Mam33 promotes cytochrome c oxidase subunit I translation in *Saccharomyces cerevisiae* mitochondria

Gabrielle A. Roloff and Michael F. Henry
Department of Molecular Biology, Rowan University School of Osteopathic Medicine, and Graduate School of Biomedical Sciences, Rowan University, Stratford, NJ 08084

**ABSTRACT** Three mitochondrial DNA–encoded proteins, Cox1, Cox2, and Cox3, comprise the core of the cytochrome c oxidase complex. Gene-specific translational activators ensure that these respiratory chain subunits are synthesized at the correct location and in stoichiometric ratios to prevent unassembled protein products from generating free oxygen radicals. In the yeast *Saccharomyces cerevisiae*, the nuclear-encoded proteins Mss51 and Pet309 specifically activate mitochondrial translation of the largest subunit, Cox1. Here we report that Mam33 is a third COX1 translational activator in yeast mitochondria. Mam33 is required for cells to adapt efficiently from fermentation to respiration. In the absence of Mam33, Cox1 translation is impaired, and cells poorly adapt to respiratory conditions because they lack basal fermentative levels of Cox1.

**INTRODUCTION**
The terminal enzyme of the mitochondrial electron transport chain, cytochrome c oxidase, is a multimeric copper-heme enzyme that catalyzes the transfer of electrons from cytochrome c to molecular oxygen. Cytochrome c oxidase biogenesis and function require the concerted expression of the nuclear and mitochondrial genomes. Three mitochondrially encoded proteins form the catalytic core, which is surrounded by 8–10 nuclear-encoded subunits in *Saccharomyces cerevisiae* and humans, respectively (Marechal et al., 2012; Soto et al., 2012a). At least 30 additional nuclear gene products are required for cytochrome c oxidase biogenesis, a subset of which is dedicated to translational activation of the mitochondrially encoded subunits (Fox, 2012; Soto et al., 2012a).

In *S. cerevisiae*, each mitochondrial gene is controlled by one or more dedicated translational activators. Proposed mechanisms of activation include interaction with the 5′ untranslated regions (UTRs) of their target transcripts, association with the mitochondrial ribosome, and anchoring to the mitochondrial inner membrane (Herrmann et al., 2013). These activities are often divided in cases in which multiple translational activators are required to promote the translation of a single mRNA transcript (Herrmann et al., 2013). Accumulating data suggest that translational activators can help couple translation with membrane insertion (Bauerschmitt et al., 2010; Gruschke et al., 2011). Consistent with this idea, translational activators can physically interact to colocalize the synthesis of different subunits of a single respiratory complex (Naithani et al., 2003). Finally, mitochondrial translational activators are part of feedback control loops that coordinate protein synthesis with assembly (Perez-Martinez et al., 2003, 2009; Barrientos et al., 2004; Mick et al., 2007; Rak and Tzagoloff, 2009; Gruschke et al., 2011). This helps prevent the accumulation of misassembled complexes, which could be deleterious to the cell.

Cox1 is a highly conserved core catalytic subunit of cytochrome c oxidase that bears copper and heme prosthetic groups for oxygen reduction. Cox1 synthesis is controlled by at least two translational activators, Pet309 and Mss51 (Decoster et al., 1990; Manthey and McEwen, 1995; Siep et al., 2000; Perez-Martinez et al., 2003). Pet309 is an integral inner-membrane protein predicted to contain up to 12 pentatricopeptide repeat (PPR) motifs within its sequence that are important for RNA binding (Manthey and McEwen, 1995; Tavares-Carreon et al., 2008; Zamudio-Ochoa et al., 2014). Pet309 physically interacts with the COX1 mRNA and the mitochondrial ribosome to promote COX1 mRNA translation (Manthey and McEwen, 1995; Bauerschmitt et al., 2010; Zamudio-Ochoa et al., 2014). The second
activator, Mss51, initiates COX1 mRNA translation by binding its 5′ UTR (Decoster et al., 1990; Siep et al., 2000; Zambrano et al., 2007; Perez-Martinez et al., 2009). In addition, Mss51 is involved in coordinating Cox1 synthesis with cytochrome c oxidase assembly through its interaction with newly synthesized Cox1 protein and a number of other assembly factors (Perez-Martinez et al., 2003, 2009; Barrientos et al., 2004; Pierrel et al., 2007; Fontanesi et al., 2010, 2011; Mick et al., 2010; Shingu-Vazquez et al., 2010). On assembly of Cox1 with other cytochrome c oxidase subunits, Mss51 is released to activate COX1 translation. Mss51 also senses heme and oxygen availability to regulate cytochrome c oxidase biogenesis (Soto et al., 2012d).

