Overexpression of *MRX9* impairs processing of RNAs encoding mitochondrial oxidative phosphorylation factors *COB* and *COX1* in yeast

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Mitochondrial translation is a highly regulated process, and newly synthesized mitochondrial products must first associate with several nuclear-encoded auxiliary factors to form oxidative phosphorylation complexes. The output of mitochondrial products should therefore be in stoichiometric equilibrium with the nuclear-encoded products to prevent unnecessary energy expense or the accumulation of pro-oxidant assembly modules. In the mitochondrial DNA of *Saccharomyces cerevisiae*, *COX1* encodes subunit 1 of the cytochrome *c* oxidase and *COB* the central core of the cytochrome *bc*1 electron transfer complex; however, factors regulating the expression of these mitochondrial products are not completely described. Here, we identified Mrx9p as a new factor that controls *COX1* and *COB* expression. We isolated *MRX*9 in a screen for mitochondrial factors that cause poor accumulation of newly synthesized Cox1p and compromised transition to the respiratory metabolism. Northern analyses indicated lower levels of *COX1* and *COB* mature mRNAs accompanied by an accumulation of unprocessed transcripts in the presence of excess Mrx9p. In a strain devoid of mitochondrial introns, *MRX9* overexpression did not affect *COX1* and *COB* translation or respiratory adaptation, implying Mrx9p regulates processing of *COX1* and *COB* RNAs. In addition, we found Mrx9p was localized in the mitochondrial inner membrane, facing the matrix, as a portion of it cosedimented with mitoribosome subunits and its removal or overexpression altered Mss51p sedimentation. Finally, we showed accumulation of newly synthesized Cox1p in the absence of Mrx9p was diminished in cox14 null mutants. Taken together, these data indicate a regulatory role of Mrx9p in *COX1* RNA processing.

Mitochondrial respiratory complexes, the ATP synthase, and the mitoribosome have a genetic hybrid origin; most of their components are encoded by the nuclear genome and a few by the mitochondrial DNA (mtDNA). The synthesis of mitochondrially encoded subunits relies on the mitochondrial gene expression system (1); in the yeast model *Saccharomyces cerevisiae*, mtDNA contains 75 kb and encodes eight polypeptides, with introns present in the genes for *COB*, *COX1*, and *21SrRNA* (2). Indeed, mitochondrial gene expression is finely regulated, and yeast studies have unveiled several regulatory events in the translation of mitochondrial encoded proteins (1–4). The regulation is necessary to accommodate the mitochondrial products with proteins that are imported from the cytosol avoiding futile energetic expenses in the translation of proteins that need the partnership of others that are being produced elsewhere. Moreover, the mitochondrial products Cox1p, Cox2p, and Cob harbor metal prosthetic groups thereof have to be properly chaperoned to avoid the uncontrolled generation of reactive oxygen species (5–7). A concerted integration of approximately 250 components (8) is required for the mitochondrial gene expression. Many of them are spatially associated in higher-order complexes named MIOREX (9), which consist of the mitoribosomes, factors involved in the mitoribosome assembly, proteins involved in mRNA and DNA metabolism, translational activators, chaperones, proteases, and proteins with unknown function; some of these factors have already more than one function described in mitochondrial RNA metabolism (10–14); their multifunctional properties facilitate regulatory loop processes that coordinate mitochondrial gene expression with protein complexes assembly (15–19).

Here, we started investigating whether the overexpression of 32 proteins of the MIOREX would unbalance the mitochondrial gene expression system resulting in deleterious effects on mitochondrial translation. Indeed, the overexpression of some components was deleterious and *MRX9* in particular called our attention due to its effects on Cox1p translation. Cox1p is a 12-transmembrane domain spanning integral IMM protein part of cytochrome *c* oxidase (COX), the terminal electron acceptor in the respiratory electron transport chain. This catalytic core subunit contains the two heme groups *a* and *a*3 and a copper ion in the CuB center (4). Afterward, we noticed that Mrx9p excess was also toxic to *COB* expression. Cytochrome *b* is the central core of the *bc*1 complex, embedded *via* eight transmembrane spans in the IMM harboring two heme *b* centers (4).

COX1 is transcribed as a polycistronic transcript containing *ATP6/ATP8* of ATP synthase (20). RNA processing requires splicing to generate the mature *COX1* transcript without

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introns, a process assisted by maturases encoded by ORFs located within some of the introns (21) and nuclear genes such as *MSS116*, *PET54*, and *COX24* (22–24). Translation of the *COX1* transcript is controlled by Pet309p, Mam33p, Mrx8p, and Mss51p (25–28); the pentatricopeptide protein, Pet309p binds to the 5′UTR of the *COX1* messenger and recruits Mss116p a DEAD-box helicase, which unwinds the 5′ UTR and facilitate the access of Mss51p to the initiation complex (26, 29). Afterward, Mss51p participates in the assembly process of the Cox1p module (30–32), it is sequestered by the Coa3p–Cox14p complex away from *COX1* mRNA; a new translation cycle only starts when the biogenesis of the Cox1 module proceeds and Mss51p is released (32, 33). Because of the loop control exerted by Mss51p, COX assembly mutants present diminished *COX1* translation with the concealed of Mss51p by Coa3p–Cox14p (30–35). Translation of *COX1* is bypassed in COX assembly mutants when *COX14* is also deleted, and therefore, Mss51p is free to proceed in new translation cycles (31, 36).

Likewise, *COB* is transcribed together with the tRNA-E gene and harbors two to six introns depending on the yeast strain (21, 37). Processing of *COB* primary transcript requires *cis*-elements such as the maturase p27 encoded by the bI4 intron (38), while the bI5 intron excision is dependent on Cbp2p (39). Translation activation of *COB* mRNA depends on Cbs1p, Cbs2p, Cbp1p, Cbp3p, and Cbp6p, the highest number of activators for a single mitochondrial RNA (3). In this study, we show some evidence that suggests a role of Mrx9p in mitochondrial RNA metabolism.

Mrx9p is a poorly described component of the MIOREX (9); it cosediments with the small subunit of the mitoribosome (SSU) (40) but also was detected close to the polypeptide tunnel exit of the mitoribosome large subunit (LSU) in proximity-dependent biotinylation studies (41). Our data support the association of Mrx9p with the mitoribosome and indicates its involvement in the control of *COB* and *COX1* RNA processing and translation.

Results

Overexpression of MIOREX components

The assembly of the OXPHOS complexes depends on a balanced output of their subunits, mitochondrially or nuclearly encoded products. Therefore, mitochondrial translation is subjected to several regulatory factors, which include mitochondria specific translation activators that have been described for all mitochondrial products (3, 19), and sophisticated feedback regulatory loops for *ATP6/8*, *COB*, *COX1*, and *VAR1* translation (15, 17–19). A new category of translation regulators includes the *ATP6/8* transcript repressor Smt1p (42). We hypothesized that overexpression of *SMT1(MRX5)* and other uncharacterized repressors would unbalance the translation of the targeted mitochondrial product. Therefore, we have cloned 32 components of the MIOREX complex (9) under the control of the *GAL10* promoter and evaluated the mitochondrial translation properties of the transformed yeast strain grown in galactose. As a proof of concept, the overexpression of *SMT1* (alias *MRX5*) resulted in a destitute translation of Atp6p and Atp8p (Fig. 1*A*). In this sense, we have prioritized the overexpression of components with unknown functions to gain insights into their role in the MIOREX complex. After evaluating the *MRX* genes, *AIM17*, *AIM18*, *AIM19*, *AIM25*, *AIM32*, *AIM34*, *MIN10*, *FMP10*, and *FMP30* overexpression (Fig. 1, *A* and *B*), we noticed a pleiotropic diminishment of mitochondrial products in the strains containing an excess of Aim19p, Aim32p, Aim34p, and Fmp30p accompanied by an elevated rate of petites formation, which can be speculated as a general impairment in the mitochondrial protein synthesis and its consequences in the mtDNA stability (43). On the other hand, *FMP10* overexpression leads to high petite formation but the accumulation of mitochondrial products seems normal. Growths of these overexpressing strains were also evaluated (Fig. S1), and they presented different rates of impairments, particularly *AIM32*, which after overnight growth on galactose, resulted in its spots revealing few viable cells. Aim32p is involved in redox quality control, its deletion results in an increased accumulation of proteins with aberrant disulfide linkages (44). Overexpression of *SMT1(MRX5)* and *MRX9* also resulted in slower growth on galactose and ethanol–glycerol media (Fig. S1).

We also tested the overexpression of some characterized components of the mitochondrial translation machinery to evaluate its effects on the output of mitochondrial products or even its toxicity to the mitochondrial translation. Except for *CBS2*, the overexpression of the tested genes here did not significantly alter the profile of mitochondrial products. Overexpression of *COB* translation activators *CBS1* and *CBS2* (45) resulted in different outcomes (Fig. 1*B*), while *CBS1* excess did not significantly change the profile of mitochondrial newly synthesized products, *CBS2* excess resulted in the higher petite formation and lower accumulation of newly synthesized Cox1p. It was reported before that Cbs2p forms a complex with Cox1p translation activator Pet309 (46), therefore extra Cbs2p may sequester Pet309 away from *COX1* mRNA processing and translation activation or interfere in Pet309p association with the DEAD-box helicase Mss116p (26, 29). Cbs1p participates in the loop control of Cob assembly and translation (18). *AEP1* overexpression, but not *AEP2*, resulted in a significant increment in petite production probably due to its impact on *ATP9* expression, although levels of Atp9p seem normal (Fig. 1*C*) (47). On the other hand, overexpression of *PET54*, *PET122*, and *PET494* (48) did not alter *COX3* translation. Pet54p is also involved in *COX1* intron processing and translation (13, 23). Atp22p excess, the *ATP6* activator (49), resulted in a small elevation of petite formation.

