Cell Metabolism
Article

The DEAD Box Protein Mrh4 Functions in the Assembly of the Mitochondrial Large Ribosomal Subunit

Dasmanthie De Silva,[1] Flavia Fontanesi,[1] and Antoni Barrientos[1,2,*]
[1]Department of Biochemistry and Molecular Biology
[2]Department of Neurology
University of Miami Miller School of Medicine, Miami, FL 33136, USA
*Correspondence: abarrientos@med.miami.edu
http://dx.doi.org/10.1016/j.cmet.2013.10.007

SUMMARY

Proteins in a cell are universally synthesized by ribosomes. Mitochondria contain their own ribosomes, which specialize in the synthesis of a handful of proteins required for oxidative phosphorylation. The pathway of mitoribosomal biogenesis and factors involved are poorly characterized. An example is the DEAD box proteins, widely known to participate in the biogenesis of bacterial and cytoplasmic eukaryotic ribosomes as either RNA helicases or RNA chaperones, whose mitochondrial counterparts remain completely unknown. Here, we have identified the *Saccharomyces cerevisiae* mitochondrial DEAD box protein Mrh4 as essential for large mitoribosome subunit biogenesis. Mrh4 interacts with the *21S* rRNA, mitoribosome subassemblies, and fully assembled mitoribosomes. In the absence of Mrh4, the *21S* rRNA is matured and forms part of a large on-pathway assembly intermediate missing proteins Mrpl16 and Mrpl39. We conclude that Mrh4 plays an essential role during the late stages of mitoribosome assembly by promoting remodeling of the *21S* rRNA-protein interactions.

INTRODUCTION

Mitochondria are semiautonomous eukaryotic organelles, descendant of an earlier aerobic prokaryote (Margulis, 1975). They have retained a small portion of their original genetic information, most having been transferred to the nucleus of the host cell. The limited number of mitochondrial DNA (mtDNA)-encoded messages (8 in yeast, 13 in human cells) is translated in membrane-bound specialized mitochondrial ribosomes (mitoribosomes). Across evolution, the proteins synthesized in mitoribosomes are essential for the conversion of energy stored in nutrients into the chemical form of ATP, aerobically by oxidative phosphorylation (OXPHOS). The mitochondrial translation system itself involves elements encoded in the mtDNA. The two mitochondrial ribosomal RNAs (rRNAs) are universally mitochondrion encoded. In *Saccharomyces cerevisiae* and human, a full set of mitochondrial tRNAs is also encoded in the mtDNA, while in some species, tRNAs are imported into mitochondria from the cytoplasm. The mitochondrial translational system is more closely related to the bacterial system than to the system present in the eukaryotic cytosol. However, significant differences exist in the genetic code and in the actual process of translation (Christian and Spremulli, 2012; Kehrein et al., 2013). Moreover, mitochondrial ribosomes differ from their bacterial ancestor in that they have a lower RNA:protein ratio, where significant amounts of RNA have been replaced by mitospecific proteins, as seen particularly in the mammalian 55S mitoribosomes (O'Brien, 2002). *S. cerevisiae* mitochondria have a 74S ribosome consisting of a small 37S subunit (SSU) formed by a *15S* ribosomal RNA (rRNA) and more than 34 proteins and a large 54S subunit (LSU) conformed by a *21S* rRNA and at least 44 proteins (Smits et al., 2007). As an exception, in *S. cerevisiae* one of the 37S subunit components, Var1, is encoded in the mtDNA.

Mitochondrial translation is known to rely mainly on nucleus-encoded proteins, such as ribosomal proteins (r-proteins), aminoacyl-tRNA synthetases, translation initiation, elongation, and termination factors, as well as mRNA-specific translational activators. Their biomedical importance is highlighted by the fact that mutations in most mtDNA-encoded tRNAs, as well as in nuclear genes encoding mitochondrial r-proteins, translation initiation, and elongation factors, are responsible for infantile multisystemic diseases frequently involving encephalomyopathy and hypertrophic cardiomyopathy (Pérez-Martínez et al., 2008).

Despite their biological and biomedical relevance, the knowledge on the molecular details of the assembly pathway and the factors involved in the biogenesis of mitoribosomes is still very limited. The factors described up to now on the biogenesis of the mitoribosome include enzymes involved in the modification of the mitochondrial rRNAs and proteins that recruit these enzymes to the mitoribosome (Cámara et al., 2011; Metodiev et al., 2009; Pintard et al., 2002; Seidel-Rogol et al., 2003; Sirum-Connolly and Mason, 1995; Surovtseva and Shadel, 2013; Wredenberg et al., 2013). They also include conserved putative guanosine triphosphatases (GTPases), which play poorly characterized roles in the assembly of the large subunit (yeast and human Mtg1 [Barrientos et al., 2003; Kotani et al., 2013], yeast Mtg2 [Datta et al., 2005], and human C7orf30 [Rorbach et al., 2012]) or small subunit (yeast Mtg3 and human C4orf14, homologs of bacterial YqeH [He et al., 2012]). The bacterial and cytoplasmic protein synthesis, which are characterized

in more detail, are known to involve the function of a large number of RNA helicases in ribosomal assembly and in the process of translation (Guenther and Jankowsky, 2009; Linder and Jankowsky, 2011). Therefore, it is somehow surprising that RNA helicases required for protein synthesis in mitochondria are yet to be identified.

This missing information led us in search of mitochondrial putative RNA helicases required for mitoribosome biogenesis and/or translation using the amenable facultative aerobe/anaerobe yeast *S. cerevisiae* as a model organism. An in silico screen among the known *S. cerevisiae* mitochondrial putative helicases directed our focus to Mrh4 (4th putative *m*itochondrial DEAD box *R*NA *h*elicase). In the single report published 10 years ago, Mrh4 was described as a suppressor of mitochondrial splicing defects in *COX1*, one of the three *S. cerevisiae* mtDNA genes that contain introns (Schmidt et al., 2002). This function is not necessarily specific for Mrh4, since several other mitochondrial helicases have been found to suppress splicing defects when overexpressed (Huang et al., 2005). A GFP fusion of Mrh4 was localized to mitochondria, and its presence was found to be important for the maintenance of the mtDNA even in an intronless background (Schmidt et al., 2002). However, mtDNA is highly unstable in strains defective in translation; hence, the genome instability could be secondary to a translation defect.

Here, to study the possible role(s) of Mrh4 in mitochondrial translation without interference of the splicing defects, we have used strains carrying intronless mtDNA in all of our experiments. We have demonstrated that Mrh4 is required during the late stages of mitoribosome assembly to promote remodeling of the *21S* rRNA-protein interactions.

RESULTS AND DISCUSSION

Mrh4 Is Required for Mitochondrial OXPHOS Biogenesis in a Strain Carrying Stable Intronless mtDNA

To study the possible role(s) of Mrh4 in mitochondrial translation, we engineered a Δ*mrh4* strain carrying intronless mtDNA. Moreover, we succeeded in stabilizing the mtDNA of this strain by using a library plasmid obtained from a screen aiming to suppress mtDNA loss in the context of a mitochondrial *pim1* protease mutation (van Dyck et al., 1998). The genomic DNA suppressor (SUP) described in this study contains three open reading frames, out of which *YCM2* is responsible for the mtDNA stabilization in *pim1* mutants (T. Langer, personal communication) as well as in the Δ*mrh4* strain. Disruption of the *MRH4* gene in diploid cells expressing the mtDNA-instability suppressor from a multicopy plasmid, followed by sporulation, tetrad dissection, and analysis of the progeny, allowed us to conclude that the Δ*mrh4* mutation did not impair the integrity of the mtDNA in cells expressing YCM2 (Table S1 available online). *YCM2* encodes a putative inner mitochondrial membrane transporter of unknown function. Although we do not currently understand the mechanisms of mtDNA stabilization in Δ*mrh4* mutants by *YCM2*, the success of this approach has enable us to define the primary functions of Mrh4 in mitochondria. In all experiments, we routinely tested mtDNA stability, and the percentage of mtDNA-containing cells (ρ^+) was consistently above 95% (Table S1).

The null *mrh4* mutant strain (Δ*mrh4*) carrying stable intronless mtDNA was found to be respiratory deficient and unable to grow in respiratory YPEG media (yeast extract, peptone, ethanol, and glycerol) (Figures 1A and 1B, see also Table S1). Δ*mrh4* yeast has undetectable levels of mtDNA-encoded OXPHOS enzyme subunits and lacks mitochondrial hemes *b* and *a*, which are prosthetic groups of respiratory complexes III and IV, respectively

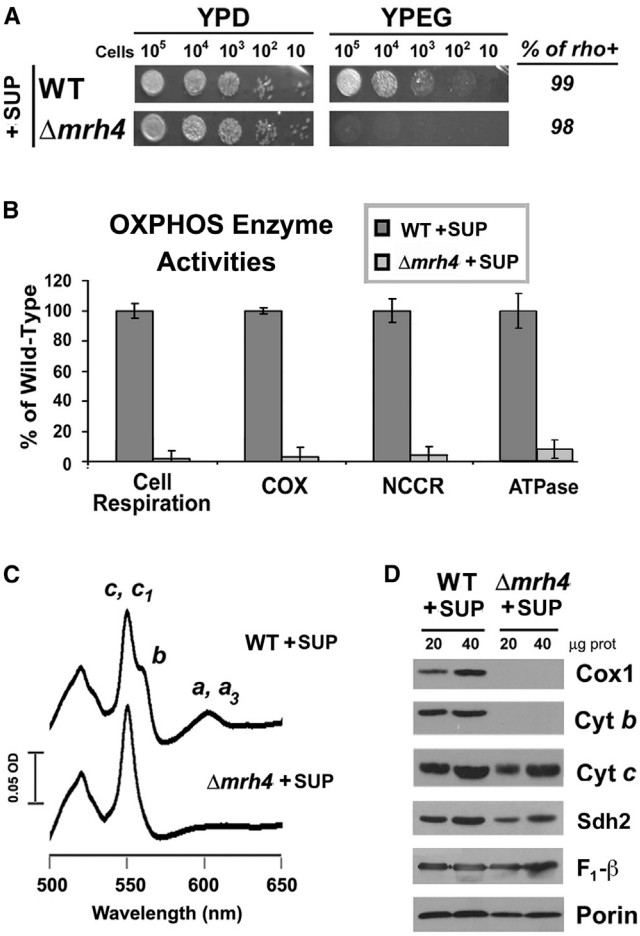

Figure 1. Mrh4 Is Essential for OXPHOS System Assembly and Function

(A) Growth test using serial dilutions of the indicated strains in complete media containing fermentable (YPD) or nonfermentable (YPEG) carbon sources. The plates were incubated at 30°C, and the pictures were taken after 2 days of growth.