In this study, we report the identification of a new Cox1 translational activator, Mam33, which is conserved in eukaryotic organisms. The factor is termed mitochondrial acidic matrix (Mam33) protein based on its cellular localization, charge, and molecular weight. The N-terminal 47 residues of Mam33 are removed upon import into mitochondria (Seytter et al., 1998). Two previous reports were unable to assign a specific function to MAM33 because cells lacking this gene remain viable and appear to retain most of their mitochondrial respiratory activity (Muta et al., 1997; Seytter et al., 1998). Here we present evidence that Mam33 promotes translation of mitochondrial COX1.

RESULTS

Cells require Mam33 to adapt efficiently from fermentative to respiratory carbon sources

S. cerevisiae prefers to ferment glucose, even in the presence of oxygen, which yields sufficient ATP for cell viability without mitochondrial respiration (the Crabtree effect; Gelade et al., 2003; Johnston and Kim, 2005). However, in nonfermentable carbon sources (e.g., glycerol, lactate, or ethanol), yeast requires mitochondrial oxidative phosphorylation for ATP synthesis. Therefore a strain lacking a gene involved in mitochondrial respiration may grow poorly or not at all on nonfermentable carbon sources. The deletion of MAM33 did not noticeably influence growth rate in media containing glucose or nonfermentable carbon sources (Supplemental Figure S1, A–C). However, when exponential-phase cells were transferred from glucose to a nonfermentable carbon source, mam33Δ cells required approximately twice the time to double their cell number as wild-type cells (Figure 1A). The reverse shift, from respiratory to fermentative growth, was unaffected by mam33Δ (Supplemental Figure S1D).

Increased adaptation time for a cell culture population may result from some cells being unable to adapt, all cells adapting slowly, or a combination of both. After a shift to respiratory media, a comparable number of mam33Δ and wild-type cells were able to adapt (Figure 1B). However, the mam33Δ cells formed colonies more slowly (Figure 1C). In sum, these results demonstrate that Mam33 is necessary for cells to adapt efficiently from fermentative to respiratory growth.

Steady-state levels of mitochondrially encoded subunits of cytochrome c oxidase are substantially reduced in fermenting mam33Δ cells

Components of the mitochondrial respiratory chain are maintained at basal levels during glucose fermentation and are up-regulated during respiratory metabolism (Ohlmeier et al., 2004). For this reason, we hypothesized that mam33Δ cells adapt less efficiently because they do not express basal levels of respiratory chain components during fermentation. Moreover, given its mitochondrial location, we speculated that Mam33 influences respiratory components expressed from the mitochondrial genome. To test this theory, we compared the levels of mitochondrial DNA (mtDNA)–encoded respiratory chain proteins in wild-type and mam33Δ cells. The yeast mitochondrial genome encodes seven essential subunits of three respiratory chain complexes: complex III (Cob1), complex IV (Cox1, Cox2, and Cox3) and complex V (Atp6, Atp8, and Atp9; Figure 2A; Fourny et al., 1998). Of interest, steady-state levels of the mtDNA-encoded subunits of complex IV (Cox1–3) were dramatically reduced in glucose-grown mam33Δ cells (Figure 2B). The mitochondrially encoded subunits of complex III (Cob1) and V (Atp6) were also decreased but to a lesser extent and these are likely indirect effects due to disruption of the electron transport chain. In sum, these results demonstrate that MAM33 is required for Cox1–3 expression when cells are grown in glucose.

The abundance of nuclear-encoded complex IV subunits tested (Cox4 and Cox5) was decreased in fermenting mam33Δ cells but to a lesser extent than Cox1–3 (Figure 2B). The loss of the highly hydrophobic core subunits (Cox1–3) of complex IV prevents its assembly and results in the down-regulation or destabilization of the remaining subunits (Soto et al., 2012a). This regulation prevents the accumulation of unassembled proteins that could form prooxidant species or aggregate and disturb membrane homeostasis. Therefore the decreases observed for the other complex subunits are likely indirect effects due to disruption of the electron transport chain.

