

**Pentatricopeptide (PPR) motifs in the N-terminal extension domain of the yeast
mitochondrial RNA polymerase Rpo41p are not essential for its function**

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ABSTRACT

The core mitochondrial RNA polymerase is a single-subunit enzyme, that in yeast *Saccharomyces cerevisiae* is encoded by the nuclear *RPO41* gene. It is an evolutionary descendant of the bacteriophage RNA polymerases, but includes an additional unconserved N-terminal extension (NTE) domain, that is unique to the organellar enzymes. This domain mediates interactions between the polymerase and accessory regulatory factors, such as yeast Sls1p and Nam1p. Previous studies demonstrated that deletion of the entire NTE domain results only in a temperature-dependent respiratory deficiency. Several sequences related to the pentatricopeptide (PPR) motifs were identified *in silico* in Rpo41p, three of which are located in the NTE domain. PPR repeat proteins are a large family of organellar RNA-binding factors, mostly involved in post-transcriptional gene expression mechanisms. In order to study their function, we analysed the phenotype of strains bearing Rpo41p variants where each of these motifs was deleted. We found that deletion of any of the three PPR motifs in the NTE domain does not affect respiratory growth at normal temperature, and results in a moderate decrease in mtDNA stability. Steady-state levels of *COX1* and *COX2* mRNAs are also moderately affected. Only the deletion of the second motif results in a partial respiratory deficiency, manifested only at the elevated temperature. Our results thus indicate that the PPR motifs do not play an essential role in the function of the NTE domain of the mitochondrial RNA polymerase.

Key words: mitochondria, RNA polymerase, pentatricopeptide motifs, yeast

Running title: PPR motifs in the mitochondrial RNA polymerase

INTRODUCTION

Mitochondria of Eukaryotes are derived from an alpha-proteobacterial endosymbiont [1, 2], but in the course of evolution their genomes were drastically reduced and acquired many unique characteristics that distinguish them from their eubacterial relatives. With the exception of the primitive mitochondria of jakobid protists that still encode a multisubunit eubacterial-type RNA polymerase in the organellar genome [3, 4], transcription and replication of the mitochondrial DNA is assured by specific single-subunit enzymes related to the proteins of the T-odd bacteriophages, and encoded in the nuclear genome [5].

The first typical mitochondrial RNA polymerase (mtRNAP) was described in yeast *Saccharomyces cerevisiae* [6]. It is a complex composed of the 153 kDa catalytic subunit encoded by the *RPO41* gene, and a single accessory 40 kDa transcription factor, encoded by the *MTF1* gene [7-12]. Whereas Rpo41p can recognize and initiate transcription from premelted promoter sequences *in vitro* [13], Mtf1p is required for transcription initiation from duplex DNA [14, 15]. The Rpo41p-Mtf1p complex recognises the promoter sequence by a mechanism reliant on induced fit and DNA bending [14]. Interaction between Rpo41p and Mtf1p depends on multiple regions of both proteins [16] and is maintained during initiation, but not elongation phase of transcription [17].

Transcription in *S. cerevisiae* mitochondria is initiated from a simple nonanucleotide promoter [18-20], giving rise to 11 polycistronic primary transcripts [21, 22]. Mitochondrial transcription by mtRNAP is coupled to respiratory activity by a simple ATP sensing kinetic mechanism [23], while RNA degradation and translational regulation provide regulatory mechanisms necessary to fine-tune the expression of particular genes [21, 24-26]. Additionally, mtRNAP primes mitochondrial DNA replication [27], and is thus essential for maintaining the functional ([rho⁺]) mtDNA.

Structural studies of the human mitochondrial RNA polymerase (POLRMT), orthologous to Rpo41p, revealed that it consists of three distinct domains: the C-terminal catalytic domain (CTD) that exhibits the typical polymerase right hand fold, the N-terminal domain (NTD) resembling the promoter-binding domain of the T7 RNA polymerase, and an N-terminal extension (NTE) that includes a domain containing PPR (pentatricopeptide) motifs [28-30]. The catalytic domain is highly conserved between the phage polymerase and the mitochondrial RNA polymerases from diverse eukaryotes. The N-terminal domain is more divergent, but a structural similarity among orthologous mtRNAP sequences and the phage protein is still apparent [29]. In the human protein this region has been implicated in transcription initiation through interactions with the promoter and the two transcription factors [28]. In the yeast Rpo41 protein this region also appears to be involved in promoter recognition and in interactions with the sole transcription factor (Mtf1p) [31, 32].

