Ribosome binding proteins Mdm38 and Mba1 display overlapping functions for regulation of mitochondrial translation

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Running title: Regulation of mitochondrial protein expression
Abbreviations: GST, glutathione S-transferase; UTR, untranslated region
**SUMMARY**

Biogenesis of respiratory chain complexes depends on the expression of mitochondrial-encoded subunits. Their synthesis occurs on membrane-associated ribosomes and is probably coupled to their membrane insertion. Defects in expression of mitochondrial translation products are among the major causes of mitochondrial disorders. Mdm38 is related to Letm1, a protein affected in Wolf-Hirschhorn syndrome patients. Like Mba1 and Oxa1, Mdm38 is an inner membrane protein that interacts with ribosomes and is involved in respiratory chain biogenesis. We find that simultaneous loss of Mba1 and Mdm38 causes severe synthetic defects in the biogenesis of cytochrome reductase and cytochrome oxidase. These defects are not due to a compromised membrane binding of ribosomes but the consequence of a mis-regulation in the synthesis of Cox1 and cytochrome \( b \). Cox1 expression is restored by replacing Cox1-specific regulatory regions in the mRNA. We conclude, that Mdm38 and Mba1 exhibit overlapping regulatory functions in translation of selected mitochondrial mRNAs.
INTRODUCTION

Cells of animals or fungi contain two translation machineries, one in the cytosol and one in mitochondria. Whereas the cytosolic translation machinery is well characterized, the process by which mitochondrial ribosomes synthesize proteins is still ill-defined. In *Saccharomyces cerevisiae*, only eight proteins are encoded by the mitochondrial genome: Subunits 1 to 3 of cytochrome oxidase (Cox1, Cox2 and Cox3); cytochrome b of cytochrome reductase; subunits 6, 8 and 9 of the F,ATPase (Atp6, Atp8 and Atp9) and the ribosomal subunit Var1. The specialization on the synthesis of a small number of hydrophobic membrane proteins might explain why the mitochondrial translation system – in contrast to that of the cytosol – is intimately associated with the inner membrane. In mitochondria, even mRNAs are bound to the inner membrane due to the presence of membrane-associated translational activators, which bind to 5’ untranslated regions of the mRNAs (Fox, 1996; Michaelis *et al.*, 1991). Each gene appears to have at least one specific translational activator. Similarly, mitochondrial ribosomes are localized to the membrane through interactions with ribosome-binding membrane proteins. In particular, the Oxa1 insertase binds to the ribosome, presumably in order to couple protein synthesis physically and functionally to membrane integration (Jia *et al.*, 2003; Szyrach *et al.*, 2003). Co-translational insertion of nascent chains is facilitated by a second mitochondrial membrane protein, Mba1. Like Oxa1, Mba1 binds to the large subunit of the mitochondrial ribosome (Ott *et al.*, 2006; Preuss *et al.*, 2001) and functions as ribosome receptor critical for coordination of protein synthesis. However, its molecular function in translation is unclear (Ott *et al.*, 2006).

Recently, we identified Mdm38 as a third integral membrane protein, which binds to mitochondrial ribosomes (Frazier *et al.*, 2006). Mdm38 and its homolog Ylh47 are
the yeast paralogs of the human protein Letm1. Deletions of the LETM1 gene are associated with Wolf-Hirschhorn syndrome (Endele et al., 1999), a disorder characterized by severe growth and mental retardation, microcephaly, seizures and hypotonia. Yeast mutants lacking Mdm38 show an altered mitochondrial morphology and defects in potassium homeostasis (Dimmer et al., 2002; Nowikovsky et al., 2004). Since nigericin, a K⁺/H⁺ exchanger, mitigates the defects of ∆mdm38 mutants, it was suggested that Mdm38 might be critical, directly or indirectly, for the K⁺/H⁺ exchange across the inner membrane of mitochondria (Nowikovsky et al., 2007). In agreement with a role of Mdm38 in ion transport, a recent study by Jiang et al. (2009) reported that Drosophila Letm1 mediates Ca²⁺/H⁺ exchange in mitochondria.