The presence of glucose triggers a global regulatory response that represses the expression of respiratory proteins (Johnston and Kim, 2005). We predicted that if glucose repression were involved, any protein compensating for Mam33 during respiratory growth would be expressed when cells were fermenting nonrepressible sugars (e.g., galactose and raffinose). To determine whether
Mam33-dependent Cox1–3 expression is due to glucose repression, we examined the steady-state levels of these proteins in cells grown in galactose and raffinose. All three cytochrome c oxidase proteins were similarly reduced in these nonrepressing sugars (Figure 2C). Thus the levels of these cytochrome c oxidase subunits are decreased in fermenting mam33Δ cells, independent of glucose repression. Although Cox1 levels are undetectable in mam33Δ mutants during these conditions, Cox2 and Cox3 levels appear to vary between experiments (Figure 2, B and C). The latter result suggests that the Cox2 and Cox3 decreases may be secondary effects.

Surprisingly, steady-state Cox1–3 levels were also decreased in mam33Δ cells grown to exponential phase in three different respiratory carbon sources (Figure 2D). The most significant decrease was observed in cells grown in glycerol. In these experiments, the reduction in Cox1 expression was the most pronounced. This result was unexpected because mam33Δ mutants do not have a respiratory growth defect during exponential phase (Supplemental Figure S1, A and B) and indicates that the Cox1 levels in these cells are enough to support wild-type growth. In previous reports, mutants with substantial steady-state Cox1 reductions displayed only minor cytochrome c oxidase activity defects (Barrientos et al., 2002; Horn et al., 2008).

Cells deleted for MAM33 have modestly reduced COX1 intron splicing

To determine whether the Cox1–3 protein reductions in mam33Δ cells are due to decreased transcription, RNA processing, or mRNA stability, we compared steady-state COX1-3 mRNA levels in wild-type and mam33Δ cells by Northern blot analysis. Whereas all three genes are transcribed as distinct polycistronic transcripts that are cleaved into individual messages before translation, only COX1 contains introns (Figure 3A; Lipinski et al., 2010). The levels of mature COX2 and COX3 transcripts were equal in mam33Δ cells, indicating that Mam33 is not required for the transcription, polycistronic cleavage, or stability of either transcript (Figure 3B). Conversely, mature COX1 transcript levels were decreased approximately twofold in the mam33Δ mutant (Figure 3B). Because COX1 contains seven introns, this analysis poorly differentiates between transcription and overall splicing defects. Inefficient intron removal will generate a multitude of low-abundance, high–molecular weight splice variants that would appear, if visible, as a faint smear by Northern analysis.

To better quantify RNA levels and detect both mature and intermediate transcripts, we used quantitative reverse transcription PCR (qRT-PCR; Figure 3, C and D). In the first experiment, an exon region present in all intermediate and mature transcripts was amplified. The RNA levels for all three genes were unchanged in mam33Δ cells, indicating that Mam33 is not required...
for COX1-3 transcription or mRNA stability (Figure 3C). In the second experiment, primers spanning COX1 exon junctions were designed to test whether COX1 intron splicing was reduced in mam33Δ cells (Figure 3A). If COX1 splicing is impaired, large introns would be present within the amplicon and prevent PCR amplification. The number of spliced transcripts decreased modestly in mam33Δ mutants, with those containing introns 1–4 being the most affected (Figure 3D). Although this result suggests that Mam33 is required for optimal COX1 intron splicing, this reduction is likely indirect due to impaired translation. This interpretation is consistent with the observation that splicing of the first two COX1 introns is most sensitive to a translation block (Decoster et al., 1990).

**Mam33 is required for efficient COX1 mRNA translation**

To ascertain whether Mam33 influences the translation of COX1-3 mRNAs, we compared mitochondrial protein synthesis in wild-type and mam33Δ cells. For this experiment, mitochondrial translation products were labeled in vivo with [35S]methionine and cysteine while blocking cytosolic translation with cycloheximide. Cox1 synthesis was strongly diminished in mam33Δ mutants during fermentation (Figure 4A). The translation of Cox2 and Cox3 was also reduced but to a lesser extent, again suggesting an indirect effect. In contrast, Cox1 synthesis was unaffected in respiring cells.

Mitochondrial gene expression is especially susceptible to indirect effects because transcription and translation are tightly coupled (Dieckmann and Staples, 1994; Wallis et al., 1994; Rouillard et al., 1996; Rodeheffer et al., 2001; Rodeheffer and Shadel, 2003; Bryan et al., 2002; Williams et al., 2007; Markov et al., 2009). Because Cox1 splicing and translation are both decreased in mam33Δ cells, it is possible that a defect in just one process indirectly disrupts the other process. For example, an increase of partially processed COX1 transcripts could delay translation of the mature mRNA. To test this possibility, we compared mitochondrial translation profiles of wild-type and mam33Δ strains carrying an intronless mitochondrial genome. The level of COX1 mRNA translation remained low in mam33Δ cells, at a level comparable to that of the intron-containing mam33Δ strain (Figure 4B). Taken together, these results suggest that Mam33 acts at the level of COX1 translation and the observed splicing defect is indirect.
Mam33 promotes COX1 translation

