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

Yash Verma[a], Upasana Mehra[a], Dharmendra Kumar Pandey[a], Joy Kar[b], Xochitl Pérez-Martinez[c], Siddhartha S. Jana[b], and Kaustuv Datta[a,*]

[a]Department of Genetics, University of Delhi South Campus, New Delhi 110021, India; [b]School of Biological Sciences, Indian Association for the Cultivation of Science, Kolkata 700032, India; [c]Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City 04510, Mexico

ABSTRACT **The synthesis of Cox1, the conserved catalytic-core subunit of Complex IV, a multisubunit machinery of the mitochondrial oxidative phosphorylation (OXPHOS) system under environmental stress, has not been sufficiently addressed. In this study, we show that the putative YihA superfamily GTPase, Mrx8, is a bona fide mitochondrial protein required for Cox1 translation initiation and elongation during suboptimal growth condition at 16°C. Mrx8 was found in a complex with mitochondrial ribosomes, consistent with a role in protein synthesis. Cells expressing mutant Mrx8 predicted to be defective in guanine nucleotide binding and hydrolysis were compromised for robust cellular respiration. We show that the 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. Significantly, we find that *GTPBP8*, the human orthologue, complements the loss of cellular respiration in Δ*mrx8* cells and GTPBP8 localizes to the mitochondria in mammalian cells. This strongly suggests a universal role of the *MRX8* family of proteins in regulating mitochondrial function.**

Monitoring Editor
Thomas Fox
Cornell University

Received: Jul 14, 2020
Revised: Aug 5, 2021
Accepted: Aug 17, 2021

INTRODUCTION

Mitochondrial proteome is a composite of proteins encoded by its genome and the nuclear genome. Cells maintain at least two distinct translation systems to achieve this; one in the cytoplasm and one in the mitochondria. The cytosolic translation apparatus is responsible for the expression of the bulk of the mitochondrial proteome, while the mitochondrial translation apparatus is required for expression of only a small subset of open reading frames that are retained in the mitochondria (Ott *et al.*, 2016). In *Saccharomyces cerevisiae*, mitochondrial DNA (mtDNA) encodes eight polypeptides, of which seven are involved in oxidative phosphorylation (OXPHOS) and ATP synthesis and one is the component of the small ribosome (Kurland and Andersson, 2000). Components that make up the mitochondrial translation system, including ribosomal proteins, are encoded by a set of nuclear genes that are separate from those encoding the cytosolic protein synthesis apparatus (Amunts *et al.*, 2014; Desai *et al.*, 2017). These are translated in the cytosol and imported into the mitochondria, where they are assembled into macromolecular complexes in a coordinated manner to incorporate mitochondrially expressed rRNAs (15S and 21S) at the correct stoichiometry. This allows for the tight regulation of the mitochondrial gene expression machinery by the nuclear genome (Couvillion *et al.*, 2016).

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E20-07-0457).

Author contributions: Y.V., U.M., D.K.P, and J.K. performed experiments. Y.V., S.S.J., and K.D. analyzed data. X.P.-M. created some strains. Y.V. and K.D. conceived the project, designed experiments, analyzed the data, and wrote the paper.

*Address correspondence to: Kaustuv Datta (kdatta@south.du.ac.in).

Abbreviations used: CDS, coding sequence; EGFP, enhanced green fluorescent protein; UTR, untranslated region.

Coordination between the synthesis of mitochondrial partners and import of nuclear-encoded proteins is the hallmark for a majority of multiprotein complexes required for electron transport chain and ATP synthesis. For example, Complex IV (COX) of the OXPHOS machinery that plays an essential role in energy production of aerobic cells has a dual genetic origin. It is composed of 11 subunits in yeast, three of which, *COX1*, *COX2*, and *COX3*, forming the catalytic core are encoded by mitochondrial genome while all others are nuclear encoded and imported from the cytosol. Cox1, which contains the two redox centers; one containing heme *A* and the other containing heme a_3-Cu_B, is conserved in the mitochondrial genome of all aerobic organisms (Khalimonchuk and Rodel, 2005; Kim *et al.*, 2012; Timon-Gomez *et al.*, 2018). Cox1 translation and its assembly into complex IV are tightly coupled and are regulated by a large number of nuclear-encoded factors as incorrect assembly would generate free radicals that are detrimental to the cell (Fontanesi *et al.*, 2008). Central to this process is Mss51, which along with Pet309 initiates Cox1 translation and remains associated with the newly synthesized Cox1 until it assembles with nuclear subunits of the preassembly complex (Decoster *et al.*, 1990; Manthey and McEwen, 1995; Perez-Martinez *et al.*, 2003; Barrientos *et al.*, 2004; Tavares-Carreon *et al.*, 2008). This allows Cox1 translation from the mitochondrial genome to be regulated with the rate of assembly of Complex IV that requires additional independent Cox2 and Cox3 assembly modules (McStay *et al.*, 2013).

GTPases that belong to the family of P-loop NTPases form the largest class of accessory factors that regulate various aspects of ribosome biogenesis and protein synthesis (Sprang, 1997; Karbstein, 2007; Clementi and Polacek, 2010; Verstraeten *et al.*, 2011; Wittinghofer and Vetter, 2011; Maracci and Rodnina, 2016; Maiti *et al.*, 2021). Of the two families of NTPases, TRAFAC and SIMBI, the TRAFAC family comprises proteins that are thought to have evolved from an ancestral GTPase involved in translation (Leipe *et al.*, 2002; Atkinson, 2015). The TRAFAC family of GTPases that function during ribosome assembly or protein synthesis generally have extensions at either their N- or C-terminus in addition to a GTPase domain. These proteins are hypothesized to function by a common mechanism whereby the energy released upon guanine nucleotide triphosphate hydrolysis powers protein conformational changes, allowing these additional domains to carry out a mechanical process (Karbstein, 2007; Strunk and Karbstein, 2009). A subset of the TRAFAC family are the translational GTPases (trGTPases) such as IF2, EF-Tu, EF-G, and RF3 with well-defined roles during protein synthesis (Maracci and Rodnina, 2016). All organisms in addition possess highly conserved GTPases classified as trGTPases, which are predicted to function during translation although their mechanism of action remains elusive. One such protein in *S. cerevisiae* is *GUF1* (GTPase of unknown function 1), which belongs to LepA family of trGTPases, present in mitochondria. Absence of Guf1 alters the mitochondrial translation rates and assembly of cytochrome oxidase complex in the cell (Bauerschmitt *et al.*, 2008).

Four members of the TRAFAC family of GTPases in addition to the trGTPases, namely, *MTG1*, *MTG2*, *MTG3,* and *MSS1*, regulate mitochondrial ribosome assembly and RNA modification in *S. cerevisiae*. Among them, *MTG1* and *MTG2* are involved in assembly of the large subunit (54S) of the mitochondrial ribosome (Barrientos *et al.*, 2003; Datta *et al.*, 2005). *MTG3* is involved in the biogenesis of the small ribosomal subunit (37S) and regulates the processing and assembly of the 15S rRNA precursors (Paul *et al.*, 2012). Mss1 in a heterodimer with Mto1 regulates modification of mitochondrial (mt)-tRNA (Decoster *et al.*, 1993; Umeda *et al.*, 2005). Previous studies of the mitochondrial proteome (Sickmann *et al.*, 2003), genome-wide localization (Huh *et al.*, 2003), phenotypic analysis of yeast knockout strains (Giaever *et al.*, 2002), and genome-wide TAP purification (Gavin *et al.*, 2002) have implicated a novel GTPase, *MRX8* (MIOREX complex component 8) encoded by *YDR336w*, to have a significant role in mitochondrial function. Mrx8 belongs to the YihA family of GTPases, which in bacteria regulates large ribosomal subunit biogenesis/stability and is essential for cell growth (Schaefer *et al.*, 2006; Cooper *et al.*, 2009). Mrx8 is largely conserved with its bacterial family member YihA in the C-terminal GTPase domain with 58% similarity. Mrx8 contains an additional 132 amino acids at its N-terminus besides the conserved GTPase domain that contain a cryptic mitochondrial targeting sequence (Claros and Vincens, 1996). The human orthologue of *MRX8* (GTPBP8-GTP binding protein 8) also contains a highly divergent N-terminal extension in addition to a conserved GTPase domain. Mrx8, which was found as a part of the MIOREX complex, is thus speculated to be involved in translation regulation (Kehrein *et al.*, 2015).

In this study we show for the first time the role of this novel protein Mrx8 in regulating optimal Cox1 synthesis during cold stress. Deletion of *MRX8* reduces the ability of cells to utilize carbon sources requiring robust cellular respiration when grown under suboptimal temperature. Consistent with a function during translation, Mrx8 peripherally localized to the inner mitochondrial membrane and associates with the mitochondrial ribosomes. Furthermore, Δ*mrx8* cells were substantially defective for both translation initiation and elongation of Cox1, and mutations in *mrx8* predicted to be deficient for guanine nucleotide binding were compromised for in vivo function. Finally, we show that the human orthologue of Mrx8 localizes to mitochondria in mammalian cells and partially rescues a glycerol growth defect under cold stress in Δ*mrx8* yeast cells, indicating functional conservation.

RESULTS

Mrx8 is localized to the mitochondrial matrix

To determine the cellular location of Mrx8, antibodies were raised against peptides within the N-terminus of Mrx8 as described in *Materials and Methods*. The specificity of the antibody was established by immunoblot analysis on isolated mitochondria from wild-type cells and compared with that of mitochondria from Δ*mrx8* cells. A novel band corresponding to the predicted size of Mrx8 (33.7 kDa) was observed in wild-type mitochondria and not in Δ*mrx8* mitochondria although the two samples expressed equivalent levels of a bona fide mitochondrial protein, Mtg2 (Figure 1A and Supplemental Figure S1 (Datta *et al.*, 2005). Moreover, Mrx8 was specifically enriched in mitochondrial fractions as was Cox2, an integral membrane protein, and not in the cytosolic fraction (Figure 1B). To determine the submitochondrial location of Mrx8, a series of protease digestions were performed. To test its association with the outer membrane, intact mitochondria were treated with the indicated concentration of proteinase K. Mrx8 was resistant to externally added proteinase K, similar to inner membrane proteins F1β and Cox2, which reflects the presence of Mrx8 inside the mitochondria (Figure 1C). To determine the presence of Mrx8 in either the inner membrane space or the matrix, mitoplasts were generated and treated with the indicated concentration of proteinase K. Mrx8 as F1β, a known peripherally associated inner membrane protein facing the matrix, remained protease protected, whereas Cox2 having epitopes facing the inner membrane space was degraded (Figure 1D). Further, to determine whether Mrx8 is associated peripherally with the inner membrane or is an integral part of the membrane, mitochondria were treated with sodium chloride, sodium carbonate, and urea, which disrupt interactions between peripheral and integral

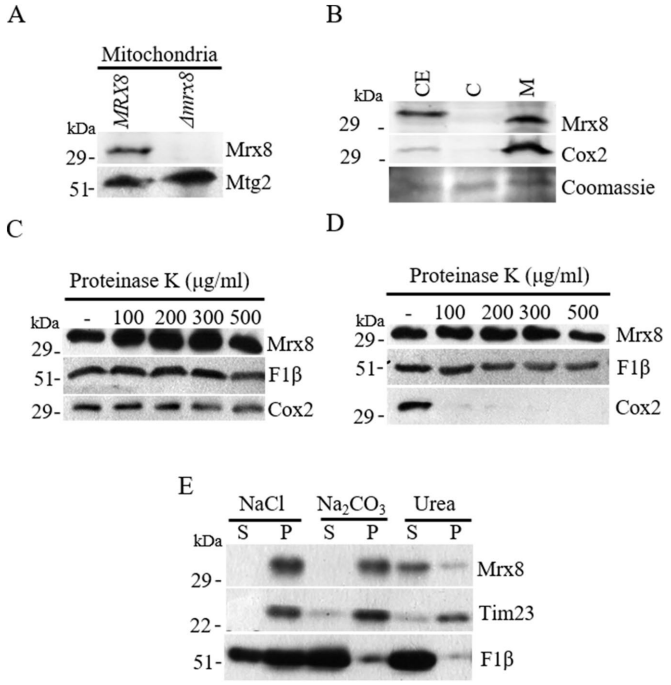


FIGURE 1: Mrx8 localizes to the mitochondrial inner membrane facing the matrix side. (A) Mitochondria were isolated from *MRX8* and Δ*mrx8* cells. Equal amounts of mitochondrial protein were separated by SDS–PAGE and subjected to immunoblot analysis. (A larger area of the immunoblot is represented in Supplemental Figure S1.) (B) Yeast cell extract (CE) was fractionated into cytosol (C) and mitochondria (M). Protein fractions were separated by SDS–PAGE and subjected to immunoblot analysis. As control a Coomassie-stained gel is shown. (C) Intact mitochondria or (D) mitoplasts were treated with 0–500 μg/ml proteinase K as indicated. The reaction was terminated by the addition of TCA, and proteins were separated by SDS–PAGE and subjected to immunoblot analysis. (E) Mitochondria were treated with either 1 M NaCl, 0.1 M Na_2CO_3, or 6 M urea as indicated. Soluble (S) and membrane (P) protein fractions were separated on SDS–PAGE and subjected to immunoblot analysis. Samples were analyzed using antibodies to Mrx8, Mtg2, Cox2, Tim23, and F1β.

