Mrpl35, A Mitospecific Component of Mitoribosomes, Plays A Key Role in Cytochrome C Oxidase Assembly

Jodie M. Box  
*Marquette University*

Jasvinder Kaur  
*Marquette University*

Rosemary A. Stuart  
*Marquette University, rosemary.stuart@marquette.edu*

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MrpL35, a mitospecific component of mitoribosomes, plays a key role in cytochrome c oxidase assembly

Jodie M. Box, Jasvinder Kaur†, and Rosemary A. Stuart*
Department of Biological Sciences, Marquette University, Milwaukee, WI 53233

ABSTRACT Mitoribosomes perform the synthesis of the core components of the oxidative phosphorylation (OXPHOS) system encoded by the mitochondrial genome. We provide evidence that MrpL35 (mL38), a mitospecific component of the yeast mitoribosomal central protuberance, assembles into a subcomplex with MrpL7 (uL5), Mrp7 (bL27), and MrpL36 (bL31) and mitospecific proteins MrpL17 (mL46) and MrpL28 (mL40). We isolated respiratory defective mrpL35 mutant yeast strains, which do not display an overall inhibition in mitochondrial protein synthesis but rather have a problem in cytochrome c oxidase complex (COX) assembly. Our findings indicate that MrpL35, with its partner Mrp7, play a key role in coordinating the synthesis of the Cox1 subunit with its assembly into the COX enzyme and in a manner that involves the Cox14 and Coa3 proteins. We propose that MrpL35 and Mrp7 are regulatory subunits of the mitoribosome acting to coordinate protein synthesis and OXPHOS assembly events and thus the bioenergetic capacity of the mitochondria.

INTRODUCTION
Mitochondrial ribosomes (mitoribosomes) perform the synthesis of a small, but important, subset of the mitochondrial proteome, which is encoded by the mitochondrial genome and represents key components of the oxidative phosphorylation (OXPHOS) system (Borst and Grivell, 1978; O’Brien, 2003; Smits et al., 2007; Kehrein et al., 2015; Greber and Ban, 2016; Ott et al., 2016; Mai et al., 2017). Thus the regulation and activity of mitoribosomes have a major potential impact on an organism’s capacity to generate its energy through aerobic respiration. Due to the endosymbiotic origin of mitochondria, the mitoribosomes are evolutionarily related to bacterial ribosomes and thus share a number of overlapping features, such as antibiotic sensitivities and similarities in protein subunit composition (O’Brien, 2002, 2003; Gruschte et al., 2010; De Silva et al., 2015; Greber and Ban, 2016; Ott et al., 2016). Despite these similarities, mitoribosomes display important compositional and functional differences from their prokaryotic ancestors. Mitoribosomes contain a higher protein:rRNA ratio than bacterial ribosomes, and this extra protein content manifests itself in two ways. First, several of the evolutionarily conserved mitoribosomal proteins are larger than their bacterial counterparts, having acquired additional, extension sequences (often C-terminal) and being termed mitochondrial-specific or “mitospecific” sequences. Second, mitoribosomes contain a number of additional novel proteins, that is, mitospecific ribosomal proteins, which are often conserved throughout eukaryotes but do not have counterparts in the prokaryotic ribosomes (Graack et al., 1988; Koc et al., 2000; O’Brien, 2002; van der Sluis et al., 2015). The significant advances in the structural analysis of mitoribosomes, including from the yeast Saccharomyces cerevisiae, have greatly enhanced our knowledge of the location of these mitospecific elements within the mitoribosome structures (Amunts et al., 2014; Brown et al., 2014; Greber et al., 2014a,b, 2015; Kaushal et al., 2014; Amunts et al., 2015; Kaushal et al., 2015; Desai et al., 2017). However, little is known about the function of these mitospecific protein features and how they may contribute to, or regulate, the translational process of the mitoribosome.

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*Address correspondence to: Rosemary A. Stuart (rosemary.stuart@marquette.edu).
†Present address: Ultragenyx Pharmaceutical, Brisbane, CA 94949.

Author contributions: J.B., J.K., and R.A.S. conceived and coordinated the study, and R.A.S. wrote the paper. J.B. designed, performed, and together with R.A.S. analyzed all the experiments shown. The data in Figure 5A represent the initial assembly events and thus the bioenergetic capacity of the mitochondria.
Compositional differences between the mitochondrial and bacterial ribosomes are thought to reflect a divergence in function of the mitochondrial translational apparatus from the bacterial. The mitochondrial proteome, in contrast to the bacterial, is mosaic in origin. The proteins synthesized by mitoribosomes are destined to become components of oligomeric complexes co-assembled with these imported nuclear encoded partners, a process facilitated by OXPHOS complex-specific chaperone proteins. Thus there is a need for a close coordination between the activity of the mitochondrial translational apparatus and the components involved in the import and assembly of their cytosol-synthesized partners (Guschke and Ott, 2010; De Silva et al., 2015; Kehrein et al., 2015; Ott et al., 2016).