A negative feedback mechanism coordinates Cox1 translation during assembly. Mss51 activates COX1 translation, binds newly synthesized unassembled Cox1 nascent polypeptide, and remains bound during complex assembly (Perez-Martinez et al., 2003; Barrientos et al., 2004; Pierrel et al., 2007; Fontanesi et al., 2010, 2011; Mick et al., 2010). At a point late in complex assembly, Mss51 is released to initiate a new round of translation. If assembly is compromised, Mss51 remains sequestered with the unassembled complex and is unable to reactivate COX1 translation. Thus Cox1 synthesis could be diminished in mam33 Δ mutants because of cytochrome c oxidase assembly defects. To test this possibility, we eliminated this feedback control mechanism using a truncated version of Cox1 ( Cox1ΔC15). Mss51 is unable to bind newly synthesized Cox1ΔC15 and is constitutively free to activate its translation (Shingu-Vazquez et al., 2010). This appears to be the only Cox1 assembly feedback mechanism, because the Cox1ΔC15 bypass significantly rescued all of the numerous assembly mutants tested (Shingu-Vazquez et al., 2010). In mam33 Δ mutants, the translational defect was not rescued by the COX1ΔC15 allele, whereas translation in the assembly mutants (cox11Δ, cox7A, mss2Δ, and pet100Δ) was significantly restored (Figure 4C). These results demonstrate that mam33 Δ mutants exhibit a Cox1 translation defect without complex assembly feedback. Of interest, the translation profiles of the assembly mutants were strikingly similar to those of the mam33 Δ mutant, in that Cox2 and Cox3 labeling was weakly reduced. Because this assembly feedback mechanism specifically targets Cox1 translation, it is likely that Mam33 also targets Cox1 and that the Cox2 and Cox3 reductions are indirect.

Our Western blot analysis and in vivo radiolabeling experiments do not exclude the possibility that Cox1 is translated normally but then rapidly degraded in mam33 Δ cells. To test Cox1 stability, we monitored expression of an ARG8 reporter under the control of the COX1 5′ and 3′ UTRs (cox1Δ::ARG8). In cells with functional translational activators, the mitochondrial cox1Δ::ARG8 gene supported growth on minimal media lacking arginine (Figure 4D). In contrast, when the COX1 translational activator PET309 was

FIGURE 4: Mam33 is required for efficient COX1 translation during fermentation. (A) Mitochondrial translation products of wild-type (BMA64-1A) and mam33 Δ (MHY1564) cells grown in glucose, galactose, or a combination of glycerol and ethanol. Cells were labeled in vivo with [35S]methionine and cysteine for 10 min in the presence of cycloheximide at 30°C (as described in Materials and Methods). Proteins were resolved by 19% SDS–PAGE and analyzed by autoradiography. The top of the gel was transferred to a nitrocellulose membrane and probed with the anti-Aco1 antibody to normalize the signals for protein loading. Each mitochondrially encoded translation product is denoted in the left margin. (B) Mitochondrial translation products in mam33 Δ cells containing an intronless mitochondrial genome. Cells were grown in galactose and processed as described in A. The denoted genotypes (from left to right lanes) correspond to the following strains (Supplemental Table S1): BMA64-1A, MHY1564, MHY1954, and MHY1985. (C) Cox1 (−) or Cox1ΔC15 (+) cells with a deletion in the indicated genes were grown in galactose, and mitochondrial translation products were radiolabeled as described for A. The denoted relevant genotypes (from left to right lanes) correspond to the following strains (Supplemental Table S1): NB40-36a, XPM295a, MHY2021, MHY2036, MHY2037, MHY2038, MHY2043, MHY2044, MHY2045, MHY2046, MHY2047, and MHY2048. (D) Growth phenotypes of strains carrying an Arg8 reporter under control of the COX1 5′ and 3′ UTRs. Relevant nuclear and mitochondrial genotypes are indicated on the left and right, respectively. Yeast cells were grown to exponential phase in yeast extract/peptone/dextrose and then spotted to glucose minimal medium containing (+Arg) or lacking (−Arg) arginine. Plates were incubated for 2 d at 30°C. The cleavage site for the pre-Arg8 matrix-targeting signal is denoted with a black triangle. The indicated relevant genotypes correspond (from top to bottom) to the following strains (Supplemental Table S1): BMA64-1A, NB40-36a, EHW463, MHY2053, and MHY2072. (E) Steady-state accumulation of the Arg8 reporter in cells grown in glucose. Total mitochondrial proteins were separated by 10% SDS–PAGE and analyzed by Western blotting. When expressed inside the mitochondrial matrix (i.e., not imported), the Arg8 mitochondrial targeting signal is inefficiently cleaved, and Arg8 migrates as two bands. Por1 was used as a loading control. The indicated relevant genotypes correspond (from top to bottom) to the following strains (Supplemental Table S1): EHW465, MHY2093, MHY2096, and MHY2095.
deleted in this strain, the cells became Arg−. The mam33Δ, cox1Δ::ARG8® strain was also Arg−, indicating that the decrease in Cox1 synthesis in fermenting mam33Δ cells is due to a defect in translation and not stability. Consistent with the Arg− growth phenotype, Arg8® steady-state protein levels were severely decreased in the mam33Δ mutant (Figure 4E).

