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Mitochondrial respiration promotes Cdc37-dependent stability of the Cdk1 homolog Cdc28

Ana Cláudia Leite, Telma S. Martins, Rute R. Cesário, Vitor Teixeira, Vitor Costa and Clara Pereira
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Original submission

First decision letter

MS ID#: JOCES/2022/260279

MS TITLE: Mitochondrial respiratory defects inhibit proliferation of yeast expressing Cdc28/Cdk1 mutations by compromising the Hsp90 co-chaperone Cdc37

AUTHORS: Ana Claudia Leite, Rute R Cesario, Telma S Martins, Vitor Teixeira, Vitor Costa, and Clara Pereira
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.

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

CDk1's involvement in mitochondrial dynamics has been well established but its connection to respiratory aspect has not been studied extensively in yeast. Using a Cdc28 mutant, this study uncovers a potential regulatory connection between mitochondrial respiratory function, Cdk1 and the Chaperone complex HSP9-Cdc37 in the context of cell division cycle, In this respect, the observations documented in this manuscript are of interest.

Comments for the author

Mitochondrial respiratory defects inhibit proliferation of yeast expressing Cdc28/Cdk1 mutations by compromising the Hsp90 co-chaperone Cdc37 Leite et al.

This manuscript uncovers a functional interaction between proteins involved in mitochondrial respiration and Cdc28 (Cdk1) in S. cerevisiae. The anchoring observation for this study is a growth defect exhibited by a degron allele of Cdc28 (cdc28td) when combined with the deficiency of ATP synthase subunit Atp2.

This defect is also seen when atp2Δ (or in other OCPHOS components Atp1, Atp4 Ucr2 and Cox4) is combined with other well-known ts alleles of CDC28, namely cdc28-1 (G1 defective) and cdc28-1N (g2 defective) at semi-permissive temperature. Mutant alleles of other cell cycle regulators Cdc5 and Cdc20 did not exhibit such growth defects. The double mutant atp2Δ cdc28td also shows higher mitochondrial fragmentation. Further explorations show that OXPHOS disruption causes reduction in the abundance of cdc28td. The atp2Δ cdc28td mutant is shown to be sensitive to the inhibition of HSP90-Cdc37 chaperon complex. In atp2Δ cdc28td mutant, some of Hsp90-Cdc37 client kinases are also low in abundance. This gives rise to the idea that Hsp90-Cdc37 chaperone activity is compromised in atp2Δ cdc28td mutant. The growth defect of atp2Δ cdc28td is suppressed by overexpression of Cdc37. The overexpression of Cdc37 also partially restores the low abundance of cdc28td in atp2Δ cdc28td double mutant. Based on these observations the authors propose that combined defect in mitochondrial respiration and Cdc28 compromise Cdc37's capacity as a chaperone.

CDk1's involvement in mitochondrial dynamics has been well established but its connection to respiratory aspect has not been studied extensively. The experiments are generally well designed and well conducted. In this respect, the observations documented in this manuscript are of interest. The authors have explored the initial growth phenotype (at semi permissive temperature) from various angles to finally home onto an involvement of Cdc37. However, their study has not delved deeper into the mechanistic aspects i.e. how compromising cdc28td and respiratory function leads to impairment of HSP90-Cdc37 system.

Following are some of issues the authors may consider addressing:

1. It would be helpful to include FACS analysis in Fig 1B do ascertain if the atp2Δ cdc28td double mutant is delayed or arrested at a specific cell cycle stage, as authors have done in Fig 2

2. Since cdc28td is a 'degron allele', it would be good for the reader to be informed of its abundance at different temperatures in Fig 1 rather than to be told about it in Fig 4.

3. From the morphology of cdc28td cells at 26C (Fig 1B), it seems that cdc28td protein's function is suboptimal. Hence, it may not be just the lower abundance of cdc28ts at 26C that is causing morphological alteration, but it's conformation that is also compromised (considering that its growth defective is suppressed by chaperone subunit Cdc37, as shown in subsequent Figs). This should be clearly mentioned in the text.

4. Relating to Point 3, Does overexpression of cdc28td alleviate the growth defect of the atp2Δ cdc28td double mutant? (this is answer the question whether the phenotype of the double mutant caused by lower expression of cdc228td in the double mutant.)

