The small GTPase KlRho5 responds to oxidative stress and affects cytokinesis

Marius Musielak, Carolin C. Sterk, Felix Schubert, Christian Meyer, Achim Paululat and Jürgen J. Heinisch
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MS TITLE: The small GTPase KlRho5 responds to oxidative stress and affects cytokinesis

AUTHORS: Marius Musielak, Carolin Sterk, Felix Schubert, Christian Meyer, Achim Paululat, and Juergen J Heinisch
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

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

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.

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 manuscript, Musielak et al. demonstrated that KlRho5, a homologue of S. cerevisiae Rho5 (ScRho5) in yeast K. lactis, have several features similar to, but partly distinct from, those of ScRho5. The authors firstly cloned KlRho5 and its binding partners KlDck1 and KlLmo1 (dimeric GEF for Rho5) by means of genome database search. They showed that KlRho5 was functionally similar to ScRho5 in that 1) Expression of KlRho5 was able to complement the Scrho5 deficiency (hyper-resistance towards H2O2) in S. cerevisiae. 2) Klrho5 deficiency increased resistance towards H2O2 in K. Lactis. 3) In response to oxidative stress and glucose starvation, KlRho5, KlDck1 and KlLmo, like their S. cerevisiae homologues, translocated to mitochondria.
On the other hand, KlRho5 was different from ScRho5 in that 1) In the nutrient sensing signaling, Klrho5 did not have genetic interactions with Klsch9 and Klgpr1, unlike their S. cerevisiae homologues. 2) Klrho5 lacked genetic interactions with 15 genes involved in various biological processes, although at least some of their S.
cerevisiae homologues have genetic interactions with Scrho5 in S. cerevisiae. 3) Deficiency of Klrho5 as well as Kldck1 and Kllmo resulted in aberrant synthesis of primary septum during cytokinesis and thicker cell wall which did not occur in S. cerevisiae deficient in Scrho5.

Comments for the author

All experiments are conducted in highly sophisticated manner and the data presented are well organized and the resultant manuscript offers several interesting findings and implications. Nevertheless, it appears to me that the present manuscript is too preliminary and lacks scientific significance enough to be published in JCS at present.

Major point:
The main topic of this manuscript is to find and elucidate possible molecular functions of KlRho5, in comparison with its homologue ScRho5. Given the increasing knowledge regarding the molecular functions of ScRho5, it appears to me that data obtained by analyses of its homologue in other yeast strain are of low scientific novelty. In addition, although the authors examined its functions from a lot of viewpoints, each experiment lacks deep analysis. Especially, although the authors have found that KlRho5 is involved in cytokinesis, underlying molecular mechanisms are not sufficiently addressed. For example possible effects of Klrho5 deficiency on KlCdc42, KlBem1 and KlCdc24, which may not yet been cloned in K. lactis but their S. cerevisiae homologues play key role in bud neck formation, should be examined.
Because involvement in cytokinesis is specific to KlRho5 but not to ScRho5, detailed molecular analysis of this process is particularly important. I feel this manuscript be suitable for other journals that deal with more specific field of interest unless above-mentioned points are sufficiently resolved.

Minor points:
1. The authors should show physical and enzymological interaction between KlRho5, KlDck1 andKlLmo1 by means of biochemical assays.

2. In Fig. S2A, the authors should add data using wild-type S. cerevisiae strain. Alternatively, exogenous expression of ScRho5 should be tested.

3. In page16, line 354, the authors described that the expression of KlRho5, KlDck1 and KlLmo1 by means of homologous recombination at the Klleu2 locus restored the growth in respective deletion strains.
However, the corresponding data are not presented.

Reviewer 2

Advance summary and potential significance to field

This paper describes genetics and biological approaches to studdy the function of the small GTPase KlRho5 (homolog of S. cerevisae Rho5 and human Rac1). They showed that similar to its homologs, this GTPase respond to oxidative stress and nutrient starvation and was implicated in spatial and temporal control of cytokinesis.
although the model is unconventional, this work allows us to confirm the involvement of GTPases in fundamental mechanisms for the cell. this work makes it possible to propose K. lactis yeasts for the understanding of the respiratory phenotype of healthy cells, as opposed to the fermentative phenotype found in cancer cells or in cells related to Alzheimer's disease for which S. cerevisae yeasts are generally used.