The cytochrome c oxidase complex (COX), the terminal electron acceptor in the mitochondrial OXPHOS machinery, is an oligomeric enzyme complex composed of mitochondrial translation products (Cox1, Cox2, and Cox3) partnered with multiple nuclearly encoded ones, imported from the cytosol. The assembly of this complex is a multi-step, chaperone-facilitated process that involves the uniting of protein subunits from two origins and in a strict stoichiometric manner. Cox1, with its metal centers ([a+a] and one copper, Cuα), forms a central aspect of the catalytic core of the COX complex. The synthesis and assembly of Cox1 into the COX complex is tightly regulated, with the proposed purpose of minimizing accumulation of potentially reactive Cox1-assembly subcomplexes (Khalimonchuk et al., 2010; Mick et al., 2011; McStay et al., 2013; Dennerlein and Rehling, 2015; Mayorga et al., 2016; Richter-Dennerlein et al., 2016; Soto and Barrientos, 2016). In yeast, Cox1 synthesis by the mitoribosomes under the strict feedback regulation from the downstream COX assembly process and directly involves the Mss51 protein (Figure 1). Mss51, a heme sensing protein, promotes the translation of Cox1 by binding to the 5′-UTR of the COX1 mRNA (Perez-Martinez et al., 2003; Barrientos et al., 2004; Pierrel et al., 2007; Perez-Martinez et al., 2009; Fontanesi et al., 2010; Mick et al., 2010; Fontanesi et al., 2011; Soto et al., 2012; Mayorga et al., 2016). The Mss51 protein can also interact with the newly synthesized Cox1 protein (via the C-terminal end of Cox1), as it is cotranslationally inserted into the membrane, where it forms an Mss51-containing Cox1-assembly intermediate (COA) with the Cox14 and Coa3(Cox25) proteins (Mick et al., 2010; Fontanesi et al., 2011; Clemente et al., 2013; McStay et al., 2013; Garcia-Villegas et al., 2017). Limiting in levels, the Mss51 remains bound to the Cox1 protein-Coa3 intermediate and thus unavailable to bind the COX1 mRNA to promote further rounds of Cox1 synthesis, until the assembly of Cox1 together with the nuclear encoded COX subunits proceeds. Further progression of Cox1 in its assembly pathway causes the release and recycling of Mss51, making it available to initiate the next round of Cox1 protein synthesis. The presence of Cox14 and Coa3 are required to trap Mss51 in the Cox1-assembly intermediate and they thus act as negative Cox1-translational regulators (Mick et al., 2010, 2011; Fontanesi et al., 2011). It is currently unclear what molecular mechanism drives the subsequent recycling of Mss51 from this Cox1-intermediate and whether, and how, the Cox14 or Coa3 proteins may be directly involved.

In this present study, we have used the yeast model S. cerevisiae to investigate the functional relevance of the mitospecific ribosomal protein, MrpL35 (also known as mL38 using the standardized mitoribosomal protein nomenclature). Conserved throughout eukaryotes (where in higher eukaryotes is referred to as MrpL38), MrpL35 (mL38) is a component of the central protuberance region of the large (54S) mitoribosomal subunit, where its functional relevance is unknown. Our evidence indicates that MrpL35 (mL38) protein is co-assembled into a subcomplex with two other mitochondrialspecific ribosomal proteins, MrpL17 (mL46) and MrpL28 (mL40), and together with proteins that are homologues of bacterial ribosomal proteins, MrpL7 (uL5), Mrp7 (bL27), and MrpL36 (bL31), respectively. MrpL35/mL38 proteins contain a C-terminal domain that displays homology to members of the conserved phosphatidylethanolamine-binding protein domain (PEBP) family. The PEBP family comprises a diverse group of proteins present in many organisms and whose functions have been linked to the regulation of key cell signaling pathways (Banfield et al., 1998; Bruun et al., 1998; Serre et al., 1998; Youssf et al., 2014). Through mutational analysis of the PEBP-like domain, we demonstrate here that MrpL35 plays a key role in coordinating the synthesis and assembly of the COX complex. Our findings demonstrate a connection among MrpL35, Cox1 synthesis, and the COX assembly components, in particular Cox14 and Coa3, and thus illustrate a link between the mitochondrial transcription apparatus and the downstream OXPHOS assembly chaperones.

**RESULTS**

**Mutation of residue Y275 of MrpL35 results in a temperature-sensitive phenotype**

The *S. cerevisiae* yeast mrpL35 null mutant strain (ΔmrpL35) expressing the wild-type MrpL35 protein (from a plasmid-borne gene) exhibited the ability to grow on the nonfermentable carbon (glycerol) source, whereas the strain lacking MrpL35 (i.e., ΔmrpL35+empty plasmid) was completely unable to do so (Figure 2A). This result illustrates the essential nature of MrpL35 for respiratory/OXPHOS-based growth. Through mL38 protein sequence alignments, we sought to identify highly conserved residues present in the PEBP-like homology domain, located at the C-terminal end of the MrpL35 (mL38) proteins. One of these residues was a conserved
Mutation of Y275 residue does not prevent mitoribosome translation

Using an in vivo radiolabeling approach in the presence of cycloheximide (to block cytosolic protein synthesis), we initially investigated if the translational capacity of the mitoribosome was inhibited in mrpL35\(^{\Delta Y275}\) and mrpL35\(^{Y275F}\) mutants grown either at the permissive and nonpermissive temperature. In S. cerevisiae, the mitoribosomes synthesize eight proteins, Var1 (a small ribosomal subunit component) and OXPHOS subunits, Cox1, Cox2, and Cox3 of the COX (core subunits of the COX enzyme), and cytochrome b (of the cytochrome bc\(_1\) enzyme) and F\(_{1}\)F\(_{0}\)-ATP synthase subunits, Atp6, Atp8, and Atp9. Both mutant strains displayed normal capacity for mitochondrial protein synthesis at the permissive temperature, and they retained the ability to synthesize also at 37°C (Figure 3A). Thus we conclude that the lack of OXPHOS-based growth of the mrpL35 mutants at 37°C is not due to an absence of mitoribosomal translation at the elevated temperature. An overall reduction in total translation levels, in particular for the mrpL35\(^{Y275F}\) mutant, was observed at 37°C, however, and may indicate a role for MrpL35 in mediating efficient mitochondrial translation or simply that the mitochondria of these cells were bioenergetically compromised after the prolonged growth period at the nonpermissive temperature, thus indirectly affecting their capacity for the energy-driven process of translation. When analyzed in isolated mitochondria, where exogenously added energy (ATP, GTP, and OXPHOS substrate NADH) were supplemented, the mrpL35\(^{Y275F}\) mitochondria displayed normal level of protein synthesis (Figure 4A).

