Two independent activities define Ccm1p as a moonlighting protein in Saccharomyces cerevisiae

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Synopsis
Ccm1p is a nuclear-encoded PPR (pentatricopeptide repeat) protein that localizes into mitochondria of Saccharomyces cerevisiae. It was first defined as an essential factor to remove the bI4 [COB (cytochrome b) fourth intron] and aI4 [COX1 (cytochrome c oxidase subunit 1) fourth intron] of pre-mRNAs, along with bI4 maturase, a protein encoded by part of bI4 and preceding exons that removes the intronic RNA sequence that codes for it. Later on, Ccm1p was described as key to maintain the steady-state levels of the mitoribosome small subunit RNA (15S rRNA). bI4 maturase is produced inside the mitochondria and therefore its activity depends on the functionality of mitochondrial translation. This report addresses the dilemma of whether Ccm1p supports bI4 maturase activity by keeping steady-state levels of 15S rRNA or separately and directly supports bI4 maturase activity per se. Experiments involving loss of Ccm1p, SMDC (sudden mitochondrial deprivation of Ccm1p) and mutations in one of the PPR (pentatricopeptide repeat) motifs revealed that the failure of bI4 maturase activity in CCM1 deletion mutants was not due to a malfunction of the translational machinery. Both functions were found to be independent, defining Ccm1p as a moonlighting protein. bI4 maturase activity was significantly more dependent on Ccm1p levels than the maintenance of 15S rRNA. The novel strategy of SMDC described here allowed the study of immediate short-term effects, before the mutant phenotype was definitively established. This approach can be also applied for further studies on 15S rRNA stability and mitoribosome assembly.

Key words: CCM1, mitochondria, moonlighting protein, splicing, stability of 15S rRNA, yeast.

INTRODUCTION

mtDNA (mitochondrial DNA) in Saccharomyces cerevisiae S288C contains nine group I introns: four in COB (cytochrome b), four in COX1 (cytochrome c oxidase subunit 1) and one in 21S RRNA [1]. Group I introns, so-called ‘molecular parasites’ or ‘infected introns’, are widespread mobile elements whose removal at RNA level is crucial for their own survival [2,3]. Although they self-splice in vitro, additional factors are required to assist this process in vivo [2–4]. Specific and non-specific nuclear-encoded proteins are required for splicing. For instance, Mss116p assists the splicing of all mtDNA introns [5], while Mne1p is only responsible for COXI aI5β intron removal [6]. Intron-encoded proteins, known as maturases, are involved in the removal of at least one intron that codes for them. Maturase mRNAs comprise part of the mature mRNA in the form of preceding exons and a large part of the intron to be removed [4]. Particularly, translation of the ORF (open reading frame) composed of the first four COB exons and part of the fourth intron [bI4 (COB fourth intron)] generates bI4 maturase. This protein participates in the excision of bI4 and aI4 (COX1 fourth intron) of their pre-mRNAs [7]. Selective pressure drives the maturases to recruit pre-existing nuclear-encoded proteins with non-related functions to assist splicing [1,3]. These factors, by acquiring a second function, become moonlighting proteins [8]. For instance, Pet54p participates in the excision of aI5β [9], but it is also required for translation of COX3 mRNA [10]. Both activities reside in a shared RNA-binding region [11]. Nam2p, a mitochondrial leucyl-tRNA synthetase is also involved in bI4 mitochondrial RNA splicing activity [12,13]. Ccm1p was reported to be essential for the removal of bI4 and aI4 [14], as well as with Nam2p [15]. Ccm1p has two PPR (pentatricopeptide repeat) motifs in tandem, located between amino acids 319 and 353 and amino

Abbreviations used: aI4, COX1 fourth intron; bI4, COB fourth intron; COB, cytochrome b; COX1, cytochrome c oxidase subunit 1; DB, dilution buffer; HRP, horseradish peroxidase; mtDNA, mitochondrial DNA; ORF, open reading frame; pAb, polyclonal antibody; PPR, pentatricopeptide repeat; qPCR, quantitative PCR; RT, reverse transcription; SD, synthetic defined; SMDC, sudden mitochondrial deprivation of Ccm1p; SPA, staphylococcal protein A; WB, washing buffer; YEP, yeast extract peptone

