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Ccm1p is a 15S rRNA primary transcript processing factor as elucidated by a novel *in vivo* system in *Saccharomyces cerevisiae*

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Abstract

In *Saccharomyces cerevisiae*, the mitoribosomal RNA of the minor subunit,15S rRNA, is transcribed as a bicistronic transcript along with $tRNA^W$. 5' and 3' sequences flanking the mature transcript must be removed by cleavage at the respective junctions before incorporating it into the mitoribosome. An *in vivo* dose-response triphasic system was created to elucidate the role of Ccm1p in the processing of 15S rRNA: Ccm1p supply ("On"), deprivation ("Off"), and resupply ("Back on"). After 72 h under "Off" status, the cells started to exhibit a complete mutant phenotype as assessed by their lack of growth in glycerol medium, while keeping their mitochondrial DNA integrity (ρ^+). Full functionality of mitochondria was reacquired upon "Back on." 15S rRNA levels and phenotype followed the Ccm1p intramitochondrial concentrations throughout the "On-Off-Back on" course. Under "Off" status, cells gradually accumulated unprocessed 5' and 3' junctions, which reached significant levels at 72–96 h, probably due to a saturation of the mitochondrial degradosome (mtEXO). The Ccm1p/mtEXO mutant (Δ*ccm1*/Δ*dss1*) showed a copious accumulation of 15S rRNA primary transcript forms, which were cleaved upon Ccm1p resupply. The gene that codes for the RNA component of RNase P was conserved in wild-type and mutant strains. Our results indicate that Ccm1p is crucial in processing the 15S rRNA primary transcript and does not *stabilize* the already mature 15S rRNA. Consequently, failure of this function in Δ*ccm1* cells results, as it happens to any other unprocessed primary transcripts, in total degradation of 15S rRNA by mtEXO, whose mechanism of action is discussed.

Keywords

Ccm1p; 15S rRNA; processing; mtEXO; mitoribosome; mitochondria

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Introduction

During the course of evolution, mitochondrial DNA (mtDNA) underwent a significant reduction by the migration of genes from mtDNA to the nuclear genome, which has been a universal phenomenon in eukaryotes (Keeling and Palmer 2008). This phenomenon also involves the loss of transcription initiation signals that results in the production of polycistronic primary transcripts, which must be processed before becoming fully functional (Anderson et al. 1981). Failures in the processing of primary transcripts cause several disorders (Van Haute et al. 2015). Specifically, problems in the nucleolytic processing of mitochondrial RNA precursors reduce the levels of the electron transport chain components and consequently may cause progressive loss of cognitive and motor function, epilepsy, retinal degeneration (Deutschmann et al. 2014; Falk et al. 2016), cardiomyopathy (Haack et al. 2013; Deutschmann et al. 2014), lactic acidosis, hypotonia, feeding difficulties, and deafness (Metodiev et al. 2016).

Although mtDNA exhibits a remarkable variation in size (Gray 2012), a shared set of genes remain in it, *COB*, *COX1*, *COX2*, *COX3*, *ATP6*, and *ATP8,* which are part of higher-order complexes (Gray 2012), and those coding for ribosomal RNAs, and transfer RNAs (Anderson et al. 1981; Foury et al. 1998). All these genes are essential for mitochondrial functionality.

The need for intramitochondrial expression of these proteins is due to their high content of hydrophobic domains, which is the cause of their mistargeting to the endoplasmic reticulum when expressed in the cytoplasm (Björkholm et al. 2015). This observation supports the notion that such proteins must be synthesized, folded, and incorporated into their corresponding complexes inside the organelle during mitochondria biogenesis. Hence the limit of migration to the nucleus imposed over mitochondrial genes. Thus, nearly 250 nuclear-encoded factors are required to produce proteins inside the organelle (Fox 2012). Interestingly, the cytoplasmic protein Puf3p regulates the translational fluctuation during metabolic adaptation to substrate availability by balancing mitochondrial and cytosolic ribosome biogenesis (Wang et al. 2019). In addition, the co-regulation of the overall network is carried out by Abf1p, an expression factor for ribosomal proteins whose binding motif is also present in mitochondrial ribosomal protein genes (de la Cruz et al. 2018).

While in humans, two initial transcripts are generated (Montoya et al. 1982), in *Saccharomyces cerevisiae,* a minimum of 11 primary transcripts has been proposed to be synthesized (Turk et al. 2013). All of them must be processed by nucleases to produce functional RNAs. Therefore, the reduction in mtDNA genes has to be compensated by a set of nuclear-encoded proteins that cleave primary transcripts at precise sites. Once released, RNA byproducts like introns must be degraded to prevent their toxicity (Margossian et al. 1996). When not processed, the *S. cerevisiae COB*, *VAR1*, and 15S rRNA primary transcripts undergo degradation as well (Dziembowski et al. 2003). The system involved in RNA degradative quality control is the mitochondrial degradosome (mtEXO). Two enzymes, a 3’→5’ exoribonuclease, and an RNA helicase, coded by the *DSS1* and *SUV3* nuclear genes respectively, compose the mtEXO (Dziembowski et al. 2003).

The 15S rRNA primary transcript (15S rRNA, $tRNA^W$; Turk et al. 2013) consists of 76 nucleotides (0.5S rRNA) at the 5'end (Osinga et al. 1981), followed by mature 15S rRNA (1646 nucleotides), a spacer or 3'-precursor (Dziembowski et al. 2003) of 1308 nucleotides and $tRNA^W$ at the 3' end, which encompasses 74 nucleotides (Naquin et al. 2018) (Fig. 1). Thus, three joints must be processed to generate the authentic 15S rRNA and $tRNA^W$. The deletion of either mtEXO subunit results in the accumulation of 3' end unprocessed forms and a subsequent reduction in processed 15S rRNA levels (Dziembowski et al. 2003). Insufficient amounts of rRNA cause the malfunction of the mitochondrial gene expression system, which results in loss of mtDNA (Merz and Westermann 2009; Guo et al. 2011). Once the $tRNA^W$ has been removed from the primary transcript by RNAse P (Hollingsworth and Martin 1986) and RNAse Z (Mörl and Marchfelder 2001), the precursor 15S rRNA may follow two routes that seem to take place simultaneously but are mutually exclusive: This molecule can be processed and integrated into the minor subunit of the mitoribosome or degraded by the quality control (QC) mtEXO. The notion that mtEXO (Gavin et al. 2002; Dziembowski et al. 2003) and Ccm1p (Möller-Hergt et al. 2018) are most probably physically associated with the mitoribosome supports this theory. Processing of the 15S rRNA primary transcript 5' terminal end is mainly controlled by Pet127p and carried out by a putative 5'→3' exonuclease (Fekete at al. 2008). Curiously, the *S. cerevisiae* 62R1 (Δ*pet127* [Cox 3–662]) double mutant is unable to cleave the 76 nucleotides at the 5' end, but it grows weakly in the presence of glycerol, which suggests that mitoribosome assembly takes place to some extent (Wiesenberger and Fox 1997).

Pentatricopeptide-repeat (PPR) proteins (Small and Peeters 2000) have been reported to be involved, among other activities, in nucleolytic processing of mitochondrial RNA (for a thorough review, see Manna 2015). PPR domains are not considered to be catalytic; instead, they mediate interactions between RNA substrates and intra- or intermolecular nuclease activity (Manna 2015). However, a subset of PPR proteins known as PRORP (protein-only RNase P) have two-domains: An N-terminal domain, composed of several PPR motifs, that confers substrate specificity (Kobayashi et al. 2012) and a C- terminal domain that harbors nucleolytic activity (Pinker et al. 2013). Examples of PRORPs are MRPP3, PRORP2, and PRORP1. These proteins participate in 5' tRNA nucleolytic processing in the mitochondria of humans, *Arabidopsis thaliana*, and *Trypanosoma brucei*, respectively (Holzmann et al. 2008; Gobert et al. 2010; Taschner et al. 2012). In *S. cerevisiae,* the participation of Ccm1p, a bifunctional PPR protein (Moreno et al. 2012) in the nucleolytic processing of *only* the 15.5S rRNA has been addressed (De Silva et al. 2015), albeit not yet demonstrated.

The present paper reports a novel *in vivo* triphasic expression system consisting of Ccm1p supply/deprivation /resupply ("On"/ "Off"/ "Back on") that causes wild-type/mutant/wild-type phenotypes. Because this system is reversible, it is amenable to conduct dual dose-response experiments that validate one another. It correlates intramitochondrial concentrations of Ccm1p and events on the 15S rRNA primary transcript that define this PPR protein as an essential processing factor. Studies involving double mutant cells (Δ*ccm1*/Δ*dss1*) showed that mtEXO and Ccm1p act concertedly but in opposition to determine the fate of the molecule. Thus, the absence of Ccm1p leads to the accumulation of unprocessed forms, which are *recognized* and degraded by the QC-surveillance mtEXO.

