MRX8, the conserved mitochondrial YihA GTPase family member is required for de novo Cox1 synthesis at suboptimal temperatures in Saccharomyces cerevisiae

Yash Verma, Upasana Mehra, Dharmendra Pandey, Joy Kar, Xochitl Perez-Martinez, Siddhartha Jana, and Kaustuv Datta

Corresponding author(s): Kaustuv Datta, University of Delhi

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E20-07-0457
TITLE: "MRX8, the mitochondrial YihA family of GTPase regulates Cox1p translation during cold stress in Saccharomyces cerevisiae"

Dear Dr. Datta,

Your manuscript entitled "MRX8, the mitochondrial YihA family of GTPase regulates Cox1p translation during cold stress in Saccharomyces cerevisiae" has been read by two expert referees. While referee #1 is more enthusiastic about the potential significance of this work, both referees identify significant shortcomings in the present manuscript, in several cases the same ones.

I am inclined to agree with #1 that a solid paper supporting your conclusions could be appropriate for publication in MBoC. However, in view of the extensive additional work and revisions necessary, I'm afraid that I have no choice but to reject the present manuscript. I am very sorry to have to communicate this bad news to you.

I hope that the reviews may be helpful to you as you continue your studies. Thank you for the opportunity to examine this work. We hope that as your studies progress you will consider submitting future manuscripts to Molecular Biology of the Cell (MBoC).

If you have any questions regarding the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

Sincerely,
Thomas D. Fox
Monitoring Editor
Molecular Biology of the Cell

Reviewer #1 (Remarks to the Author):

Mrx8 is a mitochondrial ribosome-associated protein of unknown function. In the current study, the authors present convincing evidence that Mrx8 is critical for Cox1 synthesis. Moreover, they confirm that Mrx8 is a matrix protein and show fractionation experiments with sucrose gradients. In addition, they present evidence that also the human Mrx8 homolog localizes to mitochondria. A number of interesting observations are reported here for the first time. The topic is certainly of interest. However, in its present form, this paper cannot be published. The text needs to be carefully revised! Both the English of the text and the interpretation of many of the data are a problem! I strongly recommend to give the text to a native speaker and, in addition, to an expert in mitochondrial biology for proof-reading. In addition to errors and language problems, the manuscript suffers from severe overinterpretation of the data. This is in particular a problem in the context of the point mutants in the GTPase domain. Either, the authors carefully rework the text and add more data. Or the authors cure the problem by deleting text AND weak data. Despite this harsh criticism, this study in in principle interesting and after thorough revision, might be appropriate for the MBoC.

Major points

1. Language. This manuscript needs to be carefully revised. The language is often sloppy, incorrect and difficult to understand. For example: Sentence 1: 'Complex IV (COX) of the mitochondrial OXPHOS machinery couple majority of ATP production.' Could read 'Complex IV (COX) of the mitochondrial OXPHOS machinery is of high relevance for cellular ATP production.' Sentence 2: 'Central to the COX complex is the conserved catalytic-core, Cox1p, which is tightly regulated for its synthesis and assembly.' could read: 'The synthesis and assembly of its catalytic-core subunit, Cox1p, is tightly regulated.' ... Someone has to go sentence by sentence through the entire text of the manuscript!!! This includes the title.
2. In figure 4C, a different acrylamide composition should be used to separate Arg8 from Var1. From the figure shown here, it is not possible to conclude that Mrx8 is required for Arg8 synthesis in this strain. Alternatively, the authors could use Arg8-specific antibodies in a Western blot experiment.
3. In fig. 5, the authors show many different Mrx8 point mutants. Are these proteins stable? Are their levels comparable? Since the authors have an Mrx8 antiserum, they can easily test this and show a Western blot in the supplement.
4. The authors draw many conclusions about the GTPase reaction cycle on the basis of weak and unclear phenotypes. Moreover, for none of these mutants the GTP or GDP binding properties are actually shown. This part of the study is very long and still does not reveal much information about the Mrx8 activity. This section should be strongly condensed and all speculative text needs to be deleted. On basis of the experiments shown, all the authors can conclude is that the GTPase activity is relevant. But conclusions on the specific reaction cycles cannot be drawn!
5. The authors claim that Mrx8 binds ribosomes on basis of Fig. 6. However, it migrates in deeper fractions than the large subunit. Why is this? Are these large fractions assembly intermediates of the mitochondrial ribosome? Or is this a supercomplex of the ribosome and Mss51? This should be easy to test.
6. From figure 6B, the authors conclude the following: 'We examined whether mrx8GKS145-147AAAp and mrx8TKXD253,254,256AAXAp are able to associate with mitoribosomes. Surprisingly, both mutant proteins were able to bind to the mitoribosomes to the same extent as wild type protein indicating loss of nucleotide binding is not detrimental to mitoribosome association (Figures S4C and 6B).' Again, Mrx8 is not in the ribosome fractions in 6B but further down in the gradient.

