Mdm38 interacts with ribosomes and is a component of the mitochondrial protein export machinery

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Saccharomyces cerevisiae Mdm38 and Ylh47 are homologues of human Letm1, a protein implicated in Wolf-Hirschhorn syndrome. We analyzed the function of Mdm38 and Ylh47 in yeast mitochondria to gain insight into the role of Letm1. We find that *mdm38Δ* mitochondria have reduced amounts of certain mitochondrially encoded proteins and low levels of complex III and IV and accumulate unassembled Atp6 of complex V of the respiratory chain. Mdm38 is especially required for efficient transport of Atp6 and cytochrome *b* across the inner membrane, whereas Ylh47 plays a minor role in this process. Both Mdm38 and Ylh47 form stable complexes with mitochondrial ribosomes, similar to what has been reported for Oxa1, a central component of the mitochondrial export machinery. Our results indicate that Mdm38 functions as a component of an Oxa1-independent insertion machinery in the inner membrane and that Mdm38 plays a critical role in the biogenesis of the respiratory chain by coupling ribosome function to protein transport across the inner membrane.

Introduction

Mitochondria are double membrane–bound organelles nearly ubiquitous in eukaryotic cells. One important function of mitochondria is the production of ATP through oxidative phosphorylation, and defects in this process have been found to lead to several severe human diseases (DiMauro and Schon, 2003; Zeviani and Carelli, 2003). Over 98% of mitochondrial proteins are encoded in the nucleus (Sickmann et al., 2003; Taylor et al., 2003) and transported posttranslationally into the organelle. Such proteins use the TOM (translocase of outer mitochondrial membrane) and TIM (translocase of inner mitochondrial membrane) translocation machineries in the outer and inner mitochondrial membranes for their transport into mitochondria (Neupert, 1997; Jensen and Dunn, 2002; Koehler, 2004; Rehling et al., 2004). The mitochondrial genome typically encodes a small number of proteins, most of which are polytopic membrane proteins of the respiratory chain complexes. In the yeast *Saccharomyces cerevisiae*, these are cytochrome *b* of complex III; the subunits Cox1, -2, and -3 of complex IV; and the subunits Atp6, -8, and -9 of complex V. Although much is known about how proteins are targeted to and imported into mitochondria, little is known about how mitochondrially encoded proteins are transported and assembled into the inner membrane. Only a limited number of proteins that are involved in this process have been identified, including Oxa1, Cox18/Oxa2, Pnt1, Mss2, and Mba1 (Hell et al., 1997, 1998, 2001; He and Fox, 1999; Broadley et al., 2001; Preuss et al., 2001; Nargang et al., 2002; Saracco and Fox, 2002; Stuart, 2002; Funes et al., 2004).

Oxa1 is a member of the Oxa1/YidC/Alb3 family of proteins (Stuart, 2002; Kuhn et al., 2003) and is required for growth of *S. cerevisiae* on nonfermentable carbon sources. Inactivation of Oxa1 leads to defects in the insertion of mitochondrially encoded proteins into the inner membrane. Oxa1 interacts both with newly synthesized mitochondrial proteins (Hell et al., 2001) and mitochondrial ribosomes (Jia et al., 2003; Szyrach et al., 2003). It has been suggested that Oxa1 links mitochondrial translation to membrane insertion of the newly translated proteins (Jia et al., 2003; Szyrach et al., 2003). However, it has been shown that preproteins differ in their dependency on Oxa1.

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Abbreviations used in this paper: BN, blue native; $\Delta\psi$, membrane potential; *LETM1*, *Leucine zipper–EF-hand–containing transmembrane 1*; TIM, translocase of inner mitochondrial membrane; WHS, Wolf-Hirschhorn syndrome.

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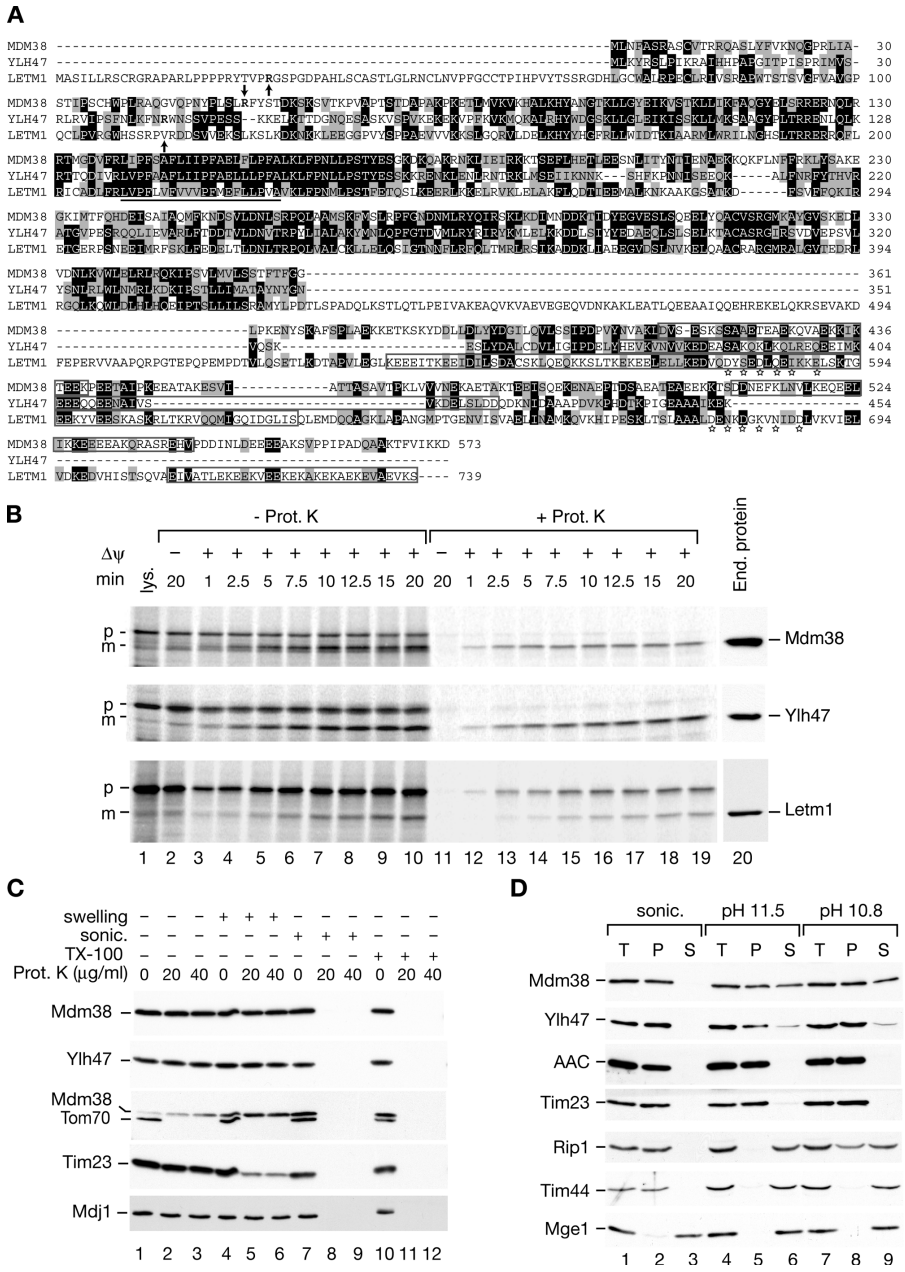

Figure 1. **Mdm38 and Ylh47 are mitochondrial inner membrane proteins.** (A) Alignment of Mdm38, Ylh47, and human Letm1. Black boxes indicate identical amino acids, and gray boxes indicate similar amino acids. Underlining represents the predicted transmembrane domain, gray outlines represent predicted coiled coils, asterisks represent critical residues for the EF-hand Ca^{2+} binding motif, and arrows represent the predicted cleavage site for mitochondrial processing peptidase. (B) ^{35}S-labeled Mdm38, Ylh47, and Letm1 were imported into isolated mitochondria in the presence or absence of $\Delta\psi$, treated with 50 μg/ml proteinase K (Prot. K) where indicated, and analyzed by SDS-PAGE and digital autoradiography. For comparison, see precursor proteins (lys.). (top and middle) Western blot of yeast mitochondria; (bottom) human HeLa cell extracts (End. protein). (C) Mitochondria were swollen, sonicated (sonic.), or solubilized in 1% Triton X-100 (TX-100); treated with proteinase K where indicated; and subjected to Western blotting. (D) Mitochondria were sonicated or treated with 0.1 M Na_2CO_3 and then left untreated (T) or separated into supernatant (S) and pellet (P) and analyzed by Western blotting.

