Published in final edited form as:

Curr Genet. 2009 August ; 55(4): 475–484. doi:10.1007/s00294-009-0260-z.

Ccm1p/Ygr150cp, a pentatricopeptide repeat protein, is essential to remove the fourth intron of both *COB* and *COX1* pre-mRNAs in *Saccharomyces cerevisiae*

J. Ignacio Moreno^{*}, Kimberley S. Buie, Rhonda E. Price, and Marta A. Piva^{*}

Department of Biological Sciences, Alcorn State University, 1000 ASU Drive # 870, Alcorn State, MS 39096, U.S.A

Abstract

YGR150C gene product (Ygr150cp) is one of the three mitochondrially located *Saccharomyces cerevisiae* proteins with pentatricopeptide repeat (PPR) motifs. Ygr150cp is essential for mitochondrial functionality but its molecular targets are still unknown. This study was undertaken to define the role of Ygr150cp in mitochondria biogenesis. Repression of Ygr150cp expression in complemented mutants prevented their use of glycerol or lactate, but allowed limited growth on ethanol-containing medium. RNA hybridization studies showed that $\Delta ygr150c$ meiotic segregants produced *COB* and *COX1* transcripts but failed to process them into the mature forms. Detailed RT-PCR assays revealed that $\Delta ygr150c$ specifically failed to remove the fourth intron of both *COB* and *COX1* pre-mRNAs while all other group I introns were excised. Expression of Ygr150cp mutants without any of the PPR motifs did not complement the growth phenotype. Accordingly, we designate *YGR150C* as *CCM1* (*COB* and *COX1* mRNA Maturation). This report provides the first evidence of PPR protein involvement in the specific removal of Group I introns in mitochondria of *Saccharomyces cerevisiae*.

Introduction

Mitochondria proliferate by incorporating components into pre-existent structures such as mitochondrial membranes and mitochondrial DNA (mtDNA) that serve as templates for growth. Continuity is assured by distributing the organelle to daughter cells before every cell division via the cytoskeleton (Yaffe 1999). During the past few decades, many of the proteins required for respiratory growth and establishment and maintenance of mitochondrial structures have been identified in yeast (Contamine and Picard 2000; Tzagoloff and Dickmann 1990). Mitochondria possess specific machinery to replicate and repair their genome, transcribe ribosomal, transfer and messenger RNAs, and translate proteins encoded by the mitochondrial genome. However, the majority of the proteins required for mitochondrial biogenesis is encoded in the nucleus, synthesized in the cytoplasm, and imported into mitochondria. Complexes I, II, III, and IV of the electron transport system are essential to aerobic respiration. *S. cerevisiae* does not possess Complex I but rather an alternative NADH dehydrogenase (Marres et al. 1991). The mitochondrial genome of *S. cerevisiae* encodes for cytochrome b (Cobp), which is one of the subunits of Complex III, and for three subunits of cytochrome c oxidase or Complex IV (Cox1p, Cox2p, and Cox3p). In addition, ATP synthase subunits 6, 8, and 9, and a ribosomal protein (Var1p) are encoded in the mtDNA (Foury et al. 1998). Depending on the strain, the yeast mitochondrial genome may contain up to thirteen introns (Luban et al. 2005). The 21S rRNA (*21S_rRNA*), cytochrome b (*COB*), and subunit I of

^{*}Corresponding authors E-mail addresses mpiva@alcorn.edu, jmoreno@alcorn.edu Telephone 1-601-877-6239, Fax 1-601-877-2328.

cytochrome c oxidase (*COX1*) genes contain one, five, and seven introns, respectively. Some of these introns are translated independently or in frame with their upstream exons to produce maturases, reverse transcriptases or site-specific endonucleases. In particular, the first four exons of the *COB* gene and 1160 bp of intron 4 (bI4), code for the bI4 maturase, which along with additional proteins like the nuclear-encoded Nam2p, are required to promote the removal of bI4 (Rho and Martinis 2000) as well as *COX1* intron 4 (aI4) (Labouesse et al. 1984; Banroques et al. 1987). Mutations that block these processes cause a secondary loss of the wild type mitochondrial (ρ^+) genome; the more stringent the block, the faster and more complete is the disappearance of the ρ^+ genome (Myers et al. 1985).

Pentatricopeptide repeat (PPR) motifs comprise a set of three related degenerated motifs of 31-36 amino acids that form two theoretical anti-parallel α -helices (Small and Peeters 2000). In recent years, it has become more accepted that PPR motifs bind RNA and act collectively to achieve high affinity and sequence specificity (Delannoy et al. 2007). For instance in *Arabidopsis thaliana*, the mitochondrial PPR protein encoded by the nuclear gene *OTP43* is absolutely required to remove the first intron of *NAD1* pre-mRNA (de Longevialle et al. 2007). Pet309p, a *S. cerevisiae* PPR protein, participates in the translation of *COX1* mRNA (Manthey and McEwen 1995; Tavares-Carreón et al. 2008) but no *in vivo* evidence of its functional association with mitochondrial transcripts has been reported so far. Our target, the *YGR150C* gene product (Ygr150cp) localizes to mitochondria (Huh et al. 2003; Reinders et al. 2006) and interacts *in vitro* with the mitochondrial ribosomal protein of the small subunit, Mrps5 (Gavin et al. 2002). However, no *in vivo* experiments that elucidate the role of this protein have been published yet. The present communication reports for the first time that Ygr150cp is specifically involved in the removal of bI4 and aI4, a function that requires the presence of both PPR motifs, and provides novel evidence of PPR motif involvement in the removal of Group I introns in *S. cerevisiae*. Accordingly, we have designated this gene as *CCM1* (*COB* and *COX1* mRNA Maturation).