Materials and Methods

Media, strains, and DNA constructs

Media, yeast manipulation, including the selection of heterozygous strains, transformation, sporulation, tetrad dissection, and further analysis of meiotic segregants were carried out as described previously (Moreno et al. 2009). *S. cerevisiae* harboring intronless mtDNA (I^0) (*MAT*a *ade1*Δ0 *lys1*03940 *ura3*Δ0) (Séraphin et al. 1987) was kindly provided by Dr. Alan M. Lambowitz (Institute for Cellular and Molecular Biology, Departments of Chemistry, Biochemistry and Microbiology, University of Texas at Austin, TX, USA. The *S. cerevisiae* Δ*dss1* strain (*MAT*α *his3*Δ1 *leu2*Δ0 *ura3*Δ0 *lys2*Δ0 *dss1*Δ0::*kanMX*) was purchased from Dharmacon (Lafayette, CO, USA). pCCM1ZZLC is a low-copy number vector that expresses the full- length *CCM1* gene fused to a ZZ tag (two IgG-binding domains in tandem) at the C-terminus as Ccm1pZZ (Moreno et al. 2012). pCCM1LC, a low-copy number vector that expresses the naturally occurring *CCM1* gene without any modifications (authentic protein construct, the gene product is Ccm1pAPC) was prepared as previously described (Moreno et al. 2009). The second *bona fide* PPR domain (PPR2) of *CCM1* open reading frame (ORF) in pCCM1ZZLC was deleted between residues 356 and 390 to generate ΔPPR2ZZ (Moreno et al. 2009). The *Lgu*I DNA fragment containing ΔPPR2ZZ ORF and all expression elements were inserted in the *Sma*I site of the low-copy vector pRS316 (ATCC, Manassas, VA, USA). The resulting vector, pΔPPR2ZZLC that expresses ΔPPR2pZZ. The constitutive expression of Ccm1p was achieved by inserting the CCM1 ORF at the *Not*I site of the 2μ-vector pDB20 (a generous gift from Dr. Leonard Guarente, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA; Becker et al. 1991) under the control of the *ADH1* promoter (pDB20-CCM1).

“On-Off-Back on” time-course experiment

Complemented meiotic segregants harboring pCCM1LC, pCCM1ZZLC, or pΔPPR2ZZLC were initially isolated on YEPG. Protein expression was induced by growing them in synthetic define medium minus uracil (SD) with galactose (SDGal) for 24 h (“On”). Cells were then washed three times with sterile deionized water at room temperature. Repression of *CCM1* expression (“Off”) was carried out by adding cells (Abs_{600} =1.0) to SD with dextrose (SDD) at a ratio of 1:20 (v/v) and incubating them for 12 h and 24 h at 30°C, 300 rpm. Subsequent “Off” times were started by adding a 24 h-culture to SDD at a ratio of 1:100 (v/v) and incubating it for an additional 24 h period (i.e., 48h). This procedure was repeated twice to achieve incubation times of 72 and 96 h. Cells from the 96 h-SDD culture (“Off”) were washed as indicated above and induced to express Ccm1pZZ or Ccm1p by adding the inoculum (Abs_{600} =1.0) to SDGal (“Back on”) at a ratio of 1:20 (v/v), for 12 and 24 h, or 1:100 (v/v), for 48h. A 48 h-culture inoculum grown in SDGal (Abs_{600} =1.0) was added to the same medium at a ratio of 1:100 (v/v) and incubated for an additional 24 h for a total of 72 h. The same procedure was carried out again for the SDD control. Samples, representing all conditions and incubation times, were collected and aliquoted. Most of the cells were stored at −70°C for further analysis, while small aliquots were collected for drop assays. They were carried out at the end of each incubation time by spotting 3 μL of 1:10 serially diluted cell suspensions (at an initial concentration of 1×10^8 cells/ mL) onto YEPG

and YEPD solid media containing geneticin (Thermo Fisher Scientific, Waltham, MA, USA). Plates were further incubated at 30°C for 48 h and photographed.

Δ*ccm1*/ Δ*dss1* haploid mutant and incubation conditions

I^0 haploids complemented with pCCM1ZZLC and carrying functional mitochondria were mated with the Δ*dss1* strain. The mating mixture was incubated for 3 hours at 30°C and spread on SSD to allow the microscopic identification of young diploids by their characteristic morphology (shmoos) (Amberg et al. 2005). Diploids were separated from the rest of the cell population by micromanipulation and allowed to form colonies, which were tested for pCCM1ZZLC content by growth on SSD. They were pooled and induced to sporulate. Tetrads were dissected on YEPG. Normal-sized and petite colonies were screened for the presence of pCCM1ZZLC, as indicated above. Segregants that harbored the expression vector were studied by colony PCR to determine whether they harbored no deletion, one (Δ*dss1* or Δ*ccm1*), or both deletions (Δ*ccm1*/Δ*dss1*). This screening used the primers indicated by the Saccharomyces Genome Deletion Project (Table 1) and the Phire Plant Direct PCR Master Mix (Thermo Fisher Scientific). For mtDNA and mtRNA hybridization analyses, suitable segregants were harvested from the tetrad dissection plate, pooled, and propagated in YEPD for 48 h. The rich medium was used to generate enough cells for the hybridization experiments. For the Δ*ccm1*/Δ*dss1* "Back on" assay, mutants that harbored pCCM1ZZLC were harvested from the tetrad dissection plate and incubated in SDD ("Off") for 48 h. At the end of the incubation period, a third of each culture was collected for reverse transcription (RT)-quantitative (q)PCR analysis using *ALG9* mRNA as the reference transcript (Teste et al. 2009). The rest of the cells were washed as mentioned above, incubated for two more days in SDGal ("Back on"), collected, and analyzed as indicated.

mtRNA, mtDNA, preparation of mitochondria, qPCR, antibodies, and protein analysis

All probes were biotinylated by PCR (Biotin PCR Labeling Core Kit, Jena Bioscience, Jena, Germany) and subsequently purified from primers and unincorporated nucleotides (QIAEX II, Qiagen, Valencia, CA, USA). RNA isolation and hybridization were performed as previously described (Moreno et al. 2009). The signals were visualized and recorded with a chemiluminescent blot scanner (C-DiGit) (Li-Cor, Lincoln, NE, USA). DNA probes that detect 15S rRNA spanned either 204 bp (Moreno et al. 2009), or 588 bp from positions 6785–7369 of the mitochondrial genome.

mtDNA was prepared and digested with *EcoR*V, as indicated previously (Defontaine et al. 1991). DNA fragments were then separated by 1% agarose gel electrophoresis, ethidium bromide-stained, photographed, and transferred to a nylon membrane. This membrane was hybridized with a biotinylated 290-bp *COB* probe (Moreno et al. 2009) or a 209-bp DNA fragment located within the 3405 bp-*EcoR*V - *EcoR*V segment that contains the full-length *RPM1* gene (9S RNA) and its promoter (Naquin et al. 2018). DNA hybridization was carried out with the North2South® Chemiluminescent Hybridization and Detection Kit (Thermo Scientific, Rockford, IL. USA), following the manufacturer's instructions. Signals were visualized and recorded, as indicated above. qPCRs were carried out in a Smart Cycler II thermal cycler (Cepheid, Sunnyvale, CA, USA) using the SensiFAST™ SYBR No-ROX

reagent (Bioline, Memphis, TN, USA). Primer sequences, annealing positions, and amplicon sizes are indicated in Fig. 1 and Table 1. RT-qPCR and qPCR products were identified by their melting temperature and endonuclease restriction patterns (Table 2). Calibration curves converted Ct (threshold cycle value) into starting amounts of template for each qPCR template–primers combination. A serial 1/10 dilution of yeast nuclear and mitochondrial DNA was used as the template for all calibration curves. Values that fit within the linear range were chosen for further calculation. The slope and R^2 of each calibration curve were determined with the SigmaStat statistical software (SPSS, Chicago, IL, USA). R^2 values were at least 0.998. mtDNA levels were assessed by qPCR using *15S_ RRNA* and *ALG9* as the target and reference genes, respectively. Levels of 15S rRNA, 5', and 3' unprocessed junctions (UJs) were assessed by RT-qPCR using 21S rRNA as the reference transcript, as previously indicated (Moreno et al. 2012). The percentage of processed 15S rRNA was calculated by subtracting the sum of 5' and 3' UJs from total 15S rRNA, which was considered 100 %. The amount of 3' processed junctions was determined by subtracting 3' UJ from total 15S rRNA levels. *ALG9* and *CCM1* mRNAs levels were measured by RT-qPCR, as mentioned above. Cytoplasm-free mitochondrial fractions were prepared as previously reported (Meisinger et al. 2006).

Two different sets of antibodies were used in this study: (i) Intramitochondrial Ccm1p was detected by immunoblot with specific polyclonal antibodies against the full-length protein produced in *E. coli* as previously described (Moreno et al. 2009). The assay limit of detection was approximately 2.5 ng of Ccm1p (Ccm1pZZ form) per lane. As no signal was displayed in mitochondrial extracts isolated from the Δ*ccm1* strain (Moreno et al. 2009), these antibodies proved to be specific; (ii) the intramitochondrial concentration of Ccm1p was measured by ELISA with affinity-purified polyclonal antibodies against a synthetic peptide (VNKKSHAKALKWEEQELN), which constitutes a potent epitope (E3) located at the C-terminus of Ccm1p. The peptide and the antibodies were obtained as previously described (Moreno et al. 2012). As 5 μg of mitochondrial proteins prepared from the Δ*ccm1* strain displayed no signal, the polyclonal antibodies showed to be specific.

For SDS/PAGE and immunoblotting, cytosol-free mitochondria samples were solubilized in cold 0.1 N NaOH (Nandakumar et al. 2003) and kept in ice for less than one hour for protein determination. Protein concentration was determined by Bradford assay and BSA as standard (Thermo Fisher Scientific).

