Atg32-dependent mitophagy sustains spermidine and nitric oxide required for heat-stress tolerance in *S. cerevisiae*

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Original submission

First decision letter

MS ID#: JOCES/2020/253781

MS TITLE: The mitophagy adaptor Atg32 sustains spermidine and nitric oxide production essential for heat stress tolerance in Saccharomyces cerevisiae

AUTHORS: Jasvinder Kaur, Juliet Goldsmith, Alexandra Tankka, Alfredo Gimenez, Jayanta Debnath, and Ariadne Vlahakis
ARTICLE TYPE: Short Report

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. I think, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this study, Kaur et al identified that Atg32-deleted strain showed respiratory growth defect during heat stress. In Atg32-deleted cells, production of spermidine and the precursor, SAM, were reduced by heat stress and the reduction of spermidine was the reason for the respiratory growth defect. Finally, the authors showed that NO was reduced in spermidine reduced cells. Taken together, the authors concluded that mitophagy have role on the production of SAM, spermidine and NO and make tolerance to the heat stress. It is important to understand the physiological role of mitophagy in yeast and this study give us great insight about it. However, there are some concerns about the data and its interpretation.

Comments for the author

1. Atg32 may have role in mitophagy independent phenomena. It is unclear whether Atg32 have role in SAM production independently of mitophagy or mitophagy is required for SAM production.

2. Why SAM was reduced in Atg32-deleted cells under heat stress condition.
The molecular aspects of this point should be clarified.

3. Spermidine have many cytoprotective roles, including NO production as the authors shown here. It is clear that the respiratory growth defect of Atg32-deleted cells under heat stress is due to reduction of spermidine, because supplementation of spermidine can recover the growth. However, it is unclear which cytoprotective role of spermidine dominantly works against heat stress.

4. Figure 1C and 1D; The authors concluded that heat stress induces mitophagy stronger than nitrogen starvation. However, starvation for 16 hours may saturate the mitophagy induction. Mitophagy induced by both nitrogen starvation and heat stress should be tested by induction time-course depend manner and shown the Om45-GFP assay on the single immuneblotting membrane.

5. Figure 3A and 3C. WT, atg32d, and spe1d should be grown on single plate like Figure 1A.

Reviewer 2

Advance summary and potential significance to field

In yeast, several organelles have been shown to be targets for selective autophagy, and the molecular mechanisms have also been elucidated, but the physiological role for each organelle degradation is only poorly understood. In this report, Kaur et al. found that respiratory growing Saccharomyces cerevisiae cells induce selective autophagy of mitochondria (mitophagy) when they are exposed to heat stress, and that cells lacking the mitophagy adaptor Atg32 exhibit defective cell growth under these conditions. Their metabolomics approaches revealed that spermidine and its precursor S-adenosyl-methionine

(SAM) significantly decrease in these cells. Externally supplying spermidine or SAM could suppress the growth defect of these cells. Moreover, the authors found that spermidine is important for the heat-induced production of cytoprotective nitric oxide (NO), and therefore ATG32 KO cells were defective in NO production and failed to gain heat stress tolerance. Thus, this study clarified an important aspect for the physiological role of mitophagy in yeast.
However, I request the authors to address the following issues to improve the manuscript.

Comments for the author

Specific comments:
(1) The authors should more carefully examine whether yeast cell growth arrest observed is specifically attributed to defective mitophagy. Do ATG11 KO cells but not ATG17 KO cells show a similar phenotype? Does a plasmid encoding ATG32 carrying mitophagy-defective mutations such as S114A S119A fail to rescue the growth arrest?

(2) It remains ambiguous which causes cell growth arrest, a decrease in SAM or that in spermidine. The authors should check SAM levels in SPE1 KO cells and ATG32 KO cells supplemented with spermidine. In addition, I wonder if the authors could examine cells lacking an enzyme that converts dcSAM to spermidine, in which the SAM level seems not to decrease.

(3) It is also unclear whether the cause of cell growth arrest is a decrease in spermidine or NO. The authors should examine whether cells lacking ATG32 and also defective in NO production are rescued by spermidine addition or not.