Western blotting of the protein extracts from the cells grown at the different temperatures indicated that the COX subunits, Cox1, Cox2, and Cox3, were strongly reduced in the mrpL35\(^{Y275A}\) and more so in the mrpL35\(^{Y275D}\) mutant strains grown at the nonpermissive temperature of 37°C (Figure 3B). Although the mrpL35 mutants display the capacity to synthesize these COX subunits at 37°C, their strongly reduced steady-state protein amounts are indicative of a possible COX assembly defect at elevated temperatures, which would promote turnover of the newly synthesized Cox1, Cox2, and Cox3 proteins. Control experiments demonstrated that the preexisting COX complex assembled in the mutant at the permissive temperature did not display an enhanced proteolytic instability (i.e. relative to the wild-type control assayed in parallel) when shifted to 37°C (Supplemental Figure S1). These results indicate that if assembled at the permissive temperature, the COX complex in the mutant cells displays a similar resilience as that of the wild-type control when shifted to 37°C. We conclude therefore that the reduced levels of COX complex in the mrpL35 mutants when grown at 37°C is indicative of a compromised ability to initially assemble the COX enzyme when the mitoribosomal protein synthesis and assembly processes occur at the nonpermissive temperature.

Although somewhat reduced, the levels of cytochrome b and Atp9 (and of Qcr7 and Atp4, two proteins dependent on the presence of cytochrome b- and Atp9-containing complexes, respectively, derivatives, mrpL35\(^{Y275A}\) and mrpL35\(^{Y275D}\), respectively, supported respiratory-based growth of the ΔmrpL35 mutant at 30°C but not at the elevated temperature of 37°C (Figure 2A). In contrast, the relatively conservative exchange of Y275 for another aromatic residue Phe(F) did not appear to impact MrpL35’s ability to support aerobic growth at both temperatures tested (Figure 2A). Thus we conclude that the highly conserved Y275 residue must play a critical structural/functional role in the MrpL35 protein that is compromised by mutation to a smaller, or negatively charged, residue.
The mrpL35 Y275 mutants display a cytochrome c oxidase defect

To analyze the OXPHOS defect in the mrpL35<sup>Y275A</sup> and mrpL35<sup>Y275D</sup> mutant strains in more depth, mitochondria were isolated from cultures grown in galactose/lactate media at the permissive temperature of 30°C. First, in organello translation in the presence of [<sup>35</sup>S]methionine and in the presence of cycloheximide (200 ug/ml). Equivalent amounts of cells were reisolated and proteins extracted and analyzed by SDS–PAGE, Western blotting, and autoradiography. (B) The indicated strains were reisolated and proteins extracted and analyzed by SDS–PAGE, Western blotting, and immunodecorated with antibodies indicated. Tim17 served as loading control.

FIGURE 3: The mrpL35 mutants maintain the ability to translate even at nonpermissive temperature. (A) MrpL35 wild-type mrpL35<sup>Y275A</sup> and mrpL35<sup>Y275D</sup> mutant strains were grown on minimal media supplemented with galactose grown at either 30°C or 37°C for 24 h, as indicated. Mitochondrial translation capacity was monitored in vivo for 10 min with [<sup>35</sup>S]methionine and in the presence of cycloheximide (200 ug/ml). Equivalent amounts of cells were reisolated and proteins extracted and analyzed by SDS–PAGE, Western blotting, and autoradiography. (B) The indicated strains were grown as described in A, total cellular proteins were extracted, and steady-state levels of indicated mitochondrial proteins were measured following SDS–PAGE, Western blotting, and immunodecorated with antibodies indicated.

for their stability, were clearly less affected in the mrpL35 mutants than the COX subunits were (Figure 3B).

The mrpL35 Y275 mutants display a cytochrome c oxidase defect

To analyze the OXPHOS defect in the mrpL35<sup>Y275A</sup> and mrpL35<sup>Y275D</sup> mutant strains in more depth, mitochondria were isolated from cultures grown in galactose/lactate media at the permissive temperature of 30°C. First, in organello translation in the presence of [<sup>35</sup>S]methionine was performed, where we observed that the overall translational capacity of the mitochondrial ribosomes was not adversely affected in the mrpL35 mutants (Figure 4A). The mrpL35<sup>Y275D</sup> mutant mitochondria, however, displayed an altered translation profile with the levels of Cox1, cytochrome b, and Atp6 elevated, relative to the wild-type control, and the levels of Cox2 were somewhat reduced (Figure 4A, left panel). The enhanced levels of newly synthesized Cox1 protein in the mrpL35 mutant mitochondria, in particular in the mrpL35<sup>Y275D</sup> mutant, indicated that the tightly regulated Cox1 synthesis pathway may be misregulated (Figure 4A, right panels).