(B) Endogenous cell respiration was measured polarographically, and cytochrome *c* oxidase (COX), NADH cytochrome *c* reductase (NCCR), and ATP synthase (ATPase) activities were measured spectrophotometrically in the indicated strains. Error bars represent the mean ± SD.

(C) Total mitochondrial cytochrome spectra. Mitochondria from WT and Δ*mrh4* strains were extracted with potassium deoxycholate under conditions that quantitatively solubilize all of the cytochromes (36). Difference spectra of the reduced (sodium dithionite) versus oxidized (potassium ferricyanide) extracts were recorded at room temperature. The absorption bands corresponding to cytochromes *a* and a_3 have maxima at 603 nm (*a* and a_3); the maxima for cytochrome *b* (*b*) and cytochrome *c* and c_1 (*c* and c_1) are 560 and 550 nm, respectively.

(D) Immunoblot analyses of the steady-state levels of Mrh4 and the indicated OXPHOS subunits in WT and Δ*mrh4* strains expressing the mtDNA instability suppressor (SUP). An antibody against Porin was used as a loading control. See also Table S1.

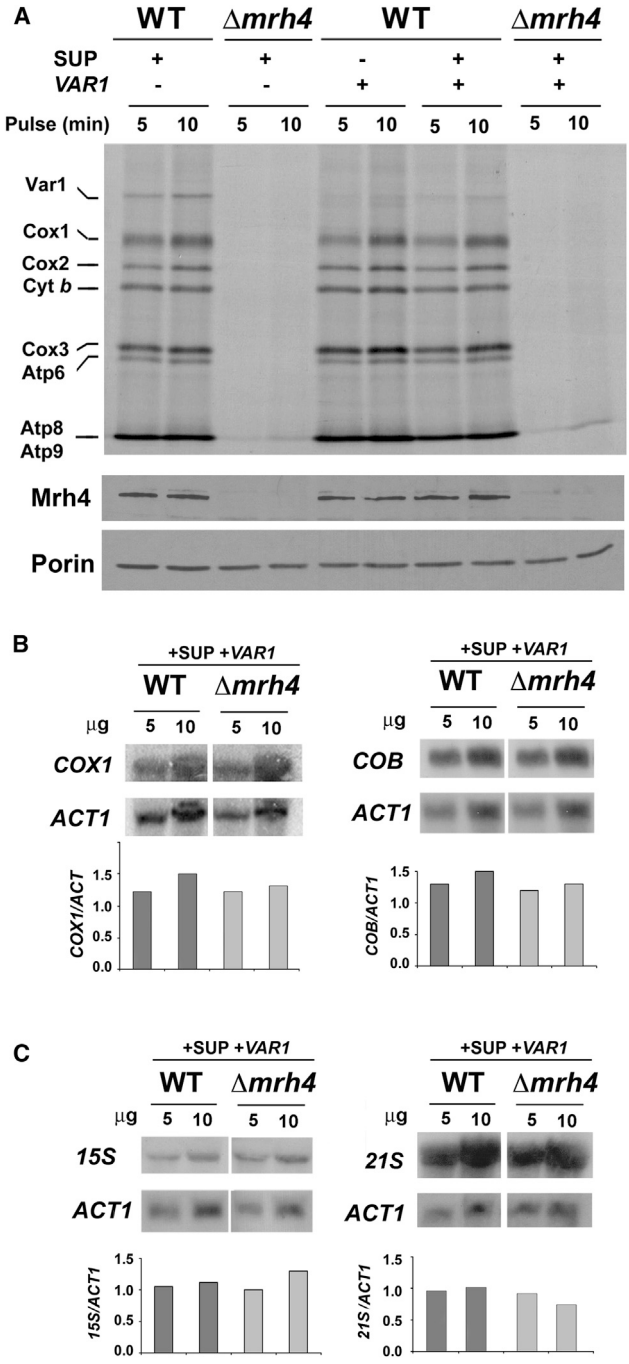

Figure 2. Mrh4 Is Required for Mitochondrial Protein Synthesis, But Not for mRNA and rRNA Processing or Stability

(A) In vivo mitochondrial protein synthesis in the indicated wild-type (WT) and Δ*mrh4* strains, expressing either the mtDNA instability suppressor (SUP), ectopic *VAR1*, or both. The strains were grown at 30°C and pulsed for 5 or 10 min at the same temperature with [^{35}S]methionine in the presence of cycloheximide to inhibit cytoplasmic protein synthesis.

(B and C) Northern blot analyses of total RNA probed for (B) *COX1*, *COB*, and (C) *15S* and *21S* rRNA. After processing, the membranes were exposed to X-ray film. The lower panels show the densitometry values obtained by using the histogram function of the Adobe Photoshop program on digitalized images. The values were normalized by the signal of *ACT1* as the loading control and expressed relative to the control. See also Figure S1.

(Figures 1C and 1D). Consistently, OXPHOS enzymatic activities were undetectable in Δ*mrh4* yeast mitochondria (Figure 1B).

Mrh4 Is Essential for Mitochondrial Protein Synthesis

To test whether the OXPHOS biogenesis defect in the Δ*mrh4* strain stems from a defect in mitochondrial gene expression, we performed in vivo mitochondrial protein synthesis experiments by following the incorporation of [^{35}S]methionine into newly synthesized mitochondrial proteins in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. Our results showed that the Δ*mrh4* strain was unable to perform mitochondrial protein synthesis even in the presence of mtDNA (Figure 2A).

The mtDNA-encoded Var1, an SSU protein translated in mitochondria, will not be synthesized in the absence of mitochondrial translation. To test the role of Mrh4 in Var1 expression, the Δ*mrh4*/*SUP* intronless strain was transformed with a construct that successfully relocates a recoded version of the *VAR1* gene to the nucleus as described previously (Sanchirico et al., 1995). The resulting strain remained respiratory deficient (data not shown) and unable to synthesize mtDNA-encoded proteins (Figure 2A), indicating that the lack of Var1 synthesis in the *mrh4* mutant is a consequence, rather than a cause, of the observed impairment in overall mitochondrial protein synthesis. Henceforth, all subsequent experiments were performed in Δ*mrh4*/*SUP* intronless strains expressing ectopic *VAR1*.

Using these strains, we further demonstrated that the protein synthesis defect in Δ*mrh4* cells is not due to a discrepancy in RNA levels, as both wild-type (WT) and Δ*mrh4* mitochondria had similar amounts of mRNAs and *15S* and *21S* rRNAs (Figures 2B and 2C, see also Figure S1).

Analysis of Temperature-Sensitive Mutants of *mrh4* Indicates a Role for Mrh4 in Mitoribosome Assembly

To discern whether the deficiency in protein synthesis observed in the Δ*mrh4* strain was the result of a defect in the biogenesis of the mitochondrial translation apparatus or in the translation process itself, we generated strains carrying *mrh4* temperature-sensitive (ts) alleles. Mrh4 contains a conserved DEAD box and an ATP-binding domain typical of RNA helicases (Figure 3A, see also Figure S2). The ts alleles were generated by site-directed mutagenesis of residues near the ATP-binding motif as described previously for the DEAD box protein Prp5 (Abu Dayyeh et al., 2002)(Figure 3A). The ts mutants (*mrh4*^{L157D} and *mrh4*^{L157D,Q158D}) were further characterized by assessing respiratory growth and translational efficiency at the restrictive temperature. The *mrh4*^{L157D,Q158D} ts mutant strain (ts2) was able to grow in respiratory media at 30°C, but not at 38°C, as expected (Figure 3B). Mitochondrial translation was assayed in whole cells by measuring [^{35}S]methionine incorporation in the presence of cycloheximide. Cells grown at either 30°C or 38°C were preconditioned at the specified translation temperature by incubating the cells for 15 min at 30°C or 38°C before the addition of [^{35}S]methionine. Mitochondrial protein synthesis was inhibited when the ts mutant was grown at the nonpermissive temperature. Growth of the ts mutant at the permissive temperature, however, did not affect mitochondrial translation at either the permissive or the restrictive temperatures (Figure 3C). The reduced translation observed in cells grown at 38°C is

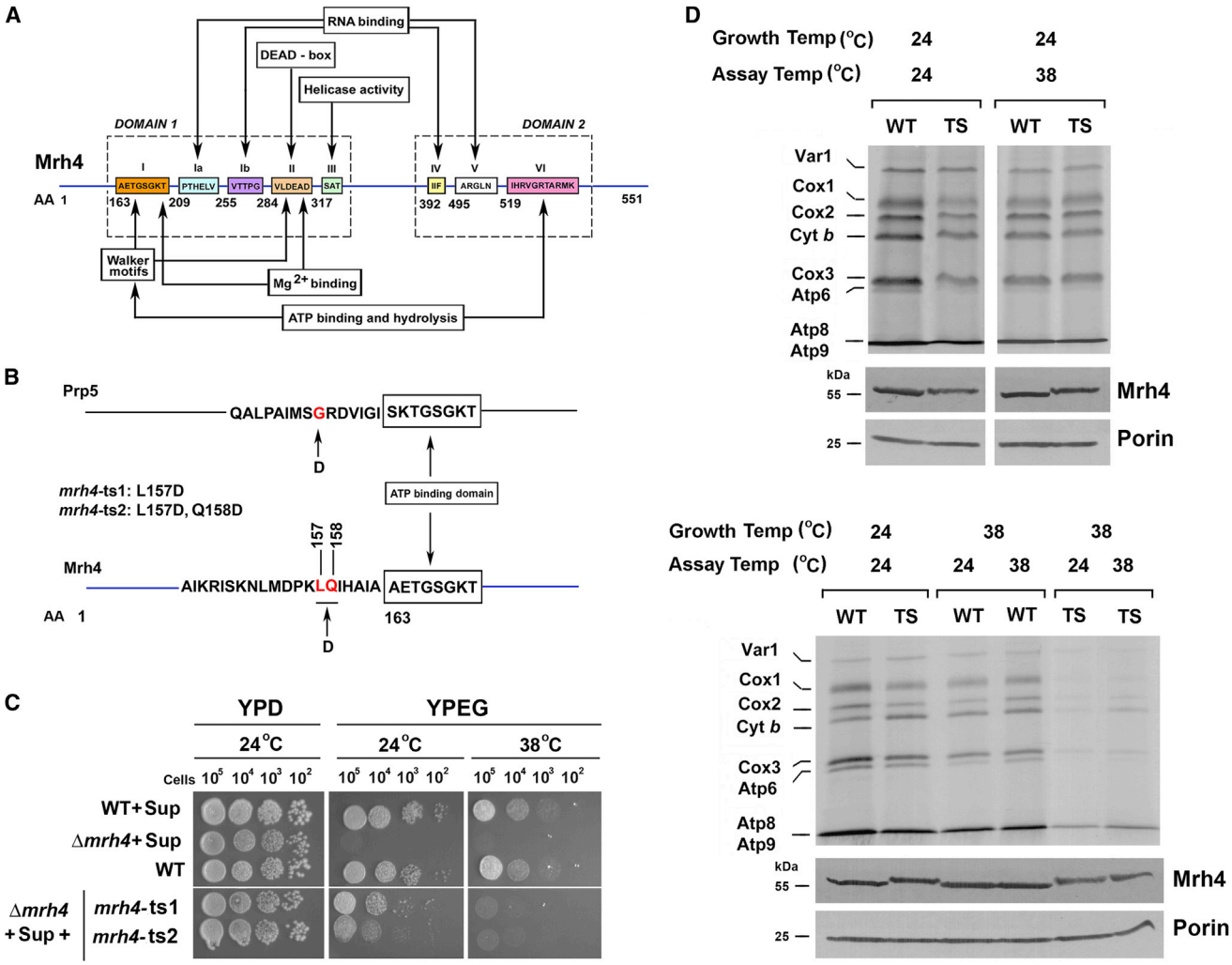

Figure 3. Characterization of Temperature-Sensitive *mrh4* Mutants Indicates a Role for Mrh4 in Assembly of the Mitochondrial Translational Apparatus

(A) Scheme representing the two domains and the conserved motifs present in Mrh4, typical of DEAD box helicases (Parsyan et al., 2011).