In some in vivo translation assays, decreased Cox1 translation is accompanied by reduced Cox2 and Cox3 labeling (Pierrel et al., 2007; Zambrano et al., 2007; Soto et al., 2012d). To test whether the affects on the other cytochrome c oxidase subunits are indirect, we chose to assay COX3 translation in mam33Δ mutants by monitoring expression of the ARG8® reporter under the control of COX3 5’ and 3’ UTRs (cox3A::ARG8®; Williams et al., 2005). Deletion of the COX1 translational activator PET309 or MAM33 did not affect cell growth on minimal medium lacking arginine (Figure 4F). As expected, deletion of a COX3 translational activator, PET54, generated Arg− cells (Figure 4F). Therefore the decrease in COX3 translation observed in mam33Δ cells (Figure 4A) is indirect and due to protein instability.

**DISCUSSION**

Here we show that Mam33 activates translation of mitochondrial encoded Cox1, a core subunit of the cytochrome c oxidase complex. Whereas cells deleted for Mam33 do not exhibit any noticeable growth defects during exponential phase, they have difficulty adapting from fermentative to respiratory growth. 

**How do we explain this distinctive phenotype?** In wild-type cells, components of the mitochondrial respiratory chain are maintained at basal levels during fermentation and are increased when cells shift to respiratory metabolism (Ohlmeier et al., 2004). When cells are rapidly shifted from a fermentable to a respiratory carbon source, this basal level of respiratory chain activity provides enough energy, as the cells increase their respiratory capacity. Respiratory adaptation is more difficult for mam33Δ cells because Cox1 is virtually absent during fermentation. Because the electron transport chain is dispensable during fermentative growth, these mutants do not exhibit a discernible growth defect when fermenting. Once acclimated, respiring mam33Δ cells are not appreciably affected because a ~50% reduction of cytochrome c oxidase activity is enough to support wild-type growth in nonfermentable media (Horn et al. 2008).

The translation of an Arg8 reporter flanked by the COX1 UTRs is Mam33 dependent (Figure 4, D and E). This result indicates that the UTRs are sufficient for Mam33 activation and strongly suggests that Mam33 has a specific target within the UTRs of COX1 mRNAs. Future work will determine which UTR is necessary and whether Mam33 acts directly on this mRNA.

Since the early 1970s, genetic screens for petite S. cerevisiae mutants have identified numerous factors critical for mitochondrial gene expression (Ebner et al., 1973; Tzagoloff et al., 1975). The analysis of respiration-deficient mutants exhibiting gene-specific translation defects led to the concept of translational activators (Muller et al., 1984; Tzagoloff and Myers, 1986; Tzagoloff and Dieckmann, 1990; Korte et al., 1989; Costanzo and Fox, 1990; Fox 1996; Haffter et al., 1990; McMullin et al., 1990; Poyton and McEwen, 1996). For the past 20 years, Mss51 and Pet309 have been the only recognized Cox1 translational activators (Decoster et al., 1990; Manthey and McEwen, 1995). However, mitochondrial translational activators with overlapping functions, or those only conditionally required, would have been missed in genetic screens for petite mutants. MAM33 is an example of such a gene.