membrane proteins using different mechanisms (Schook *et al.*, 1979; Fujiki *et al.*, 1982). Mrx8 remained in the membrane pellet as did Tim23, an integral membrane protein with four transmembrane helices (Bauer *et al.*, 1996), upon treatment with NaCl and Na_2CO_3, while F1β was found in the supernatant fraction (Figure 1E). However, on treatment with urea Mrx8 fractionates in the supernatant as did F1β, while Tim23 remained in the pellet (Figure 1E), indicating Mrx8 to be a tightly bound peripheral membrane protein, consistent with the absence of transmembrane domain(s) as shown by in silico analysis (https://embnet.vital-it.ch/software/TMPRED_form.html). Taking the results together, we can conclude that Mrx8 is tightly attached to the inner mitochondrial membrane facing the matrix side.

Mrx8 is required for growth on respiratory media during cold stress

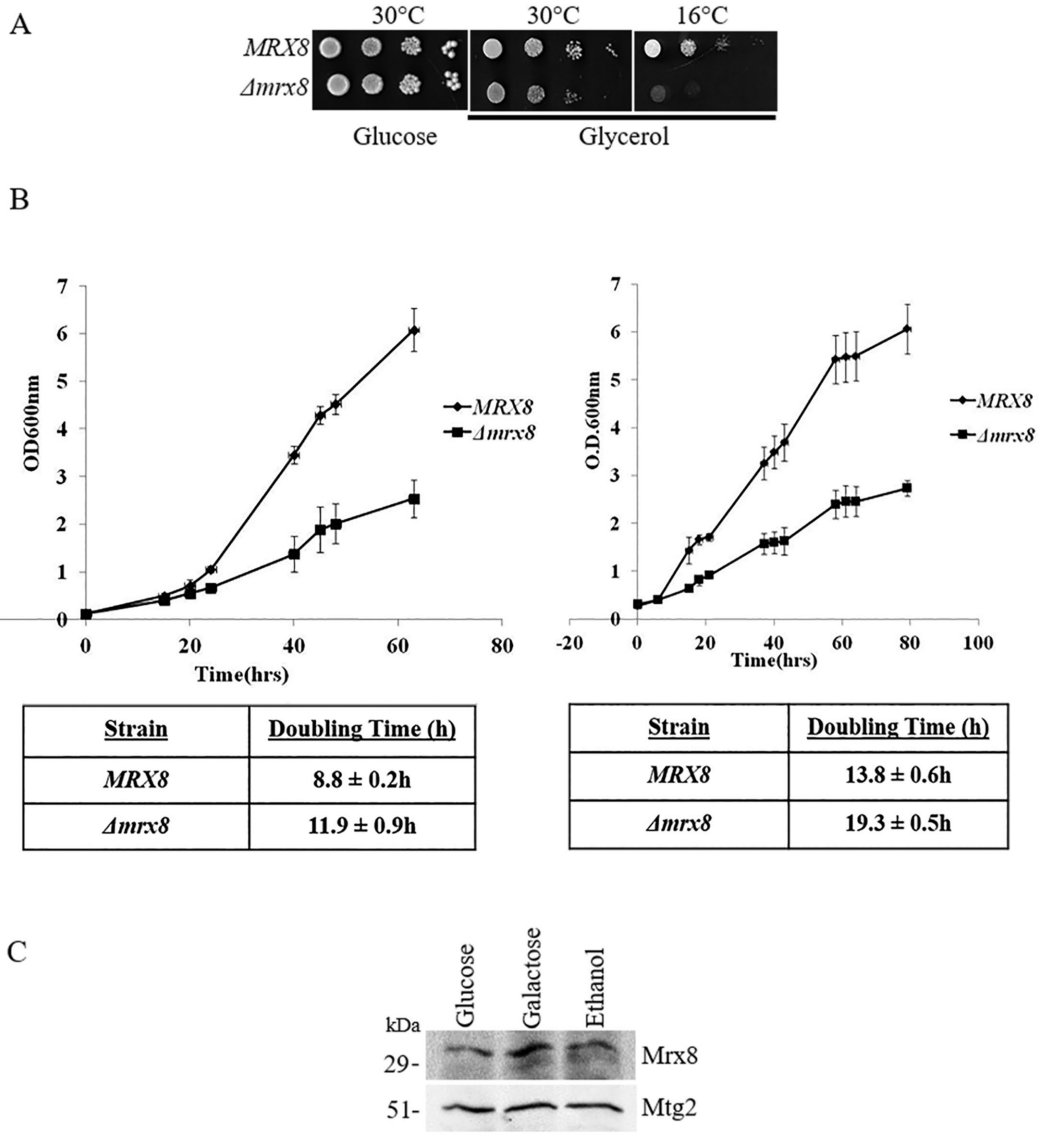
To determine whether Mrx8 plays an essential role in optimal mitochondrial activity, the ability of Δ*mrx8* cells to utilize glycerol at different temperatures was examined, as cells require robust cellular respiration for its utilization. We observed that Δ*mrx8* cells were not able to grow in media containing glycerol as efficiently as *MRX8* cells at 30°C (Figure 2, A and B). The severity of growth defect was significantly more pronounced when Δ*mrx8* cells were grown at a lower temperature (16°C), indicating that Mrx8 is required for growth on respiratory media during cold stress (Figure 2A and Supplemental Figure S2). During growth in fermentative media (glucose), the basal level of electron transport chain activity is reported (van Dijken *et al.*, 1993). A significant up-regulation of electron transport chain components, especially those encoded by the mitochondrial genome, takes place when cells are shifted from fermentative to respiratory media (glycerol) (Couvillion *et al.*, 2016; Morgenstern *et al.*, 2017). This indicates that there are regulatory proteins that maintain mitochondrial translation at a basal level during growth in fermentative media and those that up-regulate mitochondrial translation during adaptation to respiratory media. We tested whether Mrx8 has a specific role during adaptation from fermentative to respiratory media or whether it was required for both adaptation and growth on respiratory media in a temperature-dependent manner. Wild-type and Δ*mrx8* cells were either cultured in glucose and shifted to glycerol or cultured in glycerol before inoculation in fresh glycerol medium.

MRX8 and Δ*mrx8* cells show a similar growth lag when shifted from fermentative to respiratory media. However, Δ*mrx8* cells show slower utilization of glycerol as indicated by a difference of doubling time of approximately 3 h at 30°C (Figure 2B, left). Moreover, Δ*mrx8* cells adapted to respiratory media failed to utilize glycerol as efficiently as *MRX8* cells (Figure 2B, right). Similar results were obtained when Δ*mrx8* cells were constantly cultured under cold stress at 16°C (Supplemental Figure S2). Taken together, the results indicate that Mrx8 is required for optimal utilization of respiratory media.

Given that the large-scale remodeling of the mitochondrial proteome takes place upon shifting cells from fermentative to respiratory media (Couvillion *et al.*, 2016; Morgenstern *et al.*, 2017), we examined whether Mrx8 is differentially regulated during such a shift. We observed no significant change in levels of Mrx8 in mitochondria from cells cultured under different carbon sources (Figure 2C). *MRX8* transcript levels have been shown to remain constant in cells when shifted from fermentative to respiratory medium (Couvillion *et al.*, 2016). Overall, our results reflect that Mrx8 promotes cellular adaptation to utilize a nonfermentative carbon source when glucose is exhausted.

De novo Cox1 synthesis is reduced in *mrx8* null mutants

To examine whether Mrx8 is necessary for mitochondrial protein synthesis, wild-type and Δ*mrx8* cells were labeled with protein labeling mix (^{35}S-L-methionine and ^{35}S-L-cysteine) in the presence of cycloheximide to measure de novo mitochondrial translation as described in *Materials and Methods*. In Δ*mrx8* cells, no significant reduction in de novo synthesis of mitochondrially encoded proteins was observed in cells cultured at 30°C (Figure 3A and Supplemental Figure S3A). However, when newly synthesized mitochondrial proteins were labeled in cells cultured at 16°C, a severe defect in Cox1 synthesis was observed (Figure 3A and Supplemental Figure S3B). When Δ*mrx8* cells were grown at 30°C and shifted to 16°C before the addition of ^{35}S protein–labeling mix, reduction in Cox1 synthesis was observed after 4 h of the shift to 16°C (Supplemental Figure S3C). To investigate whether a reduced Cox1 level is a consequence of a defect in mitochondrial protein synthesis at 16°C in Δ*mrx8* cells rather than protein turnover, wild-type and Δ*mrx8* cells were pulse labeled at 30°C and chased at 16°C for different time intervals. The level, and therefore the stability, of the labeled proteins were comparable in the wild-type and Δ*mrx8* cells at all time points during the chase at 16°C, indicating that preexisting translation products



Strain	Doubling Time (h)
MRX8	8.8 ± 0.2h
Δmrx8	11.9 ± 0.9h

Strain	Doubling Time (h)
MRX8	13.8 ± 0.6h
Δmrx8	19.3 ± 0.5h

FIGURE 2: Mrx8 is required for efficient cellular respiration. (A) Shown are 10-fold serial dilutions of *MRX8* and Δ*mrx8* cells on glucose and glycerol plates and incubated at the indicated temperatures. (B) *MRX8* and Δ*mrx8* cells were initially cultured in either glucose (left) or glycerol (right) and then diluted into fresh glycerol media and incubated at 30°C. Optical densities were measured at 600 nm at the indicated time, and each data point is an average value of six independent colonies cultured in parallel. Tables indicate doubling time for each strain. (C) Mitochondria were isolated from wild-type cells cultured in glucose, galactose, and ethanol medium. Equal amounts of mitochondrial protein were separated by SDS–PAGE and subjected to immunoblot analysis. Samples were analyzed using antibodies to Mrx8 and Mtg2.

are equally stable (Figure 3B). Consistent with reduced de novo translation of Cox1 in Δ*mrx8* cells at 16°C, we observed a reduction in the steady state levels of Cox1 (Figure 3C). Interestingly, we also observed reduced levels of Cox2 and Cox3 at 16°C at the steady state (Figure 3C). Given that Cox1 synthesis is tightly linked to Complex IV assembly, the reduction in levels of Cox2 and Cox3 could be due to an indirect consequence of defective Complex IV biogenesis as has been previously reported (De Silva *et al.*, 2017; Mays *et al.*, 2019). However, the defect in Cox1 synthesis due to reduced *COX1* mRNA could be ruled out as levels of *COX1* mRNA were equivalent in mitochondria from *MRX8* and Δ*mrx8* cells grown at 16°C, as were *COX2*, *COX3*, and *COB* mRNA levels (Figure 3D). Taken together, our results indicate that Mrx8 acts to promote Cox1 protein synthesis.