Materials and Methods

Media and strains

All reagents were from Sigma (St. Louis, MO) unless indicated otherwise. YEP contained 1 % yeast extract (Becton, Dickinson and Co., Sparks, MD), 2 % peptone (Becton, Dickinson and Co.) YEPD, YEPGal, YEPE, and YEPG included YEP plus 2% glucose, galactose or ethanol, and 3 % glycerol, respectively. Solid media was prepared by adding 2 % agar to the components listed above. The antibiotic G418 was added to YEPD or YEPG to a final concentration of 200 μ g/mL when required. Synthetic defined medium without uracil (SD) was prepared by mixing yeast nitrogen base and uracil-free supplement medium according to the manufacturer's instructions (Sunrise Science, San Diego, CA). The pH was adjusted to 5.8 by adding 30 g of succinic acid and 50 g of NaOH per liter of SD. Glucose (SDD) or galactose (SDGal) were added to SD to a final concentration of 2 %. Wild-type BY4741 (*MATa ura3 Δ 0 leu2 Δ 0 his3 Δ 1 met15 Δ 0*) was a generous gift from Dr. Dennis R. Winge (Departments of Medicine and Biochemistry, University of Utah Health Sciences Center, Salt Lake City, UT, USA). *Δ ccm1* (*MATa ura3 Δ 0 leu2 Δ 0 his3 Δ 1 lys Δ 0 ccm1 Δ 0::kanMX*) was purchased from Invitrogen (Carlsbad, CA). The presence of group I introns in the wild type and mutant strains was confirmed by end-point PCR using their respective mtDNAs as templates. The heterozygous diploid (2nB) was obtained by crossing BY4741 with *Δ ccm1*. 2nB was selected and maintained in YEPG supplemented with G418. The homozygous diploid *Δ ccm1* (*MATa/ α ura3 Δ 0/ura3 Δ 0 leu2 Δ 0/leu2 Δ 0 his3 Δ 1/his3 Δ 1 LYS2/lys2 Δ 0 met15 Δ 0/MET15 ccm1 Δ 0::kanMX / ccm1 Δ 0::kanMX*) was from Invitrogen.

Yeast methods, Ccm1p and antibodies production

Yeast strains were cultured, mated and sporulated as previously indicated (Sherman 2002). Tetrads were treated with Zymolyase 100 T during 2-3 min (Seikagaku, Tokyo, Japan) and dissected with the MSM series 300 system (Singer Instruments, Somerset, UK) on either YEPGal or YEPG plates. All growth studies were conducted at 30 °C and when liquid media were used, flasks and tubes were shaken at 300 rpm in Innova 4230 incubators (New Brunswick Scientific, Edison, NJ). Ccm1p fused to the two synthetic IgG binding domains (ZZ) was purified as previously indicated (Moreno 1996). Using this protein, antiserum against Ccm1p was produced as described by Moreno et al. (1994). Poly his-tagged Ccm1p was produced in *Escherichia coli* Rossetta Blue (Novagen, Madison, WI) by the expression vector pET28b. The 8 M urea-based extraction procedure and Ni-NTA affinity purification were carried out following the supplier's recommendations (Qiagen, Valencia, CA). Antibodies were affinity-purified using this poly his-tagged protein as previously described (Moreno et al. 1994).

DNA manipulation, yeast transformation, and immunoblot analysis

All restriction and modification enzymes were used following the supplier's recommendations (Promega, Madison, WI). pCCM1-ZZ, a 2 µ-based vector that expresses the CCM1 open reading frame (ORF) fused to an affinity tag (attB2, polyhistidine tag, HA epitope, Protease 3C recognition site, and ZZ IgG binding domains) under the control of the *GAL1* promoter - inducible by galactose and repressible by glucose- was purchased from Open Biosystems (Huntsville, AL). pCCM1, the vector that expresses the authentic protein was prepared as follows: pCCM1-ZZ was digested with *SacII* and *KpnI* to yield a ~3.5 Kb (*GAL1* promoter and full length CCM1 ORF) and a ~6.0 Kb (rest of the vector) DNA fragments, which were gel-purified. The ~ 3.5 Kb DNA fragment was subsequently digested with *NcoI*. It yielded a ~3.0 Kb DNA fragment that spans the *GAL1* promoter and a major part of the 5' CCM1 ORF, which was gel-purified again. The minor 3' moiety was amplified by PCR using pCCM1-ZZ as template, 5'-TTTGCCCATCTAATGGTTGA-3' as forward primer and 5'-AGGAGGTACCGCGGCCGCTACATGTACATGTTAAGTTCTTGTT-3' as reverse primer, which anneals to the last 15 nucleotides of the CCM1 ORF, adds a stop signal (TAG) followed by a *KpnI* site. The 250 bp PCR- amplified DNA fragment was digested with *NcoI* and *KpnI*. The resultant 150 bp (3' moiety), ~3.0 Kb (*GAL1* and 5' moiety) and ~6.0 Kb (the rest of the shuttle vector) DNA fragments were ligated, thus generating pCCM1. This construct was confirmed by DNA sequencing. pCCM1Δ, the non-expressing vector was constructed by digestion of pCCM1-ZZ with *SacI*. This reaction excised a 1.8 Kb DNA fragment that spans the *GAL1* promoter and 1.5 Kb of the CCM1 ORF. The rest of the vector (a ~7.5 Kb DNA fragment) was gel-purified and circularized. The *CEN6/ARSH4*-based shuttle vector pRS316 (ATCC, Manassas, VA) was used as the low-copy counterpart. Two *LguI* (Fermentas, Glen Burnie, MD) sites in pCCM1 flank a 5000-bp DNA fragment (f5000) that contains the complete CCM1 ORF expression cassette plus ~500 bp upstream and ~1000 bp downstream of it. DNA fragments obtained by digesting pCCM1 with *LguI* were separated by electrophoresis in 0.5% agarose and f5000 was purified from the gel. The 5' protruding ends of f5000 were filled-in with Klenow's DNA polymerase I large fragment and ligated to the *SmaI* site of pRS316. This low-copy vector expressing Cmm1p was called pCMM1LC. PPR motifs were deleted from the CCM1 ORF as follows: a 500 bp region flanked by the *HindIII* and *SacI* restriction sites contains two in-tandem PPR motifs (PPR1 and PPR2). *HindIII*-*SacI* DNA fragments lacking PPR were synthesized and sequenced (DNA2.0 Inc, Menlo Park, CA). These fragments were swapped with the wild type CCM1 ORF in pCCM1 at the corresponding *HindIII* and *SacI* restriction sites. Strains were transformed using the MicroPulser electroporation apparatus (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Recombinants were selected on SDD plates. Yeast transformation was confirmed by plasmid content and restriction analysis. Western blot analysis was carried out following standard procedures (Sambrook and Russell 2001).