Given that Mam33 activates translation of mitochondrially encoded Cox1, how do mam33Δ cells retain respiratory competence? A likely explanation is that a second factor, which is down-regulated during fermentative growth, can compensate for Mam33 during respiration. Although the Cox1 translational activators Mss51 and Pet309 are reasonable candidate factors, both are required for Cox1 expression in fermenting cells (Decoster et al., 1990; Manthey and McEwen, 1995; Siep et al., 2000; Perez-Martinez et al., 2003, 2009; Zamudio-Ochoa et al., 2014). Another candidate is YGR021W, a protein highly homologous to human TACO1. TACO1 is a COX1 mRNA–specific translational activator whose absence causes a cytochrome c oxidase deficiency and late-onset Leigh syndrome (Weararpachai et al., 2009; Seeger et al., 2010). However, mam33Δ ygr021wΔ double mutants retain respiratory competence (unpublished data). Further studies in this lab aim to identify this factor.

Although steady-state Cox1 levels were decreased in respiring mam33Δ cells, translation was unaffected. This result suggests that Mam33 may also directly or indirectly affect complex stability during respiration. The complex may also be unstable in fermenting cells, but this phenotype would be masked because translation is impaired.

Much remains unknown regarding the molecular functions of translational activators in mitochondria. Translational activators are believed to organize the translation machinery by 1) recognizing specific mitochondrial mRNAs and altering secondary structure, 2) interacting with ribosomal proteins, and 3) tethering translation to the inner membrane (Herrmann et al., 2013). Given that Mam33 is weakly associated with the inner membrane (Seytter et al., 1998), activation of Cox1 translation likely occurs by either of the first two or a novel mechanism. Future studies in this lab will examine whether Mam33 physically interacts with the Cox1 transcript or the mitochondrial ribosome. In support of a model in which Mam33 physically interacts with mitochondrial ribosomes, a search of the STRING protein interaction database identified five mitochondrial ribosomal proteins among the 10 highest-scoring Mam33 partners. This database predicts interactions based on several methods, including gene coexpression and high-throughput interaction experiments (Jensen et al., 2009).

Contrary to our results (Supplemental Figure S1, A and B), two previous studies reported reduced mam33Δ growth on a single nonfermentable carbon source. One reported an effect on glycerol at low temperatures (Muta et al., 1997), whereas the other observed a mild defect on lactate but not glycerol at 30°C (Seytter et al., 1998). Both studies measured growth rate as the time required for cells to form colonies after plating fermenting cultures onto respiratory plates. This method does not distinguish between adaptation and growth rate.

The crystal structures of yeast Mam33 and two homologues, human p32/gC1qr/ C1QBP /HABP1 and Trypanosoma brucei p22, have been solved (Jiang et al., 1999; Sprehe et al., 2010; Pu et al., 2011). Each forms a doughnut-shaped homotrimERIC structure that displays an unusual asymmetric charge distribution such that one surface of the doughnut is covered with negatively charged residues. The most prominent feature is a central hydrophilic channel that may function as a pore. For molecules to traverse this channel, it was proposed that protein-binding partners might induce the two α-helices covering this central region to undergo a conformational change (Jiang et al., 1999). It is intriguing to envision a mechanism by which proper ribosomal alignment of COX1 mRNA would require passage through the Mam33 channel. Despite the weak sequence homology between yeast Mam33 and human p32 (E value = 0.04), the human protein complements the adaptation defect of mam33Δ cells grown in glycerol medium (Muta et al., 1997).

Although human p32 is by far the most studied Mam33 homologue, its cellular localization and proposed activities are conflicting.
The p32 protein is primarily in the mitochondria (Dietmeier et al., 1993; Dedio and Muller-Esterl, 1996; Muta et al., 1997; Matthews and Russell, 1998; Jiang et al., 1999; Muta et al., 1998; Hu et al., 2013) but has also been reported to be present in the nucleus (Matthews and Russell, 1998; van Leeuwen and O’Hare, 2001), cytoplasm (Kraimer et al., 1991; Matthews and Russell, 1998), and endoplasmic reticulum (Dedio and Muller-Esterl, 1996) and on the cell surface (Gupta et al., 1991; Ghebrehiwet et al., 1994; Manthey and McEwen, 1995; Kötlesse et al., 2000; Mahdi et al., 2001). Some of these differences may be attributed to the different tissue types or cancer cell lines examined. Furthermore, the proposed activities of p32 are ubiquitous and sometimes incompatible with its localization. These activities range from influencing cellular and viral transcription/mRNA splicing in the nucleus (Krainer et al., 1993; Dedio and Muller-Esterl, 1996) and on the cell surface (Gupta et al., 1991; Ghebrehiwet et al., 1994; Manthey and McEwen, 1995; Kötlesse et al., 2000; Mahdi et al., 2001). Gene expression (Fogal et al., 2010), and apoptosis in the mitochondria (Itahana and Zhang, 2008; Rizvi et al., 2011; Eckart et al., 2014; Xiao et al., 2014) and binding small molecules and peptides on the cell surface (Ghebrehiwet et al., 1994; Fogal et al., 2008). Of interest, p32 has also been shown to be a critical regulator of tumor metabolism via maintenance of oxidative phosphorylation, but its physiological role remains unknown (Fogal et al., 2010; Amano et al., 2011). Given that p32 complements Mam33 function in yeast, a detailed study of MAM33 in yeast cells will permit a better understanding of its underlying biological function, which is conserved in eukaryotes (Muta et al., 1997).