Specifically, Cox2 and -1 strictly require Oxa1 for membrane insertion, but cytochrome *b* and Atp6 show little Oxa1 dependency for transport across the inner membrane (Hell et al., 2001). The membrane insertion pathway for these Oxa1-independent proteins is currently unclear. Mba1 is an additional component of the mitochondrial export machinery that shares substrate specificity with Oxa1 but can either cooperate with or function independently of Oxa1 (Preuss et al., 2001). Finally, Cox18 (Oxa2 in *Neurospora crassa*) is distantly related to Oxa1 and has been shown to be required for the transport of the COOH-terminal region of the Cox2 protein across the inner membrane, together with Pnt1 and Mss2 (He and Fox, 1999; Broadley et al., 2001; Saracco and Fox, 2002; Stuart, 2002; Funes et al., 2004). Thus, given the substrate specificity of the known constituents of the export machinery, it has been proposed that alternative transport pathways must exist to handle Oxa1-independent proteins, particularly cytochrome *b* and Atp6 (Stuart, 2002).

Leucine zipper–EF-hand–containing transmembrane 1 (*LETM1*) was identified as a gene associated with Wolf-Hirschhorn syndrome (WHS; Endele et al., 1999). This disorder affects 1 in 50,000 live births and results in pre- and postnatal growth retardation, severe mental retardation, and developmental delay with microcephaly. In addition, this disease is associated with an impairment of muscular tone and seizures. WHS is caused by partial deletion of chromosome 4 at locus 4p16.3. This region encompasses multiple genes, among them *LETM1*; thus, direct genotype–phenotype correlations are difficult to determine (Zollino et al., 2003). *LETM1* deletions occur in almost all patients with WHS (Endele et al., 1999), and those patients

with mild forms of the disease, defined as the absence of microcephaly, seizures, and severe mental retardation, lack a deletion of this gene (Rauch et al., 2001; Zollino et al., 2003). Letm1 is predicted to contain two EF-hand Ca^{2+} binding domains, a transmembrane domain, a leucine zipper, and coiled-coil domains (Endele et al., 1999). Two orthologues of Letm1 exist in *S. cerevisiae*, Mdm38 (Dimmer et al., 2002) and Ypr125w (Nowikovsky et al., 2004; Schlickum et al., 2004), which we have named *YLH47* for yeast LETM1 homologue of 47 kD. Letm1, Mdm38, and Ylh47 are mitochondrial proteins, and Mdm38 has been reported to localize to the inner membrane (Sickmann et al., 2003; Nowikovsky et al., 2004; Schlickum et al., 2004). However, it is unclear how these proteins are transported into mitochondria and where in the mitochondria Ylh47 is localized. *mdm38Δ* mutant mitochondria have been reported to display various pleiotropic defects such as altered mitochondrial morphology (Dimmer et al., 2002) and defects in K^+ homeostasis (Nowikovsky et al., 2004). Yet, the specific function of Mdm38 and Ylh47 and how their function correlates to these phenotypes is unclear. Because some of the phenotypes seen in WHS patients and *mdm38Δ* are reminiscent of mitochondrial disorders (DiMauro and Schon, 2003; Zeviani and Spinazzola, 2003), we wanted to investigate the role of Mdm38 and Ylh47 in mitochondrial function.

We report that Mdm38, Ylh47, and human Letm1 are transported across the inner mitochondrial membrane and processed to a mature form in a membrane potential ($\Delta\psi$)–dependent manner. *mdm38Δ* mitochondria exhibit a severe reduction in the amounts of a subset of mitochondrially encoded proteins. Export of cytochrome *b* and Atp6 from the matrix across the inner membrane is especially affected in *mdm38Δ* mitochondria. In agreement with this defect, *mdm38Δ* mitochondria have reduced amounts of respiratory complexes III and IV and accumulate unassembled Atp6 of complex V. Moreover, both Mdm38 and Ylh47 interact with mitochondrial ribosomes. Our results indicate that Mdm38 acts as a component of the mitochondrial export machinery in an Oxa1-independent pathway and is particularly required for biogenesis of cytochrome *b* and Atp6.

Results

Letm1, Mdm38, and Ylh47 are transported across the inner mitochondrial membrane

The yeast proteins Mdm38 (Yol027c) and Ylh47 (Ypr125w) display significant sequence similarity to the human Letm1 protein (Fig. 1 A), which has been implicated in WHS. A high degree of sequence similarity is observed in the NH_2-terminal portion of the proteins, which includes a predicted single transmembrane helix that is rich in proline residues and contains a conserved glutamate residue, uncommon for transmembrane segments (Fig. 1 A, underlining). The COOH-terminal portions of the proteins contain predicted coiled-coil motifs and, in the case of Letm1, two predicted EF-hand Ca^{2+} binding motifs (Fig. 1 A). However, this region, especially the critical amino acid residues required for Ca^{2+} binding (Heizmann and Hunziker, 1991), is not conserved among the three proteins. Mitochondrial targeting signals (presequences) and potential cleavage sites for the matrix processing peptidase were identified in all three proteins (MitoProt II; Fig. 1 A). Presequences direct proteins across outer and inner mitochondrial membranes into the matrix or inner mitochondrial membrane via the TIM23 complex (Neupert, 1997; Jensen and Dunn, 2002; Koehler, 2004; Rehling et al., 2004). To assess whether Mdm38, Ylh47, and Letm1 possess targeting information for transport across the inner membrane, we synthesized the precursor proteins in rabbit reticulocyte lysate in the presence of [^{35}S]methionine/cysteine and imported them into isolated yeast mitochondria. Mdm38, Ylh47, and the human Letm1 were efficiently imported and processed to a protease-protected mature form in a $\Delta\psi$-dependent manner (Fig. 1 B). The imported mature forms of the proteins migrated similarly to the authentic proteins in yeast and human (Fig. 1 B).

Protein transport across the inner membrane requires $\Delta\psi$, suggesting that Mdm38, Ylh47, and Letm1 were localized to the mitochondrial matrix or inner membrane. To address this, we treated with proteinase K both intact mitochondria and osmotically swollen mitochondria with a disrupted outer membrane. Both Ylh47 and Mdm38 remained stable under these conditions, and no stable cleavage products of these proteins were observed (Fig. 1 C and not depicted). When mitochondrial membranes were disrupted by sonication or Triton X-100 treatment, both proteins became accessible to protease (Fig. 1 C). Thus, Mdm38 and Ylh47 are inner membrane proteins exposed to the mitochondrial matrix, which is in agreement with previous observations for the epitope-tagged Mdm38 (Nowikovsky et al., 2004).

Nowikovsky et al. (2004) showed that tagged Mdm38 was an inner membrane protein. To determine whether Ylh47 behaved in a similar way or represented a soluble protein of the matrix, mitochondria were subfractionated. After sonication, soluble proteins such as Mge1 were released into the supernatant, in contrast to Mdm38 and Ylh47, which remained associated with the membranes in the pellet fraction (Fig. 1 D, lanes 2 and 3). Carbonate treatment at both pH 11.5 and 10.8 led to a partial release of Mdm38 and Ylh47 from the membranes, whereas the peripheral membrane protein Tim44 was completely extractable (Fig. 1 D). Similar to Mdm38 and Ylh47, the single membrane–spanning Rieske Fe/S-protein (Rip1) of complex III of the respiratory chain remained partially resistant to alkaline extraction at pH 10.8, although it was fully extracted at pH 11.5. This result is explained by the fact that the transmembrane helix of the protein has been shown not to be completely embedded in the lipid environment of the inner membrane but rather located in close proximity to other proteins (Lange and Hunte, 2002), thus making it more sensitive to alkaline extraction than conventional membrane-spanning proteins. Based on these results, we conclude that Ylh47 and Mdm38 are proteins that may span the inner membrane in a complex with other proteins, as in the case of Rip1, or may not fully penetrate the inner membrane.

