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Ribosome-binding Proteins Mdm38 and Mba1 Display Overlapping Functions for Regulation of Mitochondrial Translation

Heike Bauerschmitt,*†‡ David U. Mick,‡§‖¶ Markus Deckers,¶ Christine Vollmer,¶ Soledad Funes,*†# Kirsten Kehrein,* Martin Ott,* Peter Rehling,¶ and Johannes M. Herrmann*

*Zellbiologie, Universität Kaiserslautern, 67663 Kaiserslautern, Germany; †Institut für Physiologische Chemie, Universität München, 81377 München, Germany; §Institut für Biochemie und Molekularbiologie, ZBMZ, Universität Freiburg, 79104 Freiburg, Germany; ‖Fakultät für Biologie, Universität Freiburg, 79104 Freiburg, Germany; ¶Abteilung Biochemie II, Universität Göttingen, 37073 Göttingen, Germany; and #Departamento de Genética Molecular, Instituto de Fisiología Celular, Circuito Exterior s/n, Ciudad Universitaria, Universidad Nacional Autónoma de México, Mexico, Distrito Federal, 04510, Mexico

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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 1–3 of cytochrome oxidase (Cox1, Cox2, and Cox3); cytochrome *b* (Cyt *b*) of cytochrome reductase; subunits 6, 8, and 9 of the F_oF_1-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 because of the presence of membrane-associated translational activators, which bind to 5′ untranslated regions of the mRNAs (Michaelis *et al.*, 1991; Fox, 1996). 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). Cotranslational 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 (Preuss *et al.*, 2001; Ott *et al.*, 2006) 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). Because nigericin, a K^+/H^+ exchanger, mitigates the defects of Δ*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,

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‡ These authors contributed equally to this work.

Address correspondence to: Peter Rehling (peter.rehling@medizin.uni-goettingen.de).

Abbreviations used: GST, glutathione *S*-transferase; UTR, untranslated region.

a recent study by Jiang *et al*. (2009) reported that *Drosophila* Letm1 mediates Ca^{2+}/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 because 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 *CYTB* 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-Carreón *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 (Preuss *et al.*, 2001; Frazier *et al.*, 2006; Ott *et al.*, 2006). 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 *rho*^{0} derivatives of the strains AFY25, XPM171, DaMY33, DaMY34, and DaMY48, cells were grown on ethidium bromide-containing media. 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 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 KH_2PO_4, 13 mM $MgSO_4$, 0.15 mg/ml 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 [^{35}S]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% SDS, 10% glycerol, 2.5% β-mercaptoethanol, 0.02% bromophenol blue, 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 were 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, Munich, Germany).