For both proteins, Mba1 and Mdm38, a function as membrane-associated ribosome receptors was postulated. The results shown in this study strongly support this idea since the simultaneous deletion of both proteins leads to severe synthetic defects in the biogenesis of mitochondrial translation products. We show that Mdm38 and Mba1 play a critical and selective role in the regulation of mitochondrial translation of COX1 and cytochrome b mRNA. Moreover, we provide evidence that the defect in respiratory chain biogenesis is distinct from the postulated role of Mdm38 in K⁺/H⁺ homeostasis.
MATERIALS AND METHODS

Yeast strains and growth media

Yeast strains used in this study are derivatives of W303 except for strains generated from XPM171 (Perez-Martinez et al., 2003) and SB5 (Tavares-Carreon et al., 2008) (Table 1). The deletions of MDM38, MBA1 and the sequence corresponding to the C-terminus of Oxa1 were carried out as described (Frazier et al., 2006; Ott et al., 2006; Preuss et al., 2001). For the deletions of MDM38 and MBA1 in the XPM171 background a Cre-LoxP-system for integrating and removing a kanMX4 marker was used (Güldener et al., 1996). To generate rho0 derivatives of the strains AFY25, XPM171, DaMY33, DaMY34 and DaMY48, cells were grown on ethidium bromide-containing media. In order to generate mutant strains that lack mitochondrial introns, DaMY49, DaMY50, DaMY51 and DaMY52 were mated with the strain XPM72 containing intronless mtDNA (X. Perez-Martinez) derived from CK520 (Labouesse, 1990). Cytoductants were selected by their ability to respire and to grow on media lacking adenine. Fused cells containing two nuclei were identified by their growth on media without leucine and discarded. Yeast cultures were grown at 30°C in 1% yeast extract, 2% peptone (YP) medium supplemented with 2% galactose, glucose or sucrose, or on minimal medium supplemented with 20 µg/ml adenine, uracil, histidine and tryptophan, and 30 µg/ml of leucine and lysine (Altmann et al., 2007). Mitochondria were isolated as previously described (Altmann et al., 2007).

Labeling of mitochondrial translation products (in organello and in vivo)

Translation products were labeled in isolated mitochondria as described previously (Funes and Herrmann, 2007). Mitochondria (50 µg protein) were incubated in
translation buffer (0.6 M sorbitol, 159 mM KCl, 15 mM KH₂PO₄, 13 mM MgSO₄, 0.15 mg/ml of all amino acids except methionine, 4 mM ATP, 0.5 mM GTP, 5 mM α-ketoglutarate, 5 mM phosphoenolpyruvate, 3 mg/ml fatty acid-free bovine serum albumin, 20 mM Tris/HCl pH 7.4) containing 0.6 U/ml pyruvate kinase and 10 µCi [³⁵S]methionine. Samples were incubated for indicated time points at 25°C and labeling was stopped by addition of 25 mM unlabeled methionine. The samples were further incubated for 5 min to complete synthesis of nascent chains. Mitochondria were isolated by centrifugation, washed in 1 ml 0.6 M sorbitol, 20 mM Hepes/HCl, pH 7.4, lysed in 25 µl sample buffer (2% sodium dodecyl sulfate, 10% glycerol, 2.5% β-mercaptoethanol, 0.02% bromophenolblue, 60 mM Tris/HCl pH 6.8) and subjected to SDS-PAGE. In vivo labeling of mitochondrial translation products was performed in whole cells in the presence of cycloheximide essentially as described (Barrientos et al., 2002) with the difference that cells were grown on YP medium containing 2% galactose. Proteins were precipitated in the presence of 10% trichloroacetic acid, and precipitates washed with ice-cold acetone. For quantification of Cox1 by digital autoradiography the amount of Cox1 was standardized to that of Cox3. Quantification was performed by using ImageQuant TL software (GE Healthcare).

