now been added as Figure S3 and main text (Li 187-191) has been modified accordingly.

I could imagine one simple experiment that might generate interesting and relevant results, but by no means would this be a critical experiment for this study. In yeast, exposure to Calcofluor triggers increased chitin deposition in the wall. It would be interesting to know how Calcofluor staining looks in WT or septin-mutant cells that have been growing the presence of Calcofluor for some time, particularly with regard to the localization of chitin deposition in these cells. Such experiments could help connect the idea of septins as sensors of membrane lipid status and also effectors of CWI signaling.

This is a cool idea that we will pursue in future work. Thanks!

Minor comments:

- Body text refers to Figure 1A and 1B but the figure itself does not have panels labeled A or B.

Figure 1 was revised to show panels A and B labeled clearly.

- Line 885: "S3" is missing from the beginning of the title of the figure.

“S” was added to the figure title.

Reviewer Identity: This is Michael McMurray, PhD, Associate Professor of Cell and Developmental Biology, University of Colorado Anschutz Medical Campus

Reviewer #3 (Significance (Required)):

This is an important conceptual advance in our understanding of septin function because previous work in fungal septins mostly points toward them being important in directing or restricting the localization of other proteins that modify the cell wall or plasma membrane. This new work suggests that septins can play a sensing role, as well. As a fungal (budding yeast) septin researcher myself, I think that other fungal septin researchers would be very interested in these results, and I also think the broader septin community would appreciate it. Additionally, those studying fungal cell wall and plasma membrane biogenesis and coordination, including the Cell Wall Integrity Pathway, will be interested.

© 2021. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

REFEREES CROSS COMMENTING

After reading Reviewer #1's comments, I agree that it would be appropriate to modify the wording of the authors' conclusions about where the septins lie in the CWI pathway (upstream or downstream). While they do mention that there may be other ways to interpret their results, a reader would have to search for the mention of these caveats and if the reader did not, then the strong conclusion statements might be taken as fact.

The abstract, main text, and discussion have been modified to show that while there is evidence that the septins interact with the CWI pathway, it is not clear which component is upstream vs downstream. See response to reviewer 2 above for details.

On the other hand, I don't think additional experiments looking at deletions of the other core septins will be worthwhile. I think that there is sufficient evidence to suspect that any single core septin deletion mutant will behave similar to another, and therefore that any one can be taken as representative. While it's possible that the authors might find something informative by looking at other mutants, I personally find the likelihood too low to justify additional experimentation along those lines.

Based on results from previous work from our lab, there are two subgroups of core septins in A. nidulans (hexamer and octamer) and septins within subgroups appear to behave similarly. The results from the current work support this idea with the same groups of mutants behaving in very similar ways. So, the core hexamer septins, AspA^{Cdc11}, AspB^{Cdc3}, and AspC^{Cdc12} can be used to make predictions about each other, but not about the octamer-exclusive septin AspD^{Cdc10} or the noncore septin AspE. We agree with reviewer 3 that repeating analysis on multiple septins within a subgroup is not likely to give new insight. However, we were not careful in the original version of the manuscript to distinguish between core hexamer and octamer septins. As detailed in the response to reviewer 2 above, we have modified the manuscript throughout to make clear which subgroup of septins were being examined and to put conclusions into this context.

First decision letter

MS ID#: JOCES/2020/258336

MS TITLE: Septins coordinate cell wall integrity and lipid metabolism in a sphingolipid-dependent process

AUTHORS: Alex Mela and Michelle Momany
ARTICLE TYPE: Research Article

Thank you for sending your manuscript to Journal of Cell Science through Review Commons.

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks. Congratulations on a very interesting and important manuscript.

© 2021. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).