5. In Fig 3A, why was GAL-CDC5 was used instead of a ts mutant of Cdc5, just as the authors have used ts mutants of Cdc28, Cdc20 etc? For consistency, it would better to use a ts mutant of Cdc5

6. In Fig 4B, it is shown that mitochondrial fragmentation is enhanced in atp2Δ cdc28td double mutant. Is it because the cells are halted in mitosis? If yes then enhanced mitochondrial fragmentation would be a natural consequence. This related to the Point 1 i.e. it would be appropriate to include FACS analysis/DAPI staining of these cells. If they are indeed arrested in mitosis mitochondrial fragmentation would not be a mystery. This could potentially explain an increase in $\Delta\psi m$ for the atp2Δcdc28td in Fig 4C since there is normally a spike in ATP synthesis as cells enter mitosis.

7. The interpretation of the results: The authors conclude that "the combination of both respiratory and cdc28 cause an impairment of the Hsp90-Cdc37 system". They have shown that overexpression of Cdc37 restore the abundance of cdc28td and alleviates the growth defect of the atp2Δcdc28td (Fig 7). To consolidate this conclusion, it would be appropriate to show that overexpression of Cdc37 also restores the abundance of Yck2 or Ypk1.

8. The current tile of the manuscript is too specific. The authors may consider making a bit more general (without it being a hyperbole or overblown)

Textual amendments:

The main text has some typographical errors and awkward phrases. Only some of them are listed below. The authors should comb the text to weed out such errors or awkward phrases

a. Introduction, line 90: it should be 'Cdc37' instead of 'Cdc35'
b. Result section, Line146: It should be 'cdc28-1N' instead of 'Cdc20-1N'
c. Results Section, Line 136: awkward phrases 'Atp2 has barely no impact'
d. Result section, Line 144: awkward phrase 'Likewise cdc28-1N'
e. Line 156: awkward phrase 'aggravating interaction'
f. Line 157: awkward phase 'phenotype to cell cycle arrested cells'

Reviewer 2

Advance summary and potential significance to field

The work by Leite et al. analyse how mitochondrial dysfunction affect cell cycle progression and proposes Hsp90 co-chaperone Cdc37 as important element in this scenario.

Comments for the author

The work by Leite et al. analyse how mitochondrial dysfunction affect cell cycle progression and proposes Hsp90 co-chaperone Cdc37 as important element in this scenario.

However, I consider this work speculative and some of the results do not demonstrate the conclusions that the authors state.

Major points:

1-The title should be modified as I consider that it is not proven that mitochondrial defects are responsible for inhibition of proliferation.
2- page 4: The difference in size colony indicated by authors is not clear. In addition, "…results suggest a synergistic interaction between CDC28 and ATP2.", seems to be incorrect since Atp2 is required under respiratory conditions.
3-Page 4, lines 148-150: the experiment in Fig 2B does not account for the statement that "no specific checkpoint seems to be activated in the mutants".
4-Page 6, line 178: Genetic interaction occurs between genes, not between proteins.

5- page 7, second paragraph: How to explain the significant decrease of CIT2 in cdc28 single mutants? How to explain that RTG cannot induce and impacts the phenotype? Authors should test a condition that induces RTG.
6-Page 8, line 262: "…activity is low" must be changed to "must be affected".
7-Page 6, 3rd paragraph: The protein decrease is not necessarily a consequence of a defect in Hsp90-Cdc37 activity ("impairment of ….system"). The conclusion of this paragraph should be rewritten, or shown as a hypothesis.
8-page 9, line 286: "….,indicating that Cdc37….", must be replaced by : "….,suggesting that Cdc37….", as it cannot be affirmed. Moreover, the fact that the authors consider Cdc37 as "the limiting component", cannot be concluded from these experiments.
9-page 9, line 299: "….suggesting that the inactivation of…." This assertion should be modified, as results do not account for the inactivation.
10-page 9, lines 308-308. I consider that the role of mitochondria in cell cycle progression is not clearly demonstrated in this work.
11-Microscopy images must be improved by increasing their size.

Minor points:

Abstract; line 43: Cdc28 is not the common name of this protein in all organisms (Cdc2 in S. Pombe or in humans).
Page6, line 189: "…comparing to atp2D", should be "…comparing to cdc28td".
How do authors measure protein concentration once boiled in SDS sample buffer?

First revision

Author response to reviewers' comments

We thank for the careful revision of our manuscript "JOCES/2022/260279". The comments have certainly improved the quality of the manuscript. The main corrections in the paper and the responses to the reviewer's comments are as following:

Reviewer 1 Comments for the Author:

1. It would be helpful to include FACS analysis in Fig 1B do ascertain if the atp2Δ cdc28td double mutant is delayed or arrested at a specific cell cycle stage, as authors have done in Fig 2

We thank for the suggestion. We included these results in the revised Fig.1 and its description in the results section. As reported by others, we found *cdc28td* at non-permissive temperature does not arrest in a specific cell cycle stage. We only observe a mild S phase arrest that seems to be aggravated by deletion of *ATP2*.

