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Mitochondrion. Author manuscript; available in PMC 2013 May 01.

Published in final edited form as:
Mitochondrion. 2012 May ; 12(3): 381–390. doi:10.1016/j.mito.2012.02.002.

Schizosaccharomyces pombe homologs of the *Saccharomyces cerevisiae* mitochondrial proteins Cbp6 and Mss51 function at a post-translational step of respiratory complex biogenesis

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Abstract

Complexes III and IV of the mitochondrial respiratory chain contain a few key subunits encoded by the mitochondrial genome. In *Saccharomyces cerevisiae*, fifteen mRNA-specific translational activators control mitochondrial translation, of which five are conserved in *Schizosaccharomyces pombe*. These include homologs of Cbp3, Cbp6 and Mss51 that participate in translation and the post-translational steps leading to the assembly of respiratory complexes III and IV. In this study we show that in contrast to budding yeast, Cbp3, Cbp6 and Mss51 from *S. pombe* are not required for the translation of mitochondrial mRNAs, but fulfill post-translational functions, thus probably accounting for their conservation.

Keywords

Schizosaccharomyces pombe; *Saccharomyces cerevisiae*; mitochondria; mRNA-specific translational activator; protein stability; translation-assembly coupling

1. Introduction

Mitochondria have their own genome and associated transcription and translation machineries. The respiratory complexes, embedded in the inner mitochondrial membrane are composed of subunits encoded by both nuclear and mitochondrial genes. The translation apparatus itself is also of dual genetic origin, with rRNAs and many tRNAs encoded by the mitochondrial DNA (mtDNA), whilst depending upon the species, most or, all of the protein components are encoded by the nucleus and imported into mitochondria (Towpik, 2005; Watanabe *et al.,* 2010).

The control of mitochondrial translation in *Saccharomyces cerevisiae* is very complex (Towpik, 2005). Synthesis of each of the 8 major proteins encoded by the mtDNA requires

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mRNA-specific translational activator proteins encoded in the nucleus (Table 1). Genetic suppression studies have shown that at least six of the activators needed for the translation of four mitochondrially encoded mRNAs function exclusively through the 5'-untranslated regions (UTRs) of their target mRNAs (Table 1). In these cases, the requirement for the cognate activator can be bypassed if its target ORF and 3'-UTR are fused to a 5'-UTR derived from a different mRNA and under the control of another translational activator. For example, Cbs1 and Cbs2 operate in this way on the *CYTb* mRNA encoding apo-cytochrome *b* (Cytb) (Rödel, 1986; Rödel and Fox, 1987). These activators are also indirectly required for the splicing of the *CYTb* mRNA precursors, since several of their introns encode RNA maturases that are essential for the excision of the introns that encode them (Banroques et al., 1986).

In general, translation activators that function exclusively through 5'-UTR targets are not highly conserved in amino acid sequence among budding yeast species, although the function of two of them have been shown to be orthologously conserved among members of that group (Costanzo et al., 2000). Translation activators are rate limiting for the expression of *COX1*, *COX2* and *COX3* in *S. cerevisiae* (Green-Willms et al., 2001; Perez-Martinez et al., 2009; Steele et al., 1996), and play a role in the topological organization of gene expression at the surface of the inner membrane (Krause et al., 2004; Naithani et al., 2003; Sanchirico et al., 1998b). However, except for the *COX1* mRNA-specific activator Pet309, which has experimentally verified orthologs in *Schizosaccharomyces pombe* (Ppr4) (Kühl et al., 2011) and *Neurospora crassa* (CYA-5) (Coffin et al., 1997), no clearly homologous proteins have been identified outside of the budding yeast clade.

Translation of the *S. cerevisiae COX1* mRNA also requires a more complex activator, Mss51 (Perez-Martinez et al., 2003; Siep et al., 2000). In addition to acting upon the 5'-UTR of the *COX1* mRNA (Perez-Martinez et al., 2009), Mss51 interacts with the newly synthesized Cox1 protein and is required for the synthesis of Cox1 from a chimeric mRNA bearing the *COX2* 5'-UTR (Perez-Martinez et al., 2003). Mss51 is present in early complex IV assembly intermediates containing Cox1 and other assembly proteins and is presumably required for assembly (Mick et al., 2007; Pierrel et al., 2007). Thus, Mss51 appears to couple Cox1 synthesis to complex IV assembly in an assembly-feedback loop by virtue of the fact that it cannot activate translation when sequestered in assembly intermediates (Barrientos et al., 2004; Fontanesi et al.,2010a; Mick et al., 2011; Perez-Martinez et al., 2009; Shingu-Vazquez et al.,2010).

Similarly, two factors involved in complex III biogenesis also appear to have a dual function, promoting both synthesis and assembly of cytochrome *b*: Cbp3, first described as an assembly factor (Wu and Tzagoloff, 1989; Kronekova and Rödel, 2005), and Cbp6, first proposed to be a translation factor (Dieckmann and Tzagoloff, 1985). Translation of the *S. cerevisiae CYTb* mRNA is reduced, but not eliminated by nuclear *cbp3* and *cbp6* mutations (Dieckmann and Tzagoloff, 1985; Gruschke et al., 2011). In addition, Cbp3 and Cbp6 have recently been shown to interact to form a complex that is associated both with the exit tunnel of mitochondrial ribosomes and an early assembly intermediate of respiratory complex III, containing the assembly factor Cbp4 (Gruschke et al., 2011). These two functions of the Cbp3/Cbp6 complex would allow coupled synthesis and assembly of cytochrome *b*.

Interestingly, the *S. pombe* genome encodes proteins highly homologous to Mss51, Cbp3 and Cbp6 from *S. cerevisiae*, which are all more conserved among fungi than other budding yeast translation activators (Figure S1). It is of great interest to explore the function of these proteins in *S. pombe*, since many aspects of its mitochondrial gene expression system more closely resemble that of animals than that of budding yeast. The mtDNA has two principal promoters of different strengths, yielding two overlapping primary transcripts that are

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generally processed by tRNA excision and cleavage downstream of a C-rich motif to generate the rRNAs and mRNAs containing short UTRs (Schäfer et al., 2005). Finally, *S. pombe* is a *petite*-negative yeast, whose respiratory physiology is closer to that of animal cells than *S. cerevisiae* (Chiron et al., 2007)

In this study we have investigated the function of Mss51, Cbp3 and Cbp6 in *S. pombe*, and found that they only act at post-translational steps in mitochondrial biogenesis. Since the functions of Mss51, Cbp3 and Cbp6 conserved in these two widely divergent species are post-translational, our findings suggest that their role in respiratory complex assembly may be their ancestral role, while their role in controlling budding yeast translation may be a specialized adaptation for facultative anaerobiosis.

2. Materials and Methods

2.1. Strains, plasmids, media and genetic methods

All strains are described in Table 2 and were grown at 28°C as indicated. The wild type *S. pombe strains* used were NB205-6A (*h- ade6-M216 ura4-D18 his3Δ leu1-32 rho+* [3 mitochondrial introns]) and NB34-21A (*h- ade6-M216 ura4-D18 his3Δ leu1-32 ptp1-1 rho+* [3 mitochondrial introns]) (Chiron et al., 2005). Plasmids used or constructed during this work were derivatives of pGEM-T-easy (Promega #TM042), pDUAL-FFH1 and pDUALYFH1 (Matsuyama et al., 2004; 2006), pTG1754/*Not*I (Bonnefoy et al., 1996), or pFL61 (Minet et al., 1992). Media and genetic methods were as reported previously (Bonnefoy et al., 1996; 2000). *S. pombe* transformation (Okazaki et al., 1990) was improved by (1) using single stranded salmon sperm DNA as carrier, (2) regenerating cells in complete liquid medium overnight, and (3) plating onto 5% glucose selective medium as described in Chiron et al. (2007). Yeast genomic DNA was extracted as described (Hoffman and Winston, 1987).

2.2. *Deletion of the* cbp3, cbp6 *and* mss51 *S. pombe and* S. cerevisiae *genes*

For gene deletions, PCR fragments containing the kan^R gene were generated with hybrid oligonucleotides containing 75 to 80 bases of homology with the recipient locus on both sides of the gene of interest and transformed into NB205-6A or NB34-21A as described in Chiron et al. (2007). [Kan^R] transformants able to grow in presence of the drug G418 were streaked again on selective medium, and the genomic DNA of single colonies was analyzed by PCR to look both for the correct insertion of the deletion cassette and the absence of the wild type sequences. Colonies carrying the deletion were back-crossed to a wild type strain to verify the co-segregation of the G418 resistance with the gene deletion.

In *S. cerevisiae CBP6* and *MSS51* were also deleted with the kan^R marker in the strains CW04 and CW252 using the classical PCR strategies described in (Wach, 1996). *CBP6* was also inactivated in a derivative of strain MCC60R2-16 (Costanzo and Fox, 1988) retaining only the rearranged mitochondrial genome*[MSUcbs1-2]* (see Table 1) to generate a double *Δcbs1 Δcbp6* mutant containing the rearranged genome (IK97-1). In addition, the wild type open reading frames from both genes and both yeasts were amplified by PCR, cloned into pGEMT-easy and verified to be free of mutations, then transferred into the *S. pombe* expression vector pTG1754/*Not*I or into the *S. cerevisiae* expression vector pFL61 and used for complementation tests. These showed that the corresponding wild type genes could complement all four deletions; however, no cross-species complementation could be detected.

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2.3. *Integration of FLAG versions of the* cbp6 *and* mss51 *genes*

Plasmids containing different tagged *S. pombe cbp6/mss51* genes under the control of the *nmt1* promoter (Matsuyama et al., 2006) were purchased from the RIKEN consortium and first tested for their ability to complement the corresponding mutants by transformation and selection for the *ura4* marker to maintain the plasmid in its replicative form. The tagged genes able to produce Cbp6-YFP-FLAG-His_6 and Mss51-$FLAG_2$-His_6 complemented the gene deletion, but Cbp6-$FLAG_2$-His_6 did not (data not shown). The plasmids that showed a good complementation were cut by *Not*I and transformed into the corresponding Δ*cbp6* or Δ*mss51* mutants to integrate the tagged version into the *leu1* locus (see Matsuyama et al., 2004).

2.4. Fluorescence microscopy

Fluorescence microscopy was performed on live cells in glucose culture medium, using a Zeiss Axioplan 2 microscope linked to a Cool Snap camera (Princeton Instruments). Mitotracker was obtained from Molecular Probes and used in accordance with the manufacturer's instructions.