MRX8 is required for translation initiation and elongation of Cox1

The defect in de novo Cox1 synthesis in Δ*mrx8* cells could be due to either defective translation initiation or elongation or both. To examine this aspect, we used engineered strains XPM78a, and XPM171a carrying Δ*mrx8* (Supplemental Figure S4). Both strains are deleted for the nuclear gene *ARG8*, which encodes an essential arginine biosynthetic enzyme localized to the mitochondria. XPM78a is engineered to express 512 nucleotides of intronless *COX1* fused to *ARG8*^{m} under the control of the *COX1* promoter (Perez-Martinez *et al.*, 2003) (Supplemental Figure S4A). This allows translation to be scored as a function of growth on synthetic media lacking arginine and on YPG. In comparison to wild-type cells, Δ*mrx8* cells were defective for growth on synthetic media lacking arginine and also on

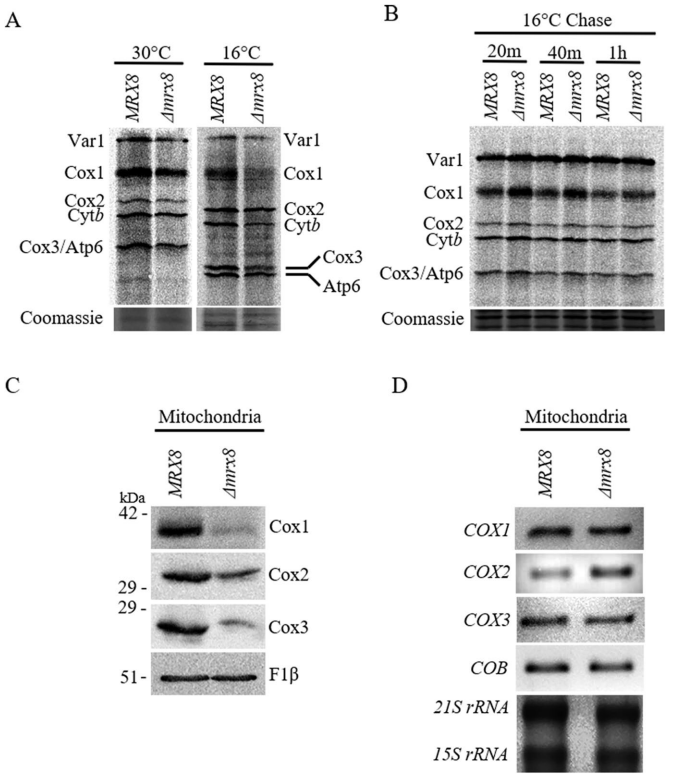


FIGURE 3: De novo Cox1 synthesis is reduced in Δ*mrx8* cells. (A) Newly synthesized mitochondrial protein products were labeled by incorporation of [^{35}S]methionine and cysteine in the presence of cycloheximide to inhibit cytosolic protein synthesis in *MRX8* and Δ*mrx8* cells at either 30°C or 16°C. (B) Newly synthesized mitochondrial protein products were labeled by incorporation of [^{35}S] methionine and cysteine in the presence of cycloheximide to inhibit cytosolic protein synthesis in *MRX8* and Δ*mrx8* cells at 30°C and chased at 16°C by the addition of casamino acid and sodium sulfate for the indicated time points. Mitochondria were isolated, and equal concentrations of mitochondrial proteins were separated on 17.5% SDS–PAGE, transferred onto a nitrocellulose membrane, exposed to BAS storage phosphor screen, and developed using phosphorimager. The positions of Var1, Cox1, Cox2, Cyt*b*, and Cox3/Atp6 are indicated. As control a Coomassie-stained gel is shown. (C) Mitochondria were isolated from *MRX8* and Δ*mrx8* cells cultured at 16°C. Equivalent amounts of mitochondrial proteins were separated via SDS–PAGE and subjected to immunoblot analysis. Samples were analyzed using antibodies to Cox1, Cox2, Cox3, and F1β. (D) Transcript levels of mitochondrially encoded genes were assayed in isolated mitochondria from *MRX8* and Δ*mrx8* cells. RNA samples were subjected to RT-PCR using primers specific for *COX1*, *COX2*, *COX3*, and *COB*. 21s rRNA and 15s rRNA levels as detected by EtBr staining. Representative images of multiple trials are shown.

YPG at 16°C but not at 30°C (Figure 4A). Aberrant de novo Cox1 synthesis and reduced steady state levels of Arg8^{m} were observed in Δ*mrx8* cells in comparison to wild-type cells at 16°C (Figure 4B). However, we did not detect a Cox1-Arg8^{m} fusion product. This is likely due to the labile nature of the Cox1-Arg8^{m} fusion product containing an internal cleavage site such that Cox1 and Arg8 can function independently (Steele *et al*., 1996; Perez-Martinez *et al*., 2003). XPM171a has a functional reengineered *ARG8^{m}* in place of the *COX1* open reading frame in the mtDNA. This strain is further engineered such that *COX1* and *COX2* open reading frames are placed downstream of the *COX2* promoter (Perez-Martinez *et al*., 2003) (Supplemental Figure S4B). The viability of this engineered strain on synthetic media lacking arginine allows us to score for translation initiation, while the viability of cells on YPG allows us to score for translation elongation. In comparison to cells harboring the wild-type allele of *MRX8*, Δ*mrx8* cells failed to grow either on synthetic media lacking arginine or on YPG at 16°C but not at 30°C, indicating the requirement of Mrx8 in Cox1 translation initiation and elongation during cold stress (Figure 4A). Consistently, both newly synthesized Cox1 and steady state levels of Arg8^{m} were reduced in Δ*mrx8* cells at 16°C in comparison to wild-type cells (Figure 4B). Further, we examined the consequences of introducing Δ*mrx8* in RGV140, an engineered strain deleted for nuclear gene *ARG8* carrying modified mtDNA, where the *COX3* promoter controls Arg8^{m} synthesis (Mays *et al*., 2019) (Supplemental Figure S5A). Growth on synthetic media lacking arginine or Arg8^{m} accumulation at 16°C remained unperturbed in Δ*mrx8* cells, indicating that Mrx8 preferentially promotes Cox1 synthesis (Supplemental Figure S5, B and C). Thus, taken together, these results indicate that Mrx8 governs optimal Cox1 translation initiation and elongation during cold stress.

Mrx8 requires nucleotide binding for its in vivo function

MRX8 belongs to the YihA/YsxC family of GTPases, with highly conserved G-domains responsible for the binding and hydrolysis of guanine nucleotide. It is well documented among GTPases involved in ribosome function that nucleotide binding and GTPase activity often power an essential function (Verstraeten *et al*., 2011). Therefore, we investigated the consequences of mutations in the G-domain of *MRX8* that are predicted to abrogate nucleotide binding and hydrolysis in vivo. The choice of the residue for mutation was based on the bacterial YihA protein as well as other TRAFAC family members, which are either biochemically characterized or for which detailed structural information is available (Lehoux *et al*., 2003; Ruzheinikov *et al*., 2004).The G1 box with the $GX_2NXGK(S/T)$ consensus is required for nucleotide binding, wherein the GKS motif is conserved across species and is responsible for binding with the α and β phosphates of GTP or GDP (Bourne *et al*., 1991; Sprang, 1997). Mutation of the GKS motif to AAA in *MRX8* is predicted to result in a complete loss of nucleotide binding and thus inhibit in vivo function as shown for several Obg family proteins in *S. cerevisiae* and *Caulobacter crescentus* (Datta *et al*., 2004; Fuentes *et al*., 2007). Cells expressing the mrx8$^{GKS145\text{-}147AAA}$ mutant protein were defective for growth on glycerol at 16°C, similar to Δ*mrx8* cells, and de novo Cox1 synthesis is defective in cells expressing mrx8$^{GKS145\text{-}147AAA}$ at 16°C (Figure 5, A and C). A reduced accumulation of steady state protein as a cause for defects observed in cells expressing mrx8$^{GKS145\text{-}147AAA}$ could be ruled out, as similar levels of Mrx8 were observed in wild-type and mutant mitochondria (Figure 5B). Therefore, our results indicate that nucleotide binding and hydrolysis are required for the in vivo function of Mrx8.

Mrx8 associates with mitochondrial ribosomes

Given that *MRX8* plays a role in optimal mitochondrial translation regulation, we examined whether Mrx8 associates with mitochondrial ribosomes. Mitochondrial ribosomal subunits were separated on sucrose gradient as described in *Materials and Methods*. The identity of individual subunits was confirmed by immunoblot analysis using the antibody to the small subunit (37S) protein Mrp13 (Partaledis and Mason, 1988) and the large subunit (54S) protein Mrp7 (Fearon and Mason, 1988). In the presence of magnesium ions and low salt (10 mM MgOAc, 100 mM NH_4Cl), Mrx8 cofractionated with both Mrp7 and Mrp13, indicating its association with the mitochondrial ribosomes (Figure 6A). Increasing the salt concentration (10 mM MgOAc, 500 mM NH_4Cl) resulted in a minor

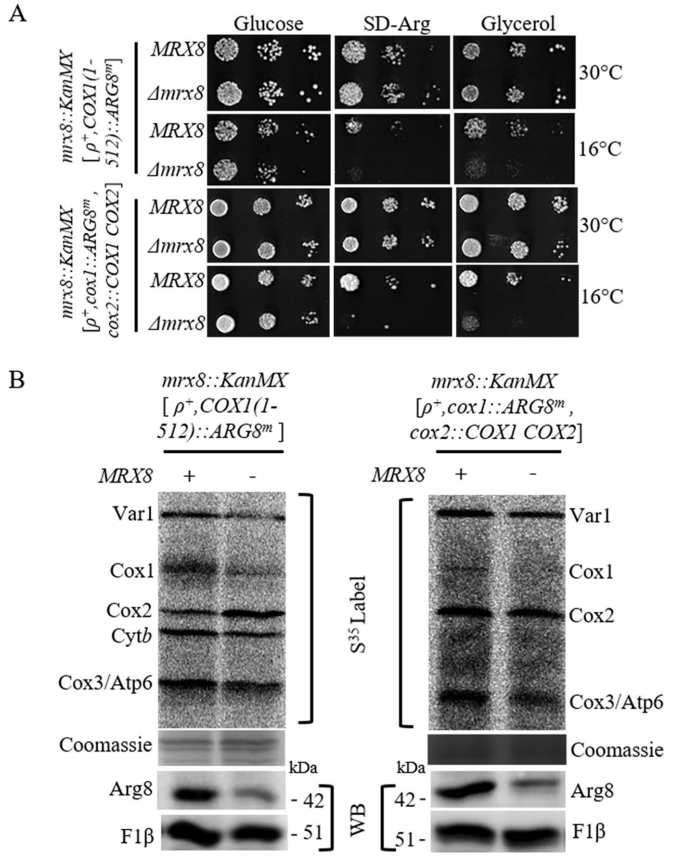


FIGURE 4: *MRX8* is essential for *COX1* translation initiation and elongation. (A) Tenfold serial dilutions of Δ*mrx8* cells in either XPM78a or XPM171a expressing either wild-type *MRX8* or vector were spotted on YPD, SD-Arg. and SGly. (B) Top: Newly synthesized mitochondrial protein products were measured in either XPM78a or XPM171a with the Δ*mrx8* allele in their nuclear genome expressing either wild-type *MRX8* or vector at 16°C by incorporation of [^{35}S] methionine and cysteine in the presence of cycloheximide to inhibit cytosolic translation. Mitochondria from labeled cells were isolated, and proteins were separated on 17.5% SDS–PAGE. Radiolabeled proteins were transferred onto a nitrocellulose membrane and visualized by phosphoimaging. The positions of mtDNA-encoded proteins are indicated. As control a Coomassie-stained gel is shown. Representative images of multiple trials are shown. Bottom: Mitochondria was isolated from Δ*mrx8* cells in either XPM78a or XPM171a expressing either wild-type *MRX8* allele or vector cultured at 16°C. Equivalent amounts of mitochondrial proteins were separated via SDS–PAGE and subjected to immunoblot analysis. Samples were analyzed using antibodies to Arg8 and F1β.