Western blotting of the isolated mrpL35 mutant mitochondrial preparations showed that the steady-state amounts of COX subunits Cox1, Cox2, and Cox3 were reduced, in particular in the mrpL35<sup>Y275D</sup> mitochondria, indicating that although synthesized at relatively normal (Cox2 and Cox3) or elevated (Cox1) levels in organello, the COX subunits fail to efficiently assemble in the mrpL35 mutant (Figure 4B). Blue native gel electrophoresis (BN–PAGE) analysis of the individual OXPHOS complexes confirmed that the levels of assembled COX complex were strongly reduced in the mrpL35 mutant mitochondria relative to the other OXPHOS complexes analyzed (Figure 4C, upper and lower panels). A parallel reduction in the level of COX enzyme activity was also measured in the mrpL35 mutants (Figure 4D). In contrast, the levels of the cytochrome bc<sub>1</sub> and F<sub>0</sub>F<sub>1</sub>-ATP synthase complexes, as indicated by steady-state analysis of individual subunits, BN–PAGE analysis of the complexes and their levels and assembly states, and the measurement of the cytochrome bc<sub>1</sub> enzyme activity were not affected to the same extent as the COX complex was in the mrpL35<sup>Y275A</sup> and mrpL35<sup>Y275D</sup> mitochondria (Figure 4, B–D).

The ability of the mrpL35<sup>Y275</sup> mutants to continue to synthesize Cox1, and moreover at elevated levels relative to the wild-type MrpL35 control, was not anticipated given the COX complex assembly defect observed in these mitochondria. As outlined earlier, synthesis of Cox1 by the mitoribosomes is under strict feedback regulation with its assembly into the COX complex (Figure 1). Hence, as previously reported, Cox1 synthesis is characteristically reduced in COX assembly mutants, for example, Δcox10 and Δcox18 mitochondria, where the steady-state levels of COX subunits are strongly reduced (Figure 4E). In contrast, the mrpL35<sup>Y275</sup> mutant, despite having strongly reduced COX subunit levels, not only retains the ability but also displays enhanced capacity to synthesize the Cox1 protein (Figure 4E).

Taken together, these data demonstrate that mutation of the MrpL35 protein at residue Y275 does not cause an overall inhibition in mitochondrial protein translation but rather results in an altered translational profile and a notable misregulated Cox1 synthesis behavior and a COX assembly defect.

Mutation in the Y275 residue of MrpL35 reveals a mitoribosomal connection to the COX assembly chaperones, Coa3 and Cox14

The observed elevated levels of Cox1 synthesis in the absence of assembly of the COX complex suggests that the Mss51 feedback regulatory system may be perturbed in the mrpL35 mutant mitochondria. Analysis of the steady-state levels of Mss51, Cox14, Shy1, and Coa3 assembly factors indicated them to be present in the mrpL35 mutants (Figure 5A). Thus the aberrant Cox1 synthesis and/or lack of efficient COX enzyme assembly observed in the mrpL35 mutant mitochondria was not due to altered levels of these key COX regulatory/assembly factors.

Following insertion into the membrane, newly synthesized Cox1 forms a COA assembly intermediate with Coa3 and other factors (including Cox14, Mss51, Coa1, and Coa2), which facilitates its subsequent assembly into the COX enzyme. We therefore tested
whether radiolabeled Cox1 in the mrpL35 mutants could be recovered in a complex with Coa3, and to do so we employed MrpL35 wild-type and mrpL35Y275D mutant mitochondria harboring a Histagged Coa3 derivative (Coa3His). Following in organello synthesis, radiolabeled Cox1 protein was recovered in a specific manner with the affinity-purified Coa3His species from both the control and mrpL35 mutant mitochondria (Figure 5B). The observed association of Cox1 with Coa3His in the mrpL35Y275D mitochondria suggests that the COX assembly defect in this mutant is downstream of the Cox1 synthesis and Coa3-Cox1 assembly intermediate formation events.
Affinity-purified Coa3<sub>His</sub> complexes were also analyzed to ascertain whether the association of Coa3 with Mss51, Shy1, and Cox14 was perturbed in the mrpL35 mutant (Figure 5C). Both Shy1 and Mss51 copurified with Coa3<sub>His</sub>, however, the levels of both proteins associated with the Coa3<sub>His</sub> complex from the mrpL35<sup>WT</sup> mitochondria were reduced, relative to those from the parallel wild-type control. Cox14 also detected in the affinity purified Coa3<sub>His</sub> complex from the mrpL35<sup>WT</sup> mitochondria; however, a significant fraction of the protein recovered was present as a novel, slower migrating species, termed here Cox14* (Figure 5C). A smaller proportion of this novel Cox14* species (relative to mature Cox14 protein) was also observed in the Coa3<sub>His</sub> complex purified from wild-type MrpL35 control mitochondria. The enrichment of this novel Cox14* species with the Coa3-complex from the mrpL35<sup>Y275D</sup> mitochondria would indicate that the behavior of the Cox14 protein is altered when the function of the MrpL35 protein is compromised.
BN–PAGE analysis indicated that the assembly of Coa3-containing complexes was significantly affected in the mrpL35Y275D mutant mitochondria (Figure 5D). In the wild-type Mrl35 control mitochondria, Coa3 was recovered in COA complexes of ∼240–480 kDa in mass, which have been proposed to also include the Cox1–Coa3 assembly intermediates of the COX complex (McStay et al., 2013; Mayorga et al., 2016). The level of these Coa3–COA complexes was strongly reduced in the mrpL35Y275D mutant mitochondria, indicating that despite the fact that newly synthesized Cox1 could associate with Coa3 (Figure 5B), the assembly state of the predominant Coa3–COA-containing complexes was strongly perturbed in this mutant. As the steady-state levels of the Coa3 protein were not reduced in the mrpL35Y275D mitochondria (Figure 5A), we conclude that the majority of the Coa3 protein in the mutant must be present in complexes smaller than 60 kDa under these conditions and thus not retained in the BN–PAGE gel. In contrast to the mrpL35Y275D mutant, the assembly state of the Coa3 complex did not appear to be affected in the mrpL35Y275A mutant mitochondria. As these mitochondria had both been isolated from cells grown at the permissive temperature, it appears that the phenotype of the mrpL35Y275D mutant is more severe and penetrated into cells grown at 30°C, in contrast to the mrpL35Y275A mutant. Consistently reduced COX levels were observed in these isolated mrpL35Y275D mitochondria and to a lesser extent in the mrpL35Y275A mitochondria (Figure 4, B–D). The aberrant assembly state of Coa3 observed in the mrpL35Y275D mutant was not simply due to the absence of an assembled COX enzyme, as Coa3-containing COA complexes were detected in mitochondria from the Δcox10 and Δcox18 COX assembly mutants, analyzed in parallel. The perturbed assembly of the Coa3–COA complexes in the mrpL35Y275D mutant mitochondria mirrored, however, that observed in the Δmas51 and wild-type/p3 mitochondria (Figure 5D). In contrast to these mitochondria where Cox1 synthesis is absent (Barrientos et al., 2004), the mrpL35Y275D mitochondria retain the capacity to synthesize Cox1 and form Cox1–Coa3 intermediates. We conclude therefore that in the ribosomal mutant mrpL35Y275D, the further (or stable) assembly of Coa3–Cox1 into larger Coa3–COA complexes was perturbed.