The first ~400 amino acids at the amino terminus of mtRNAP form the N-terminal extension (NTE), that has no counterpart in the bacteriophage RNA polymerase [33] and is generally not conserved between orthologous sequences, although weak similarity in this region can be detected in ascomycetous fungi [34]. In the human mtRNAP this extension is joined to the NTD by a helical domain containing two pentatricopeptide (PPR) motifs [29]. Whereas no significant sequence similarity can be found between the N-terminal extension of human POLRMT and yeast Rpo41p, application of an algorithm fine-tuned for detection of yeast pentatricopeptide proteins indicated that up to five putative PPR motifs can be found in the N-terminal part of Rpo41p [35].

Proteins containing the PPR motifs are a large family of RNA-binding factors that can be found in all Eukaryotes [36-42]. They are particularly abundant in plants, where they constitute the most numerous paralogous families, containing hundreds of sequences per genome [38, 42]. The vast majority of known PPR proteins are involved in various post-transcriptional

mechanisms of organellar gene expression (reviewed in [36, 37, 39, 40]). They were extensively studied in both model yeast systems – *S. cerevisiae* [39] and *S. pombe* [43], where they play significant roles in maintaining and regulating the expression of mitochondrial genes.

Typical PPR proteins contain multiple tandem repeats of a 35 amino acid motif forming two parallel alpha helices, resulting in a solenoid superhelical structure of the entire protein [37, 39, 42]. Most of them are composed solely of the PPR repeats, while some (found mostly in plants) contain additional C-terminal domains conferring enzymatic activities [37, 40]. The mitochondrial RNA polymerase is thus not a typical PPR protein, as it contains only 2 - 5 PPR motifs that constitute only a minor portion of the entire sequence.

The role of the unconserved N-terminal extension of the mitochondrial RNA polymerases, including the PPR motifs found therein, is not entirely clear. In the human POLRMT protein the PPR domain interacts with the promoter recognition loop of the NTD domain, and is essential for transcription initiation [29]. It also interacts with the upstream N-terminal extension that binds the transcription factor TFAM [28]. In yeast, deletions of the N-terminal extension of Rpo41p result in a respiratory deficiency connected with a loss of mitochondrial DNA stability that is, however, not caused by a major transcriptional defect [33]. Remarkably, while a complete or near complete (472 or 373 aa, respectively) NTE deletion resulted in an unconditional defect, deleting as many as 185 amino acids gave only a conditional (temperature sensitive) phenotype [33]. Subsequent studies suggested that this domain couples transcription to RNA processing and translation through interactions with the Nam1 and Sls1 proteins [34, 44, 45]. Deletion of the first 270 amino acids of the Rpo41p NTE domain does not affect the catalytic activity of the polymerase *in vitro*, in fact enhancing the productive/abortive ratio of RNA synthesis, whereas a 380 aa deletion decreases initiation from duplex, but not premelted promoters [46].

These results suggest that the function of the N-terminal extension domain of the yeast mitochondrial RNA polymerase is separate from the polymerase activity, but can be related to the coordination of transcription with the subsequent steps of gene expression. Available data do not, however, indicate whether the putative PPR motifs found in this domain are involved in its function. We have therefore decided to investigate their role by constructing a series of small deletions involving only the PPR motif sequences and assessing the activity of the resultant Rpo41 variants *in vivo*.

MATERIALS AND METHODS

Media and genetic techniques

Complete (YP) media supplemented with 2% glucose (YPD), 2% glycerol (YPG), or 2% glycerol and 0.1% glucose (YPDG), as well as synthetic complete (SC) media, were prepared as described in our recent work [47]. Standard yeast genetic methods were as described previously [48, 49]. Yeasts were transformed using the LiAc/PEG/ssDNA protocol [50].