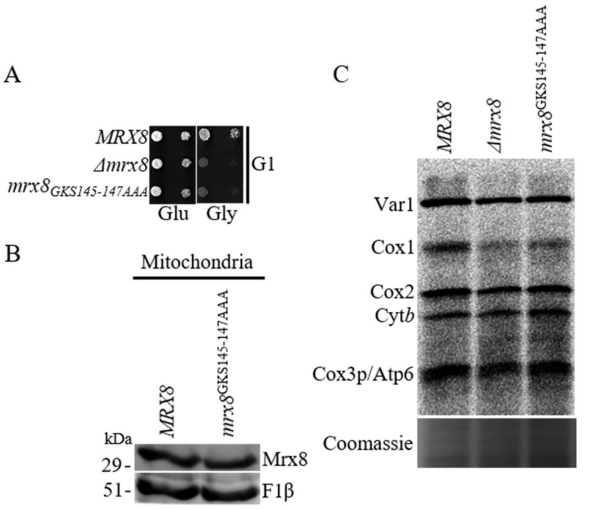


FIGURE 5: Putative nucleotide binding/hydrolysis is essential for in vivo Mrx8 function. (A) Shown are 10-fold serial dilutions of Δ*mrx8* cells expressing either wild-type *MRX8*, empty vector, or the *mrx8*$^{GKS145-147AAA}$ mutant allele on YPD and YPG at 16°C. (B) Mitochondria from Δ*mrx8* cells episomally expressing either wild-type *MRX8* or the *mrx8*$^{GKS145-147AAA}$ mutant allele were isolated. Equivalent amounts of mitochondrial proteins were separated via SDS–PAGE and subjected to immunoblot analysis. Samples were analyzed using antibodies to Mrx8 and F1β. (C) Newly synthesized mitochondrial protein products were measured in Δ*mrx8* cells episomally expressing either wild-type *MRX8*, vector, or the *mrx8*$^{GKS145-147AAA}$ mutant allele at 16°C by incorporation of [^{35}S] methionine and cysteine in the presence of cycloheximide to inhibit cytosolic translation. Mitochondria from labeled cells were isolated, and proteins were separated on 17.5% SDS–PAGE. Radiolabeled proteins were transferred onto a nitrocellulose membrane and visualized by phosphoimaging. The positions of mtDNA-encoded proteins are indicated. As control a Coomassie-stained gel is shown.

pool fractionating with ribosomal proteins while the majority of the Mrx8 were in the low-molecular-weight protein fractions (Figure 6B). To further test whether Mrx8 requires intact RNA–protein complex for migration into the sucrose gradient, we treated mitochondrial lysates with RNase A (100 μg/ml) before separation on a sucrose gradient. This led to the disruption of mitochondrial ribosomes and prevented migration of Mrx8 into the sucrose gradient (Figure 6C). Furthermore, isolation of the Mrx8-containing complex by immobilized metal affinity chromatography (IMAC) from mitochondrial lysates expressing functional Mrx8-6xHis led to copurification of Mrp7 (Figure 6D). Taken together, the results indicate that Mrx8 associates with the 54S subunit either as a part of the 74S monosome or when the 54S subunit is free from the monosome.

Mrx8 does not govern Mss51 cofractionation with the ribosomes

Cox1 translation and its assembly into Complex IV are tightly coupled to a negative feedback loop involving Mss51. In addition to its role in Cox1 translation initiation, Mss51 also interacts with newly synthesized Cox1 and remains associated until Cox1 maturation and assembly into Complex IV is initiated (Perez-Martinez *et al.*, 2003; Barrientos *et al.*, 2004; Perez-Martinez *et al.*, 2009). Defective assembly of newly synthesized Cox1 sequesters Mss51 in a higher-order Cox1 pre-assembly complex, which reduces its ability to initiate a fresh round of Cox1 translation (Perez-Martinez *et al.*, 2003, 2009). To determine whether the absence of Mrx8 affects the Mss51-mediated feedback loop, we analyzed the mobility of a functional GST-tagged Mss51 on a sucrose density gradient in wild-type and Δ*mrx8* cells. When ribosomes from wild-type cells were fractionated on a sucrose gradient in the presence of magnesium ions and low salt, we observed that only a minor pool of Mss51 migrated with ribosomal marker proteins Mrp7 and Mrp13 while the majority were in the unbound fractions (Figure 6A). On increasing the salt concentration, the majority of Mss51 migrated with fractions containing ribosomal marker proteins, and minor pools migrated into deeper fractions than the large subunit (Figure 6B). These results were contrary to the mobility of Mrx8 in a sucrose gradient. Interestingly, isolation of Mrx8-containing complex led to copurification with Mrp7 and Mss51 (Figure 6D). Taken together, the results indicate that there exists a pool of Mrx8, Mss51, and ribosomes in the mitochondria.

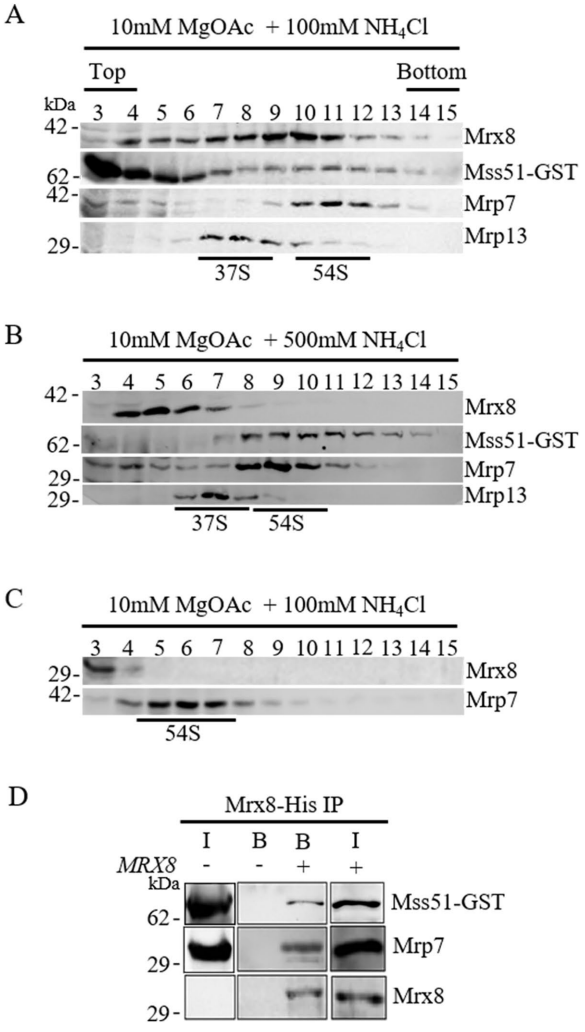


FIGURE 6: Mrx8 associates with mitochondrial ribosomes. Mitochondrial lysates from wild-type cells containing a functional *MSS51-GST* allele were separated by ultracentrifugation on a 5–30% sucrose gradient containing (A) 10 mM MgOAc, 100 mM NH_4Cl, and (B) 10 mM MgOAc, 500 mM NH_4Cl, at 135,000 × *g* for 4 h. (C) Mitochondrial lysates were incubated with RNase A before separation on a 5–30% sucrose gradient containing 10 mM MgOAc and 100 mM NH_4Cl. Fractions were TCA precipitated, separated on SDS–PAGE, and subjected to immunoblot analysis. Antibodies used were against Mrx8, GST (to detect Mss51), Mrp7(bL27m), and Mrp13(mS44). The migration of the 37S and 54S peaks were labeled based on immunoblot analysis. (D) Mitochondria from cells expressing Mss51-GST and Mrx8-6xHis were solubilized and purified using metal ion chromatography. Fifteen percent of input (I), and bound protein (B) were separated by SDS–PAGE and subject to immunoblot analysis. Antibodies used were against Mrx8, GST (to detect Mss51), and Mrp7(bL27m).

Our results indicate a condition that allows us to detect Mss51 co-fractionating with ribosomes on a sucrose density gradient in a respiratory-competent cell that is not known currently. The high-salt condition might stabilize the hydrophobic interaction of Mss51 with the ribosome including the Cox1 preassembly complex. In Δ*mrx8* cells Mss51 migration with the ribosomes under low- and high-salt conditions remained unaltered at 16°C in comparison to wild-type cells (Figures 7A and 6, A and B). This indicates that the reduced Cox1 synthesis observed in Δ*mrx8* cells is not a result of sequestration of Mss51 into a higher-order complex.

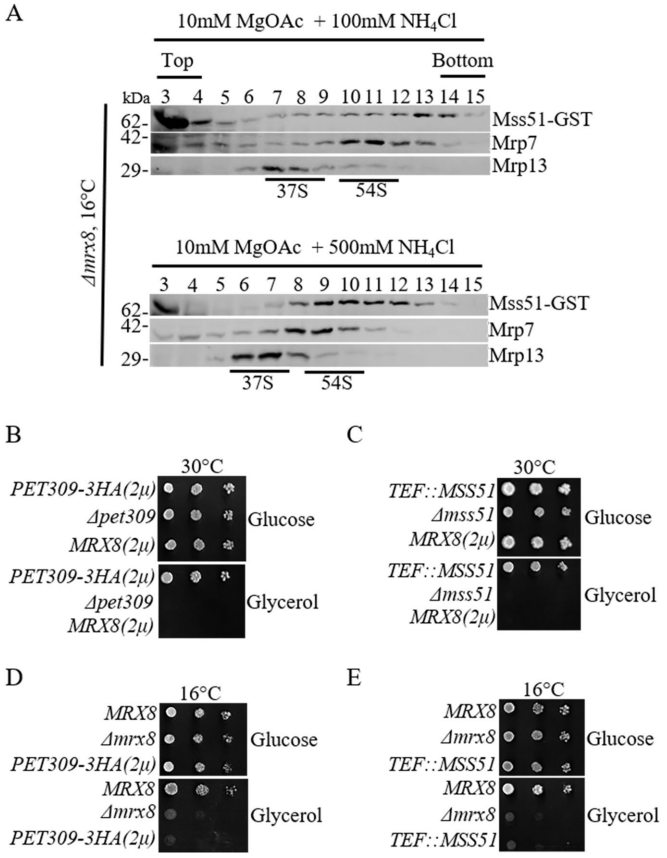


FIGURE 7: Mss51 migration on a sucrose density is independent of Mrx8. (A) Mitochondria from *MSS51-GST* cells carrying the Δ*mrx8* allele cultured at 16°C were lysed and separated by ultracentrifugation on a 5–30% sucrose gradient containing either (top) 10 mM MgOAc, 100 mM NH_4Cl, or (bottom) 10 mM MgOAc, 500 mM NH_4Cl. Fractions were TCA precipitated, separated by SDS–PAGE, and subjected to immunoblot analysis. Antibodies used were against GST (to detect Mss51), Mrp7(bL27m), and Mrp13(mS44). The migration of the 37S and 54S peaks were labeled based on immunoblot analysis. Shown are 10-fold serial dilutions of (B) Δ*pet309* cells expressing *PET309-3HA(2μ)*, empty vector, or *MRX8(2μ)*, (C) Δ*mss51* cells expressing *MSS51-GST*, empty vector, or *MRX8(2μ)*, (D) Δ*mrx8* cells expressing *MRX8*, empty vector, or *PET309-3HA(2μ)*, and (E) Δ*mrx8* cells expressing *MRX8*, empty vector, or *MSS51-GST* on glucose and glycerol media at either 30°C or 16°C.

Glycerol growth defects of Δ*mss51* and Δ*pet309* are not bypassed by multiple copies of Mrx8 and vice versa

Mss51 and Pet309 initiate Cox1 translation by binding to its 5′UTR in addition to Mss51's role during Cox1 assembly (Manthey and McEwen, 1995; Zamudio-Ochoa *et al.*, 2014). Mrx8 might function in conjugation with Pet309 and Mss51 or might serve in a redundant pathway to promote Cox1 synthesis. This was examined by testing the ability to restore glycerol growth defect in Δ*mss51* or Δ*pet309* 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 7, B–E).