MRX9 called our attention due to a diminishment of Cox1p (Fig. 1*A*) product accompanied by the appearance of an additional aberrant product. The appearance of novel mitochondrial products has been reported, the one named mp15, for instance, is found in *COX1* defective mutants in the absence of Mss51p and its expression depends on *COX1* intron composition (30, 36).

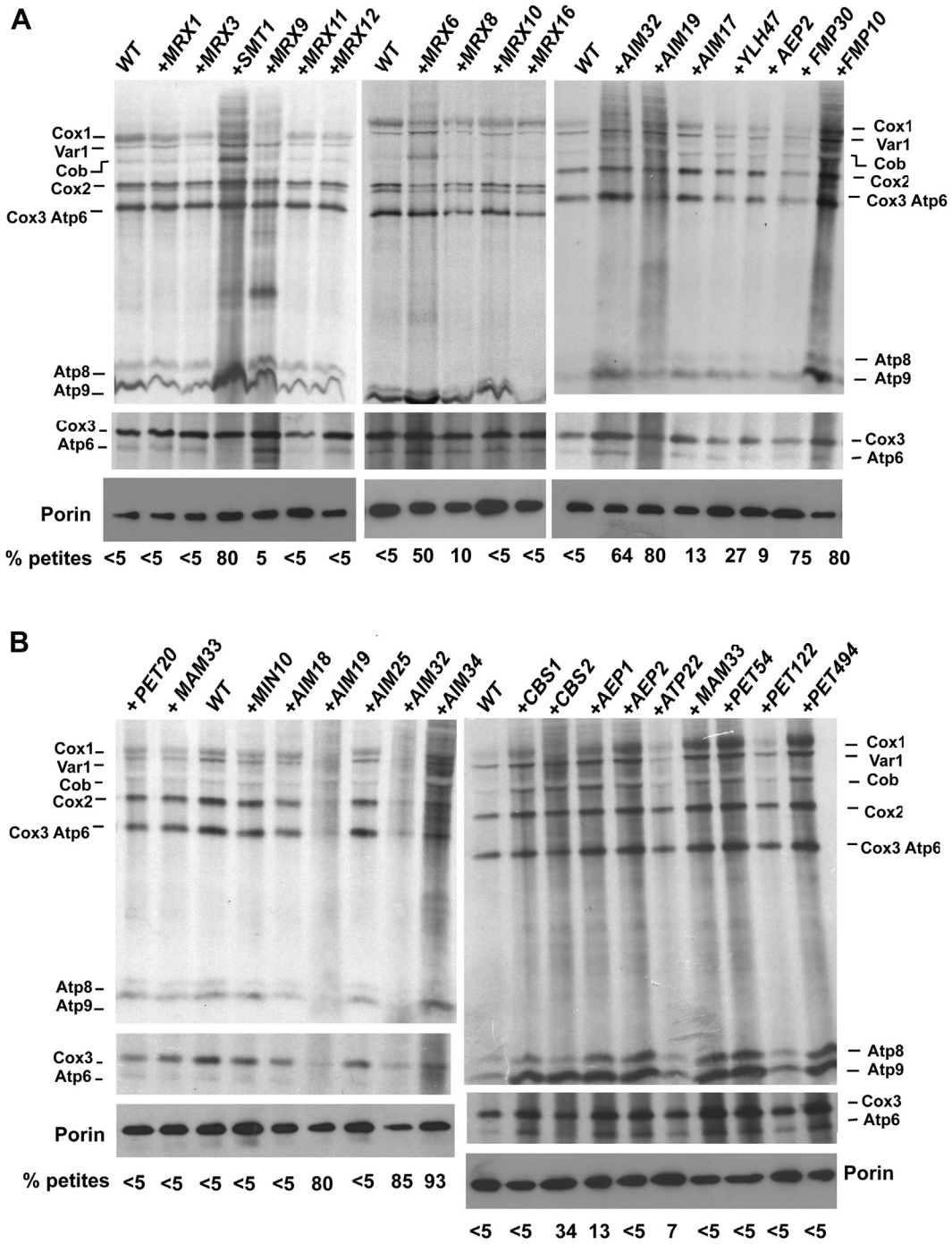

Figure 1. Overexpression of MIOREX components and analyses of mitochondrial translation properties. *A* and *B*, newly synthesized mitochondrial translation products were analyzed from the indicated strains as described in the Exprimental procedures section. The radiolabeled bands corresponding to the mitochondrial gene products are marked in the margins as indicated: subunits 1 (Cox1), subunit 2 (Cox2), subunit 3 (Cox3) of cytochrome *c* oxidase; subunit 6 (Atp6), subunit 8 (Atp8), and subunit 9 (Atp9) of ATP synthase; cytochrome *b* subunit (Cyt b) of ubiquinol cytochrome *c* reductase; the mitoribosome Var1 protein. Membranes were also probed against antiporin antibodies for loading control. The number of petites that arose in the experiments is indicated in the *bottom* of the panels.

Characterization of mrx9 null mutant and MRX9 overexpression

Albeit *MRX9* overexpression results in a lower accumulation of newly synthesized *COX1*, its deletion did not result in any growth deficiency on nonfermentable carbon sources media or changes in the mitochondrial translation properties in comparison to the WT cells (Fig. 2, *A* and *B*).

Considering the observed deficit in the accumulation of newly synthesized *COX1* under *MRX9* excess condition, we evaluated whether *MRX9* overexpression or *mrx9* deletion would impact the steady-state level of Cox1p and other mitochondrial proteins (Fig. 2*C*). The *mrx9* deletion did not change the rate of accumulation of newly synthesized Cox1p (Fig. 2*B*), and its steady-state levels was roughly equal to that

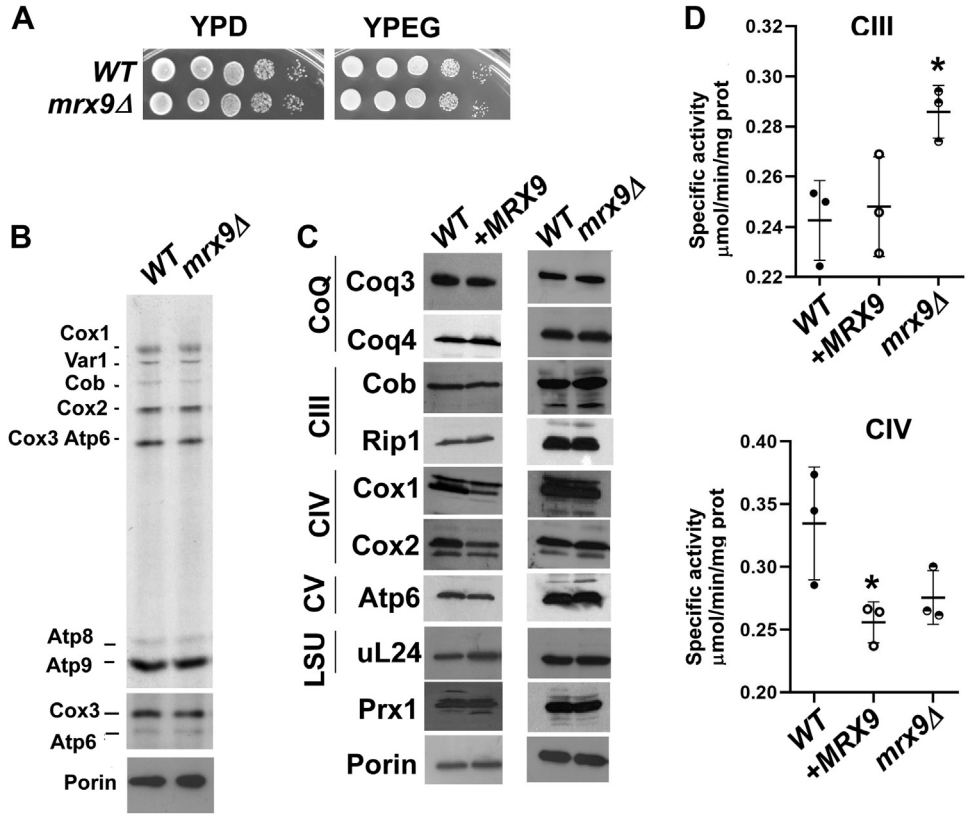

Figure 2. *MRX9* mutants phenotypes. *A*, growth properties of *mrx9Δ* mutant spotted on rich glucose (YPD) and rich nonfermentable ethanol–glycerol (YPEG) media; plates were photographed after 2 days of growth. *B*, mitochondrial newly synthesized products from the WT and *mrx9Δ* mutant were identified as in Figure1. *C*, steady-state level of the indicated mitochondrial proteins in the WT were compared to the strain overexpressing *MRX9* (+*MRX9*) and the *mrx9Δ* null mutant, Coq3p and Coq4p are components of the CoQ synthome (90), Cob and Rip1p of the respiratory complex III (CIII), Cox1p and Cox2p—complex IV (CIV), Atp6—complex V (CV), uL24 is a component of the 54S subunit of the mitoribosome (LSU), Prx1 is a soluble mitochondrial protein, and Porin is a component of the mitochondrial outer membrane. Bands intensities were evaluated and compared using the histogram for pixels densitometry of the Adobe Photoshop program. *D*, enzymatic activity of the ubiquinol cytochrome *c* reductase (CIII) measured by the rate of cytochrome *c* reduction, and the activity of the cytochrome c oxidase (CIV) measured by the rate of reduced cytochrome *c* oxidation. Data points were obtained from three independent experiments *$p < 0.05$ *versus* WT.

seen in the WT mitochondria. In contrast, the *MRX9* overexpression resulted in three times less Cox1p than the WT; Cox2p level was also diminished to a similar rate; all the other tested mitochondrial proteins did not show any significant change in the tested strains (Fig. 2*C*). Accordingly, the enzymatic activity of cytochrome *c* oxidase was diminished in the mitochondria isolated from cells overexpressing *MRX9*, while the NADH ubiquinol cytochrome *c* reductase activity elevated in the null mutant (Fig. 2, *C* and *D*). Altogether, our data indicate that *MRX9* excess alters COX biogenesis and activity.