Finally, the molecular environment of Coa3 in intact mrpL35 mutant mitochondria was independently probed with the chemical cross-linking agent disuccinimidyl glutarate (DSG) (Figure 5E). In the wild-type Mrl35 control mitochondria, endogenous Coa3 (8 kDa) was observed to form three small adducts in the presence of DSG, which corresponded to Coa3 cross-linked to partners of ∼14, 12, and 8 kDa, respectively. A reduction in the formation of the smaller two of the Coa3-adducts, and a parallel acquisition of a novel fourth Coa3-adduct, corresponding to Coa3 cross-linked to an 11-kDa protein, was observed instead in the mrpL35Y275D mitochondria. The Coa3-adduct profile in the mrpL35Y275D mitochondria bore similarities to that obtained in the Δmas51 and Δcoa14 mutant mitochondria, analyzed in parallel, where loss of the Coa3+12 kDa and Coa3+8 kDa adducts, and the gain of the novel Coa3+11 kDa adduct, was observed (Figure 5E). The altered Coa3-adduct profile in these mitochondria may be indicative of their COX-assembly defective phenotype, as it was also observed in Δcox18 mitochondria and also in the ribosomal mutant mrp20AΔC mitochondria, which are defective in mitochondrial translation and, consequently, COX assembly. Although the identities of the Coa3 cross-linked partners in these adducts are unknown at this time, the observed altered Coa3 cross-linking pattern in the mrpL35Y275D mitochondria is further evidence of an aberrant Coa3 condition in this mitoribosomal mutant.

In summary, the molecular environment of Coa3, and the nature of the Cox14 species associated with it, is altered in the mrpL35 mutant. These perturbations may impact the productive nature of the Cox1-assembly intermediate formed with the Coa3 and thus the further assembly of Cox1 into the COX enzyme.

**Mutation of Y275 residue perturbs the close relationship of Mrl35 with Mrp7 protein**

The cryo-EM structures of yeast mitoribosomes indicate Mrl35 (mL38) to be a peripheral component of the central protuberance where it is embraced by mitospecific elements of the Mrp7 (bl27) protein. To gain more insight into Mrl35 and the possible effect of the Y275 mutation on its interactions with partner proteins, we affinity purified His-tagged wild-type and mutant Mrl35 derivatives, which had been expressed in the ribosome assembly yeast mutant, mrr20AΔC (Kaur and Stuart, 2011). Stable assembly of the 545 particles has been reported to be defective in this mrr20AΔC mutant, and instead ribosomal proteins can be purified in subassemblies or subcomplexes, enabling their compositional analysis (Kaur and Stuart, 2011). The affinity purified wild-type Mrl35His was recovered in a complex with other proteins, which were shown either through specific antibody decorations or mass spectrometry analysis (Supplemental Table S1) to include Mrp7 (bl27), Mrl36 (bl31), and Mrl7 (uL5) and two mitospecific proteins, Mrl17 (mL46) and Mrl28 (ml40) (Figure 6A). Western blotting with available antibodies against Mrp7 (bl27) and Mrl36 (bl31) confirmed their specificity of their association with Mrl35His, as they were not detected in the Mrp20 ribosomal exit pore subcomplex, purified parallel via Mrl25His, (ml59), a known component of the Mrp20 export pore subcomplex (Kaur and Stuart, 2011) (Figure 6, B and C). Reciprocal affinity purifications of Mrl7His, Mrl17His, and Mrl35His from the mrr20AΔC mutant strain also confirmed their specific association with Mrp7, Mrl36, and Mrl35 proteins (Figure 6B). The ability of Mrp7 to be copurified with the His-tagged derivatives of mrl35Y275A and mrl35Y275D proteins was strongly reduced relative to the wild-type MrpL35His control (Figure 6C). The recovery of Mrl36 (bl31) in the mutant mrl35Y275 subcomplexes was also reduced, albeit not to the same extent as the Mrp7 protein. These results indicate that the interaction of Mrl35 with Mrp7 (and possibly other components of the central protuberance subcomplex) is perturbed through the mutation of the Y275 residue, such that it does not remain associated with the Y275 mutated mrl35 protein under these detergent solubilization and purification conditions.