Quantitation of intramitochondrial Ccm1pZZ levels by ELISA

The Ccm1pZZ calibrator, used for molarity measurement, is a truncated form of Ccm1pZZ that consists of the last 43 residues of Ccm1p. This peptide contains one of the strongest epitopes, VNKKSHAKALKWEEQELN (E3), and is fused at the C-terminus to the ZZ affinity tag (E3ZZ). The E3ZZ was prepared by subcloning the corresponding DNA fragment in pET28b and expressing it in *Escherichia coli* Rosetta (DE3) pLysS (Novagen, Madison, WI, USA) according to the supplier's recommendations. The bacterial cell mass was lysed by sonication in PBS, 0.5% Triton X-100, 0.1% Tween, 100 mM EDTA, 100 mM EGTA plus one tablet of Complete™ protease inhibitor (Santa Cruz Biotechnology, Dallas, TX, USA) per 10 mL of lysis buffer. E3ZZ (25.50 kDa) was affinity-purified with IgG-

Sepharose (GE Healthcare, Chicago, IL, USA), as previously described (Moreno 1996). The protein concentration was measured using the Bradford assay with BSA as standard (Thermo Fisher Scientific). E3ZZ was then aliquoted, kept frozen at −70 °C and thawed at the moment of use.

The sandwich ELISA was standardized as follows: After measuring the protein concentration in the alkaline mitochondria crude extracts, the samples were kept in ice until the moment of use, but no longer than 1h. Purified porcine IgG (Equitech-Bio, Kerrville, TX, USA) was used as the capture antibody at 2 μg per well. The E3ZZ calibrator was 1:10 diluted in 0.1 N NaOH. 5 μL of E3ZZ or mitochondria alkaline lysates were loaded on ELISA plate wells (Corning, NY, USA), which already contained 200 μL of dilution buffer (DB: 2X PBS, 130 mM EDTA, 130 mM EGTA, 1% Triton X-100, 0.1% Tween 20, 0.5% BSA and one tablet of Complete protease inhibitor per 50 mL of solution). The samples were then 1:3 serially diluted with DB and incubated for 18 h at 4°C. Plates were then washed three times (Wellwash Microplate Washer, Thermo Fisher Scientific) with washing buffer (WB: PBS, 0.5% Triton X-100, and 0.1% Tween 20). Wells were then incubated with purified polyclonal antibodies against the E3 epitope (21st Century Biochemicals, Marlboro, MA, USA), diluted 1:1000 in DB. Plates were allowed to stand at 4° C for 18 h. Wells were washed three times and incubated with 100 μL of 1:10,000 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific) in DB. Plates were incubated at room temperature for 1 h, washed with WB three times, and processed as indicated previously (Moreno et al. 2012). The limit of detection for this assay was approximately 0.14 nM.

Statistical analysis

All qPCR values represent the means ± S.E.M. of duplicates for the number of independent experiments indicated in each figure legend. When necessary, measured values were transformed into logarithms to normalize the distribution or equalize the variances. mtDNA qPCR data were analyzed by a two-sample t-test. RT-qPCR results were analyzed by one-way ANOVA, followed by Student-Newman-Keuls' post hoc test for multiple comparisons (SPSS).

Results

Deletion of *CCM1* buildups unprocessed 15S rRNA transcripts

Established Δ*ccm1* mutants lose their mtDNA (Merz and Westermann 2009) and, consequently, all the corresponding mitochondrial transcription products. To visualize the effects of the deletion on the 15S rRNA, we analyzed fresh *CCM1* and Δ*ccm1* intronless nascent meiotic segregants since the latter had inherited mtDNA, transcripts, and all the cytosolic factors from the heterozygous diploid (Moreno et al. 2012). Wild-type cells exhibited only processed 15S rRNA in predictable quantities, whereas Δ*ccm1* cells had severely diminished amounts of it. Surprisingly, the mutants also showed a longer form of approximately 3000 nucleotides of similar intensity (Fig. 2), which corresponds to the initial full-length 15S rRNA transcript (Fig. 1). The heavier transcript was the first indication that Ccm1p could be involved in a post-transcriptional modification activity that converts this

RNA to its final and functional form. This observation and previous reports (Dziembowski et al. 2003; De Silva et al. 2015) prompted us to focus our study on the processing of the 15S rRNA primary transcript by Ccm1p (Fig. 1).

Mitochondria in Δ*ccm1* non-complemented segregants keep their capability to import Ccm1p.

Non-complemented Δ*ccm1* meiotic segregants displayed, in addition to mitochondrial respiratory failure, abnormal morphology (Moreno et al. 2009). Their capacity to revert to full organelle functionality was assessed (see below) by their ability to import cytoplasmic proteins into mitochondria (Kritsiligkou et al. 2017). We have previously reported that, by deleting the second canonical PPR domain of Ccm1p, the protein loses the ability to complement Δ*ccm1* meiotic segregants (Moreno et al. 2009). Consequently, purified mitochondrial fractions from Δ*ccm1* segregants harboring the empty vector, pCCM1ZZLC, or pΔPPR2ZZLC were compared in regards to their relative levels of 15S rRNA and *CCM1* mRNA along with protein expression and import into the organelle. Cells harboring pΔPPR2ZZLC and non-complemented Δ*ccm1* (empty vector) segregants had comparable levels of 15S rRNA (Fig. 3). However, nascent Δ*ccm1* segregants harboring pΔPPR2ZZLC produced the corresponding deletant protein without apparently reduced fitness and imported it into mitochondria as efficiently as the wild- type cells (Fig. 3, *insert*). This observation indicated that mitochondria of non-complemented nascent segregants could sustain the import of a Ccm1p-like protein.

The mutant phenotype caused by the sudden mitochondrial deprivation of Ccm1p (SMDC) is reversible

We had previously complemented Δ*ccm1* cells by ectopic expression of *CCM1* ORF under the control of the *GAL1* promoter. Downregulation of *CCM1* expression ended in respiratory failure, as shown by their lack of growth on glycerol medium (Moreno et al. 2009). The “Off” (SMDC) status was enhanced by fusing a ZZ tag to the Ccm1p C-terminus (Moreno et al. 2012) since it constraints *en route* its mitochondrial import (Schülke et al. 1997). To set up our system of study, we evaluated the cells’ ability to grow again on non-fermentable substrates upon Ccm1p resupply after they had acquired the mutant phenotype. Therefore, cells were initially induced by galactose (“On”), then were incubated in glucose medium for several days (“Off”), before switching them back to galactose (“Back on”). The entire progression of events, induction/repression/reinduction, was monitored by the ability of the cells to grow on medium with glycerol. Cells in “Off” reached the full mutant phenotype after 72 h (Fig. 4a), which was consistent with our previously reported results (Moreno et al. 2012). We further extended the “Off” time up to 96 h and obtained the same results (data not shown). A complete reversion to the wild-type phenotype was observed when the cells were reinduced by switching back to the galactose medium (Fig. 4b). Growth in glycerol medium for all conditions was recorded after 48 h of incubation.Interestingly, after 72 h of additional incubation time, cells under “Off” status resumed growth and reached comparable levels to the ones that were permanently in the “On” phase, since the lack of glucose in YEPG derepressed Ccm1p expression.

Cells maintain ρ^+ status throughout the entire "On-Off-Back on" time-course experiment

The length of the most critical phase ("Off") and subsequent reversion to wild-type phenotype prompted us to determine whether the mtDNA underwent modifications. Fig. 5a depicts representative samples of mtDNA from cells subjected to the "On-Off-Back on" conditions. *COB* was used as the gene marker for genetic rearrangements (Fritsch et al. 2014), deletions (Heude et al. 1979) or depletion (Ulery et al. 1994) (Fig. 5b). All samples showed precisely the same pattern of restriction fragments without smears. Thus, neither ρ^0 nor ρ^- status, no mtDNA rearrangements, or alteration of mtDNA levels were observed throughout the entire "On-Off-Back on" experiment.

In addition to repressing the *GAL1* promoter, glucose also decreases the mtDNA copy number (Ulery et al. 1994). We had observed a transient-moderate depression of 15S rRNA and 21S rRNA levels upon the same sugar change (Moreno et al. 2012). Therefore, to factor in this swap on the mtDNA copy- number, we measured mtDNA levels in Δ*ccm1* cells complemented with a vector expressing Ccm1p under the control of a constitutive promoter (pDB20-CCM1). Subsequently, mtDNA levels in cells with the regulable expression vector were divided by mtDNA levels in cells with the constitutive expression vector, both in the presence of galactose (t = 0 h). The procedure was also carried out for cells grown in the presence of glucose (t = 96 h). The two ratios were then compared with one another: mtDNA levels in galactose did not differ from those in glucose ($p > 0.05$, Fig. 5c). Thus, differences in Ccm1p expression did not affect the mtDNA copy number.

So far, our results indicated that even though the total population of mitochondria reached a dysfunctional status, they kept the capacity to uptake Ccm1p and resume their physiological activity. Moreover, the cells re-acquired the wild-type phenotype at approximately the same rate they lost it (Fig. 4a, b). Thus, the idea of a few, still active mitochondria generating 100% of the functional population by fusion and fission (Mishra and Chan 2014) upon Ccm1p resupply could be ruled out; instead, a "stand by" condition appeared to take place during the "Off" phase.

Based on the present work, we have produced and validated a tri-phasic *in vivo* system that yielded dual dose-response results suitable to study changes on the initial 15S rRNA transcript upon modifications of the Ccm1p intramitochondrial concentrations.

