

Translational activators and mitoribosomal isoforms cooperate to mediate mRNA-specific translation in Schizosaccharomyces pombe mitochondria

Christopher J Herbert, Sylvie Labarre-Mariotte, David Cornu, Cyrielle Sophie, Cristina Panozzo, Thomas Michel, Geneviève Dujardin, Nathalie Bonnefoy

▶ **To cite this version:**

Christopher J Herbert, Sylvie Labarre-Mariotte, David Cornu, Cyrielle Sophie, Cristina Panozzo, et al.. Translational activators and mitoribosomal isoforms cooperate to mediate mRNA-specific translation in Schizosaccharomyces pombe mitochondria. Nucleic Acids Research, 2021, 49 (19), pp.11145-11166. 10.1093/nar/gkab789 . hal-03414269

HAL Id: hal-03414269
https://hal.science/hal-03414269

Submitted on 4 Nov 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Published online 11 October 2021

Nucleic Acids Research, 2021, Vol. 49, No. 19 ***11145–11166***
https://doi.org/10.1093/nar/gkab789

Translational activators and mitoribosomal isoforms cooperate to mediate mRNA-specific translation in *Schizosaccharomyces pombe* mitochondria

Christopher J. Herbert, Sylvie Labarre-Mariotte, David Cornu, Cyrielle Sophie, Cristina Panozzo, Thomas Michel, Geneviève Dujardin and Nathalie Bonnefoy*

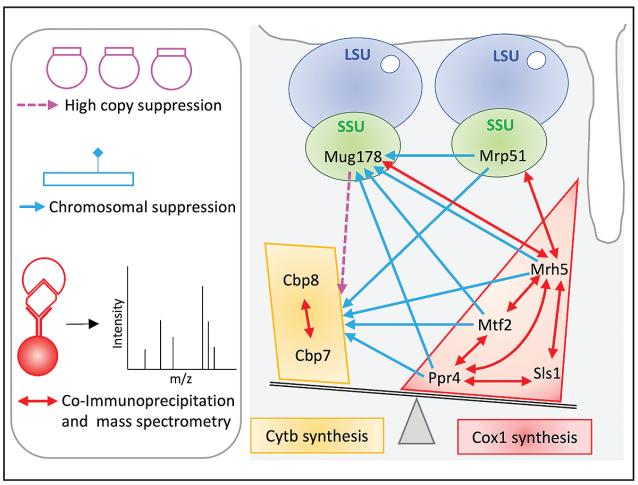
Institute of Integrative Biology of the Cell (I2BC), CEA, CNRS, Univ Paris-Sud, Université Paris-Saclay, 91198, Gif-sur-Yvette cedex, France

Received April 07, 2021; Revised August 24, 2021; Editorial Decision August 30, 2021; Accepted October 05, 2021

ABSTRACT

Mitochondrial mRNAs encode key subunits of the oxidative phosphorylation complexes that produce energy for the cell. In *Saccharomyces cerevisiae*, mitochondrial translation is under the control of translational activators, specific to each mRNA. In *Schizosaccharomyces pombe*, which more closely resembles the human system by its mitochondrial DNA structure and physiology, most translational activators appear to be either lacking, or recruited for post-translational functions. By combining bioinformatics, genetic and biochemical approaches we identified two interacting factors, Cbp7 and Cbp8, controlling Cytb production in *S. pombe*. We show that their absence affects *cytb* mRNA stability and impairs the detection of the Cytb protein. We further identified two classes of Cbp7/Cbp8 partners and showed that they modulated Cytb or Cox1 synthesis. First, two isoforms of bS1m, a protein of the small mitoribosomal subunit, that appear mutually exclusive and confer translational specificity. Second, a complex of four proteins dedicated to Cox1 synthesis, which includes an RNA helicase that interacts with the mitochondrial ribosome. Our results suggest that *S. pombe* contains, in addition to complexes of translational activators, a heterogeneous population of mitochondrial ribosomes that could specifically modulate translation depending on the mRNA translated, in order to optimally balance the production of different respiratory complex subunits.

GRAPHICAL ABSTRACT



INTRODUCTION

Translation of cellular mRNAs is a dynamic and energy consuming process whose general principles are universally conserved in prokaryotes and eukaryotes. In addition, the rate of translation is tightly controlled in response to various stimuli, in order to produce the cellular proteome in the appropriate quantity, quality, location and timeframe. This is particularly crucial in mitochondria where the oxidative phosphorylation (OXPHOS) chain complexes are of dual genetic origin: thus, the synthesis of the few mitochondrially-encoded subunits has to be tightly coordinated in stoichiometry, space and time with the production and import of the other, more numerous nuclear-encoded subunits, in order to produce the fully functional OXPHOS complexes. An imbalance in the arrival of the nuclear and

*To whom correspondence should be addressed. Tel: +33 1 69 82 31 75; Fax: +33 1 69 82 31 60; Email: nathalie.bonnefoy@i2bc.paris-saclay.fr
Present addresses:
Cyrielle Sophie, UMR Ecologie des Forêts de Guyane (EcoFoG), Campus Agronomique, BP316, 97379 Kourou cedex, Guyane.
Cristina Panozzo, Institut de Biologie Physico-Chimique, UMR 8226, CNRS, Sorbonne Université, Laboratoire de Biologie Moléculaire et Cellulaire des Eucaryotes, 75005 Paris, France.

© The Author(s) 2021. Published by Oxford University Press on behalf of Nucleic Acids Research.
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

mitochondrial subunits could block the assembly of the complexes.

In both eukaryotes and prokaryotes, the modulation of translation represents an additional subtle layer of control after transcriptional regulation. Interestingly, the general translation factors and the ribosome itself are increasingly considered as *bona fide* regulatory elements rather than just components of the synthesis machinery, as shown by various studies pointing to the regulatory role of changing the composition of the ribosomes (for review, see 1–3). Specialized cytosolic ribosomes, produced by the incorporation of variant duplicated ribosomal proteins, appear to be a conserved mechanism regulating the translation of proteins with mitochondrial functions, *e.g.* in yeast and Drosophila (4,5).

In mitochondria, translation of OXPHOS subunits occurs at the surface of the inner mitochondrial membrane and this allows co-translational insertion, due to specific factors mediating ribosome tethering and early subunit assembly (6). Mitochondrial translation is a major step of regulation for gene expression and a few reports suggest that the mitoribosome could have a filtering ability. For example, in the budding yeast *Saccharomyces cerevisiae*, at least two proteins from the mitoribosome, mS38/Cox24 and bL34, were shown to be specifically required for the production of subunits of the OXPHOS complex IV. While mS38 specificity seems to function independently of an additional translation factor (7), bL34 appears required for optimal exit from the ribosomes of extremely hydrophobic proteins such as the complex IV subunit Cox1 (8). In addition, mitoribosomal proteins can also play a specific post-translational role, closely coupled to translation; for example, yeast MrpL35, which appears to coordinate synthesis of Cox1 with its subsequent assembly (9). A large-scale proximity labeling analysis of 40 mitochondrial proteins highlights the tight connectivity of all steps of mitochondrial gene expression, where the translating ribosome appears to play a pivotal role linking RNA metabolism and OXPHOS complex assembly (10). However, recent work suggests that the sites of mitochondrial protein synthesis would be separated from the sites of mRNA processing and maturation in human cells (11).

Another unusual feature of mitochondrial translation is the apparent absence of interaction between mt-rRNA and mitochondrial mRNA leaders (mt-mRNA), which lack a canonical Shine-Dalgarno sequence to help their interaction with mitoribosomes and positioning the translation start site for initiation. Instead, in *S. cerevisiae*, translation of each of the eight mt-mRNAs appears to rely on the interaction of the long 5′ untranslated regions (5′UTR) with a specific set of translational activators (for review 12,13). These translational activators have been proposed to contribute to ribosomal heterogeneity and may be responsible for a structure of unresolved density close to the mRNA exit channel on the small ribosomal subunit (SSU) (14,15). They appear to fulfill multiple roles in mitochondrial translation by interacting with their specific target mRNA, the ribosome itself and/or the membrane, either directly or indirectly by recruiting other factors. Some of the *S. cerevisiae* translational activators are also central elements of regulatory feed-back loops adjusting the production of some subunits to their assembly into the corresponding OXPHOS complex or into the mitoribosome (16,13,17–19; and references therein).

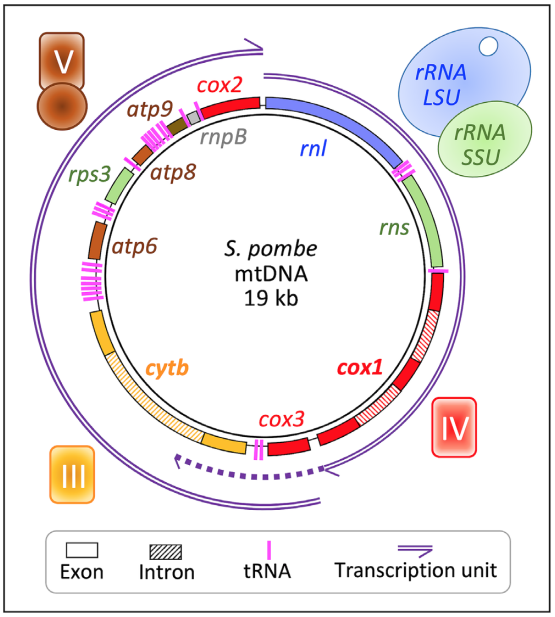


Figure 1. Map of *S. pombe* mitochondrial DNA. This 19 kb genome encodes the two rRNAs (*rnl* [21S] and *rns* [15S]), seven key subunits of the OXPHOS complexes III, IV and V as indicated, one ribosomal SSU protein, the RNase P RNA (*rnpB*) and the full set of tRNAs; *cox1* and *cytb* are mosaic genes. A major promoter is located upstream of *rnl* and a minor promoter upstream of *cox3*, generating two long precursor transcripts. Processing at the 5′ end of genes takes place only through tRNA punctuation, thus the 5′UTR of *rnl* and *cox3* are not processed further and the small amount of *cox1–cox3* bi-cistronic transcript produced from the major promoter remains stable. For more details see (22 and references therein). LSU: large ribosomal subunit.

Unlike the OXPHOS assembly factors that are generally highly conserved from yeast to human, the translational activators from *S. cerevisiae* appear to rapidly diverge in evolution, as shown by sequence homology searches in humans, or the fission yeast *Schizosaccharomyces pombe* databases (20). This could be due to co-evolution with their RNA target or to the replacement by other factors recruited to regulate mitochondrial translation. In humans and *S. pombe*, 5′UTRs are either much shorter than in *S. cerevisiae*, or absent (21,22). In this sense, *S. pombe* is an interesting intermediate model that shares many features with humans: a similar dependence for oxygen, compact mitochondrial genome (mtDNA), and an analogous mRNA production process, since mitochondrial transcription generates two large major RNAs that are processed into mature mRNAs by the removal of intervening tRNAs (Figure 1).

In *S. pombe*, we previously found sequence homologs for the Cox1 *S. cerevisiae* translational activators Pet309 and Mss51. *S. pombe* Ppr4 (23), like Pet309 (24,25), is a pentatricopeptide repeat (PPR) RNA binding protein which is a translational activator of the *cox1* mRNA. In humans, the Pet309/Ppr4 closest sequence homolog, LRPPRC, appears in complex with SLIRP to deliver mt-mRNA to the mitoribosome (26). *S. pombe* Mss51 is not required at the translational step but at a post-translational step of Cox1

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

production (27). In humans, the ablation of *MSS51* appears to enhance the muscle metabolic state by an unknown mechanism unlinked to *COX1* (28), and TACO1, an unrelated protein, appears to be a *COX1* translational activator (29). Thus, even if the protein sequences appear conserved through evolution, their function may diverge, as shown for Mss51 homologs.

Cytochrome *b* (Cytb) is the only mitochondrially encoded subunit of complex III and in *S. cerevisiae* its synthesis is regulated by an interplay of five factors: Cbp1, Cbs1 and Cbs2 which are required for Cytb synthesis, and a complex of two early assembly factors, Cbp3 and Cbp6 (30,31; and references therein). Cbp1 controls the stability and processing of the *cytb* mRNA (32,33). Alternative binding of Cbs1 or Cbp3/6 to the mitoribosomal tunnel exit allows either repression or activation of *cytb* translation, coordinated with complex III assembly. However, the precise role of Cbs2 in this regulatory feedback loop is rather enigmatic although several reports have shown that it is in the vicinity of the mitoribosome (34,14,31,10). Very recently, the purified Cbs2 protein has been crystallized and found to form an asymmetric homodimer (35) but the possible functional significance of this organization is unknown. In *S. pombe*, we found Cbp3 and Cbp6 through classical blast searches (27) and they have homologs in humans, UQCC1 and UQCC2, that form a complex with Cytb and are required for Cytb synthesis or stability (36). However, so far, homologs of Cbp1, Cbs1 and Cbs2 have not been found in fission yeasts or mammals.

In order to uncover how Cytb production is controlled in *S. pombe*, but also as a stepping stone towards the identification of new Cytb biogenesis factors in higher eukaryotes, we identified Cbp7, a homolog of Cbs2 in *S. pombe*, and further searched for its physical and genetic partners. This journey in *S. pombe* mitochondrial translation led us to identify several new factors crucial not only for Cytb biogenesis, but also Cox1 synthesis, as well as two mutually exclusive isoforms of bS1m, a component of the small subunit of the mitochondrial ribosome, which lead to different translational specificities.

MATERIALS AND METHODS

Strains, media, genetic methods and plasmids

All the *S. pombe* strains used in this study are described in Supplementary Table S1 and were grown at 28°C except for the cloning of *srd11* (see below). Unless otherwise indicated, the strains harbor two group I introns in *cox1* (*aI1* and *aI2b*) and one group II intron in *cytb* (*bI1*) (Figure 1), all three contain an ORF in frame with the preceding exon. Both *cox1* introns clearly encode endonucleases and least aI1 probably also carries a maturase function, essential for its own intron removal; in contrast the *cytb* intron seems to encode a reverse transcriptase (for review 37). In this paper, the mitochondrial genome containing all three introns is called $\sum i$ in contrast with the Δi genome which is devoid of mitochondrial introns (38).

Media and genetic methods were as described in (39,40). Complete fermentable medium generally contains 5% glucose (called 'Glc'), unless specified otherwise. The Glc medium was in some cases supplemented with antibiotics: 300 mg/l Geneticin (G418) or 300 mg/ml Hygromycin (Hygro) as marker for gene deletion or gene tagging; 0.18 μM Antimycin A (Anti) to test for ATP synthase function. Complete non-fermentable media contained either 2% galactose and 0.1% glucose (called 'Gal') or 2% glycerol and 0.1% glucose (called 'Gly'). *S. pombe* asci were microdissected directly on 5% complete glucose medium from the mixture of haploid, diploid and sporulating cells. Leucine auxotroph [Leu−] *S. pombe* cells were selected on minimal glucose medium lacking leucine after transformation either by a chemical method (41) or by electroporation as described in (42), using plasmids that were all derivatives of the high copy shuttle vector pAL-KS which carries a *leu1* gene as a selection marker (43). Most plasmids were isolated directly from the library pTN-L1 (44) purchased from the National BioRessource Project (NBRP) in Japan and amplified in our laboratory, and others correspond to the cloning of PCR-amplified isolated genes into the XhoI−NotI sites of pAL-KS.

Construction of gene deletions and double mutants in *S. pombe*

S. pombe gene deletions were constructed by the PCR method using pFA6-kanMX4 carrying the *KanR* gene that confers resistance to the antibiotics G418 (45). PCR fragments containing the *KanR* gene were generated with hybrid oligonucleotides containing 75–80 bases of homology with the recipient *locus* on both sides of the gene of interest and transformed into the wild-type strain NBp29-725, the intron-less strain P3 as in (23) or the *ptp1-1* mutant NB34-21A as described in (46). The *ptp1-1* mutation allows *S. pombe* to remain alive without mtDNA (47). [KanR] transformants able to grow in presence of the drug G418 were streaked again on selective medium, and the genomic DNA of single colonies was extracted (48) and 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. We constructed *Δcbp7*, *Δcbp8*, *Δmrp51*, *Δmrh5*, *Δmtf2* and *Δsls1* deletion mutants. *Δmrp51* could be obtained only in the *ptp1-1* background. *Δmug178* and *Δpnu1* were also constructed similarly in NBp9-725 using the hygromycin resistance gene (*hph*) instead of *KanR* (49). *Pnu1* encodes the major mitochondrial RNA nuclease and we tested the effect of this deletion in some of the sucrose gradients and immunoprecipitation (IP) experiments, however we did not notice any obvious difference in presence or absence of Pnu1. To construct the various double mutants, single mutants were first crossed with a wild type to isolate spores of the opposite mating type and then crossed with other strains carrying deletion or point mutations.

13c-Myc, 3HA, 7his, V5 or 3FLAG epitope tagging of cbp7, cbp8, mrh5, mrp51 and mug178 genes

The genes encoding Cbp7, Cbp8, Mrp51, Mug178 and Mrh5 were epitope tagged in the $\sum i$ wt NBp9-725 at their chromosomal *loci* using a PCR strategy similar to that of the gene deletion. To generate the

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

PCR fragments for transformation, appropriate plasmids (pFA6a-13Myc-KanMX6, pFA6a-3HA-KanMX6 or pFA6a-6xGLY-3FLAG-HphMX4) were used as templates in order to provide the tag and the terminator sequences (50,51). If necessary, the forward primer contained an in-frame tag coding sequence (for example to construct Mrp51-V5 or Mug178-1FLAG). The constructions were verified by PCR amplification of the 3′ section of the appropriate gene and tag followed by sequencing. The expression of the tagged proteins was then verified by western blotting of whole cell extracts or mitochondria. Single tagged strains were crossed to obtain double or triple tagged strains used for co-immunoprecipitation (co-IP) experiments. All tagged strains, either alone, or in combination were verified to grow on non-fermentable medium.