Total RNA extraction

G418^R or G418^S colonies of about 30×10^6 cells were collected from the YEPGal tetrad dissection plates. They were used to inoculate YEPGal with or without G418 and were further incubated for 16 hours. DNA-free total RNA was isolated from these cultures with the RNeasy kit (Qiagen), following the manufacturer's instructions. RNA concentration was determined spectrophotometrically at 260 nm.

RNA hybridization, reverse transcription and end-point PCR analyses

Between two and three μg of total RNA were separated on a 1.2 % agarose-formaldehyde gel, transferred to a Biotodyne Nylon membrane (Pierce, Rockford, IL), and stained with methylene blue (Sigma). Membrane blocking, hybridization and transcript detection were performed with the North2South Chemiluminescent Hybridization and Detection kit (Pierce). Signals were visualized and photographed with a gel documentation system (FluorChem SP, Alpha Innotech Corporation, San Leandro, CA). Reverse-transcription and end-point polymerase chain reactions (PCR) were performed using standard methods (Sambrook and Russell 2001). PCR primers are listed in Table 1. The 290 bp-*COB* biotinylated probe spans 229 nucleotides (nt) of *COB* exon (E) 4, all of E5, and nine nt of E6. The 146 bp-*COX1* biotinylated probe is contained within E4 (Taylor et al. 2005). Both probes were obtained by PCR, using wild-type cDNA as template, and labeled with the North2South Biotin Random Prime labeling kit (Pierce).

Quantitative PCR

The relative amount of mRNA or mtDNA was quantified by real-time PCR as indicated by Taylor et al. (2005), with minor modifications. Nuclear DNA (*ACT1*) and mtDNA (*COX1*) quantitative PCR were performed in individual tubes for each sample within the linear range of amplification in a Smart Cycler II thermal cycler (Cepheid, Sunnyvale, CA). The reaction mixtures contained cDNA or DNA as template, *ACT1* or *COX1* (E4, Table 1) primers, and 2X Absolute Blue QPCR SYBR Green Mix (Thermo Fisher Scientific), and were carried out as recommended by the reagent manufacturer. *ACT1* and *COX1* PCR products were identified by their melting temperature of 83.5 °C and 79.1 °C, respectively. mRNA quantification was performed after reverse transcription of total RNA as indicated above. Four 1:3 dilutions per sample were analyzed by real-time PCR for both target and reference genes using the indicated primers. Cycle threshold values for each sample were taken from dilutions within the linear range of amplification. The relative (*COX1/ACT1*) amount of mRNA and mtDNA were calculated for each time point using the method of Pfaffl (2001).

Fluorescence microscopy

Fluorescence microscopy was performed with an AxioImager A1 (Carl Zeiss, Inc., Thornwood, NY) fluorescence microscope equipped with an AxioCam MRc camera and a mercury short-arc lamp. 4', 6-diamino-2-phenylindole, dihydrochloride (DAPI) and MitoTracker Green FM (Invitrogen) staining were performed on ethanol-fixed and live cells, respectively according to the manufacturer's instructions. Briefly, approximately 1×10^6 cells were harvested from a 16-hour SDGal culture and washed once with phosphate buffer saline (PBS, pH 7.0). One volume of cell suspension was added to four volumes of absolute ethanol chilled at -20 °C, while vortexing at full speed and placed at -20 °C during 15 minutes. Afterwards, cells were pelleted, rehydrated in PBS and mixed with 3 μM DAPI in staining buffer (100 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.1 % Nonidet P-40). Following 15 minutes of incubation at room temperature, cells were washed with PBS and analyzed as indicated below. MitoTracker Green FM is a fluorescent dye that specifically accumulates in mitochondria regardless of mitochondrial membrane potential (Pendergrass et al. 2004). DAPI-

and MitoTracker Green FM- stained cells were visualized under a 31000 DAPI/Hoechst set and a 31001 FITC/Fluo3 set, respectively.