(4) It would be better if the authors could explain how defective mitophagy leads to a decrease in SAM.

(5) It would be interesting to examine mitophagy activity in SPE1 KO cells.

(6) Lines 106-108: Because the protein levels of OM45-GFP differ between SGE and SD-N media, it would be difficult to compare mitophagy activity between these conditions based on this assay.

(7) Figure 2A: The authors should mention and discuss the result that spermidine does not decrease in ATG32 KO cells at 30 C even though putrescine does.

(8) Figure 2B: It would be better if the authors could discuss why defective mitophagy results in a decrease in glycerol.

First revision

Author response to reviewers' comments

Dear Dr. Yoshimori,

We thank you and reviewers for your consideration and critique of this manuscript and are delighted by the overall enthusiasm in support of this study. We have incorporated the reviewer’s suggestions and performed key experiments that have significantly strengthened this study. Despite challenges posed in the current climate, the reviewer’s requests have been addressed to the best of our abilities. Below are specific replies to each reviewer comment in which we outline the nature and location of the resulting changes in the manuscript. Overlapping comments between reviewers are grouped by topic for greater ease. We look forward to your comments and final decision on the acceptance of this manuscript.

Warmest wishes,

Dr. Ariande Vlahakis and Dr. Jayanta Debnath

Is the role of Atg32 in heat stress tolerance mitophagy dependent?

Reviewer 1. "Atg32 may have a role in mitophagy independent phenomena. It is unclear whether Atg32 has a role in SAM production independently of mitophagy or mitophagy is required for SAM production."

Reviewer 2. "The authors should more carefully examine whether yeast cell growth arrest observed is specifically attributed to defective mitophagy. Do ATG11 KO cells but not ATG17 KO cells show a similar phenotype? Does a plasmid encoding ATG32 carrying mitophagy- defective mutations such as S114A S119A fail to rescue the growth arrest?"

Response: To determine if Atg32 is functioning in a mitophagy dependent manner to mediate heat stress tolerance, we performed two experiments that are described in lines 106-111 of the manuscript.

1. In Figure 1 D we performed the suggested experiment by reviewer 2 and determined that reintroduction of ATG32 S114A S119A phospho mutant to atg32Δ cells did not rescue the growth phenotype to the level that was observed following reintroduction of wild type ATG32. While the observations are statistically significant when compared to control cells, we would like to note that the utilization of SGE instead of YPGE required a relatively longer growth period and thus there is more background growth compared to figure 1B. However, we believe the effects to be clear and consistent with our findings in figure 1E.

2. In figure 1E we examined the effects of inhibiting general autophagy and determined that cells deficient for. Atg3, which is required for Atg8 conjugation and autophagosome formation, phenocopied atg32Δ cells during respiratory growth and heat stress. Taken together, these findings suggest Atg32 dependent mitophagy supports viability of respiratory growing yeast during conditions of heat stress.

Issues with comparing OM45-GFP degradation between nitrogen starvation and heat stress.

Reviewer 1. "Figure 1C and 1D; The authors concluded that heat stress induces mitophagy stronger than nitrogen starvation. However, starvation for 16 hours may saturate the mitophagy induction. Mitophagy induced by both nitrogen starvation and heat stress should be tested by induction time-course dependent manner and shown the Om45- GFP assay on the single immuneblotting membrane."

Reviewer 2. "Lines 106-108: Because the protein levels of OM45-GFP differ between SGE and SD-N media, it would be difficult to compare mitophagy activity between these conditions based on this assay."

Response: Both reviewers bring up a salient point that our conclusions cannot be met without controlling for mitophagy induction time to address the saturation of the mitophagy reporter and performing more comprehensive and controlled direct comparisons. Upon reassessment, we agree with the reviewers. Nonetheless, given the focus of the manuscript, direct comparison of which of these conditions is causing a more robust mitophagy response does not provide additional insight into the biological and metabolic functions of Atg32 during heat stress. As a

result, we chose to temper our conclusions and to more accurately reflect the original intention of the experiment. On lines 92-99 of the revised manuscript, we now simply state that our experimental heat stress condition in respiratory growing cells is sufficient to induce mitophagy. Furthermore, we state that we employ nitrogen starvation to demonstrate the fidelity of our reporter and yeast strains used in this experiment, as this is a previously established treatment known to induce mitophagy.