Mdm38 is required for respiratory chain biogenesis

In the course of analysis of the wild-type, *mdm38Δ*, *ylh47Δ*, and *ylh47Δ/mdm38Δ* strains, we determined that Mdm38 was required for efficient growth on nonfermentable carbon sources

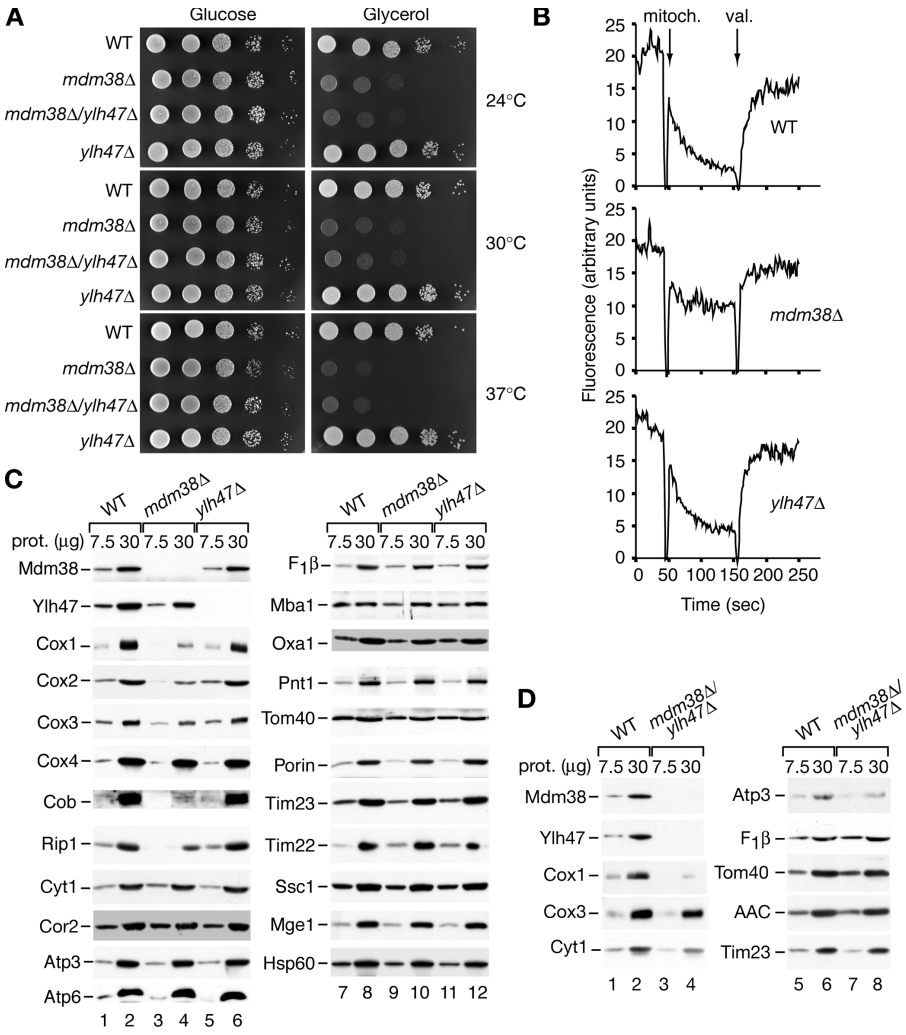

Figure 2. **Mdm38 but not Ylh47 is required for respiratory growth.** (A) Cells were cultured on YP glycerol medium to avoid the loss of mitochondrial DNA, washed, subjected to serial 10-fold dilutions, plated on YPD or YPG medium, and incubated at the indicated temperatures. (B) $\Delta\psi$ measurements by fluorescence quenching. (C) Mitochondrial proteins were subjected to SDS-PAGE and Western blotting. (D) Steady-state protein levels were analyzed as in C. Mitoch., mitochondria; prot., protein; val., valinomycin; WT, wild type.

(Fig. 2 A, right). Although *mdm38Δ* cells display slow growth on medium containing glycerol, *ylh47Δ* cells showed no significant growth defect under the same growth conditions (Fig. 2 A, right). In contrast to previous work by Nowikovsky et al. (2004), we did not observe a temperature-dependent growth defect for *mdm38Δ* cells. Surprisingly, the *ylh47Δ*/*mdm38Δ* cells displayed a growth phenotype that was similar to that of *mdm38Δ* at all temperatures. The high degree of homology between Mdm38 and Ylh47 makes it likely that these proteins perform similar functions in mitochondria. Yet, in light of our results, it seems that Mdm38 is more important for mitochondrial biogenesis than Ylh47, as Ylh47 is unable to compensate for the loss of Mdm38.

The growth defect of *mdm38Δ* cells on nonfermentable carbon sources suggested that Mdm38 was required for respiration. To support this, we assessed the $\Delta\psi$ in wild-type, *ylh47Δ*, and *mdm38Δ* mitochondria by fluorescence quenching. Although *ylh47Δ* mitochondria showed only a marginal reduction of the $\Delta\psi$ when compared with wild-type mitochondria (Fig. 2 B, top and bottom), mitochondria from *mdm38Δ* displayed a significant $\Delta\psi$ reduction (Fig. 2 B, middle).

One explanation for the observed $\Delta\psi$ defect was that the respiratory chain of *mdm38Δ* mitochondria was functionally compromised. To determine whether this was the case, we compared the steady-state protein levels of various mitochondrial proteins from wild-type, *ylh47Δ*, and *mdm38Δ* mitochondria. Most of the proteins tested, such as components of the outer and inner membrane protein translocases (Tom40, Tim23, and Tim22), complex V ($F_1\beta$, Atp3, and Atp6), porin, matrix chaperones (Ssc1, Mge1, and Hsp60), and the mitochondrial export machinery (Oxa1, Pnt1, and Mba1), were present in similar amounts in all three strains. However, *mdm38Δ* mitochondria showed a significant reduction in the steady-state levels of the mitochondrially encoded proteins Cox1, Cox2, and cytochrome *b* (Cob), the nuclear encoded Rieske Fe/S-protein (Rip1) of complex III, and a slight reduction in Cox3 levels (Fig. 2 C, lanes 3 and 4). In contrast, the steady-state protein levels in *ylh47Δ* mitochondria were similar to those of wild-type in all cases. Although growth of *ylh47Δ*/*mdm38Δ* cells was similar to *mdm38Δ* cells (Fig. 2 A), analysis of the steady-state protein levels of mitochondria from wild-type and *ylh47Δ*/*mdm38Δ* mitochondria (Fig. 2 D) showed that the *ylh47Δ*/*mdm38Δ* mitochondria had additional reductions in the amounts of both Atp3 and cytochrome c_1 (Cyt1).

Next, we solubilized mitochondria in digitonin buffer and separated respiratory chain protein complexes on blue native (BN) PAGE. Complexes III and IV form supercomplexes consisting of

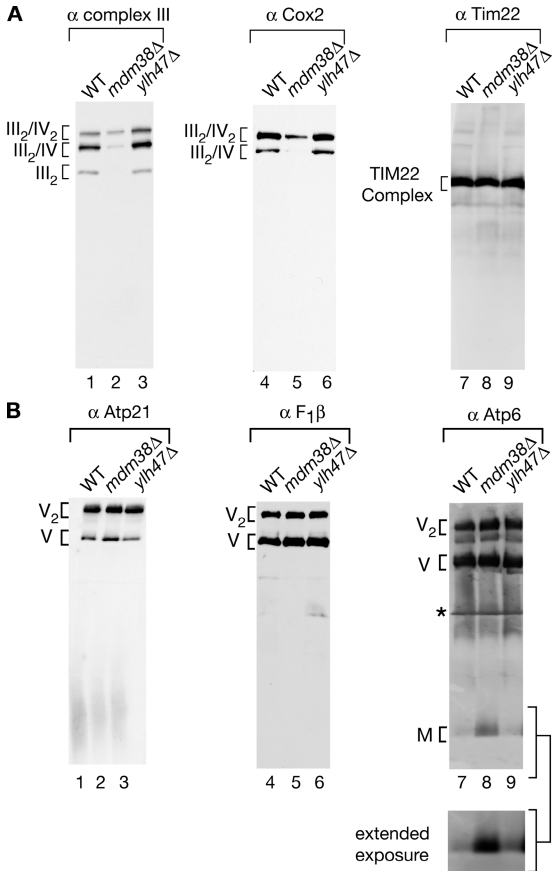

Figure 3. **Mdm38 is required for respiratory chain complex biogenesis.** (A) Mitochondria were solubilized in 1% digitonin buffer, protein complexes were separated by BN-PAGE, and respiratory chain complexes were visualized by Western blot analysis. (B) After solubilization and BN-PAGE separation as in A, the F_1F_o–ATPase complexes were analyzed by Western blot analysis. The asterisk indicates an unspecific protein band. M, monomer; WT, wild type.