Isolation of high copy and chromosomal suppressors

To search for high copy suppressors, the *Δcbp7* (CS1) or *Δcbp8* (SLM72) strains were transformed with the pTN-L1 library (44) constructed in the *leu1* vector pALKS pTN-L1 genomic library and Leu + transformants were selected and replicated onto Gal medium. Positive clones were tested for co-segregation of the [Gal+] and [Leu + phenotypes], total DNA was extracted and used to transform XL2Blue *E. coli* cells in order to recover the plasmids. After sequencing both ends to identify the chromosomal fragment carried by the plasmid, inserts were shortened, when necessary, and re-introduced into the original *Δcbp7* or *Δcbp8* strains to identify the suppressor gene.

Genetic suppressors of the $\sum i$ *Δcbp7* strain (CS1) were obtained after spreading dilutions of cells from different independent subclones onto Gal medium and exposing the plates to UV light before incubation, to select for UV-induced revertants called *srd*. After purification, the suppressor colonies were back-crossed to a *Δcbp7* mutant to verify that the [Gal+] phenotype segregated 2:2, and to the wt in order to test whether the suppressor mutations alone had a respiratory phenotype. This happened to be the case for one of the suppressors (originally called *srd11*), that caused cryo-sensitivity on Gal when combined with the wild-type *cbp7* gene. Thus, the *srd11*^{Sup} strain was transformed with the pTN-L1 library (see above) and [Leu+] transformants were screened on Gal medium at 20°C. A single colony was obtained, that contained a plasmid with several genes, among which *spbc25d12.06* clearly encoded a mitochondrial protein. In the *srd11*^{Sup} strain, a single mutation was found in *spbc25d12.06*, creating a G230E substitution in the protein.

Identification of chromosomal suppressors by full genome sequencing

For all chromosomal suppressors except *srd11*^{Sup}, no clear phenotype was obtained when the suppressor mutation was separated from the *Δcbp7* deletion, thus the genome of the suppressor strains was subjected to high throughput sequencing and compared to the starting *Δcbp7* deletion strain. Briefly, one original suppressor from each class was back-crossed to a *Δcbp7* strain, the diploids were sporulated and asci dissected to select 40 suppressed spores for each suppressor. After growth of the 40 spores in liquid medium, cells were pooled before purifying genomic DNA for whole genome sequencing at the I2BC Next Generation Sequencing (NGS) Core Facility on a MiSeq instrument (Illumina). In each case, only one mutation was present in all reads in the suppressors. The genes were PCR-amplified in the original suppressor strains and we confirmed in each case the presence of the mutation. We noticed during the course of the experiments that the *mrp51-E21K* mutation was associated to another gene mutation causing slightly slower growth on glucose medium, but having no obvious effect on non-fermentable growth and that did not appear to impair or improve the suppression of *Δcbp7*. Suppressor analysis and whole genome sequencing was carried out the same way for *Δmug178*.

Purification of mitochondria, determination of complex III and IV activities and immunoprecipitation

Mitochondria were purified as described previously (46) from *S. pombe* cells grown in complete liquid medium containing either 2% glucose (for respiratory deficient mutants and controls, used for western blot and enzyme activities) or 2% glycerol and 0.1% glucose (for tagged strains, used for localization or IP.

Decylubiquinol–cytochrome *c* reductase (complex III) activity was determined at room temperature by measuring the reduction of 20 μM of cytochrome *c* by 20 to 40 μg of mitochondria at 550 nm *minus* 540 nm over a 1-min time-course in 1 ml of 10 mM potassium phosphate pH 7, 0.01% (w/v) lauryl-maltoside and 1 mM KCN (52). Activity was initiated by the addition of decylubiquinol and initial rates were measured, calculated for 60 μg of mitochondrial proteins and expressed as a percentage of the wt activity. Two or three different mitochondrial preparations were tested for each strain, each measurement was repeated three to five times and the values obtained were averaged.

To determine cytochrome *c* oxidase activity, the rate of oxygen consumption was measured in triplicates in a stirred reaction vessel of a Clarke-type O_2 electrode at 25°C in 1 ml of 10 mM phosphate buffer pH 7, 50 mM KCl, 0.05% lauryl maltoside, 40 μM TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine), 4 mM sodium ascorbate and 20 μM cytochrome *c* as described in (53). The reaction was initiated by addition of 30–60 μg of purified mitochondria from at least three different preparations for each strain and the oxygen consumption was monitored for at least 5 min. The final activity, calculated for 60 μg of mitochondrial proteins, was determined by subtracting the basal rate from the rate obtained after addition of mitochondria to the reaction medium and expressed in percentage of the wt.

For IP 1–2 mg of purified mitochondria were washed and then lysed for 30 min at 4°C with 1% lauryl maltoside under magnesium (5 mM MgOAc) or EDTA (10 mM) conditions to maintain or dissociate the two ribosomal subunits using buffers adapted from (54) containing protease inhibitors (Roche) and 1 mM PMSF, that was extemporaneously added at each step from a freshly-made isopropanol stock. Samples were spun 30 min at 4°C and 15493 rcf and the supernatants were incubated on a rolling wheel for 3 h, at 4°C with 50 to 120 μl of agarose beads conju-

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

gated with anti-HA, anti-FLAG or anti-cMyc antibodies (Sigma). The beads were collected using a Sigma prep spin column, washed three times and eluted for 5 min at 65°C with loading buffer without DTT to yield the IP fraction. For SDS-PAGE analysis the total extract (TE) corresponds to 1/40 of the lysate.

Production of rabbit antibodies against several mitochondrial proteins from *S. pombe*

Briefly, anti-mS45 (Spbc14c8.16c, Bot1) and anti-mL58 (Spcc1393.11) were raised from N-terminal truncated proteins expressed in *Escherichia coli* following a 70-day protocol (Proteogenix) whereas polyclonal anti-Cox1 and anti-Atp6 antibodies were raised in rabbits injected at once with two synthetic peptides using the Speedy package immunization protocol from Eurogentec. Peptides were GLNGMP RRIPDYPEAF and C + PVHEHAFNTLPTKSI for Cox1 and VENQIHSSKTVSGQS + C and TPYGSGAKIVPQ KFG + C for Atp6.

PAGE and western blotting

Total, or mitochondrial protein samples were separated by SDS-PAGE on 8, 10 or 12% gels, or by Blue-native (BN)-PAGE on precast 3–12% gels from Invitrogen, before western blotting. For BN-PAGE, mitochondrial complexes were solubilized from purified mitochondria using 2% digitonin and following the instructions of the 'native PAGE sample prep kit' (Invitrogen), except that an additional centrifugation at 20 627 rcf was added before loading.

Primary antibodies used to detect *S. pombe* proteins were: anti-*S. cerevisiae* Arg8, 1/4000 (55, a gift of T.D. Fox, Ithaca, USA); anti-*S. cerevisiae* Atp2, 1/50000 (a gift of J. Velours, Bordeaux, France); anti-*S. pombe* Cox2, 1/3000 (56); anti-*S. pombe* Cytb, 1/1000 (27); anti-Cox1, 1/3000 (this work); anti-Atp6, 1/1000 (this work); anti-mS45, 1/1000 (this work), anti-mL58, 1/1000 (this work); anti-human Hsp60, 1/1000 (H3524, Sigma); anti-human alpha-Tubulin, 1/10000 (T5168, Sigma). All these proteins were mitochondrial except Tubulin which is cytosolic. The anti-Cytb antibody cannot reveal cytochrome *b* if samples are boiled. Similarly, for optimal detection of Cox1 and Atp6 with our in-house antibodies, samples are either non-boiled or heated to 60° respectively. The anti-Arg8 antibody reveals in addition to *S. pombe* Arg1 a cross-reacting non-mitochondrial protein of similar size (see Figure 6C). Anti-tag antibodies were: anti-cMyc, 1/5000 to 1/10000 (Roche clone 9E10); anti-HA, 1/1000 (Genscript Elite A01244; Boehringer Manheim clone 12CA5) or 1/2000 (monoclonal mouse antibody provided by R. Schweyen, Vienna, Austria); anti-V5, 1/5000 to 1/10000 (ab27671 Abcam, a gift of Inge Kühl); anti-FLAG, 1/1000 (F3165 and F1804, Sigma). Secondary anti-mouse or anti-rabbit HRP conjugate antibodies (Promega) were diluted 1/10000 except true-blot antibodies (Tebu-bio) used for the detection of immunoprecipitated proteins that were diluted to 1/5000.

Sucrose gradient sedimentation analysis

S. pombe cells were grown to an OD_{600} of 1–1.5 in complete or minimal glucose medium as required; the cells were then washed and resuspended in 2× the pellet volume of lysis buffer (1% Triton X-100, 20 mM HEPES pH7.6, 40 mM KCl, 1 mM PMSF, 1× protease inhibitors (Roche) and 50 mM EDTA that dissociates the ribosomes, then broken under freezing conditions using a French Press. The cell extracts were allowed to thaw on ice and then clarified by centrifugation at 20627 rcf and 4°C for 10 min. The extracts (10 OD_{260} units) were layered onto 10–45% sucrose gradients in lysis buffer (25 mM KCl) containing either EDTA or $MgCl_2$ and centrifuged at 39000 rpm and 4°C for 3.5 h in a SW41 Ti rotor (Beckman). Gradient analysis was performed using an Isco Foxy R1 fractionator and continuously monitored at 254 nm. Typically, 30 fractions were collected. For subsequent western blot analysis, the samples were treated as described in (42).

Northern blotting analysis

RNA extraction, northern blotting and probes were as in (23) except that after denaturation in formaldehyde/formamide buffer the RNAs were loaded on gels without formaldehyde, migrated in the regular 1× TBE buffer; transfer was done either following the classical 20× SSC saline method or using electro-transfer with 1× TBE buffer in a Genie electrophoretic blotter (Idea Scientific) as described in (57). The radioactive probes were PCR fragments (as in 23) and in some cases ethidium bromide stained ribosomal RNAs photographed before transfer were also shown as loading controls.

^{35}S labeling of mitochondrially synthesized proteins

S. pombe cells were grown to early exponential phase in complete or minimal (lacking leucine) 5% raffinose medium containing 0.1% glucose. Unless otherwise indicated, mitochondrial proteins were labeled at 30°C by a 3-h incubation of whole cells with ^{35}S methionine and cysteine (Hartmann Analytics) in the presence of 12 mg/ml cycloheximide (6 mg per reaction) or 1 mg/ml of anisomycin (0.5 mg/reaction). Both drugs specifically block cytosolic translation but in our hands anisomycin is more efficient in *S. pombe*. For pulse-chase analysis, cells were labeled for 3 h in the presence of anisomycin, washed with the chase buffer containing anisomycin and an excess of cold methionine and cysteine, and incubated for the indicated chase times (up to 20 h) at 30°C as described in (27).

Proteins were extracted as described in (58) and samples were separated on 16% acrylamide–0.5% bisacrylamide SDS gels. After Coomassie blue staining as a loading control and drying, gels were exposed to a film for one day or up to several weeks either at –80°C or room temperature. Alternatively, gels were transferred to a membrane that was exposed to a film before western blotting.

Cytochrome spectra

Low temperature cytochrome spectra of *S. pombe* frozen cell pastes from cells grown on solid glucose medium were recorded using a Cary 400 spectrophotometer after addition of sodium dithionite to fully reduce the cytochromes (59). The absorption maxima were 603, 560, 554 and 548 nm

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

for cytochromes *a+a3*, *b*, *c1* and *c* respectively. The *S. pombe* cytochrome *c* peak always shows a shoulder at 544 nm that disappears in a cytochrome *c* mutant.

Mass spectrometry analyses

At least three different sub-clones of Cbp7-cMyc (CS17/2), Mrh5-cMyc (SLM20) and Ppr4-cMyc (CHP182-18C) strains were used to produce separate mitochondria preparations, that were each used for independent IP experiments under Mg^{2+} conditions. IPs performed on three independent mitochondrial extracts from the untagged wild-type (NBp9-725) served as controls. The IP protocol was upscaled by using *ca.* 1.7 mg of mitochondria for each IP together with 80 μl of cMyc-agarose beads. 4 μl of the IP fraction eluted in 50 μl were tested by western blot with a cMyc antibody and the rest was loaded on a 10% polyacrylamide, 1 mm thick protein gel. After a 5 mm migration of the samples into the separating gel, standard Coomassie staining and destaining in fixing solution was performed before placing the gel in 1% acetic acid. Samples were digested as previously described (60) before submission to mass spectrometry analysis. Trypsin-generated peptides from three biological replicates of the Cbp7-cMyc, Mrh5-cMyc, Ppr4-cMyc or wt immunoprecipitates were analyzed by nanoLC–MSMS using a nanoElute liquid chromatography system (Bruker) coupled to a timsTOF Pro mass spectrometer (Bruker). Peptides were loaded with solvent A on a trap column (nanoEase C18, 100 Å, 5 μm, 180 μm × 20 mm) and separated on an Aurora analytical column (ION OPTIK, 25 cm × 75 μm, C18, 1.6 μm) with a gradient of 0–35% of solvent B for 100 minutes. Solvent A was 0.1% formic acid and 2% acetonitrile in water and solvent B was acetonitrile with 0.1% formic acid. MS and MS/MS spectra were recorded from m/z 100 to 1700 with a mobility scan range from 0.6 to 1.4 V s/cm^2. MS/MS spectra were acquired with the PASEF (Parallel Accumulation Serial Fragmentation) ion mobility-based acquisition mode using a number of PASEF MS/MS scans set as 10. MS and MSMS raw data were processed and converted into mgf files with DataAnalysis software (Bruker).

Protein identifications were performed using the MASCOT search engine (Matrix Science, London, UK) against Swissprot database and *S. pombe* taxonomy. Database searches were performed using trypsin cleavage specificity with two possible missed cleavages. Carbamidomethylation of cysteines was set as fixed modification and oxidation of methionines as variable modification. Peptide and fragment tolerances were set at 10 ppm and 0.05 Da, respectively. Proteins were validated when identified with at least two unique peptides. Only ions with a score higher than the identity threshold and a false-positive discovery rate of less than 1% (Mascot decoy option) were considered. Both mascot error tolerant and semi-specific cleavages searches were performed to characterize the N-terminal of Mrp51.

Mass spectrometry based-quantification was performed by label-free quantification using spectral count method. Total MS/MS spectral count values were extracted from Scaffold software (version Scaffold_4.11.1, Proteome software Inc, Portland, OR) filtered with 95% probability and 0.1% FDR for protein and peptide thresholds, respectively. For statistical analysis, missing values occurring in spectral count datasets at protein-level were imputed by a constant value fixed at 0.1. In order to take into account within-sample variation in spectral count datasets, a beta-binomial test was performed based on triplicates MS/MS analyses with *P*-values calculated using R package 'ibb' (version 13.06, 61). Proteins were filtered on a *P*-value <0.05 and a fold change larger than two.

RESULTS

Cbp7, a factor distantly related to the translational activator Cbs2/Cbp7 in *S. cerevisiae*, is a mitochondrial protein required for cytochrome *b* accumulation in *S. pombe*

In order to investigate how the production of cytochrome *b* is controlled in *S. pombe*, we first used as bait the two *S. cerevisiae* translational activators of *cytb*, Cbs1 and Cbs2 (also called Cbp7) (62,63) to carry out position-specific iterative (PSI)-BLAST searches, without retrieving any convincing homologs in *S. pombe* (13). However, Cbs2 shared significant sequence similarity with the dehydropanthoate reductase enzyme Pan5 (64), which is clearly present in both *S. cerevisiae* and *S. pombe* (Figure 2A). Using *S. pombe* Pan5 we ran PSI-BLAST searches on the *S. pombe* proteome. We found a distant homolog of Pan5, Spbc28e12.04, which we named Cbp7 for **c**ytochrome ***b*** **p**roduction (Supplementary Figure S1A, B).

A tagged version of Cbp7 was generated and found to be highly enriched in purified mitochondria (Figure 2B). We then deleted *cbp7* in two mitochondrial intron contexts (either $\sum i$ or Δi) and determined the respiratory phenotype; Cbp7 deletion prevented growth on non-fermentable medium in both contexts (Figure 2C and not shown), but grew normally on fermentable medium containing antimycin, showing that the ATP synthase (complex V) is not strongly affected by the mutation (Figure 2C). Cytochrome spectra showed strongly decreased peaks for cytochromes *b* and *c1* of complex III but normal to increased absorbance for cytochrome *a* + *a3* of complex IV (Figure 2D). In accordance, when mitochondria from the wt and deleted *Δcbp7* strains were purified and analyzed by western blotting, the anti-Cytb antibodies could only reveal a very faint Cytb signal, if any, in *Δcbp7* cells, whereas the complex IV subunits Cox1 and Cox2 were easily detected (Figure 2E). Thus the *Δcbp7* mutant appears to specifically affect complex III biogenesis and this was confirmed by BN PAGE (Supplementary Figure S2) that showed a strong decrease in the accumulation of supercomplexes III_2/IV and an increase of free complex IV as well as the presence of substantial levels of complex V. In accordance, complex III activity in the *Δcbp7* mutant was down to 20% of that of the wt (see section 3 of the Results).