Table 1. Yeast strains used in this study

Strain	Genotype	Source
W303-1A (wt)	*MAT**a** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1*	Thomas and Rothstein (1989)
HHY081 (*oxa1*Δ*C*)	*MAT*α *ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 oxa1-K332**	Szyrach *et al.* (2003)
HHY299 (Δ*mba1*)	*MAT**a** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 mba1::HIS5MX6*	This study
DaMY15 (*oxa1*Δ*C* Δ*mdm38*)	*MAT*α *ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 oxa1-K332* mdm38::TRP1*	This study
DaMY17 (Δ*mdm38*)	*MAT**a** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 mdm38::TRP1*	This study
DaMY18 (Δ*mdm38* Δ*mba1*)	*MAT**a** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 mba1::HIS5MX6 mdm38::TRP1*	This study
XPM171 (wt)	*MAT*α *leu2-3,112 ura3-52 lys2 arg8::hisG* [*cox1::ARG8*^{m} *cox2::COX1 COX2*]	Perez-Martinez *et al.* (2003)
DaMY33 (Δ*mba1*)	MATα *leu2-3,112 ura3-52 lys2 arg8::hisG mba1::kanMX4* [*cox1::ARG8*^{m} *cox2::COX1 COX2*]	This study
DaMY34 (Δ*mdm38*)	MATα *leu2-3,112 ura3-52 lys2 arg8::hisG mdm38::kanMX4* [*cox1::ARG8*^{m} *cox2::COX1 COX2*]	This study
DaMY48 (Δ*mdm38*/Δ*mba1*)	*MAT*α *leu2-3,112 ura3-52 lys2 arg8::hisG mdm38*Δ *mba1::kanMX4* [*cox1::ARG8*^{m} *cox2::COX1 COX2*]	This study
DaMY49 (wt)	*MAT*α *leu2-3,112 ura3-52 lys2 arg8::hisG* [*rho*^{0}]	This study
DaMY50 (Δ*mba1*)	*MAT*α *leu2-3,112 ura3-52 lys2 arg8::hisG mba1::kanMX4* [*rho*^{0}]	This study
DaMY51 (Δ*mdm38*)	*MAT*α *leu2-3,112 ura3-52 lys2 arg8::hisG mdm38::kanMX4* [*rho*^{0}]	This study
DaMY52 (Δ*mdm38*/Δ*mba1*)	*MAT*α *leu2-3,112 ura3-52 lys2 arg8::hisG mdm38*Δ *mba1::kanMX4* [*rho*^{0}]	This study
XPM72	*MAT**a** ade2-101 ura3-52 arg8::hisG kar1-1* [*rho*$^{+}$ Δ*aI* Δ*bI*]	X. Perez-Martinez
SFMeY01 (wt)	*MAT*α *leu2-3,112 ura3-52 lys2 arg8::hisG* [*rho*$^{+}$ Δ*aI* Δ*bI*]	This study
SFMeY02 (Δ*mba1*)	*MAT*α *leu2-3,112 ura3-52 lys2 arg8::hisG mba1::kanMX4* [*rho*$^{+}$ Δ*aI* Δ*bI*]	This study
SFMeY03 (Δ*mdm38*)	*MAT*α *leu2-3,112 ura3-52 lys2 arg8::hisG mdm38::kanMX4* [*rho*$^{+}$ Δ*aI* Δ*bI*]	This study
SFMeY04 (Δ*mdm38*/Δ*mba1*)	*MAT*α *leu2-3,112 ura3-52 lys2 arg8::hisG mdm38*Δ *mba1::kanMX4* [*rho*$^{+}$ Δ*aI* Δ*bI*]	This study
SB5	*MAT*α *ade2 ura3*Δ *PET309::PET309*$_{3xHA}$	Tavares-Carreón *et al.* (2008)
YPH499 (wt)	*MAT**a** ade2-101 his3-*Δ*200 leu2-*Δ*1 ura3-52 trp1-*Δ*63 lys2-801*	Sikorski and Hieter (1989)
AFY8 (Δ*mba1*)	*MAT**a** ade2-101 his3-*Δ*200 leu2-*Δ*1 ura3-52 trp1-*Δ*63 lys2-801 mba1::HIS3MX6*	This study
AFY25 (*mdm38*$_{ProtA}$)	*MAT**a** ade2-101 his3-*Δ*200 leu2-*Δ*1 ura3-52 trp1-*Δ*63 lys2-801 mdm38::MDM38*$_{ProtA}$*-HIS3MX6*	Frazier *et al.* (2006)
DaMY14 (*rho*^{0}*mdm38*$_{ProtA}$)	*MAT**a** ade2-101 his3-*Δ*200 leu2-*Δ*1 ura3-52 trp1-*Δ*63 lys2-801 mdm38::MDM38*$_{ProtA}$*-HIS3MX6* [*rho*^{0}]	This study

wt, wild-type strains.