The levels of 5' and 3' 15S rRNA unprocessed junctions inversely follow that of intramitochondrial concentration of Ccm1p

We then assessed how the *in vivo* system responded to the variation of the intramitochondrial Ccm1p concentration by measuring the levels of total 15S rRNA. The "Back on" status was a confirmatory second dose-response counterpart. Ccm1pZZ was used in this part of the study because the ZZ moiety was essential to measure the intramitochondrial protein concentration by ELISA, increase the sensitivity of the immunoblot analysis, and enhance the "Off" effect (Fig. 6a). Levels of 5' and 3' UJs were measured for both Ccm1pZZ (Fig. 6b) and Ccm1pAPC (Fig. 6c). After 24 h under "Off" status, segregants harboring pCCM1ZZLC presented a Ccm1pZZ concentration of approximately 5 fmol per μg of mitochondrial protein while the total 15S rRNA levels

remained unchanged (Fig. 6b, *insert*). Between 48 and 96 h of repression, the Ccm1pZZ concentration never reached 0, but rather maintained a constant value of approximately 1 fmol per μg of mitochondrial protein. These low levels of Ccm1p may account for the fact that the 15S rRNA transcript was never found 100% unprocessed. After 48 h under "Off" status, the total 15S rRNA levels abruptly fell more than 20-fold (Fig. 6b, c, *inserts*), while the cells' growth on YEPG was 100 times lower than that of the wild type (Fig. 4a). Additionally, the Ccm1pZZ clones presented less 15S rRNA than those harboring Ccm1pAPC over the entire "Off" period ($p < 0.05$; Fig. 6b, c), but the amount of *CCM1* transcript was similar in both cases ($p > 0.05$, data not shown). This effect agreed with the *en route* delay in the protein import caused by the ZZ tag. Remarkably, the percentages of 5' and 3' UJs that represent the unprocessed 15S rRNA progressively increased several- fold during the "Off" phase, reaching, at 96 h, their maximum value between 20 (Ccm1pAPC 3' and 5' UJs; Ccm1pZZ 5'UJ) to 50 times (Ccm1pZZ 3'UJ) over that of 0 or 24 h ($p < 0.05$). Once the Ccm1p supply was restored (right after 96 h under "Off" status) for both Ccm1p forms (Fig. 6a, *insert* "Back on"), the levels of both UJs diminished to negligible values (second dose-response) with a concomitant increase of processed 15S rRNA. This observation strongly suggests that the peaks at 96 h belong to authentic unprocessed UJs rather than abnormal forms of 15S rRNA.

In addition to the higher percentage of 3' UJ, the total 15S rRNA decay appears to be more abrupt for the ZZ form-expressing clones. Yet again, these results concur with others of SMDC, which allowed for a faster 15S rRNA primary transcript accumulation. This buildup of the unprocessed molecule might be due to a saturation of the mtEXO activity. Nevertheless, the profiles of 15S rRNA and UJs levels over the "On-Off-Back on" time-course were in agreement for both *CCM1* constructs. We did not extend the "Off" time beyond 96 hours due to the risk of damaging the mtDNA.

Ccm1p and mtEXO act concertedly but oppositely in the processing/degradation of 15S rRNA

The significant accumulation of 3' and 5' UJs (Fig. 6b, c) was investigated in more detail with a study based on Ccm1p/mtEXO double deletant cells. mtEXO is composed of *DSS1* and *SUV3* gene products (Dziembowski et al. 1998). The deletion of either gene caused substantial accumulation of not only unprocessed 15S rRNA transcripts but also of the processed form, demonstrating that mtEXO is responsible for surveying and degrading the initial 15S rRNA transcript (Dziembowski et al. 2003). RNA hybridization analysis showed that *CCM1*/*DSS1* (+/+) cells exhibited *only* processed 15S rRNA, but the Δ*ccm1/DSS1* (−/+) mutants did not display any form of 15S rRNA, not even degradation products. Single *CCM1*/ Δ*dss1* (+/−) mutants exhibited abundant processed 15S rRNA and limited amounts of non- processed transcripts (Fig. 7a, c), indicating that, as previously reported, mtEXO does not participate in the removal of intervening or flanking sequences (Dziembowski et al. 2003). Remarkably, Δ*ccm1* / Δ*dss1* (− / −) cells dramatically accumulated unprocessed transcripts, as evidenced by the abundant heavier forms. Specifically, a form that is approximately 100 nucleotides longer than the processed 15S rRNA was detected in the double mutant (Fig. 7a, 4th lane, hollow arrowhead). As the sum of the independent measurements of 3' and 5' UJ levels equaled 100% of the total unprocessed 15S rRNA (Fig.

7d, 4th set of bars), every species displayed in Δ*ccm1* / Δ*dss1* (−/ −) has at least one UJ intact. Therefore, we concluded that such a form was another degradation product from the initial transcript. Furthermore, we compared our results to the ones previously reported (see Fig. 6b, 15S rRNA panel; Dziembowski et al. 2003) in which both independent mutants, Δ*dss1* and Δ*suv3*, were wildtype for *CCM1*. Their patterns of degradation were strikingly similar to the one displayed in the fourth lane of Fig. 7a, which had a Δ*ccm1* background. These observations indicate that Ccm1p does not participate in the stabilization of the 15S rRNA primary transcript *per se*.

Importantly, the Δ*ccm1* / Δ*dss1* (−/ −) mitochondrial genome contained *RPM1*, which encodes for the RNA component (9S RNA) of the RNAse P (Stribinskis et al. 1996), as evidenced by DNA hybridization analysis. An *EcoR*V DNA fragment of approximately 3400 bp, which includes *RPM1* along with its promoter region was detected in all the genetic backgrounds examined in this experiment. (Fig. 7b). Therefore, we ruled out that the substantial accumulation of unprocessed 15S rRNA was on account of the absence of 9S RNA activity.

Quantitation by RT-qPCR detected over 30 times more total 15S rRNA in Δ*ccm1* / Δ*dss1* (−/ −) cells than in Δ*ccm1/DSS1* (−/+) mutants ($p < 0.01$) (Fig. 7c, 4th bar); the most abundant RNA was the 3’ UJ-containing transcripts ($p < 0.05$, Fig. 7d, black bar). Consequently, the results due to the lack of mtEXO agree with the peaks of 3’ and 5’ UJs levels (Fig. 6b, c) in which the surveillance system appears to be saturated, and 100 % of the cells present the mutant phenotype (Fig. 4a).

Ccm1p resupply restores the processing of the initial 15S rRNA transcript in Δ*ccm1*/Δ*dss1* cells

The Δ*ccm1*/Δ*dss1* diploid mutant was prepared with cells that harbored pCCM1ZZLC at the moment of mating, to produce Δ*ccm1*/Δ*dss1* meiotic segregants that would be competent for the “On-Off-Back on” experiment. The “Back on” approach aimed to discern whether the longer transcripts observed in the Δ*ccm1*/Δ*dss1* cells (Fig. 7a, 4th lane), were the result of the lack of Ccm1p or just aberrant RNA forms. We obtained seven Δ*ccm1*/Δ*dss1* meiotic segregants harboring pCCM1ZZLC, that under “Off” conditions, showed similar baseline levels of *CCM1* mRNA, total 15S rRNA, and processed 3’ junction (Fig. 8). When transferred to SDGal and incubated for 48h (“Back on”), we found that three of them were unresponsive to induction by galactose as they did not differ from the ones under “Off” conditions. These clones were considered as a second negative control. These results were foreseeable since Δ*ccm1*/Δ*dss1* nascent segregants harboring pCCM1ZZ in “Off” status would have a borderline reduced fitness. It is noteworthy to mention that the established Δ*ccm1* mutant (ρ^0/ρ^-) is unable to operate the *GAL1* promoter due to reduced fitness (unpublished data), but nascent non-complemented segregants still conserved that capacity (Fig. 3, *insert*). However, four clones were responsive to galactose, as shown by the significant increase in *CCM1* mRNA ($p < 0.05$), and were able to process the 15S rRNA transcript in substantial quantities ($p < 0.05$). These results confirmed that the abundant heavier species observed in the Δ*ccm1*/Δ*dss1* cells (Fig. 7a, 4th lane) were authentic unprocessed forms, which could be cleaved under “Back on” conditions.

Discussion

Several studies reported that PPR proteins are implicated in RNA processing (Manna 2015). PTCD1, a human protein that contains eight PPR motifs (Rackham et al. 2009), is involved in processing the mitochondrion heavy strand polycistronic transcript (Sánchez et al., 2011; Perks et al. 2016). This molecule includes RNA19, a precursor that consists of 16S rRNA (a component of the large mitoribosome subunit) linked at the 3' end to mt-tRNA$^{L(UUR)}$ and ND1 mRNA (Bindoff et al. 1993). When PTCD1 is not expressed, levels of 16S rRNA decrease dramatically, but heavier precursor molecules (including RNA19) become detectable by RNA hybridization analysis, albeit in much lower concentration than those of the mature rRNA (Perks et al. 2016) as it happens with 15S rRNA in baker's yeast (this study).

Similarly, another mammalian PPR protein, PTCD2, participates in the processing of the *ND5- COB* bicistronic transcript (Xu et al. 2008), which includes an intervening sequence of 600 nucleotides, not flanked by tRNAs (Anderson et al. 1981). Mice expressing a deficient PTCD2 are unable to process the bicistronic transcript; instead, they accumulate large amounts of the precursor molecule (Xu et al. 2008). However, it is not degraded by the RNA surveillance system (Szczesny et al. 2010).

Yeast Pet309p and Aep3p participate in the processing of *COX1* mRNA and the *ATP8/6* bicistronic transcript, respectively. Mutations that inactivate or delete the proteins lead to the complete disappearance of the *COX1* transcripts (Manthey and McEwen 1995) or the processed *ATP8/6* mRNA with the concomitant accumulation of trace levels of its unprocessed precursor (Ellis et al. 2004). Likewise, suppression of Ccm1p expression reduces mature *COB* and *COX1* mRNAs to undetectable levels (Moreno et al. 2009; 2012). However, the precursor remains visible in minimal amounts throughout the entire "Off" time course at approximately 5 % of the initial levels of the processed transcript (Moreno et al. 2012).