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acids 356 and 390 [16]. Both domains are required for activity [14]. The PPR family is composed of three degenerated domains that span between 31 and 36 amino acids [17]. PPR motifs occur in tandem arrays of 2–26 units per protein [18]. Each motif is predicted to comprise two anti-parallel α-helices that contain several projecting amino acidic side groups; therefore the arrays would form a superhelix with a binding surface that is suitable to interact with selected bases [19] and phosphate groups of RNA molecules [20]. PPR proteins bind to specific RNA sequences [21] and mainly participate in post-transcriptional events, such as RNA editing [22], translation [23], stability [21], processing [24] and splicing [14]. Further studies proved that Ccm1p was involved in RNA editing [22], translation [23], stability [21], processing [24] and splicing [14]. Evidence, that these two Ccm1p activities are independent of one another and that a PPR protein of fission yeast that belongs to the CCM1 family, Ppr3 [16] also stabilizes 15S rRNA [26]. These facts created a dilemma of whether Ccm1p directly participates in bI4 intron removal along with bI4 maturase, or supports the latter by stabilizing 15S rRNA, thus keeping the translational machinery functional. We undertook this work to answer the aforementioned question. Our results demonstrate, by three independent lines of evidence, that these two Ccm1p activities are independent of one another. Thus Ccm1p, a herefore unrecognized bi-functional PPR protein is, under an operational standpoint, a crucial factor that assists bI4 maturase activity and moonlights by keeping steady-state levels of mitochondrial 15S rRNA. Furthermore, lysine-conferred positive charges in the second PPR motif are required for fully efficient splicing activity but not for maintenance of 15S rRNA. Results presented in this paper exhibit the potential to tightly manipulate Ccm1p levels in order to study the pathway followed by 15S rRNA during mitoribosome assembly.

**EXPERIMENTAL**

**DNA constructs**

A 2 μm-based vector that harbours the CCM1 ORF fused at the N-terminus to a ZZ affinity tag under the control of the GAL1 promoter (pCCM1ZZ) was purchased from Open Biobiosys. A 5.5-kb DNA fragment, which contained the CCM1ZZ expression cassette plus 500 bp upstream and 1000 bp downstream of additional sequences, was produced by Lgul (Fermentas) digestion. The 5′ protruding ends of this gel-purified DNA fragment were blunt-ended with Klenow DNA polymerase (New England Biolabs) and ligated to the SmaI sites of pRS316, a low copy vector (Invitrogen). This new construct is referenced as pCCM1AAA throughout this study.

The short form of bI4 maturase, which only contained the last 254 amino acids at the C-terminal end, was synthesized by DNA 2.0, with the following modifications: five mitochondrial TrpTGA codons were changed to TrpTGG for cytoplasmic synthesis and the 12-amino acid signal peptide from a 70 kDa mitochondrial protein was fused to the N-terminus to enable mitochondrial import. The 800-bp BamHI/Xhol-flanked DNA fragment was inserted into the expression vector pYC2/CT, and named pbI4MAT. bI4 maturase expression was monitored by SDS/PAGE and Western blotting using pAb (polyclonal antibody) II (see ELISA section).

**Media and strains**

Yeast media were prepared as previously indicated [14]. The S. cerevisiae wild-type BY4741 strain (B) (MATα ura3Δ leu2Δ0 his3Δ1 met15Δ0) was a generous gift from Dr Dennis R. Winge (Departments of Medicine and Biochemistry, University of Utah, Health Science Center, Salt Lake City, UT, U.S.A.). S. cerevisiae harbouring intronless mitochondria (F’) (MATα ade1Δ0 lys1Δ0 ura3Δ0) 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, U.S.A.). B and F’ were mated with Δccm1 (MATα ura3Δ0 leu2Δ0 his3Δ1 lysΔ0 ccm1Δ0::kanMX) from Invitrogen to generate 2nB and 2nI heterozygous diploids respectively, which carry functional mitochondria. Yeast manipulation, including selection of heterozygous strains, maintenance and transformation, sporulation, tetrad dissection and further analysis of meiotic segregants were performed as described [14]. Δccm1 segregants complemented by pCCM1LC, pCCM1ZZLC, or pCCM1AAA were stored in rich medium [YEP (yeast extract peptone)] with glycerol and G418 or uracil free-SD (synthetic defined) medium with glycerol at 4°C for not longer than 3 weeks.