Plasmids and construction of deletants

Plasmids pJJ1148 and pJJ1149 [51] contained the *RPO41* gene cloned in the YCplac33 (ARS-CEN, *URA3*) and YCplac111 (ARS-CEN, *LEU2*) vectors, respectively.

Plasmid pJJ1149 (YCplac111:*RPO41*) was used as the starting point for deletion mutagenesis. PCR-mediated Site-directed, Ligase-Independent Mutagenesis (SLIM) protocol [52] was used to delete segments encoding respective PPR motifs. In this method two pairs of primers, termed R_T, F_T and R_S, F_S are used for each deletion (primers are named according to the description in [52]).

To delete the first PPR motif (PPR1, aa 47 to 82) nucleotides 139 to 446 of the *RPO41* ORF were deleted using primers dPPR1F_T (5'-ATCTGAAGACCCCCTTGTATGGAAAAATCCTTCAGAATTAGAG), dPPR1R_T (5'-

TACAAGGGGGTCTTCAGATATTAATATTGTACTTGTTGAGTCCG), dPPR1F_S (5'-TGAAAAATCCTTCAGAATTAGAG), and dPPR1R_S (ATTAATATTGTACTTGTTGAGTCCG), to yield vector YCplac111:*RPO41ΔPPR1*. To delete the second PPR motif (PPR2, aa 112 to 146) nucleotides 334 to 438 of the *RPO41* ORF were deleted using primers dPPR2F_T (5'-GACAAGAACTAGAGACGTTTTTATCGAGGATTATAACATGTAC), dPPR2R_T (5'-AACGTCTCTAGTTCTTGTCATCGAGGTTACTGCTGAGGCATG), dPPR2F_S (5'-TTTATCGAGGATTATAACATGTAC), and dPPR2R_S (5'-ATCGAGGTTACTGCTGAGGCATG), to yield vector YCplac111:*RPO41ΔPPR2*. To delete the third PPR motif (PPR3, aa 291 to 325) nucleotides 871 to 975 of the *RPO41* ORF were deleted using primers dPPR3F_T (5'-CAAAGTGGAAGCTGAGAACTCAAAGACGCTGAAAAGCTTATAG), dPPR3R_T (5'-GTTCTCAGCTTCCACTTTGTTCAAACCCTCCTCATTTTC), dPPR3F_S (5'-TCAAAGACGCTGAAAAGCTTATAG), and dPPR3R_S (5'-TTCAAACCCTCCTCATTTTC), to yield vector YCplac111:*RPO41ΔPPR3*. Numbering starts with the first amino acid (M1) and the first nucleotide (A1) of the protein and the ORF, respectively.

All the constructs were verified by Sanger sequencing performed in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics Polish Academy of Science.

Yeast strains

The *rpo41::KanMX4* cassette, amplified from the DNA of the EUROSCARF Y15657 strain (*MATα; ura3Δ0; leu2Δ0; his3Δ1; lys2Δ0; YFL036w::kanMX4*) with primers YFL036w_A (5'-TCTCCGTGAAGATATTGGTAAAAAG) and YFL036w_D (5'-ACAAAATTGTGCACTTGTCATTAGA) was used to transform strain W303-1B [53] (*MATα*,