Does spermidine's cytoprotective role function specifically through NO production?

Reviewer 1. "Spermidine has many cytoprotective roles, including NO production as the authors shown here. It is clear that the respiratory growth defect of Atg32- deleted cells under heat stress is due to reduction of spermidine, because supplementation of spermidine can recover the growth. However, it is unclear which cytoprotective role of spermidine dominantly works against heat stress."

Reviewer 2. "It is also unclear whether the cause of cell growth arrest is a decrease in spermidine or NO. The authors should examine whether cells lacking ATG32 and also defective in NO production are rescued by spermidine addition or not."

Response: To test whether the cytoprotective effects of spermidine during heat stress are directly linked to NO production, we have included an experiment that can be found in Figure 4D and is discussed in lines 221-226. We asked whether inhibiting the NO response ameliorates the benefits of spermidine addition on cellular viability during heat stress. While Tah18 is a bona fide NO synthase in yeast, genetic experiments are technically challenging as this gene is essential for viability. Instead, we utilized nitric oxide scavengers to reduce NO in yeast.
Consistent with a cytoprotective role for NO, addition of the NO scavenger CPTIO alone significantly increased cell death in respiratory growing WT cells to *atg32Δ*-like levels (Figure 4D). Importantly, while addition of spermidine significantly reduced cell death of *atg32*Δ mutants to WT-like levels, quenching cellular NO with CPTIO significantly reversed the protective effects of spermidine in both WT and atg32Δ strains (Figure 4D). Thus, we conclude that spermidine functions specifically through NO signaling to support heat stress tolerance. These findings corroborate numerous prior reports demonstrating a cytoprotective role for NO in promoting heat stress tolerance and reveal an intimate dependency between mitophagy-dependent polyamine biosynthesis and NO production for heat stress tolerance in yeast cells where mitochondrial respiration is obligatory.

What is the direct mechanism for how mitophagy regulates cellular SAM levels?

Reviewer 1. "Why SAM was reduced in Atg32-deleted cells under heat stress condition. The molecular aspects of this point should be clarified."

Reviewer 2. "It would be better if the authors could explain how defective mitophagy leads to a decrease in SAM"

Response: The reviewers ask for the mechanism through which mitophagy impacts SAM production. We agree this is an incredibly interesting, yet complex endeavor that is beyond the scope of this manuscript, which focuses on elucidating the biological functions of Atg32 in yeast cells. We believe this question is better addressed through a separate and in-depth research endeavor identifying direct mitophagy targets related to SAM biosynthesis.

Does SAM function through spermidine to rescue growth?:

Reviewer 2. "It remains ambiguous which causes cell growth arrest, a decrease in SAM or that in spermidine. The authors should check SAM levels in SPE1 KO cells and ATG32 KO cells supplemented with spermidine. In addition, I wonder if the authors could examine cells lacking an enzyme that converts dcSAM to spermidine, in which the SAM level seems not to decrease."

Response: To address this important point, we compared the growth of wild type yeast to yeast lacking the spermidine synthase Spe3, which exhibit defective production of spermidine downstream of SAM and dcSAM. Consistent with a specific role for spermidine in promoting viability in respiratory yeast during heat stress, *spe3Δ* yeast exhibited a significant heat induced respiratory growth defect similar to *atg32Δ* and *spe1Δ* cells (Figure 3C). To further address the reviewer's comment, we additionally examined growth of cells lacking the Spe4 enzyme that converts spermidine to spermine in heat stress, and found this to have no significant effect on cellular viability during heat stress (Figure 3D). These findings further corroborate a unique role for spermidine in promoting viability during heat stress in respiratory growing cells independent of SAM and spermine metabolites that are directly upstream and downstream of spermidine biosynthesis respectively. We have updated the manuscript to discuss these new findings in lines 175-179.

Is spermidine necessary for mitophagy? :

Reviewer 2. "It would be interesting to examine mitophagy activity in SPE1 KO cells."

