

# The translational activator Sov1 coordinates mitochondrial gene expression with mitoribosome biogenesis

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## ABSTRACT

**Mitoribosome biogenesis is an expensive metabolic process that is essential to maintain cellular respiratory capacity and requires the stoichiometric accumulation of rRNAs and proteins encoded in two distinct genomes. In yeast, the ribosomal protein Var1, alias uS3m, is mitochondrion-encoded. uS3m is a protein universally present in all ribosomes, where it forms part of the small subunit (SSU) mRNA entry channel and plays a pivotal role in ribosome loading onto the mRNA. However, despite its critical functional role, very little is known concerning *VAR1* gene expression. Here, we demonstrate that the protein Sov1 is an *in bona fide* *VAR1* mRNA translational activator and additionally interacts with newly synthesized Var1 polypeptide. Moreover, we show that Sov1 assists the late steps of mtSSU biogenesis involving the incorporation of Var1, an event necessary for uS14 and mS46 assembly. Notably, we have uncovered a translational regulatory mechanism by which Sov1 fine-tunes Var1 synthesis with its assembly into the mitoribosome.**

## INTRODUCTION

Ribosome biogenesis is an energetically expensive process that needs to be regulated to facilitate the stoichiometric accumulation of ribosomal RNAs (rRNAs) and proteins, and to respond to the nutritional status of the cell (1). In *Escherichia coli*, among the several mechanisms in place, synthesis of most ribosomal proteins is regulated by negative feedback loops in which specific ribosomal proteins, when in excess, act as translational repressors by binding to their own mRNAs (2). Translational autoregulation control also operates in mitochondria from the yeast *Saccharomyces cerevisiae* to coordinate the synthesis of oxidative phosphorylation (OXPHOS) complex subunits with their

assembly into functional enzymes. In yeast mitochondria, however, regulation involves a ternary element known as translational activator that binds to both a specific mRNA to promote translation and to the newly synthesized protein to stabilize it and chaperone its incorporation into the specific OXPHOS complex (3). The yeast mitochondrial genome (mtDNA) encodes for eight polypeptides, of which seven are catalytic subunits of the OXPHOS system complexes and one, Var1 (Variant protein 1), is a structural component of the mitochondrial ribosome small subunit (mtSSU) (4,5). Whether Var1 synthesis and mitoribosome assembly are translationally regulated remains to be demonstrated.

Var1, recently renamed as uS3m according to the standard nomenclature for ribosomal proteins (6), is a positively charged soluble protein universally present in all ribosomes (bacterial, cytosolic and mitochondrial), where it localizes in the mtSSU head and forms part of the mRNA entry channel (7–10). Yeast Var1 owes its name to the high degree of variability it has amongst different strains (11), which results from insertions of GC clusters and changes in the length of AT-rich stretches, mainly coding for asparagine residues (12,13). The structure and location of the *VAR1* gene are also heterogeneous across eukaryotes (14). Whereas in metazoan Var1/uS3m is always encoded in the nuclear genome, it is expressed from their mitochondrial genome in several plants and fungi. The retention of the *VAR1* gene in the mtDNA is intriguing, considering that Var1 is a soluble protein, and therefore, hydrophobicity, which could prevent mitochondrial import, should not represent a barrier to nuclear transfer of the *VAR1* gene. Indeed, it has been shown that a functional Var1 protein can be allotopically expressed from a recoded gene relocated to the nucleus (15). Alternatively, *VAR1* retention in the mtDNA could be linked to the control of gene expression by the mitochondrial functional state, as previously proposed for mitochondrion-encoded OXPHOS subunits (16).

In mitochondria, gene expression is controlled mostly at the post-transcriptional level. Yeast mitochondrial (mt)

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mRNAs contain long 5'-untranslated regions (5'-UTR), where translational activators bind to promote translational initiation. Numerous translational activators have been identified in yeast mitochondria, and their role in the biogenesis of the mitochondrion-encoded OXPHOS subunits has been extensively characterized (17). Their functions comprise multiple levels of regulation, including the coordinated synthesis of subunits from the same OXPHOS complex (18), and the assembly dependent translational control of gene expression (3). However, despite the wealth of information regarding the factors involved in the translation of mitochondrion-encoded OXPHOS subunits, very little is known concerning *VAR1* gene expression.

The open reading frame YMR066W was first identified in a random mutagenesis-based screen to encode for a mitochondrial protein required for the expression of the mitochondrial reporter gene *ARG8<sup>m</sup>* placed under the control of *VAR1* 5'-UTR (19). The encoded polypeptide was renamed Sov1 for Synthesis of Var1 and proposed to promote *VAR1* mRNA translation (19). However, both *VAR1* and *VAR1::ARG8<sup>m</sup>* transcripts were undetectable in *sov1* null mutant strains, and Sov1 involvement in Var1 synthesis was only based on the observation that the Arg8<sup>m</sup> reporter protein level was increased when *SOV1* was overexpressed (19). More recently, the deletion of *sov1* gene, as well as several mitochondrial translational activators, have been linked to Sir2-dependent life span extension, suggesting a possible regulatory function of Sov1 in nucleus-mitochondrion communication (20).

In the present study, we have been able to stabilize *VAR1* mRNA in the absence of Sov1 and demonstrate that Sov1 plays a role as a *bona fide* *VAR1* translational activator. Furthermore, we have uncovered a negative feedback loop mechanism by which Sov1 regulates not only Var1 expression but also its assembly during mitoribosome biogenesis. Sov1 promotes *VAR1* mRNA translation and, additionally, interacts with newly synthesized Var1 and mediates its assembly into the mtSSU. In the absence of Sov1, mtSSU biogenesis is impaired at the late stages that involve the incorporation of Var1/uS3m, and also its interacting partners uS14 and mS46. Based on the dual role of Sov1 in Var1 synthesis and assembly, we propose a working model according to which mitochondrial *VAR1* gene expression is coordinated with mitoribosome assembly. From an evolutionary point of view, the existence of this regulatory negative feedback loop could contribute to explain the retention of the *VAR1* gene in the yeast mitochondrial genome.

## MATERIALS AND METHODS

### Yeast strains and media

All *S. cerevisiae* strains used are listed in Supplementary Table S4. The composition of the standard culture medium used is defined in the Supplementary Material.

### Mitochondrial protein synthesis

Mitochondrial gene products were labeled with [<sup>35</sup>S]-methionine (7 mCi/mmol, Perkin Elmer) in whole cells at room temperature in the presence of 0.2 mg/ml cyclohex-

imide to inhibit cytoplasmic protein synthesis (21). Equivalent amounts of total cellular proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 17.5% polyacrylamide gel, transferred to a nitrocellulose membrane and exposed to X-ray film.

*In organello* mitochondrial translation and co-immunoprecipitation of newly synthesized polypeptides was performed as previously reported (22).

### Sucrose gradient analysis

The sedimentation properties in sucrose gradients of Sov1-HA and the mitoribosomes were analyzed essentially as described (23). Mitochondria were prepared by the method of Herrmann *et al.* (24). Four mg of protein were solubilized in 400 µl of extraction buffer (20 mM HEPES, pH 7.4, 0.5 mM PMSF, 0.8% digitonin, 0.5 mM MgCl<sub>2</sub> or 5 mM ethylenediaminetetraacetic acid (EDTA) and 25 mM KCl) on ice for 5 min. EDTA was used to analyze the 54S and 37S subunits separately, while Mg<sup>2+</sup> was added to preserve their interaction into the 74S monosome. The clarified extract obtained by centrifugation at 50 000 × g<sub>AV</sub> for 15 min at 4°C was applied to a 5 ml linear 0.3 M–1.0 M sucrose gradient containing 20 mM HEPES, 0.5 mM PMSF, 0.1% digitonin, 0.5 mM MgCl<sub>2</sub> or 5 mM EDTA and 25 mM KCl. Following centrifugation for 3 h and 10 min at 40 000 r.p.m. at 4°C using a Beckman 55Ti rotor, the gradients were collected in 14 equal fractions. Twenty µl from each fraction was used to determine the distributions of the proteins of interest by immunoblot analysis. For some experiments, the mitochondrial extracts were resolved in a 10 ml linear 10–30% sucrose gradient centrifuged for 24 h at 24 000 r.p.m. at 4°C using a Beckman 41Ti rotor. These gradients were collected in 26 equal fractions.

For protein identification, proteins from gradient fractions were methanol/chloroform precipitated as reported (25), and mass-spectrometry analysis was performed by SPARC BioCenter Molecular Analysis, The Hospital for Sick Children, Toronto, Canada.

### Sov1-HA immunoprecipitation

A functional Sov1 fused in frame to a hemagglutinin (HA) tag was expressed from an integrative vector in strains carrying a null mutant allele of *sov1*. Proteins were extracted from isolated mitochondria as for sucrose gradient analysis (see above). Clarified extracts were incubated with Anti-HA-conjugated agarose beads (Sigma) or protein A-agarose beads (Thermo) as a negative control for 4 h at 4°C with gentle rocking. After centrifugation at 500 × g<sub>AV</sub> for 5 min, supernatants were recovered, and beads washed 3 times with cold phosphate-buffered saline + 0.4% digitonin. The Sov1-HA fusion protein was eluted with Laemmli buffer, and samples were analyzed by Western blotting. All antibodies used in the study are listed in Supplementary Table S5. For Sov1-HA immunoprecipitation analysis from sucrose gradient, fractions were diluted with an equal volume of extraction buffer without digitonin prior to incubation with either Anti-HA-conjugated or protein A-conjugated agarose beads.

## Statistical analysis

All the experiments were done at least in duplicate. Quantitative data are presented as the means  $\pm$  S.D. of absolute values or percentages of control from triplicate experiments. The values for the several parameters studied were compared by Student's *t*-test.  $P < 0.05$  was considered significant.

## RESULTS

### Generation of $\Delta sov1$ strains with stable mtDNA

To determine the exact role that the Sov1 protein plays in yeast mitochondrial gene expression, we started by generating a modified *sov1* null mutant strain. In yeast, pharmacological treatments or genetic defects that abolish mitochondrial translation are known to affect mtDNA maintenance (26). However, retention of the mitochondrial genome in translation mutants can be achieved by overexpression of the *RNR1* gene (25), encoding the catalytic subunit of the ribonucleotide-diphosphate reductase, or the *YMC2* gene (23), encoding a mitochondrial glutamate transporter (27). We reasoned that if lack of Sov1 compromises *mt-VAR1* gene expression, as previously reported (19), it would, in turn, affect mitoribosome formation and overall mitochondrial protein synthesis, leading to mtDNA depletion. To prevent mtDNA loss, we replace the entire *SOV1* ORF with a KAN-MX4 cassette conferring geneticin resistance in a diploid W303 wild-type (WT) strain overexpressing either the *RNR1* or the *YMC2* genes. Additionally, the strain carries a mtDNA devoid of introns ( $I^0$ ) and the *pRS316-VAR1<sup>u</sup>* construct (15), which allows for the allotopic expression of a fully functional Var1 protein fused to the Cox4 mitochondrial targeting sequence, hereinafter referred to as universal Var1 (Var1<sup>u</sup>).