To test which step of Cytb biogenesis was affected, we analyzed both mitochondrial steady state RNA level, mitochondrial translation kinetics as well as the fate of newly synthesized mitochondrially-encoded subunits. First, the *cytb* mRNA was significantly, although never fully, destabilized (Figure 2F) whereas other mRNAs were not affected. Surprisingly, when newly synthesized mitochondrially encoded proteins were labeled with ^{35}S amino-acids in *Δcbp7* cells, no effect on mitochondrial translation was observed

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

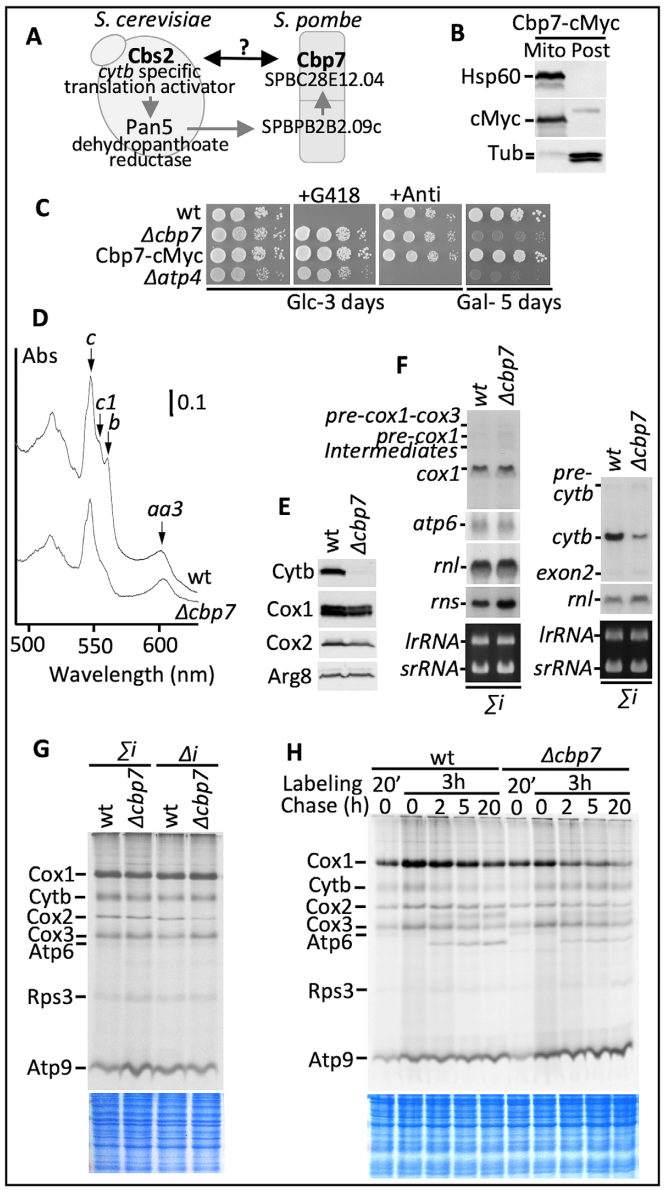


Figure 2. *S. pombe* Cbp7, a distant sequence homolog of *S. cerevisiae* Cbs2, is a mitochondrial protein required for cytochrome *b* biogenesis. (**A**) Starting from *S. cerevisiae* Cbs2, Cbp7 was found by performing successive Psi-Blast searches *via S. cerevisiae* and *S. pombe* Pan5. (**B**) Western blot analysis of purified mitochondria (Mito) and post-mitochondrial supernatant (Post) from Cbp7-cMyc cells. (**C**) Ten-fold serial dilutions of cells spotted onto complete glucose medium (Glc) supplemented or not with G418 or Antimycin A (Anti), and onto complete galactose medium (Gal). *Δatp4* cells serve as an ATP synthase deficient control. (**D**) Low temperature cytochrome spectra with peaks corresponding to cytochrome *c*, cytochromes *c1* and *b* from complex III and cytochromes *a* + *a3* from complex IV. (**E**) Western blot analysis of purified mitochondria from wt and *Δcbp7* cells. (**F**) Total RNA analyzed by Northern blotting with probes for mitochondrial mRNAs (*cytb*, *cox1, atp6*) or mitochondrial rRNAs (*rns* and *rnl* rRNA, see Figure 1). Upper panels: autoradiography; lower panels: ethidium bromide staining of total large or small cytosolic rRNAs (*lrRNA* or *srRNA*). (**G**) Newly synthesized mitochondria-encoded proteins radioactively labeled with ^{35}S and resolved by SDS-PAGE. Upper panel: autoradiography; lower panel: Coomassie staining. (**H**) Pulse-chase analysis of newly synthesized mitochondria-encoded proteins. Mitochondrial proteins were radioactively labeled with ^{35}S for 20 min or 3 h, chased for up to 20 h and resolved by SDS-PAGE. In this particular experiments wt and *Δcbp7* cells were both grown to 0.3 OD before labeling. Upper panel: autoradiography of the dried gel; lower panel: Coomassie staining.

in the mutants when compared to the isogenic wild-types (Figure 2G). The experiment was repeated many times and the Cytb protein was always efficiently labeled in the *Δcbp7* cells, sometimes less and sometimes even more strongly than in the wt. To understand the fate of this newly synthesized Cytb protein, pulse-chase experiments were carried out. After the 3h labelling, Cytb showed a clear pattern of degradation over a 20 h chase in the wt, but appeared repeatedly resistant to degradation in the *Δcbp7* mutant in several independent experiments (Figure 2H). This unexpected degradation pattern could indicate that in *Δcbp7* cells, the newly-synthetized Cytb might escape recognition and/or accessibility by mitochondrial proteases (65), possibly because of misfolding, aggregation and/or defective membrane insertion. This absence of degradation was surprising since Cytb steady state seemed dramatically decreased in the mutant compared to the wt, as revealed in purified mitochondria (Figure 2E) as well as in the total protein chase samples (Supplementary Figure S3) using our in-house antibody. We have no definitive solution to this paradox; the answer might lie with a particularity of our Cytb antibody. This only recognizes Cytb in non-boiled samples, suggesting that the epitope recognised by the antibody is a small structural motif, rather than a linear sequence. Thus, if the neo-synthesized Cytb cannot be correctly folded and inserted into the membrane, the epitope recognised by our antibody may no longer be present.

In any case, our data show that the fate and maybe the folding of the Cytb protein synthesized in the Δ*cbp7* mutant is different from that of the Cytb protein synthesized in wt cells. Thus, Cbp7, like Cbs2 in *S. cerevisiae*, appears specifically required for complex III biogenesis, but in a different way, since it compromises both the accumulation of the *cytb* mRNA and the correctly folded Cytb protein rather just than *cytb* translation. To further understand the function of Cbp7 and find other proteins controlling Cytb production in *S. pombe*, we used Cbp7 as a bait and searched for physical as well as genetic partners.

Immunoprecipitation of Cbp7 identifies Cbp8, a new factor essential for Cytb accumulation

To find physical partners of Cbp7 in *S. pombe*, we performed anti-c-Myc immunoprecipitation (IP) followed by mass spectrometry LC–MS/MS analysis on tagged *S. pombe* Cbp7-cMyc mitochondria. Mitochondria from the untagged wt served as control. Whereas Cytb itself was never enriched in IPs of Cbp7-cMyc compared to wt, a protein of unknown function, Spac22h10.09, was strongly and significantly enriched in all experiments (Figure 3A). In our mass spectrometry analyses, the absolute spectral counts for Spac22h10.09 remained low (Supplementary Table S2), which correlates with the fact that in PomBase it is reported to be of very low abundance, like Cbp7. Thus, we tentatively called this factor Cbp8, since our data suggest that it could form a complex with Cbp7. Cbp8, as well as its homologs in other *Schizosaccharomyces* species, is predicted to encode a protein with repeated helix motifs (Supplementary Figure S4A, B) and phylogenetic analyses (Supplementary Figure S4C) suggested that it could be distantly related to *S. cerevisiae* Cbp1, which is a PPR protein.

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

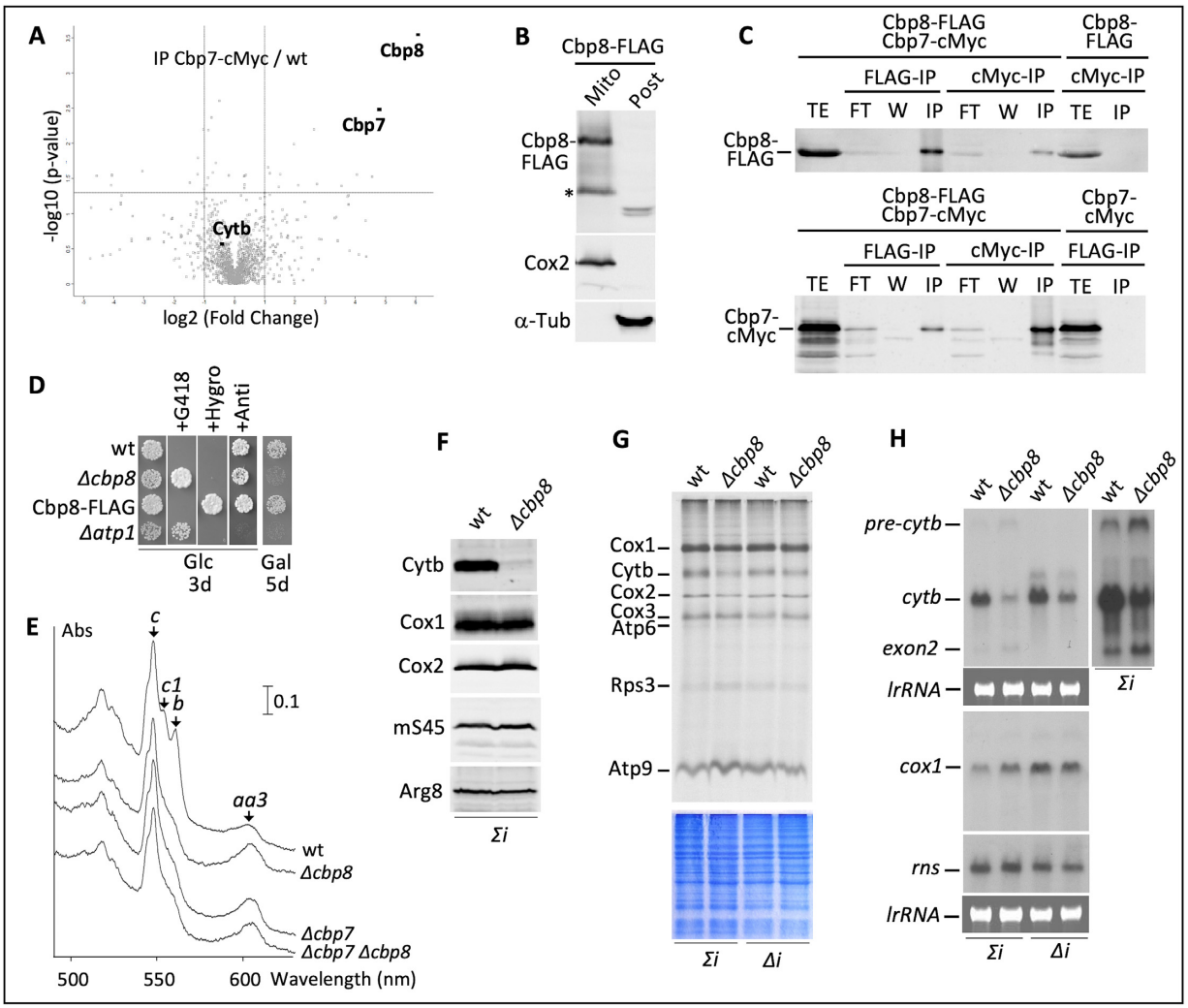


Figure 3. Cbp8, a mitochondrial protein interacting with Cbp7, is also required for cytochrome *b* biogenesis. (**A**) Volcano-plot from mass spectrometry data of the Cbp7-cMyc immunoprecipitated fraction. The *P*-value is plotted against the fold change for each protein (values are reported in log-10 and log-2 scales, respectively). Spac22h10.09 is both highly, and significantly enriched and was called Cbp8. The position of Cytb is also shown. (**B**) Western blot analysis of mitochondria (Mito) and post-mitochondrial supernatant (Post) from the Cbp8-FLAG tagged strain. (**C**) Mitochondrial extracts from a Cbp8-FLAG Cbp7-cMyc strain immunoprecipitated with anti-cMyc or anti-FLAG beads and analyzed by western blotting with the anti-tag antibodies. Strains with a single tag served as controls. (**D**) Serial dilutions of cells spotted onto Glc medium supplemented or not with G418, hygromycin (Hygro) or antimycin A (Anti), and onto Gal medium. *Δatp1* cells served as an ATPase deficient control. (**E**) Low temperature cytochrome spectra (**F**) Western blot analysis of mitochondrial proteins. (**G**) Radioactive labeling of newly synthesized mitochondrial proteins. (**H**) Northern blot analysis of total RNA.

In order to confirm the interaction between Cbp7 and Cbp8, we FLAG-tagged Cbp8 in a Cbp7-cMyc background and showed that it is a mitochondrial protein (Figure 3B); then we carried out IPs of Cbp8-FLAG and Cbp7-cMyc and observed in each case a clear co-immunoprecipitation of the other tagged protein (Figure 3C). For each experiment, we used control strains untagged for the antibodies used in the IP (Figure 3C, two last lanes) and no precipitation of the other tagged protein was observed. Thus, Cbp7 and Cbp8 strongly and specifically co-immunoprecipitate.

The deletion of the *cbp8* gene in both $\sum i$ and Δi backgrounds yielded respiratory deficient but antimycin resistant cells showing that ATP synthase was not affected (Figure 3D and not shown). To further characterize the Δ*cbp8* mutants, we performed BN-PAGE analysis of OXPHOS complexes (Supplementary Figure S2), recorded cytochrome spectra (Figure 3E), analyzed steady state and newly translated mitochondrial proteins (Figure 3F-G) and measured the steady state RNA (Figure 3H). In brief, *Δcbp8* looked similar to *Δcbp7* since complex III was barely detectable in BN-PAGE, cytochrome *b* and *c1* were not spectrally detected and a low level of *cytb* RNA was observed; the Cytb protein was still synthesized, although slightly less efficiently than in the wild-type, but only a low steady-state level could be detected. However, as with *Δcbp7* cells, the newly synthesized Cytb protein showed an aberrant degradation pattern compared to the wild-type since it remained stable for up to 20 h in a chase experiment (Supplementary Figure S5). Since the phenotype of *Δcbp8* and *Δcbp7* cells was similar, we constructed the double mutant and recorded its growth and cytochrome spectra, which appeared undistinguishable from that of either single mutant,

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

Table 1. Chromosomal suppressors of *Δcbp7*

Name	Cloning strategy	Nbr of clones	Suppressor mutation ORF → protein	Location	Known or putative function
ppr4 (*srd2*)	Genomic sequencing	1	*t1705a* → Y569N	Mito	PPR protein, *cox1* translational activator (23)
		2	*c2630t* → S877F		
		1	*c748t* → L250F		
rpl1801 (*srd8*)	Genomic sequencing	1	*c92t* → S31L	Cyto	Cytosolic ribosomal protein (LSU)
mrh5 (*srd11*)	Complementation	1	*g689a* → G230E	Mito	Mitochondrial DEAD box helicase
mrp51 (*srd12*)	Genomic sequencing	1	*g61a* → E21K	Mito	Mitochondrial ribosomal protein (SSU)

with reduced cytochrome *b* and *c1* peaks and a slightly increased cytochrome *a* + *a3* peak (Figure 3E).

All this suggests that Cbp7 and Cbp8 form a complex and that losing one or the other component of this complex leads to a similar defect in cytochrome *b* biogenesis.

Genetic suppressors of *Δcbp7* include PPR proteins, mitoribosomal proteins and a predicted mitochondrial DExD-box helicase

In parallel to physical partners, we searched for genetic suppressors that could compensate for the absence of Cbp7. Extragenic mutations allowing growth on non-fermentable medium were selected after UV mutagenesis. The corresponding chromosomal suppressor alleles were called *srd* for '**s**uppressor of **r**espiratory **d**eficiency' of *Δcbp7*. After subcloning and backcrosses, seven *srd* suppressors were retained, and genetic studies showed that they fell into four complementation groups (the *srd12* group had four members, the others each corresponded to a single clone). When separated from the *Δcbp7* allele, *srd11* conferred a phenotype that was used to clone the wild-type gene, which encodes a putative mitochondrial RNA helicase that was called Mrh5. For all the other *srd* suppressor mutations, whole genome sequencing was carried out. We identified (i) Srd2 as the PPR protein Ppr4 required for *cox1* translation (23), (ii) Srd8 as the cytosolic ribosomal protein isoform Rpl1801 and (iii) Srd12 as the probable homolog of Mrp51, a protein of the small mitoribosomal subunit (66). Rpl1801 is probably the Rpl18 paralog conferring more efficient translation of respiration related proteins as shown for other cytosolic 80S ribosomal proteins (4) and it was not studied further. In all cases, individual sequencing of several spores confirmed the identity of the mutations and the complementation groups, as summarized in Table 1.

The strength of the chromosomal suppressors was compared using drop tests, cytochrome spectra, respiratory complex activities, western blots and BN PAGE (Figure 4, panels A–E, and not shown). The suppressor mutations in *mrp51* and *ppr4* were the strongest in terms of growth on non-fermentable medium while the *mrh5* and *ppr4* suppressors showed the best restoration of Cytb spectral peak. All suppressors improved complex III activity, with a concomitant decrease in complex IV activity for the *ppr4* and *mrh5* suppressors while the *mrp51* suppressor improved both activities. Accordingly, all suppressors improved the detection of at least a small fraction of Cytb. However, the *mrh5* suppressor severely affected complex IV, both in spectra, western blot and BN PAGE, probably explaining the lower growth rate despite a good restoration of Cytb. As expected from (23), the *ppr4* mutations also affected the level of Cox1, and in fact all suppressors tended to lower the spectral cytochrome *a* + *a3*.