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 159-573) was cloned into the SalI and NotI sites of the pGEX-4T-3 vector (GE Healthcare). After expression in the *Escherichia coli* BL21(DE3) strain (Stratagene, La Jolla, CA), 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 $Pet309_{HA}$ fusion protein were lysed in lysis buffer containing 0.5 mM EDTA and 1% digitonin and subjected to coimmunoprecipitation using anti-hemagglutinin (HA; Roche, Mannheim, Germany) or anti-FLAG (Sigma, Munich, Germany) antibodies as a control. Antibodies and bound proteins were depleted from the lysate with protein G Sepharose, and beads were 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 30 s in a sonifying bath in 50 mM KCl, 10 mM $MgCl_2$, and 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}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 protein A 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 Δ*mba1* and Δ*oxa1* mutants, Δ*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 ($Oxa1^{1-331}$ or *oxa1*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 nonfermentable carbon sources (Figure 1A). In contrast, Δ*mba1*/Δ*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 Δ*mba1*/Δ*mdm38* double mutant was unable to grow on glycerol at all tested temperatures (Figure 1B). Because 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.

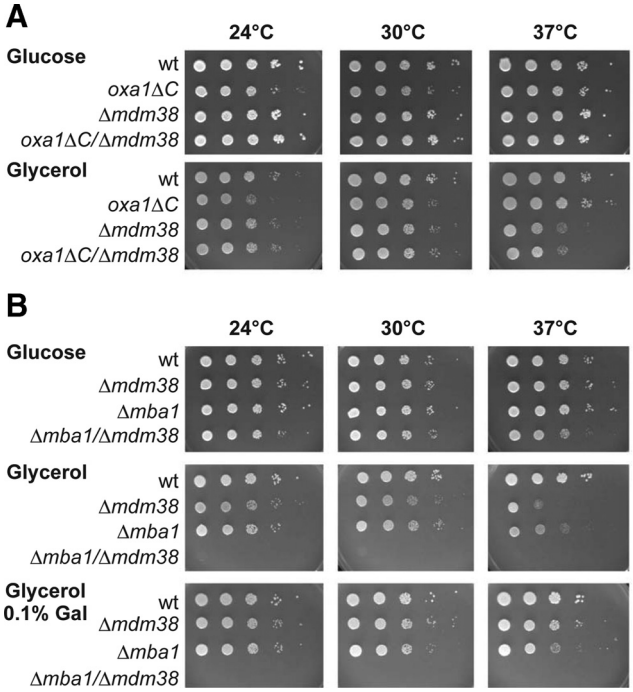

Figure 1. Yeast cells lacking Mba1 and Mdm38 show severe synthetic growth defects. (A and B) Wild-type (wt) and mutant yeast strains were grown to midlog phase, adjusted to an OD_{600} of 0.1, and 10-fold serial dilutions spotted onto plates containing 2% glucose, 2% glycerol, or 2% glycerol and 0.1% galactose (Gal). Plates were incubated for 2 (glucose) or 5 d at the indicated temperatures.

Δmdm38/Δmba1 Mutants Lack Complex III and IV of the Respiratory Chain

To identify the molecular basis for the synthetic growth defect of Δ*mba1*/Δ*mdm38* mutants, we isolated mitochondria from wild-type and mutant cells and measured cytochrome reductase (complex III) and cytochrome oxidase (complex IV) activity. Although Δ*mba1* and Δ*mdm38* mitochondria displayed reduced activities for both enzymes, Δ*mba1*/Δ*mdm38* mitochondria exhibited severe synthetic enzyme deficiencies. Only ~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 <10% did not lead to a full block of cell growth on nonfermentable medium (LaMarche *et al.,* 1992; Bauerschmitt *et al.,* 2008; Prestele *et al.,* 2009).

Defects in complexes III and IV were confirmed by Blue Native PAGE (BN-PAGE). Both complex III and complex IV were reduced in Δ*mba1* and Δ*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_oF_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 Δ*mba1* mitochondria and of Mba1 in Δ*mdm38* mitochondria possibly as a compensatory effect.