Following sporulation of the heterozygous  $\Delta sov1$  diploid and tetrad dissection, haploid *sov1* null mutant spores carrying either no plasmid, *VAR1<sup>u</sup>*, *RNR1* or *YMC2*-expressing plasmids were selected. Only  $\Delta sov1$  spores expressing Var1<sup>u</sup> retained mtDNA, while all others resulted in being  $\rho^0$  (Supplementary Table S1). The mtDNA instability phenotype appears to be associated with the complete absence of the Var1 protein since overexpression of the *RNR1*, or *YMC2* gene alone was not sufficient to promote mtDNA retention in a *var1* null mutant strain (Supplementary Table S1). Additionally, when cells were grown in glucose-containing media for 2 days ( $\sim 15$  generations), the  $\Delta sov1/VAR1^u$  strain formed *petite* colonies with a slightly increased frequency compared to control ( $\sim 10\%$  versus  $2.5\%$ , Supplementary Table S1) and neither concomitant overexpression of *RNR1* or *YMC2* genes decreased the formation of *petite* colonies to WT level (Supplementary Table S1). Thus, for all the following experiments, we utilized strains expressing Var1<sup>u</sup>, unless otherwise indicated. Moreover, to limit  $\rho^0$  accumulation, cells were routinely grown from fresh  $\rho^+$  cultures and for short periods of time.

It has been previously reported that Var1<sup>u</sup> expression in a WT strain increases *petite* accumulation (28). However, the *VAR1<sup>u</sup>-HA* construct that was used for the study was not able to complement the respiratory deficient phenotype of a

mitochondrial *var1* mutant (19), suggesting that the tagged Var1 protein is not functional and may be more prone to aggregation. In our hands, the W303  $I^0$  strain expressing the *pRS316-VAR1<sup>u</sup>* construct accumulated *petite* colonies (Supplementary Table S1) at levels comparable to the values reported for S288c derivative WT strains (29).

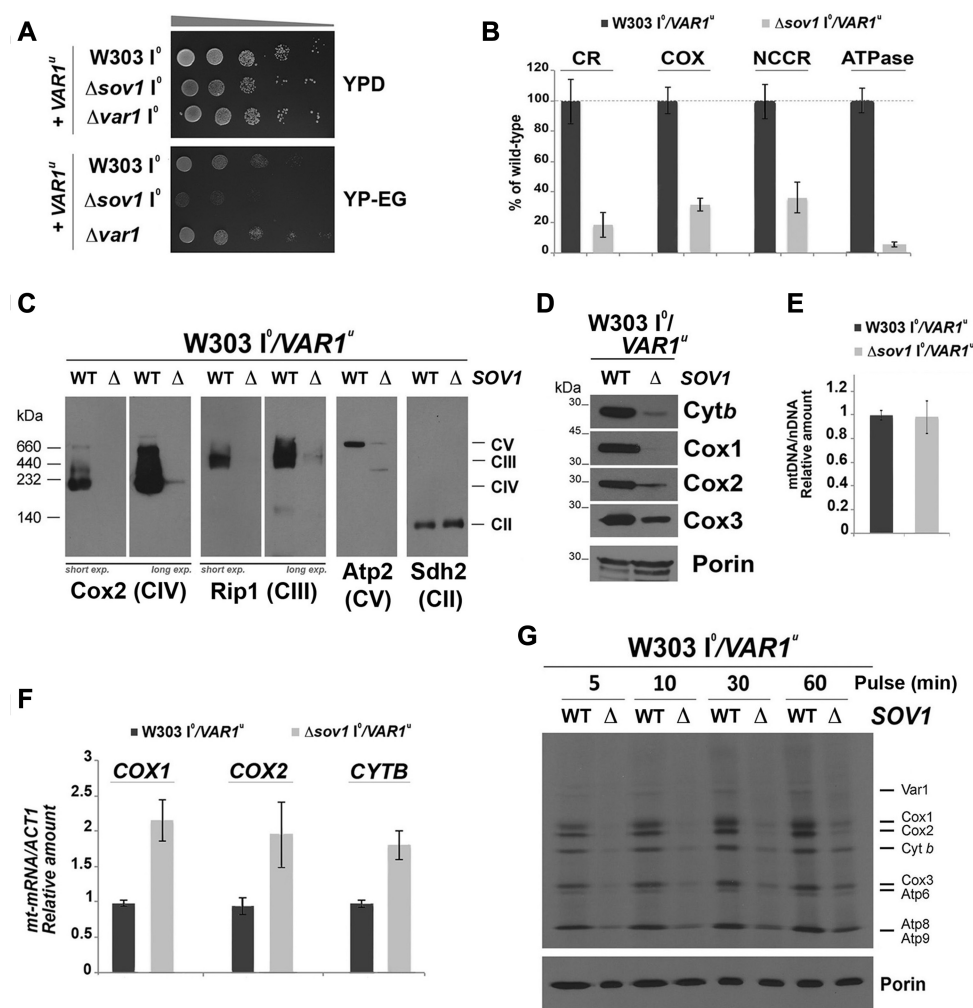
### Lack of Sov1 impairs mitochondrial translation

With the modified  $\Delta sov1$  strain available, we could ask phenotypic questions meaningfully. First, we analyzed the respiratory growth of the *sov1* null mutant strain expressing Var1<sup>u</sup> by serial dilution growth test in media containing either the respiratory substrates ethanol and glycerol (YP-EG) or the fermentable carbon source glucose (YPD) as control. The growth of the  $\Delta sov1/VAR1^u$  mutant was dramatically attenuated in non-fermentable YP-EG media (Figure 1A), due to the decrease of endogenous cell respiration to  $\sim 20\%$  of WT value (Figure 1B). The  $\Delta sov1$  respiratory defect was not due merely to the absence of *mt-VAR1* gene expression since it occurs in the presence of the Var1<sup>u</sup> protein synthesized from the nucleus, which is sufficient to fully support respiratory growth of a mitochondrial *var1* null mutant strain (Figure 1A).

To better dissect the cause of the respiratory deficient phenotype of the  $\Delta sov1/VAR1^u$  strain, we then analyzed the enzymatic activities and steady-state levels of the mitochondrial OXPHOS complexes. NADH-cytochrome *c* reductase (NCCR), cytochrome *c* oxidase (COX) and ATP synthase (ATPase) enzymatic activities, measured spectrophotometrically, were all largely reduced in the  $\Delta sov1/VAR1^u$  mutant compared to control (Figure 1B). Accordingly, the levels of fully assembled *bc1* complex (or CIII), COX (or CIV) and ATP synthase (or CV), analyzed by Blue-Native PAGE, as well as the amount of mitochondrion-encoded CIII and CIV subunits Cytb, Cox1, Cox2 and Cox3 detected by SDS-PAGE, were severely decreased in  $\Delta sov1/VAR1^u$  mitochondria (Figure 1C and D). However, succinate dehydrogenase (or CII), the only OXPHOS complex entirely formed by nucleus-encoded subunits, accumulated in the  $\Delta sov1/VAR1^u$  mutant as in the WT strain (Figure 2C). Overall, this phenotype is suggestive of a mitochondrial genome expression defect.

The gene expression defect was not related to mtDNA levels, which were similar in the  $\Delta sov1/VAR1^u$  mutant and WT strains (Figure 1E). It was not related either to compromised transcription or mRNA instability, since the steady-state levels of mitochondrial *COX1*, *COX2* and *CYTB* mRNAs were actually elevated in the null *sov1* mutant versus control (Figure 1F). This could represent a compensatory effect associated with the respiratory deficiency, similar to what was reported for another mtSSU mutation associated with a partial translational defect (30). On the contrary, analysis of mitochondrial translation by following the rate of incorporation of S<sup>35</sup>-radiolabeled methionine into mitochondrion-encoded polypeptides showed that the newly synthesized mt-Var1 protein was undetectable in the  $\Delta sov1/VAR1^u$  strain for pulses up to 1 h (Figure 1G). Additionally, overall mitochondrial protein synthesis, although not abolished, was markedly decreased in the *sov1* null mutant compared to WT despite the presence of allotopically





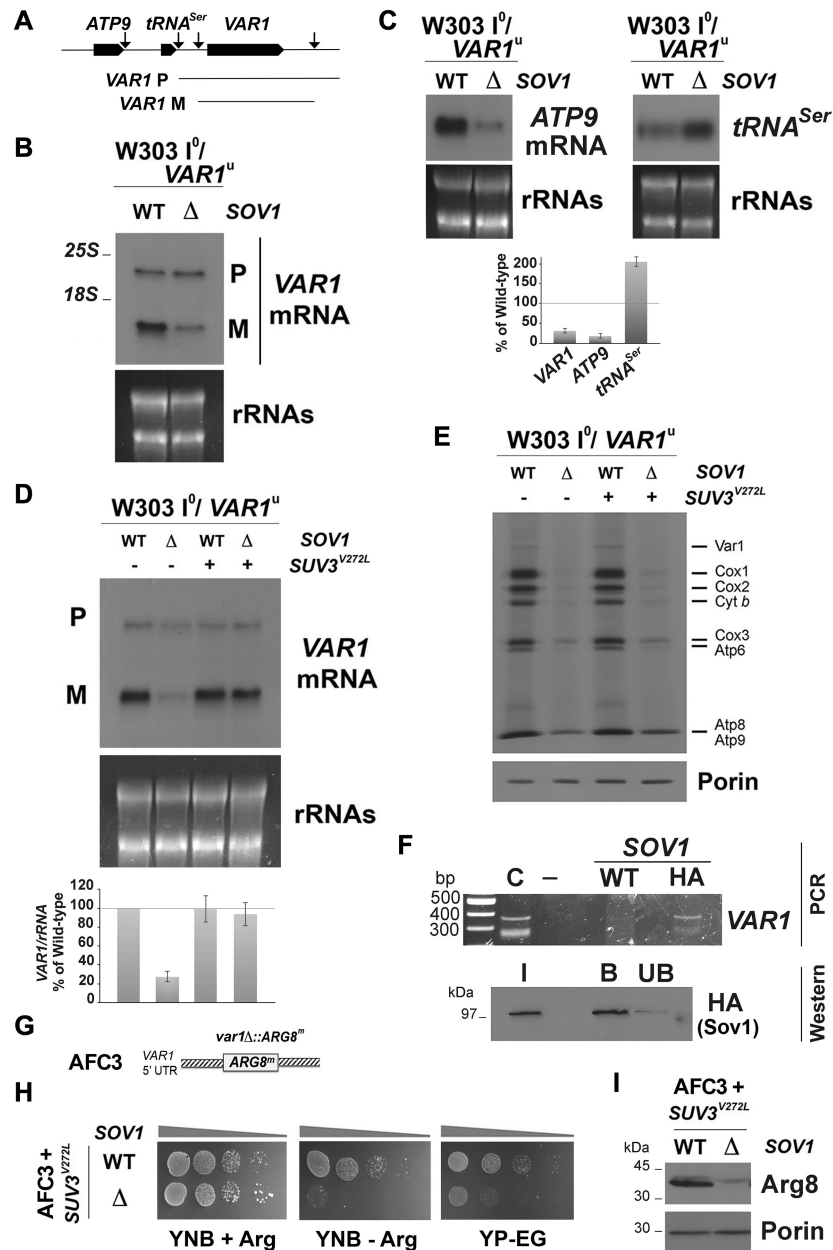
**Figure 1.** Characterization of the respiratory capacity and OXPHOS system in the absence of Sov1. (A) Serial dilution growth analysis of the indicated strains in media containing fermentable (YPD) or respiratory (YP-EG) carbon sources. Pictures were taken after 2 (YPD) or 3 (YP-EG) days of incubation at 30°C. (B) Measurement of OXPHOS parameters in the indicated strains. The graph shows endogenous cell respiration rate (CR) measured polarographically, and the enzymatic activities of cytochrome *c* oxidase (COX), NADH-cytochrome *c* reductase (NCCR) and ATPase, measured spectrophotometrically and expressed as the percentage of WT. Bars represent the mean  $\pm$  SD of three independent repetitions. (C) Steady-state levels of OXPHOS complexes extracted with lauryl maltoside from isolated mitochondria, analyzed by BN-PAGE and detected by immunoblotting with the indicated antibodies. (D) Steady-state levels of the indicated mitochondrion-encoded subunits of complex III (CIII) and complex IV (CIV) analyzed by SDS-PAGE and immunoblotting. Porin was used as loading control. (E) Mitochondrial DNA content (mtDNA) measured in whole cells by qPCR amplification of *COX1* and *ACT1* genes, markers of mtDNA and nuclear DNA (nDNA) levels, respectively. Bars represent the mean  $\pm$  SD of three independent repetitions. (F) Steady-state levels of the indicated mitochondrion-encoded mRNAs, measured by qPCR and normalized by the level of *ACT1* mRNA. Bars represent the mean  $\pm$  SD of three independent repetitions. (G) Metabolic labeling with  $^{35}\text{S}$ -methionine of newly synthesized mitochondrial products in whole cells either WT or lacking *sov1* ( $\Delta$ ) and expressing *Var1*<sup>u</sup> during increasing pulses in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. Immunoblotting for Porin was used as a loading control. Newly synthesized polypeptides are identified on the right.