In parallel, high copy suppressors were isolated. Three classes of plasmids were obtained (Table 2) encoding (i) either Mug178, annotated in PomBase as an isoform of Mrp51/bS1m, (ii) Ppr7, a PPR protein important for the stability of the *atp6* mRNA (23), or (iii) Zfs1 (67), a cytosolic/nuclear factor involved in mRNA catabolism whose overproduction probably suppressed *Δcbp7* through long range effects and that was not studied further. The strength of suppression by the *mug178* or *ppr7* plasmids was similar in terms of respiratory growth and cytochrome spectra (Figure 5A, B). However, labeling of mitochondrial translation products differed since overproduction of Mug178 clearly increased synthesis of Cytb while overproduction of Ppr7 mostly enhanced synthesis of Atp6 (Figure 5C). Also, high dosage of Mug178 slightly improved the detection of Cytb in *Δcbp7* (Figure 5D). High copy suppressors were also searched for starting from the *Δcbp8* deletion and identified the same genes (Table 2): *mug178*, *ppr7 and zfs1*, reinforcing the idea that Cbp7 and Cbp8 are acting at the same step of Cytb biogenesis. In *Δcbp8*, high dosage of *mug178* or *ppr7* led to a similar pattern of mitochondrial translation products as in *Δcbp7*: increase of Cytb or Atp6 synthesis upon overexpression of *mug178* or *ppr7* respectively (Figure 5E).

In conclusion, the same genetic suppressors can compensate for the cytochrome *b* defect of *Δcbp7* and *Δcbp8* and the study of these genetic partners of Cbp7/Cbp8 was further developed.

Mrp51 and Mug178 are mutually exclusive mitoribosomal isoforms, both important for mitochondrial translation

It was striking to isolate as suppressors of Δ*cbp7* or Δ*cbp8* two paralogs, Mrp51 and Mug178 that are predicted mitoribosomal bS1m isoforms. Although extensively remodeled compared to its bacterial and human counterparts, Mrp51 corresponds to the bS1m ribosomal protein which is nearly universally conserved (14) except *e.g.* in some particular cases, such as protist mitoribosomes (68,69). The protein bS1m is located at the mRNA exit channel, and in humans some bS1m mutations are associated in humans with a severe developmental delay (70). We found that the two isoforms are conserved in several phyla such as *Schizosaccharomyces* and *Zygomyces* (Supplementary Figures S6 and S7). In addition, *S. pombe* tagged Mrp51-HA is more abundant than Mug178-HA upon growth in glycerol and its level is further increased under glucose

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

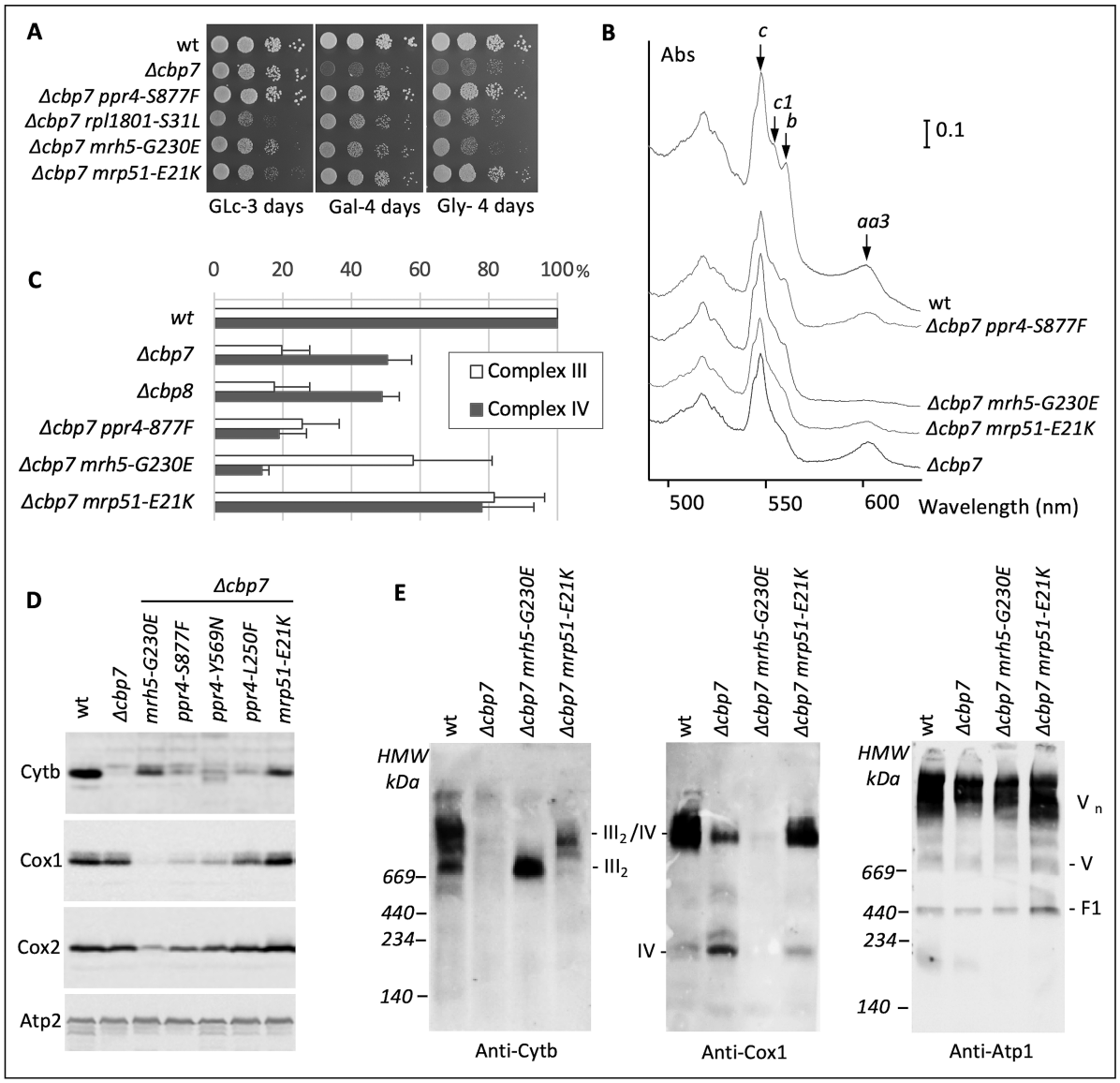


Figure 4. The respiratory deficiency of *Δcbp7* ∑i can be compensated for by mutations in several genes. (**A**) Ten-fold serial dilutions of the wt, mutant and suppressed strains spotted onto complete Glc, Gal and Gly media. (**B**) Low temperature cytochrome spectra. (**C**) Enzymatic activities of complexes III and IV in the wt, *Δcbp7* mutant and some suppressors, expressed in percentage of the wild-type. (D) Western blot analysis of mitochondrial proteins. (E) BN-PAGE analysis of digitonin-solubilized complexes in the wt, *Δcbp7* mutant and two suppressors. Positions of supercomplexes III/IV, multimers Vn as well as respiratory complexes III_2, IV, V and F1-part of complex V are indicated in comparison with a high molecular weight marker (HMW). The multimers Vn appear larger than those observed in *S. cerevisiae* under the same gel and migration conditions.

Table 2. High copy suppressors of *Δcbp7* and *Δcbp8*

Name	Cloning strategy	Nbr of clones	Suppression mode	Location	Known or putative function
mug178	Subcloning	13	Overexpression	Mito	Mrp51 isoform
ppr7	Subcloning	6	Overexpression	Mito	PPR protein, *atp6* stability (23)
zfs1	Subcloning	2	Overexpression	Cyto/Nucleus	mRNA catabolism (67)

(fermentable) conditions (Figure 6A, B). This is an atypical expression pattern, we tested six other mitoribosomal proteins and none of them showed an increased level under glucose, rather their steady state level tended to be slightly higher under glycerol conditions (data not shown). Three series of experiments were performed using tagged forms of these two proteins in order to demonstrate that they are genuine mitoribosomal proteins. First, fractionations of mitochondria and post-mitochondrial supernatant in a double tagged strain showed that both are indeed mitochondrial proteins (Figure 6C). Second, western blot analysis of mitochondrial extracts from the double tagged strain fractionated on EDTA-sucrose gradients to separate both ribosomal subunits, showed that both tagged proteins

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

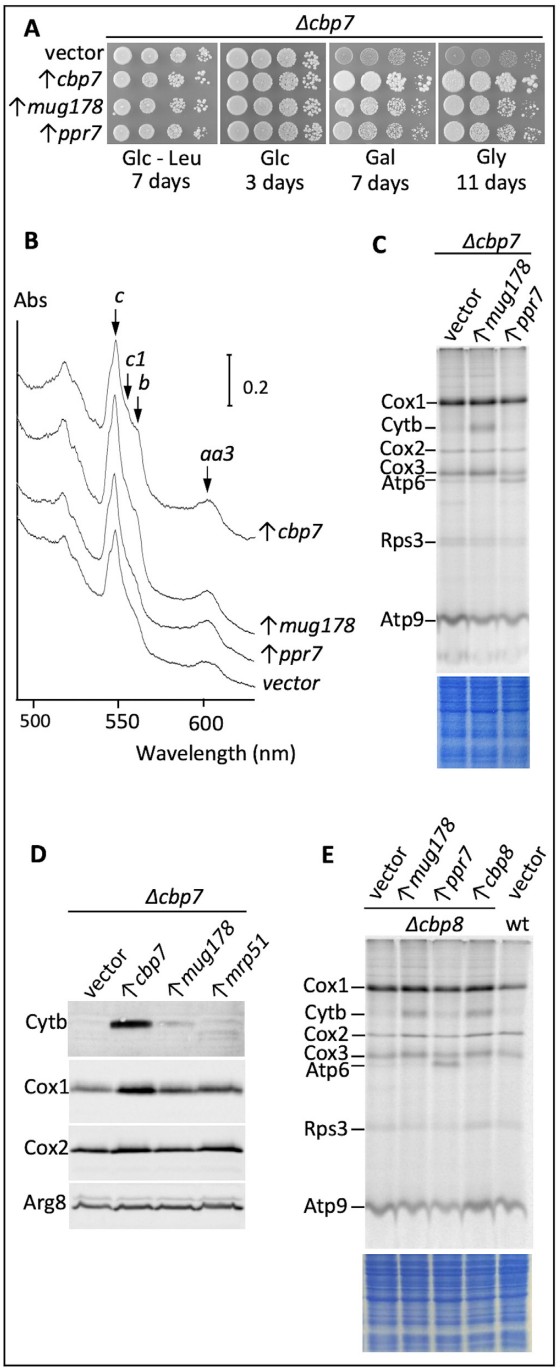


Figure 5. The respiratory deficiency of *Δcbp7* ∑i can be compensated for by overexpression of several genes. (**A**) Ten-fold serial dilutions of *Δcbp7* transformants carrying the control vector or multicopy plasmids overexpressing *cbp7*, *mug178* or *ppr7,* spotted onto minimal Glc medium lacking leucine (selecting for the plasmids) and on Glc, Gal and Gly media. (**B**) Low temperature cytochrome spectra of transformants. (**C**) Newly synthesized mitochondria-encoded proteins radioactively labeled in *Δcbp7* transformants. (**D**) Western blot analysis of mitochondrial proteins from *Δcbp7* transformants. (**E**) Newly synthesized mitochondria-encoded proteins radioactively labeled in *Δcbp8* or wt cells transformed with the control vector or multicopy plasmids overexpressing *mug178*, *ppr7* or *cbp8.*

co-sediment with the small ribosomal protein mS45 (Figure 6D). Third, immunoprecipitations performed under EDTA conditions showed that both Mrp51 and Mug178 did co-immunoprecipitate mS45 but not mL58 (Figure 6E). Strikingly these experiments also demonstrated that both tagged proteins never co-immunoprecipitated each other (Figure 6E). In addition, we also found that Cbp7-cMyc was roughly co-sedimenting with ribosomal proteins (Figure 6D and not shown); however, mS45, mL58, Mrp51 or Mug178 could not be detected upon immunoprecipitation of Cbp7-cMyc (Figure 6E). This was consistent with the mass spectrometry data of IPs of Cbp7-cMyc that detected either no or only weak enrichment for ribosomal proteins (Supplementary Table S2). Thus, Cbp7 could potentially interact with the ribosome, but rather either weakly, or transiently.

The Mrp51 mitochondrial targeting sequence does not seem to be processed upon mitochondrial import, since LC–MS/MS analysis of mitochondrial proteins did not allow detection of non-trypsinic cleavage in the Mrp51 peptides, whereas an acetylated N-terminal peptide lacking the initiator methionine could be found (Supplementary Figure S8). However, in the presence of the Mrp51-E21K mutation, partial N-terminal processing of Mrp51-E21K was induced, as a shorter discrete band was detected *via* the C-terminal HA tag (Figure 6F). Thus, the mutation Mrp51-E21K does not destabilize the tagged mutated protein but leads to the accumulation of a shorter form of the protein. This processing event does not compromise growth of Mrp51-E21K cells on non-fermentable medium in an otherwise wild-type background (Figure 7A), although the peak for complex IV cytochromes *a* + *a3* is lower (Figure 7B).

In order to further determine the relative role of Mrp51 and Mug178, we constructed *S. pombe* strains devoid of Mrp51 or Mug178. The complete absence of Mrp51 was unviable in a wild-type background. This is expected for an essential mitoribosomal protein because *S. pombe* is a *petite*-negative yeast, *i.e.* that cannot tolerate the complete loss of the mtDNA, or similarly a complete block in mitochondrial translation (71). The *mrp51* deletion could be obtained in the *ptp1-1* strain, which allows *S. pombe* to remain alive without mtDNA (47), and the spectra of the *Δptp1 Δmrp51* mutant looked like that of a *rho*° strain (Supplementary Figure S9). Thus, *mrp51* is essential in *S. pombe* and probably affects both ATP synthase and the electron transport chain, as stated by the two-component hypothesis (40,72). On the contrary, the deletion of *mug178* yielded viable cells. The *Δmug178* mutant was unable to grow on non-fermentable medium and lacked spectrally detectable cytochromes *b* and *c1* but was not sensitive to antimycin A, showing that ATP synthase is not defective (Figure 7A-B). Steady state, as well as neo-synthesized mitochondrially-encoded respiratory chain subunits, were analyzed in the different mutants (Figure 7C, D). Although *Δmug178* or *Δcbp7 mrp51-E21K* actively translated the *cytb* mRNA, the steady state Cytb protein was very low, showing that it is quickly degraded. In addition, although Cox3 synthesis was largely decreased in absence of Mug178, hemes *a* + *a3* and the subunits Cox1 and Cox2 were still detected at near wild type steady state levels. However, the cytochrome *c* oxidase activity in the *Δmug178* mutant was only 17% of that of the wt

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

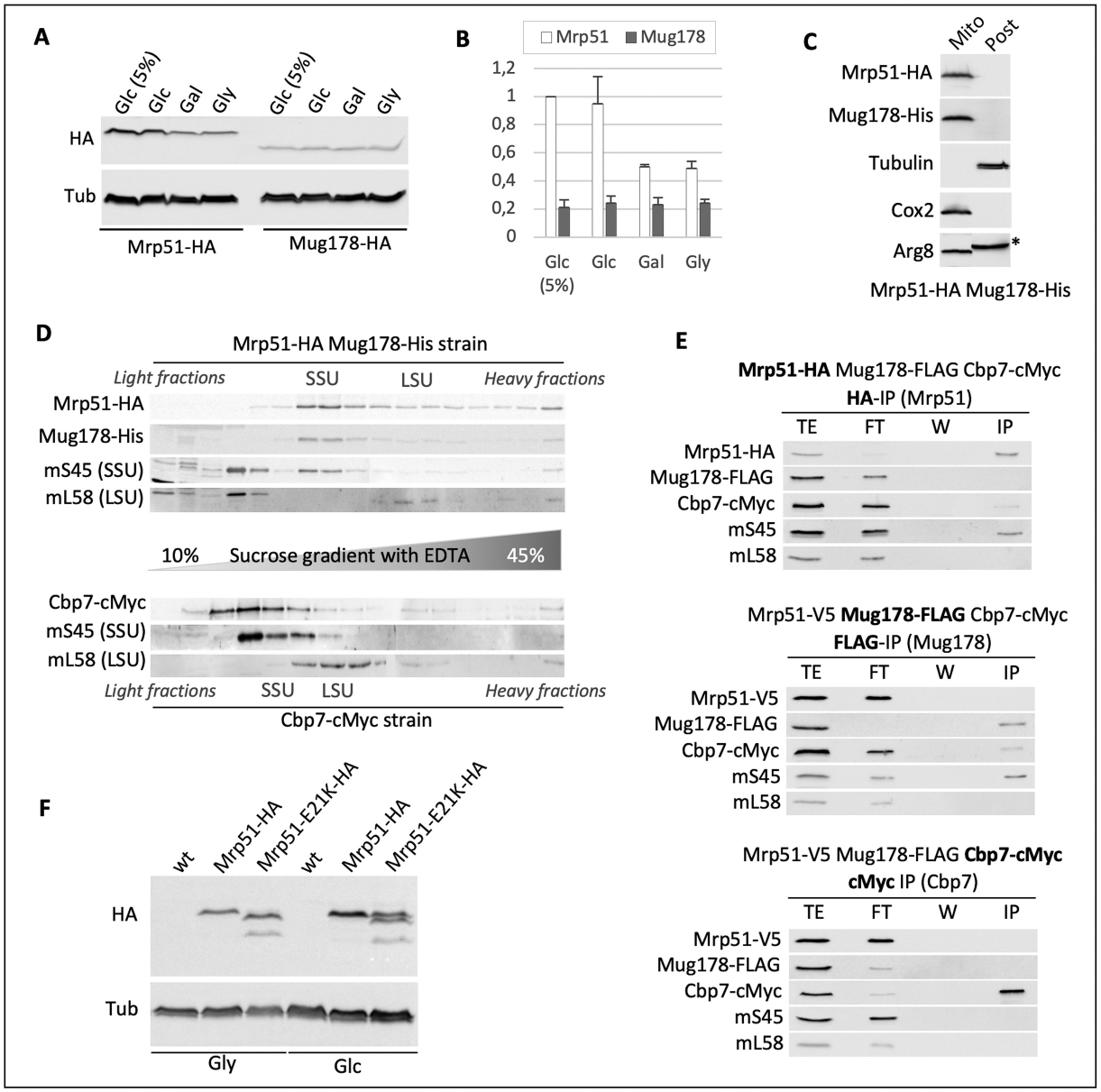


Figure 6. Mrp51 and Mug178 are mutually exclusive ribosomal isoforms. (**A**) Total proteins from Mrp51-HA or Mug178-HA cells were extracted after growth in media containing the indicated carbon sources and analyzed by western blotting. (**B**) Mean values from three repetitions of the experiment presented in panel A were plotted after determining the band densities using the Biorad Image Lab software. (**C**) Western blot analysis of mitochondria (Mito) and post-mitochondrial supernatant (post) from a tagged Mrp51-HA Mug178-His strain. (**D**) Western blot analysis of fractions resolved on a sucrose gradient loaded with Mrp51-HA Mug178-His or Cbp7-cMyc cell extracts. Sucrose gradients of the lower Cbp7 panel were spun longer. mS45: protein from the SSU; mL58: protein from the LSU. (**E**) Mitochondrial extracts from Mrp51-HA Mug178-FLAG Cbp7-cMyc or Mrp51-V5 Mug178-FLAG Cbp7-cMyc strains were subjected to IP with the indicated anti-tag antibodies coupled to agarose beads and analyzed by western blotting. (**F**) Western blot analysis of total proteins from Mrp51-HA and Mrp51-E21K-HA cells after growth in presence of the indicated carbon sources.