Response: As demonstrated in Figure 3E, inhibiting spermidine biosynthesis did not significantly affect heat stress induced mitophagy, as OM45-GFP degradation was equivalent between WT and spe1Δ strains. We have now included this in our current version of the manuscript in lines 179-181.

General manuscript suggestions:

Reviewer 1. "Figure 3A and 3C. WT, atg32d, and spe1d should be grown on a single plate like Figure 1A."

Response: In the revised figure 3 A and B we now include *atg3Δ* yeast as a control and depict all strains as they are on a single plate in the representative images.

Reviewer 2. "Figure 2A: The authors should mention and discuss the result that spermidine does not decrease in ATG32 KO cells at 30 C even though putrescine does."

Response: We now mention this with a brief discussion in the revised manuscript found in lines 159-161. Ultimately, we argue that this observation further supports SAM as the rate limiting step in spermidine biosynthesis, but not upstream polyamines, which has been previously reported by Pegg AE, 1969 (Ref. #31).

Reviewer 2. "Figure 2B: It would be better if the authors could discuss why defective mitophagy results in a decrease in glycerol.

Response: We now discuss this interesting result in lines 125-130 of the revised manuscript. Heat stress is known to trigger glycerol efflux via the yeast glyceroporin, FPS1, as a mechanism to stimulate Slt2 and high osmolarity response (HOG) MAPK signaling, which in turn is necessary for the induction of mitophagy (Refs # 19, 20). It is conceivable that cells with inhibited mitophagy hyper-activate this glycerol driven cellular response as a result of heat stress intolerance. We thus chose to focus on spermidine given the relative novelty of the interconnection between mitophagy and spermidine biosynthesis.

Second decision letter

MS ID#: JOCES/2020/253781

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AUTHORS: Jasvinder Kaur, Juliet Goldsmith, Alexandra Tankka, Sofia Bustamante Eguiguren, Alfredo A. Gimenez, Lance Vick, Jayanta Debnath, and Ariadne Vlahakis
ARTICLE TYPE: Short Report

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewer #1 still have some concerns, and I think that they are critical. Therefore, please address the concerns and send us your revised manuscript.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

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Reviewer 1

Advance summary and potential significance to field

The authors have addressed some of comments made by reviewers. However, there still remains some concerns which should be addressed before publication.

Comments for the author

Major comments,
1. To examine whether yeast cell growth arrest at high temperature is specifically attributed to defective mitophagy, the authors used Atg32-S114A/S119A mutants and explained that Atg32-S114A/S119A cannot rescue the growth arrest. Although the quantification of colony spot growth by the authors may show growth arrest in Atg32-S114A/S119A expressing cells, the size and number of colonies looks similar between Atg32(WT) and Atg32-S114A/S119A for this reviewer (fig 1D). Thus, additional experiment is required. As reviewer 2 suggested, it is essential to test ATG11KO and ATG17KO cells whether they show growth arrest at high temperature to confirm the author's conclusion.

2. The authors showed that mitophagy is induced by heat stress. It is general interest to know the level of mitophagy by heat stress compared with mitophagy by starvation (usually 6 hours in SD-N). The authors should test this point.

Minor comments,
1. The method of the quantification of colony spot growth should be explained in more detail. In some of figures, this reviewer doesn't think the quantification results reflect the size or number of colonies.

2. Figure 1B: If the p416 is plasmid, cells should be cultured on the SGE plate.

3. The method of Atg32-S114A/S119A plasmid construction should be explained. If the DNA is gift from other lab, it should be described in text and acknowledgement.

Reviewer 2

Advance summary and potential significance to field

The authors have satisfactorily addressed all the issues I raised for the original manuscript.

Comments for the author

The authors have satisfactorily addressed all the issues I raised for the original manuscript.