expressed *Var1*<sup>u</sup> (Figure 1G). Taken together, these data suggest that Sov1 is required for *mt-VAR1* gene expression and plays an additional role in overall mitochondrial protein synthesis beyond promoting *mt-VAR1* mRNA stability and/or translation.

### Sov1 is an *mt-VAR1* mRNA translational activator

The *mt-VAR1* gene is transcribed as part of a polycistronic RNA that also contains the *ATP9* and *tRNA<sup>Ser</sup>* genes (31). Endoribonucleolytic cleavage of the primary transcript releases a 2.4 kb precursor *mt-VAR1* mRNA, which is further processed to generate the mature 1.9 kb *mt-VAR1* mRNA,

including a 162 bp-long 5'-UTR and a 550 bp-long 3'-UTR (Figure 2A and (15)). It has been previously reported that while the accumulation of the precursor mRNA is independent of Sov1, the mature *mt-VAR1* mRNA is undetectable in a *sov1* null mutant (19), making it challenging to prove that Sov1 functions as an *mt-VAR1* mRNA translational activator. Likewise, in our *W303 I°* genetic background, the steady-state level of the precursor *mt-VAR1* mRNA was not affected by the lack of Sov1, whereas the accumulation of the mature *mt-VAR1* mRNA was considerably decreased in the  $\Delta\text{sov1}/\text{VAR1}^\text{u}$  mutant (Figure 2B). These data support a model in which Sov1 is not required for the precursor *mt-VAR1* mRNA processing, but for the stability of the mature



**Figure 2.** Role of *Sov1* in *VAR1* mRNA stability and expression. (A) Schematic representation of the mitochondrial primary polycistronic transcript encompassing *ATP9*, *tRNA<sup>Ser</sup>* and *VAR1* genes (in thicker bars). The sites of endoribonucleolytic cleavage are indicated by arrows. The position of the 2.4 kb precursor (P) and 1.9 kb mature (M) *mt-VAR1* mRNA are indicated by black lines. (B) Northern-blot analysis of total cellular RNAs to detect mature (M) and precursor (P) *mt-VAR1* mRNA steady-state levels in WT and *sov1* null mutant ( $\Delta$ ) strains expressing *Var1<sup>u</sup>*. Cytosolic rRNAs are used as loading control. The position of cytosolic 28S- and 18S-rRNAs (3392 and 1798 nt, respectively) relative to *mt-VAR1* mRNAs is indicated. (C) Northern-blot analysis of total cellular RNAs as in panel (B) to detect *ATP9* mRNA and *tRNA<sup>Ser</sup>*. Cytosolic rRNAs are used as loading control. Lower panel: relative amounts of mature *mt-VAR1* and *ATP9* mRNAs and *tRNA<sup>Ser</sup>* in the *sov1* null mutant strain. Signal from panels B and C were quantified and expressed as percentage of WT. Bars represent the mean  $\pm$  SD of three independent repetitions. (D) *mt-VAR1* mRNA steady-state levels in the indicated strains, as in A. Quantification of the signal for the mature *mt-VAR1* mRNA is reported below the image. Bars represent the mean  $\pm$  SD of three independent repetitions. (E) Metabolic labeling with  $^{35}$ S-methionine of newly synthesized mitochondrial products in whole cells for 10-min pulses in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. Strains included were the WT or *sov1* null mutant ( $\Delta$ ), expressing (+) or not (-) a second copy of the *SUV3* gene carrying the V272L mutation and *Var1<sup>u</sup>*. Immunoblotting for Porin was used as a loading control. Newly synthesized polypeptides are identified on the right. (F) Co-immunoprecipitation of *Sov1*-HA with *mt-VAR1* mRNA, using anti-HA-conjugated agarose beads. In the upper panel the presence of *mt-VAR1* mRNA in the bound material was analyzed by cDNA synthesis and PCR amplification. A strain carrying a non-tagged *Sov1* protein (WT) was used as negative control. Purified mtDNA was used to detect the 376 bp expected amplicon in a control PCR (C). No template DNA PCR was performed as negative control (-). The lower panel shows the analysis of immunoprecipitated fractions by immunoblotting with an anti-HA antibody. I: input. B: bound. UB: unbound. Equivalent amounts of unbound and bound samples were loaded in the gel. (G) Scheme depicting the mitochondrial genotype of strains carrying mitochondrial *ARG8* (*ARG8<sup>m</sup>*) as a reporter of mRNA translation. (H) Serial dilution growth analysis of the indicated strains in yeast nitrogen base minimum media (YNB) supplemented or not with arginine and complete respiratory (YP-EG) media. Pictures were taken after 3 days of incubation at 30°C. AFC3 = *mt-var1 $\Delta$ ::ARG8<sup>m</sup>* + *pRS316-VAR1<sup>u</sup>*. (I) Steady-state levels of the Arg8 protein in the indicated strains. Porin was used as loading control.

*mt-VAR1* mRNA. However, we failed to detect the polycistronic transcript, which is most likely a very low abundant molecule, in either the WT or *sov1* null mutant strains. Moreover, the accumulation of the mature *ATP9* mRNA and tRNA<sup>Ser</sup>, belonging to the same primary RNA that *mt-VAR1*, resulted greatly altered in the absence of Sov1. In particular, the *ATP9* mRNA level was severely decreased, while the amount of tRNA<sup>Ser</sup> was approximately double in the  $\Delta sov1/VAR1^u$  strain compared to control (Figure 2C). Taken together, these data suggest that Sov1 affects multiple aspects of the metabolism of the *mt-VAR1*-containing polycistronic transcript.

In yeast, mitochondrial translation and mRNA splicing are tightly connected since maturases required for mRNA processing are encoded in *COX1* and *CYTB* gene introns (32,33). To test whether the presence of introns in the mtDNA could affect the *sov1* null mutant phenotype, we analyzed the respiratory growth and mitochondrial translation efficiency in  $\Delta sov1/VAR1^u$  strains carrying an intron-containing ( $I^+$ ) or  $I^0$  mtDNA. No differences were observed between *sov1* null mutants irrespectively of their mtDNA intron composition in terms of growth (Supplementary Figure S1A), indicating that Sov1 is not involved in mitochondrial mRNA splicing. Although expression of *COX1* and *CYTB* in the strain harboring introns were negatively impacted by the absence of Sov1 (Supplementary Figure S1B), most likely as a consequence of decreased maturase expression owing to the general defect in mitochondrial translation. Incidentally, despite the severe decrease in *ATP9* mRNA levels in the *sov1* null mutant, the rate of Atp9 synthesis was not further affected when compared to the other mitochondrion-encoded OXPHOS subunits (Supplementary Figure S1B).

The observed instability of the *mt-VAR1* transcript could explain why no *mt-VAR1* translation is detected in the *sov1* null mutant strain. To further investigate this aspect of Sov1 function, we looked for strategies that could help to stabilize the mature *mt-VAR1* mRNA in cells lacking Sov1. In yeast, the mitochondrial genome is transcribed in polycistronic RNAs, which are then processed to generate the mature transcripts. In the case of protein-coding genes, the mRNA site of 3' cleavage is determined by a conserved stretch of 12 nt, the dodecamer sequence (34,35). In addition to defining the site of 3' processing, the dodecamer sequence has been proposed to play a role in mt-mRNA stability and translation (34). The truncation of a fragment of the *VAR1* mRNA 3'-UTR comprising the dodecamer sequence dramatically decreases transcript stability and is associated with respiratory deficiency (36). A spontaneous suppressor of this respiratory growth defect has been isolated and shown to carry a dominant missense mutation in the DEXH/D-box helicase Suv3 (36). Together with the exoribonuclease Dss1, Suv3 forms the mitochondrial RNA degradosome (37). The Suv3 suppressor missense mutation V272L occurs in the helicase motif Ia, which is involved in RNA binding (38), and is associated with alterations in mRNA steady-state levels and accumulation of excised group I introns (39). Since Suv3<sup>V272L</sup> has been shown to enhance the expression of a 3'-UTR truncated *mt-VAR1* mRNA (36), we introduced a single copy of the dominant *SUV3*<sup>V272L</sup> allele in WT and *sov1* null mutant cells. Expression of the Suv3<sup>V272L</sup> variant in the

$\Delta sov1/VAR1^u$  strain restored the accumulation of mature *mt-VAR1* mRNA to WT levels (Figure 2D). Notably, newly synthesized mt-Var1 polypeptide remained undetectable in  $\Delta sov1/VAR1^u/SUV3^{V272L}$  cells (Figure 2E), suggesting that Sov1 is required for *mt-VAR1* mRNA translation.