In vitro splicing experiments in the absence of Ccm1p showed that only minute amounts of the full-sized bI4 intron (1417 nucleotides) could be removed. Moreover, under such conditions, the bI4 intron had to be minimized down to 380 nucleotides for efficient splicing (Boniecki et al. 2009). We had proposed that Ccm1p would be able to act on long stretches of RNA that are fated to be removed, thus yielding a bI4 intron structure that is competent to undergo excision (Moreno et al. 2009). Accordingly, Ccm1p acts on the removal of long stretches of nucleotides from the 15S rRNA primary transcript (this report).

The "On-Off" approach was used by an earlier study to determine that the first activity of Ccm1p was essential to the splicing of *COB* and *COX1* pre-mRNAs (Moreno et al. 2009). Follow-up research provided clear insights into the molecular events that took place during SDMC (Moreno et al. 2012), which is the "Off" phase in the present study. During the entire "On-Off-Back on" time course, we have demonstrated that: (i) the mutant phenotype (i.e., failure to grow in the presence of glycerol as the sole source of carbon and energy) acquired during the "Off" phase was reversible upon Ccm1p resupply; (ii) the recovery of the wild-type phenotype was achieved at approximately the same rate as the preceding "Off" phase;

(iii) the phenotype changes followed the intramitochondrial Ccm1p levels; (iv) the capacity of mitochondria to import Ccm1p was maintained; (v) no ρ^- or ρ^0 cells were observed during the entire "On-Off-Back on" course which agrees with points (i) and (sii); (vi) total 15S rRNA levels directly followed the intramitochondrial concentrations of Ccm1p during the complete "On-Off-Back on" course. Consequently, we concluded that this approach was a suitable new triphasic *in vivo* dose-response system for experimentation. We performed preliminary experiments in which the "Back on" phase was started from the "Off" state at earlier times (65 h and 72 h) upon replacing glucose by galactose, thus reinducing the *GAL1* promoter. In all cases, including 96 h under "Off" status, we consistently obtained the same pattern of phenotype loss and recovery (data non-shown). Therefore, there was no credible possibility of suppressor mutation events during the off-state period.

mtEXO is a physiological complex of the RNA metabolism that is required to eliminate unprocessed and aberrant RNAs (Dziembowski et al. 2003). This present work addresses the axiom "*any unprocessed RNA molecule is destined to be degraded*." Once our *in vivo* system "On-Off-Back on" was validated, we investigated how the primary15S rRNA transcript underwent processing as the intramitochondrial concentration of Ccm1p varied. Levels of both 5' and 3' UJs gradually increased (probably due to mtEXO saturation) reaching their highest values when the cells fully acquired the mutant phenotype at a Ccm1p concentration of approximately 1 fmol per μg of mitochondrial protein. The last "Off" time point (96 h) showed the maximal accumulation of both 3' and 5' UJs. Conversely, the proportion of unprocessed 15S rRNA forms fell upon Ccm1p resupply with a simultaneous increase of total levels of 15S rRNA and recovery of the wild-type phenotype. The 15S recovery in the case of Ccm1pZZ was slower than that of Ccm1pAPC (Fig 6b and c, inner panels, respectively). This result is in agreement with the import delay scenario caused by the ZZ tag. In the case of Ccm1pAPC (no addition of extra sequences), no lag was observed as no hindrance in the import process occurred. There was a six hour-interval between measurements, long enough for the production and degradation of other proteins that may be involved in this process. Nevertheless, we do not discard the possibility of a direct action of Ccm1p on the 15S rRNA initial transcript. Furthermore, the abrupt switch "Off" using Ccm1pZZ caused a higher accumulation of 3' UJ than of 5'UJ, indicating that the 3' end processing appears to be more Ccm1p dependent than its 5' counterpart. These results suggest that Ccm1p acts on both UJs likely associated with different sets of factors.

To confirm these findings independently, we focused on the unprocessed transcripts observed in the "Off" phase. The presence of 15S rRNA and related precursors were examined in Δ*dss1* and Δ*dss1*/Δ*ccm1* mutants. The Δ*dss1* mutant exhibited mostly processed 15S rRNA and low levels of longer forms. These results confirmed that mtEXO does not participate in the removal of the 5' or 3' flanking sequences (Dziembowski et al. 2003). In agreement with our "On-Off-Back on" results, the Δ*dss1*/ Δ*ccm1* mutant exhibited a significantly higher abundance of unprocessed 15S rRNA species in which the 3' UJ was predominant. That the absence of Ccm1p causes the accumulation of such abundant quantities of precursors, which contain mature 15S rRNA, is a clear indication that Ccm1p is neither a stabilizer nor a factor to maintain the integrity of this molecule. Likewise, the absence of mtEXO activity allowed us to visualize such unprocessed forms.

Additionally, by resupplying Ccm1p to this double mutant, the amount of processed 3' junction increased significantly. These results demonstrated that mtEXO is responsible for the degradation of unprocessed 15S rRNA in the Δ*ccm1* mutant strain. Therefore, in addition to being a splicing factor (Moreno et al. 2009; 2012), this protein is essential to process the 15S rRNA primary transcript. Consequently, Ccm1p may be ruled out as a stabilizer, proper assembler, or chaperon to the mature 15S rRNA before it can be fully integrated into the mitoribosome, neither it has a *direct* role in preventing the degradation of mitochondrial rRNA.

The elimination of the 5' 0.5S rRNA is accomplished by a purportedly 5'→3' exonuclease activity associated with Pet127p (Fekete et al. 2008) that trims the 5' terminal end of the 15.5S rRNA (Wiesenberger and Fox 1997). According to our results, Ccm1p could be a molecular partner of Pet127p to control such exonucleolytic activity; it could act as a signal to anchor the enzyme associated with Pet127p or promote a putative-Ccm1p dependent endoribonuclease.

The processing of the 3' junction has some remarkable features. When released from the initial transcript, the 1308-nucleotide intervening sequence was not degraded by mtEXO since a probe that specifically hybridized to that region failed to detect a signal in RNA isolated from Δ*dss1* mutants (Dziembowski et al. 2003). A possible candidate to degrade this sequence is Dis3p, which has 3' → 5' exonuclease activity and localizes to the mitochondrion (Turk et al. 2013). However, no data regarding the elimination of the free intervening sequence has been reported so far, and no specific or consensus signal for endonucleolytic activity has been found at the 3' end of it (Turk et al. 2013). An heptakaidecamer cleavage site has been located approximately 3kb downstream of the end of the 15S rRNA- $tRNA^W$ transcript (Turk et al. 2013), too far to yield the mature 15S rRNA. In this case, as it was proposed earlier regarding the removal of the *COX1* aI4 and *COB* bI4 introns (Moreno et al. 2009), Ccm1p acts on long stretches of RNA, at the boundaries of the 3' junction, to assist the 15S rRNA precursor in acquiring a competent structure for a precise cleavage. This situation resembles that of mammalian ND6 mRNA (Van Haute et al. 2015). The mature ND6 transcript lacks a consensus sequence at the 3' end (Slomovic et al. 2005), there is a 5700-nucleotide spacer between it and closest tRNA (Anderson et al. 1981), and the processing of the 3' junction involves an RNA-binding protein, FASTK (Jourdain et al. 2015).

Like any *bona fide* PPR protein, processing the 15S rRNA primary transcript and splicing the pre- mRNAs obviously imply *in vivo* Ccm1p-RNA interactions. However, both activities reside at different PPR2 amino acids (Moreno et al. 2012). Along the same lines, replacement of *S. cerevisiae* Ccm1p by that of *S. bayanus* also dissects both functions since it reduces 15S rRNA levels without affecting the removal of introns (Jhuang et al. 2017). Interestingly, mutations of two residues in the PPR2 domain rescue the incompatibility.

In *S. cerevisiae*, the nuclear equivalent version of the cytosolic ribosome consists of one primary transcript (35S pre-RNA) that bears three of the four required rRNAs. The processing of this molecule follows a well-known pathway involving many factors and ribonucleases (Fang et al. 2005). The primary transcript has two internal spacers, ITS1 and

ITS2. Particularly, removal of ITS2 is initiated through a specific cleavage inside the spacer by the complex Las1/Crc3 followed by exoribonucleases that eliminate the ITS2 producing the 5.8S and 25S of the 60S ribosomal subunit (Pillon and Stanley 2018). In the mitochondrial case, the scenario seemed somehow related due to the presence of an intervening sequence between an rRNA and a tRNA, but the complete mechanism and identity of the factors involved are still unclear. Based on our findings, particularly the ones obtained with the Δ*dss1*/Δ*ccm1* mutant (Fig. 7), we propose that once the $tRNA^W$ has been excised by RNase P and RNase Z (Daoud et al. 2012), the spacer 3' end is exposed. When it remains bound to 15S rRNA, due to the lack of Ccm1p, this region acts as the signal recognized by mtEXO to anchor and commence the degradation of the unprocessed 15S rRNA-spacer transcript until the entire molecule is degraded. Conversely, if the spacer is removed with the assistance of Ccm1p, mtEXO cannot reach the 15S rRNA moiety, which is protected by primary binding ribosomal proteins. In fact, Nam9p, one of the first ribosomal proteins to bind 15S rRNA (Gan et al. 2002; Shajani et al. 2011), protects it from degradation (Biswas and Getz 1999). Thermosensitive *NAM9* strains lose their mature 15S rRNA at the nonpermissive temperature, which proves that the actual 15S rRNA stabilizers are the primary proteins bound to it during the biogenesis of the mitoribosome minor subunit.