Minor points
7. Introduction: The authors describe Guf1 as being essential for translation of mitochondrial proteins and assembly of cytochrome oxidase complex. This is incorrect as the deletion has only a very mild phenotype.
8. Introduction: The authors write that 'In addition to the trGTPases, an additional four GTPases have been shown to regulate mitochondrial ribosome function in S. cerevisiae.' This is incorrect as they are not regulating ribosome function but rather are relevant for ribosome assembly or for modifications of rRNA.
9. The word essential should be only used if something is strictly required. However, the authors use the word in several cases to describe very mild phenotypes.
10. 'The severity of growth defect was significantly more pronounced when Δmrx8 cells were grown at lower temperature (16{degree sign}C) indicating that Mrx8p has a role in adapting to cold stress'. Please rephrase as there is no evidence presented that Mrx8 plays a role in adaptation. Also the following sentences are overinterpretations and need to be revised carefully. The title needs to be changed accordingly.

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors propose that the GTPase Mrx8 is involved in COX1 mRNA translation initiation and elongation under cold stress, thereby allowing cellular adaptation to utilize a non-fermentable carbon source. The authors have suggested a model in which Mrx8 is sensing the nucleotide availability (GTP/GDP ratio) within mitochondria and communicating it to the translation apparatus to regulate translation. If this hypothesis were correct, one would expect a general decrease in mitochondrial translation and not a transcript-specific one. However, in the figures reported is possible to appreciate a general decline in mitochondrial translation if the absence of Mrx8, although this is not commented by the authors. The authors further claim, " we report that in vivo nucleotide bound state of Mrx8p governs its ability to associate with mitochondrial ribosomes to control Cox1p translation during cold stress." GTPases function through the binding and hydrolysis of GTP to drive a particular function. It is not entirely clear from this study how the Mrx8 GTPase activity is induced depending on its association with the ribosome (based on the nucleotide availability) and how it will regulate exclusively Cox1 translation initiation and elongation. Although Mrx8 is an uncharacterized protein and potentially relevant for mitochondrial translation, the data provided is not of enough quality and depth to warrant publication in MBC.

Major comments:
1. A main criticism is that the authors generated and analyzed a large number of mrx8 point mutants based on the proposed function of the domains from the bacterial homologs, but no data are provided showing that these mutants are compromised in GTP/GDP binding and/or hydrolysis. Moreover, the steady-state levels of mutant Mrx8 proteins are not reported, which is important to understand whether the mutations affect protein stability. Without this information, the conclusions are not fully supported by the data. Moreover, an experiment including GTP, GDP or non-hydrolyzable GTP should be performed under conditions preserving the monosome to test whether Mrx8 association with the monosome if affected.
2. Overall, the authors show that Mrx8 co-sediments with the monosome and is required for Cox1 optimal synthesis. The potential mechanism involved is not demonstrated. A general quantification of protein synthesis efficiency is missing. Could it be Mrx8 a mitochondrial ribosome assembly factor acting late in the pathway such as Mtg1 or Mtg2? Only co-sedimentation and not interaction of Mrx8 with mitoribosome is shown; immunoprecipitation assays are required. Unfortunately, all these important questions remain open.
3. There is some general problem with the quality of the data. In Fig 1B, Mrx8 and Tim23 are hardly detectable in the CE fraction. In Fig 1E, the blot for S fraction under NaCl for Mrxp8 and Tim23 has been cropped too close to the signal. Larger panels are a must, to fully appreciate the original data.
4. In Fig 2A and B, the authors claim that the mrx8 deletion mutant strains were unable to utilize respiratory media containing glycerol as a carbon source for growth, but this is not readily seen on the growth test. In the growth curves, although the authors indicate that the lag phase is the same for wt and mrx8 mutant strains when transferred for glucose to glycerol media, the division time in glycerol media is reduced in the mutant. The authors conclude from this data that Mrx8 is required for respiratory growth and adaptation to respiratory media. Regarding adaptation, the data show exactly the opposite. How is this explained?
5. The length of the pulse for protein synthesis in Fig 3 is not mentioned or indicated anywhere and the paragraph "de novo cox1p synthesis..." on page 9 is over-interpreted. The reduction in Cox1 synthesis, especially without knowing the pulse time, could be fast degradation. In any case, the conclusion that Mrx8 is required for optimal Cox1 synthesis should be move after the Arg8 experiments. The Arg8 system should also be used to assess that translation of other proteins is not affected, to support the claim of Cox1 specificity.
6. Fig 3B needs a quantification as it is difficult to see that Cox1 is severely affected as claimed by authors.