GTPBP8, the human homologue of *MRX8*, complements Δ*mrx8* cells

The closest orthologue of *MRX8* in humans is a GTPase of unknown function annotated as *GTPBP8* with nearly 45% similarity over the

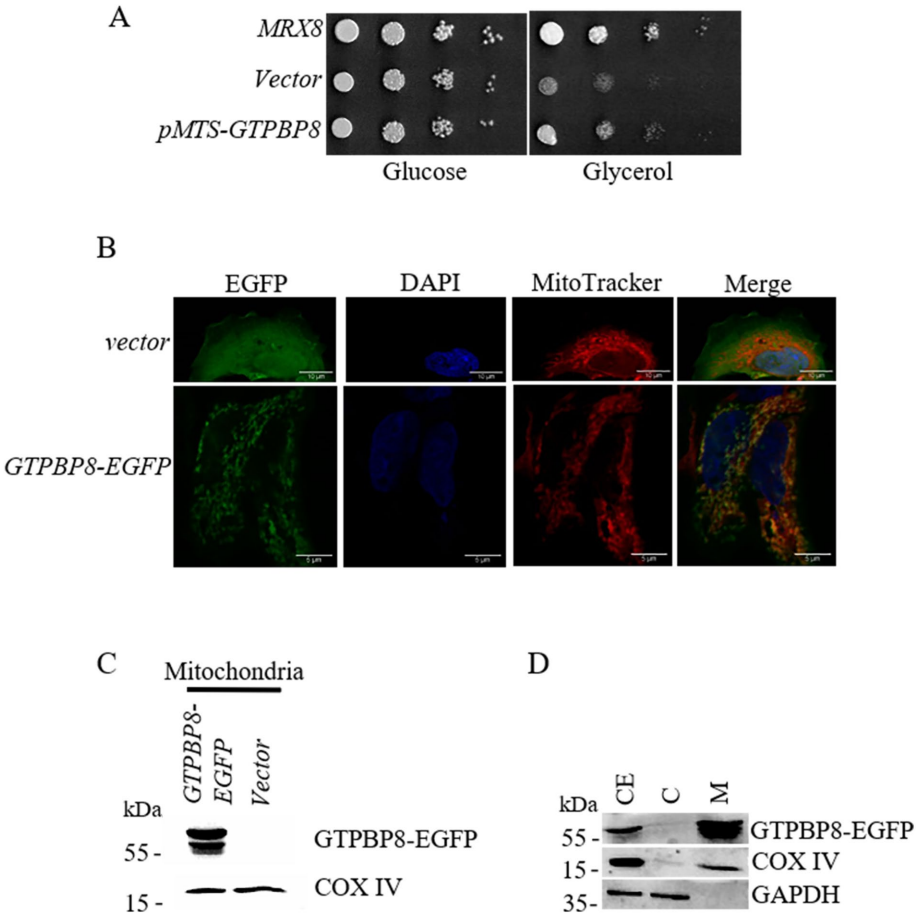


FIGURE 8: Human *MRX8* (*GTPBP8*) complements Δ*mrx8* cells and localizes to mitochondria in mammalian cell lines. (A) Shown are 10-fold serial dilutions of Δ*mrx8* cells expressing *MRX8*, empty vector, or MTS-tagged *GTPBP8* on glucose and glycerol at 16°C. (B) Shown are representative confocal images of HEK293 cells transfected with either empty vector EGFP-N1 or EGFP-tagged *GTPBP8*. Scale bars: 10 μm (vector) and 5 μm (*GTPBP8-EGFP*). (C) Mitochondria were isolated from HEK293 cells transfected with EGFP-tagged *GTPBP8* and empty vector EGFP-N1 as described in *Materials and Methods*. (D) HEK293 cell extracts (CE) were fractionated into cytosol (C) and mitochondria (M). Protein samples were separated by SDS–PAGE and subjected to immunoblot analysis. Antibodies used were against GFP (to detect GTPBP8), GAPDH, and COX IV.

entire protein length. We tested whether functional conservation exists between the two orthologous proteins. Expression of GTPBP8 in Δ*mrx8* cells failed to restore the growth defect on glycerol media at 16°C (unpublished data). This could be either because GTPBP8 is not functional in yeast or because the human mitochondrial targeting sequence on GTPBP8 is not recognized by the mitochondrial import machinery in yeast. To ensure mitochondrial localization, we fused the cleavable mitochondrial targeting sequence of *MTG3* (amino acids 1–21) at the N-terminus of GTPBP8. Interestingly, there was partial restoration of growth on glycerol at 16°C by mitochondrially targeted GTPBP8 in Δ*mrx8* cells in comparison to cells expressing wild-type *MRX8* (Figure 8A). This indicates that functional conservation exists between yeast and the human orthologue of Mrx8. The ability to restore mitochondrial function in Δ*mrx8* cells suggests that GTPBP8 is localized to the mitochondria and is involved in some aspect of cellular respiration. However, this does not rule out a differential localization of the endogenous GTPBP8. Using HEK293 cells transfected with enhanced green fluorescent protein (EGFP)-tagged GTPBP8, we demonstrated the localization of GTPBP8 in the mitochondria as it overlaps with MitoTracker red (Figure 8B). The specificity of GTPBP8-EGFP was confirmed by immunoblot analysis on mitochondria from HEK293 cells expressing EGFP-tagged GTPBP8 (63.2 kDa) or pEGFP-N1 (Figure 8C). Finally, when lysates from HEK293 cells expressing EGFP-tagged GTPBP8 were purified into cytosolic and mitochondrial fractions, we observed the enrichment of GTPBP8 in the mitochondrial fraction similar to COX IV, a bona fide mitochondrial protein, while GAPDH was enriched in the cytosolic fraction (Figure 8D). This shows that the endogenous GTPBP8 is localized to the mitochondria in mammalian cells and this function of mitochondria controlled by Mrx8 is conserved between yeast and humans. This is the first demonstration of conservation of this function of *MRX8* to the best of our knowledge.

DISCUSSION

Translation regulation in response to changing environmental signals such as temperature or nutrient availability is well documented in yeast as well as in bacteria. The process of mitochondrial translation is uniquely distinct from bacteria and cytosolic translation. Although mitochondrial translation is understood in some detail (Fox, 2012; Ott *et al.*, 2016), the adaptation of the translational machinery to changing environmental conditions that might alter the spatiotemporal requirement for the expression of various genes is not clear.

Herein, we show that *MRX8*, a protein belonging to the YihA subfamily of GTPases, promotes Cox1 synthesis during cold stress. The only prior report showing temperature-dependent regulation of mitochondrially encoded Complex IV subunits and their subsequent assembly is in the case of Δ*guf1* cells (Bauerschmitt *et al.*, 2008). We observed that Δ*mrx8* cells failed to utilize glycerol at the same rate as wild-type cells specifically at 16°C, indicating defective cellular respiration. The predicted mitochondrial localization (60% probability) (Claros and Vincens, 1996) and isolation of Mrx8 as a part of the MIOREX complex (Kehrein *et al.*, 2015) strongly indicates that Mrx8 is likely to be a mitochondrial protein. Consistently we found Mrx8 to be peripherally associated with the mitochondrial inner membrane. It is well documented that the mitochondrial inner membrane serves as a platform for mitochondrial gene expression involving steps of DNA replication, repair, transcription, RNA processing/modification, RNA degradation, ribosome biogenesis, and mRNA turnover and translation (Ott *et al.*, 2016; Singh *et al.*, 2020). Thus, not surprisingly, in Δ*mrx8* cells at 16°C we observed that de novo Cox1 synthesis and steady state accumulation were significantly reduced, without a reduction in Cox1 transcript levels, indicating a requirement of Mrx8 for proper Cox1 translation (Figure 3). Mitochondrial transcripts are recognized by specific activator proteins that interact directly with the 5′ UTR and aid its association with mitochondrial ribosomes to initiate translation (Fox, 2012). Regulation of mitochondrial translation occurs by two different mechanisms.

Strain	Genotype	Source
BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0,* ρ^+	American Type Culture Collection (ATCC)
BY4742	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* ρ^+	ATCC
CRY1	*MATa, ura3-52, trp1Δ2, leu2-3112, his3-11, ade2-1, can1-100,* ρ^+	Brickner and Fuller, 1997
KD43	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, mrx8::KanMX4,* ρ^+	ATCC
KD337	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, mrx8::KanMX, [CEN, MRX8,URA3],* ρ^+	This study
KD749	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, mrx8::KanMX4, [CEN, CYC1::MRX8, HIS3],* ρ^+	This study
KD1069	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, mrx8::KanMX4, [CEN, CYC1::mrx8*$^{GKS145\text{-}147AAA}$*, HIS3],* ρ^+	This study
KD757	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, mrx8::KanMX4, [CEN, CYC1::GTPBP8, HIS3],* ρ^+	This study
KD877	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, mrx8::KanMX4, [CEN, CYC1::*MTS-*GTPBP8, HIS3],* ρ^+	This study
KD878	*MATa, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* ρ^+	This study
KD884	*MATa, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, mrx8::KanMX4,* ρ^+	This study
XPM171a	*MATα, leu2-3,112, lys2, ura3-52, arg8::hisG, [*ρ^+*,cox1::ARG8*^{m}*, cox2::COX1 COX2]*	Perez-Martinez *et al.*, 2003
KD1512	*MATα, leu2-3,112, lys2, ura3-52, arg8::hisG, mrx8::KanMX4 [*ρ^+*,cox1::ARG8*^{m}*, cox2::COX1 COX2]*	This study
XPM78a	*MATα, leu2-3,112, lys2, ura3-52, arg8::hisG,[*ρ^+*,COX1(1-512)::ARG8*^{m}*, ΔΣaI, ΔΣbI]*	Perez-Martinez *et al.*, 2003
KD1562	*MATα, leu2-3,112, lys2, ura3-52, arg8::hisG, mrx8::KanMX4 [*ρ^+*,COX1(1-512)::ARG8*^{m}*, ΔΣaI, ΔΣbI]*	This study
RGV140	*MATa, his3-1,15 leu2-3,112, trp1-1, ura3-1 Δarg8::KanMX 4[*ρ^+*, cox3Δ::ARG8*^{m}*]*	Mays *et al.*, 2019
KD1516	*MATa, his3-1,15 leu2-3,112, trp1-1, ura3-1 Δarg8::KanMX4, mrx8::TRP1-1 [*ρ^+*, cox3Δ::ARG8*^{m}*]*	This study
KD1532	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, MSS51-GST,* ρ^+	This study
KD1567	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, MSS51-GST, mrx8::TRP1-1,* ρ^+	This study
XPM76a	*Mata, lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2 [*ρ+*, COX1(1-512)::ARG8*^{m}*, ΔΣaI, ΔΣbI]*	Perez-Martinez *et al.*, 2009
KD1608	*Mata, leu2-3,112, ura3-52, mss51Δ::LEU2,* ρ^+	This study
KD1610	*Mata, leu2-3,112, ura3-52, mss51Δ::LEU2, [TEF::MSS51-GST],*ρ^+	This study
KD1611	*Mata, leu2-3,112, ura3-52, mss51Δ::LEU2, [2μ, MRX8,URA3],* ρ^+	This study
XPM232	*Mata, lys2, leu2-3,112, arg8::hisG, ura3-52, pet309Δ::LEU2,[*ρ^+*, ΔΣaI]*	Tavares-Carreon et al., 2008
KD1612	*Mata, lys2, leu2-3,112, arg8::hisG, ura3-52, pet309Δ::LEU2, [pXP104: 2μ, PET309-3HA, URA3], [*ρ^+*, ΔΣaI]*	This study
KD1613	*Mata, lys2, leu2-3,112, arg8::hisG, ura3-52, pet309Δ::LEU2, [2μ, MRX8,URA3], [*ρ^+*, ΔΣaI]*	This study
KD1614	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, mrx8::KanMX4, [TEF::MSS51-GST]*ρ^+	This study
KD1615	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, mrx8::KanMX4, [pXP104: 2μ, PET309-3HA, URA3],* ρ^+	This study

TABLE 1: Genotypes and sources of yeast strains used in this study.

Gene	Sense primer	Antisense primer
COX1	5′GCTCTAATCCATGGTGGTTCAATTAGATTAGCACTACC3′	5′GAAAATGTCCCACCACGTAGTAAGT3′
COX2	5′AAAGTTGATGCTACTCCTGGTAGA3′	5′TGCTTCGATCTTAATTGGCA3′
COX3	5′TATGGTTCAGTATTCTATGC3′	5′TTAGACTCCTCATCAGTAGA3′
COB	5′TACTGATAGAAGTGTAGTAA3′	5′TTATTTATTAACTCTACCGA3′

TABLE 2: Primers used to quantify mRNA levels using RT-PCR.