Results

Phenotype complementation, mtDNA, and transcription analysis

Two genome-wide screenings indicated that *CCM1* is important for mitochondrial function (Dimmer et al. 2002; Steinmetz et al. 2002), but no phenotype complementation has been reported so far. Direct transformation of YEPD-grown null mutants with pCCM1 did not suppress the growth defect (Fig. 1A). Phenotype complementation as visualized by growth on media containing glycerol and ethanol was only possible when $\Delta ccm1$ meiotic segregants inherited the ρ^+ genome from a heterozygous diploid (2nB) along with pCCM1 (Fig. 1B). Finally, tetrads derived from 2nB harboring pCCM1 and dissected on YEPG, generated colonies of identical size (Fig. 1C, upper panel). Interestingly, non-complemented $\Delta ccm1$ meiotic segregants generated micro colonies of nearly 1000 cells each that could not be further propagated on medium with glycerol (Fig. 1C, bottom panel) but their growth resumed in fermentable carbon sources (i.e. glucose), albeit with reduced fitness (data not shown). Interestingly, an identical phenotype was reported for meiotic segregants lacking Mgm101p, which is involved in the maintenance of ρ^+ genomes (Zuo et al. 2002). The capacity of complemented $\Delta ccm1$ segregants to maintain the ρ^+ genome was assessed by DAPI staining (Fig. 1D). Both complemented and wild-type segregants exhibited similar features regarding mtDNA content and mitochondrial morphology. Under the same culture conditions, real-time PCR experiments revealed that mtDNA dosage in complemented segregants was not different with respect to that of the wild type (1.0 ± 0.2 , average $\pm$ SEM, $n=2$), as determined by *COX1* levels (Taylor et al. 2005). On the other hand, micro colonies (Fig. 1C, bottom panel) representing 10 generations contained approximately 20-30 % of wild-type mtDNA levels as visualized by DAPI staining. After 44 generations, 100 % of the non-complemented segregants lacked the punctuated cytoplasmic foci corresponding to mtDNA, displaying only bright nuclear DNA fluorescence, and in approximately 50 % of them, mitochondria appeared to be clumped and swollen, as visualized by MitoTracker Green FM staining. *COX1* mRNA closely paralleled the *COX1* gene as they decreased with cell doubling, indicating that the transcription *per se* was not affected by the absence of Ccm1p (Fig. 2). These observations suggest that the lack of Ccm1p first causes the failure of mitochondrial function, which is followed, after several generations, by the complete loss of the mitochondrial genome.

Ccm1p appears to be essential for the electron transport system function

The use of ethanol as source of energy by *S. cerevisiae* requires its oxidation to acetaldehyde by a cytosolic alcohol dehydrogenase 2 (Adh2p) (Marres et al. 1991). Acetaldehyde is further converted to acetylCoA, which enters the tricarboxylic acid cycle in mitochondria. On the other hand, glycerol is utilized via the oxidation of glycerol-3-phosphate to dihydroxyacetone phosphate by the FAD-dependent glycerol-3-phosphate dehydrogenase (Gut2p), which is also nuclear-encoded and located in the inner mitochondrial membrane. This oxidation takes place in the mitochondrial intermembrane space where the immediate acceptor of reduction equivalents is the coenzyme Q (Complex II of the electron transport system). Likewise, lactate is oxidized to pyruvate by L-lactate: cytochrome *c* oxidoreductase (Cyb2p), a link between Complex III and Complex IV (Mowat and Chapman, 2000). Thus, the utilization of glycerol and lactate depends more directly on the electron transport system functionality than that of ethanol, which directly requires NAD^+ inside the mitochondrial matrix to accept reduction equivalents. Therefore, in non-complemented segregants, there would be enough residual mitochondrial NAD^+ to allow limited growth. Accordingly, we hypothesized that abrupt repression of Ccm1p expression would correlate to the degree of incapacity to utilize a specific non-fermentable substrate, whose identity would hint at the targets to which Ccm1p is

functionally related. In complemented segregants harboring the multi-copy vector pCCM1, no difference in growth was detected after *GAL1* repression or induction by glucose or galactose, respectively. However, complemented segregants carrying the low-copy vector pCMM1LC responded to glucose repression (Fig. 3). Under such conditions, growth on glycerol- and lactate-containing media was dramatically reduced in comparison with galactose induction, while some limited ability to grow on ethanol was observed (Fig. 3, YEPE panel). Furthermore, the growth deficiency was slightly but consistently more evident in lactate than in glycerol. This observation led us to consider that Cmm1p is functionally related to the biogenesis of Complex III and Complex IV.

Ccm1p is involved on *COB* and *COX1* mRNAs processing

Based on our previous observations and taking into account that RNA processing falls well within the capabilities of PPR proteins, we assessed the status of mitochondrial encoded intron-containing transcripts, i.e. *COB* and *COX1* mRNAs. We took advantage of the residual mitochondrial transcripts present in the non-complemented $\Delta ccm1$ segregants after 16 hours of growth in YEPGal (Fig. 2). Northern blots of total RNA from both non-complemented $\Delta ccm1$ and *CCM1* meiotic segregants are shown in Figure 4. While *CCM1* exhibited a major band corresponding to mature *COB* mRNA, non-complemented $\Delta ccm1$ segregants did not display it, but rather two forms of higher molecular weight. The larger molecule was not present in the wild type counterpart, thus confirming the specificity of the assay. The low amounts of *COB* pre-mRNAs in $\Delta ccm1$ were expected since total RNA was obtained from a culture containing approximately 2 % of mtDNA (Fig. 2). Therefore, the filter was over-exposed in order to visualize the species of lower abundance. Similar results were observed for *COX1* transcripts. Even though the total amount of RNA loaded in the non-complemented $\Delta ccm1$ segregant lane (M) was slightly lower than in the wild type lane (W), the specific signals obtained on the mutant samples were at least two orders of magnitude lower than those of the wild type.

$\Delta ccm1$ fails to excise *al4* and *bl4*

In order to define *CCM1* function more precisely, we mapped the *COB* and *COX1* exon (E)-exon (E) joints that flank group I introns by reverse transcription-PCR (RT-PCR) which is the most sensitive method available (Clark et al. 2002). PCR products derived from *COB* and *COX1* exon 4-intron 4 joints were detected in both *CCM1* and $\Delta ccm1$ backgrounds (Fig. 5A). In $\Delta ccm1$, the exon 4-exon 5 joints generated by excision of *COB* or *COX1* intron 4 were not detected, thus demonstrating that intron 4 was not removed (Fig. 5B). All other splicing reactions involving the excision of *COX1* and *COB* group I introns took place (Fig. 5B and C). The specific nature of the mutant defect was further confirmed by its ability to splice *21S_rRNA*. Transcript examination by RT-PCR showed that mutant cells produced the mature form of 21S rRNA because a PCR product of the correct size (221 bp) derived from the exon-exon joint was detected (Fig. 5D). Based on these results, we conclude that Ccm1p is specifically involved in the removal of the fourth intron of both *COX1* and *COB* pre-mRNAs.