**In vitro binding experiments and immunoprecipitation**

For expression of a glutathione S-transferase (GST)-Mdm38 fusion protein, the open reading frame of MDM38 (encoding amino acids 159 to 573) was cloned into the SalI and NotI sites of the pGEX-4T-3 vector (GE Healthcare). Following expression in the E. coli BL21(DE3) strain (Stratagene), the fusion proteins were purified according to published procedures (Truscott et al., 2003). Purified GST-Mdm38 or GST were immobilized on glutathione sepharose and incubated with mitochondrial extracts.
essentially as described (Geissler et al., 2002). In brief, purified mitochondria were
solubilized in lysis buffer (20 mM Tris pH 7.4, 50 mM NaCl, 5 mM EDTA, 10%
glycerol and 2 mM PMSF) containing 1% digitonin. After binding the column
material was extensively washed in lysis buffer containing 0.5% Triton X-100 and
eluted with SDS sample buffer. Samples were processed for SDS-PAGE and
Western-blotting. For immunoprecipitation experiments, mitochondria expressing a
Pet309\textsubscript{HA} fusion protein were lysed in lysis buffer containing 0.5 mM EDTA and 1%
digitonin and subjected to co-immunoprecipitation using anti-HA (Roche) or anti-
FLAG (Sigma) antibodies as a control. Antibodies and bound proteins were depleted
from the lysate with ProteinG sepharose and beads washed extensively. Bound
material was eluted with SDS sample buffer. Samples were analyzed by SDS-PAGE
and Western-blotting.

**Membrane floatation assay**

Mitochondria (400 µg) were disintegrated by freeze thawing and sonified 10 times for
30s in a sonifying bath in 50 mM KCl, 10 mM MgCl\textsubscript{2}, 20 mM Tris/HCl pH 7.4. Then
the suspension was adjusted to 1.6 M sucrose and layers of 1.4 M sucrose and 0.25 M
sucrose were placed on top. After centrifugation at 255,000 x g for 2 h at 2°C the
gradient was separated into a top (membranes) and a bottom (soluble proteins)
fraction. Proteins in the fractions were precipitated by the addition of 12%
trichloroacetic acid and analyzed by Western-blotting.

**Northern Blotting**

RNA was isolated from purified mitochondria as described (Schmitt et al., 1990),
resolved on an agarose gel and transferred to a nylon membrane. The membrane was
hybridized with $^{32}\text{P}$-labeled DNA probes according to published procedures (Sambrook et al., 1989).

**Miscellaneous**

Enzyme activities were analyzed as described (Tzagoloff et al., 1975). Purification of ProteinA fusion proteins from solubilized mitochondria was performed as reported (Frazier et al., 2006), with the exception that bound proteins were released by TEV (tobacco etch virus) protease treatment. Blue native PAGE analyses were performed essentially as described (Dekker et al., 1997).
RESULTS

Loss of Mdm38 and Mba1 leads to a synthetic respiration defect

Oxa1, Mba1, and Mdm38 are ribosome-associated membrane proteins. Oxa1 and Mba1 cooperate in the coordination of mitochondrial protein insertion (Ott et al., 2006). In contrast to ∆mba1 and ∆oxa1 mutants, ∆mdm38 cells show only minor defects in the membrane insertion of mitochondrial translation products (Frazier et al., 2006). To assess whether this mild phenotype is due to a functional overlap of Mdm38 with Oxa1 or Mba1, we constructed deletion mutants lacking Mdm38 and the C-terminal 71 residues of Oxa1 (Oxa1\textsuperscript{1-331} or oxa1\textsuperscript{ΔC}) as well as Mdm38 and Mba1. Simultaneous deletion of Mdm38 and the C-terminus of Oxa1 did not aggravate the growth defect of the single mutants on non-fermentable carbon sources (Figure 1A). In contrast, ∆mba1/∆mdm38 double mutant cells displayed a respiration-deficient phenotype. Even in the presence of low concentrations of galactose, which partially rescued the single mutants, the ∆mba1/∆mdm38 double mutant was unable to grow on glycerol at all tested temperatures (Figure 1B). Since Mdm38 and Mba1 display a genetic interaction, we conclude that Mba1 and Mdm38 have overlapping important roles in the assembly, the maintenance or the function of the respiratory chain.

∆mdm38/∆mba1 mutants lack complex III and IV of the respiratory chain

To identify the molecular basis for the synthetic growth defect of ∆mba1/∆mdm38 mutants we isolated mitochondria from wild type and mutant cells and measured cytochrome reductase (complex III) and cytochrome oxidase (complex IV) activity. While ∆mba1 and ∆mdm38 mitochondria displayed reduced activities for both enzymes, ∆mba1/∆mdm38 mitochondria exhibited severe synthetic enzyme
deficiencies. Only about 14% of cytochrome reductase and virtually no cytochrome oxidase activity were detected. As a control, we measured the activity of malate dehydrogenase (MDH, Figure 2C), which was only slightly reduced in the mutants. This finding is in agreement with the observed growth phenotypes and with previous studies, which reported that even a reduction of respiratory chain activity to less than 10% did not lead to a full block of cell growth on non-fermentable medium (Bauerschmitt et al., 2008; LaMarche et al., 1992; Prestele et al., 2009).