7. Two general comments regarding the sucrose gradient experiments: 7.1- the authors need to justify the mitochondrial ribosome extraction conditions used. They have used 2% NP40 as a very strong detergent instead of digitonin or low concentrations of dodecyl maltoside. Is there any specific reason behind it? How stable are the ribosomes in these conditions? Also, the authors have not used magnesium in their buffer, which is important in the stabilization of the monosome. In addition, the authors have used 500 mM salt to dissociate the monosome into small and large subunits, whereas EDTA might be a better choice since increasing salt concentrations might decrease the interactions among different proteins. 7.2- Unfortunately, the fractionation and separation of the small, large, and monosome is not great. Changes in centrifugation time and speed seem to be necessary in order to optimize the separation conditions. For example, in fig 5B, it is unclear which fraction contains the monosome as the large subunit is overlapping with the monosome. Therefore, the association of Mrx8 with the ribosome presented in Fig 5 is not clear as the fractions for large and monosome are not well defined.
Other Points:
8. Although in old papers, yeast geneticists used Mrx8p or Cox1p, the use of the p is redundant, and most recent papers have dropped from yeast protein names.
9. The new nomenclature for mitoribosome proteins should be used.
10. The antibody against Mrx8p was raised "against N-terminus of the protein", which also includes the MTS. It would be indicated to report the antigen sequence.
11. It is a must to indicate the molecular markers for each protein on western blots
12. The old model of linear CIV assembly is referred to multiple times. The modular model should be considered.
13. Figure 7A lacks a loading control (cell growth in glucose media)
14. Citations on page 4 of the introduction on Mss51 regulatory loop should be Perez-Martinez 2003, Barrientos 2004
15. English grammar can be improved in several sections.

Dr. Matthew Welch
Editor-in-Chief,
Molecular Biology of the Cell

22nd July 2021

Dear Dr. Welch,

I am pleased to submit our original manuscript entitled, "*MRX8,* the conserved mitochondrial YihA GTPase family member is required for *de novo* Cox1 synthesis at suboptimal temperatures in *Saccharomyces cerevisiae*" for consideration as a Molecular Biology of the Cell research article.

In this manuscript, we have illuminated the role of previously uncharacterized Mrx8, a member of the YihA family of GTPases in regulating cellular respiration. This work for the first time defines the function for a protein factor to regulate Cox1 translation in response to environmental stress, namely cold shock. We have also shown that Mrx8 requires nucleotide binding to regulate mitochondrial function including Cox1 translation. Mrx8 was found in a complex with mitochondrial ribosomes, consistent with a role in protein synthesis. Interestingly, we show that the human ortholog of *MRX8* (*GTPBP8*) is able to complement the loss of *MRX8* function in yeast and also localizes to the mitochondria in mammalian cells. This poses an important question regarding the function of its ortholog in regulating COX1 translation in humans as well. *PET309* and *MSS51*, have previously been shown to regulate Cox1 translation in yeast. We show that requirement of Pet309 and Mss51 for cellular respiration is not bypassed by overexpression of Mrx8 and vice versa. Consistently the ribosomal association of Mss51 is independent of Mrx8. Interestingly no clear orthologues of *PET309* and *MSS51*are present in mammalian cells. *MRX8* on the other is conserved in both yeast and mammalian cells. This indicates *MRX8* to represent a class of proteins that have been retained universally during mitochondrial evolution for its optimal activity.

Given the central role of mitochondria to the cells, we believe that this manuscript describing the role of a gene to mitigate cold stress in yeast is of broad interest to readers of Molecular Biology of the Cell and lies well within the scope of the journal.

We had previously submitted a version of our manuscript to Molecular Biology of the Cell with reference number #E20-07-0457 which was reviewed by two experts. Additional experiments and changes in the text based on the constructive comments from these experts have allowed us to significantly enhance our manuscript. Specific details of the reviewer comments are outlined below. Direct quotes from the reviewers are followed by our indented responses. We kindly request you to initiate the peer review process of the revised manuscript at the earliest.

Thank you for your time and consideration in advance.