ade2-1, leu2-3, 112 ura3-,1 trp1-1, his3-11, 15 can1-100 [ρ^+]) to obtain a strain carrying a deletion of the entire *RPO41* ORF. This strain was subsequently crossed to two isonuclear W303 *MAT α* derivative strains CW04 [53] (*MAT α , ade2-1, leu2-3, 112 ura3-,1 trp1-1, his3-11, 15 can1-100* [ρ^+ 13 introns]) and CW252 [54] (*MAT α , ade2-1, leu2-3, 112 ura3-,1 trp1-1, his3-11, 15 can1-100* [ρ^+ intronless]), yielding heterozygous diploids HZRPO41 and HZRPO41int, respectively. The heterozygous diploids were transformed with pJJ1148 (YCplac33:*RPO41*), and the transformants sporulated. Haploid *MAT α* spores carrying the *rpo41::KanMX4* deletion and the wild-type *RPO41* gene on pJJ1148 were selected, yielding strains DRPO41 (*MAT α , ade2-1, leu2-3, 112 ura3-,1 trp1-1, his3-11, 15 can1-100, rpo41::KanMX4* [YCplac33:*RPO41*] [ρ^+ 13 introns]) and DRPO41int (*MAT α , ade2-1, leu2-3, 112 ura3-,1 trp1-1, his3-11, 15 can1-100, rpo41::KanMX4* [YCplac33:*RPO41*] [ρ^+ intronless]).

The DRPO41 and DRPO41int strains were transformed with vectors YCplac111:*RPO41* Δ PPR1, YCplac111:*RPO41* Δ PPR2, YCplac111:*RPO41* Δ PPR3. The pJJ1148 plasmid, carrying the wild-type *RPO41* gene, was then eliminated by counterselection on 5-FOA plates [55], leaving the deletion variant as the only mitochondrial RNA polymerase in the cells. The strains thus obtained were dPPR1int (*MAT α , ade2-1, leu2-3, 112 ura3-,1 trp1-1, his3-11, 15 can1-100, rpo41::KanMX4* [YCplac111:*RPO41* Δ PPR1] [ρ^+ intronless]), dPPR2int (*MAT α , ade2-1, leu2-3, 112 ura3-,1 trp1-1, his3-11, 15 can1-100, rpo41::KanMX4* [YCplac111:*RPO41* Δ PPR2] [ρ^+ intronless]), and dPPR3int (*MAT α , ade2-1, leu2-3, 112 ura3-,1 trp1-1, his3-11, 15 can1-100, rpo41::KanMX4* [YCplac111:*RPO41* Δ PPR3] [ρ^+ intronless]), dPPR1 (*MAT α , ade2-1, leu2-3, 112 ura3-,1 trp1-1, his3-11, 15 can1-100, rpo41::KanMX4* [YCplac111:*RPO41* Δ PPR1] [ρ^+ 13 introns]), dPPR2 (*MAT α , ade2-1, leu2-3, 112 ura3-,1 trp1-1, his3-11, 15 can1-100, rpo41::KanMX4* [YCplac111:*RPO41* Δ PPR2]

[rho⁺ 13 introns]), and dPPR3 (*MATa, ade2-1, leu2-3, 112 ura3-,1 trp1-1, his3-11, 15 can1-100, rpo41::KanMX4* [YCplac111:*RPO41ΔPPR3*] [rho⁺ 13 introns]).

D273-10B/51 [56] (*MATa ade5, [rho⁰]*) was used as a tester strain in mtDNA stability assays.

Respiratory growth assay

10μl of 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴ dilutions of each strain (OD₆₀₀=1) were spotted on YPD and YPG plates and incubated at 30°C (normal temperature) or 37°C (elevated temperature) for three days.

Assays of mtDNA stability

Stability of the mitochondrial genome was assessed by assaying the frequency of *petite* colony generation using the tetrazolium overlay method [57] as described previously [47]. Confirmation that the *petite* phenotype was due to mtDNA defects was obtained by crossing to the D273-10B/51 tester strain.

Mitochondrial RNA preparation and Northern blots

RNA was isolated from mitochondrial preparations enriched by differential centrifugation as described previously [58]. Total RNA Mini kit (A&A Biotechnology) was used in the RNA purification step. RNA was electrophoresed in 1% agarose-formaldehyde gel and blotted onto nylon membrane (Nytran N, GE Healthcare) as described previously [59]. Methylene blue staining of blots was used to normalise loading. Oligonucleotide and dsDNA probes were labelled with ³²P as described previously [60]. Results were visualised using the Typhoon FLA 9000 laser imaging system (GE Healthcare).