Defects in complexes III and IV were confirmed by Blue-Native polyacrylamide electrophoresis (BN-PAGE). Both complex III and complex IV were reduced in \( \Delta mba1 \) and \( \Delta mdm38 \) single mutants (Figure 2D). In the double mutant mitochondria, no complexes were detected. As a control we analyzed the levels of the inner membrane TIM22 translocase and the F\(_{\text{0}}\)F\(_{\text{1}}\)-ATPase, which were not or only slightly reduced in the mutants. In consistence, Western-blotting revealed significantly reduced steady state levels of subunits of complex III (Cyt \( b \), Rip1, Qcr10) and complex IV (Cox2) in the double mutant (Figure 2E). Moreover, we recognized a slight increase of the amount of Mdm38 in \( \Delta mba1 \) mitochondria and of Mba1 in \( \Delta mdm38 \) mitochondria possibly as a compensatory effect. We conclude that Mba1 and Mdm38 are essential for the biogenesis or stability of complex III and complex IV.

**Mba1 and Mdm38 bind to mitochondrial ribosomes**

Since Mdm38 and Mba1 displayed a genetic interaction, we analyzed if the lack of Mba1 affects ribosome binding to Mdm38. The purified soluble C-terminal domain of Mdm38 fused to glutathione-S-transferase (GST) or GST were immobilized and incubated with mitochondrial extracts from wild type or \( \Delta mba1 \) mitochondria. Both
from wild type and Δmba1 samples ribosomes specifically bound to GST-Mdm38 (Figure 3A). Also Ylh47, a protein closely related to Mdm38, was efficiently pulled out from wild type and Δmba1 mitochondria indicating a direct or indirect association with Mdm38 independent of Mba1. Most interestingly, also Mba1 bound to Mdm38. This finding suggests that Mba1 and Mdm38 physically interact with each other.

To test if Mba1 and Mdm38 were present in a common complex we isolated Mdm38ProtA from solubilized mitochondria under conditions that maintained the Mdm38-ribosome interaction (Frazier et al., 2006). The native Mdm38 complexes were released from the affinity matrix by TEV-protease treatment (Figure 3B). In addition to mitochondrial ribosomes, indicated by the presence of Mrpl39 in the eluate, Ylh47 and Mba1 were specifically recovered in complex with Mdm38. Since the observed interaction of Mba1 with Mdm38 could be indirectly mediated through the mitochondrial ribosome, we analyzed if Mba1 could be purified together with Mdm38ProtA from ribosome-deficient rho0-mitochondria. Even in the absence of intact ribosomes we observed copurification of Mba1 with Mdm38, indicating that complex formation between these proteins was not dependent on the presence of ribosomes. Thus, we conclude that by direct or indirect means Mba1 and Mdm38 form a complex in mitochondria.

Are Mba1 and Mdm38 critical for membrane localization of mitochondrial ribosomes? When mitochondria were separated into membranes and soluble proteins by floatation centrifugation, about half of the ribosomes were recovered with the membrane fraction (Figure 3C). This ratio was not significantly altered in the Δmba1 or Δmdm38 single or Δmba1/Δmdm38 double mutant mitochondria. Accordingly, we conclude that membrane association of ribosomes is not compromised in the absence of Mba1 or Mdm38.
**Mba1 and Mdm38 are critical for Cox1 and cytochrome b synthesis**

Since enzyme complexes with mitochondrially-encoded subunits were specifically affected in the Δmba1/Δmdm38 double mutant, but coupling of ribosomes to the inner membrane was not, we monitored the synthesis of translation products in mitochondria of the Δmba1 and Δmdm38 mutants. Surprisingly, the simultaneous deletion of MBAl and MDM38 selectively prevented the synthesis of Cox1 and cytochrome b (Figure 4A, arrows). In contrast, some translation products (in particular, Atp6 and Atp9) appeared to be synthesized at increased rates in this mutant. This points to a mis-regulation of mitochondrial translation in the double mutant, which could explain the absence of complex III and complex IV in this strain. Apparently, Mba1 and Mdm38 possess overlapping functions and can thus partially substitute for each other in Cox1 and cytochrome b expression. However, upon loss of both proteins the complete loss of Cox1 and cytochrome b expression becomes evident.