The first is by regulating the availability of nuclear-encoded translation activators in mitochondria, which in turn modulate rates of polypeptide formation from target mRNA (Fiori *et al.*, 2005; Couvillion *et al.*, 2016). Second, the regulation of mitochondrial translation is achieved by tightly coupling it to OXPHOS subunit assembly. If assembly fails, synthesis of key subunits expressed form mtDNA is also down-regulated (Fox, 2012). Thus, could the reduction observed in Cox1 synthesis in Δ*mrx8* cells be due to a direct role of Mrx8 in promoting Cox1 translation or a secondary consequence of sequestration of Mss51 in a higher-order complex, or a combination of both? Our results support a model whereby Mrx8 promotes Cox1 translation. First, we found Mrx8 to be associated with the ribosomes likely as a part of the 74S monosome. Second, using the $Arg8^{m}$ reporter system, we observed that Mrx8 is required for both translation initiation and elongation of Cox1 during growth under the suboptimal temperature of 16°C. Although this indicates that Mrx8 can act on UTRs and coding sequence (CDS) of Cox1 mRNA, further investigation is required to determine a direct binding of Cox1 mRNA with Mrx8. Moreover, Mss51 sequestration in a higher-order complex is not observed in Δ*mrx8* cells, arguing that reduction in Cox1 synthesis is not due to Mss51 limitation. Interestingly, we found Mrx8 to be in a complex containing Mss51 and the ribosome. However, overexpression of *MRX8* did not rescue the defective cellular respiration in Δ*mss51* or Δ*pet309* or vice versa. Taken together, these results indicate that although there might be a complex within the mitochondria containing both Mrx8 and Mss51 in association with the ribosome, however they act independently to promote Cox1 synthesis.

Nuclear-encoded GTPases have been shown to regulate various aspects of ribosome assembly and protein translation in mitochondria presumably by utilization of energy released upon GTP hydrolysis to power a mechanistic step (Karbstein, 2007; Strunk and Karbstein, 2009; Maracci and Rodnina, 2016). Cells expressing mutant Mrx8 predicted to be compromised for nucleotide binding were defective in cellular respiration including Cox1 protein synthesis. One would wonder what the rationale would be for the cell to have a putaitve GTPase specifially promoting Cox1 synthesis especially during cold stress. An answer might lie in the way Mrx8 utilizes nucleotide binding/hydrolysis to carry out its in vivo function. This aspect is under investigation.

A mitochondrial gene-expression system, especially that of the mitochondrial ribosome, is the best example of diversity in the mitochondrial proteome (Desmond *et al.*, 2011; Amunts *et al.*, 2014). Mitochondrial ribosomal proteins, assembly factors, and regulatory proteins required for mitochondrial gene expression in *S. cerevisiae* can be classified in four categories: those that have orthologues 1) in prokaryotes and all eukaryotes, 2) only in eukaryotes, 3) in prokaryotes and in some eukaryotic lineages but not all, and 4) in a narrow, lineage-specific manner such as lower eukaryotes only and absent in higher eukaryotes sequenced (Kurland and Andersson, 2000; Szklarczyk and Huynen, 2010; Gray, 2015). *MRX8* represents the third class, that is, it has orthologues in bacteria, in single cell eukaryotes such as *S. cerevisiae*, and in vertebrates including humans but in no other kingdom of life sequenced so far. Mss51 and Pet309, which are essential for Cox1 translational activation and assembly, are only yeast specific and no clear orthologues have been identified in metazoans (Perez-Martinez *et al.*, 2003; Barrientos *et al.*, 2004; Zamudio-Ochoa *et al.*, 2014; Dennerlein and Rehling, 2015). Thus, in order to maintain the synthesis of COX1 in humans, a different mechanism must exist. Significantly, we found that targeting of GTPBP8 to the mitochondria of Δ*mrx8* cells is able to largely restore growth at a reduced temperature on media requiring cellular respiration. Consistent with functional conservation, we found GTPBP8 to localize to the mitochondria in mammalian cells. Thus, it appears that *MRX8* represents a conserved pathway that has been retained during mitochondrial evolution in certain eukaryotic life forms. We are currently examining whether COX1 translation in humans is governed by *GTPBP8* and whether it has a similar role in linking optimal translation with cold stress.

MATERIALS AND METHODS

Request a protocol through *Bio-protocol*.

Yeast strains and media

Yeast strains used in this study are listed in Table 1. Complete media used were YEP (1% yeast extract, 2% peptone) containing 2% glucose (YPD) or 3% glycerol (YPG) as the carbon source. Synthetic minimal media (0.67% yeast nitrogen base) containing 2% glucose (SD), 3% glycerol (SGly) 2% galactose (SGal), 2% glucose and 0.1% 5-fluoroorotic acid (5′FOAD), and 2% glucose and 0.006% canavanine sulfate (SCAN) were supplemented with appropriate amino acids when required, as described in Guthrie and Fink (1991). Semi-synthetic lactate medium (0.3% yeast extract, 0.05% glucose, 0.05% $CaCl_2$, 0.05% NaCl, 0.06% $MgCl_2$, 0.1% KH_2PO_4, 0.1% NH_4Cl, 2% D-lactic acid, 0.8% NaOH, pH 5.5) was prepared as described (Glick and Pon, 1995).

Plasmid and strain construction

Isolation of yeast genomic DNA and yeast transformation were done as described previously (Guthrie and Fink, 1991). The *MRX8* gene along with 1 kb upstream and 1 kb downstream sequences was amplified from genomic DNA of the CRY1 strain using primers 5′TCAAATGACTGAAGAAATTT3′ and 5′CCGCATCATTCAATCCTAGT3′. The resulting 2945-base-pair PCR product was cloned in pCR 2.1-TOPO to generate pDP2 (KD290) and confirmed by sequencing from both ends. This 2945-base-pair gene product was subcloned in pRS316 (*CEN*, *URA3*) and pRS426 (*2μ*, *URA3*) as an *Eco*RI fragment to generate pDP8 (KD337) and pYV31(KD1598), respectively.

To generate *MRX8*-6xHis, the *MRX8* ORF without stop codon was PCR amplified using primers 5′CATGCCATGGTGGAACAACTGTGTAAG3′ and 5′CCGGAATTCGCTAAAATCAAACCACAGCT3′. The 945-base-pair PCR product was cloned into the pGEMT-Easy vector to generate pDP21 (KD466). *MRX8* from pDP21 was shuttled into pET-28a as a *Nco*I-*Eco*RI fragment, resulting in pDP25 (KD546). To clone *MRX8*-6xHis under the *CYC1* promoter, the 2242-base-pair gene product along with a downstream sequence from pET-28a was

cloned into p413CYC1 (*CEN*, *HIS3*) as *Xba*I and *Sma*I fragments, resulting in pDP33 (KD686).

To express *GTPBP8* (GTPBP8) in yeast, RNA isolated from a HeLa cell line was used to generate cDNA using the AMV First Strand cDNA Synthesis Kit (NEB,USA). The cDNA was then used to amplify the *GTPBP8* gene fragment using primers 5′GATATCATGGCGGCGCCCGGGCTGCGG3′ and 5′CCCATCGATTTAGTCAAGACTTCCTGTTAC3′. The resulting 855-base-pair PCR product was cloned in pGEMT-Easy (Promega) to generate pDP55 (KD754). The 855-base-pair PCR product was also directly cloned in p413CYC1 as an *Eco*RV-*Cla*I fragment, resulting in pDP52 (KD751), which was confirmed by sequencing from both ends. MTS-*GTPBP8* containing a cleavable mitochondrial targeting sequence from *MTG3* upstream of *GTPBP8* was generated by a two-step PCR with overlapping forward primers P1: 5′ATGTTGAATCTGTGTCATGCTCTTCGAGGCGTACGTCAGTTTTCCTGTTC3′, P2: 5′GTACGTCAGTTTTCCTGTTCTGTGATTGTGAAAATGGCGGCGCCCGGGCTGCG3′, and hYDR336wClaIdown: 5′CCCATCGATTTAGTCAAGACTTCCTGTTAC3′. Briefly, the first PCR amplification step used primers P2 and hYDR336wClaIdown to amplify the 855-base-pair *GTPBP8* from pDP55. A second PCR, using the 855-base-pair PCR product obtained above as template was performed using primers P1 and hYDR336wClaIdown. The 906-base-pair PCR product obtained was cloned in p413CYC1 as a *Spe*I-*Cla*I fragment to generate pDP64. To create an *GTPBP8* fused with GFP, the *GTPBP8* gene product was amplified from pDP52 using primers 5′ACGCGTCGACATGGCGGCGCCCGGGCTGCG3′ and 5′CGGGGTACCCCGTCAAGACTTCCTGTTAC3′. The 855-base-pair PCR product was cloned in pEGFP N1 (Clontech) as a *Sal*I-*Kpn*I fragment to generate pDP77 (KD986).

Cell deleted for *MRX8* were generated in a XPM171a, XPM78a, and RGV 140. Disruption of *MRX8* was carried out by using a PCR-based gene replacement approach, where either *KanMx* or *TRP1* (amplified from pFA6a-kanMX or pFA6a-TRP1) was placed between the upstream and downstream regions of *MRX8* by PCR using primers MRX8F1: 5′ATATTCCCTAATATTTTAGCGAATAGGAACCATTGGCACGGATCCCCGGGTTAATTAA3′ and MRX8R1: 5′AGCTGAAATGCAATCAGCAATATATAGATACATATTGTGAGAATTCGAGCTCGTTTAAAC3′. The linear PCR product was transformed into XPM171a, XPM78a, and RGV 140 and selected for insertion of the marker allele in place of *MRX8* to generate KD1512, KD1562, and KD1516, respectively.

Cells expressing *MSS51-GST* from its chromosomal loci was generated by transforming BY4742 with a linear PCR product obtained from pFA6a-GST-kanMX6 using primers MSS51F2: 5′CGGTTCAGAGGTAAAAGGTACCATACAATCAAGAGACAACGGATCCCCGGGTTAA TTAA3′ and MSS51R1: 5′TATATTATAAGATGAAGTTGGGCATGGCCTCCCGATAAGAATTCGAGCTCGTTTAAAC3′. Insertion of GST at the correct chromosomal position was verified by PCR using MSS51Xbalup: 5′GCTCTAGAATGACCGTGCTATATGCTCCT3′ and MSS51R1 to generate KD1532. KD1532 (*MSS51-GST*) was crossed with KD1516ρ^0 (Δ*mrx8).* Diploids were sporulated, and Δ*mrx8* haploid spores carrying *MSS51-GST* were selected to generate KD1567.

To generate the Δ*mss51* strain, XPM76aρ^0 was crossed with CRY1. Diploids were sporulated, and Δ*mss51*ρ^+ haploid spores were selected to generate KD1608. To episomally express *MSS51-GST* from a strong constitutive promoter, *MSS51-GST* was amplified from KD1532 using primers MSS51Xbalup: 5′GCTCTAGAATGACCGTGCTATATGCTCCT3′ and GSTClaIDown: 5′CCATCGATGGTCAACGCGGAACCAGATCCG3′. The 1968-base-pair PCR product was cloned in p416TEF (Mumberg *et al.*, 1995) at the *Xba*I-*Cla*I sites to generate pYV29 (KD1596).

The *mrx8GKS145-147AAA* mutant allele was generated using QuikChange (Stratagene) according to the manufacturer's instructions with variations described in Fuentes *et al.* (2007). The primers 5′TTTCTCGGAGGAACTAATGTGGCTGCAGCATCTATCTTGAACAACATAACC3′ and 5′GGTTATGTTGTTCAAGATAGATGCTGCAGCCACATTAGTTCCTCCGAGAAA3′ were used to incorporate specific mutation(s) in the GTPase domain of *MRX8* to generate pDP67 (KD975).