**DNA, RNA and protein analysis**

Nucleic acid isolation and Northern blotting were performed as previously described [14]. mtDNA levels were assessed by qPCR (quantitative PCR) using COX1 [14,27] and 15S_RRNA as mtDNA markers and ACT1 as nuclear marker in the Smart Cycler II thermal cycler (Cepheid). For Northern blotting, the 15S rRNA probe spanned 204 bp from position 654 to 857. Signals were visualized and photographed with a gel documentation system (FluorChem SP, Alpha Innotech Corporation). Levels of mitochondrial rRNAs, ACT1, CCM1 and immature COB (exon 4–intron 4 boundary) transcripts as well as maturase activity were determined by RT-qPCR (reverse transcription-qPCR) (Table 1). bI4 maturase activity was determined by priming at either COB or COX1 exon 4–exon 5 boundary [14]. Calibration curves...
converted $C_t$ (threshold cycle value) into starting amounts of template for each qPCR template–primer combination. DNA templates were obtained by purifying amplicons from end-point PCR or qPCR with the QIAEXII kit (Qiagen) following the manufacturer’s instructions, and confirmed by both $T_m$ (melting temperature) and restriction analysis. Their concentrations were determined spectrophotometrically. The slope and $R^2$ value of each calibration curve were calculated with the SigmaStat statistical software (SPSS). $R^2$ values were at least 0.994. Primer sequences, annealing positions and amplicon size are indicated in Table 1. For SDS/PAGE and Western blotting [28], yeast crude extracts were prepared in the presence of lysis buffer [130 mM EDTA, 130 mM EGTA, one tablet of Complete Protease Inhibitor (Santa Cruz Biotechnology) per ml and 2× Protease Inhibitor Cocktail (Sigma–Aldrich) in 1× PBS]. Cells were mechanically disrupted with 0.5 mm diameter glass beads for 2 min at room temperature (25°C) using a cell disruptor (Scientific Industries). Triton X-100 was then added up to 1% and disruption continued for an additional 2 min. Crude extracts were then clarified by centrifugation at 4°C for 5 min at 20000 g. Total protein contents were determined using the Bradford assay and BSA as standard (Thermo Fisher Scientific).

**Time-course experiments**

For mtDNA studies, 56 colonies (approximately 25 generations from a single cell) of Δccm1 non-complemented nascent meiotic segregants from 2nI or 2nB were harvested from YEP with galactose master plates and pooled. A fraction of the cells (1/200) were used to inoculate fresh YEP with dextrose and subsequent cultures. The remaining cells represented the initial time point ($t = 0$). Over 4 days, changes in the mitochondrial genome were monitored every 24 h as Ccm1p and functional mitochondria were thus oxidized for 1 h at room temperature in the dark. The oxidation mixture was then changed to PBS (0.1 M sodium phosphate and 0.15 M NaCl, pH 7.2) using a 2 ml-Zeba Desalt Spin Column (Thermo Fisher Scientific). Then 0.9 ml of antibody-containing effluent was combined with 0.1 ml of 50 mM EZ-link biotin hydrazide in DMSO (Thermo Fisher Scientific) and incubated for 2 h at room temperature. Biotinylated pAbII was separated from free biotin by passing the reaction mixture through a PBS equilibrated-2 ml Zeba Desalt Spin Column. The biotinylated pAbII was stored at 4°C in the presence of 0.01% thimerosal. Maximal signal for the biotinylated pAbII was obtained at a 1:5000 dilution of the final preparation indicated above. Streptavidin poly-HRP (horseradish peroxidase) (Thermo Fisher Scientific) was used as a 1:5000 dilution of the stock solution (0.5 mg/ml). The reaction was developed with TMB (3,3′,5,5′-tetramethylbenzidine) One Component HRP Microwell Substrate (BioFX Laboratories) and stopped with 0.5 M sulphuric acid. Absorbance at 414 nm was measured using a Multiskan EX ELISA reader (MTX Lab Systems).