Deletion of any of the PPR motifs abolishes Cmm1p complementation

A systematic study of Pet309p deleted one PPR motif at the time and showed that all seven motifs were essential for its biological activity (Tavares-Carreón et al. 2008). We followed a similar approach with Ccm1p because it has two in-tandem PPR motifs. We deleted the first PPR domain between amino acids 319 and 353 (Δ PPR1), the second PPR domain between amino acids 356 and 390 (Δ PPR2), and both domains between amino acids 319 and 390 (Δ PPR1/2) from *CCM1* ORF. 2nB efficiently over-expressed these three mutated proteins under standard conditions (Fig. 6A). Following sporulation, over 25 tetrads corresponding to each of the *CCM1* deletant forms were dissected and the meiotic segregants were assessed by

plasmid content (growth on SDD), G418 sensitivity/resistance (growth on YEPD + G418), growth on SDD without lysine and use of non-fermentable substrates (growth on YEPG and YEPE). None of the PPR-less constructs was able to complement the growth phenotype of $\Delta ccm1$ meiotic segregants (Fig. 6B).

Discussion

In spite of the relevance of CCM1 as a gene that encodes for a protein with PPR motifs, which are widely conserved in organelles, there is no published data about its specific role. The present study identifies for the first time the molecular targets of CCM1. Previous genome-wide reports have identified $\Delta ccm1$ as respiratory deficient (Steinmetz et al. 2002) and localized Ccm1p into mitochondria (Reinders et al. 2006), which support a mitochondrial role for CCM1. Half of the non-complemented segregants displayed abnormal mitochondrial morphology. This observation agrees with the phenotype of class II (respiratory competent) and III (respiratory incompetent) mutants, which often presented aggregated mitochondria (Dimmer et al. 2002). Class II includes, among others, $\Delta yme1$ that lacks an ATP-dependent protease located in the inner mitochondrial membrane (Hermann and Shaw, 1998), while $\Delta ccm1$ falls into class III.

In pCCM1, glucose partially repressed Ccm1p expression as assessed by immunoblot analysis (data not shown). Such background expression complemented the $\Delta ccm1$ phenotype as visualized in nascent segregants dissected on YEPG and the drop-assay results. We had to diminish the genic dosage by transferring the same expression cassette to a low-copy vector to down-regulate Ccm1p expression levels below the physiological threshold in complemented segregants. That differentially impaired their capacity to grow on non-fermentable substrates. These observations along with the observed lack of mature COB and COX1 mRNA in non-complemented mutant segregants indicated that Ccm1p is directly required in Complex III and Complex IV biogenesis. Finally, the inability of non-complemented segregants to remove bI4 and aI4 agrees with the results indicated above.

Of all proteins encoded by the *S. cerevisiae* genome that localize to mitochondria, only Aep3p, Pet309p and Ccm1p have PPR motifs (Small and Peeters, 2000). The minimal functional unit is two in-tandem α -helices that enclose a groove, whose bottom is lined with positive charges that interact with RNA phosphate groups. Therefore, one isolated PPR domain would not form a stable complex with the RNA. Our results confirm this model, since the deletion of any of the Ccm1p PPR motifs nullify its biological function. Identical conclusions were reached for Pet309p by Tavares-Carreón et al. (2008) and the human protein LPR130 (Mili and Piñol-Roma, 2003). Like Ccm1p, other PPR proteins have been implicated in RNA splicing (Schmitz-Linneweber and Small 2008). PPR proteins lack known catalytic sites, therefore the RNA processing events in which they participate must be performed by other molecules that somehow interact with the PPR-RNA complex (Delannoy et al. 2007). In this particular, Nam2p and bI4 maturase were reported to be required for bI4 and aI4 intron splicing activity in the heterologous nuclear environment (Rho and Martinis, 2000). However, the activity was so low that the splicing product was only detected by RT-PCR. Accordingly, other factors must exist in mitochondria since our data shows that naturally occurring removal of aI4 and bI4 was very efficient while non-complemented segregants did not display any detectable excision of aI4 and bI4. All maturases are translated from a single, partially processed COB transcript that comprises the upstream exons and the immediate next intron (Foury et al. 1998). Following the same pathway, the bI4 maturase should have been produced as well, since the translation initiation factor—like Pet309p for Cox1p—should be common for all COB maturases. In fact, deletion of CBS1 or CBS2 prevented the removal of bI2, bI3, and bI4 from COB pre-mRNA (Rodel 1986). Nevertheless, a role for Ccm1p in the bI4 maturase translation process cannot be ruled out. For instance, Mrf1p, a peptide chain release factor that interacts with ribosomes and mRNA stop codons (Askarian-Amiri et al. 2000), is required to complete the translation

of *COX1* and *COX2* mRNAs — which is also necessary for the excision of Group II introns - (Pel et al. 1992). Therefore, Ccm1p as a translation factor for bI4 maturase remains an open possibility: the interaction of Ccm1p with the ribosomal protein Mrps5 *in vitro* supports this idea. Regardless the intrinsic molecular mechanism by which Ccm1p acts, it is clear that this protein is essential to remove aI4 and bI4.