Sincerely,

Kaustuv Datta
Corresponding Author

Response to reviewer #1
Major Points

1. Language. This manuscript needs to be carefully revised. The language is often sloppy, incorrect and difficult to understand. For example: Sentence 1: 'Complex IV (COX) of the mitochondrial OXPHOS machinery couple majority of ATP production.' Could read 'Complex IV (COX) of the mitochondrial OXPHOS machinery is of high relevance for cellular ATP production.' Sentence 2: 'Central to the COX complex is the conserved catalytic-core, Cox1p, which is tightly regulated for its synthesis and assembly.' could read: 'The synthesis and assembly of its catalytic-core subunit, Cox1p, is tightly regulated.' ... Someone has to go sentence by sentence through the entire text of the manuscript!!! This includes the title.

> **Response**: As suggested by the reviewer we have modified the text.

2. In figure 4C, a different acrylamide composition should be used to separate Arg8 from Var1. From the figure shown here, it is not possible to conclude that Mrx8 is required for Arg8 synthesis in this strain. Alternatively, the authors could use Arg8-specific antibodies in a Western blot experiment.

> **Response**: In the current submission, experiments to address consequences on mitochondrial translation initiation and elongation from the *COX1* promoter which were represented in Figure 4C are now in Figure 4B and Figure S5C. In this we have examined consequence of deleting *MRX8* in XPM171a on Arg8 expression (Figure 4B). We have also extended the study to examine the consequence of deleting *MRX8* in XPM78a (Figure 4B) and RGV140 (Figure S5C) on Arg8 expression. Since the Arg8 and Var1 resolve very close to each other on an SDS-PAGE, we have included a immunoblot probed with anti-Arg8 antibodies. These data clearly show Arg8 synthesis under the control of *COX1* promoter is reduced in *Δmrx8* strain.

3. In fig. 5, the authors show many different Mrx8 point mutants. Are these proteins stable? Are their levels comparable? Since the authors have an Mrx8 antiserum, they can easily test this and show a Western blot in the supplement.

4. The authors draw many conclusions about the GTPase reaction cycle on the basis of weak and unclear phenotypes. Moreover, for none of these mutants the GTP or GDP binding properties are actually shown. This part of the study is very long and still does not reveal much information about the Mrx8 activity. This section should be strongly condensed and all speculative text needs to be deleted. On basis of the experiments shown, all the authors can conclude is that the GTPase activity is relevant. But conclusions on the specific reaction cycles cannot be drawn!

> **Response to point 3 and 4**: As suggested by the reviewer in point 4, we have trimmed this section of the manuscript and removed all the speculative text. In the current study we have included data of only one point mutant which is predicted to encode for a protein deficient in guanine nucleotide binding, *mrx8*$^{GKS145\text{-}147AAA}$. We show that cells expressing *mrx8*$^{GKS145\text{-}147AAA}$ are severely compromised for cellular respiration and Cox1 translation.

We have also included representative immunoblots showing that the steady state levels of mutant and wild type Mrx8 protein accumulate in the mitochondria to equivalent levels. Our findings are represented in Figure 5.

5. The authors claim that Mrx8 binds ribosomes on basis of Fig. 6. However, it migrates in deeper fractions than the large subunit. Why is this? Are these large fractions assembly intermediates of the mitochondrial ribosome? Or is this a supercomplex of the ribosome and Mss51? This should be easy to test.

Response: We have significantly revised this part of the study and revisited our conditions of lysis and gradients used for separation of mitochondrial ribosomes. Briefly, we have included 0.1% detergent used for lysis in our sucrose gradient, over our previous study based on numerous publications in the literature such as (Ott *et al.*, 2006; De Silva *et al.*, 2013). We have modified the materials and methods section accordingly and included information of all the reagents used in buffer preparation. In this revised study we have simultaneously examined the association of Mrx8 and Mss51 with mitochondrial ribosomes using both sucrose density centrifugation and metal ion chromatography (Figure 6). We observed that Mrx8 association with mitoribosomes on the sucrose density gradient is salt dependent as is Mss51. Interestingly Mrx8 and Mss51 interaction with the ribosome is inversely proportional. At low salt concentrations Mrx8 associated with the ribosome while majority of Mss51 was unbound. However, under high salt concentration we found Mss51 to fractionate with the large ribosomal subunit while majority of Mrx8 remained unbound or with the small subunit on a sucrose gradient (Figure 6). Moreover, we did not see any change in migration of Mss51 on a sucrose gradient in *Δmrx8* cells (Figure 7A). This indicates that binding of Mss51 with mitoribosomes is independent of Mrx8.

De Silva, D., Fontanesi, F., and Barrientos, A. (2013). The DEAD box protein Mrh4 functions in the assembly of the mitochondrial large ribosomal subunit. Cell Metab 18, 712-725.