**bl4 maturase protein measurement by ELISA**

The peptide epitopes LNTKQLNNFVLKFNWTKQ (I) and CPSKSNKGKRLFLIDKF (II) present in the bl4 intron-encoded maturase moiety were designed, synthesized and purified by 21st Century Biochemicals. The peptides were analysed by nanospray MS and HPLC analysis and the sequence verified by collision-induced dissociation MS/MS (tandem MS). Rabbit pAbs against these peptides, pAbI and pAbII respectively, were produced and affinity-purified by 21st Century Biochemicals. The capture antibody (pAbI) concentration was standardized for maximal signal at 40 ng/100 μl per well. The reporter antibody (pAbII) was biotinylated as follows: 0.2 mg of affinity purified antibody was mixed with 2 mg of sodium meta-periodate in 1 ml of 0.1 M sodium acetate buffer, pH 5.5. The antibody carbohydrate residues were thus oxidized for 1 h at room temperature in the dark. The oxidation mixture was then changed to PBS (0.1 M sodium phosphate and 0.15 M NaCl, pH 7.2) using a 2 ml-Zeba Desalt Spin Column (Thermo Fisher Scientific). Then 0.9 ml of antibody-containing effluent was combined with 0.1 ml of 50 mM EZ-link biotin hydrazide in DMSO (Thermo Fisher Scientific) and incubated for 2 h at room temperature. Biotinylated pAbII was separated from free biotin by passing the reaction mixture through a PBS equilibrated-2 ml Zeba Desalt Spin Column. The biotinylated pAbII was stored at 4°C in the presence of 0.01% thimerosal. Maximal signal for the biotinylated reporter pAbII was obtained at a 1:5000 dilution of the final preparation indicated above. Streptavidin poly-HRP (horseradish peroxidase) (Thermo Fisher Scientific) was used as a 1:5000 dilution of the stock solution (0.5 mg/ml). The reaction was developed with TMB (3,3′,5,5′-tetramethylbenzidine) One Component HRP Microwell Substrate (BioFX Laboratories) and stopped with 0.5 M sulphuric acid. Absorbance at 414 nm was measured using a Multiskan EX ELISA reader (MTX Lab Systems).

**Table 1 Primers used in the present study**

| Gene  | Forward primer (F) (5′→3′)          | Reverse primer (R) (5′→3′)          | Amplicon size (bp) | F/R annealing position |
|-------|-----------------------------------|-----------------------------------|--------------------|------------------------|
| ACT1  | GGACTCACCATGTCCCGAG             | AACCACCAATCCAGCAGG甘             | 127                | 904–923, 1011–1030     |
| 21S   | CGGTCGCCGGAACCTAAA             | CGGAGTTGCGAACAAATCCTT             | 221                | 2548–2567, 2553–2768   |
| COBE4I | TTTACATGCATTACATCTTT          | AAGATTCCAGCATACGACCA              | 168                | 658–681, 802–823       |
| bI2   | TGGCAAGATGTCACTATTGA          | CGTACCTCCTAAATGGAATTCT            | 71                 | 408–428, 456–478       |
| bI3   | TCTTGCCATTCCATTGAGGG         | TGCCTCAATTATCTACATTACCA           | 149                | 452–473, 578–600       |
| 1S5   | GTAAACCTAGCGCAAAGGCAA          | TGTCAATTATCTCTACACTGTT           | 108                | 270–291, 356–377       |
| CCM1  | CCAACACCTGACAGCAGCA          | TAGCGCGTCTTTGACATCAGCAG            | 120                | 2126–2144, 2224–2245   |
| COX1E4| CTACACATTAGCGATTCCAGGA       | GTCCCTGATAGATGATAATGGT            | 146                | 270–291, 393–415       |
| bI4   | AGGAGGTTGTCAGCCCAATCTT        | AATCCAAATGGAGCCATAGCA             | 174                | 672–691, 825–845       |