**MATERIALS AND METHODS**

**Strains, media, and genetic methods**

*S. cerevisiae* strains used in this study are listed in Supplemental Table S1. Yeast cells were grown at 30°C in rich medium (YP) containing 1% yeast extract and 2% peptone or synthetic complete (SC) media and incubated with gentle shaking for 40 min at 30°C. Cells were then washed once with 1.2 M sorbitol, resuspended (6.7 ml/g of cells) in Zymolyase buffer (1.2 M sorbitol, 20 mM KPi, pH 7.4, and Zymolyase 100T [1 mg/g cells; Amhsio, Abingdon, UK]), and incubated with gentle shaking for 40 min at 30°C. All of the subsequent steps and centrifugations were performed on ice or at 4°C, respectively. Spheroplasts were harvested by centrifugation (2500 × g for 5 min), resuspended in homogenization buffer (0.6 M sorbitol, 10 mM Tris-HCl, pH 7.4, 0.2% fatty acid–free bovine serum albumin [Sigma-Aldrich, St. Louis, MO], and 1 mM phenylmethylsulfonyl fluoride [Sigma-Aldrich]), and homogenized by 20 strokes in a tight-fitting glass douncer. The homogenate was centrifuged (3000 × g for 5 min), and the supernatant was transferred to a new tube. The pellet was homogenized a second time, and the supernatants, containing mitochondria, were combined. The combined supernatants were centrifuged (3000 × g for 5 min) repeatedly until no pellet was observed, and then mitochondria were harvested by centrifugation (12,000 × g for 10 min). Protein concentrations were measured using the Bio-Rad protein assay (Bradford, 1976).

**Isolation of mitochondrion**

Yeast cells were grown to exponential phase (OD_{600} = 0.6) in YP medium containing the indicated carbon source, harvested by centrifugation (3000 × g for 5 min), resuspended (2 ml/g of cells) in reducing buffer (100 mM Tris-SO_{4}, pH 9.4, and 10 mM dithiothreitol [DTT]), and then incubated with shaking for 40 min at 30°C. Cells were then centrifuged (3000 × g for 5 min), resuspended in homogenization buffer (0.6 M sorbitol, 10 mM Tris-HCl, pH 7.4, 0.2% fatty acid–free bovine serum albumin [Sigma-Aldrich, St. Louis, MO], and 1 mM phenylmethylsulfonyl fluoride [Sigma-Aldrich]), and homogenized by 20 strokes in a tight-fitting glass douncer. The homogenate was centrifuged (3000 × g for 5 min), and the supernatant was transferred to a new tube. The pellet was homogenized a second time, and the supernatants, containing mitochondria, were combined. The combined supernatants were centrifuged (3000 × g for 5 min) repeatedly until no pellet was observed, and then mitochondria were harvested by centrifugation (12,000 × g for 10 min). Protein concentrations were measured using the Bio-Rad protein assay (Bradford, 1976).

**Analysis of mitochondrial proteins**

Isolated mitochondria (20 μg of protein) were resuspended in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride [Sigma-Aldrich]), and proteins were resolved on a 10% SDS–PAGE gel (Laemmli, 1970). Proteins were transferred to a nitrocellulose membrane (Amersham Hybond ECL) and analyzed by

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**Northern blotting and qRT-PCR**

For RNA extraction, 60-ml cultures were grown to exponential phase (OD_{600} = 0.4) in YP medium containing glucose. RNA was isolated using TRIzol reagent (Life Technologies, Waltham, MA) according to the manufacturer’s instructions.

For Northern blot analysis, 20 μg of total RNA was separated on a denaturing 1% formaldehyde-agarose gel, transferred to a Hybond-N+ nylon membrane, ultraviolet cross-linked, hybridized sequentially with gene-specific 32P-labeled probes, and analyzed by autoradiography (Brown, 2004). Gene-specific probes were labeled from PCR products (Supplemental Table S2) by random oligomer labeling according to the manufacturer’s instructions (Ready-To-Go Labeling Beads dCTP; Amersham Pharmacia Biotech, Piscataway, NJ). Unincorporated nucleotides were subsequently removed using MicroSpin G-25 columns (Amersham Pharmacia Biotech).