(B) Alignment showing the ATP-binding motifs in Prp5 and Mrh4. The amino acids mutated in each case to yield temperature-sensitive (ts) proteins are labeled in red.

(C) Growth test using serial dilutions of the indicated strains in complete media containing fermentable (glucose, YPD) or nonfermentable (ethanol-glycerol, YPEG) carbon sources. The plates were incubated at the permissive 24°C or nonpermissive 38°C, and the pictures were taken after 2 days of growth.

(D) In vivo mitochondrial protein synthesis in the indicated wild-type (WT) and ts2 strains. The strains were grown at the permissive (24°C) or nonpermissive (38°C) temperatures, and aliquots were pulsed for 15 min at each temperature with [^{35}S]methionine in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. See also Figure S2.

consistent with their growth retardation at this temperature. These results are more compatible with a role of Mrh4 in the biogenesis of the translational apparatus than in translation itself, although they do not totally exclude the possibility of an involvement of Mrh4 in translation.

Mrh4 Interacts with the 54S Large Ribosomal Subunit in Dissociated and Assembled Mitoribosomes

We generated an antibody against an Mrh4 peptide, which allowed localizing the protein in mitochondrial extracts (Figure S3). Using brief sonication, alkaline carbonate extraction, and proteinase protection assays in mitochondria and mitoplasts, the ~60 kDa Mrh4 protein was sublocalized in the mitochondrial matrix, loosely associated to the inner membrane, facing the mitochondrial matrix (Figure 4A, see also Figure S3).

To determine the native size of Mrh4, the protein was extracted from isolated WT mitochondria using 1% digitonin and 25 mM KCl and analyzed by sucrose gradient sedimentation. Mrh4 cosedimented with assembled ribosomes in extracts prepared in the presence of 0.5 mM Mg^{2+} and with dissociated LSU in extracts prepared in the presence of 5 mM EDTA (Figure 4B, see also Table S2), thus suggesting an interaction of Mrh4 with the 54S mitoribosomal subunit. This result is consistent with a high-throughput study that identified an interaction of Mrh4 with Mrpl9 and several other mitochondrial r-proteins of the large subunit (Gavin et al., 2002). This interaction was disrupted in the

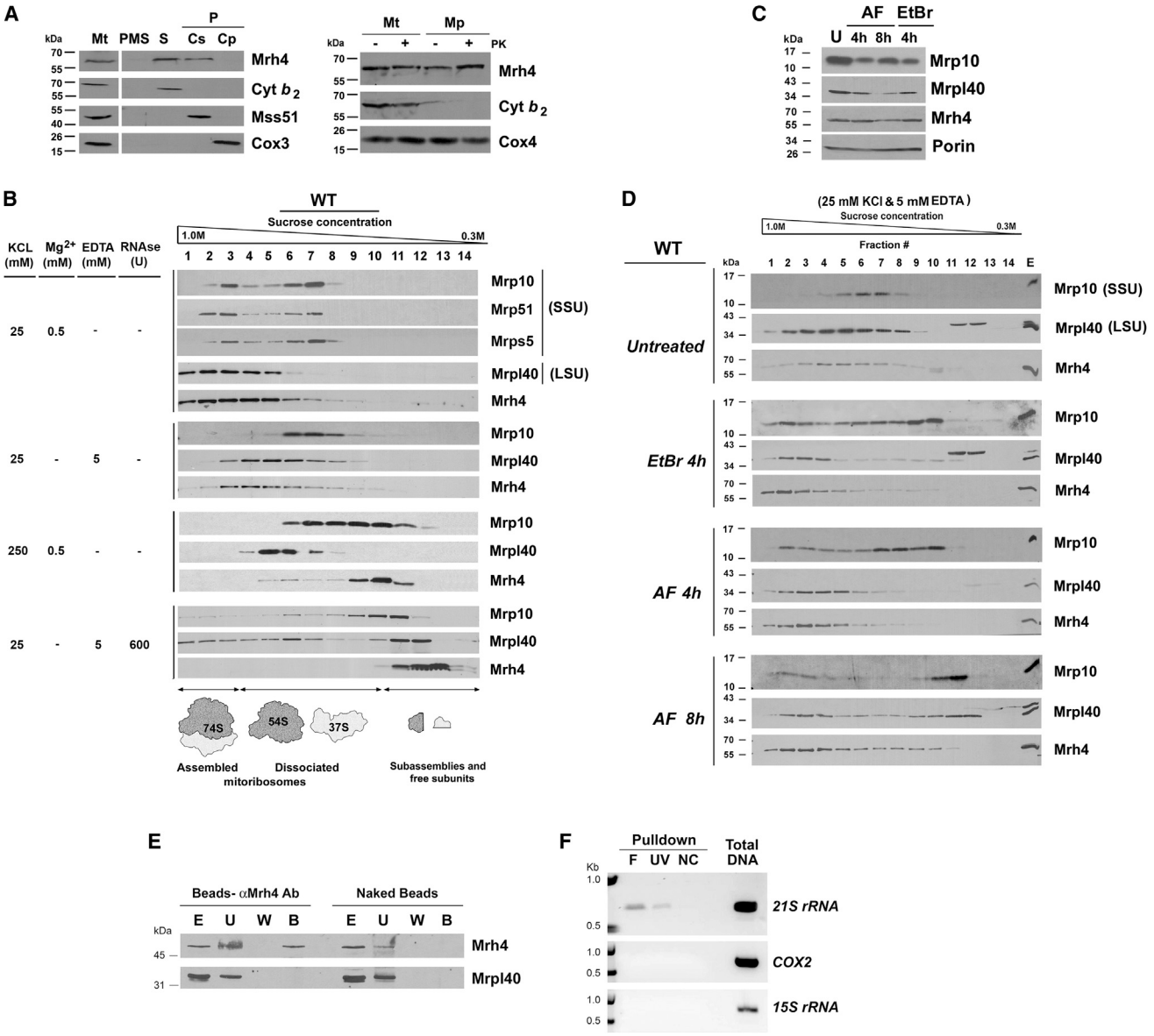

Figure 4. Mrh4 Is a Mitochondrial Matrix Protein Peripherally Bound to the Inner Membrane Interacting with the 54S Large Mitoribosomal Subunit

(A) Isolated WT mitochondria were fractionated into soluble (S) and membrane-bound (P) mitochondrial proteins by brief sonication and centrifugation. The pellet was submitted to alkaline extraction to allow the separation of the extrinsic proteins present in the supernatant (Cs) from the intrinsic proteins in the pellet (Cp). Equivalent volumes of each fraction were analyzed by immunoblotting using antibodies against Mrh4, the intermembrane space soluble protein Cyt b_2, the inner membrane extrinsic protein Mss51, and the inner membrane intrinsic protein Cox3. The right panel represents a proteinase K protection assay in mitochondria (Mt) and mitoplasts (Mp) prepared by hypotonic swelling of mitochondria. The samples were analyzed by immunoblotting, using antibodies against Mrh4, Cyt b_2, and Cox4 (protein facing the matrix).

(B) Sucrose gradient sedimentation analyses of Mrh4 and ribosomal subunits on mitochondrial extracts prepared from the WT and Δ*mrh4* strains in the presence of 1% digitonin and the conditions stated.

(C) Immunoblot analyses of the steady-state levels of Mrh4 and the indicated r-proteins in WT mitochondria isolated from untreated cells (U) or cells grown in the presence of acriflavin (AF) or ethidium bromide (EtBr) for the indicated times. An antibody against Porin was used as a loading control.

(D) Sucrose gradient sedimentation analyses of Mrh4 and ribosomal subunits on mitochondrial extracts prepared from the strains in (C) in the presence of 1% digitonin and the conditions stated.

(E) Immunoprecipitation of Mrh4 from WT extracts of chemically crosslinked mitochondria as explained in the Experimental Procedures.

(F) PCR analyses of reserve-transcribed Mrh4-coimmunoprecipitated RNA from formaldehyde- or UV-induced crosslinked or noncrosslinked mitochondria. See also Figure S3 and Table S2.

presence of high-salt concentrations, which brought Mrh4 to accumulate in a complex of ~275 kDa (Figure 4B). Moreover, when the extracts were treated with a high concentration of ribonuclease (RNase) to disrupt the ribosomal integrity, Mrh4 also accumulated as a monomer or a small complex (Figure 4B), indicating a loss of Mrh4-ribosome interaction. Mrh4 remained stable and accumulated in a complex of ~180 kDa (data not shown), even in a strain devoid of mtDNA, where rRNA and the mitochondrial ribosomes are absent.

If Mrh4 is acting exclusively as a ribosomal assembly factor, upon completion of its function it is expected to be released rather than remain bound to the growing or assembled ribosomal subunit. According to proteomics studies, the molar amount of Mrh4 is several folds lower than the amount of most LSU r-proteins (Table S2). Hence, one possibility is that Mrh4 acts on assembly and undergoes immediate recycling to assist the formation of a new LSU particle, which appears in our analyses as if it is steadily bound to ribosomes. To clarify this, we next investigated whether the interaction between Mrh4 and mitoribosomes depends on new ribosome assembly, by exposing cells to a low dosage of ethidium bromide (EtBr) or acriflavine to inhibit mitochondrial transcription as reported (Fukuhara and Kujawa, 1970). Treatments with both drugs (for 4 hr), however, induced the accumulation of petite cells to ~80% and ~20% in the presence of EtBr and acriflavine, respectively. The treatments did not affect the steady-state levels of Mrh4 significantly but affected the stability of mitoribosomal proteins (Figure 4C). Nevertheless, the sucrose gradient analysis of the treated cells revealed unexpected results in which a significant amount of the r-proteins continued to accumulate in a complex with a sedimentation similar to that of the fully assembled ribosome, even when the extracts were prepared with EDTA. Importantly, under these conditions, Mrh4 cosedimented with both Mrpl40 and Mrp10 (Figure 4D). These data indicate that the interaction of Mrh4 with the mitoribosomes occurs even in the absence of mitochondrial transcription, hence, in the absence of the mitoribosomal assembly, suggesting that Mrh4 is involved in ribosome assembly and perhaps in further steps during mitochondrial protein synthesis.