(14.1+/-2.4 nmol O_2/min for 60 μg of mitochondrial proteins for wt cells versus 2.4 +/-0.66 for *Δmug178* cells). This suggests that the limited synthesis of Cox3 leads either to the formation of a complex IV that can be detected spectrally, but has a reduced activity, or to a decreased amount of complex IV and an inactive complex that nevertheless contains hemes *a* + *a3*. Finally, we showed that overexpression of Mrp51 in *Δcbp7* or *Δcbp8* cells improved neither growth on non-fermentable medium nor Cytb detection while overexpression of Mug178 does; in addition, overproduction of Mrp51 could not suppress the absence of Mug178 (Figure 5D and not shown).

Together these results show that Mug178 and Mrp51 are mutually exclusive proteins from the small ribosomal subunit that are both important for the respiratory chain biogenesis; Mrp51 is essential for mitochondrial translation while Mug178 appears to have a more specific role, being involved in Cytb and Cox3 production.

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

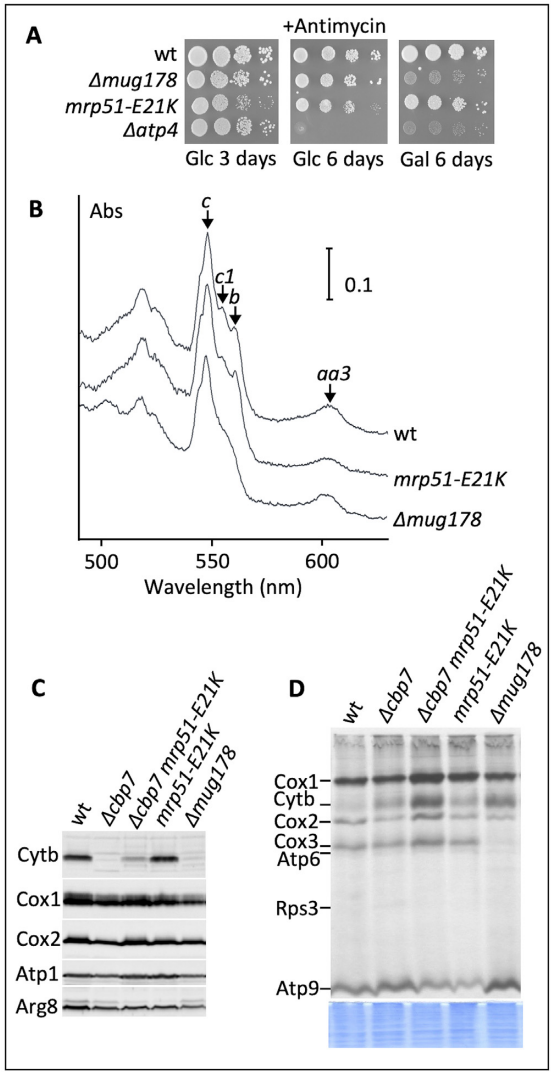


Figure 7. Mug178 is required for Cytb biogenesis and Cox3 synthesis (**A**) Ten-fold serial dilutions of cells spotted onto Glc medium supplemented, or not, with Antimycin A (Anti), and onto Gal medium. (**B**) Low temperature cytochrome spectra. (**C**) Western blot of mitochondrial proteins. (**D**) Newly synthesized mitochondrial proteins in wt, single or double mutants or in transformants.

Δcbp7/8 and *Δmug178* show additive effects, that are compensated by the same chromosomal suppressors affecting in particular Cox1 synthesis

The *Δcbp7, Δcbp8* and *Δmug178* mutants presented a similar profile in growth, cytochrome spectra and western blot, indicative of a Cytb deficiency; in addition, the double mutant *Δcbp7 Δcbp8* was identical in spectra and growth to both single mutants. Thus, we constructed the $\sum i$ *Δcbp7 Δmug178* and *Δcbp8 Δmug178* double mutants to analyze their phenotypes. We found that their growth on non-fermentable medium was more affected than any of the single mutants (Figure 8A and not shown). Radioactive labeling of newly translated mitochondrial proteins showed that the combination of *Δmug178* with either *Δcbp7* or *Δcbp8* abolished *cytb* translation while *Δcbp7 Δcbp8* was similar to *Δcbp8* (Figure 8B). Similarly, the spectra of the *Δcbp7*

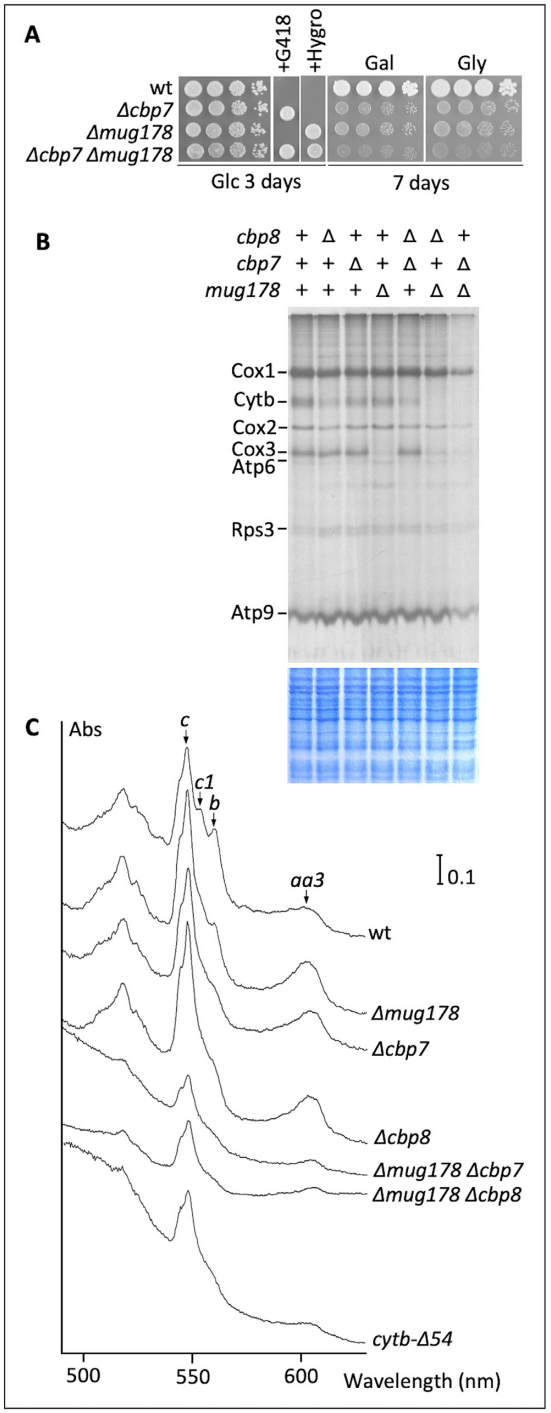


Figure 8. The absence of Mug178 and Cbp7/8 has additive effects. (**A**) Ten-fold serial dilutions of wt, single and double mutants spotted onto Glc medium supplemented or not with G418 or hygromycin, and onto Gal and Gly media. (**B**) Newly synthesized mitochondrial proteins in single or double mutants. (**C**) Low temperature cytochrome spectra. A mutant harboring a small deletion in *cytb* (*cytb-Δ54*, 26) is presented as a comparison.

Δmug178 and *Δcbp8 Δmug178* double mutants (Figure 8C) showed a strongly decreased cytochrome level and became very similar to the spectra of a *cytb* mutant, with an absence of cytochrome *b* and *c1* peaks and a decreased peak of

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

Table 3. Chromosomal suppressors of *Δmug178*

Name	Cloning strategy	Nbr of clones	Suppressor mutation ORF → protein	Location	Function	Suppression of *Δcbp7*
mtf2	Genomic sequencing	4	*453Δa* (aa 1 to 150 are wt, then 57 divergent aa)	Mito	PPR protein, *cox1* translational activator (73)	+
		1	*t754g* → W252G			
ppr4	Genomic sequencing	1	–390::39 (tandem duplication of the 39 nt from –429 to –391)	Mito	PPR protein, *cox1* translational activator (23)	+

cytochrome *a* + *a3*. Thus, there is a clear and similar additive effect between *Δmug178* and either *Δcbp7* or *Δcbp8*.

Next, we wondered whether *Δmug178* could be compensated by the different suppressors isolated from *Δcbp7*. First, no suppression was obtained with the overexpression of *cbp7* or *ppr7*. Second, we found that growth of *Δmug178* cells on non-fermentable medium was restored by the *ppr4*, *mrh5* or *mrp51* suppressor alleles of *Δcbp7* (Supplementary Figure S10A). Thus, *Δcbp7* and *Δmug178* appear to share the same chromosomal suppressors.

Since *Δmug178* easily produced spontaneous revertants on non-fermentable medium, we analyzed a few of them that fell into two complementation groups (Table 3). One group corresponded to a new allele of *ppr4* which is a small deletion in the promoter region. The second group contained five members and one of these suppressors was subjected to whole genome sequencing which identified a single mutation in the *mtf2* gene. Like *ppr4*, *mtf2* encodes a protein known to be required for Cox1 synthesis (73). Mtf2 has actually also been recently annotated as a PPR protein in PomBase and two possible motifs can be detected *in silico* with the TPRpred algorithm (74,75; not shown).

Strains containing these two suppressors alone or combined with *Δcbp7* were constructed and the *mtf2* gene was also deleted. The different strains were tested on non-fermentable medium and mitochondrial translation products were also radioactively labeled (Supplementary Figure S10B, C). Strikingly, the *ppr4* promoter mutation alone had no phenotype and a wild-type translation pattern, whereas the *mtf2* suppressor allele alone and the *Δmtf2* mutant dramatically enhanced the synthesis of Cytb while lowering that of Cox1. In addition, we found that when combined with the *Δcbp7* mutation, both *Δmug178* suppressors restored growth on non-fermentable medium and increased the synthesis ratio of Cytb compared to Cox1. Singularly, when the suppressor mutations were combined with *Δmug178*, Cytb synthesis was decreased, and thus brought closer to wild-type level. Both suppressor mutations also restored the growth of *Δcbp8* cells on non-fermentable medium (not shown).

In conclusion, *Δcbp7*, *Δcbp8* and *Δmug178* that all affect Cytb production, share many suppressors that affect Cox1 synthesis. Thus, lowering Cox1 synthesis seems to be a general mechanism of suppression. In this context, the Mrh5-G230E mutation that compensated for the absence of Cbp7 and conferred a strong complex IV defect seemed particularly interesting and we decided to investigate the function of Mrh5 further.

Mrh5 is a putative mitochondrial DExD box helicase that interacts with the ribosome and is essential for *cox1* translation

The putative helicase Mrh5 is registered in PomBase (Spbc25d12.06) as an *S. pombe* specific mitochondrial protein. It falls into the class of DExD-Box helicases with a Q motif (Supplementary Figure S11), which is unique to this protein family and might control ATP binding and hydrolysis. There is only one other mitochondrial protein in this family in *S. pombe*, Mss116. In *S. cerevisiae*, Mss116 is involved in mitochondrial splicing and has also been proposed to be a mitochondrial transcription factor modulating the action of the mitochondrial RNA polymerase Rpo41 (76 and references therein; 77,78). Thus, we decided to delete *mrh5* in both $\sum i$ and *Δi* cells in case it affected mitochondrial intron removal.

We found that *Δmrh5* was essential for respiratory growth, even in *Δi* background, suggesting that if Mrh5 is involved in intron excision, it also has another role. The *Δmrh5* mutation did not impair growth on antimycin containing medium, showing that ATPase activity is not affected (Figure 9A). However, cytochrome spectra of the deletion or point mutants showed a strong defect in complex IV and partial decrease in the complex III peaks (Figure 9B), which in *S. pombe* is often seen in complex IV deficient mutants (23). Western blot analysis was performed on the *Δmrh5* and *mrh5-G230E* mutants, as well as the tagged Mrh5-cMyc strain (Figure 9C). The results showed that Mrh5 is a mitochondrial protein and that Cox1 and Cox2 were undetectable in both mutants. Cytb level was partly decreased in *Δmrh5* and only slightly in the point mutant. Taken together, these results indicate that the complex IV defect is the primary cause of the respiratory deficiency of the Mrh5 mutants.

Next, we analyzed mitochondrial translation (Figure 9D) and RNA (Figure 9E) in both mutants as well as in the original suppressor mutation combined or not with *Δcbp7*. Whereas newly-synthesized Cox2 and Cox3 were radioactively labeled, Cox1 synthesis was abolished although the mature *cox1* mRNA was only slightly decreased in *Δi* cells, showing that Mrh5 is required for *cox1* translation. In $\sum i$ cells, the mature *cox1* mRNA was absent in *Δmrh5* and the precursor RNA containing both *cox1* introns accumulated; in the point mutant, partial splicing deficiency was observed. These observations are compatible with a role of Mrh5 in the synthesis of intron-encoded *cox1* maturase(s), although an additional independent role in splicing cannot be excluded. Together, these data show that Mrh5 is required for Cox1 synthesis, like Ppr4 and Mtf2. Strikingly, in presence of the suppressor mutation *mrh5-G230E*, Cytb synthesis was dramatically increased, and this effect was still

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

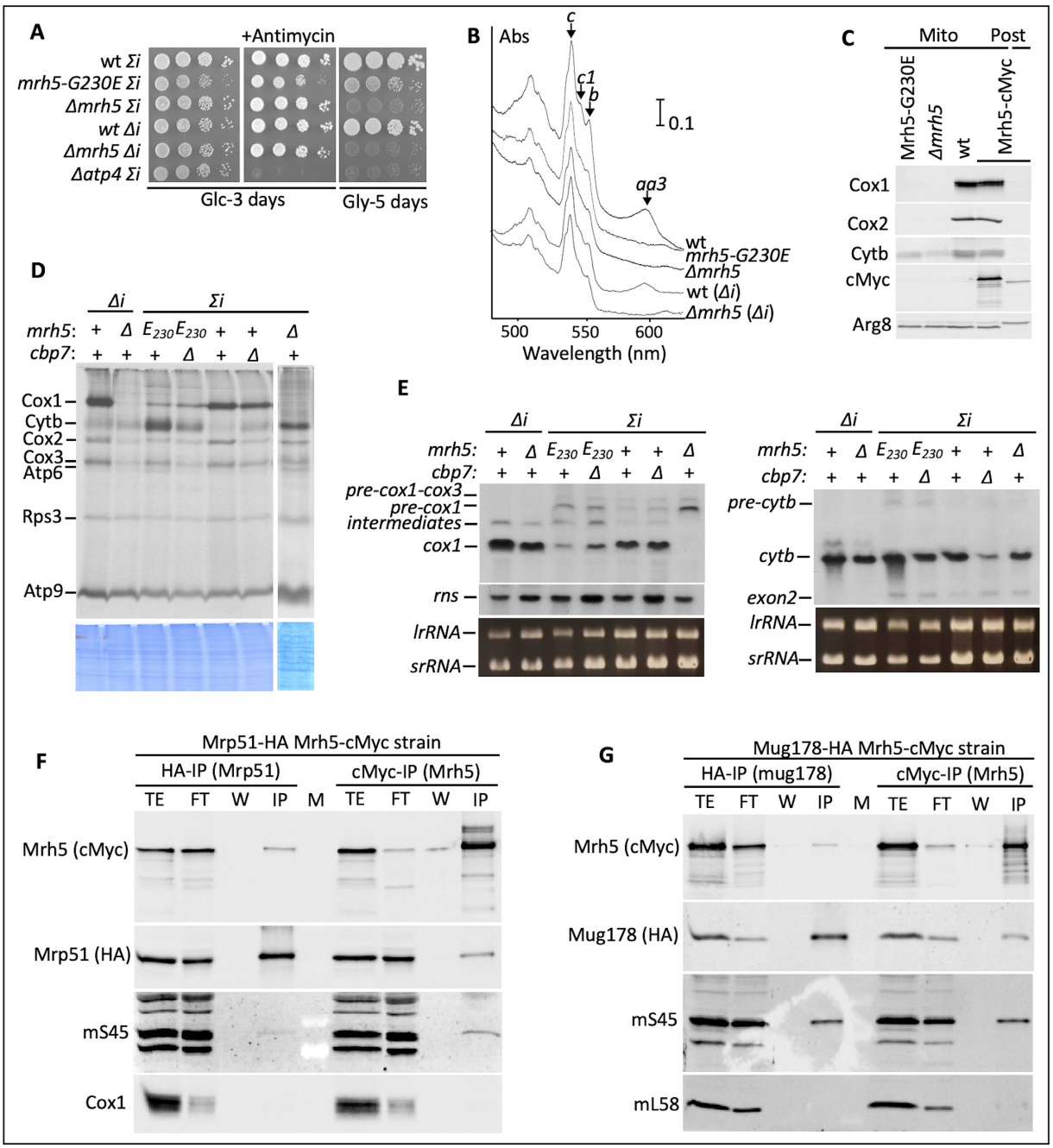


Figure 9. Mrh5 is required for *cox1* translation and interacts with the mitoribosome. (**A**) Ten-fold serial dilutions of cells spotted onto Glc medium supplemented or not with Antimycin A (Anti) and onto Gly medium. (**B**) Low temperature cytochrome spectra. (**C**) Western blot of mitochondrial (Mito) or post-mitochondrial (Post) extracts from various strains. (**D**) Newly synthesized mitochondrial proteins. (**E**) Northern blot analysis of total RNA. (**F, G**) Mitochondrial proteins from the Mrp51-HA Mrh5-cMyc or Mug178-HA Mrh5-cMyc strains immunoprecipitated with anti-HA or anti-cMyc beads under EDTA conditions and analyzed by western blotting. M corresponds to the lanes where the molecular weight marker was loaded.

visible in presence of the *Δcbp7* allele, suggesting that this might be part of the mechanism of suppression.