Sov1 is a pentatricopeptide (PPR) domain-containing protein (40). PPR motifs are degenerate 35 amino acid repeats, which mediate RNA-binding; although in a small number of cases, they have also been shown to bind to DNA or proteins (41). To test whether Sov1 role in promoting *mt-VAR1* gene expression involves its direct interaction with the *mt-VAR1* mRNA, we next performed an immunoprecipitation assay. For this purpose, we generated a Sov1-HA-tagged polypeptide (Sov1-HA)-expressing construct, which in single copy was sufficient to fully complement the respiratory deficient phenotype of a *sov1* null mutant strain (Supplementary Figure S2A). Moreover, Sov1-HA accumulated in mitochondria as a peripherally associated membrane protein (Supplementary Figure S2B-C). Mitochondrial extracts from a *SOV1-HA*  $I^0$  strain were incubated with anti-HA-conjugated agarose beads to immunoprecipitate Sov1-HA (Figure 2F, lower panel). The presence of *mt-VAR1* mRNA in the precipitate fractions was analyzed by cDNA synthesis and polymerase chain reaction (PCR) amplification of a fragment, including the *VAR1* 5'-UTR and the initial 216 bp of the coding sequence. Following PCR, we detected the *VAR1* amplicon specifically in the Sov-HA bound fraction (Figure 2F, upper panel), indicating that Sov1-HA interacts with the *mt-VAR1* mRNA. Specific RNA binding is a common feature of canonical yeast mitochondrial translational activators, including the PPR-protein Pet309 (3). Sov1 binding to the *mt-VAR1* mRNA could directly promote *mt-VAR1* mRNA stability and translational initiation. Moreover, Sov1 could interact with the unprocessed *mt-VAR1*-containing RNA, affecting its secondary structure and/or its accessibility to processing enzymes.

Lastly, to further confirm the Sov1 requirement for *mt-VAR1* mRNA translational initiation, we deleted the *SOV1* gene in a strain carrying the mitochondrial reporter *ARG8<sup>m</sup>* (42) under the control of *VAR1* 5'- and 3'-UTR (Figure 2G) in the presence of the *SUV3*<sup>V272L</sup> allele. The reporter gene is a recoded version of the nuclear *ARG8* gene that encodes for a matrix-localized arginine biosynthetic enzyme. In this way, growth in media lacking arginine represents a direct analysis of mitochondrial translation. The absence of growth on minimal medium lacking arginine and the decreased accumulation of the Arg8 protein in the AFC3- $\Delta sov1$  mutant (Figure 2H-I) indicate that *ARG8<sup>m</sup>* expression also depends on Sov1 and strengthens the specificity of Sov1 in *VAR1* expression.

### Sov1 is required for the late stages of mitoribosome SSU assembly

Sov1 acts by promoting *mt-VAR1* mRNA stability and translation. However, its function cannot be fully bypassed by allotopic Var1<sup>u</sup> expression, suggesting that Sov1 plays an additional role/s in mitochondrial protein synthesis. It has been previously shown that complementation of mutants in the mitochondrial translation factors Pet111 and Aep3 by allotopic expression of *COX2* and *ATP8* respectively de-



depends on a high level of expression achieved by the use of multicopy vectors (43). Therefore, we tested whether overexpression of *VAR1<sup>u</sup>* from the multicopy plasmid YEp351 would improve the respiratory growth of the *sov1* null mutant. Only a modest improvement in respiratory growth was observed when *VAR1<sup>u</sup>* was overexpressed (Supplementary Figure S1C), which strengthens the hypothesis that Sov1 is required for mitochondrial protein synthesis in function/s other than *mt-VAR1* translation.

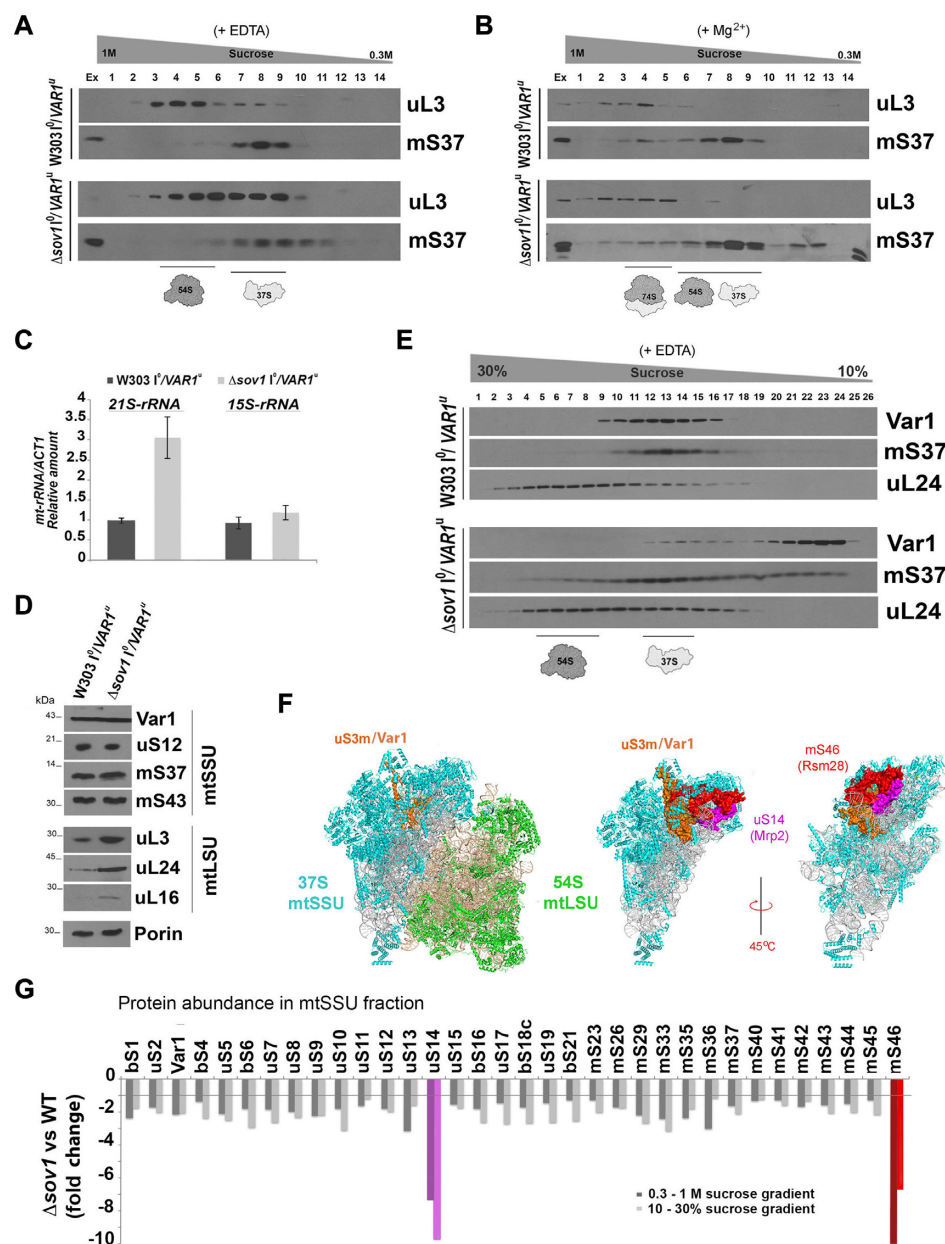
To fully understand Sov1 function and the reason why overall mitochondrial translation is compromised in the  $\Delta\text{sov1}/\text{VAR1}^u$  mutant strain, we turned our attention to the mitochondrial ribosomes. First, we investigated the mitoribosome sedimentation properties by sucrose gradient analysis using mitochondrial protein extracts prepared in the presence of either EDTA, to separate mtSSU and mtLSU, or  $\text{Mg}^{2+}$ , to preserve monosome interactions. mtSSU and mtLSU were detected by immunoblot analysis with antibodies against proteins mS37 and uL3, respectively. The sedimentation profiles of both the mtSSU and the mtLSU were similar when using WT and *sov1* mutant mitochondrial extracts (Figure 3A and B). However, when EDTA was used, we observed broader peaks in the *sov1* null mutant compared to control (Figure 3A), which could indicate ribosomal particle heterogeneity or instability. Moreover, in the *sov1* null mutant, we could detect a lower proportion of the mS37 protein signal sedimenting in the monosome fractions (Figure 3B). This observation was further confirmed by the direct comparison of mS37 and uL3 signals from the relevant sucrose gradient fractions ran in parallel in a single gel (Supplementary Figure S3). The levels of the mtSSU protein mS37 were decreased in the monosome-corresponding fractions in the *sov1* null mutant compared to WT, suggestive of an impaired association between the mtSSU and mtLSU. Additionally, we detected an increase of the mtLSU marker uL3 in the absence of Sov1 in both the fractions where the mtLSU and the monosome peak (Supplementary Figure S3). We then further explored mitoribosome subunits accumulation in WT and  $\Delta\text{sov1}/\text{VAR1}^u$  mitochondria, by detecting the mt-rRNAs by qPCR, as a marker of mitoribosome abundance. We observed a 3-fold increase in the levels of mtLSU *21S-rRNA* in cells lacking Sov1 (Figure 3C). Additionally, the steady-state levels of representative mtLSU mitoribosome proteins were also increased in *sov1* null mutant mitochondria (Figure 3D). Conversely, the mtSSU *15S-rRNA* and representative protein steady-state levels were unchanged in the  $\Delta\text{sov1}/\text{VAR1}^u$  strain compared to WT (Figure 3C and D). We have previously reported that the accumulation of the mtLSU is increased in mitochondria lacking the mtSSU protein mS38 (30). Thus, it appears that this phenotype is not strictly dependent on the absence of Sov1, since it is shared with at least another strain characterized by partially compromised mitochondrial translation. Increased mtLSU accumulation could result from a cellular response attempting to compensate for the inefficient mitoribosome performance. However, we can not exclude the possibility that the biogenesis of the two mitoribosome subunits is subject to co-regulation, a scenario that warrants future investigations.

Since Sov1 is required for Var1 expression in mitochondria, we generated an antibody against the Var1 protein.

The polyclonal antibody allowed us to detect both mitochondrially and allotopically expressed Var1 polypeptides, which can be separated by size under certain electrophoretic conditions (Supplementary Figure S4). Similar to the other mtSSU proteins, Var1 accumulated in equal amounts in WT and *sov1* null mutant mitochondria (Figure 3D), suggesting that Var1<sup>u</sup> is stable in the absence of Sov1. However, while in WT mitochondria all detected Var1 protein appeared to incorporate into the mtSSU, in the  $\Delta\text{sov1}/\text{VAR1}^u$  strain, only a small portion of Var1<sup>u</sup> sedimented in the fractions corresponding to the mtSSU (Figure 3E), suggesting that Var1 protein assembly into the mitoribosome is impaired.