Ott, M., Prestele, M., Bauerschmitt, H., Funes, S., Bonnefoy, N., and Herrmann, J.M. (2006). Mba1, a membrane-associated ribosome receptor in mitochondria. EMBO J 25, 1603-1610.

6. From figure 6B, the authors conclude the following: 'We examined whether mrx8GKS145-147AAAp and mrx8TKXD253,254,256AAXAp are able to associate with mitoribosomes. Surprisingly, both mutant proteins were able to bind to the mitoribosomes to the same extent as wild type protein indicating loss of nucleotide binding is not detrimental to mitoribosome association (Figures S4C and 6B).' Again, Mrx8 is not in the ribosome fractions in 6B but further down in the gradient.

Response: We have excluded this part of the study from the current manuscript. We intend to pursue this as a part of a study where we examine the nucleotide binding properties of purified recombinant wild type and mutant proteins.

Minor Points

7. Introduction: The authors describe Guf1 as being essential for translation of mitochondrial proteins and assembly of cytochrome oxidase complex. This is incorrect as the deletion has only a very mild phenotype.

Response: We have modified the text on page 5 to more accurately reflect the published literature.

8. Introduction: The authors write that 'In addition to the trGTPases, an additional four GTPases have been shown to regulate mitochondrial ribosome function in S. cerevisiae.' This is incorrect as they are not regulating ribosome function but rather are relevant for ribosome assembly or for modifications of rRNA.

Response: We have modified the text on page 5 to more accurately reflect the published literature.

9. The word essential should be only used if something is strictly required. However, the authors use the word in several cases to describe very mild phenotypes.

Response: We have noted the reviewers suggestion had removed all overinterpretation of published literature.

10. 'The severity of growth defect was significantly more pronounced when Δmrx8 cells were grown at lower temperature (16°C) indicating that Mrx8p has a role in adapting to cold stress'. Please rephrase as there is no evidence presented that Mrx8 plays a role in adaptation. Also the following sentences are overinterpretations and need to be revised carefully. The title needs to be changed accordingly.

Response: We have modified the text on pages 8 and 9 to more accurately reflect the data. We have also changed the manuscript title in accordance with these suggestions.

Reviewer #2

Major comments:

1. A main criticism is that the authors generated and analyzed a large number of mrx8 point mutants based on the proposed function of the domains from the bacterial homologs, but no data are provided showing that these mutants are compromised in GTP/GDP binding and/or hydrolysis. Moreover, the steady-state levels of mutant Mrx8 proteins are not reported, which is important to understand whether the mutations affect protein stability. Without this information, the conclusions are not fully supported by the data. Moreover, an experiment including GTP, GDP or non-hydrolysable GTP should be performed under conditions preserving the monosome to test whether Mrx8 association with the monosome if affected.

> **Response**: As suggested also by reviewer #1 in points 3&4, we have trimmed this section of the manuscript and removed all the speculative text. We intend to pursue role of Mrx8's nucleotide binding and its role on association with the mitochondrial ribosomes as a part of a separate study including the biochemical characterization of purified recombinant wild type and mutant proteins. So as to make it short and informative, we have included data of only one point mutant where we show that cells expressing *mrx8*$^{GKS145\text{-}147AAA}$ abolished Mrx8 function *in vivo*, hence affecting Cox1 translation. This is indicative from our serial dilution and S^{35} labeling experiments. Moreover, as suggested by the reviewer, we have shown that the steady state levels of Mrx8 and mrx8$^{GKS145\text{-}147AAA}$ in the mitochondria are comparable. Overall, this includes the possibility that Mrx8 nucleotide binding is important for Cox1 translation and our findings are represented in Figure 5.

2. Overall, the authors show that Mrx8 co-sediments with the monosome and is required for Cox1 optimal synthesis. The potential mechanism involved is not demonstrated. A general quantification of protein synthesis efficiency is missing. Could it be Mrx8 a mitochondrial ribosome assembly factor acting late in the pathway such as Mtg1 or Mtg2? Only co-sedimentation and not interaction of Mrx8 with mitoribosome is shown; immunoprecipitation assays are required. Unfortunately, all these important questions remain open.