Comments for the author

I do not ask for additional manipulations, but I wonder about the relevance of the use of the mutant labeled GFP, which, as the authors point out, does not present a complete functional activity. Moreover, wouldn't it be interesting to complete the experiments, by using the dominant negative mutant, to confirm the action of the constitutively active mutant?
I also wonder about the involvement of other small GTPases in these mechanisms due to the redundancies regularly observed in other organisms.
A small improvement could be made to improve the quality of colocalization images with enlarged views to confirm the translocation of the GTPase at the mitochondrial level.

Reviewer 3

Advance summary and potential significance to field

This paper identify Rho5 as putative functional homolog of mammalian Rac1 and its GEF components Gck1 and Lmo1 in *K. lactis* by homology search. By functional analyses, this paper shows that, first, as in *S. cerevisiae* KlRho5 is involved in oxidative stress response. Accordingly, these three components localized to mitochondria upon oxidative stress and glucose starvation as in *S. cerevisiae*. Secondly, this paper also shows for the first time the functions of KlRho5 in morphogenesis (cell wall synthesis) and cytokinesis that have not been reported for ScRho5. Deletions of each KlRho5 component led to elongated bud scar resulted from inappropriate contractile ring formed during cytokinesis, as well as increased thickness of lateral cell wall. Accordingly, KlRho5 localized to actomyosin ring during cytokinesis.

Comments for the author

This manuscript contains important findings especially regarding the mechanism of animal-type cytokinesis, and most of the data, including those on oxidative stress response, seem to be sound. Therefore, it could be published in the Journal of Cell Science after appropriate revisions. Because the descriptions regarding functions of KlRho5 on the response against oxidative stress seem to be entirely agreeable, then I would like to comment only on those on regulation of cytoskeleton and cytokinesis under stress-free conditions.

1) In fig. 3, it is described that under stress-free conditions, both KlGck1 and KlLmo1 distributed diffusely in cytosol. However, if you look carefully, both proteins seem accumulated at patches in some structure existing as one per cell that is nucleus for example. If possible, authors would take pictures after DAPI staining for nucleus for example, show them in the manuscript as larger and highly-resolved figures, and discuss the results. These distributions in interphase could be important for functions of KlRho5 during cytokinesis.

2) In fig. 7, it is described that KlRho5 is accumulated at the bud neck during cytokinesis co-localizing with contractile ring constituted by actin and myosin II. This is actually the case for actin, because in Fig. 7B, at the bud neck where KlRho5 is accumulated more densely than the other cortical regions, actin is also accumulated. However, in Fig. 7A, at the bud neck where

KlRho5 is accumulated more densely than the other cortical regions, myosin II is absent (upper panel, bottom budded cell). Conversely, at the bud neck where myosin II is accumulated, KlRho5 is present but is not more densely accumulated than the other cortical regions (upper panel, upper budded cell; bottom panel, both budded cells). Taken these together, in Fig. 7A, either myosin II are not co-localized with KlRho5 at the contractile ring. So I suggest that authors would take more pictures and show more panels, to clarify if myosin II and KlRho5 are co-localized with each other at the contractile ring.

3) In *S. cerevisiae*, it is reported that first, myosin II is recruited to the bud neck, IQGAP-like protein Cyk1 and actin are then recruited during cytokinesis (J Cell Biol. 1998 Jan 26; 140(2): 355–366. doi: 10.1083/jcb.140.2.355). In mammal, IQGAP binds to active GTP form of Rac1 and Cdc42 as an effector but not to that of RhoA. As the authors suggested, KlRho5 is more similar to mammalian Rac1 than ScRho5, therefore GTP form of KlRho5 could binds to KlCyk1 (present in the database) and be recruited to the bud neck with actin after myosin II, these consistent with Fig. 7A. Thus, the authors would be discus the possibility that KlRho5 binds to and functions with KlCyk1 during cytokinesis.
Additional minor comments are as follows.

4) In line 124, 'K. lactis' should be '*K. lactis*.'

5) In line 385, 'life-cell' might be 'live-cell.'

First revision

Author response to reviewers' comments

First of all we would like to thank the reviewers for their critical assessment of the manuscript, the time invested, and their constructive suggestions. We did our best to take into account all the points raised. For example, we investigated four more K. lactis genes whose homologs are known to be related to morphogenesis/cytokinesis regulation in S. cerevisiae, as suggested. This includes the construction of deletion mutants in all four genes, as well as the introduction of a mutation into KlCDC42 presumably encoding a constitutively active isoform of the GTPase. This turned out to suppress the morphological defects of the Klrho5 deletion in epistasis analyses. Also, we tagged KlCdc42 with GFP and studied its intracellular localization in response to oxidative stress.
While these results should largely answer the major points raised by reviewers 2 and 3, we have to admit that they only partially suffice to address the problems raised by reviewer 1. Although we clearly see the points and agree that they are surely worth following up in future investigations, we hope that the efforts invested can be somewhat appreciated, especially taken into account the more complicated handling of a non-Saccharomyces yeast in terms of genetic manipulations, within the time constraints of a revision.
In the following, please find our point by point responses to the reviewer reports.