It has been previously reported that the 37S mtSSU shifts to a slower sedimenting 30S particle in either cells treated with the mitochondrial translation inhibitor erythromycin, which leads to the lack of Var1 protein (44), or a strain carrying a thermosensitive mutation in *mt-VAR1* 5'-UTR, which also results in the complete absence of the Var1 protein at the non-permissive temperature (45). Furthermore, it has been roughly estimated by 2D electrophoresis that the 30S mtSSU particle lacks 3 to 5 proteins (44,46), one of which has been identified as uS14 (46).

Using several conditions for sucrose gradient sedimentation analysis, we were not able to resolve the 37S and 30S mtSSU particles in our  $\Delta\text{sov1}/\text{VAR1}^u$  strain (Figure 3A, B and E). One possibility is that both molecules are present in the *sov1* null mutant mitochondria: the fully assembled 37S mtSSU, which would support the detected residual mitochondrial translation, and the Var1-free 30S incomplete mtSSU particle, making it arduous to discriminate them. In order to better characterize the mtSSU molecules accumulating in the  $\Delta\text{sov1}/\text{VAR1}^u$  strain, we subjected the corresponding sucrose gradient fractions (fractions 7-8-9 from Figure 3A and fractions 12-13-14 from Figure 3E) to mass-spectrometry analysis. In WT and *sov1* mutant samples, we detected all mtSSU proteins with the exception of mS38, mS47 and mS48 (Supplementary Table S2). Most mtSSU proteins, including Var1, were present at lower levels in the *sov1* mutant fractions compared to control (1.5- to 2.5-fold decrease). Additionally, the abundance of uS14 and mS46 was 7- to 10-fold reduced in the *sov1* mutant samples in comparison to WT (Figure 3F and G; Supplementary Table S2). According to the recently reported high-resolution structure of the yeast mitoribosome, Var1/uS3m, uS14 and mS46 interact with each other's to form part of the mitoribosome head and mRNA-entry channel (Figure 3F (7)). We observed that in the absence of Sov1, the incorporation of Var1, uS14 and mS46 into the mtSSU is compromised. Taken together, our data suggest that Sov1 is required for the late stages of mtSSU biogenesis. Specifically, Sov1 could act by mediating the incorporation of an assembly module formed by Var1, uS14 and mS46 into the mtSSU. Alternatively, Sov1 may be required for the proper incorporation of the Var1 protein, which in turn is a pre-requisite for the assembly of uS14 and mS46 into the mtSSU. We predicted that, if Var1, uS14 and mS46 are sequentially assembled, Var1 incorporation into the mtSSU late-assembly intermediate would not be affected by the absence of uS14 or mS46. To better understand the interdependence in the assembly of these three mtSSU proteins, we analyzed mitoribosome



**Figure 3.** Characterization of mitochondrial ribosomes in cells lacking Sov1. (A and B) Sucrose gradient sedimentation analyses of mitoribosomal protein markers on mitochondrial extracts prepared from the indicated strains in the presence of 0.8% digitonin and either EDTA (A) or Mg<sup>2+</sup> (B). (C) Steady-state levels of mitochondrial rRNAs 21S and 15S, measured by qPCR and normalized by the level of ACT1 mRNA. Bars represent the mean  $\pm$  SD of three independent repetitions. (D) Steady-state levels of the indicated mitoribosome subunits analyzed by SDS-PAGE and immunoblotting. Porin was used as loading control. (E) Sucrose gradient sedimentation analyses of mitoribosomal protein markers on mitochondrial extracts prepared as in A. (F) Location of the mtSSU proteins Var1 (uS3m), uS14 and mS46 in the yeast mitoribosome structure (PDB ID: 5MRC) (7). (G) Mitoribosome SSU protein relative abundance determined by mass-spectrometry analysis of mtSSU fractions from the 0.3–1 M sucrose gradient in A and the 10–30% sucrose gradient in E.

function and sedimentation properties in strains lacking either uS14 or mS46. Mutant strains expressed Var1<sup>u</sup> and Rnr1 to prevent mtDNA loss. We observed that, whereas the deletion of *uS14* abolished respiratory growth and mitochondrial translation, the absence of mS46 did not compromise either of them (Supplementary Figure S5A and B), suggesting that mS46 is not essential for mitoribosome function. In line with this observation, the mtSSU sedimentation profile in a strain lacking mS46 closely resembled the WT control (Supplementary Figure S5C). Notably, in the absence of mS46, Var1 protein entirely co-sedimented with

the mtSSU marker uS12 (Supplementary Figure S5C), indicating that Var1 incorporation into the mtSSU is not dependent on mS46, but occurs prior to mS46 assembly. Conversely, when we analyzed the Var1<sup>u</sup> sedimentation profile in a strain lacking uS14, we could not detect any Var1 signal in the fractions corresponding to the mtSSU (Supplementary Figure S5C), suggesting that Var1 and uS14 assembly into the mtSSU are interdependent. Overall, our data favor a model in which Var1/uS3m and uS14 are co-inserted into the mtSSU, while mS46 is incorporated in a subsequent step.



### Sov1 interacts with newly synthesized Var1 and the mitoribosome SSU

We have established that Sov1 is required for the late stages of mtSSU biogenesis. To better understand whether Sov1 performs its function in mitoribosome assembly indirectly or through a direct physical interaction, we aimed to characterize the network of Sov1 interacting partners. For this purpose, we utilized the respiratory competent Sov1-HA-expressing strain, described above (Supplementary Figure S2A). Under different sucrose gradient conditions, Sov1-HA was detected by immunoblotting mainly in a single complex in fractions corresponding to ~200 kDa (Figure 4A and B). However, when the X-ray films were exposed for longer times, we were able to detect a small portion of Sov1-HA co-sedimenting with the mtSSU, especially when the analysis was performed in the presence of  $Mg^{2+}$  (Figure 4A and B). No Sov1-HA signal was present in the mtLSU and monosome fractions (Figure 4A and B). Lastly, a small fraction of the total Var1 signal detected in the sucrose gradients sedimented with the Sov1-HA lighter complex (Figure 4A and B). Based on the observed sedimentation profiles, we tested whether Sov1 interacts with the mtSSU and Var1 by immunoprecipitation of the Sov1-HA protein with anti-HA antibody-conjugated beads. We first performed the experiment in strains with or without expression of Var1<sup>u</sup>. Since allotopically expressed Var1<sup>u</sup> protein migrates slightly faster than mitochondrion-encoded Var1 polypeptide in 10%-SDS-PAGE (Supplementary Figure S4B), we were able to detect both in the bound material fractions (Figure 4C). Moreover, the signal of the mtSSU protein mS37 was also enriched in the Sov1-HA immunoprecipitated samples (Figure 4C), suggesting that Sov1 interacts with Var1 and the mtSSU. However, the Sov1-Var1 interaction was not preserved when the immunoprecipitation was performed in the presence of EDTA (Supplementary Figure S6A), which could be indicative of a relatively labile bond. Since Var1 is a structural component of the mtSSU, the interaction of Sov1 with Var1 could be indirect and occur only in the context of the mtSSU. To explore this possibility, we repeated the Sov1-HA immunoprecipitation experiment in a strain expressing Var1<sup>u</sup> and devoid of mtDNA, in consequence, devoid of mt-rRNAs and ultimately mitoribosomes. In this context, we were still able to immunoprecipitate Var1<sup>u</sup> protein together with Sov1-HA (Figure 4D), indicating that the interaction between Sov1 and Var1 is independent of mitoribosome integrity. Moreover, we analyzed the interaction of Sov1-HA with newly synthesized mitochondrial polypeptides by *in organello* translation in presence of S<sup>35</sup>-methionine and immunoprecipitation with an anti-HA antibody. We observed that newly synthesized Var1 was specifically co-immunoprecipitated together with the Sov1-HA protein (Figure 4E). Taken together, our data support a model in which Sov1 interacts with Var1/uS3m and mediates its incorporation in the mtSSU.

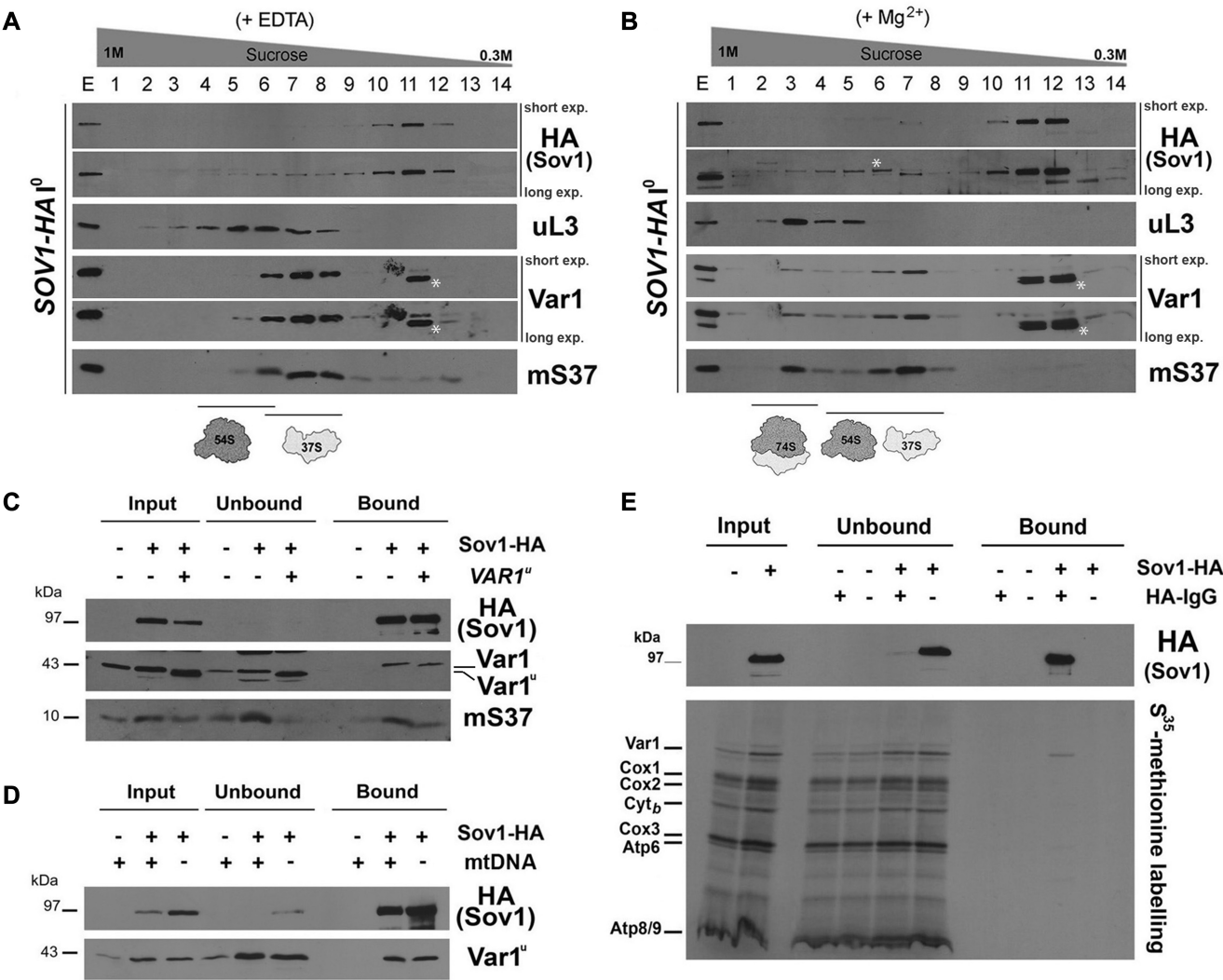
### Sov1 cooperates with the mitochondrial chaperones Tcm62 and Ssc1 to prevent newly synthesized Var1 aggregation