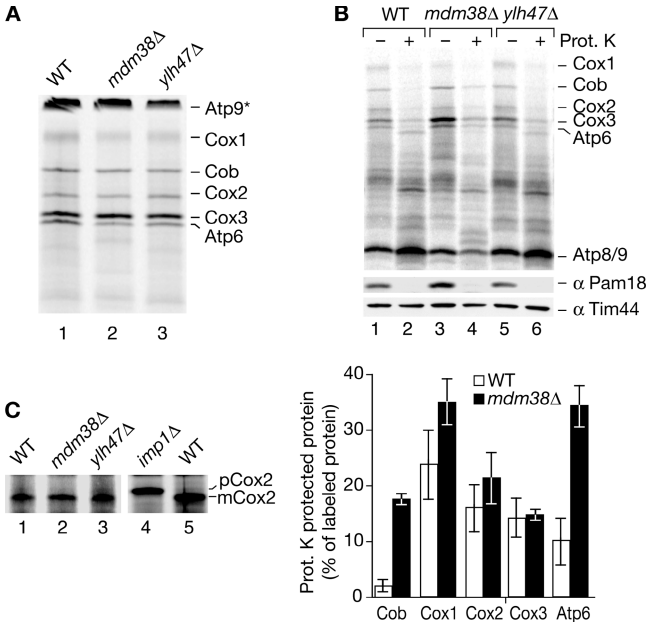

Figure 4. ***mdm38Δ* mitochondria display defects in protein export.** (A) In organello translation in the presence of [^{35}S]methionine/cysteine (30 min) was performed in isolated wild-type (WT), *mdm38Δ*, and *ylh47Δ* mitochondria and unlabeled methionine was added and incubation continued for 5 min. Mitochondria were reisolated and subjected to SDS-PAGE and digital autoradiography. Atp9* indicates an SDS-resistant form of Atp9 seen in samples not TCA precipitated (Westermann et al., 2001). (B, top) In organello translation was performed for 15 min as in A followed by a 15-min chase. Mitochondria were subjected to osmotic shock, and samples were split and mock-treated or treated with 10 μg/ml proteinase K (Prot. K) for 15 min on ice. After reisolation and TCA precipitation, samples were subjected to SDS-PAGE, Western blotting, or digital autoradiography. (bottom) Quantification of the protease-inaccessible amount of newly synthesized mitochondrial proteins as a percentage of the synthesized protein. SEM was calculated from three independent experiments. (C) In organello translations were performed as described in A and analyzed by SDS-PAGE and digital autoradiography. p, precursor; m, mature.

a dimer of complex III together with one or two complex IV monomers (III_2/IV or III_2/IV_2). We found that the levels of complexes III and IV were significantly decreased in *mdm38Δ* mitochondria (Fig. 3 A), whereas other inner membrane complexes, such as the TIM22 complex, were not affected (Fig. 3 A). In wild-type, *mdm38Δ*, and *ylh47Δ* mitochondria, the monomeric and dimeric forms of the F_1F_o–ATPase (complex V) were similar, as judged by decoration with antibodies against $F_1\beta$, Atp21, and Atp6 (Fig. 3 B). However, although Atp6 was mainly present in the monomeric and dimeric forms of the complexes in wild-type and *ylh47Δ* mitochondria, a low–molecular weight form of the protein selectively accumulated in *mdm38Δ* mitochondria (Fig. 3 B, lane 8). Because the steady-state levels of Atp6 were not reduced in the mutant (Fig. 2 C), this finding suggests that a stable pool of Atp6 accumulates in an unassembled form in the *mdm38Δ* mitochondria. We conclude that Mdm38 is required for the biogenesis of respiratory chain complexes.

mdm38Δ mutant mitochondria are affected in cytochrome *b* and Atp6 export

In yeast, complexes III, IV, and V of the respiratory chain contain seven mitochondrially encoded subunits: cytochrome *b* (complex III); Cox1, -2, and -3 (complex IV); and Atp6, -8, and -9 (complex V). Because Cox1, Cox2, Cox3, and cytochrome *b* were reduced in *mdm38Δ* mitochondria, we wanted to determine whether translation of these proteins was affected in the mutant strain. To address this, we incubated mitochondria in the presence of [^{35}S]methionine/cysteine under conditions that promote mitochondrial translation. After labeling, a chase was performed by adding excess unlabeled methionine to allow translation to go on to completion. *mdm38Δ* and *ylh47Δ* did not display a significant difference with regard to the amounts of mitochondrial translation products compared with wild type (Fig. 4 A).

An alternative explanation for the decreased steady-state protein levels in *mdm38Δ* mitochondria was that posttranslational steps in the biogenesis of mitochondrially encoded proteins involved Mdm38. We therefore tested to determine whether the mitochondrial translation products were correctly transported across the inner mitochondrial membrane. After in organello translation, wild-type and mutant mitochondria were incubated in hypotonic buffer to disrupt the outer mitochondrial membrane, generating mitoplasts. If transport across the inner membrane occurs correctly, newly synthesized proteins

are accessible to protease in mitoplasts (He and Fox, 1997; Hell et al., 2001; Preuss et al., 2001). In *mdm38Δ* mitoplasts, the membrane insertion of newly synthesized cytochrome *b* and Atp6 were affected, as these proteins remained significantly more resistant to protease treatment than in wild-type mitochondria (Fig. 4 B). In contrast to this, the *ylh47Δ* mitoplasts only displayed a small increase in the protease resistance of cytochrome *b* and Atp6 (Fig. 4 B, lanes 5 and 6). Other proteins, such as Cox1, -2, and -3 showed little or no increase in protease resistance in *mdm38Δ* or *ylh47Δ* mitochondria. To exclude the possibility that the observed protease resistance in *mdm38Δ* mitoplasts resulted from the inefficient swelling of mitochondria, we analyzed the protease accessibility of marker proteins. The intermembrane space domain of the inner membrane protein Pam18 was efficiently degraded in all strains upon protease treatment. In contrast, the matrix protein Tim44 remained protected from protease by the inner membrane in all cases (Fig. 4 B). One concern was that the reduced $\Delta\psi$ in *mdm38Δ* mitochondria could have indirectly caused the observed export defects. To address this, we first analyzed the import of proteins into wild-type and *mdm38Δ* mitochondria. Import of matrix proteins such as $F_1\beta$ or the precursor b_2(167)-DHFR (NH_2-terminal portion of cytochrome b_2 fused to dihydrofolate reductase), which possesses an inner membrane sorting signal, was similar between wild-type and mutant mitochondria (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200505060/DC1). Thus, the reduced $\Delta\psi$ in *mdm38Δ* mitochondria was sufficient for import of proteins into mitochondria, yet it did not rule out the possibility that $\Delta\psi$ was insufficient to promote protein export. Therefore, we isolated mitochondria from *cox4Δ* cells. These cells exhibit a severe reduction of $\Delta\psi$ because of loss of complex IV (Fig. S2, A and B). When *cox4Δ* mitoplasts were treated with proteinase K after in organello translation, the accessibility of newly synthesized proteins was not decreased, as compared with wild type (Fig. S2 C). This is in contrast to *mdm38Δ* mitochondria, for which an increased protease resistance for cytochrome *b* and Atp6 was observed (Fig. 4 B). Nowikovsky et al. (2004) reported that addition of the K^+/H^+ exchanger nigericin restored the $\Delta\psi$ of *mdm38Δ* mitochondria. Therefore, we added nigericin to *mdm38Δ* mitoplast and analyzed the protease accessibility of cytochrome *b* and Atp6. However, the phenotype of increased resistance of these proteins to proteinase K was not suppressed (unpublished data). Thus, we conclude that the export defects observed in *mdm38Δ* mitochondria were not due to a reduction of the $\Delta\psi$.

The NH_2 terminus of Cox2 is transported across the inner membrane into the intermembrane space and then processed by Imp1 of the inner membrane peptidase complex. When Oxa1 is defective, Cox2 accumulates as an unprocessed precursor (He and Fox, 1997; Hell et al., 2001; Preuss et al., 2001). Cox2 processing by the Imp1 protease was unaffected in *mdm38Δ* and *ylh47Δ* mitochondria, indicating that export of the NH_2 terminus of Cox2 occurred despite the lack of Mdm38 or Ylh47 (Fig. 4 C) and that Oxa1 function was not compromised. Thus, we conclude that efficient transport of cytochrome *b* and Atp6 across the inner membrane requires Mdm38, whereas the export of Cox1 and -2 was only mildly affected in *mdm38Δ* mitochondria (Fig. 4 B, bottom). Moreover, we observed a similar but much weaker effect for Ylh47.