Second revision

Author response to reviewers' comments

RESPONSE TO EDITOR AND REVIEWER COMMENTS

We thank you for your thoughtful consideration of this manuscript and overall enthusiasm for our studies. We have incorporated the suggestions made by Reviewer 1 and our point-by-point responses are outlined below. First, we have performed the requested experiment analyzing the effects of Atg11 and Atg17 deletion on heat stress, which completely supports our model that Atg32 mediates heat stress tolerance during respiratory growth through its canonical role in mitophagy. Second, as Reviewer 1 requests, we provide a direct comparison of the mitophagy response during heat stress versus nitrogen starvation. Below we describe the resulting changes to address Reviewer 1's concerns. We remain fully confident that our overall conclusions are sound and that we have thoroughly answered all additional concerns raised during this second round of review. We look forward to the acceptance of this manuscript.

Detailed response to Reviewer 1:

1. To examine whether yeast cell growth arrest at high temperature is specifically attributed to defective mitophagy, the authors used Atg32- S114A/S119A mutants and explained that Atg32-S114A/S119A cannot rescue the growth arrest. Although the quantification of colony spot growth by the authors may show growth arrest in Atg32-S114A/S119A expressing cells, the size and number of colonies looks similar between Atg32(WT) and Atg32-S114A/S119A for this reviewer (fig 1D). Thus, additional experiment is required. As reviewer 2 suggested, it is essential to test ATG11KO and ATG17KO cells whether they show growth arrest at high temperature to confirm the author's conclusion.

We have performed the exact experiment requested by reviewer 1, which was also the experiment that was initially requested by reviewer 2 during his/her review of the original manuscript. We have tested the requirement for Atg11 and Atg17 during respiratory growth

and our results are completely consistent with the model proposed by reviewer 2 in his/her original review. Previous published work demonstrates that the selective autophagy scaffold protein Atg11 is necessary for mitophagy but not macro-autophagy (Kim et al., 2001). Conversely, while the scaffold protein Atg17 is essential for macro-autophagy, it is only partially required for starvation-induced mitophagy and fully dispensable for mitophagy induced by prolonged growth in respiratory media (Kawamata et al.,2008; Kanki and Klionsky, 2008; Okamoto et al, 2009). As expected, we observed that *atg11Δ* cells completely phenocopy *atg32Δ* cells and exhibit a severe restriction in respiratory growth during heat stress. In contrast, *atg17Δ* cells exhibit WT-like growth under these conditions (Figure 1 E-F). Thus, our findings are consistent with previous studies on the genetic requirements Atg32 and Atg11, but not Atg17 for respiration-induced mitophagy. Taken together with our finding in Figure 1D that cells known to be defective in both selective mitophagy and general macro-autophagy (*atg3Δ)* exhibit a severe respiratory growth defect during heat stress, the data strongly support the model that Atg32 mediates heat stress tolerance during respiratory growth through its canonical role in mitophagy. We wish to thank both reviewers for familiarizing us to this important concept in the mitophagy field highlighting the contextual nature of the genetic requirements for mitophagy versus general macro-autophagy.

Throughout the course of these studies, our experience has been that the use of synthetic glycerol media in heat stress tolerance growth assays leads to unavoidable technical issues with all strains, including WT, and requires lengthy incubation times spanning several days. Furthermore, based on understanding of the literature, non-mitophagy roles for the Atg32 S114/S119 phosphorylation mutant have not been excluded to date. Indeed, little is known for the role of this mutant in canonical versus non-canonical Atg32 functions, whereas the roles of Atg11 and Atg17 in mitophagy are much better defined. Given the uncertainties and skepticism raised by Reviewer 1 on our growth assay studies of the Atg32 S114/S119 mutant during the previous round of review, not to mention the strict space limitations for a short report in JCS, we have chosen to remove the limiting dilution growth assays of the Atg32 S114/S119 mutant, replacing it with the new findings requested by both reviewers analyzing the genetic role of Atg11 and Atg17. The manuscript text and Figure 1 has been updated accordingly to reflect this change which can be found on lines 103-118 and Figure 1 E-F.

2. The authors showed that mitophagy is induced by heat stress. It is general interest to know the level of mitophagy by heat stress compared with mitophagy by starvation (usually 6 hours in SD-N). The authors should test this point.