> **Response:** In this revised study we have shown that Mrx8 co-migrates with the ribosome on a sucrose gradient and using IMAC we have shown Mrx8 to associate with ribosomal proteins and Mss51. In addition, we have shown that treatment of lysate with low concentrations of RNase A leads to disruption of association of Mrx8 with ribosomes (Figure 6). We have also examined whether Mrx8 might function in conjugation with Pet309 and Mss51 or might serve in redundant pathway to promote Cox1 synthesis. This was examined by testing the ability to restore glycerol growth defect in *Δmss51* or *Δpet309* cells upon introduction of multiple copies of *MRX8* or vice versa. Under all circumstances we did not observe restoration of cellular respiration in the deletion strains arguing against functional redundancy (Figure 7B, C, D and E). Moreover, we did not see any change in association of Mss51 with the ribosome on a sucrose density gradient in

Δmrx8 cells in comparison to *MRX8* (Figure 7A and Figure 6A,B). This indicates that Cox1 protein synthesis defect in *Δmrx8* is not due to Mss51 limitation. Additionally, using the $Arg8^{m}$ reporter system (also discussed below in point 5) we have observed that Mrx8 is required for both translation initiation and elongation of Cox1 during growth under sub-optimal temperature of 16°C (Figure 4 and Figure S5).

The effect on glycerol growth in cells deleted for GTPases involved in ribosome assembly (such as Mtg1, Mtg2 or Mtg3) are severe at optimal growth condition of 30°C and eventually lead to loss in mitochondrial DNA. In addition, rRNA content were compromised in ribosome assembly defect mutants such as *Δmtg1* amd *Δmtg3*. In contrast, *Δmrx8* cells have reduced growth on glycerol at sub-optimal temperature of 16°C without any loss of mitochondrial DNA. Growth on glycerol is restored similar to wild type levels in *Δmrx8* cells when incubated at optimal growth condition of 30°C, even after prolonged incubation at 16°C (data not shown). In addition we do not observe a reduction in mitochondrial transcripts (mRNA) or aberrant rRNA content in *Δmrx8* cells at 16°C (Figure 3D). Thus we believe that measuring ribosmal subunit levels would require a significant amount of work with little additional benefit.

3. There is some general problem with the quality of the data. In Fig 1B, Mrx8 and Tim23 are hardly detectable in the CE fraction. In Fig 1E, the blot for S fraction under NaCl for Mrxp8 and Tim23 has been cropped too close to the signal. Larger panels are a must, to fully appreciate the original data.

Response: We agree with these comments. For Figure 1B, we have included a western blot from a different experiment in order to show a more representative images and for Figure 1E, we have cropped a larger area.

4. In Fig 2A and B, the authors claim that the mrx8 deletion mutant strains were unable to utilize respiratory media containing glycerol as a carbon source for growth, but this is not readily seen on the growth test. In the growth curves, although the authors indicate that the lag phase is the same for wt and mrx8 mutant strains when transferred for glucose to glycerol media, the division time in glycerol media is reduced in the mutant. The authors conclude from this data that Mrx8 is required for respiratory growth and adaptation to respiratory media. Regarding adaptation, the data show exactly the opposite. How is this explained?

Response: The reviewer makes a good point. We have modified the text on pages 8 and 9 to more accurately reflect the data which indicates that Mrx8 is required for optimal utilization of respiratory media.

5. The length of the pulse for protein synthesis in Fig 3 is not mentioned or indicated anywhere and the paragraph "de novo cox1p synthesis..." on page 9 is over-interpreted. The reduction in Cox1 synthesis, especially without knowing the pulse time, could be fast degradation. In any case, the conclusion that Mrx8 is required for optimal Cox1 synthesis should be move after the

Arg8 experiments. The Arg8 system should also be used to assess that translation of other proteins is not affected, to support the claim of Cox1 specificity.

Response: We thank the reviewer for these suggestions.

- Our labeling of newly synthesized mitochondrial proteins with ^{35}S requires a pulse of 30 minutes in presence of cycloheximide to inhibit cytosolic translation followed by a chase of 10 minutes. We have included detailed experimental procedures regarding analysis of mitochondrial translation products in materials and methods section on page 23 and 24.
- We have addressed the question of whether rapid degradation of Cox1 in *Δmrx8* cells leads to reduced labeling with ^{35}S by performing an additional experiment. We have labeled the *Δmrx8* cells at 30°*C* and chased at 16°*C* for different time points. We observe no change in Cox1 labeling arguing against rapid degradation in *Δmrx8* cells as shown in Figure 3B.
- In order to further test whether Mrx8 promotes Cox1 synthesis specifically, we have introduced *Δmrx8* in strains XPM78a, XPM171a and RGV140. These Arg8 reporter strains serve as a readout of translation from Cox1 (XPM78a, XPM171a) and Cox3 loci (RGV140). As shown in Figure 4, we observed reduced Cox1 and Arg8 translation in XPM171a and XPM78a strains whereas Figure S5 shows deletion of Mrx8 does not alter Arg8 synthesis from Cox3 loci, arguing that Mrx8 specifically acts in promoting Cox1 translation.