2. Since cdc28td is a 'degron allele', it would be good for the reader to be informed of its abundance at different temperatures in Fig 1 rather than to be told about it in Fig 4.

We understand the reviewer's point and as such we included the abundance of the wt cdc28 and *cdc28td* at the tested temperatures in the revised Fig.1.

3. From the morphology of cdc28td cells at 26C (Fig 1B), it seems that cdc28td protein's function is suboptimal. Hence, it may not be just the lower abundance of cdc28ts at 26C that is causing morphological alteration, but it's conformation that is also compromised (considering that its growth defective is suppressed by chaperone subunit Cdc37, as shown in subsequent Figs). This should be clearly mentioned in the text.

We agree, since the abundance of the Cdc28td at 26C is even higer than wt Cdc28 (revised Fig1A), the degron certainly introduces some conformational alterations (which may also be true for the point mutants). We mentioned this in pag 5 lines 139-143 of the revised manuscript (marked version).

4. Relating to Point 3, Does overexpression of cdc28td alleviate the growth defect of the atp2Δ cdc28td double mutant? (this is answer the question whether the phenotype of the double mutant caused by lower expression of cdc28td in the double mutant.)

We overexpressed Cdc28 from a low copy-vector under the control of the endogenous promoter, and this reversed the *atp2Δcdc28td* double mutant growth inhibition. This result was included in the Supp. Material (Fig. S1).

5. In Fig 3A, why was GAL-CDC5 was used instead of a ts mutant of Cdc5, just as the authors have used ts mutants of Cdc28, Cdc20 etc? For consistency, it would better to use a ts mutant of Cdc5.

We agree with the reviewer. We decided to replace the *GAL-CDC5* experiments for a *cdc5-1* ts mutant but we had problems with revertants during the construction of the double mutant. As such, we tested instead an *ipl1-1* ts mutant. Both Cdc5 and Ipl1 have mitotic functions. The assays with the *ipl1-1* ts mutant also show there is no interaction between *IPL1* and *ATP2* and were used to replace *GAL-CDC5* in the Revised Fig.3.

6. In Fig 4B, it is shown that mitochondrial fragmentation is enhanced in atp2Δ cdc28td double mutant. Is it because the cells are halted in mitosis? If yes, then enhanced mitochondrial fragmentation would be a natural consequence. This related to the Point 1 i.e. it would be appropriate to include FACS analysis/DAPI staining of these cells. If they are indeed arrested in mitosis, mitochondrial fragmentation would not be a mystery. This could potentially explain an increase in Δψm for the atp2Δcdc28td in Fig 4C since there is normally a spike in ATP synthesis as cells enter mitosis.

From the cell cycle data in the revised Fig.1, we can exclude a mitotic arrest in the double mutant. Irrespective of that, in yeast, mitochondria are more interconnected than in mammalian cells and there is no evidence of mitochondrial fragmentation at any phase of the cell cycle. On the contrary, mitochondria are primarily a single continuous reticulum and the maintenance of a fused mitochondria is even deemed important for transport across the bud neck and for retention in the mother (DOI: 10.1091/mbc.E15-07-0455 and DOI: 10.1083/jcb.201611197). As such, we believe the observed fragmentation phenotype in the *atp2Δcdc28td* double mutant is unrelated to disturbances in cell cycle and more likely an indication of mitochondrial dysfunction.

7. The interpretation of the results: The authors conclude that "the combination of both respiratory and cdc28 cause an impairment of the Hsp90-Cdc37 system". They have shown that overexpression of Cdc37 restore the abundance of cdc28td and alleviates the growth defect of the atp2Δcdc28td (Fig 7). To consolidate this conclusion, it would be appropriate to show that overexpression of Cdc37 also restores the abundance of Yck2 or Ypk1.

We thank for the suggestion. We tested if overexpression of Cdc37 also restores the abundance of one of the decreased targets as suggested, by detecting Snf1p in the membranes of the Cdc37 overexpression assay. We found Snf1p levels are also restored and added this data to the revised Fig.7.

8. The current tile of the manuscript is too specific. The authors may consider making a bit more general (without it being a hyperbole or overblown)

In accordance, we proposed a more general title "Mitochondrial respiration promotes Cdc37-dependent stability of Cdc28/Cdk1"

Textual amendments:

The main text has some typographical errors and awkward phrases. Only some of them are listed below. The authors should comb the text to weed out such errors or awkward phrases

a. Introduction, line 90: it should be 'Cdc37' instead of 'Cdc35'

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d. Result section, Line 144: awkward phrase 'Likewise cdc28-1N'

e. Line 156: awkward phrase 'aggravating interaction'

f. Line 157: awkward phase 'phenotype to cell cycle arrested cells'

We thank for the amendments. We have also carefully revised the manuscript.