Although, a small fraction of Sov1-HA co-sediments with the mtSSU, most Sov1-HA protein is present in a smaller complex of ~200 kDa (Figures 4B and 5A). To better

dissect the network of Sov1 interactions, we performed Sov1-HA immunoprecipitation from the gradient fractions (fractions 11–12 in Figure 4B) corresponding to the 200 kDa Sov1-HA-containing complex and subjected the bound material to mass-spectrometry analysis. Together with Sov1-HA, we identified the putative mitochondrial chaperone Tcm62, the mitochondrial DEAD-box helicase Mss116 and Var1 (Supplementary Table S3). However, we did not detect either uS14 or mS46 polypeptides. The Tcm62 protein shares 38% sequence similarity with the mitochondrial chaperone Hsp60 and is required for the assembly of MRC CII (47). Additionally, Tcm62 was shown to prevent aggregation of mitochondrial Var1 protein at high temperatures (48). Next in order, Mss116 plays multiple roles in mitochondrial transcript metabolism (49). Specifically, we have recently reported that Mss116 interacts with the PPR-translational activator Pet309 and is required for optimal mitochondrion-encoded COX subunit 1 (Cox1) expression (50). However, no specific defect in mitochondrial *VAR1* translation was observed in an *mss116* null mutant under standard conditions (50).

Subsequently, to confirm the mass-spectrometry results, we obtained specific antibodies against Tcm62 (48) and uS14 (Supplementary Figure S4C) and first assessed their sedimentation properties by sucrose gradient analysis. It has been established before that Tcm62 forms a high molecular mass protein complex of ~850 kDa (48). Under our analysis conditions, Tcm62 sedimented in two distinct peaks, the smaller of which largely overlapped with the 200 kDa Sov1-HA-containing complex (Figure 5A). Additionally, while a minor fraction of Var1 protein also co-sedimented with the small Sov1-HA complex, no extra-mitoribosomal pool of uS14 was detected (Figure 5A). We then repeated the Sov1-HA immunoprecipitation from the gradient fractions in which Sov1-HA sediments, corresponding specifically to the mtSSU and the 200 kDa Sov1-HA complex peaks. Bound and unbound material were analyzed by immunoblotting and allowed us to confirm that Sov1-HA interacts with Tcm62 and Var1 in a complex that does not contain uS14 (Figure 5B). These results indicate that Var1 and uS14 do not form a pre-assembly module, whose incorporation into the mtSSU is mediated by Sov1. Moreover, our data suggest that Tcm62 dissociates from Sov1-HA once that the latest engages with the mtSSU to promote Var1 assembly (Figure 5A and B).

Var1 is the only soluble mitochondrion-encoded protein. It is positively charged and prone to aggregation especially at high temperatures (24). It has been previously reported that Tcm62 exerts a chaperone-like function and prevent heat-induced aggregation of mitochondrial Var1 protein (48). Since Sov1 physically interacts with Tcm62 and the extra-mitoribosomal pool of Var1, we tested whether Var1 is prone to heat-induced aggregation in the absence of Sov1. Isolated mitochondria were incubated at 24 or 37°C for up to 40 min, after which soluble and aggregated molecules were separated by centrifugation and analyzed by immunoblotting, as reported (48). Exposure of WT mitochondria to the higher temperature of 37°C induced a moderate accumulation of Var1 aggregates only after 40 min of incubation and independently on Var1<sup>u</sup> expression (Figure 5C). Otherwise, the lack of Sov1 was associated with a de-

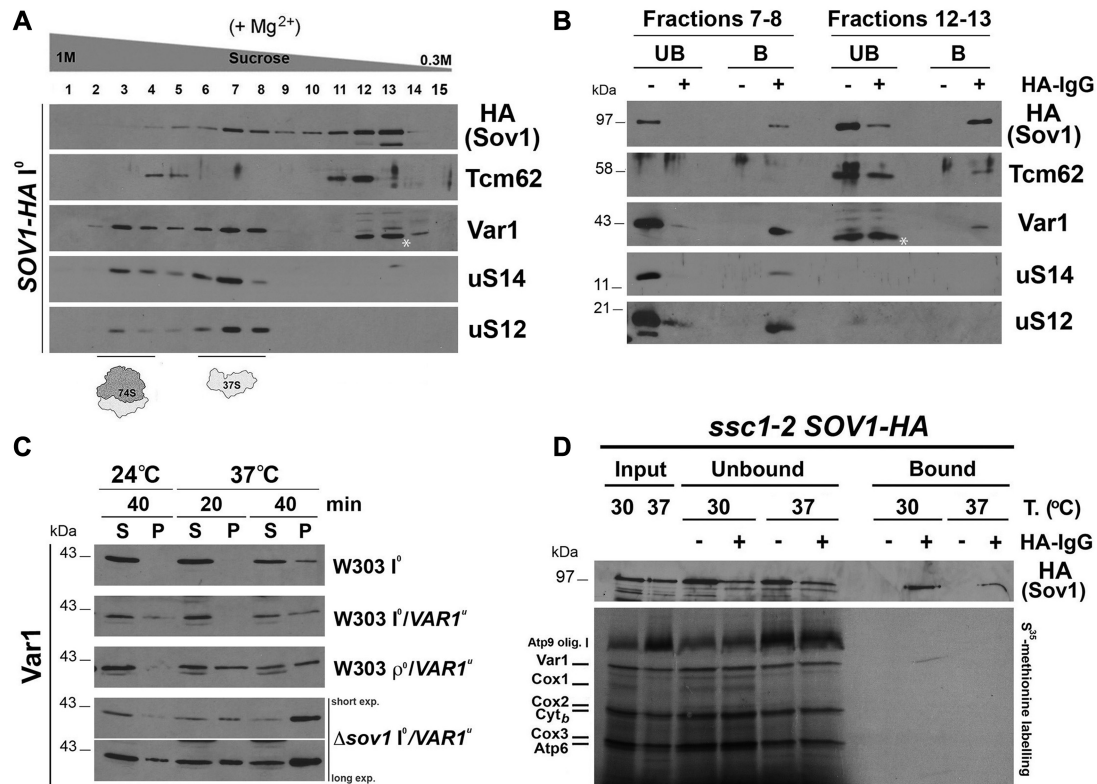


**Figure 4.** Identification of Sov1 interacting partners. (A and B) Sucrose gradient sedimentation analyses of mitoribosomal protein markers on mitochondrial extracts prepared from a strain expressing a Sov1–HA-tagged protein in the presence of 0.8% digitonin and either EDTA (A) or Mg<sup>2+</sup> (B). \* indicates Var1 and HA Abs unspecific bands. (C) Co-immunoprecipitation of Sov1–HA with Var1 and the mtSSU marker mS37, using anti-HA-conjugated agarose beads. *SOV1*–HA strains expressing (+) or not (–) Var1<sup>u</sup> were used. A strain carrying a non-tagged Sov1 protein (W303 I<sup>0</sup>) was used as negative control (–). (D) Co-immunoprecipitation of Sov1–HA with Var1 in a strain devoid of mtDNA (–). The Sov1–HA I<sup>0</sup> strain and a strain carrying a non-tagged Sov1 protein (W303 I<sup>0</sup>) were used as positive (+) and negative (–) controls respectively. In panel C and D, double amounts of bound relative to unbound samples were loaded in the gel. (E) Co-immunoprecipitation of Sov1–HA with mitochondrial newly synthesized Var1 protein, using anti-HA-conjugated agarose beads. A strain carrying a non-tagged Sov1 protein (W303 I<sup>0</sup>) and protein A-conjugated beads were used as negative controls (–). Equivalent amounts of unbound and bound samples were loaded in the gel.

tectable Var1 signal in the pellet fraction already at 24°C, which became the predominant signal at the 37°C latest time point (Figure 5C). Var1 aggregation in the *sov1* null mutant was not exclusively dependent on the lack of mitoribosome assembly, since it was largely more pronounced than in a  $\rho^0$ /VAR1<sup>u</sup> strain (Figure 5C), suggesting that Sov1 is required to prevent Var1 protein aggregation.

In addition to Tcm62, it has also been reported that the mitochondrial Hsp70 chaperone Ssc1 interacts with newly synthesized Var1 and maintains it in a soluble assembly competent state (24). Since Sov1 also interacts with newly synthesized Var1, we asked whether Sov1 and Ssc1 form a ‘chaperone-complex’ to mediate Var1 folding and assembly into the mtSSU. We first tested a possible Sov1–Ssc1 inter-

action by co-immunoprecipitation analysis. However, under all the conditions tested, we were unable to detect any Ssc1 signal in the Sov1–HA bound fractions (Supplementary Figure S6B). Neither, we detected Ssc1 in our Sov1–HA bound fraction mass-spectrometry analysis (Supplementary Table S3). If Sov1 and Ssc1 do not physically interact, they could act on Var1 sequentially. More specifically, Sov1 may be able to bind newly synthesized Var1 only once it has acquired its native conformation by Ssc1-mediated folding. In consequence, we would expect that a compromised Ssc1 chaperoning function should prevent the Sov1–Var1 interaction. To test this hypothesis, we performed a Sov1–HA immunoprecipitation analysis of *in organello* newly synthesized polypeptides using mitochondria