2.5. Northern blot analyses

S. pombe cells were grown to exponential phase (100 Klett units or 1 OD_{600}) in complete medium, total RNAs were extracted using the hot phenol protocol (Ausubel et al., 1993) and run on a formaldehyde gels before transfer onto Hybond-C extra membranes. The blots were successively hybridized with different probes at 65°C under standard saline conditions. After overnight hybridization, the blots were washed briefly several times with 6xSSPE before exposure for a few hours, or up to 2 weeks. Mitochondrial probes were PCR fragments labeled with $dATP^{32}$ using a random priming kit (Invitrogen).

2.6. S^{35} labeling of mitochondrial proteins, pulse chase experiments

S. pombe cells were grown to early exponential phase in complete 5% raffinose medium containing 0.1% glucose. Mitochondrial proteins were labeled at 30°C by a 3 hour incubation of whole cells with ^{35}S methionine and cysteine (Bioactif-Hartmann) in the presence of 10mg/ml cycloheximide, which specifically blocks cytoplasmic translation. In pulse-chase experiments, cells were labeled *in vivo* for 2.5 hours, after which they were washed and resuspended in a chase buffer consisting of complete 5% raffinose medium containing 0.1% glucose, 10 mg/ml cycloheximide, 3 mg/ml cysteine, and 10 mg/ml methionine, and divided into four equal aliquots (we found that the chase had to be carried out in the presence of cycloheximide even after several washes, since removing the drug from the cold chase buffer led to a strong background of radio-active amino-acid incorporation into cytoplasmic proteins). The zero point was spun down directly and frozen at -18°C. The other tubes were incubated at 28°C with shaking, time points were generally taken after 1, 3 and 6 hours. As a control, cells were plated after 24h in chase buffer, to verify that they were still viable. For *S. cerevisiae*, galactose grown cells were labeled in presence of 0.6 mg/ml cycloheximide for 20 minutes, washed and chased with non-radioactive methionine and cysteine (3 and 10 mg/ml of cysteine and methionine respectively in complete galactose medium) for one hour in the absence of cycloheximide. *S. pombe* and *S. cerevisiae* proteins were extracted as described in Gouget et al., (2008), samples were run on 16% acrylamide – 0.5% bisacrylamide SDS gels and the dried gels were exposed to a film for one day, or up to several weeks at -70°C, or onto a phosphorimager screen for up to several days at room temperature.

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2.7. Purification of mitochondria and enzymatic activity measurements

Mitochondria were purified from *S. pombe* cells grown in complete glucose medium (Chiron et al., 2007). For the measurement of enzymatic activities, the resuspension buffer was supplemented with 0.5% BSA. Cytochrome *c* oxidase activity was measured on isolated mitochondria as described previously (Lemaire and Dujardin, 2008).

2.8. Antibody production: anti-Cytb

An *S. pombe* cytochrome *b* specific antibody was obtained from Eurogentec after the immunization of two rabbits, simultaneously with two synthetic peptides. These peptides were chosen based on the results obtained when raising an anti-Cytb from *S. cerevisiae* (Nouet et al., 2007) and by comparison of the *S. pombe* Cytb sequence to the predicted structure for the *S. cerevisiae* Cytb (Fisher and Meunier 2008). The peptides synthesized were: $_{101}$LYYGSYKYPRTMT$_{113}$, which is predicted to be in an inter-membrane space loop and $_{256}$CALPADPLKTPMS$_{268}$ which is expected to be in the matrix. Pre-immune rabbit sera were verified to be free of background around the expected band size (25-42 kDa) by western blotting of wild type *S. pombe* mitochondria. The rabbits were then subjected to the AS-DOUB-LX standard protocol from Eurogentec that included five immunizations over 87 days, final bleeding was 15 weeks after the first immunization. Working dilutions between 1/1 000 - 1/2 500 were shown to give the best signals on non-heated samples.

2.9. Alkali carbonate treatment and western blots

Alkali carbonate treatment to isolate membrane and soluble mitochondrial fractions was performed as described previously (Lemaire and Dujardin, 2008). Samples were run on 10 or 12% SDS-PAGE before Western blotting. Primary antibodies were: anti-human Hsp60, 1/1 000, (Sigma H3524); anti-*S. cerevisiae* Arg8: 1/4 000 (Steele et al., 1996); anti-*S. pombe* Cox2, 1/2 500 (Gaisne and Bonnefoy, 2006); anti-*S. pombe* Cytb, 1/1 000; anti-Flag, 1/1000 (Sigma F185); Secondary antibodies were diluted 1/10 000 fold.

2.10. Blue-native gels and in gel activities

BN-PAGE was carried out according to (Schägger and von Jagow, 1991) modified as described in (Lemaire and Dujardin, 2008). The respiratory complexes were separated on 5-8% or 5-10% polyacrylamide gradient gels. *S. pombe* mitochondria were solubilized with 2% digitonin. Western blots of blue native gels were performed as reported by (Lemaire and Dujardin, 2008).

2.11. Cytochrome spectra

Low temperature cytochrome spectra of *S. pombe* cell pastes were recorded using a Cary 400 spectrophotometer after addition of sodium dithionite to fully reduce the cytochromes (Claisse et al., 1970). The absorption maxima were 603, 560, 554 and 548 nm for cytochromes *aa3*, *b*, *c1* and *c* respectively. The *S. pombe* cytochrome *c* peak always shows a 544 nm shoulder that disappears in a cytochrome *c* mutant (N. Bonnefoy, unpublished).

3. Results

3.1. Only a few of the mRNA-specific translational activators from budding yeast appear to be conserved in S. pombe

Blast searches for *S. pombe* homologs were run on the fourteen proteins classified in *S. cerevisiae* as mitochondrial translation activators specifically required for the synthesis of a given mitochondrial protein (see Table 1 and introduction). Only five resulted in a clear match in the *S. pombe* database: Pet309 with Spac8C9.06c and Spac1093.01, Aep2 with Spcc11E10.04, Mss51 with Spac25B8.04c, Cbp6 with Spbc947.14c, and Cbp3, recently

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classified as a translation factor (Gruschke et al., 2011), with Spcc4B3.17. The function of the homologs of Pet309 (Ppr4) and Aep2 (Ppr6) has been reported elsewhere (Kühl et al., 2011). In this study we have focused on the homologs of Cbp3, Cbp6 and Mss51; these are conserved in fungal genomes (Figure S1-3). In addition there is a clear human sequence homolog for Cbp3 (Figure S1); a potential Cbp6 homolog is also found in higher eukaryotes including humans (Figure S2), however Cbp6 is a rather small protein, which makes searches for sequence homologies beyond fungi less reliable. For Mss51, a possible human homolog has also been detected, ZMNYD-17 (Mick et al., 2011; Perez-Martinez et al., 2009, Figure S3). This has a conserved zinc finger MNYD domain proposed to mediate protein-protein interactions (Matthews et al., 2009).

3.2. In S. pombe Cbp3 and Cbp6 are important for complex III but not for cytochrome b *translation*

In *S. cerevisiae*, Cbp3 and Cbp6 have been shown to form a complex involved both in the translation of cytochrome *b* and the early assembly of complex III. To investigate the function of the Cbp3 and Cbp6 proteins in *S. pombe*, each gene was disrupted in several wild type strains. Irrespective of the strain background, both deletion mutants showed a similar stringent growth defect on galactose medium (Figure 1A and data not shown), which indicates a strong mitochondrial respiratory defect in *S. pombe* (Chiron et al., 2007). In *S. pombe*, when the electron transfer is blocked by antimycin A, the hydrolysis of ATP by complex V becomes essential for viability, even on fermentable medium as it is the only way to generate a membrane potential. Neither Δ*cbp3* nor Δ*cbp6* mutants were sensitive to antimycin A on glucose medium (Figure 1A), demonstrating that they contain at least some level of complex V activity, unlike Δ*ppr6* cells, which are defective for complex V (Kühl et al., 2011).

As expected from the respiratory growth defects, the cytochrome spectra of the two deletion mutants differed from wild type (Figure 1B): both Δ*cbp3* and Δ*cbp6* cells showed strongly reduced peaks of cytochrome *b* and *c1* (specific for complex III) and reduced cytochrome *aa3* (specific for complex IV), like a Δ*cytb* mutant. Such a decrease in the cytochrome *aa3* peak has already been observed in another *S. pombe* mutant affected in complex III, Δ*abc1*. This suggests that this is a secondary consequence of the complex III defect. Thus both Cbp3 and Cbp6 appear to be primarily required for the biogenesis of complex III as in *S. cerevisiae*.

Since the *S. cerevisiae* Δ*cbp3* and Δ*cbp6* mutants are required for efficient translation of cytochrome *b* (Gruschke et al., 2011), the synthesis of mitochondrial proteins was investigated in Δ*cbp3* and Δ*cbp6* cells by *in vivo* labeling with ^{35}S methionine and cysteine in the presence of cycloheximide. Both mutants produced mitochondrial protein patterns similar to the wild type: Cytb, Cox1, 2 and 3 were clearly visible, although Cox2 was less strongly labeled in both mutants (Figure 1C). As a control, Δ*cytb* cells were also labeled and found to lack the Cytb protein as expected. Thus, unlike *S.* cerevisiae, *S. pombe* strains lacking Cbp3 or Cbp6 appear to synthesize cytochrome *b* at normal levels.

Since Cbp3 and Cbp6 appeared to have a similar phenotype, we chose to pursue the study of Cbp6, in order to further understand its role in *S. pombe* and *S. cerevisiae*. Like its *S. cerevisiae* counterpart (Dieckmann and Tzagoloff 1985), *S. pombe* Cbp6 is a mitochondrial protein as shown by a *Cbp6-YFP* reporter integrated at the ectopic *leu1* locus under the control of the *nmt1* promoter (Matsuyama et al., 2006; Figure S4A, B, C). After carbonate treatment of purified mitochondria, the tagged Cbp6 protein was generally difficult to detect, but appeared to partition into the soluble fraction like Arg1 (Figure S4D). This suggests that Cbp6 is not an integral membrane protein.