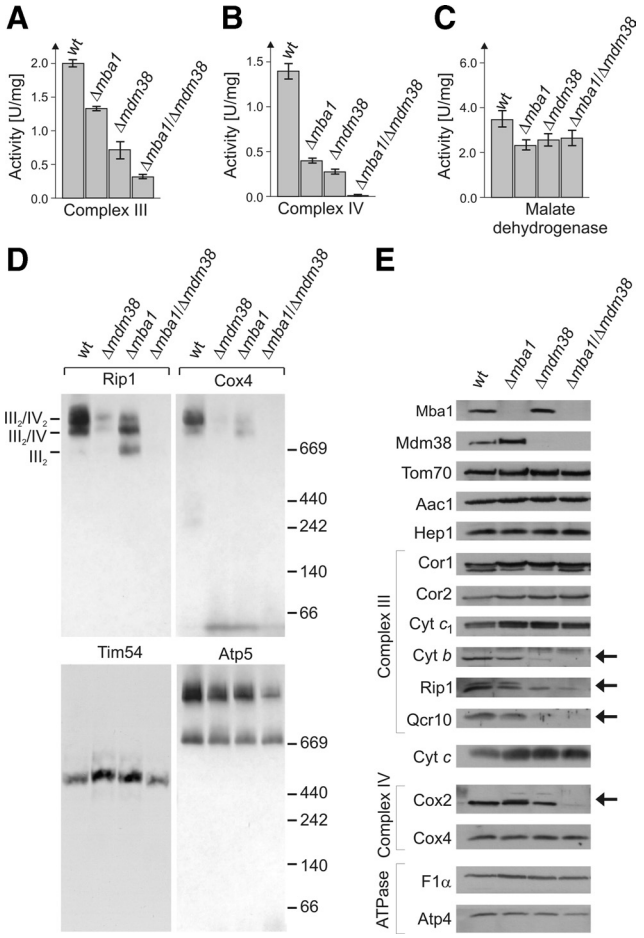

Figure 2. Δ*mba1*/Δ*mdm38* mitochondria show severe defects in complexes III and IV of the respiratory chain. (A–C). Complex III, complex IV, and malate dehydrogenase activities were measured in isolated mitochondria from the strains indicated. SEs were calculated from three independent experiments. (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 in kDa 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*; Cytc_1, cytochrome c_1; F1α, α subunit of the F_oF_1-ATPase. The arrows indicate proteins that show diminished levels in the double mutant.

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

Because 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 GST or GST were immobilized and incubated with mitochondrial extracts from wild-type or Δ*mba1* mitochondria. Both from wild-type and Δ*mba1* 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 Δ*mba1* 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.