The inability of the double mutant to synthesize Cox1 and cytochrome b might be due to a transcriptional defect, instability of the respective mRNAs, or a specific defect in their translation. To distinguish between both possibilities we isolated RNA from yeast mitochondria and assessed the levels of different mRNAs by Northern-blotting (Figure 4B). In yeast mitochondria the COX1 and cytochrome b transcripts undergo splicing. The Δmba1/Δmdm38 double mutant showed severely reduced mRNA levels of COX1 and cytochrome b, while other mRNAs were not affected or even increased (COX3). However, in the mutant mitochondria we detected higher molecular weight COX1 and cytochrome b transcript species (data not shown), indicating that transcription occurred in the mutant mitochondria.
Since mitochondrial RNA splicing has been found to be affected by defects in mitochondrial ion homeostasis, we introduced intron-less mitochondrial DNA into cells carrying deletions of \( \text{MBA1, MDM38, or MBA1/MDM38} \) by cytoduction. These mutant strains displayed similar growth defects on non-fermentable carbon sources as seen for the intron-containing mutant strains (Figure 4C). Thus, the observed growth defect of the mutant strains under conditions that require respiration cannot be attributed to defective RNA splicing. In agreement with this, when we analyzed mitochondrial translation in these strains \textit{in vivo} or \textit{in organello} the previously observed defect in Cox1 synthesis was not cured. We therefore conclude that the translational defects observed in \( \Delta \text{mba1/\Delta mdm38} \) double mutant mitochondria are independent of RNA splicing.

Mitochondrial protein synthesis is tightly regulated by translational activators, which bind to the 5'-UTRs of mRNAs (Fox, 1996; Michaelis et al., 1991). To assess a function of Mba1 and Mdm38 in translational regulation, we tested for Cox1 expression in cells in which the reading frame of \( \text{COX1} \) was flanked by 5’- and 3’-UTRs of \( \text{COX2} \). Yeast cells were grown in the presence of cycloheximide to inhibit cytoplasmic translation, and mitochondrial translation products were labelled with \(^{35}\text{S}-\text{methionine}. \) From the wild type mitochondrial genome, Cox1 was not expressed in \( \Delta \text{mba1/\Delta mdm38} \) mutant cells (Figure 4D). In contrast, Cox1 synthesis was independent of Mba1 and Mdm38 when the \( \text{COX1} \) reading frame was flanked by the untranslated regions of \( \text{COX2} \) (Figure 4E). Thus, by changing the \( \text{COX1}\)-specific UTR into a \( \text{COX2}\)-specific UTR (Perez-Martinez et al., 2003) we were able to render Cox1 expression independent of Mba1 and Mdm38. A quantification of the translation products revealed that compared to the wild type control ~105% of Cox1 was synthesized in \( \Delta \text{mba1/\Delta mdm38} \) mutant cells. We therefore conclude that Mba1
and Mdm38 are specifically involved in translational regulation of Cox1 and cytochrome b.

Translation of the \textit{COXI} mRNA is under control of the translational regulator Pet309, which specifically interacts with the 5’UTR of the \textit{COXI} transcript (Manthey and McEwen, 1995). Similar to Mdm38 and Mba1, Pet309 is associated with the mitochondrial inner membrane (Manthey \textit{et al}., 1998) and found in large protein complexes (Krause \textit{et al}., 2004; Naithani \textit{et al}., 2003). To address if Mdm38 was in complex with the translational activator Pet309, we performed coimmunoprecipitation experiments using a HA-tagged version of Pet309 (Tavares-Carreón \textit{et al}., 2008). Mitochondria containing Pet309_{HA} were solubilized in digitonin-containing buffer and incubated with anti-HA or anti-FLAG antibodies as a control. Pet309 was efficiently precipitated from the extract with HA antibodies but not recovered in the control sample. Moreover, Mdm38 and mitochondrial ribosomes, were specifically coimmunoprecipitated with Pet309_{HA} (Figure 4F, lanes 4 vs. 5). In contrast, Pam18, a mitochondrial membrane protein that exposes a domain into the matrix, was not recovered. We interpret this finding as indication that Mdm38 and Pet309 interact directly or indirectly as parts of a protein complex. The fact, that ribosomes can be detected in the Pet309_{HA} precipitate suggests that Mdm38 and Pet309 both act at the mitochondrial ribosomes. Taken together, these observations support a role of Mdm38 and Mba1 in translational processes at the ribosome.