Reviewer 2 Comments for the Author:

Major points:

1- The title should be modified as I consider that it is not proven that mitochondrial defects are responsible for inhibition of proliferation.

In the revised version we proposed a more general title "Mitochondrial respiration promotes Cdc37-dependent stability of Cdc28/Cdk1"

2- page 4: The difference in size colony indicated by authors is not clear. In addition, "…results suggest a synergistic interaction between CDC28 and ATP2.", seems to be incorrect since Atp2 is required under respiratory conditions.

The synergistic interaction we are reporting concerns the growth in fermenting conditions (glucose media) at semi-repressive conditions. This was the growth condition used throughout all the work. In this media, atp2 had no effect on growth, cdc28td at 35ºC had only a minor effect on growth, but the double mutant exhibited no growth at all. The growth under respiratory conditions (and at 37ºC) were shown only as controls. We decided to remove this figure to prevent a potential confounding effect when presenting the negative interaction between the genes.

3- Page 4, lines 148-150: the experiment in Fig 2B does not account for the statement that "no specific checkpoint seems to be activated in the mutants".

Considering the reviewer's comment, we removed this sentence from the revised version.

4- Page 6, line 178: Genetic interaction occurs between genes, not between proteins.

We thank the reviewer for pointing it out. The sentence was corrected to "genetic interaction between OXPHOS encoding genes and *CDC28"*

5- page 7, second paragraph: How to explain the significant decrease of CIT2 in cdc28 single mutants? How to explain that RTG cannot induce and impacts the phenotype? Authors should test a condition that induces RTG.

CIT2 basal expression is reported to be affected by the cell cycle phase with a peak at G1 (https://doi.org/10.1073/pnas.1919535117 and 10.1016/j.csbj.2022.03.033). We can only speculate that the observed alteration in the *CIT2* basal levels in the mutants may be due to the distinct cell cycle phase at which they arrest. We added this information to the revised manuscript (marked version page8, line247-249). *CIT2* was not affected in Cdc28-1 and yet, the loss of proliferation phenotype in the double mutants was identical, suggesting the low Cit2 basal levels in the Cdc28td and 1N are likely unrelated to the observed phenotype.

As suggested, we tested growth in the complete absence of glutamate in the media, conditions that induce the RTG response (10.1093/emboj/20.24.7209), and no alterations in growth were observed. This result was added to the Fig. S6.

6- Page 8, line 262: "…activity is low" must be changed to "must be affected".

In accordance, we re-written this sentence in the revised version.

7- Page 6, 3rd paragraph: The protein decrease is not necessarily a consequence of a defect in Hsp90- Cdc37 activity ("impairment of ….system"). The conclusion of this paragraph should be rewritten, or shown as a hypothesis.

In accordance, we re-written this conclusion from the revised version.

8- page 9, line 286: "…,indicating that Cdc37…." , must be replaced by :"…,suggesting that Cdc37…." , as it cannot be affirmed. Moreover, the fact that the authors consider Cdc37 as "the limiting component", cannot be concluded from these experiments.

In accordance, we removed this conclusion from the revised version.

9- page 9, line 299: "….suggesting that the inactivation of…." This assertion should be modified, as results do not account for the inactivation.

In accordance, we re-written this sentence in the revised version.

10- page 9, lines 308-308. I consider that the role of mitochondria in cell cycle progression is not clearly demonstrated in this work.

As mentioned in the response to major point 2, under fermentation conditions, the absence of Atp2, or other OXPHOS components, had a strong impact on cell proliferation when combined with Cdc28 mutants, at semi-restrictive temperatures. However, the proliferation of the OXPHOS single mutants was not affected, indicating that OXPHOS is not critical for cell cycle progression in normal conditions but it is vital in conditions of low Cdc28 activity. Plus, OXPHOS/cdc28td mutants, but not single mutants, were highly sensitive to Hsp90-Cdc37 inhibition at permissive temperature, suggesting a role of mitochondria in the regulation of Hsp90-Cdc37 and, as consequence, of Cdc28 and cell cycle progression.

11- Microscopy images must be improved by increasing their size.

We increased the size of the microscopy images in the revised Fig.4.

Minor points:

Abstract; line 43: Cdc28 is not the common name of this protein in all organisms (Cdc2 in S. Pombe or in humans).