In spite of the significance of PPR proteins, few studies about their molecular mechanism have been reported. Representative findings were achieved on PPR4 from maize (*Zea mays*) a chloroplast-targeted PPR protein (Schmitz-Linneweber et al. 2006). PPR4 was shown to facilitate *in vivo* splicing of the first intron of the plastid *rps12* pre-mRNA, a Group II intron. It has been previously reported that Ccm1p is not involved in the excision of Group II introns (Luban et al. 2005). To the best of our knowledge, the present study describes for the first time Ccm1p as a PPR protein involved in the removal of Group I introns. It also introduces the possible role of this protein as a modulator of the electron transport system biogenesis in *S. cerevisiae* and offers a novel *in vivo* model to study still unknown interactions between PPR proteins and RNA in mitochondria.

Acknowledgements

The authors thank Drs. B. Bequette, R.C. Sizemore, B. Patlolla, K. McGee, L. Johnson, G.M. Santangelo and G. Shearer for their support and encouragement. This work was funded by Public Health Service Grant 5P20RR016476 from the National Center for Research Resources.

References

- Askarian-Amiri ME, Pel HJ, Guévremont D, McCaughan KK, Poole ES, Sumpter VG, Tate WP. Functional characterization of yeast mitochondrial release factor 1. *J Biol Chem* 2000;275:17241–17248. [PubMed: 10748224]
- Banroques J, Perea J, Jacq C. Efficient splicing of two yeast mitochondrial introns controlled by a nuclear-encoded maturase. *EMBO J* 1987;6:1085–1091. [PubMed: 3036491]
- Clark TA, Sugnet CW, Ares M Jr. Genomewide analysis of mRNA processing in yeast using splicing-specific microarrays. *Science* 2002;296:907–910. [PubMed: 11988574]
- Contamine V, Picard M. Maintenance and integrity of the mitochondrial genome: a plethora of nuclear genes in the budding yeast. *Microbiol Mol Biol Rev* 2000;64:281–315. [PubMed: 10839818]
- Delannoy E, Stanley WA, Bond CS, Small ID. Pentatricopeptide repeat (PPR) proteins as sequence-specificity factors in post-transcriptional processes in organelles. *Biochem Soc Trans* 2007;35:1643–1637. [PubMed: 18031283]
- de Longevialle AF, Meyer EH, Andrés C, Taylor NL, Lurin C, Millar AH, Small ID. The pentatricopeptide repeat gene *OTP43* is required for trans-splicing of the mitochondrial *nad1* intron 1 in *Arabidopsis thaliana*. *Plant Cell* 2007;19:3256–3265. [PubMed: 17965268]
- Dimmer KS, Fritz S, Fuchs F, Messerschmitt M, Weinbach N, Neupert W, Westermann B. Genetic basis of mitochondrial function and morphology in *Saccharomyces cerevisiae*. *Mol Biol Cell* 2002;13:847–853. [PubMed: 11907266]
- Easlon E, Tsang F, Skinner C, Wang C, Lin SJ. The malate-aspartate NADH shuttle components are novel metabolic longevity regulators required for calorie restriction-mediated life span extension in yeast. *Genes Dev* 2008;22:931–44. [PubMed: 18381895]
- Foury F, Roganti T, Lecrenier N, Purnelle B. The complete sequence of the mitochondrial genome of *Saccharomyces cerevisiae*. *FEBS Lett* 1998;440:325–331. [PubMed: 9872396]
- Gavin AC, Bösch M, Krause R, Grandi P, Marzioch M, Bauer A, Schultz J, Rick JM, Michon AM, Cruciat CM, Remor M, Höfert C, Schelder M, Brajenovic M, Ruffner H, Merino A, Klein K, Hudak M, Dickson D, Rudi T, Gnau V, Bauch A, Bastuck S, Huhse B, Leutwein C, Heurtier MA, Copley RR, Edelmann A, Querfurth E, Rybin V, Drewes G, Raida M, Bouwmeester T, Bork P, Séraphin B, Kuster B, Neubauer G, Superti-Furga G. Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* 2002;415:141–147. [PubMed: 11805826]