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3.3. Δ cbp6 contains normal levels of mitochondrial mRNA but destabilizes Cytb and prevents complex III assembly

In *S. cerevisiae*, the absence of a specific translation activator does not strongly compromise the stability of its target RNA, but can prevent the excision of RNA maturase encoding-introns from precursor RNAs (Fox, 1996). We observed a similar phenotype for the *S. pombe* Δ*ppr4* mutant, which is the Pet309 homolog. This mutant contains substantial amounts of *cox1* mRNA if the *cox1* gene lacks introns, but accumulates the *cox1* precursor in an intron-containing background (Kühl et al., 2011). We therefore investigated the state of the mitochondrially encoded *cytb* mRNA in the Δ*cbp6* mutant in an intron-containing background. The *cox2* mRNA was also analyzed since ^{35}S labeling experiments showed a slightly reduced level of Cox2 protein. Both the *cytb* and *cox2* mRNAs were present at normal levels in the Δ*cbp6* mutant (Figure 2A), showing that the effect on the Cox2 protein is not a consequence of a lower mRNA level, and that the intron encoded maturase from *cytb* is correctly synthesized leading to normal splicing in Δ*cbp6* cells. This shows that Cbp6 is not required for translation of the intronic region of the *cytb* precursor RNA.

To analyze the stability of newly synthesized mitochondrial proteins in the Δ*cbp6* mutant, a pulse-chase analysis was performed (Figure 2B). After 6 hours of chase, cytochrome *b* was still stable in both the mutant and the wild-type, while Cox1 and Cox3 appeared slightly more unstable in the mutant. Longer chase experiments were difficult to interpret because after 12h of chase in presence of cycloheximide all signals started to collapse in both strains. Given this behaviour of newly synthesized Cytb in *S. pombe*, we decided to perform a western blot analysis rather than a pulse chase experiment to look at the stability of Cytb. Thus, polyclonal antibodies recognizing an inter-membrane space and a matrix peptide were raised and used to probe Western blots of purified mitochondria (see Materials and Methods). Strikingly, Cytb was undetectable in the Δ*cbp6* mutant (Figure 2C). Thus, virtually all of the Cytb protein synthesized in the Δ*cbp6* mutant is degraded. The complex IV subunit Cox2 was easily detectable in Δ*cbp6* mitochondria, although its level is reduced (Figure 2C), consistent with the reduced cytochrome aa_3 spectral absorption (Figure 1B). Taken together with the normal Cytb synthesis observed in the experiment of Figure 1C, these results indicate that in *S. pombe*, Cbp6 is primarily required after the synthesis of Cytb, either for its long term stabilization, or for its assembly into complex III.

Our results cannot formally exclude a partial effect on the stability of cytochrome *c* oxidase subunits including Cox2, whether it is direct or indirect. As a comparison, we found that Cox2 level is wild-type in the *Δcytb* mutant used in this work whereas Cytb is undetectable (data not shown). However, a study of various mitochondrial mutants from *S. pombe* (Ahne et al., 1984) shows that among 12 strains harbouring different *cytb* mutations, 11 show a decrease of cytochrome *c* oxidase activity, which can reach only 3% of the wild-type level. Thus, secondary effects of complex III defects on complex IV must be analyzed individually.

Probing of BN-PAGE Western blots with the anti-Cytb antibody showed that complex III was present as three bands in the wild-type, probably corresponding to dimers of complex III with, or without monomers or dimers of complex IV (Figure 2D, lane 2). In both Δ*cbp6* and control Δ*cytb* mitochondria, complex III was completely lacking (lanes 3 and 4). As expected, the higher molecular weight bands of complex III were absent in the Δ*ppr4* mutant, which lacks complex IV. These data are the first evidence for the existence of respiratory supercomplexes containing complexes III and IV in *S. pombe* mitochondria and confirm that complex III is not assembled in Δ*cbp6* mitochondria, despite a strong synthesis of Cytb.

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3.4. S. cerevisiae Cbp6 does not function solely through a target in the 5'-untranslated region of the mRNA encoding Cytb

Since our data pointed towards a strictly post-translational role for Cpb6 in *S. pombe*, we revisited the function of the relatively poorly characterized *S. cerevisiae* Cbp6, heretofore considered a translation activator of Cytb synthesis. Two other *S. cerevisiae* mRNA-specific translation activators of Cytb synthesis, Cbs1 and Cbs2, have been shown to function through targets in the 5'-untranslated region (UTR) of the Cytb mRNA (Rödel, 1986; Rödel and Fox, 1987). This was demonstrated by showing that in the absence of Cbs1 or Cbs2, the Cytb coding sequence could be translated from chimeric mRNAs bearing the 5'-UTRs of other mitochondrial mRNAs, leading to respiratory growth. To test whether *S. cerevisiae* Cbp6 could be similarly bypassed we placed a rearranged *rho*$^-$ mtDNA encoding a chimeric mRNA, bearing the *COX3* 5'-UTR fused to the Cytb coding region (Costanzo and Fox, 1988) in a Δ*cbs1* Δ*cbp6* double mutant. When this strain was crossed to a *rho*$^+$ Δ*cbs1* single mutant, with a functional *CBP6* gene, the resulting diploids were respiratory competent, demonstrating the expected bypass of the *cbs1/cbs1* homozygous deletion (Figure 3A). However, when the Δ*cbs1* Δ*cbp6* double mutant strain containing the chimeric *CYTb* mRNA was crossed with a *rho*$^+$ Δ*cbp6* single mutant harboring a functional *CBS1* gene, the resulting *cbp6/cbp6* homozygous diploids remained respiratory defective (Figure 3A). Thus, at least part of the Cbp6 function could not be bypassed by the presence of a heterologous 5'-UTR fused to the Cytb coding sequence. This shows that the *CYTb* 5'-UTR cannot be the sole target of Cbp6 in *S. cerevisiae* mitochondria.

3.5. S. cerevisiae Cbp6 is not essential for the synthesis of Cytb but is required for its stability

The first study of Cbp6Sc reported that Cytb synthesis was reduced but not abolished by a point mutation carried by the strain E158, that had been isolated in an intron-containing mtDNA context (Dieckman and Tzagoloff, 1985). We sequenced the *S. cerevisiae CBP6* gene in the E158 mutant and found that the mutation changes the initiation codon of *CBP6* from ATG to ATA. Thus, in the mutant E158, *CBP6* may not be fully inactivated, since ATA has been shown to serve as an alternative initiation codon in yeast (Chang et al., 2010): this could explain the residual synthesis of Cytb in this mutant. Therefore, we reinvestigated this question by deleting the *CBP6* gene in *S. cerevisiae* with mitochondria containing either the 13-intron *CYTb* gene or the intronless *CYTb* gene. Growth on non-fermentable medium was strictly abolished in both backgrounds (Figure 3B). Next we examined the synthesis and stability of the mitochondrially encoded proteins by a short *in vivo* labeling followed by a one-hour chase. The results in Figure 3C show that in the absence of Cbp6Sc, synthesis of Cytb is reduced but detectable in both mtDNA backgrounds. However, Cytb made in the mutants was fully degraded after the chase, in contrast to wild-type where no degradation could be observed. Thus, in *S. cerevisiae*, Cbp6 is not essential for the *CYTb* mRNA translation although it might be required for a normal rate of synthesis. However, it is essential for the stability of the Cytb protein. Thus, the functions of Cbp6 in *S. pombe* and in *S. cerevisiae* appear to be largely similar, despite the absence of functional cross-complementation.

3.6. Mss51 is a membrane associated mitochondrial protein important for complex IV biogenesis

The role of *S. cerevisiae* Mss51 in the translational activation of the *COX1* mRNA through its 5'-UTR is firmly established, along with its post-translational interaction with newly synthesized Cox1 protein (Perez-Martinez *et al.,* 2003; Barrientos *et al.,* 2004; Perez-Martinez *et al.,* 2009). It is also clear that in *S. cerevisiae* Mss51 is a mitochondrial protein. Strikingly, the YFP fusion signal obtained for *S. pombe* Mss51 during the proteome study by the RIKEN consortium (Matsuyama et al., 2006) was a staining of both the plasma and

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nuclear membranes, and the conclusion drawn was a localization of the protein in the endoplasmic reticulum. Due to this unexpected result, we did not use the Mss51-YFP-FLAG construct suspecting that it might not be functional, we used the Mss51-$FLAG_2$-His_6 construct, which we found could complement the *mss51* deletion; this was integrated into Δ*mss51* cells for the cell fractionation experiments. The tagged Mss51 was detected only in the mitochondrial fraction and like Cox2 was strongly resistant to carbonate extraction (Figure 4A), indicating that it is a membrane protein. *S. cerevisiae* Mss51 is also membrane associated, but only peripherally (Siep et al., 2000; Fontanesi et al., 2010b).

To investigate the function of Mss51 in *S. pombe*, the gene was disrupted in different wild type strains. Whatever the strain background, the deletion mutants showed a clear growth defect on galactose medium (Figure 4B). A very slight growth was observed upon longer incubations (data not shown). Similarly to the Δ*cbp3 and Δcbp6* mutants, Δ*mss51* cells were resistant to antimycin A on glucose medium, showing that they contain a functional complex V.

As expected from the respiratory growth defect, the cytochrome spectra of the deletion mutant differed from wild type (Figure 4C): Δ*mss51* cells showed normal cytochrome *b* and *c1* peaks, but cytochromes *aa3* were not detectable. The spectra from Δ*ppr4* cells appeared even more affected, with less remaining cytochrome *b* and a complete lack of cytochromes *aa3*. Thus Mss51 appears to be primarily required for the biogenesis of complex IV, although the phenotype of its deletion is not as stringent as that of Δ*ppr4*.

3.7. In S. pombe Mss51 is required only at a post-translational step of complex IV biogenesis

The synthesis of mitochondrial proteins was investigated in Δ*mss51* and Δ*ppr4* cells by *in vivo* labeling with ^{35}S methionine and cysteine in the presence of cycloheximide. The Δ*mss51* mutant produced mitochondrial protein patterns similar to the wild type: Cytb, Cox1, 2 and 3 were clearly visible, although Cox2 was less strongly labeled in both mutants, especially Δ*mss51* (Figure 5A). As expected, Δ*ppr4* cells clearly lacked Cox1 (Kühl et al., 2011). Thus, in *S. pombe*, Mss51 appears to be required at a post-translational step of complex IV biogenesis; this contrasts with the role of Mss51 in *S. cerevisiae*, where it is absolutely required for the synthesis of Cox1. As for Cbp6, no functional cross-complementation could be obtained between *S. cerevisiae* and *S. pombe*.