Reviewer 1 Comments for the author

All experiments are conducted in highly sophisticated manner and the data presented are well organized, and the resultant manuscript offers several interesting findings and implications. Nevertheless, it appears to me that the present manuscript is too preliminary and lacks scientific significance enough to be published in JCS at present.

Major point:
The main topic of this manuscript is to find and elucidate possible molecular functions of KlRho5, in comparison with its homologue ScRho5. Given the increasing knowledge regarding the molecular functions of ScRho5, it appears to me that data obtained by analyses of its homologue in other yeast strain are of low scientific novelty.

Acutally, using a different yeast species is key to this work: a major function of the human homolog, namely its influence on cytoskeleton dynamics, cannot be reproduced so far with Rho5 in S. cerevisiae, whereas our data indicate that there is such an influence during cytokinesis in K. lactis.

In addition, although the authors examined its functions from a lot of viewpoints, each experiment lacks deep analysis. Especially, although the authors have found that KlRho5 is involved in cytokinesis, underlying molecular mechanisms are not sufficiently addressed. For example, possible effects of Klrho5 deficiency on KlCdc42, KlBem1 and KlCdc24, which may not yet been cloned in K. lactis but their S. cerevisiae homologues play key role in bud neck formation, should be examined.

We cloned KlCDC42 and produced a hyperactive allele to test for suppression of the cytokinesis defect of Klrho5. KlCDC42-G12V was stably integrated at the Klleu2 locus and crossed to a Klrho5 deletion strain. In fact, there appears to be a functional overlap, as the activated allele can suppress the abberant morphology. This has been included in the revised manuscript.
Heterozygous deletions in KlCDC42 have also been obtained in a diploid strain but cannot be further analyzed, as the gene is essential and there is no stringent conditional promoter available for K. lactis, as outlined in our previous works on cytokinesis. This information is also included in the new manuscript.
Moreover, we constructed a GFP-KlCdc42 fusion and observed that it also translocates to the mitochondria upon oxidative stress, indicating that there may be a functional overlap in this response as well. However, as stated in the revised manuscript, this GFP fusion cannot complement the lethality of the deletion, indicating that it is not sufficiently functional in vivo. Therefore, we took caution in the interpretation of the localization results.
We also cloned and deleted the genes KlBEM1 and KlCDC24 in a heterozygous diploid strain. Further analyses were again impeeded by the lethality of either deletion as demonstrated by tetrad analyses and briefly stated in the revised manuscript. The respective data are presented in supplementary Fig. S4.

Because involvement in cytokinesis is specific to KlRho5 but not to ScRho5, detailed molecular analysis of this process is particularly important. I feel this manuscript be suitable for other journals that deal with more specific field of interest unless above-mentioned points are sufficiently resolved.

As stated above, we added data on the homologs of genes with known relations to cytokinesis in S. cerevisiae. Clearly, we agree with the other two referees, and believe that the demonstration that K. lactis could be a better model to study these functions of the human homolog than S. cerevisiae is of sufficient interest to a broader readership. While the reviewer is completely right in that it would be of utmost interest to reveal all the detailed relationships in cytokinesis regulation in K. lactis, and compare them to the overwhelming literature available for S. cerevisiae, this is subject to extensive future investigations and would be far beyond the scope of this one manuscript.

Minor points:

1. The authors should show physical and enzymological interaction between KlRho5, KlDck1 and KlLmo1 by means of biochemical assays.

Interaction of the GEF subunits with Rho5 has been shown by CoIPs in S. cerevisiae in the paper of Schmitz et al., 2015. Given the high degree of identities at the protein level and the functional complementation in heterologous expression, we do not think that what could be gained in knowledge would justify the repetition of these experiments for the K. lactis proteins. On the other hand, we agree that demonstrating that Dck1/Lmo1 indeed functions as a GEF would be quite helpfull from a biochemical point of view. However, to our knowledge that has not been achieved for any Rho5 homologs of fungi or humans, as such experiments are quite laborious. We further believe that these experiments, while certainly interesting, are beyond the scope and the point of this manuscript, which centers on the physiological roles of KlRho5, rather than its intrinsic biochemical properties. The latter are likely to be very similar to those of ScRho5, whose analysis may be much more easy to perform, and thus one should concentrate on that yeast, since studies in K. lactis will probably not provide important new insights with regard to subunit interactions and GEF activity.