Subcellular localization of *MRX8*

Mitochondria were isolated as described previously (Datta *et al.*, 2005), except that cells were incubated with LongLife Zymolyase (2.5 mg/g of dry cell weight) in 1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4, at 30°C for 2 h to produce spheroplasts. Mitochondria were subjected to extraction as described previously with sodium chloride or sodium carbonate (Fujiki *et al.*, 1982) or urea (Schook *et al.*, 1979). Soluble fractions containing peripherally associated membrane proteins were separated by centrifugation at 150,000 × *g* for 1 h in a Beckman 70Ti rotor from integral membrane proteins. Mitoplasts were generated by subjecting mitochondria to osmotic shock as described previously (Datta *et al.*, 2005). Intact mitochondria or mitoplasts were treated with 0–500 µg/ml proteinase K and incubated on ice for 30 min. Proteinase K was inactivated by the addition of trichloroacetic acid (TCA) to 15%.

Separation of mitochondrial ribosome

Mitochondrial ribosomes were separated by sucrose density gradient as previously described (Fearon and Mason, 1992; Datta *et al.*, 2005) with slight modification. Mitochondria were lysed by the addition of 10 mM Tris-Cl, pH 7.4, 10 mM MgOAc,100 mM NH_4Cl, 7 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2% NP-40. Lysates were clarified by centrifugation at 40,000 × *g* for 25 min at 4°C. Where indicated, lysates were incubated with 200 U/ml RNase A at room temperature for 30 min to disrupt RNA–protein complexes as described in Ott *et al.* (2006). Mitochondrial ribosomes were separated on a 5–30% sucrose density gradient containing 10 mM Tris-Cl, pH 7.4, 10 mM MgOAc, 7 mM β-mercaptoethanol, 0.1% NP-40, and 100 or 500 mM NH_4Cl, as indicated, by ultracentrifugation at 135,000 × *g* for 4 h in a Beckman SW 41 Ti rotor (Datta *et al.*, 2005). Equivalent fractions were collected, and proteins samples were precipitated by the addition of TCA to 15%.

Analysis of mitochondrial translation products

Mitochondrial translation products were labeled as previously described with slight modifications (Fox *et al.*, 1991). Cells were grown in SGal lacking methionine at 30°C or 16°C to an OD_{600} of 1. Mitochondrial translation products were labeled by the addition of 0.1 mCi [^{35}S]methionine and cysteine (Easy Tag Express^{35}S protein labeling mix, Perkin Elmer NEG77200 [specific activity: 1175 Ci/mmol]) in the presence of 0.1 mg/ml cycloheximide to inhibit cytosolic protein synthesis at either 30°C or 16°C for 30 min (Fox *et al.*, 1991). Labeled cells were chased by the addition of chase solution (1% casamino acids and 2 mg/ml sodium sulfate) for 10 min or as indicated, and mitochondria were isolated. The mitochondrial protein concentration was estimated by the Bradford method (Bradford, 1976). Equal amounts of mitochondrial proteins were separated on 17.5% SDS–PAGE and transferred onto a nitrocellulose membrane. The membrane was exposed to BAS storage phosphor screen (GE Healthcare Life Sciences) and developed using FUJIFILM FLA9000 phosphroimager (GE Healthcare Life Sciences).

Purification of Mrx8-6xHis using metal ion chromatography

Mitochondria (4 mg), isolated from cells expressing Mrx8-6xHis were lysed in buffer containing 50 mM Na_2HPO_4, 300 mM NaCl, 0.5 mM PMSF, 5 mM imidazole, pH 8.0, 1% Triton X-100, and 0.6% *n*-dodecyl-β-D-maltoside (DDM) for 30 min on ice. The lysate was clarified by centrifugation at 40,000 × *g* for 30 min. Clarified lysate was incubated with 75 µl of precalibrated Co-NTA resin (Cat #786932; G-Biosciences) for 2 h at 4°C. Subsequently, to remove unbound protein, beads were incubated with buffer containing 50 mM Na_2HPO_4, 300 mM NaCl, 0.5 mM PMSF, 10 mM imidazole, pH 8.0, for 5 min, and this step was repeated five times. Proteins bound to beads were eluted using buffer containing 50 mM Na_2HPO_4, 300 mM NaCl, 0.5 mM PMSF, 400 mM imidazole, pH 8.0. Eluted protein was concentrated by the addition of TCA to 15%, separated by SDS–PAGE, and subjected to immunoblot analysis.

Mitochondrial RNA isolation and RT-PCR analysis

Mitochondria were isolated from cells that were incubated with LongLife Zymolyase (2.5 mg/g of dry cell weight) in SB3 buffer (50 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 3 mM dithiothreitol, and 1 M sorbitol) at 37°C for 2 h to produce spheroplasts. The cells were further lysed using 30–50 stokes of tight-fitting pestle in a Dounce homogenizer and centrifuged to remove any insoluble debris. Mitochondrial RNA was isolated from intact mitochondria using phenol chloroform as described previously (Turk and Caprara, 2010). Random hexamers as primers were used to generate cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). For reverse transcript PCR (RT-PCR), sense and antisense primers specific to *COX1*, *COX2*, *COX3*, and *COB* are shown in Table 2. Equal amounts of total mitochondrial RNA were mixed with high-molecular-weight (HMW) RNA loading dye (95% formamide, 0.025% SDS, 0.1 µg/µl ethidium bromide, 0.5 mM EDTA, 1.25× MOPS buffer, 0.25% bromophenol blue, and 0.25% xylene cyanol) and separated on a 1.5% agarose formaldehyde denaturing gel. HMW RNA gel loading dye was prepared as described previously (Bhardwaj *et al*., 2012).

Isolation of mitochondrial fractions from HEK293 cells

HEK293 cells were transfected with either pDP77 (GTPBP8-EGFP) or vector alone using Lipofectamine 2000 (Thermo Scientific) according to the manufacturer's protocol. Five 100 mm culture dishes of transfected cells with approximately 100% confluency were used to isolate mitochondria. Cells were scraped and lysed by being passed through a narrow 25-guage needle 15 times. Cells were washed and lysed in mitochondrial lysis buffer containing 250 mM sucrose, 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 10 mM $MgCl_2$, 1 mM PMSF, protease inhibitor cocktail, and phosphatase inhibitor. Mitochondrial enriched fractions were obtained using a differential centrifugation method described previously (Zhao *et al*., 2019).

Fluorescence microscopy

HEK293 cells were transfected using Lipofectamine 2000 (Thermo Scientific) with either pDP77 (GTPBP8-GFP) or vector alone. Cells were fixed and stained with MitoTracker Red CMXRos (Invitrogen) and 4′,6-diamidino-2-phenylindole (DAPI) (Roche) according to the manufacturer's protocol. Images were captured using a Zeiss LSM 880 confocal laser-scanning microscope and analyzed using ZEN software.

Antibodies used for immunoblot analysis

Mrx8-specific polyclonal antibodies (Link Biotech) were raised in rabbits against epitopes ALKKKLNSRPKERLPNWLK and YEKPSNSDINKVNRFFNK, which correspond to amino acids 45–63 and 72–89, respectively, within the N-terminus, and used at a dilution of 1:500. The peptide sequence chosen were based on the likelihood of being after a cleavable mitochondrial targeting sequence. Antibodies used in this study, Mtg2 (1:2000) (Datta *et al*., 2005); Cox2 (1:50) (Pinkham *et al*., 1994); F1β (1:5000) (Emtage and Jensen, 1993); Tim23 (1:5000) (Emtage and Jensen, 1993); Cox1 (1:1000): mouse monoclonal (11D8B7), Cox3 (1:1000): mouse monoclonal (DA5BC4) and COX IV (1:5000): mouse monoclonal (20E8C12) from Abcam; GFP (1:2000): mouse monoclonal (sc-9996), GAPDH (1:4000): mouse monoclonal (sc-32233) from Santa Cruz Biotechnology; GST (1:5000): mouse monoclonal (GST.B6) from Epitope Biotech. Proteins were separated on either 10% or 12.5% SDS–PAGE and processed for immunoblot as described previously (Lin *et al*., 2004).

ACKNOWLEDGMENTS

We are extremely grateful to Janine Maddock for strains, plasmids, and antibodies and Tom Fox for the Arg8 antibody. We also thank Vani Brahmachari and K. Natarajan for critical reading of the manuscript and Anagha Nair for technical assistance. Y. V. acknowledges University Grants Commission (UGC) for fellowship support. U. M. acknowledges Council Of Scientific And Industrial Research (CSIR), Science and Engineering Research Board (SERB), and Indian Council of Medical Research (ICMR) for fellowship support. D.K.P. acknowledges ICMR for fellowship support. We also acknowledge instrumentation facilities at Central Instrumentation Facility, University of Delhi South Campus (CIF-UDSC), and UGC:Special Assistance Programme/Department Of Science & Technology:Fund for Improvement of S&T Infrastructure (UGC-SAP/DST-FIST)–supported CIF, Genetics. This work was supported by grants from Department of Atomic Energy-Board of Research In Nuclear Sciences (DAE-BRNS) (Grant number: 37(1)/14/37/2016-brns/37272), and CSIR (Grant number: 38 (1297)/111EMR-II), SERB (CRG/2020/001932) and a Delhi University, Research and Development (DU-R&D) grant to K.D.

REFERENCES

Amunts A, Brown A, Bai XC, Llacer JL, Hussain T, Emsley P, Long F, Murshudov G, Scheres SH, Ramakrishnan V (2014). Structure of the yeast mitochondrial large ribosomal subunit. Science 343, 1485–1489.

Atkinson GC (2015). The evolutionary and functional diversity of classical and lesser-known cytoplasmic and organellar translational GTPases across the tree of life. BMC Genomics 16, 78.

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

Barrientos A, Zambrano A, Tzagoloff A (2004). Mss51p and Cox14p jointly regulate mitochondrial Cox1p expression in Saccharomyces cerevisiae. EMBO J 23, 3472–3482.

Bauer MF, Sirrenberg C, Neupert W, Brunner M (1996). Role of Tim23 as voltage sensor and presequence receptor in protein import into mitochondria. Cell 87, 33–41.

Bauerschmitt H, Funes S, Herrmann JM (2008). The membrane-bound GTPase Guf1 promotes mitochondrial protein synthesis under suboptimal conditions. J Biol Chem 283, 17139–17146.

Bhardwaj AR, Pandey R, Agarwal M, Katiyar-Agarwal S (2012). Northern blot analysis for expression profiling of mRNAs and small RNAs. Methods Mol Biol 883, 19–45.

Bourne HR, Sanders DA, McCormick F (1991). The GTPase superfamily: conserved structure and molecular mechanism. Nature 349, 117–127.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248–254.

Brickner JH, Fuller RS (1997). SOI1 encodes a novel, conserved protein that promotes TGN-endosomal cycling of Kex2p and other membrane proteins by modulating the function of two TGN localization signals. J Cell Biol 139, 23–36.

Claros MG, Vincens P (1996). Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur J Biochem 241, 779–786.