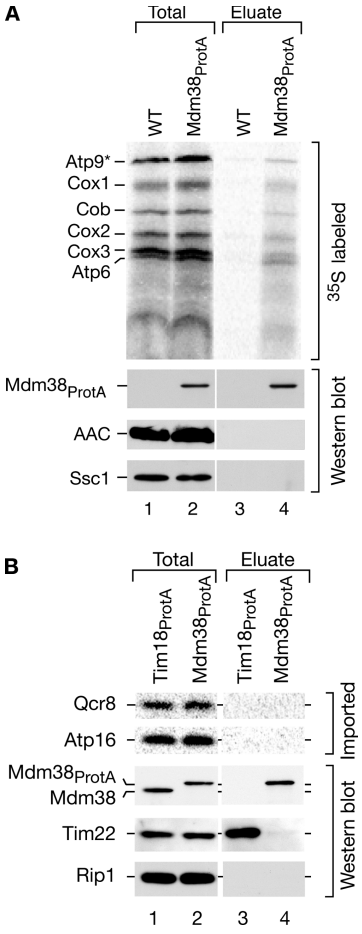

Figure 5. **Mdm38 interacts with mitochondrially encoded proteins.** (A) In organello translations were performed with wild-type (WT) and Mdm38 Protein A–tagged mitochondria ($Mdm38_{ProtA}$). After translation, mitochondria were lysed in digitonin buffer and subjected to IgG chromatography. Samples were eluted and subjected to SDS-PAGE and digital autoradiography or Western blotting. 10% of total and 100% of eluate were loaded. Atp9* indicates SDS-resistant Atp9. (B) ^{35}S-labeled Qcr8 and Atp16 were imported into $Tim18_{ProtA}$ and $Mdm38_{ProtA}$ mitochondria. Mitochondria were treated with proteinase K, solubilized in digitonin buffer, and subjected to IgG chromatography. Bound proteins were analyzed as in A. 10% of total and 100% of eluate were loaded. White lines indicate that intervening lanes have been spliced out.

Mdm38 interacts with newly synthesized mitochondrial proteins

We next addressed whether newly synthesized mitochondrially encoded proteins interact with Mdm38. Mitochondrial translation products were labeled as described in the previous section in wild-type mitochondria and mitochondria containing Mdm38 with a COOH-terminal Protein A tag. Mitochondria were then solubilized in digitonin-containing buffer, and $Mdm38_{ProtA}$, together with associated proteins, was isolated (Geissler et al., 2002; Rehling et al., 2003). As a control for specificity of the purification procedure, Western blots were performed and decorated for $Mdm38_{ProtA}$, the abundant inner membrane protein

AAC, and Ssc1 (matrix Hsp70). As expected, neither AAC nor Ssc1 were purified with $Mdm38_{ProtA}$ (Fig. 5 A). In contrast to the mock-purification where no radiolabeled proteins were purified (Fig. 5 A), we found that Cox1, Cox2, Cox3, cytochrome *b*, Atp6, and Atp9 were all associated with $Mdm38_{ProtA}$ (Fig. 5 A).

To determine whether the interaction between Mdm38 and newly synthesized proteins reflected a possible chaperone-like activity for Mdm38, Qcr8 and Atp16, subunits of complex III and V, respectively, were imported into mitochondria containing $Mdm38_{ProtA}$ or $Tim18_{ProtA}$ of the TIM22 complex (Rehling et al., 2003). After removal of unimported precursor proteins by protease treatment, mitochondria were lysed and Protein A–tagged proteins were isolated. None of the imported proteins was found to be associated with Mdm38 or the TIM22 complex (Fig. 5 B).

In summary, our analyses indicate that Mdm38 interacts specifically with newly synthesized mitochondrially encoded proteins and points to a role of Mdm38 in the transport of a subset of these proteins across the inner membrane.

Mdm38 and Ylh47 associate with mitochondrial ribosomes

There are several reasons why Mdm38 could copurify with newly synthesized proteins. One of these possibilities is that Mdm38 could be directly associated with respiratory chain complexes. To test this, mitochondria expressing $Mdm38_{ProtA}$ or $Ylh47_{ProtA}$ were solubilized in digitonin buffer and subjected to IgG chromatography. Bound proteins were eluted, separated by SDS-PAGE, and subjected to Western blot analyses. Both Mdm38 and Ylh47 efficiently bound to and eluted from the IgG-Sepharose (Fig. 6 A). To develop a control, we tested for the presence of Ssc1 and AAC, neither of which were found in the eluate fractions. Interestingly, a fraction of Ylh47 coeluted with $Mdm38_{ProtA}$ and vice versa, suggesting that these two proteins interact either directly or indirectly. Although newly synthesized Cox2 and -3 were found to be associated with Mdm38 (Fig. 5 A), components of complex IV were not copurified with Mdm38 or Ylh47 at steady state (Fig. 6 A). Similarly, Cyt1 and Rip1 of complex III were not detected in the eluate fraction (Fig. 6 A), indicating that neither complex III nor complex IV was significantly bound to Mdm38 or Ylh47 at steady state. In light of this observation, we speculate that the interaction of Mdm38 with newly synthesized mitochondrial proteins reflects a role for this protein in the early stages of membrane insertion before assembly of these proteins into the respiratory chain complexes. Oxa1, a central component of the export machinery, had been found to interact with mitochondrial translation products and to associate with ribosomes (Hell et al., 2001; Jia et al., 2003; Szyrach et al., 2003). Thus, we tested to determine whether mitochondrial ribosomes were bound to Mdm38 and Ylh47. Indeed, both $Mdm38_{ProtA}$ and $Ylh47_{ProtA}$ copurified with Mrp49, a component of the large subunit of the mitochondrial ribosome (Fig. 6 A). Surprisingly, the interaction of Mdm38 and Ylh47 with ribosomal proteins was stable enough to be maintained over the course of the purification. We estimated from the Western blot analyses of $Mdm38_{ProtA}$ purifications that only a fraction

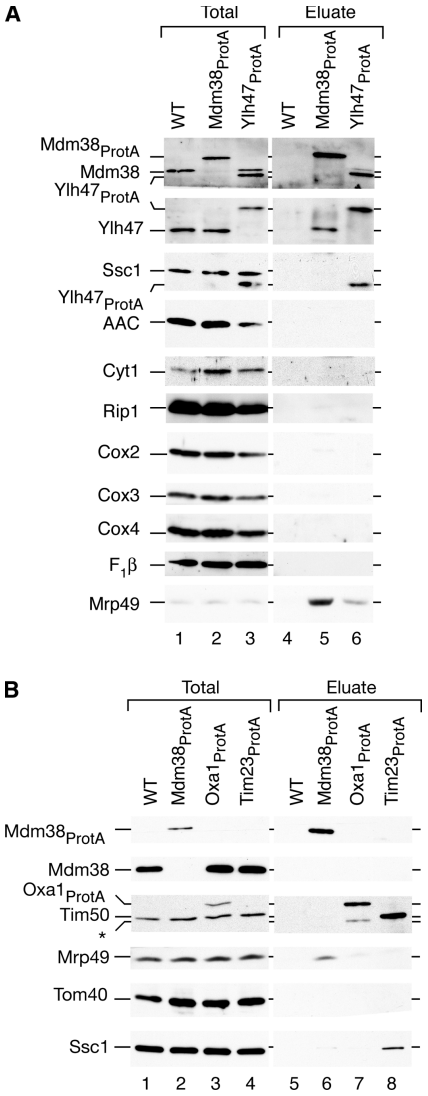

Figure 6. **Protein interactions of Mdm38 and Ylh47.** (A) Wild-type (WT), $Mdm38_{ProtA}$, and $Ylh47_{ProtA}$ mitochondria were solubilized in digitonin buffer and subjected to IgG chromatography. Bound proteins were eluted with SDS sample buffer and analyzed by SDS-PAGE and Western blotting. 7% of total and 100% of eluate were loaded. (B) Mitochondria containing $Mdm38_{ProtA}$, $Oxa1_{ProtA}$, or $Tim23_{ProtA}$ were solubilized in digitonin buffer as described in A. The asterisk indicates the breakdown product of $Oxa1_{ProtA}$. 7% of total and 100% of eluate were loaded. White lines indicate that intervening lanes have been spliced out.

of ribosomal proteins copurified with $Mdm38_{ProtA}$ under our experimental conditions. Previous analyses have shown that, similar to what we found for Mdm38, Oxa1 associates with a subfraction of the mitochondrial ribosomes (Szyrach et al., 2003). To obtain further support for the ribosome association, we performed sucrose density–gradient centrifugation of mitochondrial lysates. While soluble mitochondrial proteins such as Mge1 migrated at the top of the gradient, Mdm38 was found at higher sucrose concentrations. It comigrated with the ribosomal peak, which included proteins such as Mrpl22, a result that is consistent with the idea that Mdm38 and ribosomes form a complex (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200505060/DC1).