2. In Fig. S2A, the authors should add data using wild-type S. cerevisiae strain. Alternatively, exogenous expression of ScRho5 should be tested.

We included the data from a wild-type S. cerevisiae strain.

3. In page16, line 354, the authors described that the expression of KlRho5, KlDck1 and KlLmo1 by means of homologous recombination at the Klleu2 locus restored the growth in respective deletion strains.
However, the corresponding data are not presented.
We included a sentence stating the exact numbers of segregants examined. Images would be no different from what is already shown for wild-type and deletion mutants, so that their inclusion would be more distracting than helpfull.

Reviewer 2 Comments for the author

I do not ask for additional manipulations, but I wonder about the relevance of the use of the mutant labeled GFP, which, as the authors point out, does not present a complete functional activity.
We are aware of this problem, which is why it is mentioned for reasons of sincerety. As stated in the discussion, the same restriction applies to the GFP-ScRho5 fusion and the studies performed in S. cerevisiae. However, we believe the localization data are valid, since the GTPase is clearly partially functional in the fusion protein, as it shows an intermediary growth behaviour between wild-type and rho5 deletions under oxidative stress. Several attempts to modify the linker region between GFP and Rho5 to get a fully functional fusion protein have failed so far in our laboratory with the S. cerevisiae constructs, indicating that it is an intrinsic problem with N-terminal fluorophore fusions of GTPases. This is also examplified with the data now added on the GFP-KlCdc42 fusion, which unfortunately appears to be even less functional and is therefore treated with caution in the revised manuscript.

Moreover, wouldn't it be interesting to complete the experiments, by using the dominant negative mutant, to confirm the action of the constitutively active mutant?
We followed this suggestion and constructed two putatively dominant negative KlRHO5 mutants, which carry either the mutation (T17N) claimed to confer the dominant negative phenotype in human Rac1, or the K16N substitution, claimed to be effective in ScRHO5 (Singh et al., 2019). Unfortunately, our phenotypic analyses did not produce any evidence that these mutants negatively affect the morphology of the cells carrying either the mutant alleles alone or in strains still carrying a wild-type KlRHO5 allele. In contrast to other researchers who employed strong promoters for overexpression, we expressed the mutant alleles under the control of the native KlRHO5 promoter, which may explain these findings.
Given the time constraints and these disappointing preliminary results, we decided not to pursue this further. We would prefer not to comment on that in the manuscript. However, if the reviewer believes that it would be of value for a general audience (rather than distracting), we are prepared to include the preliminary data in the supplementary material.

I also wonder about the involvement of other small GTPases in these mechanisms, due to the redundancies regularly observed in other organisms.
As this was also a point raised by the first referee, we included data on KlCdc42, as outlined above.

A small improvement could be made to improve the quality of colocalization images, with enlarged views to confirm the translocation of the GTPase at the mitochondrial level.
In principle this is a good idea. However, since we now added the data on the GFP-KlCdc42 fusion to figure 3, it seems fairly crowded as it is. We find it hard to position enlarged views. The current images would have to be reduced in size to add another panel, making it even more difficult to follow the overall cellular localizations. Also, images are of sufficiently high quality to be enlarged from the PDF file on a screen to any size necessary to inspect the colocalizations more closely.

Reviewer 3 Comments for the author
This manuscript contains important findings especially regarding the mechanism of animal-type cytokinesis, and most of the data, including those on oxidative stress response, seem to be sound. Therefore, it could be published in the Journal of Cell Science after appropriate revisions.

Because the descriptions regarding functions of KlRho5 on the response against oxidative stress seem to be entirely agreeable, then I would like to comment only on those on regulation of cytoskeleton and cytokinesis under stress-free conditions.

1) In fig. 3, it is described that under stress-free conditions, both KlDck1 and KlLmo1 distributed diffusely in cytosol. However, if you look carefully, both proteins seem accumulated at patches in some structure existing as one per cell that is nucleus for example. If possible, authors would take pictures after DAPI staining for nucleus for example, show them in the manuscript as larger and highly-resolved figures, and discuss the results. These distributions in interphase could be important for functions of KlRho5 during cytokinesis.
We included colocalization studies with a nuclear mCherry marker and staining of vacuoles. No colocalization could be observed in these experiments, as shown in supplementary Fig. S6 and now stated in the main text.