Mrh4 Interacts with the *21S* rRNA

Since we observed an interaction of Mrh4 with the LSU, as a DEAD box protein, it would be expected to bind the *21S* rRNA. To test this, highly purified WT mitochondria were subjected to either formaldehyde- or UV-mediated protein-RNA crosslinking or no treatment before disrupting them with 1% SDS, diluting the extract to final 0.05% SDS, proceeding to Mrh4 immunoprecipitation (Figure 4E), and isolation of the coimmunoprecipitated RNA. Following reverse transcription, PCR analysis showed that in both treated and control mitochondria, the *15S* rRNA or *COX1* mRNA were not detected in any sample. In contrast, *21S* rRNA was detected exclusively in crosslinked samples (Figure 4F), thus demonstrating an interaction of Mrh4 with the *21S* rRNA in vivo.

The ATP-Dependent Helicase Activity of Mrh4 Is Essential for Its Function

We next asked whether the ATP-dependent helicase activity of Mrh4 is necessary for its role in mitochondrial translation. An affirmative answer to this question was already suggested by the fact that mutations altering the environment of its ATP binding domain produce ts phenotypes (Figure 3). To further investigate this possibility, we performed site-directed mutagenesis of the ATP-binding and DEAD box conserved regions in Mrh4 (Figure 5A). Mutation of alanine A163 to valine in the ATPase-A motif and a change of aspartate D287 in the DEAD box motif to asparagine (but not to glutamate) abolished the function of Mrh4 (Figures 5B and 5C), as reported for other helicases (Pause and Sonenberg, 1992). We conclude that the ATP-dependent helicase activity of Mrh4 is necessary for its function in vivo.

Mrh4 Is Not Required for the Processing or Modification of the *21S* rRNA

In all systems, the rRNAs are synthesized as precursors that need to undergo a series of processing and modifications prior to becoming a mature transcript. Acquiring proper RNA secondary structures and identifying the correct modification sites followed by a conformational change are considered crucial steps in this process. The key players assisting these processes in both *Escherichia coli* (Srivastava and Schlessinger, 1988) and *S. cerevisiae* (Bohnsack et al., 2009) systems are RNA helicases. Therefore, we decided to test a possible role of Mrh4 in assisting the processing or modification of the *21S* rRNA. The *S. cerevisiae* intronless *21S* rRNA precursor is an ~3.9–4.1 kb transcript that is matured into a 3.1 kb transcript by the removal of an ~900 bp extension from the 3′ end (no apparent information available on 5′ end processing) (Merten et al., 1980). Northern blot analysis (Figure 2C, see also Figure S1) showed a complete processing at the 3′ end of the *21S* rRNA in the Δ*mrh4* intronless strain. A possible defect in microprocessing at the 5′ and the 3′ end of the *21S* rRNA was subsequently tested by primer extension analysis at the 5′ end and RNase H digestion at the 3′ end in the presence of a DNA/RNA chimeric oligonucleotide followed by northern blot analysis (Li et al., 1999). Our results discarded any processing defect at both 3′ and 5′ ends of the *21S* rRNA in the absence of Mrh4 (Figures S1A and S1B). In yeast mitochondria, the *21S* mRNA contains three modified nucleotides: one pseudouridine ($\Psi_{2,819}$) and two 2′-*O*-methylated nucleotides ($Gm_{2,270}$ and $Um_{2,791}$). Gm2,270 and Um2,791 are catalyzed by the 2′-*O*-ribose methyltransferases Mrm1 (or Pet56; Sirum-Connolly and Mason, 1993) and Mrm2 (Pintard et al., 2002), respectively. As the phenotype of *mrm1* mutants resembles that of the Δ*mrh4*, an aberration in the site-specific methylation of the *21S* rRNA in the Δ*mrh4* strain was speculated. However, primer extension analyses failed to show any defect in both $G_{2,270}$ and $U_{2,791}$ methylations in the Δ*mrh4* strain (Figure S1C).

A Large LSU Assembly Intermediate Accumulates in the Absence of Mrh4

Another essential step in ribosome biogenesis is the actual formation of the ribonucleoprotein particle. In *E. coli*, the r-proteins assemble with the rRNA concomitantly with their synthesis and processing in a stepwise manner, progressing through a series of subassembly particles. The r-proteins directly interacting with the 23S rRNA create binding sites for additional r-proteins during this assembly process (Shajani et al., 2011). Hence, we investigated whether any ribosome assembly intermediate, the potential Mrh4 substrate, accumulates in the absence of Mrh4.

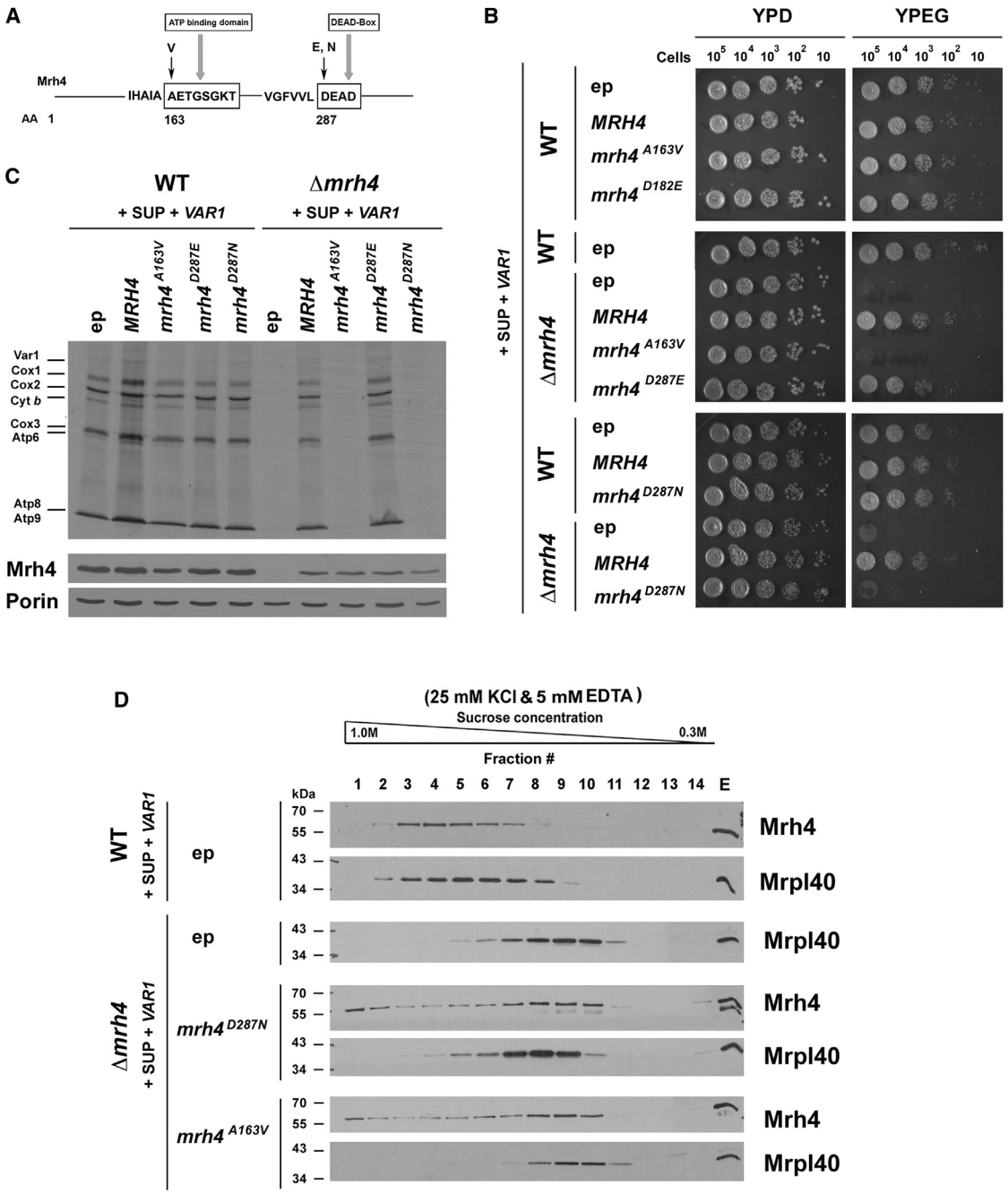

Figure 5. The Helicase Activity of Mrh4 Is Required for Function In Vivo

(A) Scheme showing the ATP-binding and DEAD Box motifs in Mrh4 and the mutated amino acids.

(B) Growth test using serial dilutions of the indicated strains in complete media containing fermentable (YPD) or nonfermentable (YPEG) carbon sources. The plates were incubated at 30°C, and the pictures were taken after 2–3 days of growth. ep, empty plasmid.

(C) In vivo mitochondrial protein synthesis (MPS) in wild-type (WT) and Δ*mrh4* strains expressing the indicated *MRH4* alleles. The bottom panel shows immunoblot analysis of Mrh4 levels using porin as a loading control.

Sucrose gradient analyses showed that both the SSU and the LSU from WT mitochondria sediment faster than the ribosomal particles from Δ*mrh4* mitochondria. A slower sedimentation profile (30S ribonucleoparticle) for the SSU was expected for the Δ*mrh4/SUP* strain due to the absence of Var1 (Sanchirico et al., 1995), although upon expressing the ectopic *VAR1*, complete assembly of the 37S mitoribosomal subunit was restored (Figure 6A). However, the strain remained unable to synthesize mtDNA-encoded proteins (Figure 2A), since the complete assembly of the 54S subunit is still impaired as we only detected a 54S-precursor particle (pre-54S) with a slower sedimentation profile (similar to the SSU) (Figure 6A).

Analyses of the levels of r-proteins in Δ*mrh4* mitochondria indicated that they were similar, or elevated, in comparison with those of the WT mitochondria (Figure 6B, see also Table S3). The accumulation of r-proteins in Δ*mrh4* mitochondria differs from ρ^0 mitochondria (Figure 6B), devoid of mtDNA, in which the turnover of these proteins, the early-stage assembly proteins in particular, is enhanced in the absence of rRNA, as reported (Kaur and Stuart, 2011). These results further indicated that in Δ*mrh4* mitochondria, at least a set of r-proteins is capable of forming a proteolysis-protected, partially assembled ribosome particle (Figure 6A), presumably containing the *21S* rRNA.