Given these surprising ^{35}S labeling results, we analyzed the mitochondrially encoded *cox1* and *cox2* mRNAs from a Δ*mss51* strain containing introns in *cox1*. Northern blots revealed no defect in the accumulation of mature messengers (Figure 5B). Thus, the reduced labeling of the Cox2 protein in Δ*mss51* cells is not due to a low level of *cox2* mRNA, and second, the intron encoded maturases of *cox1* are synthesized as well as Cox1 itself in the absence of Mss51.

Next we looked at the stability of the Cox2 and Cox1 proteins in Δ*mss51* cells. Cox2 was detectable in Δ*mss51* purified mitochondria, although its level was greatly reduced (Figure 5C), consistent with the reduced ^{35}S labeling (Figure 5A). No antibodies against *S. pombe* Cox1 are available. Therefore, we undertook a pulse-chase experiment to study the stability of newly synthesized mitochondrially-encoded proteins in the Δ*mss51* mutant (Figure 5D). After 6 hours of chase, Cox1 was clearly less stable in the Δ*mss51* mutant than in the wild-type, while Cox2 was poorly labeled in the mutant even before starting the chase, as noted before (Figure 5C).

To confirm the specificity of Mss51 function in *S. pombe*, we examined respiratory complex assembly by enzymatic activity measurements. A strong, but not complete, decrease in

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complex IV activity was observed in purified mitochondria from the Δ*mss51* mutant (Figure 5E). Similarly, complex IV activity was barely detectable when assayed in the gel after BN-PAGE (not shown). In contrast, the control Δ*ppr4* mutant, that is unable to synthesize Cox1 (Kühl et al., 2011), completely lacks complex IV activity (Figure 5D). Thus, the low level of Cox2 and the unstable Cox1 subunit of Δ*mss51* cells seem to partly assemble into complex IV to produce a weak residual activity, consistent with the slightly leaky growth of Δ*mss51* cells on galactose medium.

4. Discussion

Mitochondrial genomes encode very few proteins, 8 in yeasts and 13 in mammals. This small number of proteins makes it possible to specifically control the translation of each mt-mRNA. Over the years budding yeast has been used as a model system to identify factors involved in such translational control and its coupling to the assembly of the newly-synthesized subunits into the final respiratory complexes. It is fascinating that at present, so many factors have been found to be required for the production of single mitochondrial proteins, their binding with prosthetic groups, their insertion into the membrane and within their enzymatic complex (Fontanesi et al., 2008). The control of mitochondrial translation in *S. cerevisiae* requires at least fourteen nuclear encoded proteins that activate the translation of individual mRNAs, by acting, at least in part, on the 5'UTR of their target mRNAs (Table 1). This raises the question of whether *S. pombe* uses equally complex mechanisms to regulate its mitochondrial biogenesis.

Searching the predicted proteome of *S. pombe*, we have only found five sequence homologs of *S. cerevisiae* mRNA-specific translational activators: Pet309, Mss51, Cbp6, Cbp3 and Aep2. Interestingly, in *S. cerevisiae* most of these proteins fulfill a dual function. Pet309 and Mss51 both activate translation of the *COX1* mRNA through binding the 5' UTR. In addition, Pet309 is also required for the stability of intron-containing *COX1* mRNAs (Manthey and McEwen, 1995), whereas Mss51 also binds the Cox1 protein until it is assembled. Thus, as described in the introduction, Mss51 participates in an assembly-feedback regulation of Cox1 synthesis, a phenomenon called Control by Epistasy of Synthesis that has been well described in chloroplast biogenesis (CES, see Choquet and Wollman, 2008). Cbp6 and Cbp3, were first proposed to be *S. cerevisiae* translation and assembly factors, respectively (Dieckman and Tzagoloff 1985; Wu and Tzagoloff 1989), but have recently been reported to form a complex that associates with the exit tunnel of the mitochondrial ribosome (Gruschke et al., 2011). In addition, the Cbp3-Cbp6 complex is also part of a none-ribosome-bound assembly intermediate of the *bc1* complex, containing newly synthesized cytochrome *b* and the assembly factor Cbp4. This is consistent with our observations that in *S. cerevisiae*, Cbp6 is important not only for translation but also for the stability of the Cytb protein, and that replacing the 5' UTR of cytochrome *b* by that of another mitochondrial gene does not suppress the deletion of *CBP6*. Thus, Cbp6, like Mss51, has a dual function and targets in *S. cerevisiae*. Finally, Aep2 is the least well characterized; it interacts with the *ATP9* 5'UTR (Ellis et al., 1999), but it is unknown whether it participates in assembly of the Atp9 protein.

In this paper, we have focused on the function of three *S. pombe* proteins, homologous to three *S. cerevisiae* proteins that function both in mitochondrial translation and assembly of the respiratory complexes: in mitochondria, these two steps have been shown to play a key role in the regulation of mitochondrial gene expression. Beyond the sequence conservation, do the fission yeast factors play the same role as their budding yeast homologs? Whereas this seems to be the case for Ppr4/Pet309 and Ppr6/Aep2 (Kühl et al., 2011), we show here that the *S. pombe* Cbp3, Cbp6 and Mss51 genes carry out only post-translational functions that may be similar to their *S. cerevisiae* counterparts; they are not detectably required for

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cytb or *cox1* mRNA translation. These findings suggest that the stability/assembly function of these three factors might represent their ancestral roles, shared by the homologous genes from different organisms. Budding yeast might have recruited these proteins for translational activation as a later adaptation, allowing the coupling of protein synthesis and complex assembly in response to its specific energetic needs.

This would suggest that in *S. pombe* assembly-feedback control of synthesis mediated by Mss51 for Cox1 may not exist. In this case, in *S. pombe* Mss51 would only have a role in protein stability, insertion of prosthetic groups (*e.g.* in Cox1 or Cox2), or assembly of complex IV. In *S. pombe* as well as in human cells, mitochondrial translation might be regulated through general factors rather than specific factors. For example mtEF-Ts and mtIF3 are mitochondrial translation factors with a regulatory rather than protein synthesis role in the general steps of translation, and they are conserved in *S. pombe* and humans, but not in *S. cerevisiae* (Chiron et al., 2005). In addition, we have discovered in *S. pombe* a negative regulator of mitochondrial translation, Ppr5 (Kühl et al., 2011), which, like the human PTCD1 (Rackham et al., 2009) that inhibits the stability of the mitochondrial leucine tRNAs, might play an important function in down-regulating translation of nearly all mt-mRNAs in *S. pombe*, possibly in response to environmental conditions.

Alternatively, an assembly-feedback mechanism might occur in *S. pombe*, but the actors could be different to *S. cerevisiae*. First, Cox1 could be a subunit subject to such control in *S. pombe*, but factors other than Mss51, *e.g.* Ppr4 the homolog of Pet309, could mediate the regulatory feedback loop, possibly by interacting with Mss51. In humans, the *COX1* translational activator TACO1 (Weraarpachai et al., 2009) could play this role together with the possible Mss51 homolog, ZMYND17. A search for other components of the *S. cerevisiae* Cox1 assembly feedback regulation does not retrieve reasonable homologs of Coa3 and Cox14 in *S. pombe* (Mick *et al.*, 2010); but these are very small proteins, which makes sequence comparisons rather unreliable. However, Coa1 which forms a complex with Mss51 and Cox1 in *S. cerevisiae* (Pierrel *et al.*, 2007) is clearly conserved in *S. pombe*, with an N-terminal extension that does not exist in the *S. cerevisiae* protein and could take on the role of a missing component, such as Cox14 or Coa3.

Second, the subunit controlled for complex IV in *S. pombe* could be Cox2 rather than Cox1, since we obtained a much less efficient labeling of Cox2 in the absence of Mss51. However, in our hands the labeling of *S. pombe* Cox2 appears generally decreased in a wide variety of respiratory mutants such as the strains used in this work (Δ*cytb*, Δ*cbp3/6*, Δ*ppr4*), or other *Δppr* mutants (Kühl et al., 2011). In addition labeling of mitochondrial proteins in *S. pombe* is much less efficient than in *S. cerevisiae*. In our experiments we had to label mitochondrial proteins for up to 3 hours, this means that the results of the protein labeling experiments are a mixture of synthesis and turnover and not a simple measure of the neo-synthesis of the mitochondrial proteins. Thus additional experiments are needed to determine whether the effect of Δ*mss51* on Cox2 labeling is specific and is really synthesis or stability.

Finally, our data also raise the question of the exact function of Cbp3 and Cbp6 in both *S. pombe* and *S. cerevisiae* and whether an assembly-feedback process involving these factors could occur for Cytb, linking it to the assembly of complex III. It is clear that in *S. cerevisiae* CES regulation is not restricted to complex IV, as it has also been described for the F_0 subunits of complex V, although the mechanism and factors involved in this case are less clear (Rak et al., 2009). It would be interesting to look at mutants that fail to assemble complex III and determine whether this impacts Cytb synthesis, and whether Cbp6 could play a role in this control at least in *S. cerevisiae*. Cbp6 could also be required for the insertion of heme into the newly synthesized Cytb, or act at a later step in complex III assembly, explaining the defect in Cytb stability in its absence.

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5. Conclusion

Only one third of the specific translational activators found in *S. cerevisiae* mitochondria appear conserved in sequence in the distant yeast *S. pombe*, and probably also in higher eucaryotes. These conserved *S. cerevisiae* factors generally fulfill dual functions, *e.g.* both in mRNA translation and early complex assembly. We show in this study that three of the *S. pombe* sequence homologs act only at a post-translational step, *i.e.* have a more limited role than their *S. cerevisiae* counterparts although still active within the same biogenesis pathway. This might also be true for the potential human homologs of these factors. Such difference might be correlated to the more compact structure of *S. pombe* mRNA and/or differences in metabolism. Thus the question of how translation and its coupling with assembly are mediated in organisms other than *S. cerevisiae* remains open, suggesting that additional components or mechanisms are yet to be discovered, probably through specific screens rather than sequence homology searches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Mauricette Gaisne for constant and skillful technical assistance, Cristina Panozzo for help with fluorescent imaging and Sophie Marsy for sharing her expertise on Blue-Native Gels and enzyme activity measurements. We thank Geneviève Dujardin and Christopher J. Herbert for fruitful discussions and critical reading of the manuscript, G.D. for the gift of antibodies. We thank B. Meunier for advice on the choice of Cytb peptides and B. Schäfer for the gift of the original *Δcytb* strain. FLAG-tagged versions of the *cbp6* and *mss51* genes were distributed by the RIKEN Bioresource Center. This work was supported by a grant of the Agence Nationale pour la Recherche (ANR) JCJC06-0163 to NB and a grant from the US National Institutes of Health (GM29362) to TDF. IK was supported by the ANR and by a grant FDT20091217787 from the Fondation pour la Recherche Médicale.