- Hermann GJ, Shaw JM. Mitochondrial dynamics in yeast. *Ann Rev Cell Dev Biol* 1998;14:265–303. [PubMed: 9891785]
- Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS, O'Shea EK. Global analysis of protein localization in budding yeast. *Nature* 2003;425:686–691. [PubMed: 14562095]
- Labouesse M, Netter P, Schroeder R. Molecular basis of the 'box effect'. A maturase deficiency leading to the absence of splicing of two introns located in two split genes of yeast mitochondrial DNA. *Eur J Biochem* 1984;144:85–93. [PubMed: 6207024]
- Luban C, Beutel M, Stahl U, Schmidt U. Systematic screening of nuclear encoded proteins involved in the splicing metabolism of group II introns in yeast mitochondria. *Gene* 2005;354:72–79. [PubMed: 15908144]
- Manthey GM, McEwen JE. The product of the nuclear gene *PET309* is required for translation of mature mRNA and stability or production of intron-containing RNAs derived from the mitochondrial *COX1* locus of *Saccharomyces cerevisiae*. *EMBO J* 1995;14:4031–4043. [PubMed: 7664742]
- Marres CA, de Vries S, Grivell LA. Isolation and inactivation of the nuclear gene encoding the rotenone-insensitive internal NADH: ubiquinone oxidoreductase of mitochondria from *Saccharomyces cerevisiae*. *Eur J Biochem* 1991;195:857–862. [PubMed: 1900238]
- Mili S, Piñol-Roma S. LRP130, a pentatricopeptide motif protein with a noncanonical RNA-binding domain, is bound in vivo to mitochondrial and nuclear RNAs. *Mol Cell Biol* 2003;23:4972–4982. [PubMed: 12832482]
- Moreno JI. A *Trypanosoma cruzi* polyantigen obtained by gene fusion: its expression in *Staphylococcus aureus* and rapid purification. *Protein Expr Purif* 1996;8:332–340. [PubMed: 8936595]
- Moreno JI, Piva MA, Miralles CP, De Blas AL. Immunocytochemical localization of the beta 2 subunit of the gamma-aminobutyric acidA receptor in the rat brain. *J Comp Neurol* 1994;350:260–271. [PubMed: 7884042]
- Mowat, CG.; Chapman, SK. Flavocytochrome b2. In: Holzenburg, A.; Scrutton, NS., editors. *Enzyme-Catalyzed Electron and Radical Transfer*. Kluwer Academic/Plenum Publishers; New York: 2000. p. 279-295.
- Myers AM, Pape LK, Tzagoloff A. Mitochondrial protein synthesis is required for maintenance of intact mitochondrial genomes in *Saccharomyces cerevisiae*. *EMBO J* 1985;4:2087–2092. [PubMed: 3905388]
- Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;29:2002–2007.
- Pendergrass W, Wolf N, Poot M. Efficacy of MitoTracker Green and CMXRosamine to measure changes in mitochondrial membrane potentials in living cells and tissues. *Cytometry* 2004;61:162–169. [PubMed: 15382028]
- Reinders J, Zahedi RP, Pfanner N, Meisinger C, Sickmann A. Toward the complete yeast mitochondrial proteome: multidimensional separation techniques for mitochondrial proteomics. *J Proteome Res* 2006;5:1543–1554. [PubMed: 16823961]
- Rho SB, Martinis SA. The bI4 group I intron binds directly to both its protein splicing partners, a tRNA synthetase and maturase, to facilitate RNA splicing activity. *RNA* 2000;6:1882–1894. [PubMed: 11142386]
- Rödel G. Two yeast nuclear genes, CBS1 and CBS2, are required for translation of mitochondrial transcripts bearing the 5'-untranslated COB leader. *Curr Genet* 1986;11:41–45. [PubMed: 3329045]
- Sambrook, J.; Russell, DW. *A Laboratory Manual*. Vol. 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor; New York: 2001. Molecular Cloning.
- Schmitz-Linneweber C, Williams-Carrier RE, Williams-Voelker PM, Kroeger TS, Vichas A, Barkan A. A pentatricopeptide repeat protein facilitates the trans-splicing of the maize chloroplast *rps12* pre-mRNA. *Plant Cell* 2006;18:2650–2663. [PubMed: 17041147]
- Schmitz-Linneweber C, Small I. Pentatricopeptide repeat proteins: a socket set for organelle gene expression. *Trends Plant Sci* 2008;13:663–670. [PubMed: 19004664]
- Sherman F. Getting started with yeast. *Methods Enzymol* 2002;350:3–41. [PubMed: 12073320]
- Small ID, Peeters N. The PPR motif - a TPR-related motif prevalent in plant organellar proteins. *Trends Biochem Sci* 2000;25:46–47. [PubMed: 10664580]

- Steinmetz LM, Scharfe C, Deutschbauer AM, Mokranjac D, Herman ZS, Jones T, Chu AM, Giaever G, Prokisch H, Oefner PJ, Davis RW. Systematic screen for human disease genes in yeast. *Nat Genet* 2002;31:400–404. [PubMed: 12134146]
- Tavares-Carreón F, Camacho-Villasana Y, Zamudio-Ochoa A, Shingú-Vázquez M, Torres-Larios A, Pérez-Martínez X. The pentatricopeptide repeats present in Pet309 are necessary for translation but not for stability of the mitochondrial *COX1* mRNA in yeast. *J Biol Chem* 2008;283:1472–1479. [PubMed: 18039654]
- Taylor SD, Zhang H, Eaton JS, Rodeheffer MS, Lebedeva MA, O'Rourke TW, Siede W, Shadel GS. The conserved Mec1/Rad53 nuclear checkpoint pathway regulates mitochondrial DNA copy number in *Saccharomyces cerevisiae*. *Mol Biol Cell* 2005;16:3010–3018. [PubMed: 15829566]
- Tzagoloff A, Dieckmann CL. *PET* genes of *Saccharomyces cerevisiae*. *Microbiol Rev* 1990;54:211–225. [PubMed: 2215420]
- Yaffe MP. The machinery of mitochondrial inheritance and behavior. *Science* 1999;283:1493–1497. [PubMed: 10066164]
- Zuo XM, Clark-Walker GD, Chen XJ. The mitochondrial nucleoid protein, Mgm101p, of *Saccharomyces cerevisiae* is involved in the maintenance of ρ^+ and ori/rep-devoid petite genomes but is not required for hypersuppressive ρ^- mtDNA. *Genetics* 2002;160:1389–1400. [PubMed: 11973295]

Fig. 1. Phenotype complementation

A: Transformed strains were collected from master plates and incubated at 30 °C during 24 h in SDD. Cells were washed three times with distilled water and incubated in SDGal for additional 24 h. Afterwards, the cultures were centrifuged and pellets were resuspended to reach a concentration of 10^8 cells per mL. 10 μ L were streaked out on the indicated media and incubated at 30 °C during three days. B: Tetrads were dissected on YEPGal and G418^R meiotic segregants harboring the indicated plasmids were directly resuspended in sterile water and handled as in A. C: *Left*: Transformed segregants were dissected on YEPG. *Right*: Tables indicate the segregant phenotype as R (resistant to G418, i.e. *Δccm1*) or S (sensitive to G418, i.e. *CCM1*) or growth (+) / no growth (-) on SDD without lysine. Bottom picture depicts a micro colony. Plates were incubated at 30 °C during four days. Longer periods of incubation did not increase the size of the micro colonies. D: Cells from a 30-generation colony were grown for 14 additional generations in SDGal and stained with DAPI for DNA or MitoTracker Green FM for mitochondria. Representative images were obtained by fluorescence microscopy and differential interference contrast (DIC).