The 54S Precursor that Accumulates in Δ*mrh4* Mitochondria Is Bound to the Inner Membrane

Mitochondrial ribosomes are tethered to the inner membrane. It has been proposed that the actual process of mitoribosome assembly occurs in contact with the membrane (Kaur and Stuart, 2011). To investigate whether the Δ*mrh4* pre-54S particle is membrane bound, we mildly sonicated Δ*mrh4* mitochondria, recovered the mitochondrial membranes by low-speed centrifugation, and subjected the membranes to alkaline carbonate extraction to disrupt the ionic interactions between the proteins peripherally bound to the membranes. Similar to WT mitochondria, the r-proteins Mrpl40, Mrp20, Mrpl4, Mrpl13, Mrpl36, Mrpl22, Mrp7, Mrpl32, and Mrp49 cofractionated with Mss51, a marker for inner membrane proteins loosely associated to the membrane (Figure S4). A similar membrane association behavior was reported for nonassembled r-proteins in ρ^0 cells and in cells that accumulate a ribosomal assembly subcomplex composed of tunnel-exit-site proteins due to the expression of a truncated form of Mrp20 (Kaur and Stuart, 2011). Together with these results, our data further support the possibility of mitoribosome assembly occurring on the matrix surface of the inner mitochondrial membrane.

Mrh4 Is Required for a Late-Stage Assembly Step of the 54S Ribonucleoprotein Particle that Affects Its Association with the Small Subunit

To gain an insight into the composition of the pre-54S particle accumulated in Δ*mrh4* mitochondria, sucrose gradients were analyzed for RNA and protein content by measuring the RNA concentration at 260 nm and probing immunoblots with all available antibodies against 54S r-proteins, respectively. As expected, equivalent amounts of *15S* rRNA were measured, forming the 37S SSU in mitochondrial extracts from WT and Δ*mrh4* cells expressing ectopic *VAR1*. Importantly, the levels of *21S* rRNA in WT 54S and Δ*mrh4* 54S precursor were also similar (Figures 6C and 6D), indicating the ribonucleoprotein nature of the 54S precursor that accumulates in the absence of Mrh4. In WT mitochondrial extracts prepared in the presence of Mg^{2+}, all of the r-proteins cosedimented in the same pool of fractions. Approximately 30%–40% of LSU proteins sedimented faster together with a portion of SSU proteins, indicating the presence of fully assembled ribosomes, whereas the rest of proteins sedimented in fractions corresponding to dissociated 54S subunits (Figure 6C, see also Figure S4). In EDTA-containing gradients, most proteins sedimented as part of the 54S subunit, although smaller subassemblies were also detected for Mrpl36, Mrp7, and Mrp49, indicating an instability due to the extraction conditions used (Figure 6D). On the other hand, in the Δ*mrh4* mitochondrial extracts prepared with Mg^{2+}, the pre-54S LSU particle was not detected cosedimenting with the SSU. Even though this pre-54S forms a large complex that sediments in fractions similar to the WT 54S subunit extracted in the presence of EDTA (Figure 6C), it is still impaired from associating with the SSU. When the extracts were prepared with EDTA, a large assembly intermediate (yet with slower sedimentation than the 54S subunit) was detected containing all the r-proteins tested by immunoblotting, even though significant amounts of Mrpl36, Mrp7, and Mrp49 were also found in smaller complexes (Figure 6C). These results suggested that the pre-54S could be heterogeneous and perhaps missing small sets of certain r-proteins.

It has been proposed that a late-stage 54S precursor completes its assembly at the inner membrane where it incorporates the r-protein Mrpl32 (Nolden et al., 2005). The Δ*mrh4* pre-54S contains Mrpl32, which supports the late-stage nature of the intermediate and suggests the requirement of Mrh4 subsequently to the incorporation of Mrpl32. However, based on the sucrose gradient analyses in the presence of Mg^{2+} or EDTA (Figures 6C and 6D), it is possible that in Δ*mrh4* mitochondria, most, if not all, proteins incorporate into the pre-54S, but are unable to acquire the correct conformation, as described in some bacterial mutants (Dohme and Nierhaus, 1976), thus becoming unstable. In support of this view, the sucrose gradient sedimentation of purified ribosomes in the presence of Mg^{2+} and high salt concentrations (500 mM NH_4Cl) showed that even though the SSU remained essentially intact, a portion of the pre-54S formed in the absence of Mrh4 was markedly disintegrated. This disintegration presumably caused the degradation of some *21S* rRNA and led to the accumulation of 40%–50% 54S r-proteins (Mrpl40 and Mrpl4) as monomers (Figure 6E). These results indicate the unstable nature of the 54S-precursor particle and further confirm the requirement of Mrh4 to stabilize and perhaps promote proper conformation of the 54S preassemblies.

To determine precisely if any proteins are missing in the pre-54S complex, fractions 6, 9, and 12 from the Δ*mrh4* sucrose gradient and fraction 5 from the WT sucrose gradient in Figure 6D were methanol/chloroform precipitated and analyzed by mass spectrometry. Rtc6 (*Eco*L36) was not detected in any sample, probably due to its smaller size, as seen in *E. coli* samples (Sharpe Elles et al., 2009). Mrpl36, Mrp7, and Mrp49 were consistently detected in both Δ*mrh4* and WT fractions. All the other LSU r-proteins were also detected, with three exceptions: Mrpl50 (*Eco*L9), Mrpl16 (*Eco*L16), and Mrpl39 (*Eco*L33) were essentially absent exclusively from the Δ*mrh4* fractions (Table S3, see also Figure 6D). These subunits are either inefficiently incorporated or particularly susceptible to dissociation from the LSU in the absence of Mrh4.

Interestingly, the bacterial counterparts of these proteins are incorporated during late steps of the LSU assembly process (Nierhaus, 1991), which further supports a role of Mrh4 at a late stage of 54S assembly. Mrpl50 is known to be dispensable for respiratory growth (Gan et al., 2002), although mutations in *Eco*L9 increase levels of ribosome hopping (Herbst et al., 1994). *Eco*L16 and *Eco*L33 play important structural and perhaps catalytic roles. tRNA crosslinking studies have shown that in the 50S subunit, *Eco*L16 was crosslinked from the A site, *Eco*L16 and *Eco*L33 from the P site, and *Eco*L33 from the

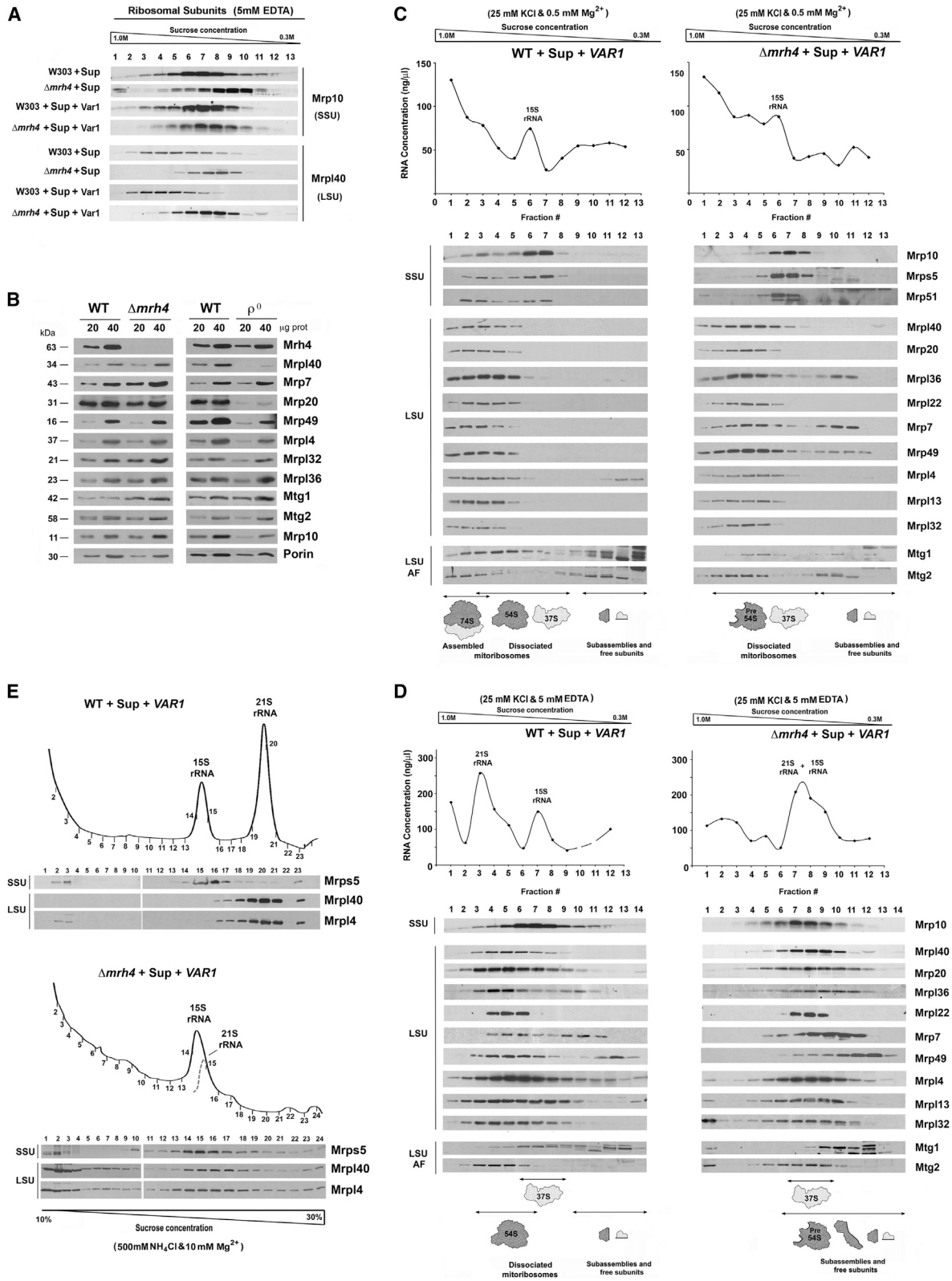

Figure 6. Mrh4 Is Essential for a Late-Stage Step in 54S Mitoribosome Assembly

(A) Sucrose gradient sedimentation profiles of Mrp10 from the small ribosomal subunit (SSU) and Mrpl40 from the large ribosomal subunit (LSU) in mitochondrial extracts prepared in the presence of 1% digitonin and the indicated conditions from the WT and Δ*mrh4* strains expressing SUP and ectopic *VAR1*.

(B) Steady-state levels of LSU proteins and assembly factors in the indicated strains analyzed by immunoblotting.