Finally, cMyc-tagged Mrh5 was subjected to IP in presence of EDTA, in strains also HA-tagged for Mrp51 or Mug178. The results of the cMyc- and HA-IPs showed that Mrh5 clearly co-precipitates with proteins of the small ribosomal subunit, including mS45, Mug178 and Mrp51, but no co-IP signal was observed with Cox1. Thus, Mrh5 interacts with the mitoribosomal SSU and is required primarily for *cox1* translation.

Mrh5 and Ppr4 belong to a *cox1* translation complex that interacts with the mitochondrial ribosome

To get a genome-wide view of the physical partners of Mrh5, we decided to perform IPs of cMyc-tagged Mrh5

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

under Mg^{2+} conditions, that maintain association of the ribosome, followed by LC-MS/MS analysis. Results presented in Supplementary Table S3 were statistically analyzed (Supplementary Table S4) and volcano-plots were drawn to highlight proteins that were both highly and significantly enriched in the Mrh5-cMyc IP fractions (Figure 10A). The strongest interactors (in red) were Ppr4 and Mtf2 as well as the *S. pombe* homolog of the *S. cerevisiae* Sls1 protein. The vast majority of the other significantly enriched proteins included almost all components of the small (in green) and large (in blue) mitoribosomal subunits. Importantly, Cox1 was very well detected in the analysis but was not enriched in the Mrh5 IP, supporting our western blot results.

In parallel, the same IP and LC–MS/MS experiment was carried out with the tagged Ppr4-cMyc strain. The raw results presented in Supplementary Table S3 and statistically analyzed (Supplementary Table S5) were also made into volcano plots (Figure 10B). Ppr4 strongly interacted with Mrh5, Mtf2 and Sls1 and there was also a slight enrichment in some ribosomal proteins. Retrieving the same four highly enriched proteins starting from either Mrh5-cMyc and Ppr4-cMyc is a strong argument for the existence of a stable Mrh5/Ppr4/Mtf2/Sls1 complex.

Mrh5, Ppr4 and Mtf2 are all essential for Cox1 translation (see above, 23,73). The fourth partner, Sls1, is an integral membrane protein that was found as a suppressor of a point mutation in the mitochondrial RNA polymerase and also of a deletion in the *NAM1/MTF2* gene in *S. cerevisiae* (79,80). To decipher the role of Sls1 in *S. pombe*, we constructed and analyzed deletion mutants in $\sum i$ and Δi cells. In both cases, *Δsls1* cells were respiratory deficient and antimycin-resistant (Figure 11A). Spectral and western blot analysis showed that *Δsls1* cells lacked spectral *a+a3* peak, were devoid of Cox1 and very depleted in Cox2 (Figure 11B, C). Northern blot analysis (Figure 11D) revealed that the *cox1* mRNA was absent in *Δsls1* $\sum i$ cells while the *Δi* genome allowed the recovery of mature *cox1* mRNA. However, in both contexts there was no Cox1 synthesis (Figure 11E).

Thus, our mass spectrometry and phenotypic analyses show that Mrh5, Ppr4, Mtf2 and Sls1 are part of a *cox1* translational complex interacting with the mitoribosome, at least through Mrh5. Interestingly, mutations in Mrh5, Ppr4 and Mtf2 leading to a decrease of Cox1 synthesis partially compensate for the absence of Cbp7, Cbp8 or Mug178 that all appear to control proper Cytb production, stressing the importance of the balance between Cox1 and Cytb synthesis.

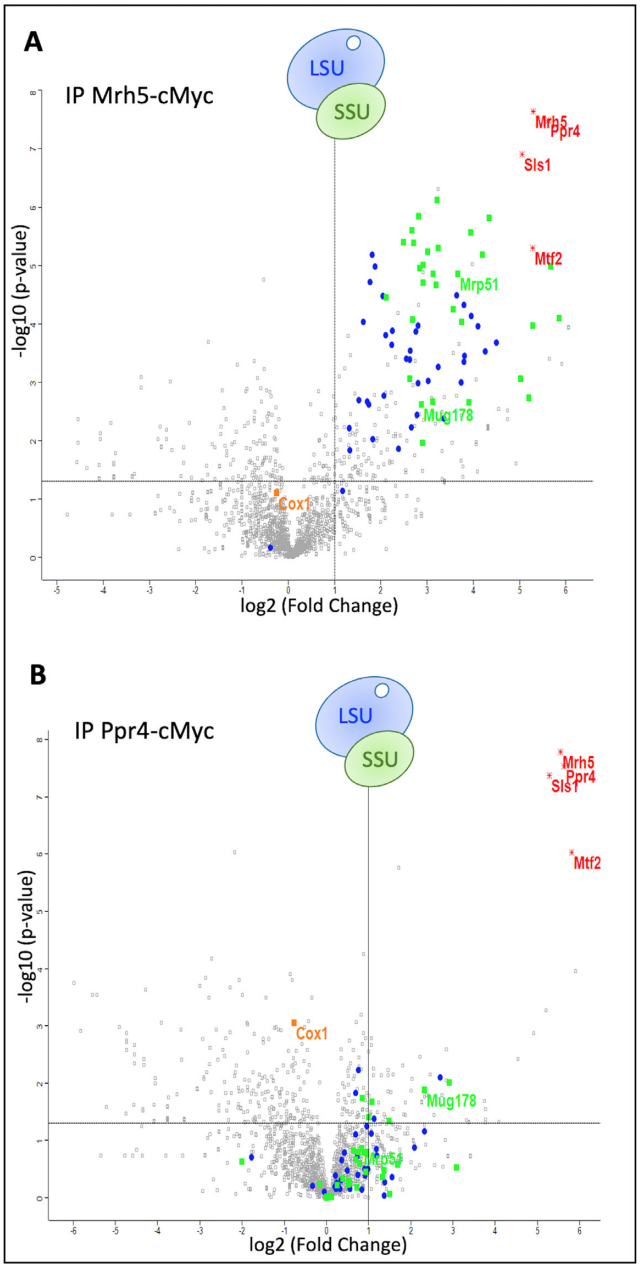


Figure 10. Volcano plot from mass spectrometry data of (**A**) Mrh5-cMyc or (**B**) Ppr4-cMyc immunoprecipitated fractions reveals common interacting proteins. Spectra obtained in both IPs could be assigned to 2386 and 2380 proteins respectively. The *P*-value is plotted against the fold change for each protein (values are reported in log-10 and log-2 scales, respectively). Proteins in the upper right corner are both highly and significantly enriched. Colors are as follows: Ppr4/Mrh5/Sls1/Mtf2 in red; mitochondrial SSU proteins in green; mitochondrial LSU proteins in blue; Cox1 in orange; other proteins in grey. SSU et LSU protein assignment were determined using a combination of Blast searches and PomBase annotations.

DISCUSSION

In this paper, we have shown that *cbp7* and *cbp8* encode two new factors controlling cytochrome *b* production in *S. pombe* and that their absence can be compensated for by mutating (Mrp51), or overexpressing (Mug178) a pair of mutually exclusive ribosomal proteins, as well as by mutations in different members of a *cox1* translation complex.

Both deletion mutants *Δcbp7* and *Δcbp8* show a very comparable phenotype, with a stronger destabilization of the *cytb* mRNA in *Δcbp8* and a similar level of Cytb labeling in *Δcbp8* or in the double mutant, suggesting that Δ*cbp8* is epistatic to Δ*cbp7*. In addition, all the high-copy and extragenic suppressors of the *Δcbp7* mutation are able to suppress the *Δcbp8* mutation, suggesting that they act in close cooperation. Our immunoprecipitation and mass spectrometry (IP-LC-MS/MS) data show that Cbp7 and

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

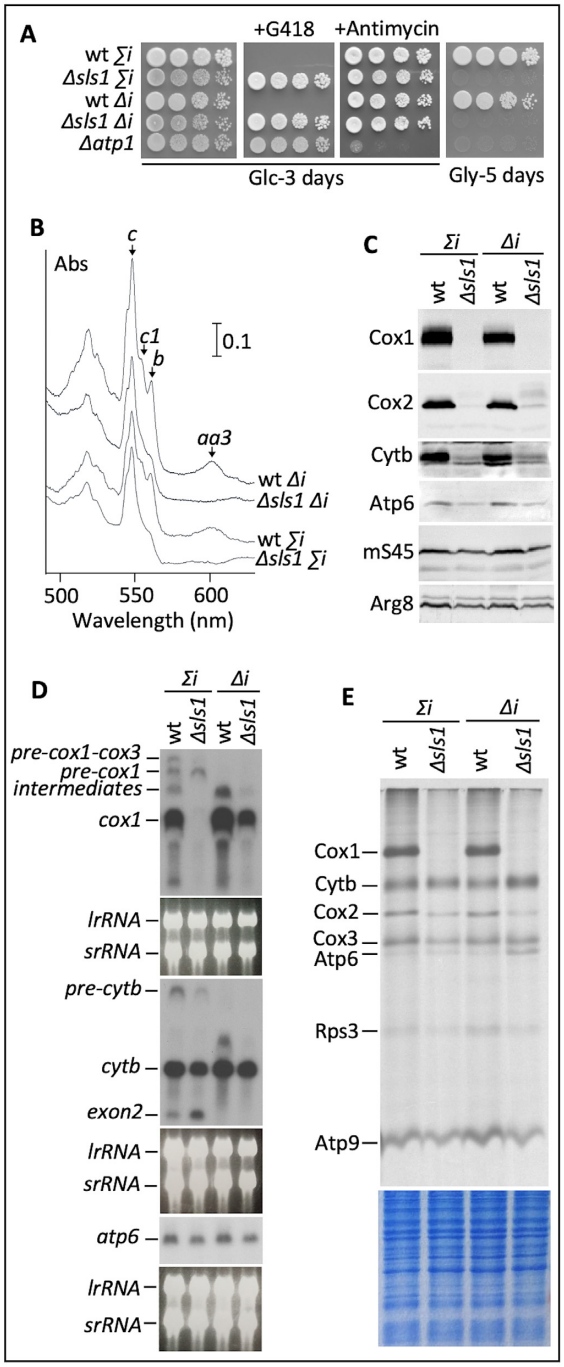


Figure 11. Sls1 is essential for Cox1 synthesis. (**A**) Ten-fold serial dilutions of wt, *Δsls1*, or *Δatp4* cells spotted onto Glc medium supplemented or not with G418 or Antimycin A (Anti), or onto Gly medium. (**B**) Low temperature cytochrome spectra. (**C**) Total RNA analyzed by northern blot. (**D**) Newly synthesized mitochondrial proteins. (**E**) Western blot of mitochondrial proteins.

Cbp8 physically interact, and that they are very low abundance proteins, like the translational activators described in *S. cerevisiae*.

It is puzzling that *Δcbp7* and *Δcbp8* strongly decrease both *cytb* mRNA and the steady state level of the Cytb protein, with little effect on the translation kinetics but an aberrant degradation pattern for the newly synthesized protein. This raises the question of whether Cbp7/8 operates before, or after Cytb synthesis, or at both steps. Our data is consistent with the hypothesis that Cbp7 and Cbp8 act before translation for two main reasons. First, in our IP-LC–MS/MS experiments, we do not detect an interaction between Cbp7 and Cytb or Cbp3/6 (Supplementary Table S2). Second, Cbp8 from *S. pombe* and other *Schizosacharomyces* species share some similarity with the PPR RNA binding protein Cbp1. In *S. cerevisiae*, Cbp1 is involved in the stability/processing/translation of the *cytb* mRNA (81,82,31–33). Thus, we hypothesize that Cbp8 could be distantly related to Cbp1, and could bind and stabilize the *cytb* mRNA, this is consistent with the strongly decreased level of *cytb* mRNA when Cbp8 is lacking. Taking all these data into consideration, we would like to propose that the role of Cbp7 and Cbp8 is to channel the *cytb* mRNA onto 'appropriate' ribosomes. Translation on such ribosomes would ensure that synthesis is coupled to assembly, this could happen *via* (i) a specific ribosomal composition, (ii) their location in a sub-region of the mitochondrial membrane devoted to complex III assembly and/or (iii) their preloading with early assembly factors, possibly Cbp3/Cbp6.

Interestingly, the suppressors of *Δcbp7* and *Δcbp8* include a mutated Mrp51 and the overproduction of Mug178, two isoforms of the mitoribosomal protein bS1m. We found that Mug178 and Mrp51 are mutually exclusive ribosomal proteins, strongly suggesting that at least two types of ribosomes coexist in *S. pombe* mitochondria, depending on the bS1m isoform they harbor. In *S. cerevisiae*, bS1m is strategically placed to interact with translational activators, since it is part of the wall of the mRNA exit channel (14,15). This channel serves as a platform for an unresolved density in the SSU structure, proposed to correspond to a heterogenous and dynamic pool of *S. cerevisiae* translational activators, including Cbs2 and Cpb1 which copurify with the mitochondrial ribosome (14). Cbs2 has also been shown to be in close proximity to bS1m through bioID analysis (10).

The mutation Mrp51-E21K that suppresses the *Δcbp7* and *Δcbp8* mutants creates a partly processed new N-terminal cleavage site, most probably downstream of the mutation. This might either partly disable the Mrp51-ribosomes, thus favoring Mug178-ribosomes, or generate an additional new bS1m isoform that could mimic Mug178. The surface of bS1m facing the channel is electropositive and the E21K mutation which introduces a positive charge instead of a negative one could also modify the interaction of Mrp51 with the mRNAs during translation. Since the Mrp51-E21K mutation does not appear deleterious and can suppress *Δmug178* cells, we rather favor the hypothesis of a new 'Mug178-like' functionality in Mrp51-E21K. Thus, globally, the mechanism of suppression by the E21K variant of Mrp51 would have effects similar to overexpressing *mug178*.