Abbreviations

aa	amino acids
bp	base pair
mt-mRNA	mitochondrial mRNA
mtDNA	mitochondrial DNA
nt	nucleotides
ORF	open reading frame
UTR	untranslated region

References

Ahne A, Muller-Derlich J, Merlos-Lange AM, Kanbay F, Wolf K, Lang BF. Two distinct mechanisms for deletion in mitochondrial DNA of *Schizosaccharomyces pombe* mutator strains. Slipped mispairing mediated by direct repeats and erroneous intron splicing. J Mol Biol. 1988; 202:725–734. [PubMed: 3172236]

Ahne F, Merlos-Lange AM, Lang BF, Wolf K. The mitochondrial genome of the fission yeast *Schizosaccharomyces pombe*. 5. Characterization of mitochondrial deletion mutants. Curr. Genet. 1984; 8:517–524.

Ausubel, FM.; Brent, R.; Kingston, RE.; Moore, DD.; Seidman, JG.; Struhl, K. Current protocols in Molecular Biology. John Wiley & sons; Boston, Mass.: 1993.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Banroques J, Delahodde A, Jacq C. A mitochondrial RNA maturase gene transferred to the yeast nucleus can control mitochondrial mRNA splicing. Cell. 1986; 46:837–844. [PubMed: 2875797]

Barrientos A, Zambrano A, Tzagoloff A. Mss51p and Cox14p jointly regulate mitochondrial Cox1p expression in *Saccharomyces cerevisiae*. Embo J. 2004; 23:3472–3482. [PubMed: 15306853]

Bonnefoy N, Kermorgant M, Brivet-Chevillotte P, Dujardin G. Cloning by functional complementation, and inactivation, of the *Schizosaccharomyces pombe* homologue of the *Saccharomyces cerevisiae* gene *ABC1*. Mol Gen Genet. 1996; 251:204–210. [PubMed: 8668131]

Bonnefoy N, Kermorgant M, Groudinsky O, Dujardin G. The respiratory gene *OXA1* has two fission yeast orthologues which together encode a function essential for cellular viability. Mol Microbiol. 2000; 35:1135–1145. [PubMed: 10712694]

Chang CP, Chen SJ, Lin CH, Wang TL, Wang CC. A single sequence context cannot satisfy all non-AUG initiator codons in yeast dagger. BMC Microbiol. 2010; 10:188. [PubMed: 20618922]

Chen W, Dieckmann CL. Genetic evidence for interaction between Cbp1 and specific nucleotides in the *5′* untranslated région of mitochondrial cytochrome *b* mRNA in *Saccharomyces cerevisiae*. Mol Cell Biol. 1997; 17:6203–6211. [PubMed: 9343381]

Chiron S, Gaisne M, Guillou E, Belenguer P, Clark-Walker GD, Bonnefoy N. Studying mitochondria in an attractive model: *Schizosaccharomyces pombe*. Methods Mol Biol. 2007; 372:91–105. [PubMed: 18314720]

Chiron S, Suleau A, Bonnefoy N. Mitochondrial translation: elongation factor tu is essential in fission yeast and depends on an exchange factor conserved in humans but not in budding yeast. Genetics. 2005; 169:1891–1901. [PubMed: 15695360]

Choquet, Y.; Wollman, FA. The CES process. In: D., S., editor. The chlamydomonas source book. Academic press; 2008. p. 1027-1063.

Claisse ML, Pere-Aubert GA, Clavilier LP, Slonimski PP. [Method for the determination of cytochrome concentrations in whole yeast cells]. Eur J Biochem. 1970; 16:430–438. [PubMed: 5477288]

Coffin JW, Dhillon R, Ritzel RG, Nargang FE. The *Neurospora crassa cya-5* nuclear gene encodes a protein with a region of homology to the *Saccharomyces cerevisiae* PET309 protein and is required in a post-transcriptional step for the expression of the mitochondrially encoded COXI protein. Curr Genet. 1997; 32:273–280. [PubMed: 9342407]

Costanzo MC, Bonnefoy N, Williams EH, Clark-Walker GD, Fox TD. Highly diverged homologs of *Saccharomyces cerevisiae* mitochondrial mRNA-specific translational activators have orthologous functions in other budding yeasts. Genetics. 2000; 154:999–1012. [PubMed: 10757749]

Costanzo MC, Fox TD. Specific translational activation by nuclear gene products occurs in the 5' untranslated leader of a yeast mitochondrial mRNA. Proc Natl Acad Sci U S A. 1988; 85:2677–2681. [PubMed: 2833749]

Dieckmann CL, Tzagoloff A. Assembly of the mitochondrial membrane system. *CBP6*, a yeast nuclear gene necessary for synthesis of cytochrome *b*. J Biol Chem. 1985; 260:1513–1520. [PubMed: 2981859]

Dunstan HM, Green-Willms NS, Fox TD. *In vivo* analysis of *Saccharomyces cerevisiae COX2* mRNA 5'-untranslated leader functions in mitochondrial translation initiation and translational activation. Genetics. 1997; 147:87–100. [PubMed: 9286670]

Ellis TP, Lukins HB, Nagley P, Corner BE. Suppression of a nuclear *aep2* mutation in *Saccharomyces cerevisiae* by a base substitution in the 5'-untranslated region of the mitochondrial *oli1* gene encoding subunit 9 of ATP synthase. Genetics. 1999; 151:1353–1363. [PubMed: 10101162]

Fisher N, Meunier B. Molecular basis of resistance to cytochrome *bc1* inhibitors. FEMS Yeast Res. 2008; 8:183–192. [PubMed: 18093133]

Fontanesi F, Clemente P, Barrientos A. Cox25 teams up with Mss51, Ssc1, and Cox14 to regulate mitochondrial cytochrome *c* oxidase subunit 1 expression and assembly in *Saccharomyces cerevisiae*. J Biol Chem. 2010a; 286:555–566. [PubMed: 21068384]

Fontanesi F, Soto IC, Barrientos A. Cytochrome *c* oxidase biogenesis: new levels of regulation. IUBMB Life. 2008; 60:557–568. [PubMed: 18465791]

Fontanesi F, Soto IC, Horn D, Barrientos A. Mss51 and Ssc1 facilitate translational regulation of cytochrome *c* oxidase biogenesis. Mol Cell Biol. 2010b; 30:245–259. [PubMed: 19858289]

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NIH-PA Author Manuscript

Fox TD. Translational control of endogenous and recoded nuclear genes in yeast mitochondria: regulation and membrane targeting. Experientia. 1996; 52:1130–1135. [PubMed: 8988256]

Gaisne M, Bonnefoy N. The *COX18* gene, involved in mitochondrial biogenesis, is functionally conserved and tightly regulated in humans and fission yeast. FEMS Yeast Res. 2006; 6:869–882. [PubMed: 16911509]

Gouget K, Verde F, Barrientos A. *In vivo* labeling and analysis of mitochondrial translation products in budding and in fission yeasts. Methods Mol Biol. 2008; 457:113–124. [PubMed: 19066022]

Green-Willms NS, Butler CA, Dunstan HM, Fox TD. Pet111p, an inner membrane-bound translational activator that limits expression of the *Saccharomyces cerevisiae* mitochondrial gene *COX2*. J Biol Chem. 2001; 276:6392–6397. [PubMed: 11106667]

Gruschke S, Kehrein K, Rompler K, Grone K, Israel L, Imhof A, Herrmann JM, Ott M. Cbp3-Cbp6 interacts with the yeast mitochondrial ribosomal tunnel exit and promotes cytochrome *b* synthesis and assembly. J Cell Biol. 2011; 193:1101–1114. [PubMed: 21670217]

Hoffman CS, Winston F. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene. 1987; 57:267–272. [PubMed: 3319781]

Islas-Osuna MA, Ellis TP, Marnell LL, Mittelmeier TM, Dieckmann CL. Cbp1 is required for translation of the mitochondrial cytochrome *b* mRNA of *Saccharomyces cerevisiae*. J Biol Chem. 2002; 277:37987–37990. [PubMed: 12149267]

Krause K, Lopes de Souza R, Roberts DG, Dieckmann CL. The mitochondrial message-specific mRNA protectors Cbp1 and Pet309 are associated in a high-molecular weight complex. Mol Biol Cell. 2004; 15:2674–2683. [PubMed: 15047869]

Kronekova Z, Rödel G. Organization of assembly factors Cbp3p and Cbp4p and their effect on bc_1 complex assembly in *Saccharomyces cerevisiae*. Curr Genet. 2005; 47:203–212. [PubMed: 15759116]

Kühl I, Dujeancourt L, Gaisne M, Herbert CJ, Bonnefoy N. A genome wide study in fission yeast reveals eight PPR proteins that regulate mitochondrial gene expression. Molecular and Cellular Biology. 2011; 39:80–29-8041.