6. Fig 3B needs a quantification as it is difficult to see that Cox1 is severely affected as claimed by authors

Response: In the current submission, we have included quantification of our labeling experiments shown in Figure 3A and Figure S3B. The quantifications are represented in Figure S3. In all cases we see a reduction in Cox1 synthesis in *Δmrx8* cells. We have modified the text based on our data at various places in the manuscript on pages 9 and 10.

7. Two general comments regarding the sucrose gradient experiments: 7.1- the authors need to justify the mitochondrial ribosome extraction conditions used. They have used 2% NP40 as a very strong detergent instead of digitonin or low concentrations of dodecyl maltoside. Is there any specific reason behind it? How stable are the ribosomes in these conditions? Also, the authors have not used magnesium in their buffer, which is important in the stabilization of the monosome. In addition, the authors have used 500 mM salt to dissociate the monosome into small and large subunits, whereas EDTA might be a better choice since increasing salt concentrations might decrease the interactions among different proteins. 7.2- Unfortunately, the fractionation and separation of the small, large, and monosome is not great. Changes in centrifugation time and speed seem to be necessary in order to optimize the separation conditions. For example, in fig 5B, it is unclear which fraction contains the monosome as the large subunit is overlapping with the monosome. Therefore, the association of Mrx8 with the ribosome presented in Fig 5 is not clear as the fractions for large and monosome are not well defined.

Response: Choice of detergent for solubilizing mitochondria was based on the detergent being a non-ionic. NP-40, TX-100 and Digitonin all fall in this category and have previously been used to solubilize mitochondria in order to extract mitochondrial ribosome followed by separation on a sucrose density gradient (Datta *et al.*, 2005; Ott *et al.*, 2006; De Silva *et al.*, 2013). We have significantly revised this part of the study and revisited our conditions of lysis and gradients used for separation of mitochondrial ribosomes. Briefly, we have included 0.1% detergent used for lysis in our sucrose gradient, over our previous study based on numerous publications in the literature such as (Ott *et al.*, 2006; De Silva *et al.*, 2013). In addition, our lysis and gradient contain 10mM Mg^{2+} ions. We have modified the materials and methods section accordingly and included information of all the reagents used in buffer preparation. 500mM NH_4Cl has been used extensively in publications showing separation of small and large mitochondrial ribosomal subunits. Some of these are (Jin *et al.*, 1997; Barrientos *et al.*, 2003; Williams *et al.*, 2004; Williams *et al.*, 2005; Williams *et al.*, 2007)

Barrientos, A., Korr, D., Barwell, K.J., Sjulsen, C., Gajewski, C.D., Manfredi, G., Ackerman, S., and Tzagoloff, A. (2003). MTG1 codes for a conserved protein required for mitochondrial translation. Mol Biol Cell 14, 2292-2302.

Datta, K., Fuentes, J.L., and Maddock, J.R. (2005). The yeast GTPase Mtg2p is required for mitochondrial translation and partially suppresses an rRNA methyltransferase mutant, mrm2. Mol Biol Cell 16, 954-963.

De Silva, D., Fontanesi, F., and Barrientos, A. (2013). The DEAD box protein Mrh4 functions in the assembly of the mitochondrial large ribosomal subunit. Cell Metab 18, 712-725.

Jin, C., Myers, A.M., and Tzagoloff, A. (1997). Cloning and characterization of MRP10, a yeast gene coding for a mitochondrial ribosomal protein. Curr Genet 31, 228-234.

Ott, M., Prestele, M., Bauerschmitt, H., Funes, S., Bonnefoy, N., and Herrmann, J.M. (2006). Mba1, a membrane-associated ribosome receptor in mitochondria. EMBO J 25, 1603-1610.

Williams, E.H., Bsat, N., Bonnefoy, N., Butler, C.A., and Fox, T.D. (2005). Alteration of a novel dispensable mitochondrial ribosomal small-subunit protein, Rsm28p, allows translation of defective COX2 mRNAs. Eukaryot Cell 4, 337-345.

Williams, E.H., Butler, C.A., Bonnefoy, N., and Fox, T.D. (2007). Translation initiation in Saccharomyces cerevisiae mitochondria: functional interactions among mitochondrial ribosomal protein Rsm28p, initiation factor 2, methionyl-tRNA-formyltransferase and novel protein Rmd9p. Genetics 175, 1117-1126.