**cDNA synthesis and subsequent qRT-PCR** were performed with 30 ng of total RNA using an Applied Biosystems StepOne Real-Time PCR system according to the manufacturer’s instructions (Taq Universal SYBR Green One-Step Kit; Bio-Rad, Hercules, CA). After a reverse transcription cycle at 50°C for 10 min, DNA was amplified by 1 cycle at 95°C for 1 min and then 40 cycles consisting of 95°C for 15 s followed by 60°C for 1 min. A melt-curve analysis was performed to confirm primer specificity. Reactions were run in triplicate in three independent experiments. The primer sequences are listed in Supplemental Table S2.

To determine the time required for cells to adapt from a fermentative to respiratory carbon source, log-phase cells in SC glucose medium were harvested with gentle shaking for 40 min at 30°C. Cells were then centrifuged (3000 × g for 5 min), resuspended in homogenization buffer (0.6 M sorbitol, 10 mM Tris-HCl, pH 7.4, 0.2% fatty acid–free bovine serum albumin [Sigma-Aldrich, St. Louis, MO], and 1 mM phenylmethylsulfonyl fluoride [Sigma-Aldrich]), and homogenized by 20 strokes in a tight-fitting glass douncer. The homogenate was centrifuged (3000 × g for 5 min), and the supernatant was transferred to a new tube. The pellet was homogenized a second time, and the supernatants, containing mitochondria, were combined. The combined supernatants were centrifuged (3000 × g for 5 min) repeatedly until no pellet was observed, and then mitochondria were harvested by centrifugation (12,000 × g for 10 min). Protein concentrations were measured using the Bio-Rad protein assay (Bradford, 1976).

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**Adaptation experiments**

To determine the time required for cells to adapt to a fermentative to respiratory carbon source, log-phase (OD_{600} = 0.3) cells grown in 50 ml of SC glucose at 30°C were washed twice with distilled H_{2}O (dH_{2}O) and resuspended in the same medium containing either glycerol or ethanol. The cells were diluted in half (OD_{600} = 0.15) in the same medium, incubation with shaking was continued, and the time required for the cell density to double was determined by measuring OD_{600}. To determine the time required for cells to shift from respiratory to a fermentative growth, the same experiment was performed, except that cells were first grown in SC medium containing either glycerol or ethanol and then switched to SC medium containing glucose.

To assay cell viability and colony formation after transfer to a non-fermentable carbon source, log-phase cells in SC glucose medium (OD_{600} = 0.2) were washed twice with dH_{2}O and diluted. Approximately 200 cells were spread onto SC glucose or SC glycerol medium and incubated at 30°C for 2 or 3 d, respectively. Viability (percent) was calculated as the number of glycerol colonies divided by glucose colonies. Wild type was set equal to 100%.
Western blotting (Towbin et al., 1979). The antibodies were gifts from the following people and used at the indicated dilutions: anti-Arg8 rabbit polyclonal antibody (1:1000) from Tom Fox at Cornell University (Steele et al., 1996), anti-Cob1 rabbit polyclonal antibody (1:10,000) from Brian Robinson at the Hospital for Sick Children Research Institute (Lee et al., 2001), anti-Cor1 (1:10,000), anti-Cyt1 (1:2000), anti-Qcr6 (1:5000), and anti-Qcr2 (1:10,000) rabbit polyclonal antibodies from Rosemary Stuart at Marquette University (Cruiat et al., 2000), anti-Cox5a (1:2000), anti-Rip1 (1:2000), anti-Cyc1 (1:1000), and anti-Sdh2 (1:5000) rabbit polyclonal antibodies from Antoni Barrientos at the University of Miami (Fontanesi et al., 2011), anti-Atp6p (1:10,000) and anti-Atp7 (1:50,000) rabbit polyclonal antibodies from Jean Velours and Ophry Pines for strains and antisera. This work was supported by a grant from the New Jersey Health Foundation.

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Mam33 promotes COX1 translation

Mam33 promotes COX1 translation in mitochondria by interacting with the COX1 mRNA-specific translational activator proteins. Mam33 interacts directly with the COX1 mRNA and promotes its translation through the mitochondrial ribosome. This interaction is essential for the proper assembly and function of the cytochrome c oxidase complex. Mam33 is a novel mitochondrial protein that is specifically involved in the translation of COX1 mRNA, ensuring the proper expression and assembly of the cytochrome c oxidase complex in mitochondria.