\textbf{The \textit{Δmba1/Δmdm38} growth defect cannot be rescued by nigericin treatment}

Mdm38 was proposed to be critical for mitochondrial potassium homeostasis since the growth of \textit{Δmdm38} mutant cells on glycerol-containing media is improved by nigericin, a K⁺/H⁺ antiporter (Nowikovsky \textit{et al}., 2007). We therefore analyzed if the
growth defect of Δmba1/Δmdm38 mutants could be similarly rescued by nigericin (Figure 5A). While the growth defect of Δmdm38 cells on non-fermentable medium could be compensated by nigericin as reported, the growth defect of the Δmba1/Δmdm38 double mutants was not improved. Accordingly, nigericin was unable to substitute for the lack of Mba1 and Mdm38. Thus, the respiratory chain assembly defect, which is already apparent in Δmdm38 mutant mitochondria but aggravated in Δmba1/Δmdm38 double mutant cells, cannot be suppressed by nigericin and thus is unrelated to a role of Mdm38 in K⁺/H⁺ homeostasis.
DISCUSSION

Here we show that Mba1 and Mdm38 have an overlapping function in mitochondrial protein synthesis. Double mutants lacking both proteins display synthetic growth defects on non-fermentable carbon sources. The levels of COXI and cytochrome b mRNAs are significantly reduced in these strains and the corresponding proteins are therefore not synthesized. This causes specific defects in complexes III and IV of the respiratory chain explaining the respiration deficiency.

In addition to the genetic interaction between Mba1 and Mdm38, coisolation experiments suggest a physical association of both proteins. This interaction does not require the presence of ribosomes. However, ribosomes apparently stimulate or stabilize the binding since only reduced amounts of Mba1 were found in association with Mdm38 in rho⁰ cells. Our observations are consistent with a role of Mba1 and Mdm38 in recruiting components to the ribosome, which are critical for the stabilization and translation of specific mRNAs (Figure 5B). Recently, a 900 kDa multisubunit complex was identified that contains Cbp1, Pet309 as well as several non-identified subunits (Krause et al., 2004). Cbp1 is a factor that is specifically required for the translation and stability of the mRNA of cytochrome b (Dieckmann et al., 1984). Pet309 plays a comparable role for COXI transcripts (Manthey and McEwen, 1995). Interestingly, Cbp1 and Pet309 were identified together in a genetic screen for components critical for the co-translational translocation of protein domains across the inner membrane (Saracco and Fox, 2002). Here we show, that Pet309, the translational activator for COXI mRNA, is present in a complex with Mdm38 as well as with mitochondrial ribosomes in agreement with a function of Mdm38 in mitochondrial translation.
While our results demonstrate a role of Mdm38 and Mba1 in translation, they do not rule out a role of Mdm38 in K⁺ homeostasis. Since nigericin did not rescue the double mutant, it is likely that Mdm38 is involved in two processes, regulation of translation and, indirectly or directly, ion transport. We report that the defects found here for mitochondrial translation are not indirectly caused by defective splicing. In agreement with this is the fact that Mdm38 is conserved in human although mitochondrial RNAs do not undergo splicing in human mitochondria.

A recent study showed that, in human cells, the Mdm38 homolog Letm1 is associated with mitochondrial ribosomes (Piao et al., 2009). Similar to the situation in yeast, a knockdown of Letm1 by RNAi leads to defects in respiratory chain biogenesis (Tamai et al., 2008). Interestingly, also overexpression of Letm1 caused a problem in the biogenesis of respiratory chain complexes from which it was concluded that Letm1 could function as a regulator in translation (Piao et al., 2009). It is tempting to speculate that loss of Letm1 in Wolf-Hirschhorn syndrome patients leads to a mis-regulated translation, which contributes to the pathological phenotype. It will be exciting to explore the potential role of Mba1 and Mdm38 in ribosome binding and their interaction with translational activators in further detail in the future.