Paromomycin has been used to evaluate the quality of the mitochondrial translation process by inducing translational misreading and proteotoxic stress in *S. cerevisiae* mitochondria (50, 51). The strain overexpressing *MRX9* showed an increased resistance of paromomycin compared to the WT and the *mrx9* null mutant strains (Fig. S2). Similar increments in paromomycin resistance were observed before with mitoribosome mutants (52).

We also tested whether *mrx9* deletion would impact growth or Cox1p translation in the COX mutants: *shy1*, *cox14*, *mss51*, *pet111*, *pet309*, and *pet494*. COX assembly mutants usually present a destitute translation of *COX1*, mostly because Mss51p stays trapped by Cox14p–Coa3p complex in the Cox1p assembly module and cannot reinitiate a new translation cycle (30, 31). An exception is the *cox14* mutant, which is defective in COX assembly (53), with a high accumulation of newly synthesized Cox1p and epistatic over other mutations affecting Cox1p module assembly (30, 33). However, the *cox14* null mutant is not epistatic over genes involved in *COX1* 5′UTR control expression (14, 22, 36). Here, we showed that *mrx9* deletion in *cox14Δ* cells impacts negatively in *COX1* translation, suggesting Mrx9p is involved with *COX1* 5′ UTR control (Fig. 3*A*), and the released Mss51p did not optimally perform new cycles of *COX1* translation if Mrx9p is not present, even in the absence of Cox14p. The transformation of the double mutant *mrx9Δcox14Δ* with pG82/T1, a multicopy plasmid harboring *MSS51* and *COX24* (22), did not rescue the accumulation of Cox1p to the *cox14Δ* single mutant level (Fig. S3).

Deletion of *shy1* results in respiratory growth impairment with a residual COX activity that can be rescued by an extra copy of *MSS51* (54, 55); *pet111* and *pet494* were included as control of defective *COX2* and *COX3* translation activation (4, 49). The *mrx9* deletion in the *shy1* null mutant worsens its slow growth on respiratory selective media, which can only be observed after 7 days of incubation (Fig. S4).

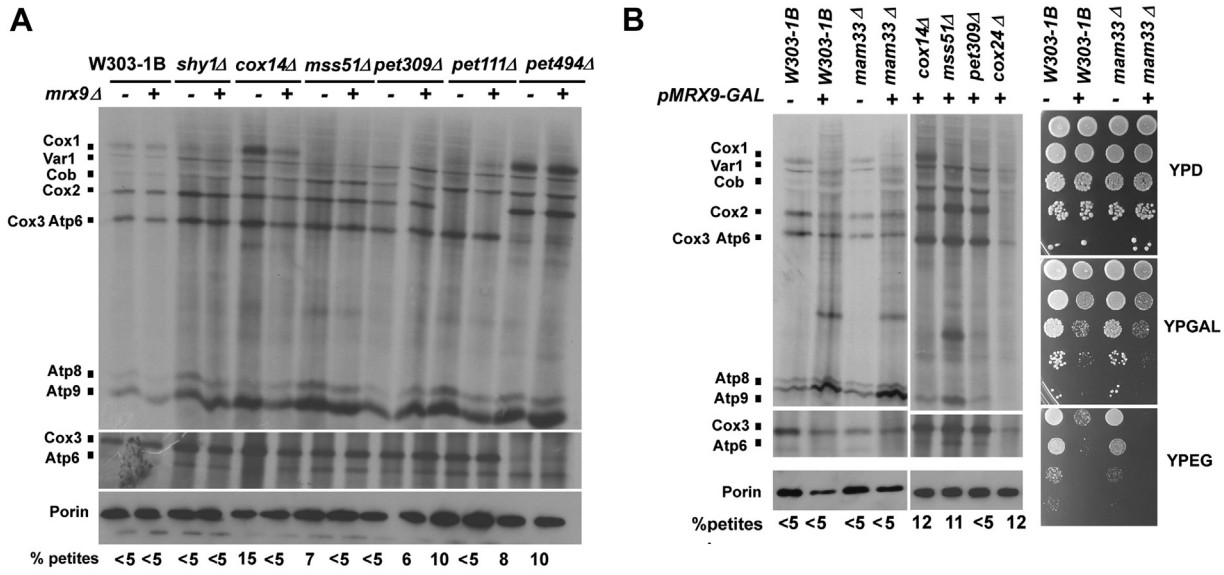

Figure 3. Comparison of mitochondrial protein synthesis in COX mutants harboring or not *mrx9* deletion and overexpressing *MRX9*. *A*, the indicated strains containing the *mrx9Δ* deletion (+) and the original strains (–) were grown in rich galactose media and cells labeled for the analyses of mitochondrially encoded newly synthesized products as indicated in the methods sections. *B*, newly synthesized mitochondrial products from COX mutants transformants harboring the pMRX9-GAL (+) were also analyzed from cells grown in galactose-rich media The respective mitochondrial products were identified as in Figure 1. Growth of *mam33* mutant transformed or not with *MRX9* was also compared to the WT strains. Membranes were also probed against antiporin antibodies for loading control.

MRX9 overexpression was also tested in *mss51*, *pet309*, *cox14*, *mam33*, and *cox24* null mutants, which except for *cox14* and *mam33* are defective in *COX1* translation in respiratory conditions. In these transformed mutants, *MRX9* excess was toxic to mitochondrial translation in the *cox24Δ* background (Fig. 3*B*). The toxicity was not enough to cause an exacerbated mtDNA loss, and the transformed mutants were still able to spontaneously revert the respiratory deficient phenotype, similarly to the untransformed *cox24Δ* mutant (22). In the *mam33* mutant, *MRX9* overexpression leads to destitute *COX1* translation reflecting in the absence of any noticeable growth on ethanol–glycerol media. *MAM33* was firstly characterized as required for *COX1* translation and adaptation to the respiratory metabolism (27). Therefore, it seems that *MRX9* excess is still toxic to cells devoid either of Cox24p or Mam33p. Absence or excess of Mrx9p did not alter Cox24p endogenous level (Fig. S5). In these assays we observed again that extra *MRX9* intensified the accumulation of an uncommon peptide in the *mss51* mutant overexpressing *MRX9*; the higher accumulation of this peptide occurs independently of Mss51p presence, but in the *cox14Δ*, or *pet309Δ* backgrounds, Mrx9p excess did not lead to its synthesis. A rational explanation for that are the increment of free Mss51p in *cox14Δ* cells and the poor stability of *COX1* transcripts in *pet309Δ* (26). Similar accumulation properties of mp15, an uncommon peptide, was described before in *mss51* mutant cells (36), which strengthens the hypothesis that the aberrant peptide detected in the presence of *GAL-MRX9* overexpression and mp15 have the same nature.

We also evaluate whether the *mrx9* deletion would have an additive effect on mitoribosome mutants. Recently, we described new LSU mitoribosome mutants, some of them with poor growth on respiratory media (52); considering the proximity of Mrx9p with the LSU tunnel exit components, particularly uL23, mL57, and uL29 (41), we hypothesized that Mrx9p may play a role in the mitoribosome biogenesis. If this was the case, then its removal should have an additive effect over the slow respiratory growth of uL16, uL23, or uL29 mutants (52); likewise the aforementioned phenotype of *shy1 mrx9* double mutant, *mrx9* removal marginally worsens the respiratory growth of the uL23 mutant (Fig. S6).

MRX9 overexpression delays the adaptation to the respiratory metabolism

S. cerevisiae is a Crabtree-positive cell that presents repression of aerobic catabolism in the presence of glucose (56). When the glucose is exhausted from the media, the yeast cells start to transit from the fermentative metabolism to the respiratory metabolism with drastic changes in the expression of tricarboxylic acid cycle enzymes and components of the mitochondrial electron transport chain (57). It was recently demonstrated that mitoribosomal protein synthesis occurs at high levels during glucose fermentation, which depends on bL27 mitochondria-specific C-terminal extension and its binding partner Mam33p (58), as mentioned before; it is also required for *COX1* translation and adaptation to the diauxic shift (27). As it was already described for *mam33* mutants, we investigated whether, besides Cox1p synthesis deficiency, the overexpression of Mrx9p would also impact the transition from fermentative to respiratory metabolism.

To a better accompaniment of Mrx9p levels, a hemagglutinin (HA)-tagged version of *MRX9* was cloned under the control of different promoters not repressed by glucose and its expression was compared. Curiously, the steady-state level of the Mrx9p-HA product was higher when expressed under the

control of the endogenous promoter in comparison to the version under the control of the alcohol dehydrogenase *ADH* promoter, which was previously shown to be stronger than a weak version of the cytochrome *c* gene, *CYC1*, promoter (59). The *MRX9-HA* under the control of the translation elongation factor 1α (*TEF1*) promoter and the glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter resulted in higher contents of *MRX9-HA*. In cells grown in galactose media, the transformant harboring the *GAL10-MRX9* fusion accumulate more Mrx9-HA than the *GPD-MRX9* or *TEF-MRX9* constructs (Fig. 4*A*).