By two independent approaches, the "On-Off-Back on" time course and the Δ*ccm1*/Δ*dss1* double mutant, we have demonstrated that the 15S RNA primary transcript has only two alternatives: *Either* it is processed and incorporated into the mitoribosome minor subunit, *or* it is degraded by the mitochondrial RNA surveillance system as a consequence of processing failure in Δ*ccm1* strains.

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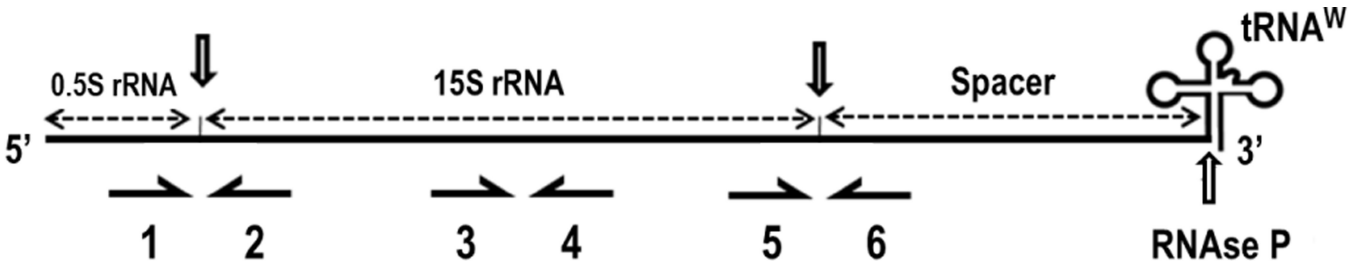

Fig. 1.

Scheme of the 15S rRNA initial transcript. Downward pointing arrows indicate the processing sites studied in this paper. Horizontal arrows (1 to 6) show the location of the primers used to assess the levels of unprocessed joints and total 15S rRNA

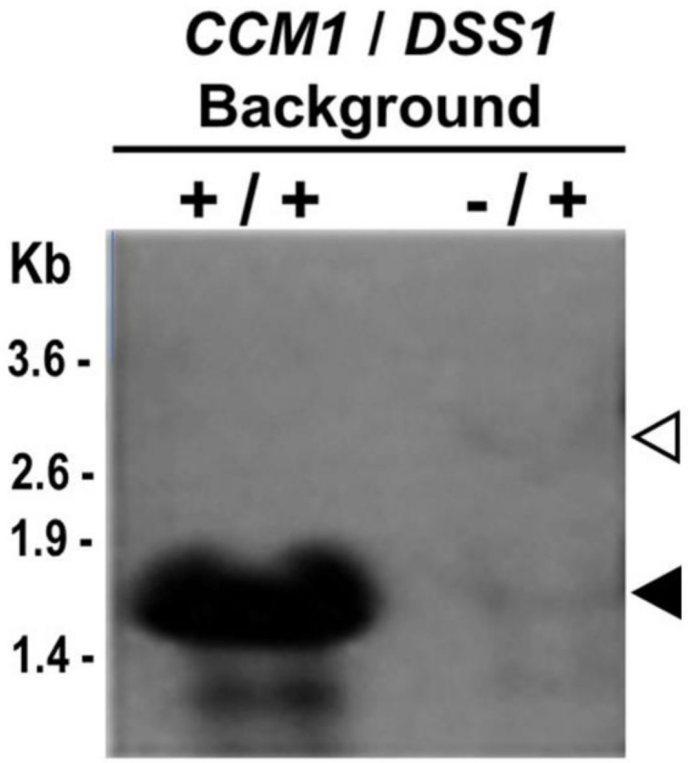

Fig. 2.

Non-complemented Δ*ccm1* nascent meiotic segregants exhibit unprocessed 15S rRNA. Hybridization analysis of total RNA of 10 and 15 μg per well from *CCM1* and Δ*ccm1* strains, respectively. The filter was probed with a 204 bp-biotinylated *15S R_RNA* DNA probe that spans positions 7167–7370 of the mitochondrial genome, within the mature transcript. Filled and hollow arrowheads indicate the position of processed and non-processed forms, respectively

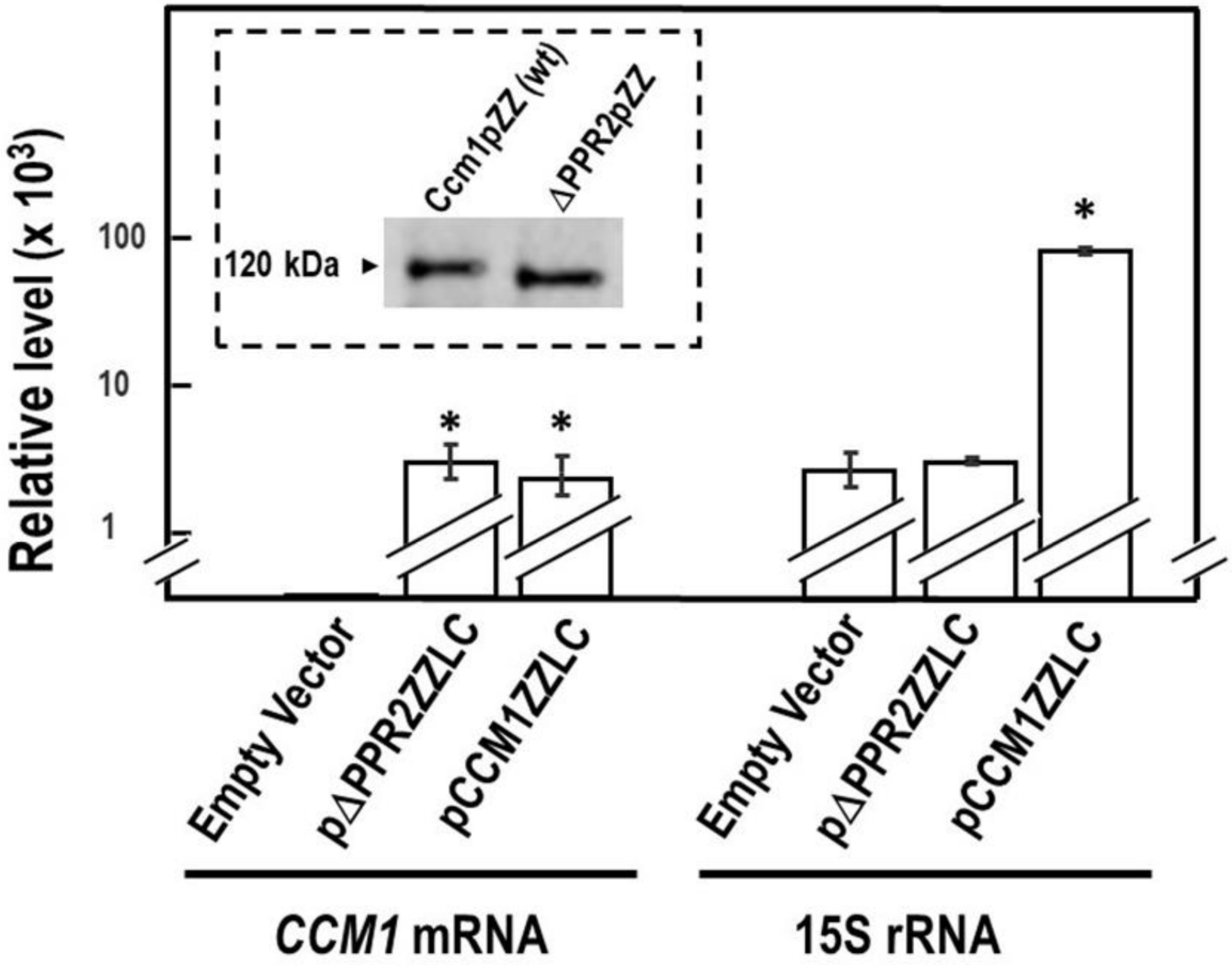

Fig. 3.

Ccm1p is imported by non-functional mitochondria. Δ*ccm1* cells harboring the empty vector, pΔPPR2ZZLC, or pCCM1ZZLC were grown in SDGalfthe ones previously reported or 24h. Their relative levels of 15S rRNA and *CCM1* mRNA were measured by RT-qPCR. *Columns in association with vertical bars* represent the means ± SEM of three independent clones measured in duplicate. *ALG9* mRNA was the reference transcript. *, $p < 0.05$; one-way analysis of variance, Student-Newman- Keuls' post hoc test. *Insert:* Crude extracts of purified mitochondria from strains expressing wild-type Ccm1pZZ and Ccm1ΔPPR2p (ΔPPR2pZZ) were electrophoresed in a 5–20 % gradient SDS-PAGE until the 35 kDa marker ran off. Proteins were transferred to a nitrocellulose membrane and probed with polyclonal antibodies against Ccm1p produced in *E. coli*