**Note:** $t$ (threshold cycle value) was determined using the SigmaStat statistical software (SPSS). $R^2$ values were at least 0.994. Primer sequences, annealing positions and amplicon size are indicated in Table 1. For SDS/PAGE and Western blotting [28], yeast crude extracts were prepared in the presence of lysis buffer [130 mM EDTA, 130 mM EGTA, one tablet of Complete Protease Inhibitor (Santa Cruz Biotechnology) per ml and 2× Protease Inhibitor Cocktail (Sigma–Aldrich) in 1× PBS]. Cells were mechanically disrupted with 0.5 mm diameter glass beads for 2 min at room temperature (25°C) using a cell disruptor (Scientific Industries). Triton X-100 was then added up to 1% and disruption continued for an additional 2 min. Crude extracts were then clarified by centrifugation at 4°C for 5 min at 20000 g. Total protein contents were determined using the Bradford assay and BSA as standard (Thermo Fisher Scientific).

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Yeast crude extracts were prepared by resuspending cell pellets that had been stored at −70 °C in lysis buffer as described for SDS/PAGE and Western blotting samples. Then 120 μg of protein in 100 μl were serially diluted 1:2 with 100 μl of DB (dilution buffer: lysis buffer plus 1 % Triton X-100, 0.1 % Tween 20 and 0.5 % BSA) in pAbI-coated 96-well Costar ELISA plates (Corning) and incubated at 4 °C for 18 h. After three washes with WB (washing buffer: 1 % Triton X-100 and 0.1 % Tween 20 in 1×PBS), 100 μl of biotinylated pAbII in DB was added per well. Plates were then incubated at 4 °C for 18 h. Wells were then washed three times with WB followed by incubation with 100 μl of Streptavidin Poly-HRP in DB for 2 h at room temperature. After the wells were washed three times with WB, followed by three washes with 0.05 % Tween 20 in 1×PBS, reactions were developed and processed as indicated above.

Statistical analysis
All qPCR values represent the means ± S.E.M. of two or three replicates from the number of independent experiments indicated in each Figure legend. Time course results (amount of target mRNA over ACT1 mRNA or target gene over ACT1) were expressed as a percentage of the value obtained at t = 0 (control). RT-qPCR data were analysed by one-way ANOVA followed by Dunnett’s post hoc test for multiple comparisons with the control group (SPSS). The strength of association between the relative levels of CCM1 mRNA and bi4 maturase activity or 15S rRNA levels was measured with the Pearson Product Moment Correlation. Values of target mRNA from cells harbouring pCCM1AAA were compared with those of the wild-type plasmid using the Student’s t test.

RESULTS AND DISCUSSION
Intron-containing mtDNA cells became rho−mutants dramatically faster than their intronless counterparts
We have determined the rate of mtDNA decay with which nascent non-complemented Δccm1 meiotic segregants from 2nB (B cells) and 2nI (I0 cells) diploids became rho− mutants. After being cytoplasmically inherited by the segregants, Ccm1p and functional mitochondria were diluted as cell proliferation progressed [14]. At t = 0, relative levels of mtDNA in B segregants as determined by 15S_ RRNA and COX1 [27] were 0.5 and 1.5 orders of magnitude lower than those of their I0 counterparts respectively (Figure 1A). No decrease in mtDNA levels was detected in I0 segregants over the particular time-frame of this experiment. However, further reductions were observed in B cells, in which mtDNA levels were 0.5 (15S_ RRNA) and 1 (COX1) order of magnitude lower at t = 96 h than the corresponding levels at t = 0. Overall, these results agree with our previous observations in B cells [14]. Another comparable case is NAM1, a gene involved in intron removal from COB and COX1 pre-mRNAs which, when deleted, generated a large proportion of rho− cells in strains with intron-containing genomes, while strains with intronless mtDNA showed a normal pattern of mitochondrial translation and kept their genomes intact [29,30]. Therefore the presence of introns makes mtDNA stability significantly more Ccm1p-dependent than the intronless mtDNA counterpart regardless of the 15S rRNA steady-state levels (see below). This is a first line of evidence showing that Ccm1p plays a key role in COB and COX1 mRNA maturation independently from 15S rRNA. Ccm1p deletion did not affect transcription or stability of COB and COX1
Ccm1p is a moonlighting protein