(legend continued on next page)

E site (Osswald et al., 1995). Whereas *Eco*L33 does not seem to play a major role in ribosome assembly (Maguire and Wild, 1997), *Eco*L16 has been shown to accelerate the late steps of in vitro assembly (Franceschi and Nierhaus, 1990) and to induce a conformational change in the 50S, which may in turn affect the peptidyltransferase activity and subunit association of the ribosome (Martín-Marcos et al., 2007).

The Large Ribosomal Subunit Assembly Factors Mtg1 and Mtg2 Cosediment with the Δ*mrh4* 54S Precursor

Several proteins relevant to ribosome assembly and translation were also detected in our mass spectrometry studies. Two LSU assembly factors, the GTPases Mtg1 and Mtg2, were present in both the WT and Δ*mrh4* fractions. The *21S* rRNA methyltransferase Mrm1 was detected in both fractions, whereas Mrm2 was found exclusively in the mutant fractions (Table S4, see also Figure 6D). Additionally, most mRNA-specific translational activators were detected in WT and Δ*mrh4* fractions, with the exception of Mss51 and Pet494, which were detected exclusively in the WT fraction (Table S5, see also Figure 6D).

Focusing on Mtg1 and Mtg2, their steady-state levels, as for Mrh4, were similar in the presence and absence of mtDNA (Figure 6B). However, Mtg1 levels were increased in Δ*mrh4* mitochondria (Figure 6B), perhaps reflecting a compensatory or regulatory mechanism occurring during the assembly of the 54S ribosomal subunit. Sucrose gradient sedimentation profiles in WT mitochondria confirmed the cosedimentation of Mtg1 and Mtg2 with assembled ribosomes and the 54S LSU, even though Mtg1 showed a slight instability in EDTA extracts (Figures 6C and 6D). In Δ*mrh4*, when extracts were prepared with Mg^{2+}, both Mtg1 and Mtg2 cosedimented with the pre-54S, while in extracts prepared in the presence of EDTA, only Mtg2 cosedimented with the pre-54S (Figures 6C and 6D). These results indicate that Mtg1 and Mtg2 are recruited to the 54S assembly line either prior to or independently of the action of Mrh4.

Several bacterial ribosomal mutants accumulate 40S–45S particles, each lacking small sets of proteins that overlap with those missing in the Δ*mrh4* pre-54S particle. One of them is a *Bacillus subtilis* mutant of YlqF, homolog to yeast Mtg1. YlqF homologs are widely present in gram-positive bacteria, archaea, and all eukaryotes and also found in a few gram-negative bacteria (but not *E. coli*). Quantitative mass spectrometry and cryo-electron microscopy (cryo-EM) structural analyses of the 45S particles accumulated in YlqF-depleted *B. subtilis* cells revealed that the r-proteins L16, L27, L28, L33, L35, and L36 are missing or dramatically underrepresented in 45S intermediates (Li et al., 2013). This spectrum is similar to several 50S precursors analyzed from *E. coli*, indicating the presence of global rate-limiting steps in the late-stage assembly of bacterial 50S subunit (Li et al., 2013). The structural analysis of the 45S particles revealed several major conformations of the 23S rRNA, differing in the stability of the functional centers of the 50S subunit and the orientation of a long helix H38, indicating that these particles are defective in both subunit association and tRNA binding (Li et al., 2013). H38, known as the aminoacyl-binding site (A site) finger (ASF), is located in the intersubunit space of the ribosomal 50S subunit, forms one of the RNA-protein bridges (bridge B1a) with the 30S subunit, and interacts directly with both A site and peptidyl-binding site (P site) tRNAs throughout the decoding process (Komoda et al., 2006). YlqF was proposed to act as an rRNA chaperone to facilitate the reorientation of H38 into its native conformation, which would lead to a global stabilization of the whole central protuberance of the 50S subunit (Li et al., 2013). H38 is truncated in the mammalian mitoribosome, where bridge B1a is replaced by a protein-protein bridge (Sharma et al., 2003). On the contrary, H38 is well conserved in the *S. cerevisiae* mitochondrial 21S rRNA. In yeast mitochondria, if Mrh4 is acting subsequently to Mtg1, it could play a role in stabilizing a 54S intermediate, perhaps directly involving H38 or adjacent helices, to facilitate late r-protein binding.

Another bacterial protein particularly relevant to our studies is DbpA, an *E. coli* 3′–5′ RNA DEAD box helicase, whose helicase and ATPase activities are dependent on a specific region in the 23S rRNA, helix 92, located within the peptidyl transferase center (PTC) of the ribosome (Diges and Uhlenbeck, 2001). Overexpression of a dominant-negative *dbpa* mutant induced a deficit in 50S subunits and gave rise to a 45S particle containing reduced levels of L16, L25, L27, L28, L33, L34, and L35 (Sharpe Elles et al., 2009). Several of the missing r-proteins bind near the PTC, where DbpA also binds, suggesting the requirement of a DbpA-mediated conformational change for the binding of these r-proteins at this site. As mentioned earlier, Mrh4 could play a similar role on the assembly of the 54S mitochondrial LSU subunit, perhaps in cooperation with Mtg1. Future work will be devoted to precisely mapping the *21S* rRNA helix target of Mrh4.

The Δ*mrh4* 54S Precursor Is an On-Pathway Intermediate

Mrh4 variants carrying mutations in the ATP-binding and DEAD box motifs cosedimented with the pre-54S particle in sucrose gradients (Figure 5D). This indicates that even when Mrh4 is catalytically inactive, it is still capable of binding to its substrate. It further suggests that the pre-54S particle could be an intermediate in the 54S assembly pathway upon which Mrh4 normally acts. To test this possibility, we performed two independent experiments. In the first experiment, highly purified WT and Δ*mrh4* mitochondria were used for in organello transcription in the presence of [^{3}H]UTP. All the samples were extensively washed and submitted to a brief sonication, after which one aliquot of disrupted Δ*mrh4* mitochondria was mixed with a ρ^0 cell extract containing Mrh4 and r-subunits. Following incubation for 30 min at room temperature, proteins were extracted from the three samples, and their sucrose gradient sedimentation profiles

(C and D) Sucrose gradient sedimentation analyses of SSU and LSU proteins and LSU assembly factors (AF) in mitochondrial extracts from the indicated strains prepared in the presence of 1% digitonin, 25 mM KCl, and either (C) 0.5 mM Mg^{2+} or (D) 5 mM EDTA. The fractions were used to measure total RNA concentration (top) and to analyze the distribution of Mrh4 and the ribosomal proteins by immunoblotting (bottom).
(E) Mitoribosomes from WT and Δ*mrh4* strains purified in the presence of 10 mM Mg^{2+} and 500 mM NH_4Cl were fractionated on a sucrose gradient using a BRB-188 Density Gradient Fractionator (Brandel). For each strain, a continuous A254 nm record is presented (top). The dashed line indicates the probable position of the 21S rRNA peak, based on the immunoblotting results presented at the bottom. See also Figure S4. and Tables S2–S5

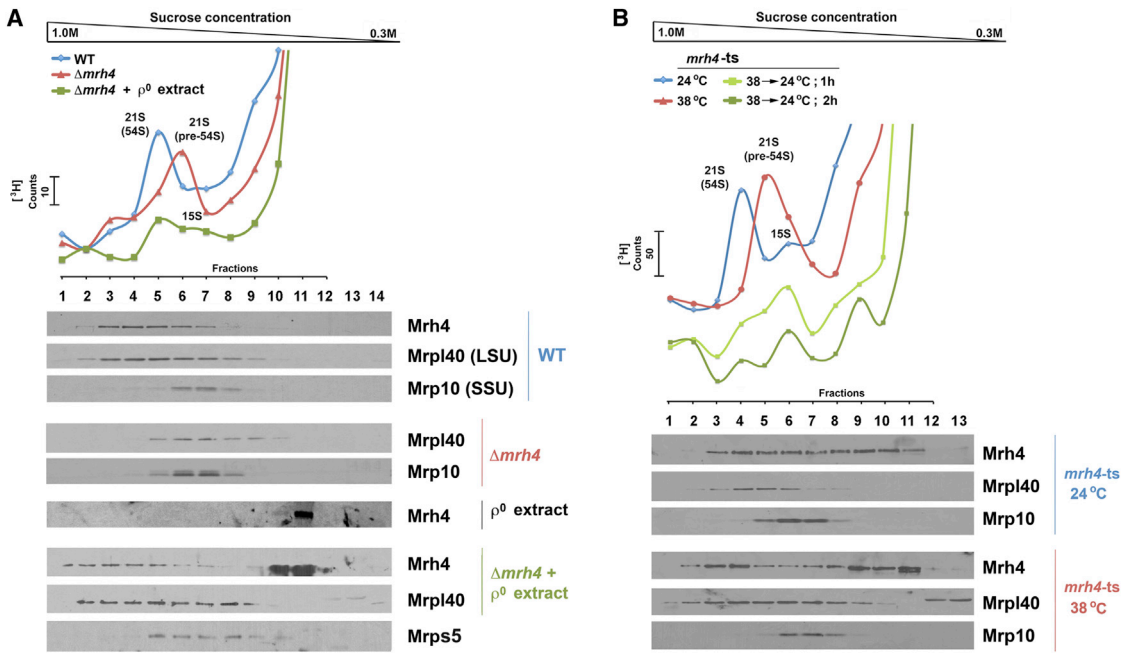

Figure 7. The Δ*mrh4* Pre-54S Particle Is an On-Pathway Intermediate

(A and B) Sucrose gradient sedimentation analyses of [^{3}H]UTP-labeled rRNA in (A) WT and Δ*mrh4* mitochondrial extracts and Δ*mrh4* extracts incubated with ρ^0 extracts containing Mrh4 and r-proteins and (B) mitochondria purified from the *mrh4*-ts2 mutant strain grown at either 24°C or 38°C and aliquots of 38°C-grown mitochondria incubated at 24°C for 1 or 2 hr. The graph represents the [^{3}H] count in each fraction. In the lower panels, the fractions in each case were used for immunoblot analysis of Mrh4 and the indicated r-proteins.

were analyzed by measuring the incorporation of [^{3}H]UTP into rRNA and immunostaining. In the second approach, mitochondria purified from the *mrh4*-ts2 mutant strain grown at either 24°C or 38°C were used for in organello transcription in the presence of [^{3}H]UTP. Following extensive wash, aliquots of 38°C-grown mitochondria were incubated at 24°C for 1 or 2 hr prior to processing as explained earlier. In both experiments, a portion of the pre-54S particle was chased into a particle with 54S sedimentation properties (Figures 7A and 7B), demonstrating that it is an intermediate in the 54S assembly pathway.

Is Mrh4 Conserved in Humans?