2) In fig. 7, it is described that KlRho5 is accumulated at the bud neck during cytokinesis co-localizing with contractile ring constituted by actin and myosin II. This is actually the case for actin, because in Fig. 7B, at the bud neck where KlRho5 is accumulated more densely than the other cortical regions, actin is also accumulated. However, in Fig. 7A, at the bud neck where KlRho5 is accumulated more densely than the other cortical regions, myosin II is absent (upper panel, bottom budded cell). Conversely, at the bud neck where myosin II is accumulated, KlRho5 is present but is not more densely accumulated than the other cortical regions (upper panel, upper budded cell; bottom panel, both budded cells). Taken these together, in Fig. 7A, either myosin II are not co-localized with KlRho5 at the contractile ring. So I suggest that authors would take more pictures and show more panels, to clarify if myosin II and KlRho5 are co-localized with each other at the contractile ring.
This was a very good point and we included more images in supplementary materials (Fig. S5). In fact, close inspection of these images from the GFP channel showed an even distribution of KlRho5 in the plasma membrane of small budded cells, without a specific accumulation at the bud neck. Colocalization with KlMyo1-mCherry indeed can thus be attributed to the appearance of the latter at the bud neck, without an increase of KlRho5 concentration. In contrast, large budded cells do accumulate KlRho5 at the bud neck, as shown in Fig. 7B (more images have been re-inspected and confirm this). This information has been added to the respective paragraphs of the results and discussion sections. We also determined the Pearson-coefficients to substantiate our point.

3) In S. cerevisiae , it is reported that first, myosin II is recruited to the bud neck, IQGAP-like protein Cyk1 and actin are then recruited during cytokinesis (J Cell Biol. 1998 Jan 26; 140(2): 355–366. doi: 10.1083/jcb.140.2.355). In mammal, IQGAP binds to active GTP form of Rac1 and Cdc42 as an effector but not to that of RhoA. As the authors suggested, KlRho5 is more similar to mammalian Rac1 than ScRho5, therefore GTP form of KlRho5 could binds to KlCyk1 (present in the database) and be recruited to the bud neck with actin after myosin II, these consistent with Fig. 7A. Thus, the authors would be discus the possibility that KlRho5 binds to and functions with KlCyk1 during cytokinesis.
We thank the reviewer for this valuable input and added a respective paragraph to the discussion. Thus, a possible connection of KlRho5 and KlCdc42 with KlIQG1 (= KlCYK1) has been added, citing the paper mentioned and related works.
Data on KlCDC42 have been added to the manuscript, as also suggested by the other two referees. We have deleted one allele of KlIQG1 in a heterozygous diploid. However, as also stated now and demonstrated in Fig. S4, experimental evidence is hard to provide, since KlIQG1 is an essential gene and stringently controlled promoters are still not available for K. lactis. Work on such a promoter is in progress in our laboratory, but not yet advanced to a point which would allow its application in the near future.

Additional minor comments are as follows.
4) In line 124, 'K. lactis' should be 'K. lactis.'
5) In line 385, 'life-cell' might be 'live-cell.'
both typos have been corrected.

Second decision letter

MS ID#: JOCES/2020/258301

MS TITLE: The small GTPase KlRho5 responds to oxidative stress and affects cytokinesis

AUTHORS: Marius Musielak, Carolin Sterk, Felix Schubert, Christian Meyer, Achim Paululat, and Juergen J Heinisch
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out because I would like to be able to accept your paper, depending on further comments from reviewers.

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

In response to my previous comments, Musielak et al. have newly shown in the revised manuscript that KlCdc42, whose S. cerevisiae homologue play key role in bud neck formation, functionally interacts with KlRho5 because its hyperactive form (KlCdc42 G12V) suppressed the aberrant morphology of Klrho5-deleted K. lactis strain and, in response to oxidative stress, GFP-tagged KlCDC42 translocated to mitochondria as KlRHO5 did. The authors also cloned KlBem1 and KlCdc24, whose S. cerevisiae homologues are known to play key role in bud neck formation, and tried to address their functional interaction with KlRho5. However, this attempt was unsuccessful because their genetic deletions were lethal. Nevertheless, these studies collectively greatly improved our understanding of molecular functions of KlRho5 in K. lactis.

Comments for the author

I understand the authors' claim against my previous comment. Although not all points raised in my previous comment have been addressed, with the newly added and improved data based on my comment as well as other reviewers' comments, I think that the revised manuscript is now acceptable for publication in JCS.