The differential expression of *MRX9-HA* under these different promoters allowed the evaluation of the adaptation of the transformants to the diauxic shift and the respiratory metabolism. In this experiment, cells were grown to Abs ∼1,0 at 600 nm and then spotted on glucose and ethanol–glycerol rich media. The faster the adaptation to the respiratory metabolism, the faster they will grow on ethanol–glycerol. After 1 day of growth, we were able to see that the cells expressing the *GPD-MRX9-HA* construct have a clear delay in their respiratory growth, which was also confirmed by the analyses of the growth curve of this transformed strain in ethanol–glycerol liquid media (Fig. 4*B*). A slightly lower growth rate was also noticed in cells expressing the *TEF-MRX9-HA* construct. The *mrx9* deletion, however, did not significantly change the adaptation to the respiratory media (Fig. 4*B*). We also tested whether the *GPD-MRX9* transformed cells have delayed adaptation to the respiratory metabolism rather than a respiratory deficiency itself; therefore, we compared growth on ethanol–glycerol media from fermentative cells pregrown on glucose and spotted at their logarithmic growth *versus* cells already adapted to the respiratory metabolism after pregrowth on galactose. When pregrown in galactose, cells harboring the *GPD-MRX9* construct show similar growth on ethanol–glycerol media in comparison to untransformed cells (Fig. 4*D*).

MRX9-HA expression was also evaluated in cells at logarithmic growth in glucose, that is, fermentative condition and

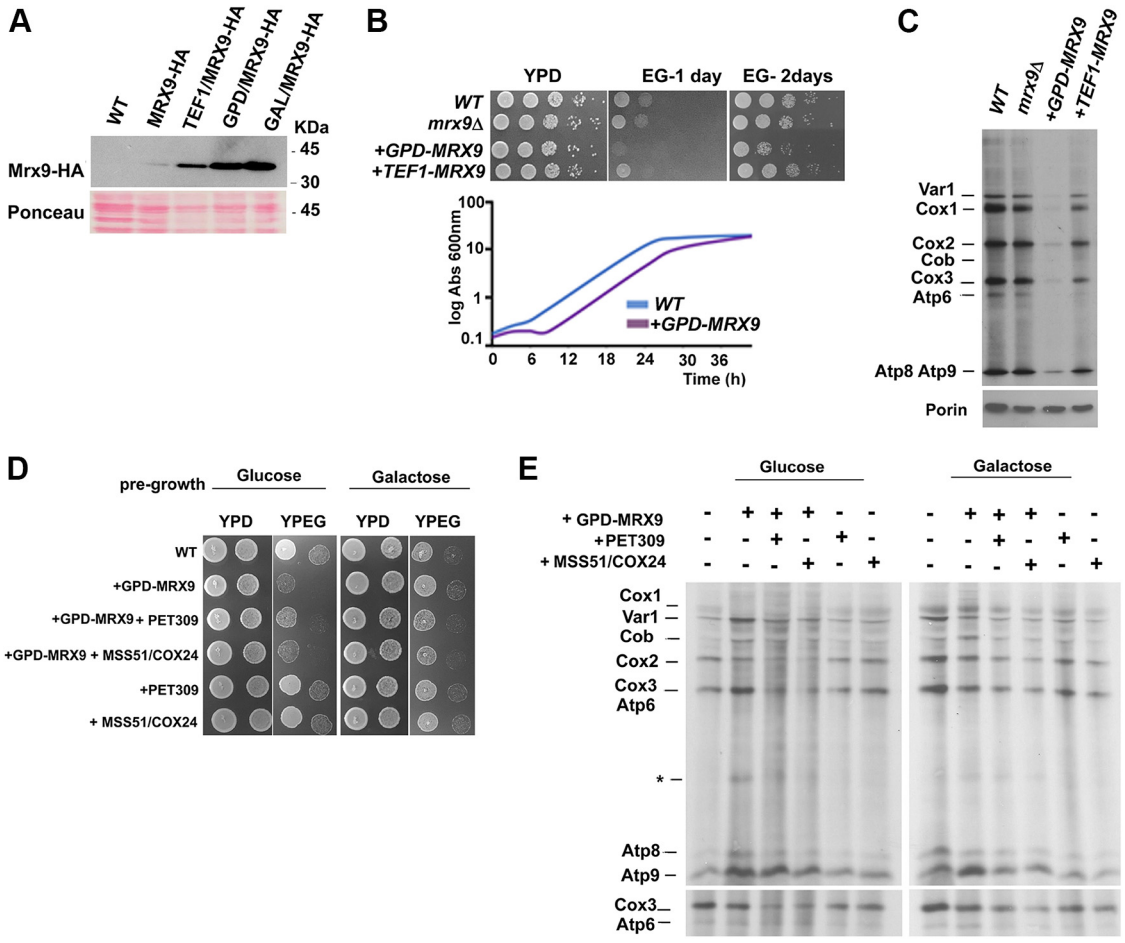

Figure 4. Evaluation of *MRX9* overexpression and adaptation to the respiratory metabolism. *A*, Mrx9p-HA content obtained from mitochondria isolated from the indicated strains grown in galactose media. Mitochondrial protein extracts were separated on SDS-PAGE for membrane transfer and immunodecoration with anti-HA. Ponceau staining is shown below. *B*, growth properties of the indicated strains spotted on rich glucose (YPD) and rich nonfermentable ethanol–glycerol (EG) media from cells at exponential fermentative growth. The EG plates were photographed after 1 day and 2 days as indicated; the YPD plate was photographed after 2 days of growth. *C*, mitochondrial newly synthesized products of the indicated strains were labeled from glucose-grown cells at exponential phase. The indicated products on the *left* are the same described in Figure 1. *D*, growth of *GPD-MRX9* transformants were also assessed from culture cells pregrown on glucose or galactose and spotted on YPD and YPEG at exponential logarithmic phase. Plates were photographed after 1 day at 30 °C. *E*, mitochondrial translation properties from glucose or galactose grown cells as indicated. The signals "–" and "+" indicate the presence of the correspondent plasmid in the strain. The respective mitochondrial products were identified as in Figure 1.

in ethanol–glycerol media. In these conditions, the *TEF-MRX9-HA* and *GPD-MRX9-HA* transformants showed a high yield of Mrx9p-HA, and its unprocessed version is also accumulated in both growth conditions. In glucose-grown cells, the unprocessed Mrx9-HAp signal is stronger, probably due to a mitochondrial import limitation present in glucose fermentative growth conditions. Accordingly, the Mrx9p-HA level with the endogenous promoter was three times higher in respiratory conditions, while the porin level was roughly 20 times stronger in cells growing in the respiratory condition (Fig. S7), indicating that *MRX9* endogenous expression is less repressed in glucose than porin and other mitochondrial proteins (57).

The delay in the adaptation to the respiratory metabolism caused by *MRX9* overexpression was also confirmed when we assessed newly synthesized mitochondrial gene products in glucose fermentative cells at exponential growth (Fig. 4*C*). Under this condition, cells expressing the *GPD-MRX9-HA* fusion resulted in a poor accumulation of mitochondrial products, while as for the cells harboring the *TEF-MRX9-HA* fusion, the mitochondrial translation is also diminished. The deletion of *mrx9* did not significantly alter the accumulation of mitochondrial products in comparison to the WT cells (Fig. 4*C*).

Considering the deleterious effect of *MRX9* excess on *COX1* translation, we tried to circumvent it by overexpressing *PET309* in a multicopy plasmid and the couple *MSS51–COX24*, initially cloned in the same multicopy plasmid pG82/T1 (22). Neither *PET309* nor *MSS51/COX24* multicopy plasmids were able to ameliorate the growth rate of *GPD-MRX9* transformants on ethanol–glycerol when pregrown culture was conducted in glucose (Fig. 4*D*). Analyses of newly synthesized mitochondrial product of these transformants strains, in the same growth conditions described previously, indicate that in fermenting cells *COX1* synthesis is the most reduced product when *GPD-MRX9* construct is present. Again, extra *PET309* or *MSS51/COX24* did not significantly change the translation properties caused by Mrx9p excess (Fig. 4*E*). Probably because of the level of *MRX9* expression, in galactose grown cells the *GPD-MRX9* construct was less toxic to *COX1* translation (Fig. 4*E*), in agreement with the presented growth results (Fig. 4*D*). Altogether, these results strongly support the idea that the deficit in *COX1* translation caused by Mrx9p excess retards the adaptation to the respiratory metabolism.

Finally, we compared the effects of the *GPD-MRX9* overexpression in the *mam33Δ* background, under fermentative and respiratory conditions. As previously reported, Mam33p is required for proper Cox1p translation in fermentative conditions (27); the *MRX9* excess did not change the accumulation of newly synthesized mitochondrial products in comparison with the untransformed *mam33Δ*, except that in respiratory conditions the uncommon peptide between Atp6p and Atp8p products were once again accumulated with the presence of extra Mrx9p. Curiously, the accumulation of newly synthesized Cob and Atp9p seems augmented in the double mutant *mam33Δ mrx9Δ*, the effect on Atp9p is difficult to interpret due to its electrophoretic properties, but it seems that Mrx9p also regulates Cob translation (Fig. S8).

Mrx9p intramitochondrial localization and distribution

To gain a better topology localization of the Mrx9p protein, we followed the Mrx9p-HA solubilization properties after sonication with or without carbonate, as well its distribution in mitochondria and mitoplasts subjected to proteinase K digestion. Mrx9p is a hydrophobic inner membrane protein facing the matrix compartment that is recovered in the membrane fraction following disruption of mitochondria by sonic irradiation in the presence of carbonate (Fig. 5*A*) and resistant to proteinase K in mitochondria and in mitoplasts, the latter lacking an intact outer membrane (Fig. 5*B*). Conversion of mitochondria to mitoplasts by hypotonic shock was confirmed from the substantial loss of the soluble intermembrane marker cytochrome b2 and supported by the decrease of Sco1p as a result of the proteinase K treatment of mitoplasts but not mitochondria. Sco1p is an inner membrane protein facing the intermembrane space (60). The matrix protein α-ketoglutarate dehydrogenase (61) was protected against proteinase K in mitochondria and mitoplasts confirming the intactness of the inner membrane in the mitoplasts.