Steady-state analysis of protein levels indicated that a reduction in Mrp7 levels was observed in the mrl35Y275D mutant (Figure 6D, upper and lower panels). Mutation of the Y275 to Ala(A) residue did not greatly compromise the stability of the Mrl35 protein, though a decrease in Mrl35 steady-state levels was seen with the mrrpL35Y275D mitochondria. In contrast to Mrp7, the levels of Mrl36, another central protuberance constituent, or other 545 particle proteins, for example, Mrp20 and MrpL4, were relatively unaffected in the MrpL35 mitochondria (Figure 6D).

Taken together, we conclude that the Mrl35 can assemble into a central protuberance subcomplex with the Mrl36, Mrp7, MrlL7, MrlP17, and MrlP28 proteins and that the association with, and possibly stability of, Mrp7 is perturbed through the mutation of the Y275 residue in Mrl35.

**DISCUSSION**

Mitochondrial translation and the assembly of the OXPHOS complexes are processes of critical importance to aerobic metabolism and cell viability. Mutation and impairment of mitoribosome capacity has been shown to be directly associated with a range of
pathophysiological neuromuscular conditions (Lightowlers et al., 2014; De Silva et al., 2015). Investigating the molecular details of mitoribosome activity, regulation, and coupling of translation to the downstream OXPHOS assembly events is therefore critical for our understanding of normal and dysfunctional cellular metabolism. MrpL35 (mL38) is a mitospecific component of the yeast mitoribosomal 54S particle. Located in the central protuberance region, aspects of MrpL35 and the mitospecific domain of its partner protein Mrp7 (bL27) form an externally exposed element of this region of the mitoribosome (Amunts et al., 2014; Desai et al., 2017). Mutation of residue Tyr(Y)275 of the MrpL35 protein was found to compromise its ability to support aerobic growth and OXPHOS assembly, in particular at elevated temperatures, with the exchange for the negatively charged Asp(D) residue being more deleterious than Ala(A). Surprisingly, the molecular basis for the defective OXPHOS function in the mrpL35 mutants was not due to a primary inhibition in mitochondrial protein translation, but rather our OXPHOS complex analysis supports a pronounced COX assembly defect in these mrpL35 mutants. The consequences of the mrpL35 mutation for the assembly of the cytochrome bc1 or F1F0-ATP synthase complexes, which both also contain mitoribosome synthesized subunits, were not as pronounced as they were for the COX complex assembly. As the mrpL35 mutants retained the capacity to synthesize the mitochondrial encoded COX subunits, the strong reduction in COX complex steady-state levels indicates that a defect may lie in a posttranslational step required for the assembly of the COX complex. Furthermore, the elevated levels of Cox1 synthesized in the mutants, despite their gross COX assembly defect, points to a breakdown in the Mss51/Cox14/Coa3-dependent feedback loop used to negatively regulate Cox1 synthesis under conditions when further assembly of Cox1 into a COX complex is perturbed.

Our data indicate that newly synthesized Cox1 can form the Cox1–Coa3-containing assembly intermediate in the mrpL35 mutant, but the further progression of Cox1 assembly from this intermediate into the Coa3–COA intermediate complex, and thus subsequently to the fully assembled COX enzyme, are defective. Both Cox14 and Coa3 act as negative regulators of the Cox1 synthesis regulatory pathway (Mick et al., 2010; Fontanesi et al., 2011). Thus the elevated levels of Cox1 synthesized in the mrpL35 may also be indicative of a breakdown in the function of the Cox14 and/or Coa3 proteins. We observed the presence of a novel form of proteins identified to copurify with the MrpL35ΔHis and MrpL25ΔHis proteins are indicated. Note Rim1 and Mam33 were found to copurify with both the MrpL35ΔHis and MrpL25ΔHis subcomplexes, which was confirmed by Western blotting (Rim1) and reciprocal Mam33ΔHis purification studies (not shown). M, molecular mass marker proteins (kDa) used for SDS–PAGE. (B) Affinity purification of His-tagged MrpL7, MrpL17, MrpL35, Mrp7, and mrp20ΔC derivatives from mrp20ΔC mitochondria was performed as described in A with the exception that following SDS–PAGE, Western blotting and immunodecoration with indicated specific antibodies was performed. Total, 5% of solubilized material; Bound, 100% of affinity purified material recovered from the Ni-NTA beads. (C) Affinity purification of His-tagged MrpL35WT, mrpL35Y275A, or mrpL35Y275D derivatives or MrpL25 from mrp20ΔC mitochondria was performed as described in A with the exception that following SDS–PAGE, Western blotting and immunodecoration with indicated specific antibodies was performed. Total, 5% of solubilized material; Bound, 100% of affinity purified material recovered from the Ni-NTA beads. (D) The steady-state amounts of mitoribosomal proteins were determined by analyzing indicated mitochondria, as described in Figure 4B (upper panel). The amounts of Mrp7, MrpL35, and MrpL4 were quantified, normalized to Tim44, and expressed as a percentage of the wild-type control (lower panel).
Cox14, the Cox14* species enriched with the purified Coa3 complex and observed in the wild type, and at greatly increased proportions in Coa3 complexes from the mrpL35 mutant. The Cox14* species has not been reported in the literature to date and may represent a novel, post-translationally modified form of the Cox14 protein. The abundance of Cox14* with Coa3 complex in the mrpL35 mutant would indicate its recycling to its regular Cox14 status is perturbed in this mitoribosomal mutant, which in turn may stall the progression of Cox1–Coa3 assembly intermediate. Both BN–PAGE and chemical cross-linking data further illustrate that the stable assembly state of the Coa3–COA complexes is defective in the mrpL35Y275D mutant. Although the nature of this modification is currently unknown, we speculate here that the formation of Cox14*, and its possible recycling to Cox14, may influence the productive nature of the Cox1–Coa3 complex as an assembly intermediate and/or its ability to recruit or retain Miss51 and thus Cox14’s ability to negatively control the Cox1 synthesis levels.