To support the idea that the interaction of Mrp49 with Mdm38 was specific, we compared the purification of $Mdm38_{ProtA}$ to that of $Tim23_{ProtA}$ and $Oxa1_{ProtA}$. Although Tim50 and Ssc1 selectively copurified with $Tim23_{ProtA}$ (Geissler et al., 2002; Chacinska et al., 2005), Mrp49 was found predominantly in association with $Mdm38_{ProtA}$ (Fig. 6 B). Thus, it appeared that ribosomal proteins such as Mrp49 were specifically associated in a protein complex with Mdm38. We did not detect significant amounts of Mrp49 associated with $Oxa1_{ProtA}$, although the protein had been previously reported to associate with ribosomes (Jia et al., 2003; Szyrach et al., 2003). Conceivable explanations for this discrepancy are that the ribosome–Oxa1 interaction is too labile to survive the purification procedure and that tagging of Oxa1 at the COOH terminus had a destabilizing effect on the ribosome interaction.

The copurification of Mrp49 with $Mdm38_{ProtA}$ and $Ylh47_{ProtA}$ suggested that mitochondrial ribosomes were associated with Mdm38 and Ylh47. To further verify this, we performed Protein A purifications of $Mdm38_{ProtA}$ and $Ylh47_{ProtA}$ and separated the eluted proteins by SDS-PAGE. The gels were either stained with Coomassie or subjected to Western blotting with antibodies against Protein A. The Coomassie-stained gels showed a large number of proteins that selectively copurified with both $Mdm38_{ProtA}$ and $Ylh47_{ProtA}$ but were not present in the mock-isolation (Fig. 7 A). Western blotting for Protein A demonstrated that the bands observed by Coomassie staining were not breakdown products of the tagged proteins (Fig. 7 A, lanes 5 and 6). To determine the identity of the coisolated proteins, the gel lanes were cut into pieces, subjected to in-gel digestion, and subsequently analyzed by mass spectrometry. The major proteins identified were constituents of both the large and small subunits of the mitochondrial ribosome (Table I and Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200505060/DC1).

To determine whether Mdm38 interacts with translating ribosomes, $Mdm38_{ProtA}$ mitochondria were incubated before initiation of translation with or without puromycin, an antibiotic that inhibits protein synthesis and induces the release of nascent chains from ribosomes. After treatment, mitochondria were solubilized and $Mdm38_{ProtA}$ was isolated by IgG chromatography. The puromycin treatment efficiently inhibited translation under these conditions (Fig. 7 B, top). In contrast, copurification of the ribosome was not significantly affected (Fig. 7 B, bottom). Thus, association of ribosomes with Mdm38 occurs in the absence of nascent chains.

In light of this finding, we asked whether the association of newly synthesized precursors with Mdm38 reflected its association with translating ribosomes. Although mitochondrial translation products copurified with $Mdm38_{ProtA}$ in control isolations, treatment with puromycin after translation and subsequent isolation under low-salt conditions significantly reduced the amount of radiolabeled proteins that copurified with Mdm38 (Fig. 7 C). In contrast, puromycin treatment did not affect the association of ribosomes with Mdm38 (Fig. 7 C). When isolations were performed in the presence of high salt, even in the absence of puromycin, both ribosomes and translation products were no longer associated with Mdm38 (Fig. 7 C, lane 6). However, after chemical cross-linking,

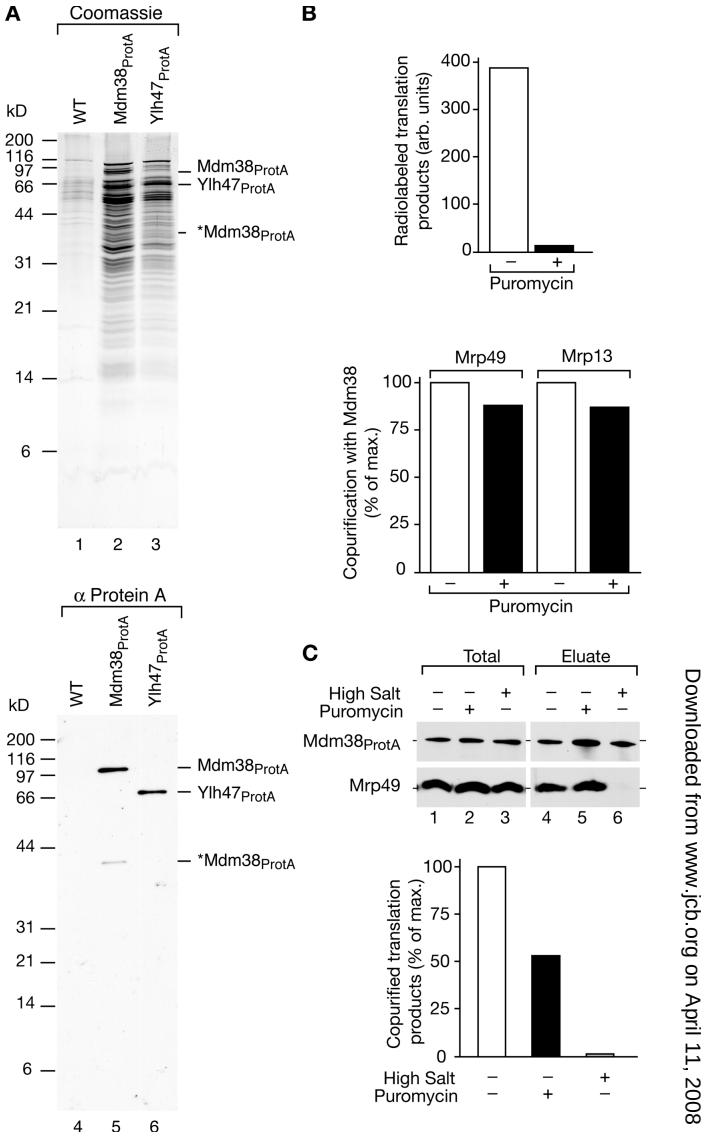

Figure 7. **Mdm38 and Ylh47 form a complex with mitochondrial ribosomes.** (A) Mitochondria were solubilized in digitonin buffer and subjected to IgG chromatography. The eluate was separated on urea SDS-PAGE and stained with colloidal Coomassie or analyzed by Western blotting with a Protein A antiserum. Lanes of the Coomassie-stained SDS gel were cut into slices and analyzed by mass spectrometry (Table I). *$Mdm38_{ProtA}$ indicates the breakdown product of $Mdm38_{ProtA}$. (B) In organello translation was performed in isolated $Mdm38_{ProtA}$ mitochondria in the presence or absence of puromycin for 30 min. $Mdm38_{ProtA}$ was isolated as described in A. Samples were analyzed by SDS-PAGE, digital autoradiography, or Western blotting. $n = 1$. (C) After in organello translation, mitochondria were left untreated (lanes 1 and 3) or treated with puromycin (lane 2). After solubilization in low-salt (lanes 1 and 2) or high-salt (lanes 3) buffer, extracts were subjected to IgG chromatography and analyzed by SDS-PAGE and digital autoradiography or Western blotting. $n = 1$. 10% of total and 100% of eluate were loaded. White lines indicate that intervening lanes have been spliced out.

Mdm38 could be coisolated with mitochondrial ribosomes via $Mrp20_{TAP}$, even in the presence of high salt (Fig. S3 B). Thus, based on the puromycin treatment, we conclude that the copurification of translation products with Mdm38 reflects a transient interaction that is mediated through association with translating ribosomes.