Williams, E.H., Perez-Martinez, X., and Fox, T.D. (2004). MrpL36p, a highly diverged L31 ribosomal protein homolog with additional functional domains in Saccharomyces cerevisiae mitochondria. Genetics 167, 65-75.

Other points:

8. Although in old papers, yeast geneticists used Mrx8p or Cox1p, the use of the p is redundant, and most recent papers have dropped from yeast protein names.

9. The new nomenclature for mitoribosome proteins should be used.

> **Response to point 8 and 9**: We have noted the reviewers suggestion and made modifications in the manuscript accordingly.

10. The antibody against Mrx8p was raised "against N-terminus of the protein", which also includes the MTS. It would be indicated to report the antigen sequence.

> **Response**: The antigenic sequences we have chosen to generate Mrx8 antibodies does not fall in the MTS region. We have addressed this comment in the material and methods section page 26.

11. It is a must to indicate the molecular markers for each protein on western blots

> **Response**: We have made modifications in the Figures based on reviewer's suggestion.

12. The old model of linear CIV assembly is referred to multiple times. The modular model should be considered.

> **Response:** We have modified the text on page 4 to more accurately reflect the published literature.

13. Figure 7A lacks a loading control (cell growth in glucose media)

> **Response:** We have modified the Figure 8A to accurately reflect the loading control.

14. Citations on page 4 of the introduction on Mss51 regulatory loop should be Perez-Martinez 2003, Barrientos 2004

> **Response:** We have modified the text on page 4 to more accurately reflect the published literature.

15. English grammar can be improved in several sections.

> **Response**: We have made modifications in the manuscript and multiple independent PI's have had a chance to read the draft.

RE: Manuscript #E20-07-0457R-A
TITLE: "MRX8, the conserved mitochondrial YihA GTPase family member is required for de novo Cox1 synthesis at suboptimal temperatures in Saccharomyces cerevisiae"

Dear Dr. Datta:

I have read you revised manuscript entitled "MRX8, the conserved mitochondrial YihA GTPase family member is required for de novo Cox1 synthesis at suboptimal temperatures in Saccharomyces cerevisiae." I believe that you have addressed the comments of the referees of your original submission, and that the paper can be published in MBoC with some minor alterations.

1. The statement on page 12 that Mrx8 is associated with the 74S monosome is not clearly supported by Fig 6A. It seems at least as likely that Mrx8 is associated with the large (54S) ribosomal subunit, since there isn't a clear peak of monosomes (large and small subunits peaking together). The strongest evidence for ribosome association is the immune precipitation experiment, which only tests the large subunit. The statement on page 12 should point out this ambiguity.

2. Fig S5 B needs labels indicating MRX8/mrx8.

Thank you for submitting this interesting work to MBoC.

Sincerely,
Thomas Fox
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Datta,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Prof. Thomas Fox
Monitoring Editor,
Molecular Biology of the Cell

5th August 2021

Dear Prof. Fox,

I am pleased to submit our revised manuscript entitled, "*MRX8,* the conserved mitochondrial YihA GTPase family member is required for *de novo* Cox1 synthesis at suboptimal temperatures in *Saccharomyces cerevisiae*" with reference number E20-07-0457R-A for consideration as a Molecular Biology of the Cell research article.

Specific details of the Editor/reviewers comments are outlined below. Direct quotes from the reviews are followed by our indented responses.

Thank you for your time and consideration in advance.

Sincerely,

Kaustuv Datta
Corresponding Author

Response to Monitoring Editor

1. The statement on page 12 that Mrx8 is associated with the 74S monosome is not clearly supported by Fig 6A. It seems at least as likely that Mrx8 is associated with the large (54S) ribosomal subunit, since there isn't a clear peak of monosomes (large and small subunits peaking together). The strongest evidence for ribosome association is the immune precipitation experiment, which only tests the large subunit. The statement on page 12 should point out this ambiguity.

> As suggested by the reviewer we have modified the text on Page 12 and page 13 to more accurately interpret Figure 6A. Based on this suggestion by the reviewer, we have also modified Figure 6A and 7A such that labeling of 74S monosome on the sucrose gradient has been removed. This has been done to more accurately reflect our findings in the modified text.

2. Fig S5 B needs labels indicating MRX8/mrx8.

> As suggested by the reviewer we have correctly labeled Fig S5B indicating *MRX8* and *Δmrx8*.

RE: Manuscript #E20-07-0457RR
TITLE: "MRX8, the conserved mitochondrial YihA GTPase family member is required for de novo Cox1 synthesis at suboptimal temperatures in Saccharomyces cerevisiae"

Dear Dr. Datta:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,

Thomas Fox
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Datta:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

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We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
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