We thank for the suggestion. We added information on the protein name in other organisms. Pag. 3 line 75 "Cdc28 (CDK1 in mammals; cdc2 in *S. pombe*) targets more than 200 substrates, and its activity..."

Page6, line 189: "…comparing to atp2D", should be "…comparing to cdc28td".

This sentence was re-wrote in the revised manuscript.

How do authors measure protein concentration once boiled in SDS sample buffer?

Cells are boiled in SDS sample buffer without β-mercapthoethanol and protein concentration measured using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific). β-mercapthoethanol is added after since if interferes with the quantification. We added this information to the M&M section (page 15 of the marked version).

Second decision letter

MS ID#: JOCES/2022/260279

MS TITLE: Mitochondrial respiration promotes Cdc37-dependent stability of Cdc28/Cdk1

AUTHORS: Ana Claudia Leite, Telma S Martins, Rute R Cesario, Vitor Teixeira, Vitor Costa, and Clara Pereira
ARTICLE TYPE: Research Article

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As you will see, the reviewers gave favourable reports but suggest some minor modificaitons. I hope that you will be able to carry these out because I would like to be able to accept your paper.

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Reviewer 1

Advance summary and potential significance to field

CDk1's involvement in mitochondrial dynamics has been well established but its connection to respiratory aspect has not been studied extensively. This manuscript uncovers a functional interaction between proteins involved in mitochondrial respiration and Cdc28 (Cdk1) in S. cerevisiae

The authors have revised the manuscript in accordance with this reviewer's suggestions and have responded to all the questions satisfactorily.

Comments for the author

No additional revisions are required.

Reviewer 2

Advance summary and potential significance to field

The manuscript has been largely improved and most of my request have been answered,

Comments for the author

Although the manuscript has been largely improved and most of my request have been answered, I consider some important point:

-In response to my comment 5, Figure S7 (and not S6 as indicated) AND CONTRARY THAN AUTHORS STATE, shows that low glutamate (that induces RTG) causes lethality for the double mutant with respect to the control. Consequently, RTG induction can impact growth and the conclusion

“phenotype of the atp2Δcdc28td mutant at semi-restrictive temperature is not due to activationof the RTG signalling pathway” is not supported.

In addition, I do not undernstand how growth is different for double mutant under control growing conditions in upper and lower panels.

Minor points:

-Figure 1D shows 35ºC while text and figure legend indicate 37ºC.

-In Dicussion, ““The present work further reinforces the role of…” must be changed to “The present work point to …”.

Second revision

Author response to reviewers' comments

Reviewer 2 Comments for the author

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-In response to my comment 5, Figure S7 (and not S6 as indicated) AND CONTRARY THAN AUTHORS STATE, shows that low glutamate (that induces RTG) causes lethality for the double mutant with respect to the control. Consequently, RTG induction can impact growth and the conclusion “phenotype of the atp2Δcdc28td mutant at semi-restrictive temperature is not due to activation of the RTG signalling pathway” is not supported.
In addition, I do not undernstand how growth is different for double mutant under control growing conditions in upper and lower panels.”

We understand the reviewer’s point. A minor discrepant effect seems apparent at this replicate. In fact there are always some minor variations between replicas (which justifies the differences in the controls which were not performed simultaneously), since we are working with temperature sensitive mutants which are extremely sensitive to small fluctuations in the incubator temperature. Someone opening the incubator during the assay is sufficient to cause minor differences in the growth of cdc28td and the double mutant. We replaced these results for another replicate with a more similar control to the assay in the lower panel. Considering all the results, though we cannot completely discard an effect of the RTG response, if it is involved in atp2cdc28 phenotype, it only plays a minor role. Considering this matter, we decided to replace “phenotype of the atp2Δcdc28td mutant at semi-restrictive temperature is not due to activation of the RTG signalling pathway” for a more cautious “activation of the RTG signalling pathway does not play a significant role in the lethal phenotype of the atp2Δcdc28td mutant at semi-restrictive temperature” pag. 8 line 249-251.

"Minor points:
-Figure 1D shows 35ºC while text and figure legend indicate 37ºC.
-In Dicussion, ““The present work further reinforces the role of…” must be changed to “The present work point to …”."

We thank for the amendments. We have corrected the legend and the indicated sentence.

Third decision letter

MS ID#: JOCES/2022/260279

MS TITLE: Mitochondrial respiration promotes Cdc37-dependent stability of Cdc28/Cdk1

AUTHORS: Ana Claudia Leite, Telma S Martins, Rute R Cesario, Vitor Teixeira, Vitor Costa, and Clara Pereira
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.