Fig. 2. Steady-state transcript levels in non-complemented segregants over time

Total DNA and RNA were isolated from 16-hour YEPD cultures of G418^R ($\Delta ccm1$) and G418^S (*CCM1*) nascent meiotic colonies (0 incubation time), and subsequent YEPD cultures. mtDNA levels (■) were determined by real-time PCR, using *COX1* and *ACT1* as the target and reference genes, respectively. For mRNA quantification (□), four 1:3 dilutions per reverse transcription sample were analyzed by real-time PCR for *COX1* and *ACT1* as previously indicated. Cycle threshold values for each incubation time were obtained from dilutions within the linear range of amplification. Results were expressed as percentage of wild type's relative amount of mtDNA and mRNA, which were considered 100 % at each time point. Symbols $\pm$ vertical bars represent the average of two independent experiments $\pm$ SEM.

Fig 3. Repression of Cmm1p expression differentially affects the utilization of specific non-fermentable substrates by complemented *AccmI* segregants

Complemented sister segregants harboring Ccm1p expressing vectors and one non-complemented segregant harboring pCCM1Δ or pRS316 were grown in SDD for *GALI* promoter repression [Glu (R)] and in SDGal for *GALI* promoter induction [Gal (I)] during 24 h. The cells were washed three times with water and counted using a cell counter (Multisizer 3, Beckman Coulter, Fullerton, CA). Starting with a concentration of 10^8 cells/mL, further 1/10 serial dilutions were performed. Three μ L of each dilution were spotted onto solid media. The first two panels are controls for plasmid content (SDD) and G418 resistance.

Fig. 4. Non-complemented mutant segregants do not produce mature *COB* or *COX1* mRNA
 Northern blot analysis of total RNA (between 2-3 μ g per lane) isolated from 16 hour-YEPGal cultures of *CCMI* (W) and Δ *ccm1* (M) nascent meiotic segregants. Left panel: Hybridization with a biotinylated 290-bp *COB* probe that spans part of exon 4 and exon 6, and all of exon 5. Right panel: Hybridization with a biotinylated 146-bp *COX1* probe that spans part of exon 4. Arrowheads indicate the mature forms.

Fig. 5. Non-complemented mutant meiotic segregants do not excise aI4 and bI4

Total RNA was isolated from 16 hour-YEPGal cultures of *CCM1* (W) and $\Delta ccm1$ (M) nascent meiotic segregants, and reverse-transcribed. The resulting cDNAs were amplified by end-point

PCR using the primers symbolized by the horizontal arrows in the figure. A: Presence and removal of aI4 and bI4. B and C: Removal of all other Group I introns. D: Removal of omega intron. nc: PCR negative control. The number of bp indicates the PCR product size.

Fig. 6. Deletions of any PPR motifs failed to complement *Δccm1* phenotype

A: Immunoblot analysis of recombinant proteins with purified polyclonal antibodies against Ccm1p. 25 μ g of total protein in clarified crude extracts from galactose-induced 2nB cells carrying the indicated plasmids were run in 12% SDS-PAGE and transferred to nitrocellulose membrane. Ccm1p expressed in *E. coli* was used as control. B: G418^R meiotic segregants harboring vectors that express wild-type Ccm1p (pCMM1), PPR1-deleted Ccm1p (Δ PPR1), PPR2-deleted Ccm1p (Δ PPR2), and PPR1/PPR2-deleted Ccm1p (Δ PPR1/2). The strains were incubated at 30 °C for 2 days (YPD + G418) or 3 days (SDD, YEPG, YEPE).

TABLE 1

Primers used in this study

Template	Forward primer	Reverse primer
<i>ACT1</i>	GTATGTGTAAAGCCGGTTTGG	CATGATACCTTGGTGTCTTGG
<i>COB</i> E2-E3 joint	TGGACAGATGTCACATTGAGG	CGTTACCTACAAATGGAATTGCT
<i>COB</i> E3-E4 joint	TCTCAGCAATTCCATTTGTAGG	TGCCATTAAATGCATAATAACCA
<i>COB</i> E4-E5 joint	TCCAGAGATTCTTTGCGTTACA	TTCAGGTACAATAGATGCTGGTG
<i>COB</i> E5-E6 joint	GGTAATCCTTTAGTAACACCAGCA	CTTCTACATGGCATGCTCCA
<i>COB</i> E4-I4 joint	TTCCAATGCATTCATACTTTATTTT	AAGATTTCCAGCATACAGCACA
<i>COX1</i> E4	CTACAGATACAGCATTTCCAAGA	GTGCCTGAATAGATGATAATGGT
<i>COX1</i> E3-E4 joint	TGCCTGCTTTAATTGGAGGT	GGTCCTGAATGTGCCTGAAT
<i>COX1</i> E4-E5 joint	AGGAGGTGGTGACCCAATCT	AATCCAATTGAAGCCATAGCA
<i>COX1</i> E5-E6 joint	TGCTATGGCTTCAATTGGATT	CCAGTTAAACCACCCATTGTG
<i>COX1</i> E6-E7-E8 joint	CAATGGGTGGTTAACTGGTG	GCCCCAATGAAAATTAATCAGA
<i>COX1</i> E4-I4 joint	TGATCAATTTTCATTACAGCGTTC	ATAAGGGCCACTGTTTGACC
21S rRNA E1-E2 joint	CGGGTCCCGAACTTAAATA	CGAGGTGGCAAACATAGCTT
15 S rRNA	GAGGGCGAGCGTTAATCATA	TCGAATCCGTTTCGCCTACTC