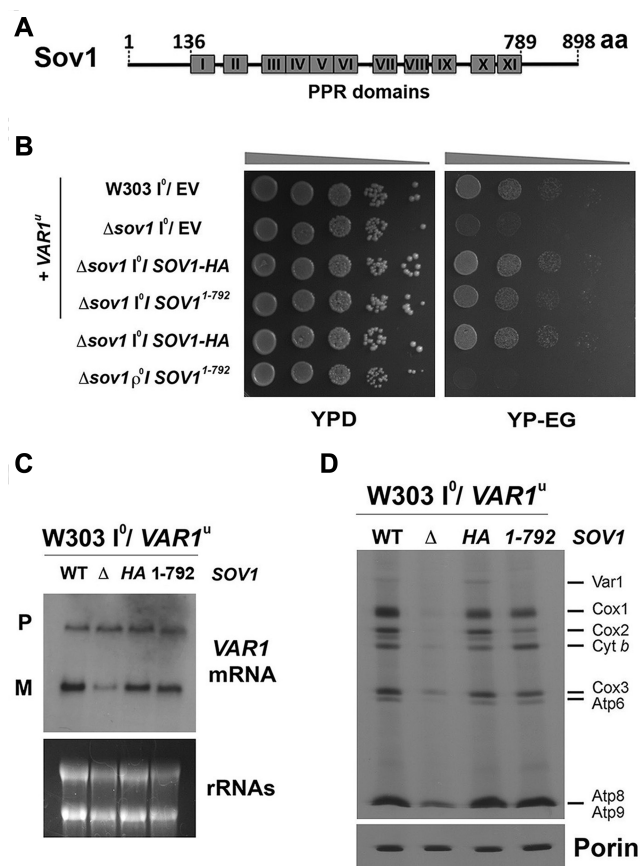
**Figure 5.** Role of Sov1 and the mitochondrial chaperones Tcm62 and Ssc1 in Var1 biogenesis. (A) Sucrose gradient sedimentation analyses of mitochondrial extracts prepared in the presence of  $Mg^{2+}$  from a strain expressing a Sov1-HA tagged protein. \* indicates Var1 Ab unspecific bands. (B) Co-immunoprecipitation of Sov1-HA protein with the mitochondrial chaperone Tcm62 and the indicated mtSSU proteins from sucrose gradient fractions. Fractions 7-8 and 12-13 from the gradient reported in panel (A) were used for immunoprecipitation with either Anti-HA-conjugated (+) or protein A-conjugated (-) agarose beads. Equivalent amounts of unbound (UB) and bound (B) samples were loaded in the gel. (C) Analysis of Var1 protein aggregation in the indicated strains. Isolated mitochondria were incubated at either 24°C or 37°C for the indicated amount of time. Soluble (S) and insoluble (pellet, P) material were separated by centrifugation and analyzed by immunoblotting. (D) Interaction of Sov1-HA with mitochondrial newly synthesized Var1 protein in a *ssc1-2* thermosensitive mutant. *In organello* mitochondrial translation was performed at the permissive (30°C) or not permissive (37°C) temperature for 30 min. Anti-HA-conjugated (+) and protein A-conjugated (-) agarose beads were used for immunoprecipitation. Equivalent amounts of unbound and bound samples were loaded in the gel.

isolated from the thermosensitive mutant *ssc1.2*. The mt-Hsp70 chaperone plays multiple key roles in mitochondrial biogenesis, including mitochondrial import, translation and protein folding. The *ssc1.2* mutation was originally described to compromise mitochondrial import at the non-permissive temperature of 37°C (51). Additionally, we reported that already at the permissive temperature (30°C), the *ssc1.2* mutant strain is partially respiratory deficient, mainly due to a decrease in mitochondrial Cox1 translation (52). The Cox1 synthesis defect and the accumulation of a newly synthesized Atp9 oligomers were further exacerbated at 37°C, while the rate of Var1 synthesis was not affected (Figure 5D and (24)). Moreover, it has been shown that the *ssc1.2* mutation does not affect the capacity of the chaperone to bind newly synthesized Var1, but compromises its release, leading to Var1 sequestration (24). Immunoprecipitation of the Sov1-HA tagged protein in the *ssc1.2* genetic background led to the recovery of newly synthesized Var1, when *in organello* translation was performed at 30°C. This interaction was completely lost at the non-permissive temperature of 37°C (Figure 5D), suggesting that Sov1 binds to Var1 only after it is released from Ssc1.

### Sov1 C-terminus is required to promote *mtVAR1* mRNA translation

The Sov1 protein contains eleven PPR domains (Figure 6A, (40)). PPR proteins are present exclusively in eukaryotes and function mainly in organellar post-transcriptional RNA metabolism, including RNA processing, stability, and expression (41). While in yeast mitochondria only 15 PPR proteins have been so far identified (40), in terrestrial plants, they represent a large family with more than 500 known polypeptides (41). Domain analysis of known plant PPR proteins has highlighted a common organization with any additional no-PPR functional domain localized in the C-terminus portion of the polypeptide (53). Following the last PPR domain, Sov1 also contains a 109 amino acid long C-terminus stretch (Figure 6A). To investigate the functional relevance of this portion of the Sov1 protein, we introduced by site-directed mutagenesis 2 stop codons after nucleotide 2376 of the *SOV1-HA* gene in order to generate a Sov1 truncated polypeptide lacking amino acids 793-898. Single copy expression of the *SOV1<sup>1-792</sup>* construct in the  $\Delta$ *sov1/VAR1<sup>u</sup>* strain restored the respiratory capacity





**Figure 6.** Analysis of Sov1 protein domains. (A) Schematic representation of Sov1 protein PPR domains. (B) Serial dilution growth analysis of the indicated strains in media containing fermentable (YPD) or respiratory (YP-EG) carbon sources. Pictures were taken after 2 (YPD) or 3 (YP-EG) days of incubation at 30°C. (C) Steady-state levels of mature (M) and precursor (P) *mt-VAR1* mRNA in the indicated strains as in Figure 2A. rRNAs are used as loading control. (D) Metabolic labeling with <sup>35</sup>S-methionine of newly synthesized mitochondrial products in whole cells for 10-min pulses in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. Immunoblotting for Porin was used as a loading control. Newly synthesized polypeptides are identified on the right. WT Sov1. Δ: *soy1* null mutant. HA: Sov1-HA tagged. 1-792: C-terminus truncated Sov1 lacking aminoacids 790–898.

of the mutant almost to WT level, as seen by the growth in respiratory substrate-containing media (Figure 6B). Moreover, the C-terminus truncated Sov1 protein was sufficient to stabilize the mature *VARI* mRNA (Figure 6C) and to support overall mitochondrial translation in the presence of allotopically expressed Var1<sup>u</sup> (Figure 6D). However, cells expressing the *SOV1*<sup>1-792</sup> variant were unable to synthesize mitochondrion-encoded Var1 protein (Figure 6D), suggesting that the C-terminus domain of Sov1 is required to promote *VARI* mRNA translation. Indeed, when the Δ*soy1*/*VARI*<sup>u</sup>/*SOV1*<sup>1-792</sup> strain was plated in non-selective media, all cells that lost the *VARI*<sup>u</sup> construct also lost mtDNA (Figure 6B), due to their incapacity to synthesize the mt-Var1 protein. In this way, we were able to separate the function of Sov1 in promoting *VARI* mRNA translation from its role in mediating Var1 protein assembly into the mtSSU.

### Sov1 regulates the rate of *mtVARI* mRNA translation

In yeast mitochondria, gene expression is regulated by negative feedback loops, which couple the rate of translation of key structural subunits with their assembly into OXPHOS complexes (3). So far, assembly dependent translational regulatory systems have been described to control the expression of CIV subunit Cox1, CIII subunit Cytb and CV subunits Atp6 and Atp8 (3). These regulatory loops rely on the dual function of a tertiary factor that acts on the mRNA 5'-UTR to promote translation and binds to the newly synthesized polypeptide to mediate assembly. Since the two activities are mutually exclusive, the distribution of the tertiary factor between two functionally distinct pools determines its availability as a translational activator. In other words, if the assembly of the target subunit is compromised, the tertiary factor remains bound to it and is prevented from binding to the mRNA and promoting translational initiation (3).

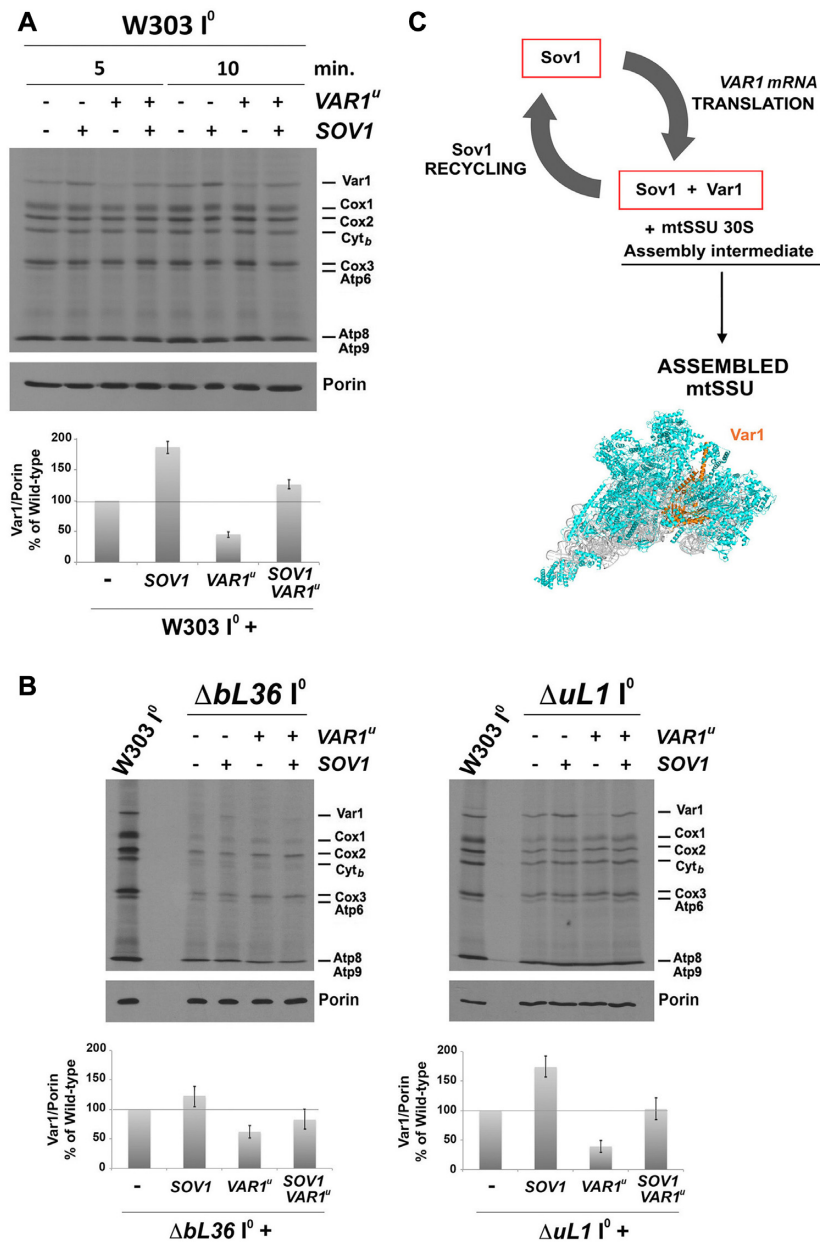
The dual capacity of Sov1 to promote mt-*VARI* mRNA translation and to mediate newly synthesized Var1 protein assembly into the mtSSU reminded us of the property of a translational regulation tertiary factor. To investigate whether Sov1 plays a role in the regulation of Var1 expression, we analyzed the rate of *mt-VARI* translation in relation to the amount of Sov1 protein. Specifically, we over-expressed Sov1 using an episomal plasmid in a WT strain, independently or in combination with allotopically expressed Var1<sup>u</sup>. The increase in the amount of Sov1 was associated with a higher rate of mt-Var1 synthesis (Figure 7A). On the contrary, the increase in Var1<sup>u</sup> protein levels resulted in a decrease in *mt-VARI* mRNA translation (Figure 7A). Whereas, co-expression of both Sov1 and Var1<sup>u</sup>, restored mt-Var1 synthesis to levels comparable to control (Figure 7A). The observed changes in *mt-VARI* translation were reflected in the Var1 protein steady-state level, while other mtSSU proteins were unaffected (Supplementary Figure S5A). Moreover, the different rates of Var1 expression in the strains analyzed were not due to alterations in the accumulation of *mt-VARI* mRNA (Supplementary Figure S7B).