As presented in the model from Figure 12, we postulate that Mrp51 and Mug178 in *S. pombe* could preferentially interact with different translational activators, thus mediating a ribosomal translational specificity. Consequently, a change in the ratio of Mug178 to Mrp51, or a muta-

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

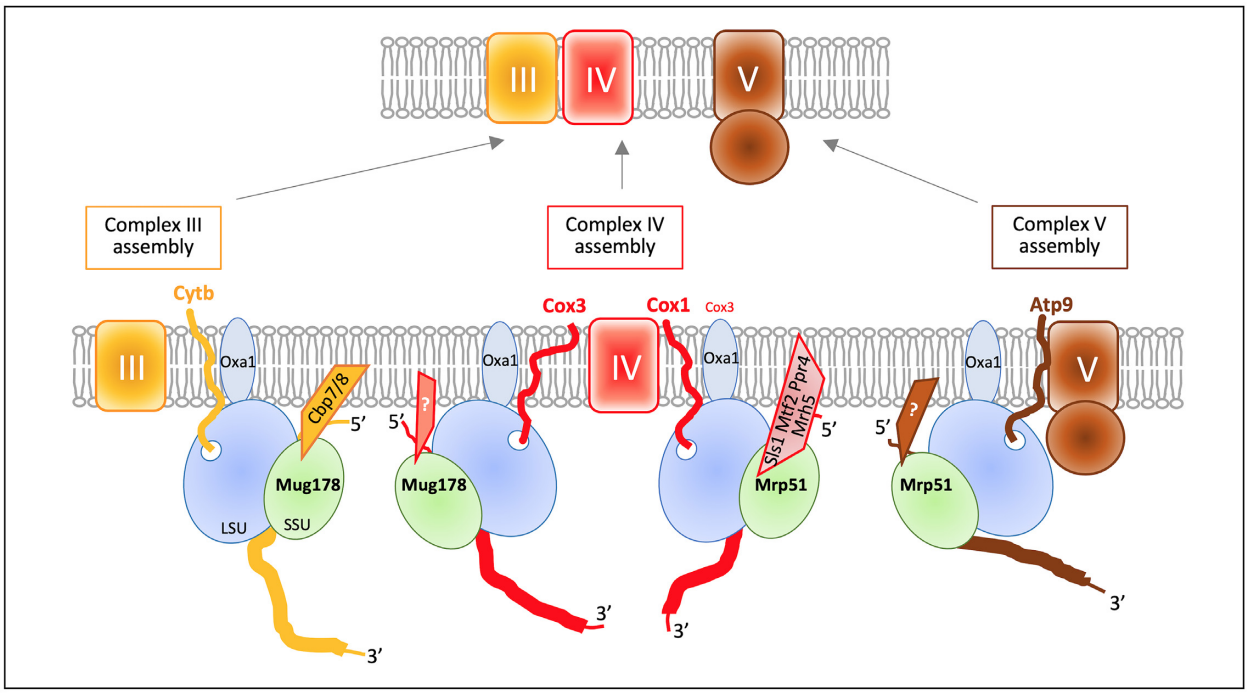


Figure 12. Model of the cooperative interplay between ribosomal isoforms and translational activators to localize and coordinate synthesis and assembly of OXPHOS subunits. Translational activators of *cytb* and *cox1*, Cbp7/8 and Mrh5/Ppr4/Mtf2/Sls1 respectively, could preferentially interact with either Mug178 or Mrp51 ribosomes, the translated mRNA and the membrane, in order to localize Cytb and Cox1 synthesis into the sub-regions optimal for subsequent assembly. Similarly, *cox3* translation initiation would be favored on Mug178-containing SSU. Mrp51 ribosomes are proposed to synthesize most of the other proteins, including a low amount of Cox3 protein, maybe through translation of the bi-cistronic *cox1-cox3* mRNA. Small (SSU) and large (LSU) ribosomal subunits have been indicated.

tion in one of the proteins could modify the stoichiometry of the newly synthesized subunits. Given the phenotype of the *Δmug178* mutants, for which Cytb synthesis and assembly is dysregulated, we would hypothesize that Cbp7/8 and the *cytb* mRNA preferentially interact with Mug178-containing ribosomes. Our direct IP and IP-LC–MS/MS analyses do not reveal a favored interaction of Cbp7 with Mug178. However, one would expect such an interaction to be transient, and thus not necessarily strong enough to co-IP the ribosome, since the complex has to dissociate when translation is engaged, in order to allow protein synthesis to proceed. Following the model, if Cbp7/8 or Mug178 are absent, translation of Cytb would no-longer be restricted to Mug178-ribosomes and would thus initiate erratically on the more abundant Mrp51-ribosomes. Enhanced Cytb synthesis in the *Δmug178* mutant shows that Mrp51-ribosomes can translate the *cytb* mRNA but are probably unable to properly convey the newly made protein to the assembly machinery. It is unlikely that they cannot be loaded with Cbp3/Cbp6 since in *S. cerevisiae* this couple of factors is able to interact with ribosomes at the tunnel exit independently of the translated mRNA 5′UTR (31). We rather think that Mrp51-ribosomes might not be located in the areas of the mitochondrial membrane where assembly of Cytb with the nuclear-encoded subunits of complex III occurs. Indeed, several studies have shown that there is a dynamic sub-compartmentalization of the mitochondrial membrane, allowing different functional specificities, and it is likely that localizing mitochondrial translation plays an important role in building this membrane microarchitecture (83–85,11; for review 12). Finally, the synthesis of the Atp9 subunit of ATP synthase is repeatedly decreased or enhanced in the Mrp51-E21K or *Δmug178* mutants respectively, suggesting that Mrp51 ribosomes could be important for the synthesis of subunits of complex V in addition to complex IV. Such a dual defect in both a respiratory complex and ATP synthase would explain the lethality of Mrp51; as would a defect in the synthesis of the ribosomal protein Rps3 (40,72).

In addition to the effects on Cytb, Cox3 synthesis is strongly reduced when Mug178 is absent. The simplest hypothesis is that Mug178-ribosomes are also used to produce the Cox3 protein. Alternatively, *cox3* translation could depend on Mrp51-ribosomes, but suffer a specific decrease due to the competition with the enhanced translation of *cytb* observed when Mug178-ribosomes are lacking. However, when *Δmug178* is combined with *Δcbp7* or *Δcbp8*, *cytb* translation is abolished without restoring Cox3 synthesis. In addition, Mug178 overproduction also stimulates Cox3 synthesis. Thus Mug178-ribosomes appear to be the most important site for Cox3 production in the wild-type. The observation that Cox3 is made on the same ribosomes as Cytb, but different to Cox1 and Cox2 might suggest that this translational 'separation' is part of a mechanism to coordinate the relative levels of complex III and IV. The residual cytochrome *c* oxidase activity measured in the *Δmug178* mutant could be due to the synthesis of Cox3 from the low abundance *cox1-cox3* precursor RNA (Figure 1) on Mrp51-

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

ribosomes, while the major monocistronic *cox3* mRNA is translated on Mug178-ribosomes.

A second important class of suppressors is constituted by a series of mutations in factors required for *cox1* synthesis: Ppr4, Mtf2, Mrh5. These mutations alone generally have opposite effects on Cox1 and Cytb by lowering and increasing synthesis respectively, and tend to normalize the production of Cytb when associated to *Δcbp7/8* or *Δmug178.* These suppressors highlight the importance of the translational balance between *cox1* and *cytb* for optimal complex and supercomplex assembly. An attractive hypothesis to explain the suppression is that decreasing the rate of *cox1* translation and slowing down complex IV formation would allow illegitimate access of Cbp7/8 to Mrp51-ribosomes and recruit them to areas devoted to *cytb* translation, thus allowing some synthesis of Cytb and its further assembly into complex III.

IP-LC–MS/MS experiments have revealed a complex of four interacting proteins, two of them being previously uncharacterized in *S. pombe*, Sls1 (SPAP8A3.14c) and Mhr5 (SPBC25D12.06). In *S. cerevisiae* Sls1 is an integral membrane protein that has been proposed to act together with Nam1/Mtf2 in channeling the nascent mRNAs to the translation machinery (86), and for example to transfer the *COX1* mRNA to Pet309, since this activator interacts physically with Mtf2 (12). However, BioID analysis of Mtf2 and Sls1 suggests that transcription and translation are rather spatially separated, since no direct connection was found between Mtf2 or Sls1 and the SSU (10). In *S. pombe* we found that both Sls1 and Mtf2 are fully essential only for *cox1* translation, suggesting that they could be more specific than in *S. cerevisiae*, in accordance with their strong interaction with Ppr4 and Mrh5. The DEAD-box helicase Mrh5 is required for Cox1 synthesis and we have shown that it co-precipitates with mitoribosomal proteins. In *S. cerevisiae*, four mitochondrial DExH/D-box helicases are known (for review 87), including Mss116 and Mrh4 which harbor a canonical DEAD motif: (i) Mss116 fulfills multiple roles in mitochondrial intron splicing, transcription under cold stress, *cox1* translation initiation and elongation and ribosome interaction (88 and references therein); (ii) Mrh4 is essential to mitoribosome assembly (89). Our results suggest that Mrh5 is the real homolog of Mss116, despite some differences, maybe linked with the different mitochondrial intron content of *S. pombe* and *S. cerevisiae*. Supporting this hypothesis, *S. cerevisiae* Mss116 physically interacts with Pet309, like Mrh5 with Ppr4, the Pet309 homolog (88).

In conclusion, to our knowledge, our work represents the first example of isoforms of the mitochondrial ribosome that appear to confer a translational specificity, in close cooperation with extra-ribosomal factors required for mRNA-specific translation. In *S. cerevisiae*, no isoforms of mitoribosomal proteins have been described and in plants, eight genes coding organellar uL18 are found, however only uL18m and uL18c have so far been confirmed to be mitochondrial and chloroplastic ribosomal proteins respectively (90). In humans, there are three mitoribosomal proteins related to bS18, however these are not mutually exclusive isoforms like Mrp51 and Mug178. Instead, all three proteins are associated at the same time with the ribosome but at different locations, and correspond to the canonical bS18c and two proteins specific to humans, mS40 and mL66 (91). Interestingly, for the human bS1m, seven smaller computationally mapped isoforms of various sizes (83 to 162 residues) are currently reported in the UniProt database, in addition to the representative 187 aa sequence and their possible roles, if any, is unknown. It is thus tempting to hypothesize that in humans and *S. pombe*, bS1m, that occupies a central position in the early assembly of mitoribosome (92), could contribute to the heterogeneity of the SSU and maybe compensate for the lack of some translational activators found in *S. cerevisiae*. Further work will be needed to elucidate how the interplay of translation and OXPHOS assembly factors functions in time and space to build functional OXPHOS complexes in *S. pombe* and to compare the role played by the different bS1m isoforms in *S. pombe* and also human mitoribosomes.

DATA AVAILABILITY

The Mass Spectrometry data are available at the database Pride of ProteomeXchange under the accession number PXD025400. Supplementary Tables S2, S4 and S5 provide the statistical analysis for the IPs of Cbp7-cMyc versus wt, Mrh5-cMyc versus wt and Ppr4-cMyc versus wt respectively. Raw data for LC–MS/MS analyses of IPs from Mrh5-cMyc, Ppr4-cMyc and wt are provided in Supplementary Table S3.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We are grateful to Thomas D. Fox, Rudolf Schweyen, Inge Kühl and Jean Velours for the generous gift of antibodies, to Bénédicte Barrault for providing some of the plasmids used for gene tagging, to Brigitte Meunier for all the supplies to measure respiratory complex activities and to Isabelle Hatin for help with accessing a Typhoon Phosphorimager. The National BioResource Project (Japan) dispatched the pAL-KS plasmid and pTN-L1 library. This work has benefited from the facilities and expertise of the I2BC High-throughput Sequencing Platform (Next Generation Sequencing Core facility), as well as from the facilities and expertise of the I2BC proteomic platform (Proteomic-Gif, SICaPS) supported by IBiSA, Ile de France Region, Plan Cancer, CNRS and Paris-Sud University. Free software was used on the following servers: TPRpred on the Tübingen MPI toolkit server; I-Tasser on the Zhang-server; T-Coffee and Clustal-W on the EMBL-EBI server; BoxShade on the Expasy server. We thank Johannes Herrmann for a stimulating discussion and Valerie Wood from PomBase for excellent support and reactivity, including gene name reservation and personal communications.

Author contributions: C.J.H. performed sucrose gradients, IPs, genetic and molecular work to construct strains and identification of suppressors, as well as supervising C.S.; S.L.M. constructed numerous deleted and tagged strains, measured cytochrome spectra, extracted mitochondrial

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

proteins and performed western blots. D.C. performed LC–MS/MS experiments. C.S. performed initial strain constructions and *Δcbp7* suppressor search and identification. C.P. extracted and blotted RNA. T.M. measured enzyme activities. G.D. did RNA blot hybridizations and BN-PAGE analyses. N.B. did cytochrome spectra, mitochondria purification, ^{35}S labeling and pulse-chases, IPs and western blots, and supervised S.L.M. N.B., G.D. and C.J.H. wrote the paper. N.B. designed the project and supervised the work.

FUNDING

French National Center for Scientific Research (CNRS); Association Française contre les Myopathies [15680, 16863 to N.B.].
Conflict of interest statement. None declared.

REFERENCES

1. Filipovska,A. and Rackham,O. (2013) Specialization from synthesis: how ribosome diversity can customize protein function. *FEBS Lett.*, **587**, 1189–1197.
2. Sonneveld,S., Verhagen,B.M.P. and Tanenbaum,M.E. (2020) Heterogeneity in mRNA Translation. *Trends Cell Biol.*, **30**, 606–618.
3. Gościńska,K. and Topf,U. (2020) The evolving role of ribosomes in the regulation of protein synthesis. *Acta Biochim. Pol.,* **67**, 465–473.
4. Segev,N. and Gerst,J.E. (2018) Specialized ribosomes and specific ribosomal protein paralogs control translation of mitochondrial proteins. *J. Cell Biol.*, **217**, 117–126.
5. Kong,J., Han,H., Bergalet,J., Bouvrette,L.P.B., Hernández,G., Moon,N.-S., Vali,H., Lécuyer,É. and Lasko,P. (2019) A ribosomal protein S5 isoform is essential for oogenesis and interacts with distinct RNAs in Drosophila melanogaster. *Sci. Rep.*, **9**, 13779.
6. Itoh,Y., Andréll,J., Choi,A., Richter,U., Maiti,P., Best,R.B., Barrientos,A., Battersby,B.J. and Amunts,A. (2021) Mechanism of membrane-tethered mitochondrial protein synthesis. *Science*, **371**, 846–849.
7. Mays,J.-N., Camacho- Villasana,Y., Garcia- Villegas,R., Perez-Martinez,X., Barrientos,A. and Fontanesi,F. (2019) The mitoribosome-specific protein mS38 is preferentially required for synthesis of cytochrome *c* oxidase subunits. *Nucleic Acids. Res*, **47**, 5746–5760.
8. Guedes- Monteiro,R.F., Ferreira- Junior,J.R., Bleicher,L., Nóbrega,F.G., Barrientos,A. and Barros,M.H. (2018) Mitochondrial ribosome bL34 mutants present diminished translation of cytochrome *c* oxidase subunits. *Cell Biol. Int.*, **42**, 630–642.
9. Box,J.M., Kaur,J. and Stuart,R.A. (2017) MrpL35, a mitospecific component of mitoribosomes, plays a key role in cytochrome *c* oxidase assembly. *Mol. Biol. Cell*, **28**, 3489–3499.
10. Singh,A.P., Salvatori,R., Aftab,W., Aufschnaiter,A., Carlström,A., Forne,I., Imhof,A. and Ott,M. (2020) Molecular connectivity of mitochondrial gene expression and OXPHOS biogenesis. *Mol. Cell*, **79**, 1051–1065.
11. Zorkau,M., Albus,C.A., Berlinguer- Palmini,R., Chrzanowska-Lightowlers,Z.M.A. and Lightowlers,R.N. (2021) High-resolution imaging reveals compartmentalization of mitochondrial protein synthesis in cultured human cells. *Proc. Natl Acad. Sci. U.S.A*, **118**, e2008778118.
12. Fox,T.D. (2012) Mitochondrial protein synthesis, import, and assembly. *Genetics*, **192**, 1203–1234.
13. Herrmann,J.M., Woellhaf,M.W. and Bonnefoy,N. (2013) Control of protein synthesis in yeast mitochondria: the concept of translational activators. *Biochim. Biophys. Acta*, **1833**, 286–294.
14. Desai,N., Brown,A., Amunts,A. and Ramakrishnan,V. (2017) The structure of the yeast mitochondrial ribosome. *Science*, **355**, 528–531.
15. Itoh,Y., Naschberger,A., Mortezaei,N., Herrmann,J.M. and Amunts,A. (2020) Analysis of translating mitoribosome reveals functional characteristics of translation in mitochondria of fungi. *Nat. Commun*, **11**, 5187.
16. Perez- Martinez,X., Butler,C.A., Shingu- Vazquez,M. and Fox,T.D. (2009) Dual functions of Mss51 couple synthesis of Cox1 to assembly of cytochrome *c* oxidase in *Saccharomyces cerevisiae* mitochondria. *MBoC*, **20**, 4371–4380.
17. Fontanesi,F. (2013) Mechanisms of mitochondrial translational regulation. *IUBMB Life*, **65**, 397–408.
18. Franco,L.V.R., Su,C.H. and Tzagoloff,A. (2020) Modular assembly of yeast mitochondrial ATP synthase and cytochrome oxidase. *Biol. Chem.*, **401**, 835–853.
19. Seshadri,S.R., Banarjee,C., Barros,M.H. and Fontanesi,F. (2020) The translational activator Sov1 coordinates mitochondrial gene expression with mitoribosome biogenesis. *Nucleic Acids Res.*, **48**, 6759–6774.
20. Atkinson,G.C., Kuzmenko,A., Kamenski,P., Vysokikh,M.Y., Lakunina,V., Tankov,S., Smirnova,E., Soosaar,A., Tenson,T. and Hauryliuk,V. (2012) Evolutionary and genetic analyses of mitochondrial translation initiation factors identify the missing mitochondrial IF3 in *S. cerevisiae*. *Nucleic Acids Res.*, **40**, 6122–6134.
21. Temperley,R.J., Wydro,M., Lightowlers,R.N. and Chrzanowska-Lightowlers,Z.M. (2010) Human mitochondrial mRNAs–like members of all families, similar but different. *Biochim. Biophys. Acta*, **1797**, 1081–1085.
22. Schäfer,B. (2005) RNA maturation in mitochondria of *S. cerevisiae* and *S. pombe*. *Gene*, **354**, 80–85.
23. Kühl,I., Dujeancourt,L., Gaisne,M., Herbert,C.J. and Bonnefoy,N. (2011) A genome wide study in fission yeast reveals nine PPR proteins that regulate mitochondrial gene expression. *Nucleic Acids Res.*, **39**, 8029–8041.
24. Tavares- Carreón,F., Camacho- Villasana,Y., Zamudio- Ochoa,A., Shingú- Vázquez,M., Torres- Larios,A. and Pérez- Martínez,X. (2008) The pentatricopeptide repeats present in Pet309 are necessary for translation but not for stability of the mitochondrial *COX1* mRNA in yeast. *J Biol. Chem.*, **283**, 1472–1479.
25. Zamudio- Ochoa,A., Camacho- Villasana,Y., García- Guerrero,A.E. and Pérez- Martínez,X. (2014) The Pet309 pentatricopeptide repeat motifs mediate efficient binding to the mitochondrial *COX1* transcript in yeast. *RNA Biol*., **11**, 953–967.
26. Aibara,S., Singh,V., Modelska,A. and Amunts,A. (2020) Structural basis of mitochondrial translation. *Elife*, **9**, e58362.
27. Kühl,I., Fox,T.D. and Bonnefoy,N. (2012) *Schizosaccharomyces pombe* homologs of the *Saccharomyces cerevisiae* mitochondrial proteins Cbp6 and Mss51 function at a post-translational step of respiratory complex biogenesis. *Mitochondrion*, **12**, 381–390.
28. Rovira Gonzalez,Y.I., Moyer,A.L., LeTexier,N.J., Bratti,A.D., Feng,S., Peña,V., Sun,C., Pulcastro,H., Liu,T., Iyer,S.R. *et al.* (2021) *Mss51* deletion increases endurance and ameliorates histopathology in the *mdx* mouse model of Duchenne muscular dystrophy. *FASEB J.*, **35**, e21276.
29. Weraarpachai,W., Antonicka,H., Sasarman,F., Seeger,J., Schrank,B., Kolesar,J.E., Lochmüller,H., Chevrette,M., Kaufman,B.A., Horvath,R. *et al.* (2009) Mutation in *TACO1*, encoding a translational activator of COXI, results in cytochrome *c* oxidase deficiency and late-onset Leigh syndrome. *Nat. Genet.*, **41**, 833–837.
30. García- Guerrero,A.E., Camacho- Villasana,Y., Zamudio- Ochoa,A., Winge,D.R. and Pérez- Martínez,X. (2018) Cbp3 and Cbp6 are dispensable for synthesis regulation of cytochrome *b* in yeast mitochondria. *J. Biol. Chem.*, **293**, 5585–5599.
31. Salvatori,R., Kehrein,K., Singh,A.P., Aftab,W., Möller- Hergt,B.V., Forne,I., Imhof,A. and Ott,M. (2020) Molecular wiring of a mitochondrial translational feedback loop. *Mol. Cell*, **77**, 887–900.
32. Mittelmeier,T.M. and Dieckmann,C.L. (1993) *In vivo* analysis of sequences necessary for CBP1-dependent accumulation of cytochrome *b* transcripts in yeast mitochondria. *Mol. Cell. Biol.*, **13**, 4203–4213.
33. Ellis,T.P., Schonauer,M.S. and Dieckmann,C.L. (2005) *CBT1* interacts genetically with *CBP1* and the mitochondrially encoded cytochrome *b* gene and is required to stabilize the mature cytochrome *b* mRNA of *Saccharomyces cerevisiae*. *Genetics*, **171**, 949–957.
34. Rödel,G. (1997) Translational activator proteins required for cytochrome *b* synthesis in *Saccharomyces cerevisiae*. *Curr. Genet.*, **31**, 375–379.
35. Wu,D., Zhu,G., Zhang,Y., Wu,Y., Zhang,C., Shi,J., Zhu,X. and Yuan,X. (2021) Expression, purification, crystallization and preliminary X-ray crystallographic studies of a mitochondrial membrane-associated protein Cbs2 from *Saccharomyces cerevisiae*. *Peer J.*, **9**, e10901.