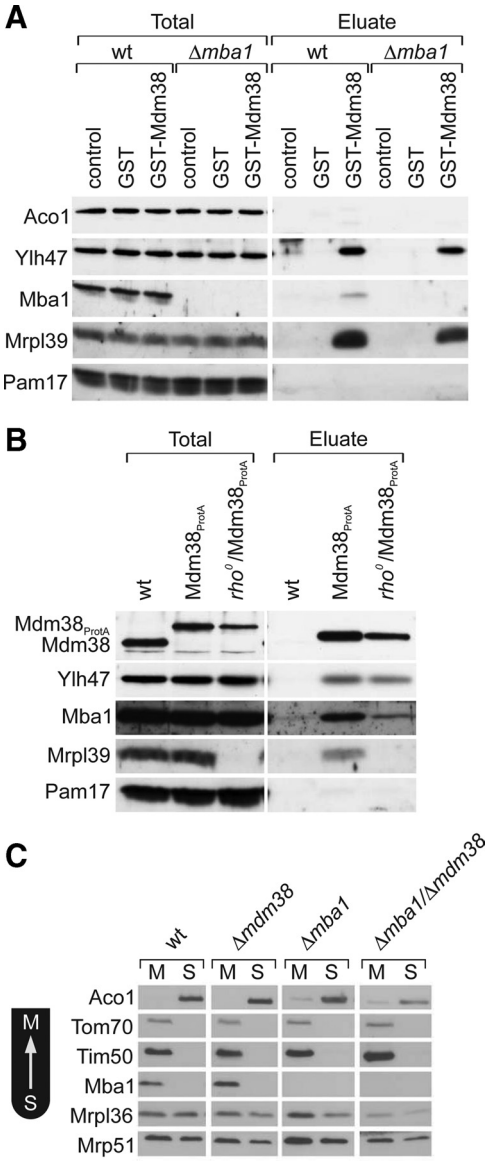

Figure 3. Mba1 and Mdm38 physically interact with each other and with mitochondrial ribosomes. (A) Wild-type (wt) and Δ*mba1* mitochondria were lysed and incubated with purified GST or GST-Mdm38 bound to glutathione Sepharose. After extensive washing, bound proteins were eluted and visualized by Western blotting using antibodies against the indicated proteins. Control lanes show an aliquot of the mitochondrial extracts. (B) Wild-type or $Mdm38_{ProtA}$-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. Four percent 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.

To test if Mba1 and Mdm38 were present in a common complex, we isolated $Mdm38_{ProtA}$ 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. Because the observed interaction of Mba1 with Mdm38 could be indirectly mediated through the mitochondrial ribosome, we analyzed if Mba1 could be purified together with $Mdm38_{ProtA}$ from ribosome-deficient *rho*^{0}-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 Δ*mba1* or Δ*mdm38* single or Δ*mba1*/Δ*mdm38* 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 Cyt b Synthesis

Because enzyme complexes with mitochondrially encoded subunits were specifically affected in the Δ*mba1*/Δ*mdm38* double mutant, but coupling of ribosomes to the inner membrane was not, we monitored the synthesis of translation products in mitochondria of the Δ*mba1* and Δ*mdm38* mutants. Surprisingly, the simultaneous deletion of *MBA1* and *MDM38* selectively prevented the synthesis of Cox1 and Cyt *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 Cyt *b* expression. However, upon loss of both proteins the complete loss of Cox1 and Cyt *b* expression becomes evident.

The inability of the double mutant to synthesize Cox1 and Cyt *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 *CYTB* transcripts undergo splicing. The Δ*mba1*/Δ*mdm38* double mutant showed severely reduced mRNA levels of *COX1* and *CYTB*, whereas other mRNAs were not affected or even increased (*COX3*). However, in the mutant mitochondria we detected higher molecular weight *COX1* and *CYTB* transcript species (data not shown), indicating that transcription occurred in the mutant mitochondria.

Because 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 *MBA1, MDM38*, or *MBA1/MDM38* by cytoduction. These mutant strains displayed similar growth defects on nonfermentable 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 in vivo or in organello, the previously observed defect in Cox1 synthesis