Reviewer 2

Advance summary and potential significance to field

This work confirm the involvement of GTPases in oxidative stress, and control of cytokinesis in an unusual model K. lactis yeasts. This model could be great for the understanding of the respiratory phenotype of healthy cells, as opposed to the fermentative phenotype found in cancer cells or in cells related to Alzheimer's disease for which S. cerevisae yeasts are generally used.

Comments for the author

In response of reviewers, the authors provided news data, and news comments in the manuscript. They investigated homologs of K. lactis genes whose are known to be related to morphogenesis/cytokinesis regulation in S. cerevisiae, as suggested by reviewers. In this way, they constructed deleted mutants and activated form of KlCDC42. For example, they showed that KlCDC42-G12V can suppress the morphological defects of the Klrho5 deletion. These results were now in the revised manuscript and confirmed overlapping function of GTPases.
For mutant labelled GFP, this is a recurrent problem for studies on GTPases, and should not be against the publication of this article. Especially since the non-functionality of these tagged proteins is highlighted in the text, and give some localization information.
I am not surprised by the failure of the modification of the linker the region between GFP and Rho5 to get a fully functional fusion, and this supports my conviction that GFP prevent interaction of the GTPase with its partners.
For dominant negative KlRHO5 mutants, the results could have supported the conclusions if they had been conclusive. But again, it is common that these negative dominants are not always as effective as expected for GTPases. It is therefore not necessary to add the preliminary data in the supplementary material.
For enlarged views, it's a question of form, I let the editorial board judge, in spite of the lack of positive answers to my requests I confirm that this article can be published because of the new data it brings on these unconventional but interesting model, as KiRho5 seems to be more similar ta mammalian Rac1 than ScRho5.

Reviewer 3

Advance summary and potential significance to field

This paper identify Rho5 as putative functional homolog of mammalian Rac1 and its GEF components Gck1 and Lmo1 in *K. lactis* by homology search. By functional analyses, this paper shows that, first, as in *S. cerevisiae* KlRho5 is involved in oxidative stress response. Accordingly, these three components localized to mitochondria upon oxidative stress and glucose starvation as in *S. cerevisiae*. This was also observed for the small GTPase KlCdc42 which may serve overlapping functions. Secondly, this paper also shows for the first time the functions of KlRho5 in morphogenesis (cell wall synthesis) and cytokinesis that have not been reported for ScRho5. Deletions of each KlRho5 component led to elongated bud scar resulted from inappropriate contractile ring formed during cytokinesis, as well as increased thickness of lateral cell wall. Accordingly, KlRho5 localized to actin ring during cytokinesis.

Comments for the author

This revised version of the manuscript corrected thoroughly by the authors according to my suggestions made toward the previous version. Therefore, now, it should be published in the Journal of Cell Science after minor expression correction described below, and without performing additional experiments.

1) In the previous version, authors just described that under stress-free conditions, both KlGck1 and KlLmo1 distributed diffusely in cytosol. In the revised version, however, authors observed occasional accumulation of KlDck1 and KlLmo1 at patches in the cytosol other than nucleus or vacuole using a new figure S4. At this stage, identity of the patch is unclear, but it could be a cue to further elucidation of the function of KlRho5.

2) In the previous version, authors described that KlRho5 is accumulated at the bud neck during cytokinesis co-localizing with contractile ring constituted by actin and myosin II. Then I pointed out that this is actually the case for actin, but is not always the case for myosin II. Because as shown in Fig. 7A (Fig. 7B in revised version; same figures), in small budded cells (three of four), KlRho5 is actually present at the bud neck in the similar level as that in the other cortical regions, but not so accumulated there, whereas myosin II is clearly accumulated at the bud neck. And because in large budded cell (one of four), KlRho5 is accumulated at the bud neck, whereas myosin II is absent there. To clarify whether KlRho5 and myosin II are co-localized at the bud neck, authors collected more images and showed in new figure S6. In all cases, myosin II is accumulated at the bud neck regardless of the size of bud. In contrast, KlRho5 is not accumulated at the neck of small bud but at the large bud. Therefore it is reasonable to describe that KlRho5 co-localized with myosin II at the neck of large bud during later stages of cytokinesis. Accordingly, it is suggested to insert a sentence in line 447, "It should be noted that at the neck of small bud KlRho5 is actually present but is not so accumulated unlike large bud during later stages of cytokinesis."