Lemaire C, Dujardin G. Preparation of respiratory chain complexes from *Saccharomyces cerevisiae* wild-type and mutant mitochondria : activity measurement and subunit composition analysis. Methods Mol Biol. 2008; 432:65–81. [PubMed: 18370011]

Lipinski KA, Puchta O, Surandranath V, Kudla M, Golik P. Revisiting the yeast PPR Proteins - Application of an iterative Hidden Markov model algorithm reveals new members of the rapidly evolving family. Mol Biol Evol. 2011; 28:2935–2948. [PubMed: 21546354]

Manthey GM, McEwen JE. The product of the nuclear gene *PET309* is required for translation of mature mRNA and stability or production of intron-containing RNAs derived from the mitochondrial *COX1* locus of *Saccharomyces cerevisiae*. EMBO J. 1995; 14:4031–4043. [PubMed: 7664742]

Matsuyama A, Arai R, Yashiroda Y, Shirai A, Kamata A, Sekido S, Kobayashi Y, Hashimoto A, Hamamoto M, Hiraoka Y, Horinouchi S, Yoshida M. ORFeome cloning and global analysis of protein localization in the fission yeast *Schizosaccharomyces pombe*. Nat Biotechnol. 2006; 24:841–847. [PubMed: 16823372]

Matsuyama A, Shirai A, Yashiroda Y, Kamata A, Horinouchi S, Yoshida M. pDUAL, a multipurpose, multicopy vector capable of chromosomal integration in fission yeast. Yeast. 2004; 21:1289–1305. [PubMed: 15546162]

Matthews JM, Bhati M, Lehtomaki E, Mansfield RE, Cubeddu L, Mackay JP. It takes two to tango: the structure and function of LIM, RING, PHD and MYND domains. Curr Pharm Des. 2009; 15:3681–3696. [PubMed: 19925420]

Mick DU, Fox TD, Rehling P. Inventory control: cytochrome *c* oxidase assembly regulates mitochondrial translation. Nat Rev Mol Cell Biol. 2011; 12:14–20. [PubMed: 21179059]

Mick DU, Vukotic M, Piechura H, Meyer HE, Warscheid B, Deckers M, Rehling P. Coa3 and Cox14 are essential for negative feedback regulation of *COX1* translation in mitochondria. J Cell Biol. 2010; 191:141–154. [PubMed: 20876281]

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NIH-PA Author Manuscript

Mick DU, Wagner K, van der Laan M, Frazier AE, Perschil I, Pawlas M, Meyer HE, Warscheid B, Rehling P. Shy1 couples Cox1 translational regulation to cytochrome *c* oxidase assembly. EMBO J. 2007; 26:4347–4358. [PubMed: 17882259]

Minet M, Dufour ME, Lacroute F. Complementation of *Saccharomyces cerevisiae* auxotrophic mutants by *Arabidopsis thaliana* cDNAs. Plant J. 1992; 2:417–422. [PubMed: 1303803]

Naithani S, Saracco SA, Butler CA, Fox TD. Interactions among *COX1*, *COX2*, and *COX3* mRNA-specific translational activator proteins on the inner surface of the mitochondrial inner membrane of *Saccharomyces cerevisiae*. Mol Biol Cell. 2003; 14:324–333. [PubMed: 12529447]

Nouet C, Bourens M, Hlavacek O, Marsy S, Lemaire C, Dujardin G. Rmd9p controls the processing/stability of mitochondrial mRNAs and its overexpression compensates for a partial deficiency of Oxa1p in *Saccharomyces cerevisiae*. Genetics. 2007; 175:1105–1115. [PubMed: 17194787]

Okazaki K, Okazaki N, Kume K, Jinno S, Tanaka K, Okayama H. High-frequency transformation method and library transducing vectors for cloning mammalian cDNAs by trans-complementation of *Schizosaccharomyces pombe*. Nucleic Acids Res. 1990; 18:6485–6489. [PubMed: 2251111]

Payne MJ, Finnegan PM, Smooker PM, Lukins HB. Characterization of a second nuclear gene, *AEP1*, required for expression of the mitochondrial *OLI1* gene in *Saccharomyces cerevisiae*. Curr Genet. 1993; 24:126–135. [PubMed: 8358819]

Perez-Martinez X, Broadley SA, Fox TD. Mss51p promotes mitochondrial Cox1p synthesis and interacts with newly synthesized Cox1p. EMBO J. 2003; 22:5951–5961. [PubMed: 14592991]

Perez-Martinez X, Butler CA, Shingu-Vazquez M, Fox TD. Dual functions of Mss51 couple synthesis of Cox1 to assembly of cytochrome *c* oxidase in *Saccharomyces cerevisiae* mitochondria. Mol Biol Cell. 2009; 20:4371–4380. [PubMed: 19710419]

Pierrel F, Bestwick ML, Cobine PA, Khalimonchuk O, Cricco JA, Winge DR. Coa1 links the Mss51 post-translational function to Cox1 cofactor insertion in cytochrome *c* oxidase assembly. EMBO J. 2007; 26:4335–4346. [PubMed: 17882260]

Rackham O, Davies SM, Shearwood AM, Hamilton KL, Whelan J, Filipovska A. Pentatricopeptide repeat domain protein 1 lowers the levels of mitochondrial leucine tRNAs in cells. Nucleic Acids Res. 2009; 37:5859–5867. [PubMed: 19651879]

Rak M, Tzagoloff A. F1-dependent translation of mitochondrially encoded Atp6p and Atp8p subunits of yeast ATP synthase. Proc Natl Acad Sci U S A. 2009; 106:18509–18514. [PubMed: 19841266]

Rödel G. Two yeast nuclear genes, *CBS1* and *CBS2*, are required for translation of mitochondrial transcripts bearing the 5'-untranslated *COB* leader. Curr Genet. 1986; 11:41–45. [PubMed: 3329045]

Rödel G, Fox TD. The yeast nuclear gene *CBS1* is required for translation of mitochondrial mRNAs bearing the *cob* 5' untranslated leader. Mol Gen Genet. 1987; 206:45–50. [PubMed: 3033440]

Saint-Georges Y, Bonnefoy N, di Rago JP, Chiron S, Dujardin G. A pathogenic cytochrome *b* mutation reveals new interactions between subunits of the mitochondrial *bc1* complex. J Biol Chem. 2002; 277:49397–49402. [PubMed: 12384503]

Sanchirico, ME. PhD thesis. University of massachusetts; Amherst, MA: 1998a. Understanding mitochondrial biogenesis through gene relocation..

Sanchirico ME, Fox TD, Mason TL. Accumulation of mitochondrially synthesized *Saccharomyces cerevisiae* Cox2p and Cox3p depends on targeting information in untranslated portions of their mRNAs. EMBO J. 1998b; 17:5796–5804. [PubMed: 9755179]

Schäfer B. RNA maturation in mitochondria of *S. cerevisiae* and *S. pombe*. Gene. 2005; 354:80–85. [PubMed: 15913924]

Schägger H, von Jagow G. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal Biochem. 1991; 199:223–231. [PubMed: 1812789]

Shingu-Vazquez M, Camacho-Villasana Y, Sandoval-Romero L, Butler CA, Fox TD, Perez-Martinez X. The carboxyl-terminal end of Cox1 is required for feedback-assembly regulation of Cox1 synthesis in *Saccharomyces cerevisiae* mitochondria. J Biol Chem. 2010; 285:34382–34389. [PubMed: 20807763]

Siep M, van Oosterum K, Neufeglise H, van der Spek H, Grivell LA. Mss51p, a putative translational activator of cytochrome *c* oxidase subunit-1 (COX1) mRNA, is required for synthesis of Cox1p in *Saccharomyces cerevisiae*. Curr Genet. 2000; 37:213–220. [PubMed: 10803883]

NIH-PA Author Manuscript

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Steele DF, Butler CA, Fox TD. Expression of a recoded nuclear gene inserted into yeast mitochondrial DNA is limited by mRNA-specific translational activation. Proc Natl Acad Sci U S A. 1996; 93:5253–5257. [PubMed: 8643562]

Towpik J. Regulation of mitochondrial translation in yeast. Cell Mol Biol Lett. 2005; 10:571–594. [PubMed: 16341268]

Wach A. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. Yeast. 1996; 12:259–265. [PubMed: 8904338]

Watanabe K. Unique features of animal mitochondrial translation systems. The non-universal genetic code, unusual features of the translational apparatus and their relevance to human mitochondrial diseases. Proc Jpn Acad Ser B Phys Biol Sci. 2010; 86:11–39.

Weraarpachai W, Antonicka H, Sasarman F, Seeger J, Schrank B, Kolesar JE, Lochmuller H, Chevrette M, Kaufman BA, Horvath R, Shoubridge EA. Mutation in *TACO1*, encoding a translational activator of *COXI*, results in cytochrome *c* oxidase deficiency and late-onset Leigh syndrome. Nat Genet. 2009; 41:833–837. [PubMed: 19503089]

Wu M, Tzagoloff A. Identification and characterization of a new gene (*CBP3*) required for the expression of yeast coenzyme QH2-cytochrome *c* reductase. J Biol Chem. 1989; 264:11122–11130. [PubMed: 2544586]

Zeng X, Hourset A, Tzagoloff A. The *Saccharomyces cerevisiae ATP22* gene codes for the mitochondrial ATPase subunit 6-specific translation factor. Genetics. 2007; 175:55–63. [PubMed: 17110482]

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Highlights

>*S. cerevisiae* contains mRNA-specific activators controlling mitochondrial translation. >Only a few of these factors seem conserved in the yeast *S. pombe* and in human. >Conserved *S. cerevisiae* factors have an additional role *e.g.* in complex assembly >We find that the homologs in *S. pombe* fulfil only this complex assembly function. >The complex assembly function is thus probably the ancestral one.

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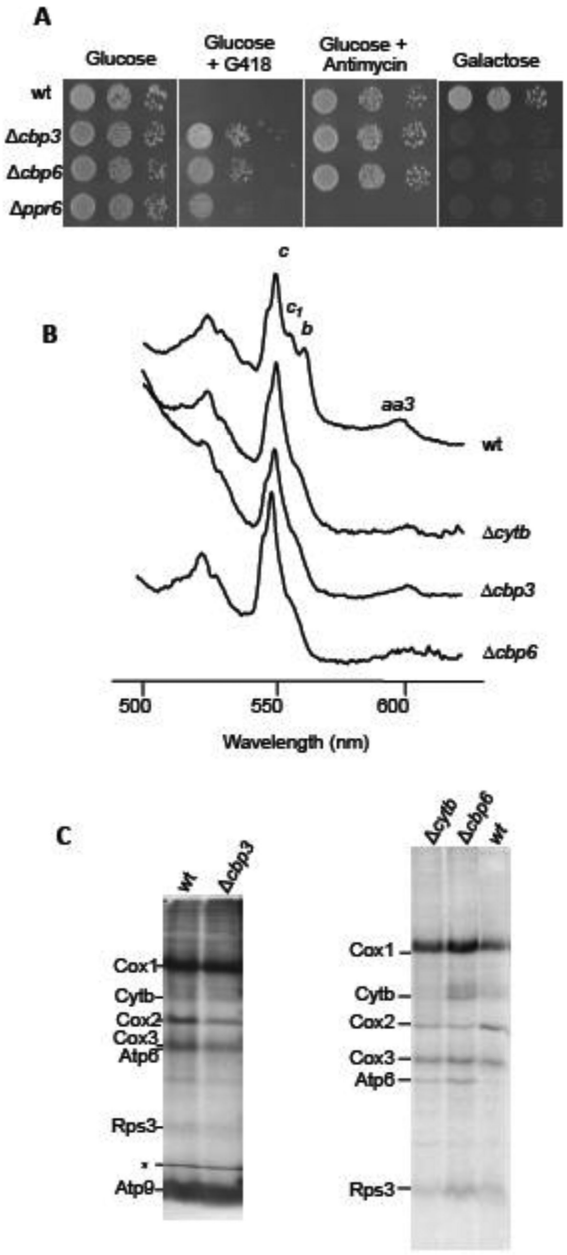

Figure 1.