Mrx9p is a component of the MIOREX complex (9); it cosediments with the small subunit of the mitoribosome (SSU) (40), and it is also close to the polypeptide tunnel exit of the mitoribosome LSU in proximity-dependent biotinylation studies (41). Here, we evaluate the distribution of Mrx9-HAp in the WT strain and also assessed the mitoribosome assembly status in the null mutant *mrx9Δ* and in the strain overexpressing *MRX9-HA* under the control of the *GAL10* promoter. Mitochondria were isolated and mitoribosomes extracted in the presence of 0.5 mM $MgCl_2$ (12) either using Triton, a stringent detergent, or digitonin, a mild detergent. The extracts were subjected to sedimentation in a sucrose gradient as previously described (62). We used the bL31 antibody to follow the sedimentation of the 54S subunit and anti-mS37 to follow the 37S subunit (Fig. 6).

In the tested strains (WT+ *MRX9HA*, *mrx9Δ*, WT+*MRX9HA-GAL10*), bL31 sediments at fractions 1 and 2 at the bottom of the sucrose gradient, fraction 1 is probably enriched with the assembled mitoribosome and fraction 2 with the 54S subunit (Fig. 6). The mS37, and therefore 37S subunit, is enriched in fractions 3, 4, and 5 in all tested strains. In both cases, the sedimentation properties of bL31 and mS37 did not change significantly in Triton or digitonin conditions (Fig. 6*B*). On the contrary, for Mrx9-HA, the two detergents lead to distinct outcomes; with Triton, the Mrx9-HA distribution peaks in the lighter fractions 10 and 11 (Fig. 6*A*); with digitonin Mrx9-HA, it was spread from fractions 1 to 10 (Fig. 6*B*). These results indicate that Mrx9p is associated with the mitoribosome subunits as previously suggested (9, 40, 41) but its association is lost in stringent extraction conditions. In the strain overexpressing *MRX9-HA* using digitonin, the

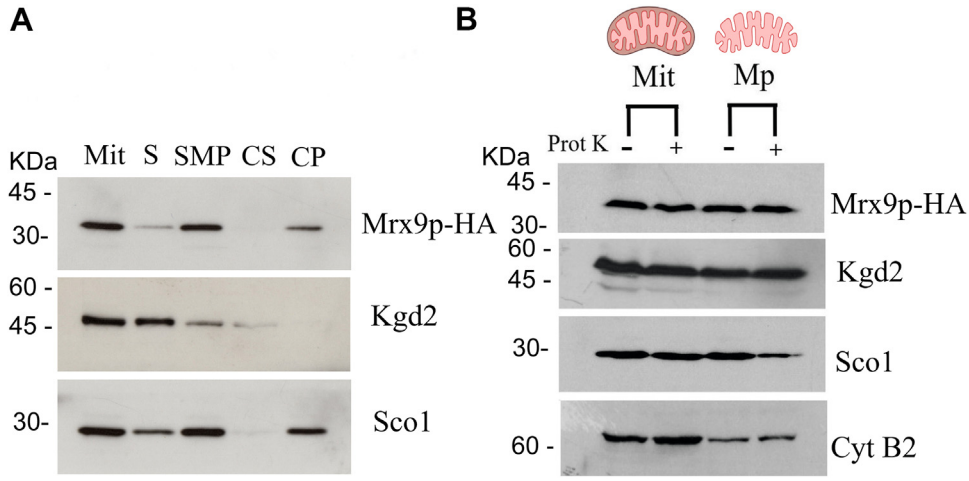

Figure 5. Mrx9p is in the mitochondrial inner membrane facing the matrix side. *A*, mitochondrial proteins isolated from cells expressing the Mrx9p-HA constructs (Mit) were sonicated and centrifuged, obtaining supernatant (S) and pellet (SMP). The pellet was suspended in carbonate salt and centrifuged again, with a new super (CS) and pellet (CP). The distribution of Mrx9p-HA in the different fractions was assessed using an anti-HA antibody; the solubilization was also checked using an antibody against the matrix soluble Kgd2p and the inner membrane protein Sco1p. *B*, mitochondria containing the Mrx9-HA (Mit) were osmotically challenged with Hepes buffer generating mitoplasts devoid of the outer membrane and the intermembrane compartment (Mp). Mitochondria and mitoplasts were digested (+) or not (–) with proteinase K to test the availability of the indicated tested proteins to digestion.

Mrx9-HA product is also found in heavier fractions but it peaks in fraction 7 (Fig. 6*B*).

As shown before (Figs. 1 and 3), yeast cells overexpressing *MRX9* presented a deficiency in *COX1* translation and the exacerbated accumulation of an aberrant translation product that resembles the reported mp15 (36). Therefore, we hypothesized that if mp15 and the aforementioned product are the same, then *MRX9* excess is affecting Mss51p accessibility for translation activation and elongation control (35). Although Mss51p was not found in the MIOREX complexes (1, 9), its association to the mitoribosome has been reported in some special high salt conditions (41). Here, we investigated Mss51p distribution in the tested sucrose gradients; with the WT levels of Mrx9-HA, Mss51p is found in heavier fractions (f1 and f2) coincident to the distribution of the mitoribosome subunit bL31 and to its sedimentation in another study (14). Two other peaks of Mss51p distribution were also detected: one in fraction 9 and the second in fraction 12 (Fig. 6*B*). The fraction 9 peak may correspond to the 420 KDa Cox1 module assembly (63) and the second, in fraction 12, to the reported 120 KDa complex hypothesized to be responsible for, or source of, Mss51p function on *COX1* translation (33, 63).

In *mrx9Δ* mitochondria digitonin extraction, Mss51p is no longer found together with bL31; it shows a peak again in fraction 9 and a new peak in a lighter fraction (f12-f13). Finally, when *MRX9-HA* is overexpressed, Mss51p is mostly found in the lighter complex, and a small amount peaks in fraction 7, which is coincident with Mrx9p-HA distribution. Indeed, biotin proximity studies suggested an interaction of Mss51p with Mrx9p (41). The Mss51p concentrated in the lighter fraction can be interpreted as an accumulation of translationally active Mss51p complex hampered to proceed in new *COX1* translation cycles by the Mrx9p excess. In pulled-down experiments, we were unable to detect a copurification of a tagged version of Mrx9 and Mss51p (not shown).

Anti-Prx1 was used as a sedimentation control of lighter fractions; Prx1p is a soluble mitochondrial protein, subjected to Oct1p and Imp2p processing, present in the matrix and in the intermembrane space (64), and an association of Prx1p with mitoribosome subcomplexes has been reported (65). In the *mrx9Δ* strain gradient, some Prx1p is also found spread in the bottom fractions, indicating a possible interaction with such subcomplexes in these cells. It was proposed that Prx1p might prevent oxidative damage during the assembly of ribosomal subcomplexes (65). The major Prx1p band in the panels can be interpreted as the Prx1p processed by Oct1p (64), the lighter upper band as the unprocessed product. In some panels, a smaller Prx1p band is also detected and it has been interpreted as a degradation product.

MRX9 excess in mtDNA intronless strains

The mitochondrial *COX1* gene is transcribed as a polycistronic precursor RNA containing *COX1* with variable but multiple introns (20, 66). Considering that the uncommon mp15 peptide synthesis depends on the constitution of *COX1* introns (36), we tested how Mrx9p excess would affect mitochondrial translation in mtDNA intronless strain. Contrary to our previous results, in the intronless background, overexpression of *MRX9* did not result in impairments in *COX1* translation or a diauxic shift delay. Cells expressing *GPD-MRX9* fusion were grown on glucose and spotted at the exponential growth phase on respiratory ethanol–glycerol media, forcing the respiratory adaptation. While in the mtDNA intronless background, the overexpression of *MRX9* did not cause any growth delay; in the strain harboring introns, a slow growth is observed, confirming previous results (Fig. 7*A*). The Mrx9p-HA levels derived from the *GPD-MRX9* fusion were similar independently of the intron presence or media composition (Fig. S9). Moreover, in galactose grown cells transformed with the