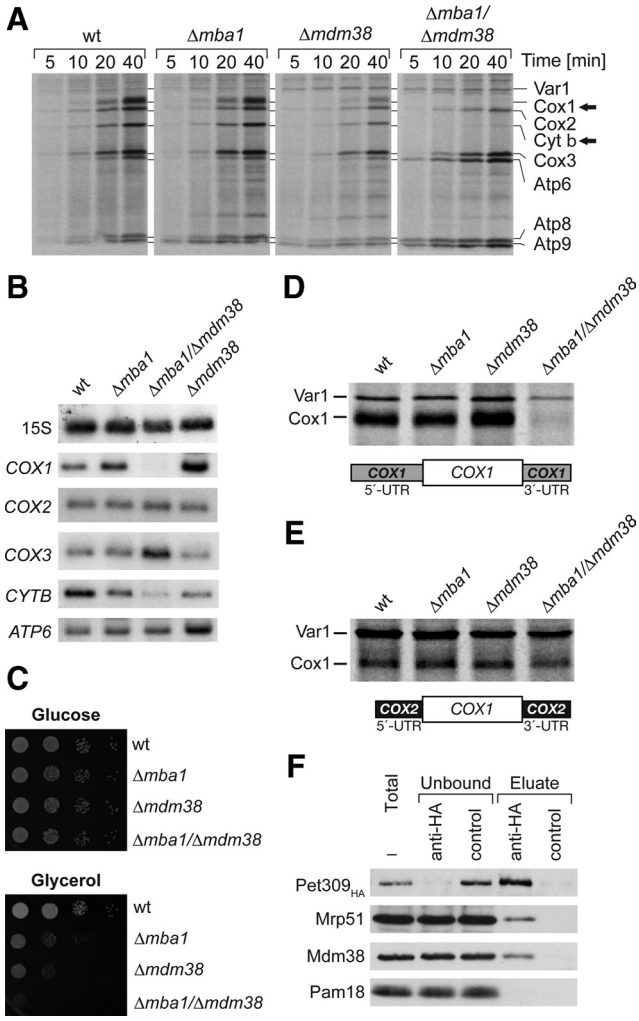

Figure 4. Δ*mba1*/Δ*mdm38* mitochondria synthesize severely reduced amounts of Cox1 and Cyt *b*. (A) Mitochondria isolated from the strains indicated were incubated in [^{35}S]methionine-containing translation buffer for the times indicated. Mitochondria were reisolated, washed, and subjected to SDS-PAGE and autoradiography. Arrows depict Cox1 and Cyt *b*. (B) Δ*mba1*/Δ*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 midlog phase, and 10-fold serial dilutions were 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 $Pet309_{HA}$ were solubilized in digitonin buffer and subjected to immunoprecipitation with anti-HA or anti-FLAG antibodies (control). Bound protein was analyzed by Western blotting. Five percent of total and unbound samples and 100% of the eluates were loaded on the gels.

was not cured. We therefore conclude that the translational defects observed in Δ*mba1*/Δ*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 (Michaelis *et al.*, 1991; Fox, 1996). To assess a func-

tion of Mba1 and Mdm38 in translational regulation, we tested for Cox1 expression in cells in which the reading frame of *COX1* was flanked by 5′- and 3′-UTRs of *COX2*. Yeast cells were grown in the presence of cycloheximide to inhibit cytoplasmic translation, and mitochondrial translation products were labeled with [^{35}S]methionine. From the wild-type mitochondrial genome, Cox1 was not expressed in Δ*mba1*/Δ*mdm38* mutant cells (Figure 4D). In contrast, Cox1 synthesis was independent of Mba1 and Mdm38 when the *COX1* reading frame was flanked by the UTRs of *COX2* (Figure 4E). Thus, by changing the *COX1*-specific UTR into a *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 with the wild-type control ~105% of Cox1 was synthesized in Δ*mba1*/Δ*mdm38* mutant cells. We therefore conclude that Mba1 and Mdm38 are specifically involved in translational regulation of Cox1 and Cyt *b*.

Translation of the *COX1* mRNA is under control of the translational regulator Pet309, which specifically interacts with the 5′UTR of the *COX1* transcript (Manthey and McEwen, 1995). Similar to Mdm38 and Mba1, Pet309 is associated with the mitochondrial inner membrane (Manthey *et al.*, 1998) and is found in large protein complexes (Naithani *et al.*, 2003; Krause *et al.*, 2004). 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 *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.

The Δmba1/Δmdm38 Growth Defect Cannot Be Rescued by Nigericin Treatment

Mdm38 was proposed to be critical for mitochondrial potassium homeostasis because the growth of Δ*mdm38* mutant cells on glycerol-containing media is improved by nigericin, a K^+/H^+ antiporter (Nowikovsky *et al.*, 2007). We therefore analyzed if the growth defect of Δ*mba1*/Δ*mdm38* mutants could be similarly rescued by nigericin (Figure 5A). Although the growth defect of Δ*mdm38* cells on nonfermentable medium could be compensated by nigericin as reported, the growth defect of the Δ*mba1*/Δ*mdm38* 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 Δ*mdm38* mutant mitochondria but aggravated in Δ*mba1*/Δ*mdm38* double mutant cells, cannot be suppressed by nigericin and thus is unrelated to a role of Mdm38 in K^+/H^+ homeostasis.