A. Growth phenotype of the Δ*cbp3*, Δ*cbp6* and Δ*ppr6* mutants. Serial dilutions of the mutants and isogenic wild type were spotted onto complete media containing the indicated carbon sources and supplements and incubated for 4 days at 28°C. **B. Cytochrome spectra of Δ*cbp3*, Δ*cbp6* and Δ*cytb*mutants.** Cells of the wild type and the mutants were grown for 2 nights on glucose medium and low temperature cytochrome spectra were recorded after the addition of dithionite to fully reduce the cytochromes. Peaks for cytochromes *aa3*, *b*, *c1* and *c* are indicated. **C. *In vivo* ^{35}S labeling of the Δ*cbp3*, Δ*cbp6* and Δ*cytb* mutants.** Mutant and wild type cells were labeled with ^{35}S methionine/cysteine in the presence of cycloheximide that blocks cytoplasmic translation. For Δ*cbp3*, the band marked with the asterisk is an artifact observed after fast migrations. For Δ*cbp6*, the labeling presented is obtained after a long migration time to separate the high molecular weight proteins. As a

result of this Atp8 and 9 have run off the gel; however, Atp8 and 9 were clearly present in the mutant on other gels. For reasons that are not clear, Atp6 is generally labeled better in electron transport chain mutants than in the wild type.

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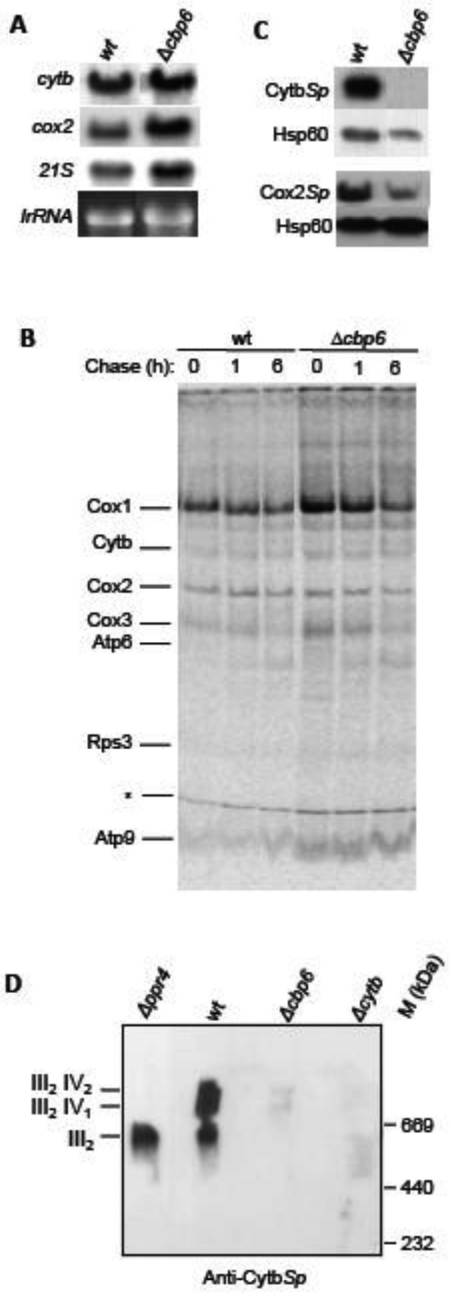

Figure 2.

A. Analysis of the steady state levels of some mtRNAs in the ***Δcbp6* mutant.** Total RNA from the intron-containing NB205-6A wild type and isogenic *Δcbp6* mutant were hybridized to *cytb* and *cox2* probes as indicated. Loading controls are hybridizations of the *21S* ribosomal RNA and a UV photograph of the cytoplasmic large ribosomal RNA. **B. Pulse chase analysis of Δ*cbp6* cells.** Wild type and Δ*cbp6* cells were labeled as in Figure 1C (time 0) and a chase was performed after washes and addition of non-radioactive methionine and cysteine for 1 or 6h before extraction of total proteins and analysis by SDS-PAGE. C. Steady state level of mitochondrial proteins in the ***Δcbp6* mutant.** Purified mitochondria from the mutant and the wild type were analyzed by SDS-PAGE and Western blot. Proteins were hybridized to *S. pombe* antibodies raised against Cox2 and Cytb. The

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loading control is Hsp60 that recognizes a mitochondrial matrix protein. D. Western blot analysis of respiratory complexes III and IV in Δ***cbp6*** **cells.** Blue-Native-PAGE analysis of purified mitochondria from the Δ*ppr4*, Δ*cbp6* or Δ*cytb* mutant compared to the wild type NB205-6A was conducted and the samples were analyzed by western blotting with the anti-Cytb antibodies. Positions for dimers of complex III with, or without monomers or dimers of complex IV are indicated.

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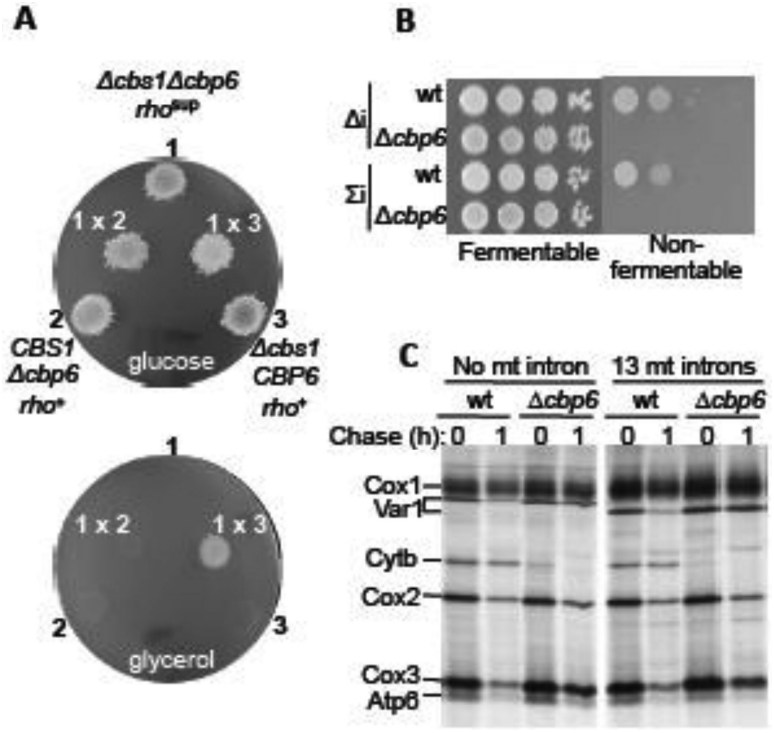

Figure 3.

A. Suppression test of the *Δcbp6* mutation by a rearranged *COX3::CYTb* genome. A *Δcbp6 Δcbs1* strain (1) containing a rearranged suppressor mitochondrial genome *COX3::CYTb* (*rho*^{sup}) was crossed either with a *Δcbs1 rho*$^{+}$ strain (2) or with a *Δcbp6 rho*$^{+}$ strain (3) on a complete glucose medium (upper plate), and the diploids were tested for growth on glycerol (lower plate). **B. Effect of intron content on the growth of Δ*cbp6* cells.** Serial dilutions of the intron-less (*Δi*) and intron-containing (*Σi*) Δ*cbp6* mutants were spotted onto complete media containing fermentable (glucose) or nonfermentable (glycerol) carbon sources and incubated for 2 and 3 days respectively. **C. *In vivo* ^{35}S pulse-chase analysis of the *S. cerevisiae Δcbp6* mutant.** The Δ*cbp6* mutant and the isogenic wild type were labeled with ^{35}S methionine/cysteine in presence of cycloheximide for 20 minutes (time 0) followed by a one-hour chase (1) in a buffer devoid of cycloheximide and radioactive amino-acids.

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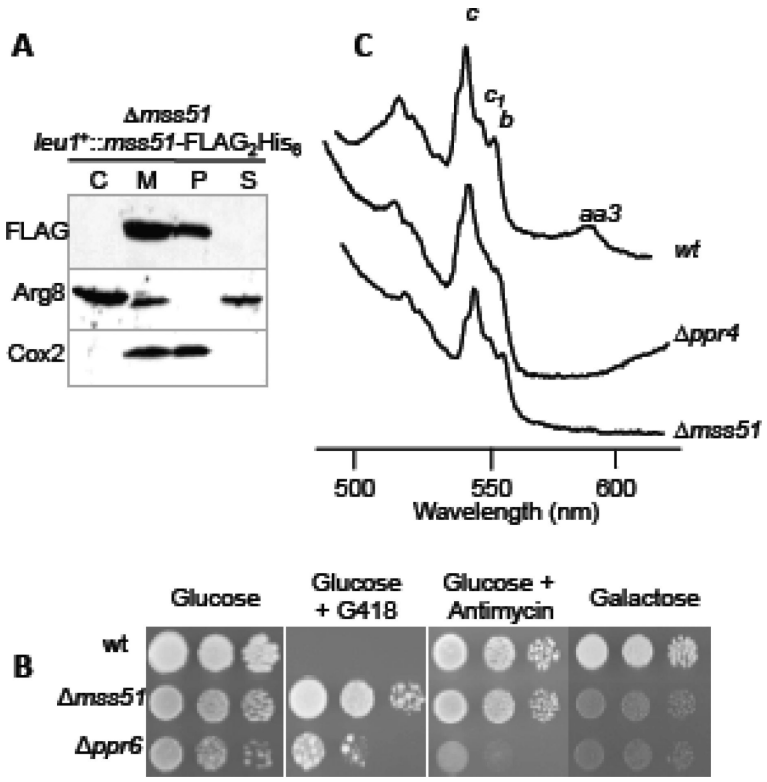

Figure 4.