Figure 2 Assessment of SMDC in B and I^0 complemented Δccm1 segregants
(A) Splicing failure in COB mRNA due to Ccm1p loss. A portion (15 μg) of total RNA from B segregants was analysed by Northern blotting using a COB E4–E5 probe. (B) Ccm1p (Ccm1p-zz) monitored in 10 μg of clarified crude extracts by immunoblotting with an antibody against Ccm1p [14]. 15S rRNA assessment by Northern blotting of 5 μg of total RNA from the same samples using 15S rRNA and 21S rRNA probes. (C) Decay rate of 15S rRNA measured by RT-qPCR in I^0 and B segregants. Results are means ± S.E.M. of duplicate measurements.
Figure 3  Dissection of bi4 maturase and 15S rRNA stabilizing activities by SMDC
and I0 analysis of the samples described in Figure 2(C) revealed that B rates, reaching 3.0 ± 0.5 h in comparison with the value at Figure 3A), reaching practically zero at 48 h. However, no significant difference in 15S rRNA levels was detected at either 3 or 6 h in comparison with the value at t = 0 (P < 0.05; Figure 3A), reaching practically zero at 48 h. However, no significant difference in 15S rRNA levels was detected at either 3 or 6 h in comparison with the value at t = 0 (P < 0.05). Additional factors might be interacting with 15S rRNA and thus ameliorating the SMDc effect. As Ccm1p is reportedly not part of the ribosome [35], it might be considered as an RNA chaperone/carrier that protects and/or delivers 15S rRNA for assembly of the small subunit. This precise function has its human counterpart GTPase ERAL1 protein [36]. However, the role of Ccm1p as 15S rRNA transcription factor cannot be ruled out yet. Actually, a previous report states that human mitochondrial RNA polymerase has also two PPR motifs in tandem, located between amino acids 263–296 and 297–330; when deleted, the polymerase cannot initiate transcription [37]. That bI4 maturase activity drop preceded the 15S rRNA decay (see also Figures 2(A) and 2(B), lanes 48 h in Δccm1 segregants) clearly indicated that the failure in the activity of bI4 maturase was not due to a translation malfunction. Moreover, bI4 intron removal was shown to be significantly more Ccm1p-dependent than the maintenance of 15S rRNA steady-state levels. The strong statistical association detected between relative levels of CCM1 mRNA and bI4 maturase activity (correlation coefficient = 0.988, P = 0.01) also confirmed a direct assistance of Ccm1p on bI4 maturase activity. Interestingly, in vitro experiments reported limited efficiency in the removal of the naturally occurring, full-size bI4 intron (∼ 1600 nt) by a Nam2p–bI4 maturase–RNA ternary complex [38]. Ccm1p would perfectly fit this scenario as a factor that assists the full-length bI4 intron in acquiring the competent structure towards a catalytic form. Finally, the activities of bI2 and bI3 maturase partially declined, but recovered at 24 h as did 215S rRNA levels (Figure 3B and inset), but not those of 15S rRNA. This ‘pit-shape’ pattern was most likely due to repression of mitochondrial transcription by dextrose [39].