3) In *S. cerevisiae*, it is reported that first, myosin II is recruited to the bud neck, IQGAP-like protein Cyk1/Iqg1 and actin are then recruited during cytokinesis (J Cell Biol. 1998 Jan 26; 140(2): 355–366. doi: 10.1083/jcb.140.2.355). In mammal, IQGAP binds to active GTP form of Rac1 and Cdc42 as an effector but not to that of RhoA. As the authors suggested, KlRho5 is more similar to mammalian Rac1 than ScRho5, therefore GTP form of KlRho5 could binds to KlCyk1 and be recruited to the bud neck with actin after myosin II. In the previous version, the authors did not discuss on the relationship between KlRho5 and KlCdc42 or KlRho5 and Iqg1, and on difference between systems including KlRho5 and ScRho5. So it was suggested that these relationship chould be mentioned. Accordingly, in the revised version, the authors not only discuss on these topics, but also added experiments regarding subcellular localization of KlCdc42 and knockout phenotypes of KlIqg1. These have greatly improved this paper.

Second revision

Author response to reviewers' comments

Response to reviewer comments

Reviewer 1 Advance Summary and Potential Significance to Field:
In response to my previous comments, Musielak et al. have newly shown in the revised manuscript that KlCdc42, whose S. cerevisiae homologue play key role in bud neck formation, functionally interacts with KlRho5 because its hyperactive form (KlCdc42 G12V) suppressed the aberrant morphology of Klrho5-deleted K. lactis strain and, in response to oxidative stress, GFP- tagged KlCDC42 translocated to mitochondria as KlRHO5 did. The authors also cloned KlBem1 and KlCdc24, whose S. cerevisiae homologues are known to play key role in bud neck formation, and tried to address their functional interaction with KlRho5. However, this attempt was unsuccessful because their genetic deletions were lethal. Nevertheless, these studies collectively greatly improved our understanding of molecular functions of KlRho5 in K. lactis.

Reviewer 1 Comments for the Author:
I understand the authors' claim against my previous comment. Although not all points raised in my previous comment have been addressed, with the newly added and improved data based on my comment as well as other reviewers' comments, I think that the revised manuscript is now acceptable for publication in JCS.

We thank the reviewer for his valuable input and the kind remarks on the revised manuscript. No action is required in these comments regarding the second revision.

Reviewer 2 Advance Summary and Potential Significance to Field:
This work confirm the involvement of GTPases in oxidative stress, and control of cytokinesis in an unusual model K. lactis yeasts. This model could be great for the understanding of the respiratory

phenotype of healthy cells, as opposed to the fermentative phenotype found in cancer cells or in cells related to Alzheimer's disease for which S. cerevisae yeasts are generally used.

Reviewer 2 Comments for the Author:
In response of reviewers, the authors provided news data, and news comments in the manuscript. They investigated homologs of K. lactis genes whose are known to be related to morphogenesis/ cytokinesis regulation in S. cerevisiae, as suggested by reviewers. In this way, they constructed deleted mutants and activated form of KlCDC42. For example, they showed that KlCDC42- G12V can suppress the morphological defects of the Klrho5 deletion. These results were now in the revised manuscript, and confirmed overlapping function of GTPases.
For mutant labelled GFP, this is a recurrent problem for studies on GTPases, and should not be against the publication of this article. Especially since the non-functionality of these tagged proteins is highlighted in the text, and give some localization information.
I am not surprised by the failure of the modification of the linker the region between GFP and Rho5 to get a fully functional fusion, and this supports my conviction that GFP prevent interaction of the GTPase with its partners.
For dominant negative KlRHO5 mutants, the results could have supported the conclusions if they had been conclusive. But again, it is common that these negative dominants are not always as effective as expected for GTPases. It is therefore not necessary to add the preliminary data in the supplementary material.
For enlarged views, it's a question of form, I let the editorial board judge, in spite of the lack of positive answers to my requests.
As explained in the first revision, we believe that specific enlargements are hard to fit in the already overloaded figures in a manner not conflicting with the labels and size bars. Since we confirmed that pictures are of high quality and can be enlarged on the computer screen from the PDF, there is no loss of information. However, if the editor should insist, we could add some enlargements to the supplementary material, just presenting the overlay images.
I confirm that this article can be published because of the new data it brings on these unconventional but interesting model, as KiRho5 seems to be more similar ta mammalian Rac1 than ScRho5.

We also thank this reviewer and appreciate the insight in the difficulties of working with tagged and mutant versions of GTPases. Again, from these comments no action is required regarding the second revision.