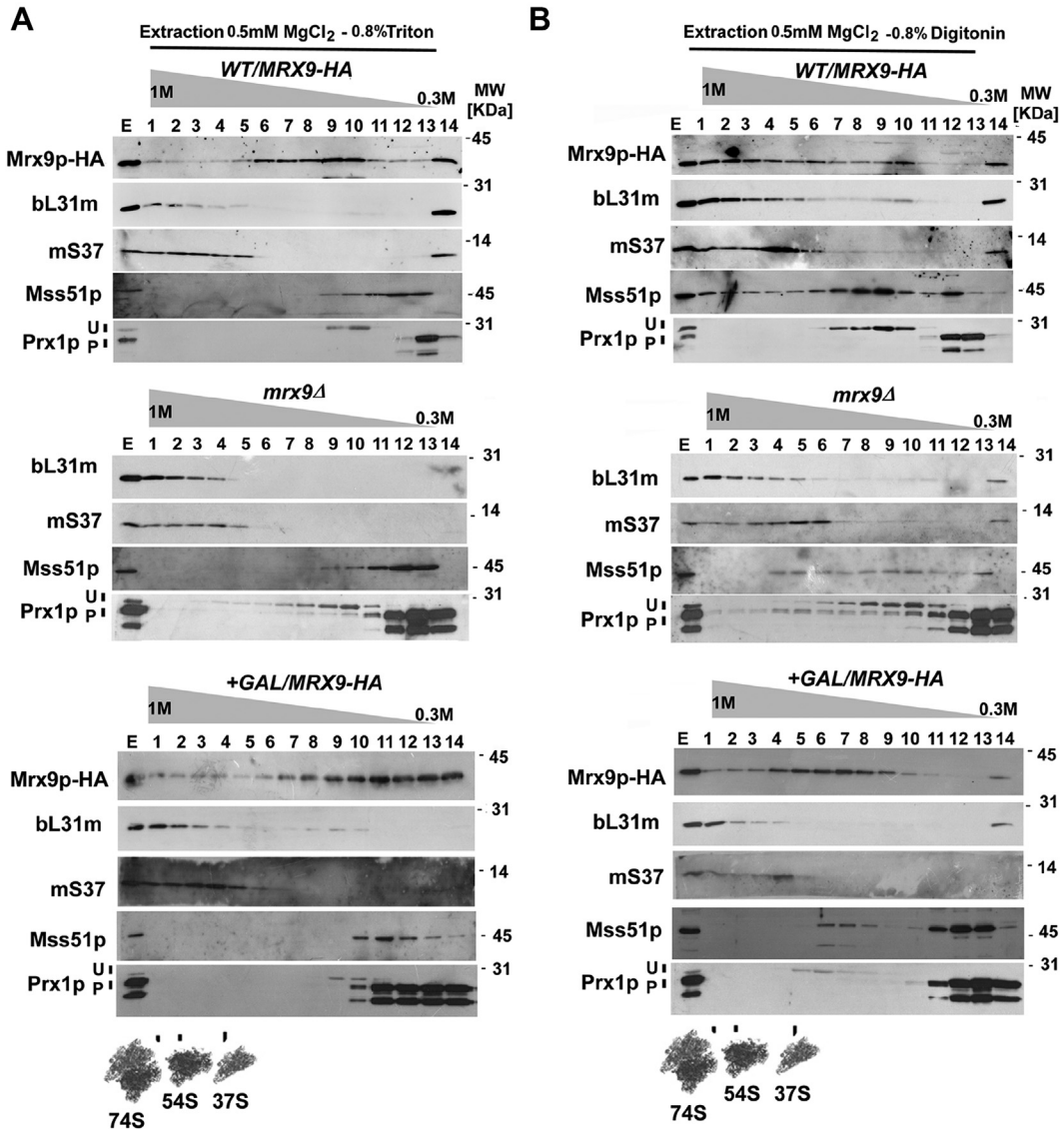

Figure 6. The Mrx9-HA sedimentation pattern and the assembly of the mitoribosome 54S and 37S subunits were assessed from the strain expressing *MRX9-HA* construct with its endogenous promoter (WT/MRX9-HA) and with the *GAL10* promoter (+GAL/MRX9-HA) and in the *mrx9* null mutant (*mrx9Δ*). Mitochondrial proteins were obtained from galactose grown cells and extracted in the presence of 0.5 mM $MgCl_2$ and the indicated detergent (0.8% Triton – A, 0.8% digitonin B). The extracts were sedimented in a linear sucrose gradient (1M to 0.3 M as represented by the *gray* bar). Fractions were collected from the *bottom* (1) to *top* (14). The Western blots were assayed for anti-HA, anti-bL31 (54S component), anti-mS37 (37S component), anti-Mss51p, and anti-Prx1p (soluble—sedimentation control). Prx1p is shown in two bands U (unprocessed) and P (processed), which are dependent on Oct1p/Imp processing state (65). The structure of the mitoribosome (74S), its large (54S), and small subunit (37S) are represented on the bottom of panels (*A* and *B*), based on the sedimentation properties of bL31 and mS37.

GAL-MRX9 construct, the accumulation of newly synthesized mitochondrial products is not affected in the strain devoid of mitochondrial introns, but, once again, *COX1* translation is impaired with Mrx9p excess in the strain harboring introns (Fig. 7*B*). Mitochondrial products were also analyzed from cells grown in glucose at exponential fermentative growth; similarly to Figure 4*D*, the *GPD-MRX9* expression resulted in a significant reduction of the mitochondrial products but not in the intronless strain (Fig. 7*C*). Curiously, *MRX9* overexpression leads to a higher accumulation of newly synthesized Cob in the intronless strains either in fermentative cells with the *GPD-MRX9* construct and in galactose grown cells with the *GAL-MRX9* fusion (Fig. 7, *B* and *C*).

Processing of the mtDNA genes harboring introns *COX1* and *COB* were also evaluated in the *mrx9* null mutant and in the strain overexpressing *MRX9*. Northern blots from isolated mitochondria were probed against *COB*, *COX1*, and *COX2* probes. *COX2* mRNA levels were similar in the tested strains, but mature *COB* and *COX1* mRNAs were poorly accumulated and unprocessed intermediates detected in both cases (Fig. 7*D*). Therefore, we can conclude that *MRX9* excess interferes in the processing of *COX1* and *COB* primary transcripts and that is likely the main cause of the phenotypes previously described here. The diminishment of these transcripts can be either a direct effect of the Mrx9p excess on the mRNAs or due to a titration on splicing factors. *COB* and

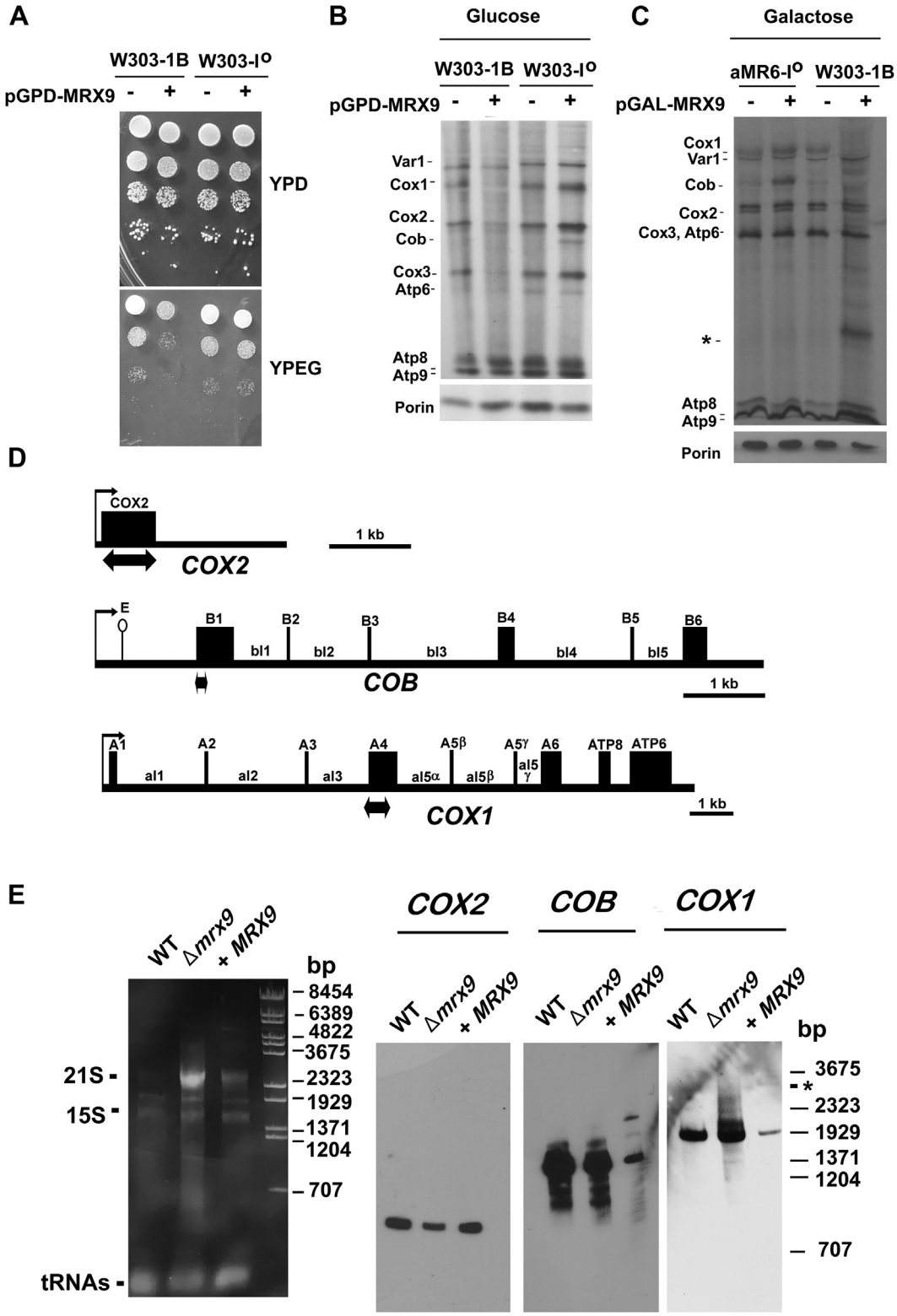

Figure 7. *MRX9* excess toxicity is dependent on the presence of mitochondrial introns. *A*, glucose-grown cells of the indicated strains were spotted on rich glucose media (YPD) and rich ethanol–glycerol media (YPEG) and photographed after 2 days at 30 °C. *B* and *C*, mitochondrial newly synthesized products were labeled from the indicated strains harboring (+) or not (-) the indicated *MRX9* plasmid. Cells at exponential logarithmic growth (*B* in glucose, *C* in galactose) were incubated in the presence of methionine-cysteine ^{35}S mixture and the mitochondrial products identified as in Figure 1. *D*, schematic representation of the W303-1B yeast strain mitochondrial primary RNA transcripts. *COB* and *COX1* exons are represented with *capital letters* and the introns with *lowercases letters*, *double arrows* positioned the probes employed in the Northern blots as follows: *COX2* entire coding sequence, *COB* a 23 nucleotide primer positioned just after the first ATG (Table S2, (95)); *COX1* exon a4 entire sequence. Scale and graphics were based on a previous review (77). *E*, mitochondrial RNAs from the indicated strains were extracted and separated in a 1% agarose gels with a BstEII digest of λ phage serving as a size standard. The ethidium-bromide stained gel on the *left* is representative of one extraction, the base pairs sizes of the standard fragments are indicated on the *right*. RNAs were transferred to a nylon membrane, hybridized to 3′end biotin-conjugated probes for *COB*, *COX1*, and *COX2* depicted in (*D*). The asterisk * between 3675 and 2323 indicates a faint band of a partially processed *COX1*.