MrpL35, like the other members of the mL38 family, contains a PEBP-like domain in its C-terminal region. PEBP proteins are a highly diverse group of proteins (i.e., not exclusively mitoribosomal proteins) involved in signaling pathways. PEBP-domains form anion ligand-binding pockets, which can interfere with kinase-based signaling pathways when they form complexes with anionic ligands (Banfield et al., 1998; Banfield and Brady, 2000; Yousuf et al., 2014). The cryoEM structural analyses of mitoribosomes indicate that the PEBP-region of MrpL35/mL38 proteins is exposed to an exterior surface of the CP (Amunts et al., 2014, 2015; Greber et al., 2015; Desai et al., 2017). This aspect of the MrpL35 may represent a binding site for an external regulatory ligand or feature of a nonribosomal protein (e.g., a phosphorylated residue) in the central protuberance of the mitoribosome. Residue Y275 is conserved in all MrpL35 (mL38) proteins and is located in the PEBP-like domain of MrpL35 (mL38). It is unclear whether the PEBP-like domain of MrpL35 binds anionic ligands, but the conserved nature of this domain, and the detrimental effect of the Y275 mutation, may suggest a role for MrpL35 in a signaling pathway involving the mitoribosome. It is important to note that the region of MrpL35 where residue Y275 is located is directly adjacent to the fungal-specific, mitospecific domain of Mrp7 (bL27), which embraces the PEBP-like domain of MrpL35 (Amunts et al., 2014; Desai et al., 2017). We observed that mutation of residue Y275 destabilized the association of MrpL35 with its partner protein Mrp7 and led to a reduction in Mrp7 levels in the mutant mitochondria. Some of the reported phenotypes in the mrpL35 mutants could therefore be attributed to a disturbance in the relationship of MrpL35 with Mrp7 protein rather than (or in addition to) a defect in MrpL35.

How does mutation of MrpL35, a mitoribosomal protein of the central protuberature, affect the COX assembly pathway and at the level of the Coa3/Cox14 proteins? One possibility is a physical connection between elements of the central protuberature, involving the PEBP-domain of MrpL35 and/or Mrp7, and the COX assembly chaperones, such as Cox14 and Coa3 proteins in the membrane, which is important for the correct operation of the COX assembly chaperones. The mitoribosomes are physically tethered to the inner membrane; however, to date this association has been shown to involve aspects of the 54S particle in the vicinity of the polypeptide exit channel and thus distanced from the central protuberature region (Jia et al., 2009; Gruschke et al., 2010; Haque et al., 2010; Pfeffer et al., 2015). It is therefore unlikely that MrpL35 in context of the translating ribosome can have a direct physical connection to the Cox14/Coa3 proteins; however, we cannot rule out that MrpL35 (and/or Mrp7) independent of the assembled ribosome (e.g., alone or in context of a central protuberature subcomplex) could do so. Furthermore, it is also possible that MrpL35 and/or components of the central protuberature may serve to recruit Miss51 to the ribosome and thus modulate its capacity to act as a Cox1 translational activator; however, we have not obtained data to support a Miss51–MrpL35 (or Mrp7) interaction to date. Another possibility is that MrpL35, through its PEBP-like domain, serves to affect COX assembly by recruiting a common anionic factor important for signaling between the ribosome and the Coa3–Cox14/Miss51 proteins and thereby to balance the Cox1 synthesis rate with the downstream chaperoned assembly events. Mutation of the Y275 residue may impair this ligand binding and thus promote more Cox1 synthesis in a manner that is uncoupled from the downstream assembly events. Finally, the mutation of the MrpL35 protein could also cause alterations in the ribosomal structure/organization that affects the accessibility of the newly synthesized Cox1 protein to the Miss51 protein following its insertion into the membrane. Miss51 binds to the extreme C-terminal tail of the newly synthesized Cox1 protein, a step required to recruit the chaperone into the Cox1–Coa3 assembly intermediate (Shingu-Vazquez et al., 2010; Garcia-Villegas et al., 2017). Factors that hinder the access to the C-terminal region of the newly synthesized Cox1 may influence the recruitment of Miss51 to the Cox1–Coa3 complex. Delayed recycling of the ribosome at the termination of translation and/or impaired disengaging of the ribosome from Oxa1, the membrane site of cotranslational insertion of proteins, may both prevent Miss51’s timely access to the C-tail of Cox1. Interestingly, yeast mutants deficient in the ribosome-recycling factor (Rf1), which promotes ribosomal subunit dissociation and recycling, display an OXPHOS assembly defect that was not caused by an inhibition in mitoribosomal translation per se (Ostojic et al., 2016). Rather, similarly to the mrpL35 mutants, the rf1 mutant mitochondria displayed an altered profile of synthesis products, including increased levels of newly synthesized Cox1, and reduction in COX complex assembly levels. However, in contrast to the mrpL35 mutants, the yeast rf1 mutants also exhibit a strong impairment in the assembly of the F1F0–ATP synthase complex, in addition to the observed COX assembly defect (Ostojic et al., 2016). The molecular defect underpinning the COX assembly deficiency in the mrpL35 Y275 mutant would thus appear to be different from that of the rf1 mutant and ribosome recycling where a more pleiotropic OXPHOS assembly defect is described. Taking this together with other mrpL35 results reported here, namely i) the maintained ability to form the Cox1–Coa3 intermediate, ii) the enrichment of Cox14* with Coa3, and iii) the altered assembly state of the Coa3 complex (as evidenced by chemical cross-linking and BN–PAGE approaches), we favor that the mrpL35Y275 mutation does not cause a general ribosome translation inhibition defect but rather has direct consequences for functional integrity of the Miss51, Coa3, or Cox14 proteins.