Reviewer 3 Advance Summary and Potential Significance to Field:
This paper identify Rho5 as putative functional homolog of mammalian Rac1 and its GEF components Gck1 and Lmo1 in *K. lactis* by homology search. By functional analyses, this paper shows that, first, as in *S. cerevisiae* KlRho5 is involved in oxidative stress response. Accordingly, these three components localized to mitochondria upon oxidative stress and glucose starvation as in *S. cerevisiae*. This was also observed for the small GTPase KlCdc42 which may serve overlapping functions. Secondly, this paper also shows for the first time the functions of KlRho5 in morphogenesis (cell wall synthesis) and cytokinesis that have not been reported for ScRho5. Deletions of each KlRho5 component led to elongated bud scar resulted from inappropriate contractile ring formed during cytokinesis, as well as increased thickness of lateral cell wall. Accordingly, KlRho5 localized to actin ring during cytokinesis.

Reviewer 3 Comments for the Author:
This revised version of the manuscript corrected thoroughly by the authors according to my suggestions made toward the previous version. Therefore, now, it should be published in the Journal of Cell Science after minor expression correction described below, and without performing additional experiments.

1) In the previous version, authors just described that under stress-free conditions, both KlGck1 and KlLmo1 distributed diffusely in cytosol. In the revised version, however, authors observed occasional accumulation of KlDck1 and KlLmo1 at patches in the cytosol other than nucleus or vacuole using a new figure S4. At this stage, identity of the patch is unclear, but it could be a cue to further elucidation of the function of KlRho5.
We are aware of the problem and keep it in mind. Patchy distribution has already been observed for the *S. cerevisiae* homologs and efforts have been made to identify the

intracellular compartments they associate with, so far without success (Schmitz et al., 2015; 2018). Thus, we expect that in *K. lactis* it will be even more difficult to address this problem and appreciate that the reviewer does not insist and does not require further experiments.

2) In the previous version, authors described that KlRho5 is accumulated at the bud neck during cytokinesis co-localizing with contractile ring constituted by actin and myosin II. Then I pointed out that this is actually the case for actin, but is not always the case for myosin Ⅱ. Because as shown in Fig. 7A (Fig. 7B in revised version; same figures), in small budded cells (three of four), KlRho5 is actually present at the bud neck in the similar level as that in the other cortical regions, but not so accumulated there, whereas myosin II is clearly accumulated at the bud neck. And because in large budded cell (one of four), KlRho5 is accumulated at the bud neck, whereas myosin II is absent there. To clarify whether KlRho5 and myosin II are co-localized at the bud neck, authors collected more images and showed in new figure S6. In all cases, myosin II is accumulated at the bud neck regardless of the size of bud. In contrast, KlRho5 is not accumulated at the neck of small bud but at the large bud. Therefore it is reasonable to describe that KlRho5 co-localized with myosin II at the neck of large bud during later stages of cytokinesis.
Accordingly, it is suggested to insert a sentence in line 447, "It should be noted that at the neck of small bud KlRho5 is actually present but is not so accumulated unlike large bud during later stages of cytokinesis."
A slightly modified sentence capturing this conclusion has been added, as suggested.

3) In *S. cerevisiae* , it is reported that first, myosin II is recruited to the bud neck, IQGAP-like protein Cyk1/Iqg1 and actin are then recruited during cytokinesis (J Cell Biol. 1998 Jan 26; 140(2): 355–366. doi: 10.1083/jcb.140.2.355). In mammal, IQGAP binds to active GTP form of Rac1 and Cdc42 as an effector but not to that of RhoA. As the authors suggested, KlRho5 is more similar to mammalian Rac1 than ScRho5, therefore GTP form of KlRho5 could binds to KlCyk1 and be recruited to the bud neck with actin after myosin II. In the previous version, the authors did not discuss on the relationship between KlRho5 and KlCdc42 or KlRho5 and Iqg1, and on difference between systems including KlRho5 and ScRho5. So it was suggested that these relationship chould be mentioned. Accordingly, in the revised version, the authors not only discuss on these topics, but also added experiments regarding subcellular localization of KlCdc42 and knockout phenotypes of Kllqg1. These have greatly improved this paper.
We thank the reviewer for this positive judgement.

Suggestions of this and the other reviewers were indeed extremely helpfull and we appreciate their inputs. We agree that the efforts invested in the additional experiments to draw up the revision significantly improved the manuscript and wish to convey our sincere gratidude to the reviewers.

Third decision letter

MS ID#: JOCES/2020/258301

MS TITLE: The small GTPase KlRho5 responds to oxidative stress and affects cytokinesis

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ARTICLE TYPE: Research Article

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