As requested by the Reviewer, we have directly compared the mitophagy response, measured using Om45-GFP cleavage, between nitrogen starvation (SD-N) and respiratory growth during heat stress. which is now shown in the right most panel in figure 1C. We did attempt this experiment at shorter timepoints, as suggested by the reviewer, but we had difficulty reproducibly detecting the free GFP band after only 6h of heat stress. As a result, we extended this experiment to 24 hours, which allow us to robustly detect the free GFP band in both the heat stress and SD-N conditions. Most importantly, this allowed us to make a rigorous and direct comparison of these two condition as specifically requested by this reviewer. At this timepoint, we clearly observe that nitrogen starvation elicits higher levels of Om45-GFP cleavage versus respiratory growth during heat stress (Figure 1C, right). The manuscript has been updated to describe this additional experiment on lines 95-96.

Minor comments,

1. The method of the quantification of colony spot growth should be explained in more detail. In some of figures, this reviewer doesn't think the quantification results reflect the size or number of colonies.

We agree with the reviewer that quantifying such growth assays is not routine, so we now

provide more detailed information in our Materials and Methods section on lines 295-301. Briefly, the quantification of colony spot growth was performed with Fiji to determine the density/intensity of each patch of yeast for each dilution. The images were converted to 8-bit tiffs and the rectangle selection was used to select the regions of interest. The Analyze> Gels>Plot Lanes function was then used to generate a profile plot of intensity peaks. Relevant peaks were isolated from background using the line tool, and the relative density/intensity for each dilution patch was quantified using the wand selection tool. The quantifications represent fold change relative to WT (or empty vector) yeast at each of the respective dilutions.

In certain cases, such as Figure 1B in particular, as the degree of dilution increases, we see do greater variability (evidenced by larger error bars) with this method of quantification. Further, this method does not take into account the size of individual colonies, but rather the entire yeast dilution spot as a whole. Despite these caveats, we still believe these quantifications provided are a useful resource to accompany the representative growth images that are more traditionally shown for these experiments. We hope that the clarification and more detailed methods we have provided in the revision will help readers when considering the dilution series growth data throughout the paper.

2. Figure 1B: If the p416 is plasmid, cells should be cultured on the SGE plate.

This particular experiment was technically challenging due to the need for synthetic -Uracil media to select for the WT ATG32 expressing plasmid. As mentioned in point 1 above, synthetic glycerol media harbors significant technical issues for heat stress tolerance growth assays likely due to the relatively nutrient replete nature of synthetic media. As a result, we did use YPGE media for this experiment. We fully realized that this created the risk of losing selection for the WT ATG32 plasmid over the course of the growth assay. Despite the caveats created by the lack of selection for the rescue plasmid, we found that ATG32 restoration to *atg32Δ* cells is able to enhance respiratory growth during heat stress compared to the empty vector (Figure 1B). To allay the reviewers concerns, we have provide a new image clearly showing this rescue in Figure 1B in the latest revision.

3. The method of Atg32-S114A/S119A plasmid construction should be explained. If the DNA is gift from other lab, it should be described in text and acknowledgement.

We previously includes all of the acknowledgements of gifts, including from other labs in the materials and methods section, which is now on lines 252-253 in the revised manuscript. This is due to the strict word limitations needed for a short report in JCS; the word count which includes the acknowledgements section but not the methods. As mentioned above, we have removed the previous findings using the Atg32-S114A/S119A plasmid, replacing it with the analysis of *atg11Δ* and *atg17Δ* cells. Thus, we no longer utilize (or describe) this specific plasmid in this latest revision.

References:

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Third decision letter

MS ID#: JOCES/2020/253781

MS TITLE: Atg32 dependent mitophagy sustains spermidine and nitric oxide required for heat stress tolerance in S. cerevisiae

AUTHORS: Jasvinder Kaur, Juliet Goldsmith, Alexandra Tankka, Sofia Bustamante Eguiguren, Alfredo A. Gimenez, Lance Vick, Jayanta Debnath, and Ariadne Vlahakis
ARTICLE TYPE: Short Report

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors have addressed comments made by this reviewer. I believe that the current manuscript becomes suitable for publication.

Comments for the author

There are no specific comments.