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REFERENCES


FIGURE LEGENDS

**Figure 1.** Yeast cells lacking Mba1 and Mdm38 show severe synthetic growth defects. (A) and (B) Wild type (wt) and mutant yeast strains were grown to mid log phase, adjusted to an OD$_{600}$ of 0.1, and tenfold serial dilutions spotted onto plates containing 2% glucose, 2% glycerol or 2% glycerol and 0.1% galactose (Gal). Plates were incubated for 2 (glucose) or 5 days at indicated temperatures.

**Figure 2.** $\Delta mba1/\Delta mdm38$ mitochondria show severe defects in complexes III and IV of the respiratory chain. (A) to (C). Complex III, complex IV and malate dehydrogenase activities were measured in isolated mitochondria from the strains indicated. Standard errors were calculated from three independent experiments. (D) Mitochondrial protein complexes were resolved by BN-PAGE, transferred to PVDF membranes and probed with antibodies against Rip1 (complex III), Cox4 (complex IV), Tim54 (TIM22 complex) and Atp5 ($F_oF_1$-ATPase). Positions of molecular weight markers are indicated. (E) Mitochondria (50 µg) of the indicated strains were analyzed by Western-blotting with antibodies against the indicated proteins. Cyt $b$, cytochrome $b$; Cyt $c_1$, cytochrome $c_1$; $F_1\alpha$, $\alpha$ subunit of the $F_oF_1$-ATPase. The arrows depict proteins which show diminished levels in the double mutant.

**Figure 3.** Mba1 and Mdm38 physically interact with each other and with mitochondrial ribosomes. (A) Wild type (wt) and $\Delta mba1$ mitochondria were lysed and incubated with purified GST or GST-Mdm38 bound to glutathione Sepharose. After extensive washing, bound proteins were eluted and visualized by Western-blotting using antibodies against the indicated proteins. Control lanes show an aliquot of the
mitochondrial extracts. (B) Wild type or Mdm38ProtA-expressing mitochondria that contain or lack a mitochondrial genome were lysed with buffer containing 1% digitonin. The extracts were incubated with IgG sepharose. The resin was washed and bound proteins were eluted and analyzed by Western-blotting. 4% of the total sample and 100% of the eluate were loaded on the gels. (C) Mitochondria of the indicated strains were fractionated into membrane (M) and soluble (S) fractions by freeze-thawing and floatation. Proteins of these fractions were analyzed by Western-blotting.

**Figure 4.** ∆mba1/∆mdm38 mitochondria synthesize severely reduced amounts of Cox1 and cytochrome b. (A) Mitochondria isolated from the strains indicated were incubated in 35S-methionine-containing translation buffer for the times indicated. Mitochondria were reisolated, washed and subjected to SDS-PAGE and autoradiography. Arrows depict Cox1 and cytochrome b. (B) ∆mba1/∆mdm38 mitochondria lack COX1 mRNA. RNA was isolated from the indicated mitochondria and analyzed by Northern-blotting using radioactive probes for the indicated transcripts. (C) Wild type (wt) and mutant yeast strains lacking mitochondrial introns were grown to mid log phase, and tenfold serial dilutions spotted onto plates containing glucose or glycerol/ethanol. Plates were incubated at 30°C. (D) and (E). Expression of Cox1 under the COX2 promoter bypasses the need for Mba1 and Mdm38. Mitochondrial translation products were radiolabeled in the indicated deletion mutants. Cells contained a wild type mitochondrial genome (D) or the reading frame of COX1 was flanked by UTRs from COX2 (E). (F) Mdm38 interacts with the Cox1-specific translational activator Pet309. Mitochondria carrying Pet309<sup>HA</sup> were solubilized in digitonin buffer and subjected to immunoprecipitation with anti-HA or anti-FLAG antibodies (control). Bound protein was analyzed by Western-
blotting. 5% of total and unbound samples and 100% of the eluates were loaded on the gels.