Whereas DEAD box proteins contain a conserved core region, their primary sequence is significantly divergent. Currently, it is uncertain whether Mrh4 is conserved in humans. The best BLAST match of Mrh4 in the human proteome is the DEAD box helicase DDX28, a protein with dual location in the nucleus and mitochondria (Valgardsdottir et al., 2001) that shares 23.4% identity and 39% similarity with Mrh4, particularly outside the helicase domains. Although heterologously expressed human DDX28 is imported into yeast mitochondria, it does not complement a Δ*mrh4* strain (Figure S2). Ongoing research efforts will help us in determining whether DDX28 functions in human mitoribosome biogenesis and mitochondrial translation.

Conclusion

Thus far, only a few RNA helicases have been shown to be localized to mitochondria and to be involved in mitochondrial RNA metabolism, either in splicing or RNA degradation (Szczesny et al., 2013). Together, our data identify the DEAD box protein Mrh4 as a putative RNA helicase shown to play a role in mitochondrial ribosome biogenesis. The pre-54S particles that accumulate in the absence of Mrh4 contain no, or reduced amounts of, Mrpl16 and Mrpl39, two late-assembly r-proteins. We propose that by interacting with the *21S* rRNA, Mrh4 could act as an RNA chaperone to facilitate RNA-protein interactions or to catalyze protein displacement from RNA, thereby participating in the structural reorganization of late 54S ribonucleoprotein assemblies to allow efficient binding of at least Mrpl16 and Mrpl39. The stable incorporation of these proteins is essential for ribosomal subunit association. In this way, Mrh4 is essential for mitochondrial protein synthesis and, consequently, the biogenesis of the OXPHOS system.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media

All *S. cerevisiae* strains used are listed in Table S6. The construction of strains expressing mutant alleles of *mrh4* and the composition of the standard culture media used are defined in the Supplemental Information.

Peptide Antibody against Mrh4

We have used the services of Open Biosystems and Thermo Scientific to generate an affinity-purified rabbit polyclonal peptide antibody against Mrh4. The peptide, KTKSWAKALPKIIKKHQRLS, comprises amino acids 542–561 on Mrh4.

Characterization of the Mitochondrial Respiratory Chain and Oxidative Phosphorylation System

Endogenous cell respiration was assayed in whole cells in the presence of galactose using a Clark Type polarographic oxygen electrode from Hansatech Instruments at 24°C, as described (Barrientos et al., 2002).

Mitochondria prepared from the different strains were used for spectrophotometric assays carried out at 24°C to measure KCN-sensitive COX activity, antimycin A-sensitive NADH cytochrome *c* reductase, and succinate cytochrome *c* reductase activities and oligomycin-sensitive ATP synthase activity, as described (Barrientos et al., 2002). Total mitochondrial cytochrome spectra were obtained as reported (Barrientos et al., 2002).

In Vivo Mitochondrial Protein Synthesis

Mitochondrial gene products were labeled with [^{35}S]methionine (7 mCi/mmol; PerkinElmer) in whole cells at 30°C in the presence of 0.2 mg/ml cycloheximide to inhibit cytoplasmic protein synthesis (Barrientos et al., 2002). Equivalent amounts of total cellular proteins were separated by SDS-PAGE on a 17.5% polyacrylamide gel, transferred to a nitrocellulose membrane, and exposed to Kodak X-OMAT X-ray film.

Sucrose Gradients

The sedimentation properties in sucrose gradients of Mrh4 and ribosomal proteins from total mitochondrial extracts were analyzed essentially as described (Barrientos et al., 2004). Mitochondria were prepared by the method of Herrmann et al. (1994). Protein from WT and *Δmrh4* (4 mg) was solubilized in 400 μl of extraction buffer (20 mM HEPES [pH 7.4], 0.5 mM phenylmethanesulfonylfluoride (PMSF), 1% digitonin, 0.5 mM $MgCl_2$ or 5 mM EDTA, and 25 mM KCl) on ice for 10 min. The clarified extract obtained by centrifugation at 50,000 × *g* for 15 min was applied to 5 ml of linear 0.3–1.0 M sucrose gradient containing 20 mM HEPES, 0.5 mM PMSF, 0.1% digitonin, 0.5 mM $MgCl_2$ or 5 mM EDTA, and 25 mM KCl. Following centrifugation for 3 hr and 10 min at 40,000 rpm using a Beckman 55 Ti rotor, the gradients were collected in 14 equal fractions. A total of 40 μl from each fraction was used to determine the distribution of Mrh4 and the ribosomal proteins by immunoblot analysis. Simultaneously, 200 μl from each fraction was used to extract RNA using phenol-chloroform and measured using the NanoDrop 2000 spectrophotometer. For some experiments, the mitochondrial extracts were incubated with 600 U/ml RNase (Fermentas) for 30 min on ice prior to adding on to the sucrose gradients. To test the salt sensitivity of the Mrh4-ribosome interaction, some gradients were performed on extracts prepared in the presence of 250 mM KCl. All of the gradients were performed at least in triplicate using independent mitochondrial preparations. The gradients reported are representative of each strain because the patterns observed were reproducible.

Mitoribosomes prepared from mitochondrial fractions that were highly purified using a sucrose step gradient as reported (Meisinger et al., 2006) to minimize contamination from cytoplasmic ribosomes were also analyzed by sucrose density gradient sedimentation as described (Fearon and Mason, 1992), with slight modifications described in the Supplemental Experimental Procedures. In this case, ribosomal extracts were layered on a 10 ml, 10%–30% linear sucrose gradient containing 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 7 mM β-mercaptoethanol, and 500 mM NH_4Cl. The gradients were centrifuged at 40,000 rpm for 4 hr and 40 min at 4°C in a Beckman SW 41Ti rotor. The gradients were fractionated into 400 μl aliquots, and the UV absorbance at 254 nm was monitored using an ISCO continuous-flow cuvette using a BRB-188 Density Gradient Fractionator (Brandel). Protein samples were precipitated by addition of trichloroacetic acid (TCA) to 15%, separated by SDS-PAGE, and subjected to immunoblot analysis.

RNA Analysis

For in organello transcription assays followed by chase of newly synthesized transcripts, RNA synthesis in isolated yeast mitochondria was performed in the presence of uridine 5′-triphosphate, tetrasodium salt, [5.6-^{3}H] ([^{3}H]UTP) as reported (Groot et al., 1981). RNA-protein crosslinking with UV or formaldehyde followed by immunoprecipitation assays were performed essentially as described (Antonicka et al., 2013). In both cases, the methods were slightly modified, as described in the Supplemental Experimental Procedures.

Methods for RNA isolation, primer extension analyses of the 21S rRNA, and site-directed cleavage of the 3′ end of 21S rRNA by RNase H are also described in the Supplemental Experimental Procedures.

Statistical Analysis

All of the experiments were done at least in triplicate. The data are presented as the means ± SD of absolute values or percentages of control. The values obtained for WT and Δ*mrh4* mutant strains for the different parameters studied were compared by Student's t test. $p < 0.05$ was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2013.10.007.

ACKNOWLEDGMENTS

We thank Dr. S. Sultana (University of Miami) for technical assistance on primer extension analyses. We thank Dr. M. Boguta (Warsaw University of Technology), Dr. T. Fox (Cornell University), Dr. M. Ott (Stockholm University), Dr. R. Stuart (Marquette University), and Dr. A. Tzagoloff (Columbia University) for providing reagents. This research was supported by an NIH-RO1 GM071775-06 Grant (to A.B.), an MDA Research Grant (to A.B.), an MDA Development Grant (to F.F.), and an American Heart Association predoctoral fellowship (to D.D.). The authors declare that they do not have any conflict of interest.

Received: May 20, 2013
Revised: September 4, 2013
Accepted: October 8, 2013
Published: November 5, 2013

REFERENCES

Abu Dayyeh, B.K., Quan, T.K., Castro, M., and Ruby, S.W. (2002). Probing interactions between the U2 small nuclear ribonucleoprotein and the DEAD-box protein, Prp5. J. Biol. Chem. *277*, 20221–20233.

Antonicka, H., Sasarman, F., Nishimura, T., Paupe, V., and Shoubridge, E.A. (2013). The mitochondrial RNA-binding protein GRSF1 localizes to RNA granules and is required for posttranscriptional mitochondrial gene expression. Cell Metab. *17*, 386–398.

Barrientos, A., Korr, D., and Tzagoloff, A. (2002). Shy1p is necessary for full expression of mitochondrial *COX1* in the yeast model of Leigh's syndrome. EMBO J. *21*, 43–52.

Barrientos, A., Korr, D., Barwell, K.J., Sjulsen, C., Gajewski, C.D., Manfredi, G., Ackerman, S., and Tzagoloff, A. (2003). *MTG1* codes for a conserved protein required for mitochondrial translation. Mol. Biol. Cell *14*, 2292–2302.

Barrientos, A., Zambrano, A., and Tzagoloff, A. (2004). Mss51p and Cox14p jointly regulate mitochondrial Cox1p expression in *Saccharomyces cerevisiae*. EMBO J. *23*, 3472–3482.

Bohnsack, M.T., Martin, R., Granneman, S., Ruprecht, M., Schleiff, E., and Tollervey, D. (2009). Prp43 bound at different sites on the pre-rRNA performs distinct functions in ribosome synthesis. Mol. Cell *36*, 583–592.

Cámara, Y., Asin-Cayuela, J., Park, C.B., Metodiev, M.D., Shi, Y., Ruzzenente, B., Kukat, C., Habermann, B., Wibom, R., Hultenby, K., et al. (2011). MTERF4 regulates translation by targeting the methyltransferase NSUN4 to the mammalian mitochondrial ribosome. Cell Metab. *13*, 527–539.

Christian, B.E., and Spremulli, L.L. (2012). Mechanism of protein biosynthesis in mammalian mitochondria. Biochim. Biophys. Acta *1819*, 1035–1054.

Datta, K., Fuentes, J.L., and Maddock, J.R. (2005). The yeast GTPase Mtg2p is required for mitochondrial translation and partially suppresses an rRNA methyltransferase mutant, *mrm2*. Mol. Biol. Cell *16*, 954–963.

Diges, C.M., and Uhlenbeck, O.C. (2001). *Escherichia coli* DbpA is an RNA helicase that requires hairpin 92 of 23S rRNA. EMBO J. *20*, 5503–5512.

Dohme, F., and Nierhaus, K.H. (1976). Total reconstitution and assembly of 50 S subunits from *Escherichia coli* Ribosomes in vitro. J. Mol. Biol. *107*, 585–599.

Fearon, K., and Mason, T.L. (1992). Structure and function of MRP20 and MRP49, the nuclear genes for two proteins of the 54 S subunit of the yeast mitochondrial ribosome. J. Biol. Chem. *267*, 5162–5170.

Franceschi, F.J., and Nierhaus, K.H. (1990). Ribosomal proteins L15 and L16 are mere late assembly proteins of the large ribosomal subunit. Analysis of an *Escherichia coli* mutant lacking L15. J. Biol. Chem. *265*, 16676–16682.