In summary, our findings here unexpectedly highlight that components of the mitoribosome, MrpL35, and possibly its closely associated Mrp7 protein, play a role in regulating the COX assembly process, in particular by influencing the molecular environments of the Miss51, Coa3, and Cox14 proteins. Furthermore, this study also represents the first report of a novel Cox14 species, Cox14*, associated with the Cox1–Coa3 complex, suggesting that Cox14 may be regulated by a posttranslational event. Future experimentation will ascertain the nature of the Cox14 modification and the role it plays in Cox14’s ability to support Coa3, Miss51, and the productive Cox1–assembly pathway and how this may involve the mitoribosomes and MrpL35.
Yeast strains and growth conditions
All S. cerevisiae strains used in this study are in the haploid W303-1A genetic background (W303-1A, mat a leu2, trp1, ura3, his3, ade2). They include the ΔmrpL35 (MRPL35::KAN) mutant and those harboring plasmid borne copies of the wild-type MRPL35 gene or mrpL35 mutant derivatives, created through a plasmid shuffling approach, as described below. The W303-1A-based strain mrp20A·C + GAL10-mrp20ΔC (termed here mrp20ΔC) was also employed (Kaur and Stuart, 2011). Yeast strains were cultured using standard protocols on either minimal medium supplemented as appropriate with uracil, tryptophan, leucine, adenine, histidine, or on full YP media, as indicated. Mitochondria were isolated from cultures grown at 30°C in minimal medium and containing 0.5% lactate and 2% galactose as carbon sources.

Generation of mrpL35 mutants
The yeast chromosomal MrpL35 open reading frame (ORF) was replaced with the Kan cassette in a haploid W303-1A yeast strain harboring a wild-type MRPL35 (MRPL35 WT) genomic insert (corresponding to the MRPL35 ORF plus 650 base pairs 5’ and 300 base pairs 3’) cloned into a pRS316 URA3-based plasmid. The resulting strain then transformed with the centromeric plasmid pRS413 (HIS3) harboring either a similar genomic fragment containing the wild-type or mutant mrpL35 derivative or no insert (i.e., the empty plasmid). Following plasmid shuffling process, involving growth on 5FOA to promote the loss of the pRS316(URA3)-MRPL35 WT plasmid, ΔmrpL35 transformants harboring only the pRS413(HIS3) plasmids were selected. The resulting strains (ΔmrpL35+MRPL35 WT or ΔmrpL35+mrpL35 Y275 derivatives) are referred to here as the ΔmrpL35 null or MrpL35 Y275 (wild-type) and the mrpL35 Y275A, mrpL35 Y275D, or mrpL35 Y275F mutant strains. Mutations in the Y275 residue of MRPL35 gene in the pRS413(HIS3)-MRPL35 plasmid were generated using a PCR site-directed mutagenesis strategy.

Generation of His-tagged MrpL35 and Coa3 derivatives
The Yip351-LEU2 vector containing the open reading frame encoding either the wild-type MrpL35 or mrpL35 Y275D derivatives or the Coa3 protein as C-terminal His-specific antibody. Following expression of the tagged proteins, transformation of the LEU2-kanamycin resistant strain harboring a wild-type MRPL35 (MRPL35 WT) derivative strain with BstEII and integrated into the leu2 loci of indicated yeast strains and LEU2+ transformants were selected. The presence of His-tagged derivatives in isolated mitochondria was confirmed using Western blot approaches with a His-specific antibody.

Affinity purification of His-tagged proteins
Isolated mitochondria (200 μg protein) harboring the His-tagged protein derivatives were solubilized in lysis buffer (160 mM KCl, 20 mM HEPES-KOH, 10 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.4), with 1.0% Triton X-100, 0.6% n-dodecyl β-D-maltoside (DDM), or 1% digitonin, as indicated, for 30 min on ice. Following a clarifying spin, Ni-NTA purification of His-tagged proteins was performed as previously described (Jia et al., 2007).

BN-PAGE analysis
BN-PAGE analysis of digitonin (1%) or DDM (0.6%) solubilized mitochondrial extracts (30 μg protein) was performed using Invitrogen Nu-PAGE gradient (3–12%) gels according to the manufacturer’s protocol and followed by Western blotting and antibody detection, as indicated.

Miscellaneous
All experiments presented were performed at least three times; results were consistently reproducible, and representative examples of data are given in the figures. Spectral measurements of specific enzyme activities of the cytochrome bc1 and COX complexes were performed as previously described (Tzagoloff et al., 1975). In organello translation with [35S]methionine and chemical cross-linking with DSG (7.7 Å, homobifunctional, amine-specific), were performed as previously described (Helling et al., 2001). Mitochondrial isolation, protein determination, and SDS–PAGE were performed as previously described (Dienhart and Stuart, 2008). The Cox1 and Cox3 antibodies used in this study were commercially obtained (Cox1: Molecular Probes, anti-yeast Cox1, mouse monoclonal 11D8-B7, lot# 6251-1; Cox3: Invitrogen/Novex Anti-Cox3 monoclonal, 459300, lot#H3578). All other antibodies used were rabbit polyclonal against the respective purified yeast proteins and generated either in the Stuart lab or received as gifts (see Acknowledgments). Antigen–antibody complexes were detected by horseradish peroxidase–coupled secondary antibodies and chemiluminescence detection on x-ray films. Resulting signals were quantified following scanning using Image Studio software.

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