**Figure 5.** The ∆mba1/∆mdm38 growth defect is independent of a role of Mdm38 in the maintenance of mitochondrial K⁺/H⁺ homeostasis. (A) Growth of the indicated strains was analyzed as described for Fig. 1A. Plates labeled “+Nig” contained 2 µM nigericin. (B) Hypothetic model for the role of Mdm38 and Mba1 in translational control. Ribosome-associated membrane proteins are depicted. Translational activators (black sphere) associate specifically with the 5’-UTR of mitochondrial transcripts and might employ Mba1 and Mdm38 for their recruitment to mitochondrial ribosomes.
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<td>XPM171 (wt)</td>
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<td>(Perez-Martinez et al., 2003)</td>
</tr>
<tr>
<td>DaMY33 (Δmba1)</td>
<td>MATa leu2-3,112 ura3-52 lys2 arg8::hisG mba1::kanMX4 [cox1::ARG8m cox2::COX1 COX2]</td>
<td>This study</td>
</tr>
<tr>
<td>DaMY34 (Δmdm38)</td>
<td>MATa leu2-3,112 ura3-52 lys2 arg8::hisG mba1::kanMX4 [cox1::ARG8m cox2::COX1 COX2]</td>
<td>This study</td>
</tr>
<tr>
<td>DaMY48 (Δmdm38 Δmba1)</td>
<td>MATa leu2-3,112 ura3-52 lys2 arg8::hisG mba1::kanMX4 [cox1::ARG8m cox2::COX1 COX2]</td>
<td>This study</td>
</tr>
<tr>
<td>DaMY49 (wt)</td>
<td>MATa leu2-3,112 ura3-52 lys2 arg8::hisG [rho0]</td>
<td>This study</td>
</tr>
<tr>
<td>DaMY50 (Δmba1)</td>
<td>MATa leu2-3,112 ura3-52 lys2 arg8::hisG mba1::kanMX4 [rho0]</td>
<td>This study</td>
</tr>
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<td>DaMY51 (Δmdm38)</td>
<td>MATa leu2-3,112 ura3-52 lys2 arg8::hisG mba1::kanMX4 [rho0]</td>
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</tr>
<tr>
<td>DaMY52 (Δmdm38 Δmba1)</td>
<td>MATa leu2-3,112 ura3-52 lys2 arg8::hisG mba1::kanMX4 [rho0]</td>
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</tr>
<tr>
<td>XPM72</td>
<td>MATa ade2-101 ura3-52 arg8::hisG kar1-1 [rho+ ΔΣα1 ΔΣβl]</td>
<td>X. Perez-Martinez</td>
</tr>
<tr>
<td>SFMeY01 (wt)</td>
<td>MATa leu2-3,112 ura3-52 lys2 arg8::hisG [rho+ ΔΣα1 ΔΣβl]</td>
<td>This study</td>
</tr>
<tr>
<td>SFMeY02 (Δmba1)</td>
<td>MATa leu2-3,112 ura3-52 lys2 arg8::hisG mba1::kanMX4 [rho+ ΔΣα1 ΔΣβl]</td>
<td>This study</td>
</tr>
<tr>
<td>SFMeY03 (Δmdm38)</td>
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<td>This study</td>
</tr>
<tr>
<td>SFMeY04 (Δmdm38 Δmba1)</td>
<td>MATa leu2-3,112 ura3-52 lys2 arg8::hisG mba1::kanMX4 [rho+ ΔΣα1 ΔΣβl]</td>
<td>This study</td>
</tr>
<tr>
<td>SB5</td>
<td>MATa ade2 ura3Δ PET309::PET309,ΔHA</td>
<td>(Tavares-Carreon et al., 2008)</td>
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<tr>
<td>YPH499 (wt)</td>
<td>MATa ade2-101 his3-Δ200 leu2-Δ11 ura3-52 trp1-Δ63 lys2-801</td>
<td>(Sikorski and Hieter, 1989)</td>
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<tr>
<td>AFY8 (Δmba1)</td>
<td>MATa ade2-101 his3-Δ200 leu2-Δ11 ura3-52 trp1-Δ63 lys2-801 mba1::HIS3MX6</td>
<td>This study</td>
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<td>AFY25 (mdm38Pros)</td>
<td>MATa ade2-101 his3-Δ200 leu2-Δ11 ura3-52 trp1-Δ63 lys2-801 mba1::HIS3MX6</td>
<td>This study</td>
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<tr>
<td>DaMY14 (rho0 mba1)</td>
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<td>This study</td>
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wt, wild type strains.