Table I. **Proteins of the mitochondrial ribosome that were found in complex with Mdm38 and Ylh47 by affinity purification and mass spectrometry**

Proteins of small ribosomal subunit	Mdm38	Ylh47	Proteins of large ribosomal subunit	Mdm38	Ylh47
MRP1/YDR347			IMG1/YCR046C	√	√
MRP10/YDL045W-A			IMG2/YCR071C	√	√
MRP13/YGR084C			MRP20/YDR405W	√	√
MRP17/YKL003C	√	√	MRP49/YKL167C	√	√
MRP2/YPR166C	√	√	MRP7/YNL005C	√	√
MRP21/YBL090W		√	MRPL1/YDR116C	√	√
MRP4/YHL004W	√	√	MRPL10/YNL284C	√	√
MRP51/YPL118W		√	MRPL11/YDL202W	√	√
MRPS16/YPL013C	√	√	MRPL13/YKR006C	√	√
MRPS17/YMR188C	√	√	MRPL15/YLR312W-A	√	√
MRPS18/YNL306W	√	√	MRPL16/YBL038W	√	
MRPS28/YDR337W	√	√	MRPL17/YNL252C	√	√
MRPS35/YGR165W	√	√	MRPL19/YNL185C		
MRPS5/YBR251W	√	√	MRPL20/YKR085C	√	√
MRPS8/YMR158W		√	MRPL22/YNL177C		
MRPS9/YBR146W	√	√	MRPL23/YOR150W	√	
NAM9/YNL137C	√	√	MRPL24/YMR193W	√	√
PET123/YOR158W	√	√	MRPL25/YGR076C	√	√
PPE1/YHR075C			MRPL27/YBR282W	√	
RSM10/YDR041W	√	√	MRPL28/YDR462W	√	√
RSM18/YER050C	√	√	MRPL3/YMR024W	√	√
RSM19/YNR037C			MRPL31/YKL138C		
RSM22/YKL155C	√	√	MRPL32/YCR003W		
RSM23/YGL129C	√	√	MRPL33/YMR286W		
RSM24/YDR175C	√	√	MRPL35/YDR322W	√	√
RSM25/YIL093C			MRPL36/YBR122C	√	√
RSM26/YJR101W	√		MRPL37/YBR268W		
RSM27/YGR215W	√	√	MRPL38/YKL170W	√	
RSM28/YDR494W	√	√	MRPL39/YML009C		
RSM7/YJR113C	√	√	MRPL4/YLR439W	√	√
SWS2/YNL081C	√		MRPL40/YPL173W	√	
VAR1/Q0140	√	√	MRPL44/YMR225C		
YMR31/YFR049W			MRPL49/YJL096W	√	√
YNR036C			MRPL50/YNR022C		
			MRPL51/YPR100W	√	√
			MRPL6/YHR147C		
			MRPL7/YDR237W	√	
			MRPL8/YJL063C	√	√
			MRPL9/YGR220C	√	√
			RML2/YEL050C	√	
			YML6/YML025C	√	√
			YDR115W		
			YPL183W-A		

Discussion

To gain insight into the molecular role of Letm1 in mitochondria, we analyzed the function of its orthologues, Mdm38 and Ylh47, in *S. cerevisiae* mitochondria. First, we addressed the transport of the human Letm1, Mdm38, and Ylh47 into mitochondria and found that all three proteins could be efficiently transported into yeast mitochondria in a $\Delta\psi$-dependent manner. Transport of the human protein, as well as Mdm38 and Ylh47, is accompanied by proteolytic cleavage of the predicted presequence, indicative of protein translocation across the inner membrane. Further analyses of the submitochondrial localization of the yeast proteins showed that both Ylh47 and Mdm38 were inner membrane proteins exposed to the mitochondrial matrix. This finding is in agreement with a recent analysis in which a tagged Mdm38 protein was shown to be an inner mitochondrial membrane protein (Nowikovsky et al., 2004). Thus, Ylh47 and Mdm38 are both tightly associated with

the inner membrane and exhibit similar topologies. Letm1 has been previously localized to mitochondria, and subsequent analyses of truncation constructs have suggested that targeting information resides in the NH_2-terminal portion of the protein (Schlickum et al., 2004). Based on these findings and our analyses, it appears likely that the localization of Letm1 in human cells is similar to that of Mdm38 and Ylh47 in yeast.

Dimmer et al. (2002) identified Mdm38 in a screen for yeast cells with defects in mitochondrial distribution and morphology. Moreover, work by Nowikovsky et al. (2004) suggested that Mdm38 is involved in mitochondrial K^+ homeostasis. Therefore, we wanted to analyze the function of Mdm38 and Ylh47 in more detail. Our analyses have demonstrated that Mdm38 is associated with newly synthesized mitochondrial proteins and that this interaction occurs via the ribosome. Furthermore, Mdm38 is required for efficient membrane insertion of cytochrome *b* and Atp6, whereas other proteins appear to be less dependent on Mdm38 function. In agreement with a role for Mdm38 in export, the steady-state levels of cytochrome *b* were reduced in *mdm38Δ* mitochondria, leading to reduced amounts of complexes III and IV. Defects in human and yeast Atp6 have been found to only mildly affect the stability of the F_1F_o–ATPase (Hadikusumo et al., 1988; Jesina et al., 2004). However, besides the fraction of Atp6 in the F_1F_o–ATPase, a stable pool of Atp6 was also found to accumulate in an unassembled state in *mdm38Δ* mitochondria. In contrast, mitochondria lacking Ylh47 exhibited a significantly weaker phenotype with regard to protein insertion and did not display a growth defect on nonfermentable carbon sources. In addition to the observed defect in the biogenesis of cytochrome *b* and Atp6, *mdm38Δ* cells also displayed reduced steady-state amounts of other respiratory chain proteins. The reduction of both nuclear and mitochondrially encoded proteins can be explained by an increased instability of unassembled proteins (Yang and Trumpower, 1994; Lemaire et al., 2000). In this regard, it is interesting to note that the stability of Atp6 is decreased in *oxa1Δ* mitochondria because of increased turnover via the membrane-embedded protease Yme1 (Lemaire et al., 2000). The accumulation of a stable Atp6 pool in *mdm38Δ* mitochondria suggests that the protein is inaccessible to the protease in the membrane.

Mdm38 and Ylh47 were found in physical association with mitochondrial ribosomes in the absence of translation products. Yet, this interaction was not critical for the synthesis of mitochondrial proteins. Because a subset of newly synthesized mitochondrial proteins showed defects in membrane insertion and Mdm38 was shown to interact with newly synthesized proteins indirectly via the ribosome, it is likely that ribosome binding to Mdm38 is important for Oxa1-independent protein export. We found that $Mdm38_{ProtA}$ lacking amino acids 361–573 was still able to bind ribosomes (unpublished data), implicating the most conserved part of the protein in this function. Given that not all Mdm38 molecules seem to be bound to ribosomes at steady state, it is conceivable that a free pool of Mdm38 exists or that Mdm38 forms additional protein complexes with as-yet-undefined proteins. Dimmer et al. (2002) reported morphological defects for yeast cells with compromised respiratory chain assembly and/or function. This agrees well with the observed respiratory chain defects in *mdm38Δ* mitochondria. Moreover, the respiratory chain defects might also account for an indirect effect on ion transport (Nowikovsky et al., 2004).

Mdm38 displays similarities to the mitochondrial Oxa1 protein in several regards. Oxa1 is a central component of the mitochondrial protein insertion machinery and is highly conserved between yeast and man (Bonnefoy et al., 1994; He and Fox, 1997; Hell et al., 1998; Stuart, 2002). Oxa1 interacts with newly synthesized mitochondrial proteins (Hell et al., 2001) and associates with mitochondrial ribosomes (Jia et al., 2003; Szyrach et al., 2003), similar to Mdm38. In addition, *oxa1* mutant mitochondria are defective in inner membrane insertion of mitochondrially encoded proteins. Export of Cox2 strictly depends on Oxa1 function, whereas membrane insertion of cytochrome *b*, Cox3, and Atp6 are only mildly affected in *oxa1* mutants (He and Fox, 1997; Hell et al., 1998, 2001). This suggests the existence of alternative pathways for inner membrane protein insertion (Hell et al., 2001; Stuart, 2002). In contrast to *oxa1* mutant mitochondria, *mdm38Δ* mitochondria display selective protein insertion defects for cytochrome *b* and Atp6. Thus, it appears that Mdm38 is a candidate protein for an Oxa1-alternative protein insertion pathway in mitochondria. Further support for this hypothesis comes from the fact that Cox2 transport and processing were not affected in *mdm38Δ* mitochondria, whereas *oxa1* mutant mitochondria accumulate the precursor of Cox2 (He and Fox, 1997; Hell et al., 1997, 1998).

The biogenesis of respiratory chain complexes requires the synthesis of the proteins encoded by the mitochondrial genome and their subsequent insertion into the inner membrane. Several human diseases have been linked to mutations in mitochondrial genes encoding structural components of complexes I, III, IV, and V of the respiratory chain. In addition, a limited number of nuclear encoded proteins that are mainly involved in the assembly of the respiratory chain complexes have been identified and linked to human diseases (DiMauro and Schon, 2003; Zeviani and Carelli, 2003). *LETM1* defects likely contribute to the neuromuscular features characteristic of most WHS patients, as patients with smaller chromosomal deletions that excluded the *LETM1* gene showed weaker phenotypes and lacked the WHS characteristic seizures (Endele et al., 1999; Rauch et al., 2001). Based on these findings, it appears likely that these neuromuscular features result from defects in mitochondrial function because of the loss of functional Letm1. However, the molecular function of the Letm1 protein remains unclear. The Letm1 orthologue Mdm38 plays a role in respiratory chain function at the cellular level, as demonstrated by the growth defects and reduced $\Delta\psi$ observed in *mdm38Δ* mitochondria. Letm1 partially rescues the growth defect of *mdm38Δ* cells, suggesting that both proteins fulfill similar cellular functions (Nowikovsky et al., 2004). Thus, it is probable that Letm1 is involved in the biogenesis of the respiratory chain in humans similar to Mdm38 in yeast. Therefore, the phenotype of WHS patients, especially the neuromuscular defects and the seizures, likely reflects defects in oxidative phosphorylation and thus

resembles classical mitochondrial encephalomyopathies such as MELAS (mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes), MERRF (myoclonic epilepsy with ragged-red fibers), or MILS (maternally inherited Leigh's syndrome; DiMauro and Schon, 2003). However, future work on Letm1 in human cells and especially in WHS patient cells is clearly needed to support this hypothesis.