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

36. Tucker,E.J., Wanschers,B.F.J., Szklarczyk,R., Mountford,H.S., Wijeyeratne,X.W., van den Brand,M.A.M., Leenders,A.M., Rodenburg,R.J., Reljić,B., Compton,A.G. *et al.* (2013) Mutations in the UQCC1-interacting protein, UQCC2, cause human complex III deficiency associated with perturbed cytochrome *b* protein expression. *PLoS Genet.*, **9**, e1004034.
37. Schäfer,B. (2003) Genetic conservation versus variability in mitochondria: the architecture of the mitochondrial genome in the *petite*-negative yeast *Schizosaccharomyces pombe*. *Curr. Genet.*, **43**, 311–326.
38. Schäfer,B., Merlos- Lange,A.M., Anderl,C., Welser,F., Zimmer,M. and Wolf,K. (1991) The mitochondrial genome of fission yeast: inability of all introns to splice autocatalytically, and construction and characterization of an intronless genome. *Mol. Gen. Genet.*, **225**, 158–167.
39. Bonnefoy,N., Kermorgant,M., Dujardin,G. and Brivet- Chevillotte,P. (1996) Cloning by functional complementation, and inactivation, of the *Schizosaccharomyces pombe* homologue of the *Saccharomyces cerevisiae* gene *ABC1*. *Molec. Gen. Genet.*, **251**, 204–210.
40. Bonnefoy,N., Kermorgant,M., Groudinsky,O. and Dujardin,G. (2000) The respiratory gene *OXA1* has two fission yeast orthologues which together encode a function essential for cellular viability. *Mol. Microbiol.*, **35**, 1135–1145.
41. Chen,D.C., Yang,B.C. and Kuo,T.T. (1992) One-step transformation of yeast in stationary phase. *Curr. Genet.*, **21**, 83–84.
42. Dujeancourt,L., Richter,R., Chrzanowska- Lightowlers,Z.M., Bonnefoy,N. and Herbert,C.J. (2013) Interactions between peptidyl tRNA hydrolase homologs and the ribosomal release factor Mrf1 in *S. pombe* mitochondria. *Mitochondrion*, **13**, 871–880.
43. Tanaka,K., Yonekawa,T., Kawasaki,Y., Kai,M., Furuya,K., Iwasaki,M., Murakami,H., Yanagida,M. and Okayama,H. (2000) Fission yeast Eso1p is required for establishing sister chromatid cohesion during S phase. *Mol. Cell Biol.*, **20**, 3459–3469.
44. Nakamura,T., Nakamura- Kubo,M., Hirata,A. and Shimoda,C. (2001) The *Schizosaccharomyces pombe spo3+* gene is required for assembly of the forespore membrane and genetically interacts with *psy1(+)*-encoding syntaxin-like protein. *Mol. Biol. Cell*, **12**, 3955–3972.
45. Wach,A., Brachat,A., Pöhlmann,R. and Philippsen,P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast*, **10**, 1793–1808.
46. Chiron,S., Gaisne,M., Guillou,E., Belenguer,P., Clark- Walker,G.D. and Bonnefoy,N. (2007) Studying mitochondria in an attractive model: *Schizosaccharomyces pombe*. *Methods Mol. Biol.*, **372**, 91–105.
47. Haffter,P. and Fox,T.D. (1992) Nuclear mutations in the *petite*-negative yeast *Schizosaccharomyces pombe* allow growth of cells lacking mitochondrial DNA. *Genetics*, **131**, 255–260.
48. Hoffman,C.S. and Winston,F. (1987) A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene*, **57**, 267–272.
49. Goldstein,A.L. and McCusker,J.H. (1999) Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast*, **15**, 1541–1553.
50. Bähler,J., Wu,J.Q., Longtine,M.S., Shah,N.G., McKenzie,A., Steever,A.B., Wach,A., Philippsen,P. and Pringle,J.R. (1998) Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast*, **14**, 943–951.
51. Funakoshi,M. and Hochstrasser,M. (2009) Small epitope-linker modules for PCR-based C-terminal tagging in *Saccharomyces cerevisiae*. *Yeast*, **26**, 185–192.
52. Song,Z., Iorga,B.I., Mounkoro,P., Fisher,N. and Meunier,B. (2018) The antimalarial compound ELQ-400 is an unusual inhibitor of the *bc1* complex, targeting both Q_o and Q_i sites. *FEBS Lett.*, **592**, 1346–1356.
53. Dodia,R., Meunier,B., Kay,C. and Rich,P.R. (2014) Comparisons of subunit 5A and 5B isoenzymes of yeast cytochrome *c* oxidase, *Biochem. J.*, **464**, 335–342.
54. Kehrein,K., Schilling,R., Möller- Hergt,B.V., Wurm,C.A., Jakobs,S., Lamkemeyer,T., Langer,T. and Ott,M. (2015) Organization of mitochondrial gene expression in two distinct ribosome-containing assemblies. *Cell Rep.*, **10**, 843–853.
55. Steele,D.F., Butler,C.A. and Fox,T.D. (1996) 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.*, **93**, 5253–5257.
56. Gaisne,M. and Bonnefoy,N. (2006) The *COX18* gene, involved in mitochondrial biogenesis, is functionally conserved and tightly regulated in humans and fission yeast. *FEMS Yeast Res.*, **6**, 869–882.
57. Kevil,C.G., Walsh,L., Laroux,F.S., Kalogeris,T., Grisham,M.B. and Alexander,J.S. (1997) An improved, rapid northern protocol. *Biochem. Biophys. Res. Commun.*, **238**, 277–279.
58. Gouget,K., Verde,F. and Barrientos,A. (2008) *In vivo* labeling and analysis of mitochondrial translation products in budding and in fission yeasts. *Methods Mol. Biol.*, **457**, 113–124.
59. Claisse,M.L., Péré- Aubert,G.A., Clavilier,L.P. and Slonimski,P.P. (1970) Method for the determination of cytochrome concentrations in whole yeast cells. *Eur. J. Biochem.*, **16**, 430–438.
60. Szabó,Á., Papin,C., Cornu,D., Chélot,E., Lipinszki,Z., Udvardy,A., Redeker,V., Mayor,U. and Rouyer,F. (2018) Ubiquitylation dynamics of the clock cell proteome and TIMELESS during a circadian cycle. *Cell Rep.*, **23**, 2273–2282.
61. Pham,T.V., Piersma,S.R., Warmoes,M. and Jimenez,C.R. (2010) On the beta-binomial model for analysis of spectral count data in label-free tandem mass spectrometry-based proteomics. *Bioinformatics*, **26**, 363–369.
62. Rödel,G. (1986) Two yeast nuclear genes, *CBS1* and *CBS2*, are required for translation of mitochondrial transcripts bearing the 5′-untranslated *COB* leader. *Curr. Genet.*, **11**, 41–45.
63. Muroff,I. and Tzagoloff,A. (1990) *CBP7* codes for a co-factor required in conjunction with a mitochondrial maturase for splicing of its cognate intervening sequence. *EMBO J.*, **9**, 2765–2773.
64. Tzschoppe,K., Krause- Buchholz,U. and Rödel,G. (1999) Mutational analysis of yeast mitochondrial translational activator Cbs2p and of YHR063Cp, a protein with similarity to Cbs2p. *Curr. Genet.*, **36**, 201–207.
65. Vasquez- Calvo,C., Suhm,T., Büttner,S. and Ott,M. (2020) The basic machineries for mitochondrial protein quality control. *Mitochondrion*, **50**, 121–131.
66. Green- Willms,N.S., Fox,T.D. and Costanzo,M.C. (1998) Functional interactions between yeast mitochondrial ribosomes and mRNA 5′ untranslated leaders. *Mol. Cell. Biol.*, **18**, 1826–1834.
67. Navarro,F.J., Chakravarty,P. and Nurse,P. (2017) Phosphorylation of the RNA-binding protein Zfs1 modulates sexual differentiation in fission yeast. *J. Cell Sci.*, **130**, 4144–4154.
68. Saurer,M., Ramrath,D.J.F., Niemann,M., Calderaro,S., Prange,C., Mattei,S., Scaiola,A., Leitner,A., Bieri,P., Horn,E.K. *et al.* (2019) Mitoribosomal small subunit biogenesis in trypanosomes involves an extensive assembly machinery. *Science*, **365**, 1144–1149.
69. Tobiasson,V. and Amunts,A. (2020) Ciliate mitoribosome illuminates evolutionary steps of mitochondrial translation. *Elife*, **9**, e59264.
70. Pulman,J., Ruzzenente,B., Bianchi,L., Rio,M., Boddaert,N., Munnich,A., Rötig,A. and Metodiev,M.D. (2019) Mutations in the *MRPS28* gene encoding the small mitoribosomal subunit protein bS1m in a patient with intrauterine growth retardation, craniofacial dysmorphism and multisystemic involvement. *Hum. Mol. Genet.*, **28**, 1445–1462.
71. Chiron,S., Suleau,A. and Bonnefoy,N. (2005) 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*, **169**, 1891–1901.
72. Chen,X.J. and Clark- Walker,G.D. (2000) The petite mutation in yeasts: 50 years on. *Int. Rev. Cytol.*, **194**, 197–238.
73. Liu,J., Li,Y., Chen,J., Wang,Y., Zou,M., Su,R. and Huang,Y. (2018) The fission yeast *Schizosaccharomyces pombe* Mtf2 is required for mitochondrial *cox1* gene expression. *Microbiology*, **164**, 400–409.
74. Karpenahalli,M.R., Lupas,A.N. and Söding,J. (2007) TPRpred: a tool for prediction of TPR-, PPR- and SEL1-like repeats from protein sequences. *BMC Bioinformatics*, **8**, 2.
75. Zimmermann,L., Stephens,A., Nam,S.-Z., Rau,D., Kübler,J., Lozajic,M., Gabler,F., Söding,J., Lupas,A.N. and Alva,V. (2018) A completely reimplemented MPI bioinformatics toolkit with a new HHpred server at its core. *J. Mol. Biol.*, **430**, 2237–2243.
76. Halls,C., Mohr,S., Del Campo,M., Yang,Q., Jankowsky,E. and Lambowitz,A.M. (2007) Involvement of DEAD-box proteins in group I and group II intron splicing. Biochemical characterization of Mss116p, ATP hydrolysis-dependent and -independent mechanisms, and general RNA chaperone activity. *J. Mol. Biol.*, **365**, 835–855.

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021

77. Fedorova,O. and Pyle,A.M. (2012) The brace for a growing scaffold: Mss116 protein promotes RNA folding by stabilizing an early assembly intermediate. *J. Mol. Biol.*, **422**, 347–365.
78. Markov,D.A., Wojtas,I.D., Tessitore,K., Henderson,S. and McAllister,W.T. (2014) Yeast DEAD box protein Mss116p is a transcription elongation factor that modulates the activity of mitochondrial RNA polymerase. *Mol. Cell Biol.*, **34**, 2360–2369.
79. Rouillard,J.M., Dufour,M.E., Theunissen,B., Mandart,E., Dujardin,G. and Lacroute,F. (1996) *SLS1*, a new *Saccharomyces cerevisiae* gene involved in mitochondrial metabolism, isolated as a synthetic lethal in association with an *SSM4* deletion. *Mol. Gen. Genet.*, **252**, 700–708.
80. Bryan,A.C., Rodeheffer,M.S., Wearn,C.M. and Shadel,G.S. (2002) Sls1p is a membrane-bound regulator of transcription-coupled processes involved in *Saccharomyces cerevisiae* mitochondrial gene expression. *Genetics*, **160**, 75–82.
81. Islas- Osuna,M.A., Ellis,T.P., Mittelmeier,T.M. and Dieckmann,C.L. (2003) Suppressor mutations define two regions in the Cbp1 protein important for mitochondrial cytochrome *b* mRNA stability in *Saccharomyces cerevisiae*. *Curr. Genet.*, **43**, 327–336.
82. Lipinski,K.A., Puchta,O., Surendranath,V., Kudla,M. and Golik,P. (2011) Revisiting the yeast PPR proteins—application of an iterative hidden Markov model algorithm reveals new members of the rapidly evolving family. *Mol. Biol. Evol.*, **28**, 2935–2948.
83. Sanchirico,M.E., Fox,T.D. and Mason,T.L. (1998) Accumulation of mitochondrially synthesized *Saccharomyces cerevisiae* Cox2p and Cox3p depends on targeting information in untranslated portions of their mRNAs. *EMBO J.*, **17**, 5796–5804.
84. Vogel,F., Bornhövd,C., Neupert,W. and Reichert,A.S. (2006) Dynamic subcompartmentalization of the mitochondrial inner membrane. *J. Cell Biol.*, **175**, 237–247.
85. Fiori,A., Perez- Martinez,X. and Fox,T.D. (2005) Overexpression of the *COX2* translational activator, Pet111p, prevents translation of *COX1* mRNA and cytochrome *c* oxidase assembly in mitochondria of *Saccharomyces cerevisiae*. *Mol. Microbiol.*, **56**, 1689–1704.
86. Bonawitz,N.D., Clayton,D.A. and Shadel,G.S. (2006) Initiation and beyond: multiple functions of the human mitochondrial transcription machinery. *Mol. Cell*, **24**, 813–825.
87. Szczesny,R.J., Wojcik,M.A., Borowski,L.S., Szewczyk,M.J., Skrok,M.M., Golik,P. and Stepien,P.P. (2013) Yeast and human mitochondrial helicases. *Biochim. Biophys. Acta (BBA) - Gene Regul. Mech.*, **1829**, 842–853.
88. De Silva,D., Poliquin,S., Zeng,R., Zamudio- Ochoa,A., Marrero,N., Perez- Martinez,X., Fontanesi,F. and Barrientos,A. (2017) The DEAD-box helicase Mss116 plays distinct roles in mitochondrial ribogenesis and mRNA-specific translation. *Nucleic Acids Res.*, **45**, 6628–6643.
89. De Silva,D., Fontanesi,F. and Barrientos,A. (2013) The DEAD box protein Mrh4 functions in the assembly of the mitochondrial large ribosomal subunit. *Cell. Metab.*, **18**, 712–725.
90. Wang,C., Fourdin,R., Quadrado,M., Dargel- Graffin,C., Tolleter,D., Macherel,D. and Mireau,H. (2020) Rerouting of ribosomal proteins into splicing in plant organelles. *Proc. Natl Acad. Sci. U.S.A.*, **117**, 29979–29987.
91. Greber,B.J. and Ban,N. (2016) Structure and function of the mitochondrial ribosome. *Annu. Rev. Biochem.*, **85**, 103–132.
92. Ferrari,A., Del' Olio,S. and Barrientos,A. (2020) The diseased mitoribosome. *FEBS Lett.*, **595**, 1025–1061.

Downloaded from https://academic.oup.com/nar/article/49/19/11145/6389634 by CNRS user on 04 November 2021