Fukuhara, H., and Kujawa, C. (1970). Selective inhibition of the in vivo transcription of mitochondrial DNA by ethidium bromide and by acriflavin. Biochem. Biophys. Res. Commun. *41*, 1002–1008.

Gan, X., Kitakawa, M., Yoshino, K., Oshiro, N., Yonezawa, K., and Isono, K. (2002). Tag-mediated isolation of yeast mitochondrial ribosome and mass spectrometric identification of its new components. Eur. J. Biochem. *269*, 5203–5214.

Gavin, A.C., Bösche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J.M., Michon, A.M., Cruciat, C.M., et al. (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature *415*, 141–147.

Groot, G.S., van Harten-Loosbroek, N., van Ommen, G.J., and Pijst, H.L. (1981). RNA synthesis in isolated yeast mitochondria. Nucleic Acids Res. *9*, 6369–6377.

Guenther, U.P., and Jankowsky, E. (2009). Helicase multitasking in ribosome assembly. Mol. Cell *36*, 537–538.

He, J., Cooper, H.M., Reyes, A., Di Re, M., Kazak, L., Wood, S.R., Mao, C.C., Fearnley, I.M., Walker, J.E., and Holt, I.J. (2012). Human C4orf14 interacts with the mitochondrial nucleoid and is involved in the biogenesis of the small mitochondrial ribosomal subunit. Nucleic Acids Res. *40*, 6097–6108.

Herbst, K.L., Nichols, L.M., Gesteland, R.F., and Weiss, R.B. (1994). A mutation in ribosomal protein L9 affects ribosomal hopping during translation of gene 60 from bacteriophage T4. Proc. Natl. Acad. Sci. USA *91*, 12525–12529.

Herrmann, J.M., Stuart, R.A., Craig, E.A., and Neupert, W. (1994). Mitochondrial heat shock protein 70, a molecular chaperone for proteins encoded by mitochondrial DNA. J. Cell Biol. *127*, 893–902.

Huang, H.R., Rowe, C.E., Mohr, S., Jiang, Y., Lambowitz, A.M., and Perlman, P.S. (2005). The splicing of yeast mitochondrial group I and group II introns requires a DEAD-box protein with RNA chaperone function. Proc. Natl. Acad. Sci. USA *102*, 163–168.

Kaur, J., and Stuart, R.A. (2011). Truncation of the Mrp20 protein reveals new ribosome-assembly subcomplex in mitochondria. EMBO Rep. *12*, 950–955.

Kehrein, K., Bonnefoy, N., and Ott, M. (2013). Mitochondrial Protein Synthesis: Efficiency and Accuracy. Antioxid. Redox Signal. http://dx.doi.org/10.1089/ars.2012.4896.

Komoda, T., Sato, N.S., Phelps, S.S., Namba, N., Joseph, S., and Suzuki, T. (2006). The A-site finger in 23 S rRNA acts as a functional attenuator for translocation. J. Biol. Chem. *281*, 32303–32309.

Kotani, T., Akabane, S., Takeyasu, K., Ueda, T., and Takeuchi, N. (2013). Human G-proteins, ObgH1 and Mtg1, associate with the large mitochondrial ribosome subunit and are involved in translation and assembly of respiratory complexes. Nucleic Acids Res. *41*, 3713–3722.

Li, Z., Pandit, S., and Deutscher, M.P. (1999). Maturation of 23S ribosomal RNA requires the exoribonuclease RNase T. RNA *5*, 139–146.

Li, N., Chen, Y., Guo, Q., Zhang, Y., Yuan, Y., Ma, C., Deng, H., Lei, J., and Gao, N. (2013). Cryo-EM structures of the late-stage assembly intermediates of the bacterial 50S ribosomal subunit. Nucleic Acids Res. *41*, 7073–7083.

Linder, P., and Jankowsky, E. (2011). From unwinding to clamping - the DEAD box RNA helicase family. Nat. Rev. Mol. Cell Biol. *12*, 505–516.

Maguire, B.A., and Wild, D.G. (1997). The roles of proteins L28 and L33 in the assembly and function of *Escherichia coli* ribosomes in vivo. Mol. Microbiol. *23*, 237–245.

Margulis, L. (1975). Symbiotic theory of the origin of eukaryotic organelles; criteria for proof. Symp. Soc. Exp. Biol. *1975*, 21–38.

Martín-Marcos, P., Hinnebusch, A.G., and Tamame, M. (2007). Ribosomal protein L33 is required for ribosome biogenesis, subunit joining, and repression of GCN4 translation. Mol. Cell. Biol. *27*, 5968–5985.

Meisinger, C., Pfanner, N., and Truscott, K.N. (2006). Isolation of yeast mitochondria. Methods Mol. Biol. *313*, 33–39.

Merten, S., Synenki, R.M., Locker, J., Christianson, T., and Rabinowitz, M. (1980). Processing of precursors of 21S ribosomal RNA from yeast mitochondria. Proc. Natl. Acad. Sci. USA *77*, 1417–1421.

Metodiev, M.D., Lesko, N., Park, C.B., Cámara, Y., Shi, Y., Wibom, R., Hultenby, K., Gustafsson, C.M., and Larsson, N.G. (2009). Methylation of 12S rRNA is necessary for in vivo stability of the small subunit of the mammalian mitochondrial ribosome. Cell Metab. *9*, 386–397.

Nierhaus, K.H. (1991). The assembly of prokaryotic ribosomes. Biochimie *73*, 739–755.

Nolden, M., Ehses, S., Koppen, M., Bernacchia, A., Rugarli, E.I., and Langer, T. (2005). The m-AAA protease defective in hereditary spastic paraplegia controls ribosome assembly in mitochondria. Cell *123*, 277–289.

O'Brien, T.W. (2002). Evolution of a protein-rich mitochondrial ribosome: implications for human genetic disease. Gene *286*, 73–79.

Osswald, M., Döring, T., and Brimacombe, R. (1995). The ribosomal neighbourhood of the central fold of tRNA: cross-links from position 47 of tRNA located at the A, P or E site. Nucleic Acids Res. *23*, 4635–4641.

Parsyan, A., Svitkin, Y., Shahbazian, D., Gkogkas, C., Lasko, P., Merrick, W.C., and Sonenberg, N. (2011). mRNA helicases: the tacticians of translational control. Nat. Rev. Mol. Cell Biol. *12*, 235–245.

Pause, A., and Sonenberg, N. (1992). Mutational analysis of a DEAD box RNA helicase: the mammalian translation initiation factor eIF-4A. EMBO J. *11*, 2643–2654.

Pérez-Martínez, X., Funes, S., Camacho-Villasana, Y., Marjavaara, S., Tavares-Carreón, F., and Shingú-Vázquez, M. (2008). Protein synthesis and assembly in mitochondrial disorders. Curr. Top. Med. Chem. *8*, 1335–1350.

Pintard, L., Bujnicki, J.M., Lapeyre, B., and Bonnerot, C. (2002). MRM2 encodes a novel yeast mitochondrial 21S rRNA methyltransferase. EMBO J. *21*, 1139–1147.

Rorbach, J., Gammage, P.A., and Minczuk, M. (2012). C7orf30 is necessary for biogenesis of the large subunit of the mitochondrial ribosome. Nucleic Acids Res. *40*, 4097–4109.

Sanchirico, M., Tzellas, A., Fox, T.D., Conrad-Webb, H., Periman, P.S., and Mason, T.L. (1995). Relocation of the unusual *VAR1* gene from the mitochondrion to the nucleus. Biochem. Cell Biol. *73*, 987–995.

Schmidt, U., Lehmann, K., and Stahl, U. (2002). A novel mitochondrial DEAD box protein (Mrh4) required for maintenance of mtDNA in *Saccharomyces cerevisiae*. FEMS Yeast Res. *2*, 267–276.

Seidel-Rogol, B.L., McCulloch, V., and Shadel, G.S. (2003). Human mitochondrial transcription factor B1 methylates ribosomal RNA at a conserved stem-loop. Nat. Genet. *33*, 23–24.

Shajani, Z., Sykes, M.T., and Williamson, J.R. (2011). Assembly of bacterial ribosomes. Annu. Rev. Biochem. *80*, 501–526.

Sharma, M.R., Koc, E.C., Datta, P.P., Booth, T.M., Spremulli, L.L., and Agrawal, R.K. (2003). Structure of the mammalian mitochondrial ribosome reveals an expanded functional role for its component proteins. Cell *115*, 97–108.

Sharpe Elles, L.M., Sykes, M.T., Williamson, J.R., and Uhlenbeck, O.C. (2009). A dominant negative mutant of the *E. coli* RNA helicase DbpA blocks assembly of the 50S ribosomal subunit. Nucleic Acids Res. *37*, 6503–6514.

Sirum-Connolly, K., and Mason, T.L. (1993). Functional requirement of a site-specific ribose methylation in ribosomal RNA. Science *262*, 1886–1889.

Sirum-Connolly, K., and Mason, T.L. (1995). The role of nucleotide modifications in the yeast mitochondrial ribosome. Nucleic Acids Symp. Ser. *1995*, 73–75.

Smits, P., Smeitink, J.A., van den Heuvel, L.P., Huynen, M.A., and Ettema, T.J. (2007). Reconstructing the evolution of the mitochondrial ribosomal proteome. Nucleic Acids Res. *35*, 4686–4703.

Srivastava, A.K., and Schlessinger, D. (1988). Coregulation of processing and translation: mature 5′ termini of *Escherichia coli* 23S ribosomal RNA form in polysomes. Proc. Natl. Acad. Sci. USA *85*, 7144–7148.

Surovtseva, Y.V., and Shadel, G.S. (2013). Transcription-independent role for human mitochondrial RNA polymerase in mitochondrial ribosome biogenesis. Nucleic Acids Res. *41*, 2479–2488.

Szczesny, R.J., Wojcik, M.A., Borowski, L.S., Szewczyk, M.J., Skrok, M.M., Golik, P., and Stepien, P.P. (2013). Yeast and human mitochondrial helicases. Biochim. Biophys. Acta *1829*, 842–853, http://dx.doi.org/10.1016/j.bbagrm.2013.1002.1009.

Valgardsdottir, R., Brede, G., Eide, L.G., Frengen, E., and Prydz, H. (2001). Cloning and characterization of MDDX28, a putative dead-box helicase with mitochondrial and nuclear localization. J. Biol. Chem. *276*, 32056–32063.

van Dyck, L., Neupert, W., and Langer, T. (1998). The ATP-dependent PIM1 protease is required for the expression of intron-containing genes in mitochondria. Genes Dev. *12*, 1515–1524.

Wredenberg, A., Lagouge, M., Bratic, A., Metodiev, M.D., Spåhr, H., Mourier, A., Freyer, C., Ruzzenente, B., Tain, L., Grönke, S., et al. (2013). MTERF3 regulates mitochondrial ribosome biogenesis in invertebrates and mammals. PLoS Genet. *9*, e1003178.