Materials and methods

Yeast strains and growth conditions

S. cerevisiae strains used in this study were derivatives of YPH499, with the exception of $Mrp20_{TAP}$ (Open Biosystems), which is a derivative of BY4741. For the isolation of mitochondria, cells were grown in liquid YPG medium (1% [wt/vol] yeast extract, 2% [wt/vol] bacto-peptone, and 3% [wt/vol] glycerol). For mitochondrial isolation, *cox4Δ* cells and the corresponding wild type were grown on media containing 2% sucrose as a carbon source. Tagging of Mdm38, Ylh47, and Oxa1 was performed by chromosomal integration in YPH499. Deletion of the *MDM38* (AFY23) and *YLH47* (AFY24) open reading frames in YPH499 was performed by homologous recombination using a PCR-generated cassette containing the *kanMX6* marker gene flanked by regions homologous to the 5′ and 3′ end of the coding region. To generate an *mdm38Δ/ylh47Δ* strain (AFY27), the *MDM38* open reading frame was replaced by *HISMX6* in the *ylh47Δ* strain.

Protein import into isolated mitochondria

Mitochondrial precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine/cysteine (GE Healthcare). Import of radiolabeled precursor proteins into mitochondria was performed as previously described (Ryan et al., 2001).

Subcellular localization analyses

For localization of Ylh47 and Mdm38 in mitochondria, mitochondria that had been subjected to osmotic shock in a buffer containing 1 mM EDTA and 10 mM MOPS, pH 7.2 (mitoplasts), or mitochondria sonicated (3 × 30 s with 40% duty cycle in a sonifier [model 250; Branson]) in buffer containing 10 mM Tris/HCl, pH 7.4, and 500 mM NaCl, were treated with proteinase K at the indicated concentrations. Samples were subjected to TCA precipitation and analyzed by SDS-PAGE and Western blotting. To determine whether Mdm38 and Ylh47 were membrane proteins, mitochondria were subjected to carbonate extraction essentially as described previously (Frazier et al., 2004). In brief, mitochondria were suspended in 0.1 M sodium carbonate (pH 11.5 or 10.8) for 30 min at 0°C and centrifuged at 45,000 rpm in a TLA45 rotor (Beckman Coulter) for 30 min at 2°C. Samples were precipitated with TCA and subjected to SDS-PAGE and Western blotting for various marker proteins.

Labeling of mitochondrial translation products

For in organello translation of mitochondrially encoded proteins, mitochondria were isolated from cells grown in media containing glycerol as the carbon source. Translation was performed in the presence of [^{35}S]methionine/cysteine essentially as described previously (Westermann et al., 2001). After translation and reisolation, mitochondrial proteins were either precipitated with TCA and directly analyzed by tris-tricine SDS-PAGE or swollen in EM buffer (1 mM EDTA and 10 mM MOPS, pH 7.2) and divided, and one half was treated with 10 μg/ml proteinase K for 15 min on ice before TCA precipitation.

Mass spectrometric analysis

After staining with Coomassie brilliant blue, gels were cut into slices. In-gel digestion of proteins was performed with trypsin in 25 mM of ammonium bicarbonate buffer (pH 7.8) at 37°C overnight. Tryptic peptide fragments were extracted twice with 50:50 acetonitrile/5% formic acid and subjected to online reversed-phase capillary HPLC separation with HPLC systems (Dionex/LC Packings; Schaefer et al., 2004). Tandem mass spectrometry (MS/MS) spectra were obtained using a QStar XL (Applied Biosystems) system equipped with a nano electrospray ionization source (MDS Sciex). Alternatively, MS/MS experiments were performed with a Finnigan LCQ XP instrument (Thermo Electron Corporation) equipped with a nano electrospray ionization source (PicoView 100; New Objective) and distal coated SilicaTips (FS360–20–10-D; New Objective). Data-dependent software (QStar XL, Analyst QS [Applied Biosystems], LCQ XP, and Xcalibur [Thermo Electron Corporation]) was used for online MS/MS analyses. Mitochondrial proteins with a known function that were identified with >25% sequence coverage were selected, whereas proteins frequently found as contaminants in Protein A purifications were omitted. For protein identification, uninterpreted MS/MS spectra were correlated with the National Center for Biotechnology Information protein sequence database (www.ncbi.nlm.nih.gov) restricted to *S. cerevisiae* applying the SEQUEST algorithm.

Chemical cross-linking

$Mrp20_{TAP}$ mitochondria were suspended in 20 mM Hepes/KOH, pH 7.4, 50 mM NaCl, 1% digitonin, and 10% glycerol and were incubated with the thio-cleavable cross-linker dithiobis(succinimidyl propionate) or left untreated for 30 min at 4°C. The cross-linker was quenched, and samples were subjected to a clarifying spin. Subsequently, the samples were loaded on IgG-Sepharose. Bound proteins were washed with buffer (20 mM Hepes/KOH, pH 7.4, 750 mM NaCl, 0.3% Triton X-100, and 10% glycerol) and eluted from IgG-Sepharose with SDS sample buffer containing 1% β-mercaptoethanol.

Sucrose-gradient analysis and complex isolation

Isolated mitochondria (4 mg protein) from YPH499 cells grown on YP medium supplemented with 2% galactose were lysed in buffer containing 1.5% digitonin, 20 mM Tris, pH 7.5, 10 mM MgOAc, 5 mM DTT, and 1 mM PMSF for 30 min at 4°C. After a clarifying spin, the lysate was applied to a linear 15–30% sucrose gradient containing a total volume of 11.2 ml. After centrifugation for 16 h at 25,500 rpm in a TH-641 rotor (Sorvall), the gradient was fractionated from the top and absorption at 254 nm was determined for each fraction. Aliquots were TCA precipitated and analyzed by SDS-PAGE and Western blot. Western blot signals were quantified with Scion Image 1.62a (Scion Co.).

Protein A–tagged proteins were isolated from mitochondria essentially as described previously (Geissler et al., 2002). In brief, mitochondria (8–10 mg for preparative scale, 1 mg for analytical scale, and 0.5 mg for in organello labeling and subsequent isolation) were solubilized in solubilization buffer (30 mM Tris/HCl, pH 7.4, 80 mM KCl, 5% [wt/vol] glycerol, 5 mM $MgCl_2$, and 1% digitonin) at 4°C, subjected to a clarifying spin, and applied to IgG-Sepharose. After binding to the column and extensive washing with solubilization buffer containing 0.3% digitonin, bound proteins were eluted with SDS sample buffer without β-mercaptoethanol to avoid release of the IgG chains from the Sepharose. After elution from the column, samples received reducing reagents and were subsequently analyzed by SDS-PAGE, Western blotting, or digital autoradiography.

Miscellaneous

Antibodies were generated in rabbits against selected peptides of Letm1, Ylh47, Mdm38, Oxa1, Pnt1, and Mba1. Antibodies against Cox1, -2, and -3 were purchased from Invitrogen. For the separation of proteins after in organello translations, tris-tricine SDS-PAGE was used. Western blots were performed with PVDF or nitrocellulose membranes according to standard procedures and detected by ECL. $\Delta\psi$ measurements were determined using the potential-sensitive fluorescent dye $DiSC_3(5)$ (Rehling et al., 2003). BN-PAGE analyses were performed essentially as described previously (Ryan et al., 2001).

Online supplemental material

Fig. S1 shows that the import of nuclear encoded proteins into *mdm38Δ* mitochondria is not affected. Fig. S2 demonstrates that *cox4Δ* mitochondria lack intact complex IV and have a reduced $\Delta\psi$ but are not affected in the export of cytochrome *b* or Atp6. Fig. S3 shows that Mdm38 comigrates with mitochondrial ribosomes on a sucrose gradient and that it can be cross-linked to mitochondrial ribosomes. The supplemental text gives a list of nonribosomal proteins identified by mass spectrometry in the Protein A purification analyses. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200505060/DC1.

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