COX1 RNAs processing and translation control share some proteins in common, such as the bI4 maturase, Mss116p, Mrs1p, and the *COB* translation factor Cbs2p, which is also necessary for bI4 maturase function (2, 38, 67, 68).

The overexpression of *MRX9* was also tested on the expression of mitochondrial $ARG8^{m}$ at the *COX1*, *COX2*, *COB*, and *ATP9* loci of mtDNA in the *arg8Δ* mutant strain. *COX2* and *ATP9* are mitochondrial intronless genes. $ARG8^{m}$ is a version of nuclear *ARG8* but modified to accommodate for the differences in the genetic code of yeast mitochondria (69) (Fig. 8). Overexpression of *GAL-MRX9* in *cox2Δ*, and *atp9Δ* in minimal galactose media did not impair growth in the absence of arginine, indicating that the reporter gene $ARG8^{m}$ is being sufficiently produced and *MRX9* excess is not toxic to its expression in galactose. However, in the study of the 5′ UTR of *cox1Δ* and *cobΔ* strains, the $ARG8^{m}$ expression was not sufficient to warrant optimal growth when *MRX9* is overexpressed. In the glucose spots, the *GAL-MRX9* construct is shut off, and the transformant strain grows as much as the originally deleted *cox1Δ* and *cobΔ* strains. These results strengthen the hypothesis that Mrx9p interferes in 5′UTR activation or processing of these two mitochondrial genes.

Discussion

Mitochondria OXPHOS complexes can form large structures called supercomplexes (70), whose formation and stoichiometry is modulated by the metabolic state and energetic demand of the cell (71). In yeast, the two observed supercomplexes consist of the dimeric *bc*1 complex associated with one or two cytochrome *c* oxidase complexes on either side of *bc*1, while the ATP synthase dimers and oligomers are required for cristae formation (70–72). Mitochondrial supercomplexes are the final elements of a highly ordered orchestration of events, tightly regulated, involving mtDNA gene expression, mitochondrial protein import, and OXPHOS complexes assembly. Mitochondrial translation control is critical for gene expression; it depends on nuclearly encoded imported proteins to be active. Moreover, translation activations of mitochondrial mRNAs are coupled to complexes assembly by feedback loops control (4, 15–19), unveiling a strong functional interplay between the two existing genomes. Moreover, intermediate OXPHOS modules can exert control in the translation of components of a different OXPHOS complex module (17, 73, 74). In this work, we identified Mrx9p as a new component of the regulatory conundrum, ultimately required for the final stoichiometry of the OXPHOS complexes.

Mrx9p excess toxicity is dependent on the presence of mtDNA introns; it interferes in the processing of *COB* and *COX1* introns during the translation of the respective mRNAs (Fig. 8). Although the evolutionary significance of intervening sequences in yeast mtDNAs is still under debate (2), the removal of all mitochondrial introns results in altered mitochondrial morphology, gene expression, and metabolism impacting growth and lifespan (75). Indeed, the presence of introns provides an additional step of communication between nuclear and mitochondrial genomes, in which nuclearly encoded proteins regulate the expression of mitochondrial genes. For instance, our labeling experiments showed the regulatory effect of *COB* introns in translation, the strains devoid of introns clearly accumulated more of the newly synthesized Cob.

Translation of *COX1* and *COB* transcripts, the correct inner membrane insertion of the newly synthesized polypeptides, and the association with assembly factors are dependent on the function of a multitude of proteins, with different levels of regulation, from RNA processing to protein chaperones (18, 37, 76). Indeed, many of these proteins affect both *COX1* and *COB* expression, including Mrs1p, Mss116p, Cbs2p, Nam1p, Nam2p, and even the maturase encoded by bI4 (77). Accordingly, we propose that Mrx9p also belongs to this group of factors affecting the expression of both genes.

The overexpression experiments assumed the premise that an eventual excess of translation repressor or some sort of downregulator would result in loss of translation of a specific polypeptide. Indeed, we showed that *SMT1(MRX5)* overexpression resulted in the diminished translation of the *ATP6/8* transcript as expected (42), while the excess of *CBS2* and *AEP1* were putatively toxic to the gene expression system as

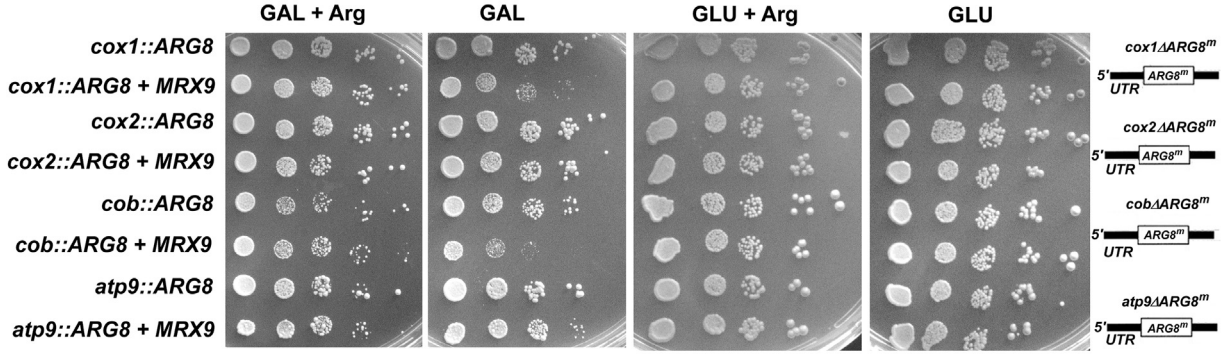

Figure 8. *MRX9* excess impairs proper activation of the reporter $ARG8^{m}$ under control of *COB* and *COX1* 5′ UTR. Mitochondrial mutants strains cox1Δ (*cox1::ARG8*), cox2Δ (*cox2::ARG8*), cobΔ (*cob::ARG8*), and atp9Δ (*atp::ARG8*) and the same strains transformed with the *GAL-MRX9* fusion (+*MRX9*) were grown overnight in rich galactose media. Cells were spotted on minimal galactose (GAL) and minimal glucose without supplementation (GLU) and supplemented with arginine (GAL + Arg) (GLU + ARG). On the right, a depicted panel of the respective disrupted gene. Plates were photographed after 3 days of growth at 30 °C.

suggested by the elevated number of petites, confirming previous results observed for *CBS2* (78). Overexpression of poorly characterized MIOREX components *FMP10*, *FMP30*, and *AIM19* (9) also lead to elevated petite formation and a pleiotropic diminishment of mitochondrial products, and in these cases, the understanding of the toxicity of their gene products may help unveil their specific function. Overexpression of *MRX9* was further evaluated using tagged proteins and different promoters that lead to a variable yield of the protein; clearly, Mrx9p excess in the fermentative grown cells was toxic to respiratory adaptation. Therefore, considering the regulatory effect of Mrx9p in *COX1* and *COB* mRNAs, the presence of intervening sequences can be an evolutionary advantage for the optimal adaptation to the respiratory metabolism.

In our working model (Fig. 9), Mrx9p facilitates the access of translation activators to *COB* and *COX1* 5′ UTR and regulates the access of splicing factors to their introns. In the WT strain growing under respiratory conditions, occurs the optimal recruitment of translation activators and the mRNAs are properly processed. Fermentative and respiratory grown cells expressing, respectively, *GPD-MRX9* construct and *GAL-MRX9* construct present the excess of Mrx9p interfering in the 5′UTR and in intron removal of *COB* and *COX1*. If Mrx9p, translation activators, and splicing factors are competing for the same mRNA sites, then extra Mrx9p would ultimately unbalance the proper modular assembly of cytochrome *c* oxidase and the *bc*1 complex (79–82), impairing respiratory adaptation (*GPD-MRX9*) and respiratory growth (*GAL-MRX9*).

Improper adaptation to the diauxic shift together with destitute Cox1p translation in fermentative conditions was also observed in *mam33Δ* mutants together with the loss of optimal *COX1* splicing (27); indeed, Mam33p has multiple functions: it chaperones newly imported mitoribosomal proteins to ensure proper assembly of the mitoribosome (11), interacts with bL27 mitospecific domain and controls optimal mitochondrial translation under glucose (58), and together with Mrx6p and Pim1p regulates mtDNA copy number (83). The phenotypes resemblance between *mam33* deletion and *MRX9* excess suggests that Mrx9p may interfere in Mam33p function.

Overexpression of *MRX9* in pos-diauxic grown cells diminishes specifically the accumulation of newly synthesized Cox1p as well elicit the appearance of an aberrant translation product, resembling mp15 properties, which is usually found in the absence of translationally active Mss51p and dependent on *COX1* intron composition (30, 36). The regulatory loop performed by Mss51p (15) is bypassed in *cox14Δ* cells (30) but not in *cox14Δcox24Δ* double mutants (14, 22); similarly, in the *cox14Δmrx9Δ* double mutant, the bypass is not as efficient as in the *cox14Δ* mutant. This result suggests that translationally active Mss51p did not efficiently perform new cycles of *COX1* translation in the double mutant *cox14Δmrx9Δ*. In our model, the absence of Mrx9p leads to a suboptimal association of Mss51p with the mitoribosome and the translational activation complex (Fig. 9).

Moreover, Mrx9p removal or overexpression altered the Mss51p sedimentation pattern, similar to that reported for the *cox24Δ* mutants (14) with a concentration of Mss51p in the lighter fractions of the gradient. Cox24p (mS38) is a component of the mitoribosome (84) required for proper *COX1* splicing and *COX1*, *COX2*, and *COX3* translation (14, 22) When we tested the overexpression of *MRX9* in some *COX* mutants, it was clearly more harmful to the mitochondrial protein synthesis of *cox24Δ* null mutant.