Lastly, similar tendencies in the rate of *mt-VARI* translation were observed upon Sov1 and/or Var1<sup>u</sup> expression in mtLSU mutants, in which mitochondrial translation is only partially compromised (Figure 7B). An increase in Var1 expression in these mutants, however, was not sufficient to restore respiratory growth (Supplementary Figure S5C).

Taken together, our data support a working model (Figure 7C) in which the amount of Sov1 not-bound to Var1 protein determines the rate of mt-Var1 expression. In this way, Sov1 regulates Var1 synthesis in an mtSSU assembly dependent manner.

### DISCUSSION

Constructing mitochondrial ribosomes is an expensive metabolic process that is essential to maintain cellular aerobic bioenergetics. In *S. cerevisiae*, it involves two mtDNA-encoded rRNAs and 73 proteins, only one of which, Var1 or uS3m is encoded in the mitochondrial genome and synthesized in mitoribosomes. In this manuscript, we have uncovered a regulatory mechanism that coordinates the synthesis



**Figure 7.** Sov1-dependent regulation of Var1 translation. (A) Metabolic labeling with <sup>35</sup>S-methionine of newly synthesized mitochondrial products of the indicated strains in whole cells for 5- and 10-min pulses in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. Immunoblotting for Porin was used as a loading control. Newly synthesized polypeptides are identified on the right. Quantification of newly synthesized Var1 for 10-min pulse is reported as % of WT in the lower panel. Bars represent the mean ± SD of three independent repetitions. (B). Metabolic labeling with <sup>35</sup>S-methionine of newly synthesized mitochondrial products of the indicated strains as in A for 10-min pulses. (C) Model of Sov1 role in Var1 translational regulation. See text for explanation.

and assembly of Var1 into the maturing mtSSU, have gained insight into the actual Var1 assembly process, and have provided a potential explanation for the retention of the *VAR1* gene in the mitochondrial genome.

Var1/uS3m is a component of the mitoribosome mRNA entry channel. In all living organisms, ribosomes are responsible for translating the information encoded by the mRNAs into proteins. Despite fundamental differences in the mechanisms of transcript-ribosome recognition, initiation of translation universally involves the loading of the

mtSSU onto the mRNA. In yeast mitochondrial ribosomes, similarly to bacteria, the mRNA entry channel is located between the head and the shoulder of the mtSSU and is formed by Var1/uS3m, and also proteins uS4 and uS5 (7). Although more extensive remodelings of the mRNA entry channel have occurred in cytosolic and mammalian mitochondrial ribosomes; nevertheless, the uS3m protein remains a conserved structural component (8,10). Moreover, with the exception of trypanosomal mitochondrial ribosomes (54), uS3m also plays a key functional role by directly

interacting with the mRNA. For example, it has been shown that in human cytosolic ribosomes, uS3m can interact with abasic sites in the mRNA and play a role in translation quality control (55). In prokaryotes, the helicase activity of uS3 and uS4 proteins guarantees that mRNA secondary structures are resolved in order for single-strand transcripts to enter the narrow mRNA tunnel (56). A function that in yeast mitochondria could be performed by 5'-UTR RNA-binding proteins, as we recently proposed for the RNA helicase Mss116 (50).

Although recent high-resolution cryo-electron microscopic structures of mitochondrial ribosomes have provided a notable amount of information regarding their composition and function (7–8,54), mitoribosome assembly pathways and factors involved have just started to emerge (25,57–58). Here, we show that in yeast mitochondria Var1/uS3m protein is incorporated into the mtSSU in the late-assembly stages, which is in line with earlier observations reporting the accumulation of a 30S particle in yeast strains lacking Var1 (44,45). Additionally, the process appears to be conserved in bacteria, where uS3 was classified as a member of the fourth and last ribosomal protein assembly cluster (59). Our data indicate that Var1/uS3m biogenesis requires the function of the mitochondrial protein Sov1, which interacts with newly synthesized Var1 and mediates its incorporation into the mtSSU. In a similar manner, the specific chaperone Yar1 binds to the yeast cytosolic ribosomal protein uS3m to prevent its aggregation and promotes its assembly into the immature pre-40S particle to generate the functional ribosomal small subunit (60). Otherwise, in *Trypanosoma brucei* mitoribosome uS3m is part of the early assemblosome, although a cluster of nine assembly factors prevents it from interacting with the other mitoribosome proteins until the late stages of maturation (58). Mitochondrial Var1/uS3m protein is prone to aggregation, which here we have shown to be prevented by three mitochondrial proteins acting in concert, the mt-Hsp70 chaperone Ssc1, the putative chaperone Tcm62 and Sov1. These chaperones also cooperates to promote Var1 post-translational biogenetic steps. Sov1 and Tcm62 form a chaperonin complex that interacts with Var1 protein after its mt-Hsp70-dependent folding. The molecular mass of Sov1+Var1+Tcm62 roughly correspond to the 200 kDa size of the complex. Moreover, Tcm62 is released before Sov1-mediated incorporation of Var1 into the mtSSU. In this way, two mitochondrial multifunctional chaperones and one Var1-specific assembly factor coordinate Var1 folding and assembly.

Our mass-spectrometry analysis of mitochondrial fractions isolated from a strain lacking Sov1 suggests that uS14 and mS46 protein incorporation into mitoribosomes is also compromised in the absence of Sov1. Our data are in agreement with previous observations regarding uS14 assembly (46). However, a fraction of Var1/uS3m can still be incorporated in the mtSSU in a Sov1-independent manner. A possibility is that the Var1/uS3m protein assembled in the mtSSU in the absence of Sov1 does not fully acquired the proper native conformation, which is, in turn, required for efficient incorporation of uS14 and mS46. Indeed, we determined that Var1/uS3m, uS14 and mS46 do not interact to form a late assembly module prior to their incorporation

into the mtSSU. However, Var1/uS3m assembly into the mtSSU is uS14-dependent and we cannot exclude that Sov1 recruits uS14 after interacting with the mtSSU. On the contrary, mS46 incorporation into the mtSSU occurs in a Sov1-independent manner, after Var1 and uS14 assembly. Moreover, the assembly of the mtSSU protein mS38, which is not detected by mass-spectrometry, also occurs after Var1, uS14 and mS46 have been incorporated, since these three proteins are present in the mtSSU that accumulates in a  $\Delta mS38$  strain (30). In the case of the mammalian mitoribosome, uS3m and uS14 are part of the same late-binding assembly group, together with uS10m and mS33 (57).

Ultimately, the late formation of a complete mRNA entry channel during assembly appears to be a conserved feature in ribosome biogenesis. Since the mRNA entry channel grants the mtSSU the capacity to interact with transcripts and to potentially initiate translation, it is plausible that its formation could represent a target for regulation. Notably, the yeast cytosolic ribosomal protein uS14 is subject to a feedback translational control system, in which by binding to its own mRNA, uS14 represses its expression in the absence of assembly partners (61).

In this study, we provide experimental evidence demonstrating that Sov1 acts as *in bona fide* *mt-VAR1* mRNA translational activator. These include: (i) The complete absence of Var1 synthesis in a *sov1* null mutant strain in which mature *mt-VAR1* mRNA is stable (Figure 2D and E). (ii) The lack of expression in a *sov1* null mutant strain of the mitochondrial *ARG8<sup>m</sup>* reporter gene under the control of *mt-VAR1* 5'-UTR and the consequent lack of growth in the absence of arginine (Figure 2G–I). (iii) The binding of Sov1 protein to *mt-VAR1* mRNA (Figure 2F). (iv) The exclusive requirement of the Sov1 C-terminus domain for *mt-VAR1* translation, despite the presence of mature *mt-VAR1* mRNA and Var1<sup>u</sup>-containing mitoribosomes (Figure 6). (v) Lastly, the direct correlation between Sov1 protein levels and the rate of Var1 synthesis (Figure 7). Furthermore, we suggest that *mt-VAR1* expression is controlled by the amount of Var1-unbound Sov1 protein. In this way, Sov1 establishes a negative feedback regulatory loop that coordinates mitochondrial Var1 synthesis and mtSSU assembly.

In the yeast *S. cerevisiae*, it has been well established that mitochondrion-encoded key subunits of the OXPHOS complexes are the subject of assembly dependent control of gene expression. This type of translational regulation plays a pivotal role in the biogenesis of the yeast mitochondrial OXPHOS system and is achieved through a distinctive mechanism that involves dual function tertiary factors binding to the mRNA and the newly synthesized protein in a mutually exclusive manner (3). Now, with the characterization of Sov1 function in *mt-VAR1* gene expression, we extend assembly dependent translation regulation to yeast mitoribosome biogenesis.

Which is then the possible physiological significance of this regulatory system? Since Var1 is prone to aggregation, coupling its expression to the amount of protein that can be assembled into the mitoribosomes could contribute to preventing the deleterious effects associated with an excess of free protein. Moreover, whereas the protein stoichiometry in the assembled and functional mtSSU has to be respected, an exceptional imbalanced in gene copy number



exists between nuclear and mitochondrion-encoded components. Translational regulation could contribute to counteracting this imbalance during the biogenetic process. In general, the complexity and abundance of ribosomes entails several levels of regulation to avoid unnecessary waste of cellular energy in the production of excess components. In this frame, to pace the rate of Var1 synthesis with its assembly makes the mitoribosome biogenesis a more efficient process. Alternatively, it has been shown for mammalian mitoribosomes that an excess of nuclear-encoded ribosomal proteins is synthesized and imported into mitochondria (57). It is tempting to speculate that by controlling the rate of Var1 synthesis and incorporation into available mitoribosome late assembly intermediates, the amount of functional mtSSU could more rapidly vary in response to changes in cellular metabolism. The gain in system flexibility would ultimately contribute to explain the retention of the *VAR1* gene in the mtDNA.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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