A. Mitochondrial and membrane localization of Mss51 in ***S. pombe***. The *mss51-FLAG$_2$His$_6$* construct under the control of the *nmt1* promoter was stably integrated into the *leu1* locus of Δ*mss51* cells. Mitochondria (M) from these cells were purified and either analyzed directly by western blotting together with the post-mitochondrial supernatant (C), or subjected to alkaline carbonate extraction to generate pellet (P) and supernatant (S) samples. Mss51 was detected with the FLAG antibody, control antibodies were anti-Cox2 that reveals the mitochondrial fraction, while the anti-Arg8 produces in addition to a mitochondrial labeling a band in both the mitochondrial and cytoplasmic fractions (antibodies against the *S. cerevisiae* Arg8 recognize the *S. pombe* Arg1 protein). **B. Growth phenotype of the Δ*mss51* mutants.** Serial dilutions of the two mutants as well as the isogenic wild type and a control complex V deficient mutant, Δ*ppr6* (Kühl et al., 2011) were spotted onto complete media containing the indicated carbon sources and supplements and incubated for 4 days at 28°C. **C. Cytochrome spectra of the Δ*mss51* and *Δppr4* mutants.** Cells of the wild type and the mutants were grown for 2 to 3 nights on glucose medium and low temperature cytochrome spectra were recorded after the addition of dithionite to fully reduce the cytochromes. Peaks for cytochromes *aa3*, *b*, *c1* and *c* are indicated.

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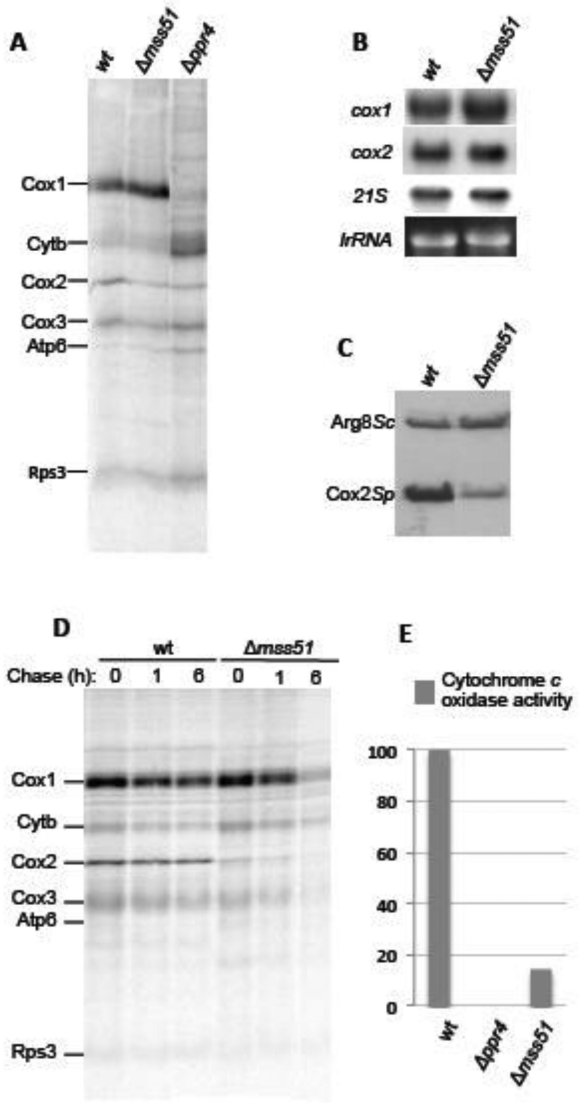

Figure 5.

A. *In vivo* ^{35}S labeling of *Δmss51* mutants. Mitochondrially-encoded proteins of the mutant together with the wild type and a control strains lacking the *cox1* translational activator Ppr4 (Kühl et al., 2011) were labeled with ^{35}S methionine/cysteine and a typical result is shown. As for Δ*cbp6* in Figure-1C, Atp8/9 were clearly labeled in other gels. B. Analysis of the steady state levels of the mtRNAs in the ***Δmss51* mutant.** Total RNA from the intron-containing NB205-6A wild type and isogenic *Δmss51* mutants were hybridized to *cox1* and *cox2* probes as indicated. Loading controls are a hybridization of the *21S* ribosomal RNA and a UV photograph of the cytoplasmic large ribosomal RNA. **C. Steady state level of Cox2 in the *Δmss51* mutant.** Purified mitochondria from the mutant and the wild type were analyzed by SDS-PAGE and Western blot and hybridized to an antibody raised against *S. pombe* Cox2. The control antibody is anti-Arg8 (see Figure 4A). **D. Pulse chase analysis of Δ*mss51*cells.** Wild type and Δ*mss51* cells were labeled as in panel A (time 0) and a chase was performed after washes and addition of non-radioactive methionine and cysteine for 1 or 6h before extraction of total proteins and analysis by SDS-PAGE. **E. Cytochrome *c* oxidase activity in Δ*mss51* mitochondria.** Cyanide- sensitive cytochrome *c* oxidase

activity was measured in equal amounts of mitochondria of Δ*mss51*, Δ*ppr4* (that lack Cox1) and the isogenic wild type cells (wild type activity was set as 100%).

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Table 1

mt-mRNA specific factors acting in *S. cerevisiae* mitochondrial translation and their conservation in fission yeast and humans.

S. cerevisiae					***S. pombe***
Target mRNA	**Translational activator**	**PPR motifs**	**Acts through:**		**Homolog (PPR motifs)**
			5'UTR	**other sequences**	
VAR1	Sov1	3	?	?	-
CYTb	Cbs1	-	+	-	-
	Cbs2	-	+	-	-
	Cbp1	3	+	?	-
	Cbp3	**-**	**+**	**Cytb protein**	**Spcc4b3.17**[1]
	Cbp6	**-**	**+**	**Cytb protein**	**Spbc947.14c**[2]
COX1	Pet309	13	+	?	Ppr4 (13)
	Mss51	**-**	**+**	**Cox1 protein**	**Spac25b8.04c**[3]
COX2	Pet111	5	+	-	-
COX3	Pet54	-	+	-	-
	Pet122	-	+	-	-
	Pet494	-	+	-	-
ATP6/8	Atp22	2	+	?	
ATP9	Aep1/Nca1	2	?	?	-
	Aep2/Atp13	4	+	-	Ppr6 (5)

Cbp3, Cbp6 and Mss51 data are in bold.

Alignment parameters between the *S. cerevisiae* and *S. pombe* homologs using the PIR server (http://pir.georgetown.edu/pirwww/search/pairwise.shtml) were the following :

Data extracted from: Chen and Dieckmann 1997; Dunstan et al., 1997; Ellis et al., 1999; Fox, 1996; Gruschke et al., 2011; Islas-Osuna et al., 2002; Kühl et al., 2011; Lipinski et al., 2011; Manthey and McEwen, 1995; Mick et al., 2011; Payne et al., 1993; Sanchirico, 1998a; Zeng et al., 2007; for review, see Towpik, 2005.

[1]Z-score: 603.7; E-value: 8.1e-32; Identity : 35.2% (38.9% ungapped) in 284 aa overlap (16-275:11-291)

[2]Z-score: 137.3; E-value: 7.7e-06; Identity : 26.3% (27.0% ungapped) in 76 aa overlap (23-96:77-152).

[3]Z-score: 1035.8; E-value: 6.9e-56; Identity : 36.7% (39.7% ungapped) in 398 aa overlap (1-371:37-431)

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Table 2

Strains used and constructed in this work

Name	Nuclear genotype	Reference
S. pombe		
NB205-6A	*h- ade6-M216 ura4-D1.8 his3Δ leu 1-32 [rho+]*	Chiron et al., 2005
IK107-7	*h- ade6-M216 ura4-D1.8 his3Δ leu 1-32 Δcbp3::kan^R [rho+]*	This work
IK1-4	*h- ade6-M216 ura4-D1.8 his3Δ leu1-32 Δcbp6::kan^R [rho+]*	This work
IK3-1	*h- ade6-M216 ura4-D1.8 his3Δ leu1-32 Δmss51::kan^R [rho+]*	This work
IK38-B	*h- ade6-M216 ura4-D1.8 his3Δ leu1-32 Δcbp6::kan^R leu1+::cbp6-YFP-FlagHis$_6$ [rho+]*	This work
IK42-6	*h- ade6-M216 ura4-D1.8 his3Δ leu1-32 Δmss51::kan^R leu1+::mss51-Flag$_2$His$_6$ rho+*	This work
IK39-3	*h- ade6-M216 ura4-D1.8 his3Δ leu1-32 Δppr4::kan^R [rho+]*	Kühl et al., 2011
IK91-4	*h- ade6-M216 ura4-D1.8 his3Δ leu1-32 Δppr6::kan^R [rho+]*	Kühl et al., 2011
NB324-11D	*h? ade7-50 leu1-32 [cytb-Δ541^1]*	N. Bonnefoy
S. cerevisiae		
CW252	MATx *ade2-1, his3-11,15, trp1-1, leu2-3,112 ura3-1 can1-1 [rho+ Δi^2]*	Saint-Georges et al., 2002
IK43-1	MATx *ade2-1, his3-11,15, trp1-1, leu2-3,112 ura3-1 can1-1 Δcbp6 ::kan^R [rho+ Δi^2]*	This work
CW04	MATx *ade2-1, his3-11,15, trp1-1, leu2-3,112 ura3-1 can1-1 [rho+ Σi^3]*	Banroques et al., 1986
IK44-5	MATx *ade2-1, his3-11,15, trp1-1, leu2-3,112 ura3-1 can1-1 Δcbp6 ::kan^R [rho+ Σi^3]*	This work
MCC62^4	MATx *his3Δ1 cbs1::TRP1 [rho+^{5}]*	Costanzo and Fox, 1988
MCC60R2-16^4	*MATa ade2 his3 Δ 1 leu2-3,112 ura3-52 cbs1::TRP1 [rho+^{5}, MSUcbs1-2^6]*	Costanzo and Fox, 1988
IK97-1	*MATa ade2 his3Δ1 leu2-3,112 ura3-52 cbs1::TRP1 Δcbp6 ::kan^R [MSUcbs1-2^6]*	This work

[1] 541 bp deletion in the *cytb* gene derived from strain EB7 (Ahne et al., 1988).

[2] Intron-less genome with corrected *CYTb* sequence

[3] 13 Intron-containing genome, W303 background

[4] May contain the *trpl-289* mutation

[5] 8 Intron-containing genome, D273 background

[6] rearranged mitochondrial genome, with sequences coding the 5'UTR region of *COX3* down to -173 fused to sequences coding the *CYTb* 5'UTR starting at -6.