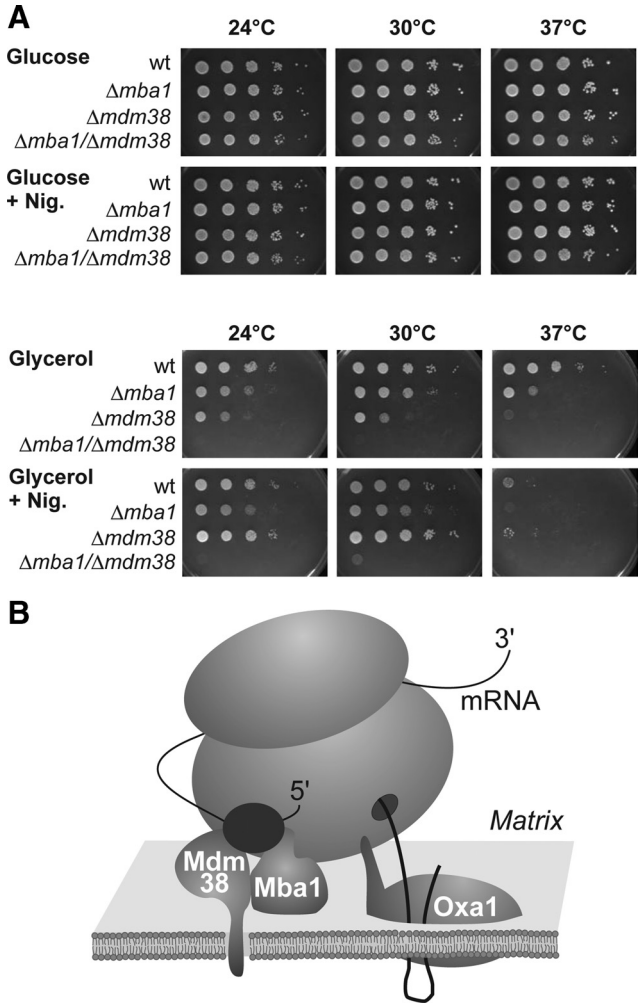

Figure 5. The Δ*mba1*/Δ*mdm38* growth defect is independent of a role of Mdm38 in the maintenance of mitochondrial K^+/H^+ homeostasis. (A) Growth of the indicated strains was analyzed as described for Figure 1A. Plates labeled +Nig contained 2 μM nigericin. (B) Hypothetic model for the role of Mdm38 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.

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 nonfermentable carbon sources. The levels of *COX1* and *CYTB* 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 because only reduced amounts of Mba1 were found in association with Mdm38 in *rho*^{0} 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 and Pet309 as well as several nonidentified subunits (Krause *et al.*, 2004).

Cbp1 is a factor that is specifically required for the translation and stability of the mRNA of *CYTB* (Dieckmann *et al.*, 1984). Pet309 plays a comparable role for *COX1* transcripts (Manthey and McEwen, 1995). Interestingly, Cbp1 and Pet309 were identified together in a genetic screen for components critical for the cotranslational translocation of protein domains across the inner membrane (Saracco and Fox, 2002). Here we show that Pet309, the translational activator for *COX1* mRNA, is present in a complex with Mdm38 as well as with mitochondrial ribosomes in agreement with a function of Mdm38 in mitochondrial translation.

Although our results demonstrate a role of Mdm38 and Mba1 in translation, they do not rule out a role of Mdm38 in K^+ homeostasis. Because 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 RNA interference 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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