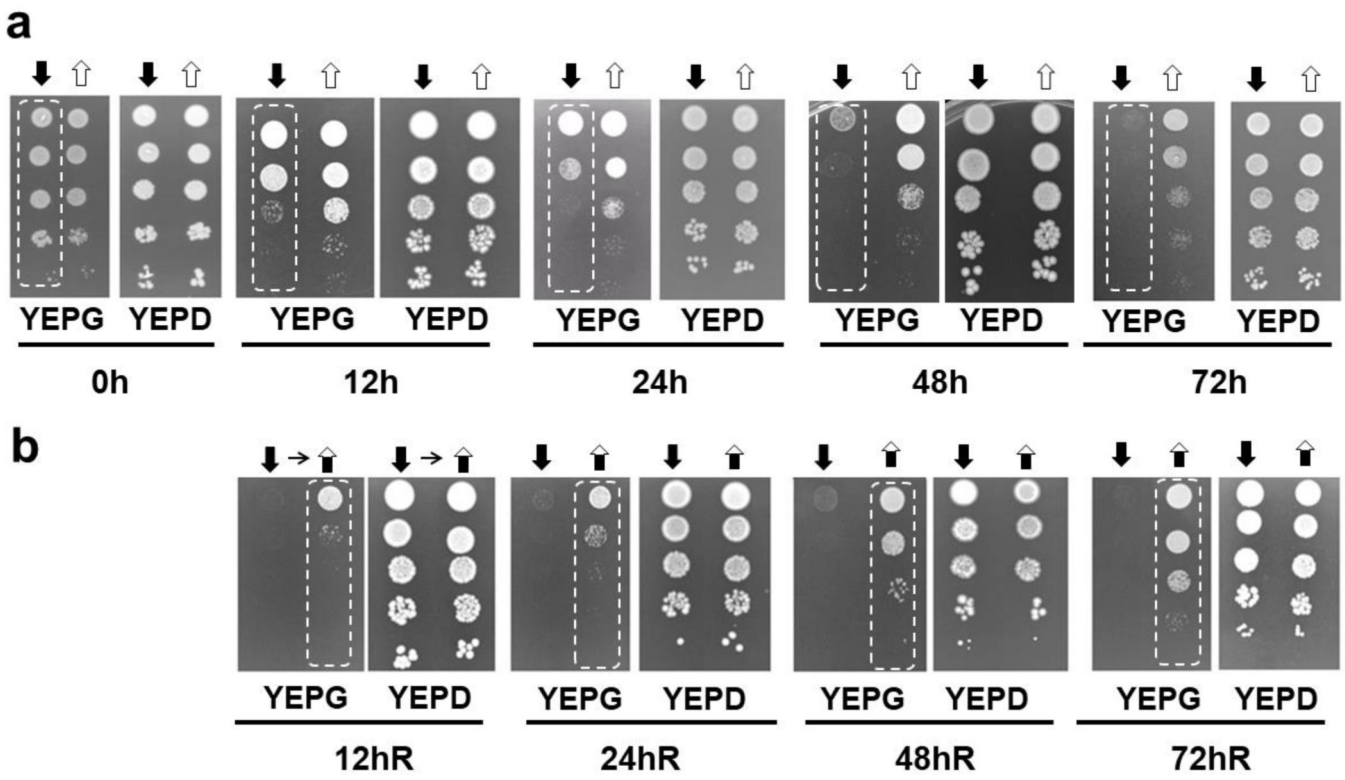

Fig. 4.

Progression of the phenotype throughout the "On-Off-Back on" time-course. **a** A inoculum was taken from a master plate of YEPG-geneticin and grown in SDGal for 48 h ("On"; t = 0 h). Cultures in SSD ("Off" [⬇]) and SDGal ("On" [⇧]). **b** From the 72h-SDD culture ("Off"), cells were either grown in SDD ("Off" [⬇] or SDGal ("Back on" [⬆]), as shown by the small horizontal arrow. White-dashed rectangles indicate the acquired transient mutant and wild- type phenotype during the entire experimental course

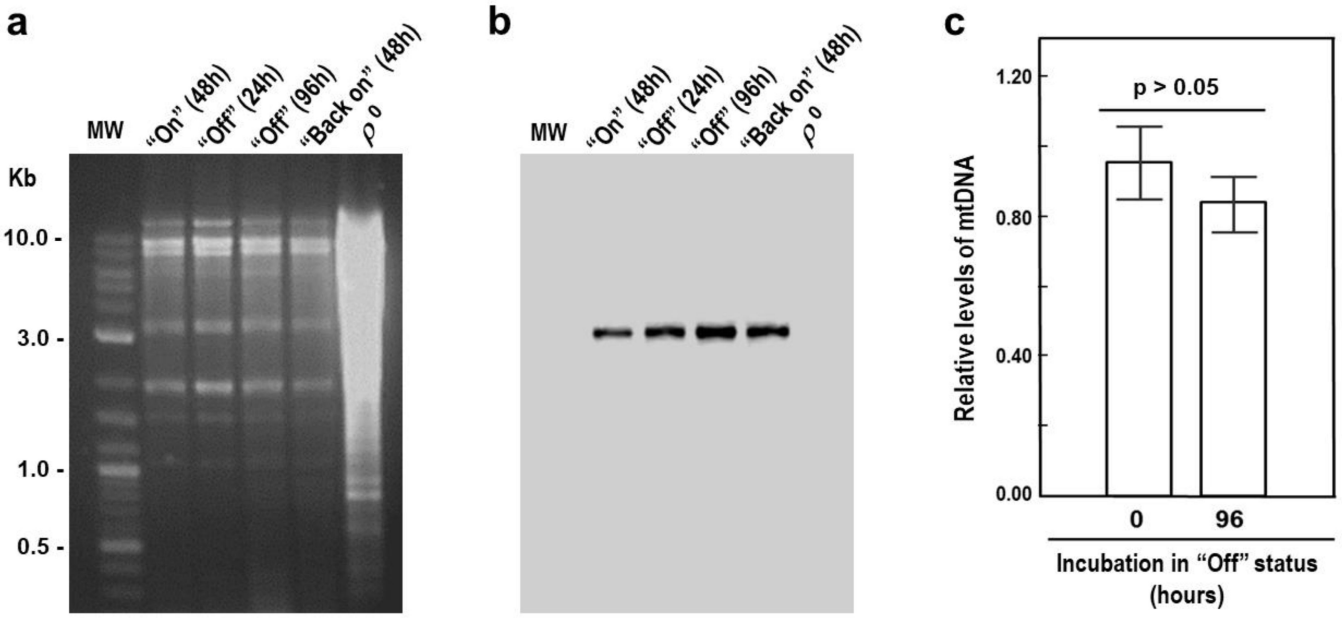

Fig. 5.

Mitochondrial DNA (mtDNA) does not undergo modifications during the "On-Off-Back on" time-course experiment. **a** Ethidium bromide-stained agarose gel electrophoresis of *EcoR*V-digested mtDNA isolated from clones grown under "On," "Off," and "Back on" conditions. MW: 2-log DNA ladder (New England Biolabs). ρ^0 lane: total DNA was purified from an established Δ*ccm1* strain. **b** DNA hybridization analysis of **a** using a 290 bp-biotinylated *COB* DNA probe that spans positions 28033–28322 of the mitochondrial genome. **c** Relative levels of mtDNA were assessed by qPCR using *15S R_RNA* and *ALG9* as target and reference genes, respectively. *Columns in association with vertical bars* represent the means ± SEM of three independent Ccm1pZZ-expressing clones measured in duplicate. The values were not found significantly different by a two-sample *t*-test.

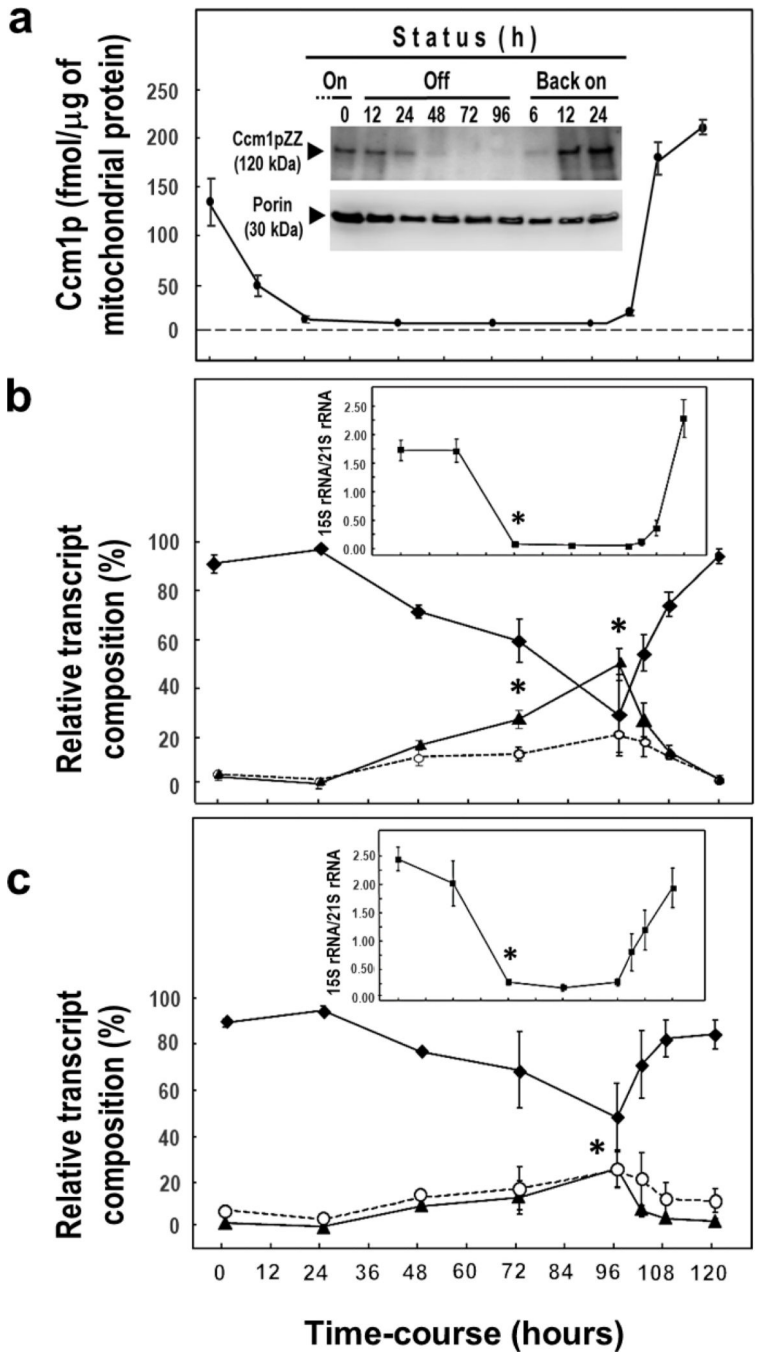

Fig. 6.