Cytoplasmic expression and import of bI4 maturase by Δccm1 segregants were performed as previously described [7], but it failed to remove the bI4 intron. This observation hinted that, without Ccm1p, bI4 maturase was present but inactive. Since an active bI4 maturase eliminates its own mRNA (i.e. the mRNA precursor to synthesize Cob1p), under SMDc conditions, bI4 maturase mRNA should accumulate, thus increasing bI4 maturase synthesis. In agreement with this rationale, we found that at t = 3 h, bI4 maturase synthesis was boosted ~ 4-fold (Figure 3C) with respect to t = 0. Furthermore, relative levels of bI4 maturase protein remained at least two-times higher during the entire experimental timeframe. The boost in maturase levels along with a concomitant drop in activity (Figure 3A) rules out that SMDc increased maturase stability. Surprisingly, a second and even larger boost of bI4 maturase synthesis was detected at t = 72 h when 12% of 15S rRNA remained, after a progressive accumulation of its own mRNA (i.e. the first four exons of COB mRNA and part of bI4), indicating that de novo protein synthesis took place at later SMDc stages. As a matter of fact, we have never observed that 15S rRNA levels were reduced to 0 during the timeframe of all our present and previously reported experiments [14]. It has long been accepted that both rRNAs and ribosomal proteins are readily degraded unless they are incorporated into a ribosomal subunit [40]. Shorter SMDc times showed that the boost event took place as early as 1 h, supporting the idea that this effect occurs at translational level (Figure 3C inset). These results along with the ones depicted in Figure 3(A) suggest that the main bulk of 15S rRNA could be in transit, stabilized by Ccm1p. Thus, this system might be a powerful tool to study mitoribosome assembly.

Finally, the transient nature of the first boost (Figure 3C) suggests that bI4 maturase is under a high turnover. In agreement with this observation, Western blotting analysis of the boost revealed that while a major form of ~ 55 kDa was visualized at t = 0, a ~ 75 kDa form, consistent with the full-length molecule appeared at 3 h when the boost took place (Figure 3D). In addition, the ~ 55 kDa molecule, other species of lower molecular mass were also visualized when crude extracts were prepared in the presence of high concentration of protease inhibitors. Therefore the boost might overcome this high turnover of bI4 maturase, making visible the 75 kDa full-length form. We observed a unique ~ 30 kDa band when crude extracts from the very same
samples were prepared without protease inhibitors. Thus, the previously described ~30 kDa fragment with maturase activity corresponding to the protein C-terminal end [7] could probably be a degradation product rather than a physiological form. We conclude that the SMDC strategy that yielded the central line of experimental evidence also introduces an improved system to dissect in vivo molecular interactions that, otherwise, would be considered cause–effect events.

The three lysine residues of Ccm1p PPR2 are required for full bI4 maturase activity but not to maintain 15S rRNA levels

The CCM1 ORF lacking PPR2, which is not the canonical motif in the full length Ccm1p, complemented neither B [14] nor Δccm1 segregants (J. I. Moreno and M.A. Piva, unpublished work). Based on the role of positive charges in these motifs [23], we cumulatively replaced the three lysines of PPR2 (between amino acids 356 and 390) by alanine residues. No difference between bI4 maturase activity and 15S rRNA levels was observed in B Δccm1 segregants complemented by single Lys356Ala or double Lys356Ala/Lys357Ala mutants in comparison with the wild-type ORF. However, a significant decrease in bI4 maturase activity along with a concomitant increase in bI4 maturase synthesis was observed in B Δccm1 segregants complemented with triple-mutated Ccm1p (Lys356Ala/Lys357Ala/Lys358Ala, i.e. pCCM1LC-AAA) with respect to wild-type ORF (P < 0.05, Figure 4). Cells harbouring wild-type and triple mutated ORFs expressed similar levels of CCM1 mRNA. Importantly, no statistical differences in the activity of the other two maturases or the levels of 15S rRNA were detected between the triple-mutated and the wild-type ORFs (P > 0.05). These results contribute to identify which amino acids are involved in one of the activities [19] and provided the final line of evidence that characterizes, for the first time, Ccm1p as a PPR protein with moonlighting capabilities.

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