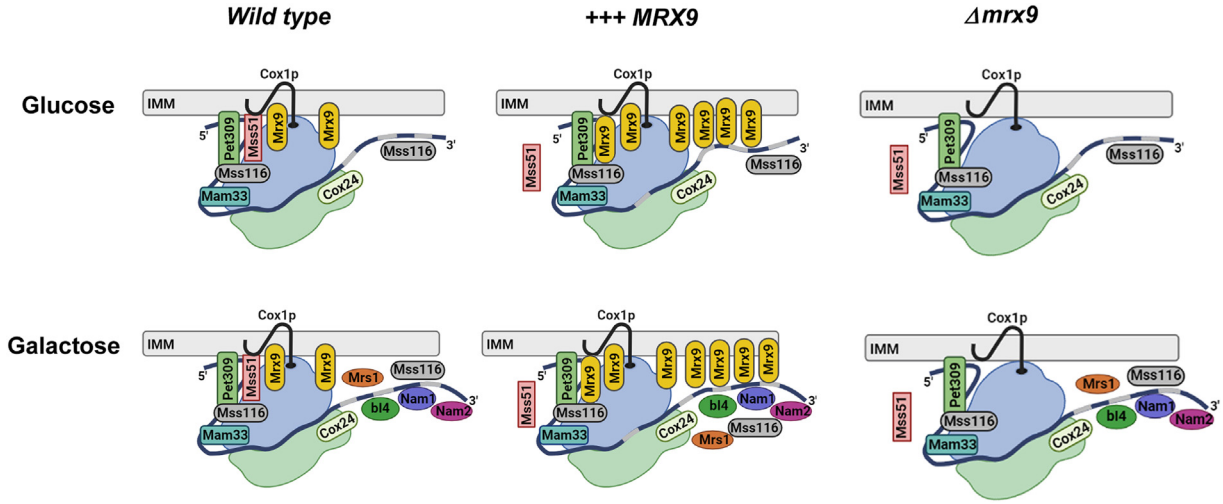

Figure 9. Model for Mrx9p function in *COX1* processing and translation. In the WT, Mrx9p associated with the mitochondrial inner membrane (IMM) and the mitoribosome. *COX1* mRNA starts its translation before all intervening sequences (in *gray*) are processed and concomitantly with the insertion of the nascent polypeptide (Cox1p) in the IMM (1, 4, 40). Optimal Cox1p translation requires Pet309p, Mss51p, Mss116p, Mam33p, Mrx8p, and Cox24p (4, 22, 25–29). In galactose, expression of nuclearly encoded mitochondrial proteins is not repressed and their steady-state level elevated inside the organelle, including those involved in intron processing (77). Some of them depicted in the panel: Mrs1p, Mss116p, Cox24p, Nam1p, Nam2p, and the mitochondrial bI4 maturase, which function depends on Cbs2p (68). The accumulation of these factors intensifies the mitochondrial protein synthesis. When *MRX9* is overexpressed (+++MRX9) in glucose (*GPD-MRX9* construct), our data show destitute mitochondrial protein synthesis; in galactose (*GAL-MRX9* construct), its excess lead to dislocation of Mss51p from the activation complex, interference in the 5′ UTR sequence and impairment in intron processing. Finally, in the null mutant (*Δmrx9*), Mss51p is not optimally recruited to the 5′ UTR activation complex.

We hypothesized that Mrx9p is part of the regulatory system that the mitochondrial introns exert on gene expression based on the following phenotypes of *MRX9* excess toxicity: defective *COX1* and *COB* mRNAs processing; poor arginine complementation by $ARG8^{m}$ placed under the control of *COX1* and *COB* 5′ UTR; the slower adaptation to the respiratory metabolism depends on the presence of mitochondrial introns; interference in Mss51p distribution and availability for new *COX1* translation cycles, as also observed in the *mrx9* null mutant. Although a direct interaction of a tagged version of Mrx9p with Mss51p was not observed (data not shown), previous studies have indicated their physical proximity (41). In the cell map obtained from systematic synthetic genetic array studies (85), Mrx9p function is clustered together with proteins known to have a role in RNA metabolism. In the future, Mrx9p interaction studies are necessary to determine whether the toxicity of Mrx9p excess arose from its effects on splicing factors or directly on RNA primary transcripts.

Experimental procedures

Yeast strains and growth media

The genotypes and sources of yeast strains used in this study are listed in Table S1. Yeast strains were maintained in YPD (1% yeast extract, 2% peptone, 2% glucose), YPGal (1% yeast extract, 2% peptone, 2% galactose), YPEG (1% yeast extract, 2% peptone, 2% glycerol, 2% ethanol), minimal glucose (2% glucose, 0.67% yeast nitrogen base without amino acids, supplemented with auxotrophic requirements), and sporulation media (0.5% yeast extract, 1% peptone, 0.05% glucose). All reagents, unless otherwise indicated, were obtained from Sigma–Aldrich.

Overexpression of MIOREX components

The MIOREX components *AEP1*, *AEP2*, *AIM17*, *AIM18*, *AIM19*, *AIM25*, *AIM32*, *AIM34*, *ATP22*, *CBS1*, *CBS2*, *FMP10*, *FMP30*, *MAM33*, *MIN10*, *MRX1*, *MRX2(COQ11)*, *MRX3*, *MRX4*, *MRX5(SMT1)*, *MRX6*, *MRX8*, *MRX9*, *MRX10*, *MRX11*, *MRX12*, *MRX13*, *MRX16 PET20*, *PET54*, *PET122*, *PET494*, *and YLH47* were amplified by PCR with the proper pairs of oligonucleotides listed in Table S2 and cut with the indicated restriction enzyme. The digested PCR products were cloned into YIp351-GAL or YIplac204-GAL plasmids (86) in which the coding sequence of the cloned gene is under control of the *GAL10* promoter.

Construction of MRX9 alleles

The *MRX9* WT allele was amplified from nuclear DNA with primers MRX9-F0 and MRX9-2 (Table S2); the 2042 bp PCR product was doubled, digested with BamH1 and Sal1, and cloned in pUC18 and YIp35I (87), both previously cut with the same enzymes yielding pUC18/MRX9 and pMRX9/ST1. The pUC18/MRX9 was used as a template for a new PCR cycle with the bidirectional primers MRX9-D1 and MRX9-D2 resulting in a clean deletion of *MRX9*. The linear product containing *MRX9* flanking region in pUC18 was digested with Bcl1 and ligated to *HIS3* on a 1 kb BamH1 fragment of DNA. The *mrx9::HIS3* null allele was recovered from this plasmid as a 1.7 kb Sal1-BamH1 fragment and was substituted by one-step gene replacement for the WT allele in the yeast strain W303-1B.

MRX9 C-terminal was fused to the HA-tag by PCR amplification with MRX9-F0 and MRX9-HA primers. This PCR product contains the *MRX9* endogenous promoter and it was cloned into YIp352 (87). A second PCR amplification using MRX9-HA and MRX9-1 primers amplified only the *MRX9* coding sequence. This PCR product was cloned into YIp352-ADH, YIp351-TEF1, YIp351-GPD, and YIp351-GAL (86), in which the coding sequence of *MRX9-HA* allele is under control of the *GAL10* promoter, alcohol dehydrogenase promoter (*ADH*), the translation elongation factor 1α promoter (*TEF1*), and the GAPDH promoter (*GPD*) (59).

Mitochondrial protein synthesis

Depending on the experiments, yeast cell cultures were grown in rich galactose media or rich glucose media. Mitochondrial translation products were labeled in whole cells with a mixture of [^{35}S] methionine and [^{35}S] cysteine (7 mCi/mmol) in the presence of cycloheximide. Cells were lysed and protein extracted as previously described (52). Total cellular proteins were separated by SDS-PAGE on 17.5% polyacrylamide or 6M UREA-PAGE on 12.5% polyacrylamide gel systems.

Mitochondria isolation and fractionation

Yeast mitochondria were prepared by the method of Herrmann *et al.* (88) using Zymolyase 20T, Zymo Research to obtain spheroplasts. Four milligram of mitochondrial preparation were solubilized in 400 μl of an extraction buffer (20 mM Hepes pH 7.4, 25 mM KCl, 0.5 mM PMSF, 0.8% Triton X100 [or 2% Digitonin], 5 mM $MgCl_2$) and were centrifuged at 27,000*g* for 15 min and applied onto a linear sucrose gradient 0.3 to 1.0 M, with the same constitution of the correspondent extraction buffer. The linear sucrose gradients were centrifuged for 3 h at 40,000 rpm in the 55Ti Beckmann rotor, Beckmann Coulter (12). Antibodies and sources are listed in Table S3.

RNA was extracted from the mitochondria at a protein concentration of 5 mg/ml as described (89). Total mitochondrial RNAs were separated on a 1% agarose gel. The gel was stained with ethidium bromide, photographed, and the RNAs blotted to a nylon membrane. Following UV crosslinking, the nylon membrane was hybridized overnight at 42 °C with COB and COX1 probes (Table S1) that were 3′ end labeled with a biotin kit (Thermo Scientific) as described elsewhere (89).

Enzymatic assays of mitochondrial respiratory complexes

Ubiquinol-cytochrome *c* reductase activity was measured by following the increase in absorbance at 550 nm due to the reduction of cytochrome *c* in the presence of 10 mM NADH (NCCR). COX was assayed by following the oxidation of ferrocytochrome at 550 nM (90). GraphPad Prism software was used for graphs and data analyses by Turkey's statistical test.

Data availability

All data discussed here are presented in the manuscript.

Supporting information—This article contains supporting information (91–100).

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Conflicts of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: H, hemagglutinin; LSU, large subunit; mtDNA, mitochondrial DNA.

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