Accumulation of unprocessed 15S rRNA transcripts during the “Off” status reaches significant levels at 96 h. “Time course (hours)” at the bottom of the chart represents the total span of the “On-Off-Back on” experiment. **a** Intramitochondrial Ccm1p levels at “On” (24 h in SDGal medium is t = 0 h), “Off” (t =12 to 96 h), and “Back on” (t =102 to 120 h) during the time-course experiment. Quantitation was carried out by ELISA. *Insert*: Ccmp1 was visualized by 5–20% PAGE and immunoblot analysis with an anti-Ccm1p polyclonal antibody. Times for each status are designated at the top of the immunoblot. Porin was used

as the loading control. **b** Percentage of 3’ unprocessed junctions (−▲−), 5’ unprocessed junctions (---○---), and mature 15S rRNA [i.e. total 15S rRNA – (3’ unprocessed junctions + 5’ unprocessed junctions)] (−◆−) was measured by RT-qPCR as the levels of Ccm1pZZ or (**c**) Ccm1pAPC changed. *Inserts in **b** and **c*** Relative levels of total 15S rRNA, 21S rRNA was used as the reference transcript. Samples for analyses in **a**, **b**, and **c** came from a common set of cultures. *Symbols in association with vertical bars* represent the means ± SEM of three independent clones measured in duplicate. For statistical analysis, data were log-transformed when necessary to normalize the variables or homogenize the variances. *, $p < 0.05$, one-way ANOVA, Student-Newman-Keuls post hoc test. Downward pointing arrowheads indicate the last “On” time point and the initiation of the “Back on” status

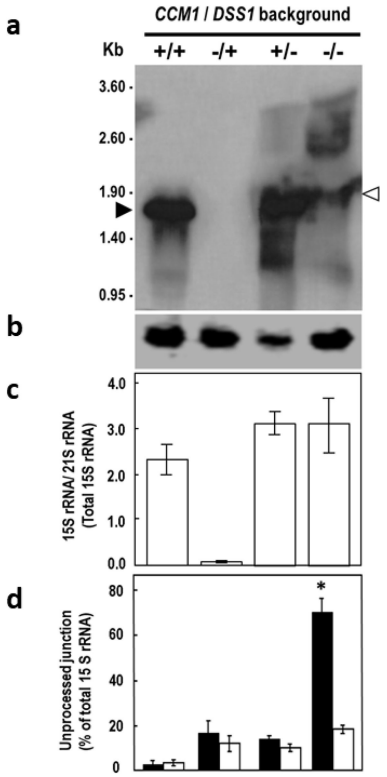

Fig. 7.

The Δ*ccm1* / Δ*dss1* (−/ −) mutant displays unprocessed, rather than mature 15S rRNA. **a** Hybridization analysis of mitochondrial RNA. Total RNA (10–15 μg per well) was isolated from strains of *CCM1/DSS1* (+/+), Δ*ccm1*/ *DSS1* (−/+), CCM1/ Δ*dss1* (+/−), and Δ*ccm1* / Δ*dss1* (−/ −) genetic backgrounds and probed with a 585 bp-biotinylated *15S R_RNA* DNA fragment that spans positions 6785–7369 of the mitochondrial genome, inside the mature transcript. **b** Hybridization analysis of *EcoR*V-digested mtDNA (4 μg per well) with a 209 bp-DNA probe that anneals to a fragment that contains the full-length *RPM1* gene and its promoter. RT-qPCR assessment of (**c**) relative levels of total 15S rRNA and (**d**) 3' UJ (black columns) and 5' UJ (white columns). 21S rRNA was the reference transcript. The assays depicted in **b**, **c,** and **d** correspond to samples from the set of mutants shown in **a**. Measurements in **c** and **d** for each strain were independent. *Columns in association with vertical bars* represent the means ± SEM of three independent clones measured in duplicate. *, $p < 0.05$, one-way ANOVA, Student-Newman-Keuls post hoc test

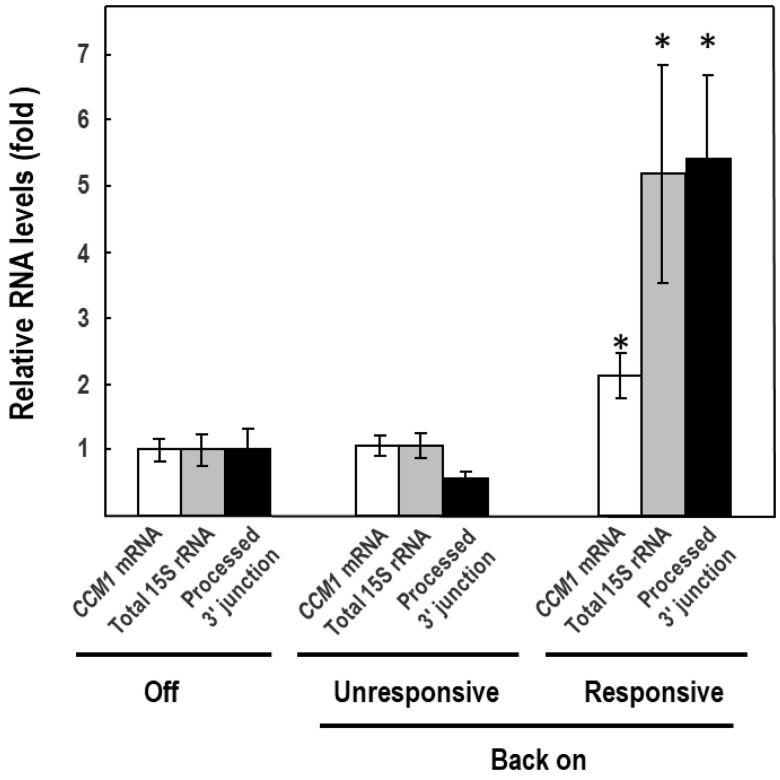

Fig. 8.

Processing of 3’ UJ is restored in Δ*ccm1*Δ*dss1* (−/ −) segregants upon Ccm1pZZ resupply (Back on). Relative levels of *CCM1* mRNA, total 15S rRNA, and 3’ processed junction (total 15S rRNA – 3’ UJ) were assessed by RT-qPCR. “Off” represents the baseline values obtained under SDD conditions (n = 7). *ALG9* mRNA was the reference transcript. *Columns in association with vertical bars* represent the means SEM of three independent clones measured in duplicate. Data were log-transformed to normalize the variables or homogenize the variances.*, $p < 0.05$, one-way ANOVA, Student-Newman-Keuls post hoc test

Table 1:

Primers used in this study

Template	Forward primer	Reverse primer	Amplicon size (bp)	Annealing position *(a)*	Reference
15S_RRNA 5'unprocessed junction	AGTTATATAATAAGGAAAAG (n°1)	AGCTTATTCTATAGTTCATT (n°2)	173	6527–6546, 6680–6699	This article
Total *15S_RRNA*	GTTAAACCTAGCCAACGATCCA (n°3)	TGTCCAATATTCCTCACTGCTG (n°4)	108	270–291, 356–377	Moreno et al. 2009
15S_RRNA 3'unprocessed junction	GCGAAGTTGAAATACAGTTACCG (n°5)	GGGCCCCGGAACTATTAATA (n°6)	123	8126–8148, 8229–8248	This article
ALG9	CACGGATAGTGGCTTTGGTGAACAATTAC	TATGATTATCTGGCAGCAGGAAAGAACTTGGG	161	236545–236573 236413–236443 (Chromosome XIV)	Teste et al. 2009
CCM1	CCAAACCTGAGACCACGGA	TAGCGGCTTTCATCACCAGCTC	509	793380–793404 792871–792895 (Chromosome VII)	Moreno et al. 2012
21S rRNA	CGGGTCCCGGAACTTAAATA	CGAGGTGGCAAACATAGCTT	221	2548–2567, 2553–2768	Moreno et al. 2012
DSS1	GTTTACAAATTGAATCGGATGACTC	TCAATATCAGGCTCTAATCCTTTTG	517	845573–845597, 845080–845104 (Chromosome XIII)	*Saccharomyces* Genome Deletion Project
kanMX4 module	N / A	CTGCAGCGAGGAGCCGTAAT	576 (*DSS1*) 675 (*CCM1*)	250 bp downstream of 5 '-end of the kanMX4 module	*Saccharomyces* Genome Deletion Project

(a) Mitochondrial genome unless otherwise indicated

Table 2:

Characteristics of qPCR products

qPCR amplicon	Melting temperature (°C)	Restriction endonuclease	Cleavage position
15S_RRNA 5'unprocessed junction	77.0	*Hinf*I	85
15S_RRNA	78.0	*Hinf*I	55
15S_RRNA 3'unprocessed junction	78.5	*Ssp*I	60
21S_RRNA	79.0	*Bfa*I	161
ALG9	83.0	*Age*I	89
CCM1	77.0	*Hinf*I	58