Clementi N, Polacek N (2010). Ribosome-associated GTPases: the role of RNA for GTPase activation. RNA Biol 7, 521–527.
Cooper EL, Garcia-Lara J, Foster SJ (2009). YsxC, an essential protein in Staphylococcus aureus crucial for ribosome assembly/stability. BMC Microbiol 9, 266.
Couvillion MT, Soto IC, Shipkovenska G, Churchman LS (2016). Synchronized mitochondrial and cytosolic translation programs. Nature 533, 499–503.
Datta K, Fuentes JL, Maddock JR (2005). The yeast GTPase Mtg2p is required for mitochondrial translation and partially suppresses an rRNA methyltransferase mutant, mrm2. Mol Biol Cell 16, 954–963.
Datta K, Skidmore JM, Pu K, Maddock JR (2004). The Caulobacter crescentus GTPase CgtAC is required for progression through the cell cycle and for maintaining 50S ribosomal subunit levels. Mol Microbiol 54, 1379–1392.
Decoster E, Simon M, Hatat D, Faye G (1990). The MSS51 gene product is required for the translation of the COX1 mRNA in yeast mitochondria. Mol Gen Genet 224, 111–118.
Decoster E, Vassal A, Faye G (1993). MSS1, a nuclear-encoded mitochondrial GTPase involved in the expression of COX1 subunit of cytochrome c oxidase. J Mol Biol 232, 79–88.
Dennerlein S, Rehling P (2015). Human mitochondrial COX1 assembly into cytochrome c oxidase at a glance. J Cell Sci 128, 833–837.
Desai N, Brown A, Amunts A, Ramakrishnan V (2017). The structure of the yeast mitochondrial ribosome. Science 355, 528–531.
De Silva D, Poliquin S, Zeng R, Zamudio-Ochoa A, Marrero N, Perez-Martinez X, Fontanesi F, Barrientos A (2017). The DEAD-box helicase Mss116 plays distinct roles in mitochondrial ribogenesis and mRNA-specific translation. Nucleic Acids Res 45, 6628–6643.
Desmond E, Brochier-Armanet C, Forterre P, Gribaldo S (2011). On the last common ancestor and early evolution of eukaryotes: reconstructing the history of mitochondrial ribosomes. Res Microbiol 162, 53–70.
Emtage JL, Jensen RE (1993). MAS6 encodes an essential inner membrane component of the yeast mitochondrial protein import pathway. J Cell Biol 122, 1003–1012.
Fearon K, Mason TL (1988). Structure and regulation of a nuclear gene in Saccharomyces cerevisiae that specifies MRP7, a protein of the large subunit of the mitochondrial ribosome. Mol Cell Biol 8, 3636–3646.
Fearon K, Mason TL (1992). Structure and function of MRP20 and MRP49, the nuclear genes for two proteins of the 54 S subunit of the yeast mitochondrial ribosome. J Biol Chem 267, 5162–5170.
Fiori A, Perez-Martinez X, Fox TD (2005). Overexpression of the COX2 translational activator, Pet111p, prevents translation of COX1 mRNA and cytochrome c oxidase assembly in mitochondria of Saccharomyces cerevisiae. Mol Microbiol 56, 1689–1704.
Fontanesi F, Soto IC, Barrientos A (2008). Cytochrome c oxidase biogenesis: new levels of regulation. IUBMB Life 60, 557–568.
Fox TD (2012). Mitochondrial protein synthesis, import, and assembly. Genetics 192, 1203–1234.
Fox TD, Folley LS, Mulero JJ, McMullin TW, Thorsness PE, Hedin LO, Costanzo MC (1991). Analysis and manipulation of yeast mitochondrial genes. Methods Enzymol 194, 149–165.
Fuentes JL, Datta K, Sullivan SM, Walker A, Maddock JR (2007). In vivo functional characterization of the Saccharomyces cerevisiae 60S biogenesis GTPase Nog1. Mol Genet Genomics 278, 105–123.
Fujiki Y, Hubbard AL, Fowler S, Lazarow PB (1982). Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. J Cell Biol 93, 97–102.
Gavin AC, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, Schultz J, Rick JM, Michon AM, Cruciat CM, *et al*. (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature 415, 141–147.
Giaever G, Chu AM, Ni L, Connelly C, Riles L, Veronneau S, Dow S, Lucau-Danila A, Anderson K, Andre B, *et al*. (2002). Functional profiling of the Saccharomyces cerevisiae genome. Nature 418, 387–391.
Glick BS, Pon LA (1995). Isolation of highly purified mitochondria from Saccharomyces cerevisiae. Methods Enzymol 260, 213–223.
Gray MW (2015). Mosaic nature of the mitochondrial proteome: implications for the origin and evolution of mitochondria. Proc Natl Acad Sci USA 112, 10133–10138.
Guthrie C, Fink GR (1991). Guide to yeast genetics and molecular biology. Methods Enzymol 194, 1–863.
Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS, O'Shea EK (2003). Global analysis of protein localization in budding yeast. Nature 425, 686–691.
Karbstein K (2007). Role of GTPases in ribosome assembly. Biopolymers 87, 1–11.
Kehrein K, Schilling R, Moller-Hergt BV, Wurm CA, Jakobs S, Lamkemeyer T, Langer T, Ott M (2015). Organization of mitochondrial gene expression in two distinct ribosome-containing assemblies. Cell Rep 10, 843–853.
Khalimonchuk O, Rodel G (2005). Biogenesis of cytochrome c oxidase. Mitochondrion 5, 363–388.
Kim HJ, Khalimonchuk O, Smith PM, Winge DR (2012). Structure, function, and assembly of heme centers in mitochondrial respiratory complexes. Biochim Biophys Acta 1823, 1604–1616.
Kurland CG, Andersson SG (2000). Origin and evolution of the mitochondrial proteome. Microbiol Mol Biol Rev 64, 786–820.
Lehoux IE, Mazzulla MJ, Baker A, Petit CM (2003). Purification and characterization of YihA, an essential GTP-binding protein from Escherichia coli. Protein Expr Purif 30, 203–209.
Leipe DD, Wolf YI, Koonin EV, Aravind L (2002). Classification and evolution of P-loop GTPases and related ATPases. J Mol Biol 317, 41–72.
Lin B, Thayer DA, Maddock JR (2004). The Caulobacter crescentus CgtAC protein cosediments with the free 50S ribosomal subunit. J Bacteriol 186, 481–489.
Maiti P, Lavdovskaia E, Barrientos A, Richter-Dennerlein R (2021). Role of GTPases in driving mitoribosome assembly. Trends Cell Biol 31, 284–297.
Manthey GM, McEwen JE (1995). The product of the nuclear gene PET309 is required for translation of mature mRNA and stability or production of intron-containing RNAs derived from the mitochondrial COX1 locus of Saccharomyces cerevisiae. EMBO J 14, 4031–4043.
Maracci C, Rodnina MV (2016). Review: translational GTPases. Biopolymers 105, 463–475.
Mays JN, Camacho-Villasana Y, Garcia-Villegas R, Perez-Martinez X, Barrientos A, Fontanesi F (2019). The mitoribosome-specific protein mS38 is preferentially required for synthesis of cytochrome c oxidase subunits. Nucleic Acids Res 47, 5746–5760.
McStay GP, Su CH, Tzagoloff A (2013). Modular assembly of yeast cytochrome oxidase. Mol Biol Cell 24, 440–452.
Morgenstern M, Stiller SB, Lubbert P, Peikert CD, Dannenmaier S, Drepper F, Weill U, Hoss P, Feuerstein R, Gebert M, *et al*. (2017). Definition of a high-confidence mitochondrial proteome at quantitative scale. Cell Rep 19, 2836–2852.
Mumberg D, Muller R, Funk M (1995). Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156, 119–122.
Ott M, Amunts A, Brown A (2016). Organization and regulation of mitochondrial protein synthesis. Annu Rev Biochem 85, 77–101.
Ott M, Prestele M, Bauerschmitt H, Funes S, Bonnefoy N, Herrmann JM (2006). Mba1, a membrane-associated ribosome receptor in mitochondria. EMBO J 25, 1603–1610.
Partaledis JA, Mason TL (1988). Structure and regulation of a nuclear gene in Saccharomyces cerevisiae that specifies MRP13, a protein of the small subunit of the mitochondrial ribosome. Mol Cell Biol 8, 3647–3660.
Paul MF, Alushin GM, Barros MH, Rak M, Tzagoloff A (2012). The putative GTPase encoded by MTG3 functions in a novel pathway for regulating assembly of the small subunit of yeast mitochondrial ribosomes. J Biol Chem 287, 24346–24355.
Perez-Martinez X, Broadley SA, Fox TD (2003). Mss51p promotes mitochondrial Cox1p synthesis and interacts with newly synthesized Cox1p. EMBO J 22, 5951–5961.
Perez-Martinez X, Butler CA, Shingu-Vazquez M, Fox TD (2009). Dual functions of Mss51 couple synthesis of Cox1 to assembly of cytochrome c oxidase in Saccharomyces cerevisiae mitochondria. Mol Biol Cell 20, 4371–4380.
Pinkham JL, Dudley AM, Mason TL (1994). T7 RNA polymerase-dependent expression of COXII in yeast mitochondria. Mol Cell Biol 14, 4643–4652.
Ruzheinikov SN, Das SK, Sedelnikova SE, Baker PJ, Artymiuk PJ, Garcia-Lara J, Foster SJ, Rice DW (2004). Analysis of the open and closed conformations of the GTP-binding protein YsxC from Bacillus subtilis. J Mol Biol 339, 265–278.
Schaefer L, Uicker WC, Wicker-Planquart C, Foucher AE, Jault JM, Britton RA (2006). Multiple GTPases participate in the assembly of the large ribosomal subunit in Bacillus subtilis. J Bacteriol 188, 8252–8258.
Schook W, Puszkin S, Bloom W, Ores C, Kochwa S (1979). Mechanochemical properties of brain clathrin: interactions with actin and alpha-actinin and polymerization into basketlike structures or filaments. Proc Natl Acad Sci USA 76, 116–120.

Sickmann A, Reinders J, Wagner Y, Joppich C, Zahedi R, Meyer HE, Schonfisch B, Perschil I, Chacinska A, Guiard B, *et al.* (2003). The proteome of Saccharomyces cerevisiae mitochondria. Proc Natl Acad Sci USA 100, 13207–13212.

Singh AP, Salvatori R, Aftab W, Aufschnaiter A, Carlstrom A, Forne I, Imhof A, Ott M (2020). Molecular connectivity of mitochondrial gene expression and OXPHOS biogenesis. Mol Cell 79, 1051–1065.e1010.

Sprang SR (1997). G protein mechanisms: insights from structural analysis. Annu Rev Biochem 66, 639–678.

Steele DF, Butler CA, Fox TD (1996). Expression of a recoded nuclear gene inserted into yeast mitochondrial DNA is limited by mRNA-specific translational activation. Proc Natl Acad Sci USA 93, 5253–5257.

Strunk BS, Karbstein K (2009). Powering through ribosome assembly. RNA 15, 2083–2104.

Szklarczyk R, Huynen MA (2010). Mosaic origin of the mitochondrial proteome. Proteomics 10, 4012–4024.

Tavares-Carreon F, Camacho-Villasana Y, Zamudio-Ochoa A, Shingu-Vazquez M, Torres-Larios A, Perez-Martinez X (2008). The pentatricopeptide repeats present in Pet309 are necessary for translation but not for stability of the mitochondrial COX1 mRNA in yeast. J Biol Chem 283, 1472–1479.

Timon-Gomez A, Nyvltova E, Abriata LA, Vila AJ, Hosler J, Barrientos A (2018). Mitochondrial cytochrome c oxidase biogenesis: recent developments. Semin Cell Dev Biol 76, 163–178.

Turk EM, Caprara MG (2010). Splicing of yeast al5beta group I intron requires SUV3 to recycle MRS1 via mitochondrial degradosome-promoted decay of excised intron ribonucleoprotein (RNP). J Biol Chem 285, 8585–8594.

Umeda N, Suzuki T, Yukawa M, Ohya Y, Shindo H, Watanabe K, Suzuki T (2005). Mitochondria-specific RNA-modifying enzymes responsible for the biosynthesis of the wobble base in mitochondrial tRNAs. Implications for the molecular pathogenesis of human mitochondrial diseases. J Biol Chem 280, 1613–1624.

van Dijken JP, Weusthuis RA, Pronk JT (1993). Kinetics of growth and sugar consumption in yeasts. Antonie Van Leeuwenhoek 63, 343–352.

Verstraeten N, Fauvart M, Versees W, Michiels J (2011). The universally conserved prokaryotic GTPases. Microbiol Mol Biol Rev 75, 507–542, second and third pages of table of contents.

Wittinghofer A, Vetter IR (2011). Structure-function relationships of the G domain, a canonical switch motif. Annu Rev Biochem 80, 943–971.

Zamudio-Ochoa A, Camacho-Villasana Y, Garcia-Guerrero AE, Perez-Martinez X (2014). The Pet309 pentatricopeptide repeat motifs mediate efficient binding to the mitochondrial COX1 transcript in yeast. RNA Biol 11, 953–967.

Zhao Y, Sun X, Hu D, Prosdocimo DA, Hoppel C, Jain MK, Ramachandran R, Qi X (2019). ATAD3A oligomerization causes neurodegeneration by coupling mitochondrial fragmentation and bioenergetics defects. Nat Commun 10, 1371.