The following probes were used: for *COX1*, plasmid pYGT21 containing exon sequences [61]; for *COX2*, plasmid pJM2 [62]; for *COB*, oligonucleotide 5'-CATGGCATGCTCCAATTTGTCCTAATAATACGAAATTGAAA; for *ATP6/8*, plasmid pBZ6 (B. Zapisek) – *ATP6* ORF cloned into pGEM®-T Easy (Promega); for *ATP9*, PCR

product (325 bp) amplified using primers Atp9F (5'- TATGCAATTAGTATTAGCAGC) and Atp9R (5'- GAATGTTATTAATTTAATCAAATGAG).

RESULTS

Construction of the deletions of the PPR motifs in the N-terminal extension domain of Rpo41p

Unlike in human POLRMT [29], PPR motifs in the yeast Rpo41 sequence are not readily detected by profile matching tools. Using yeast-specific profiles and orthologous alignments from different yeast genomes [35], five divergent PPR motifs can be, however, identified in *S. cerevisiae* Rpo41p (Figure 1). Three of them, named PPR1, PPR2 and PPR3 here, are located within the unconserved N-terminal extension domain. The fourth motif lies at the boundary of the N-terminal extension and the conserved N-terminal domain, and the fifth one is close to the beginning of the C-terminal conserved catalytic domain. As the N-terminal extension is functionally independent from the bacteriophage-like RNA polymerase activity confined to the conserved regions of the protein, we decided to investigate the function of the three PPR motifs located therein by generating deletions encompassing each of these motifs.

To obtain variants bearing deletions of the first three PPR motifs, sequences encoding 35 aa (beginning at the first amino acid of each motif, as shown in Figure 1) were deleted from the *RPO41* ORF in pJJ1149 (see Materials and methods for details of plasmid and strain construction) using the PCR-mediated Site-directed, Ligase-Independent Mutagenesis (SLIM) protocol [52], yielding the YCplac111:RPO41dPPR1, YCplac111:RPO41dPPR2, and YCplac111:RPO41dPPR3 vectors.

As deletion of *RPO41* results in a complete and irreversible loss of mtDNA [63], heterozygous diploid strains were used as a starting point. In order to test possible involvement of the PPR motifs in Rpo41 in intronic RNA processing, strains with two variants of the mitochondrial genome were used – one containing 13 introns (CW04), and one devoid of all

introns (CW252). Wild-type *RPO41* was introduced into the heterozygous strains by transformation with the pJJ1148 vector. The resulting transformants were subsequently sporulated, and haploid strains DRPO41 and DRPO41int carrying the deletion of *RPO41* in the genomic DNA, with the pJJ1148 plasmid providing the functional wild-type allele, and a [ρ^+] mitochondrial genome containing 13 introns, or devoid of all introns, respectively, were selected.

DRPO41 and DRPO41int strains were subsequently transformed with the ARS-CEN, *LEU2* vectors encoding the PPR deletion variants of the Rpo41 protein (YCplac111:RPO41dPPR1, YCplac111:RPO41dPPR2, and YCplac111:RPO41dPPR3). The pJJ1148 vector encoding the wild-type Rpo41p was then removed by counter-selection on 5-FOA plates [55], resulting in strains where the deletion variants were the only Rpo41 proteins in the cell. These strains were named dPPR1int, dPPR2int, and dPPR3int (strains with the intronless mtDNA) and dPPR1, dPPR2, and dPPR3 (strains with intron-containing mtDNA).

Effect of PPR motif deletions on the respiratory growth of yeast

Respiratory capacity of the strains carrying the deletion variants of the Rpo41p RNA polymerase was verified by testing their growth on a non-fermentable carbon source (glycerol) at normal (30°C) and elevated (37°C) temperature (Figure 2). Deletions of the first and third PPR motifs had no observable effect on respiratory growth of the relevant strains at either temperature. Only in the dPPR2 and dPPR2int strains, bearing a deletion of the second PPR motif, a moderate respiratory defect could be observed. This strain still grows on glycerol, but the resultant colonies are visibly smaller (an effect observable also on glucose), suggesting a hypomorphic *petite* phenotype, similar to the defect observed in deletants missing the first 90 amino acids of Rpo41p [34]. This phenotype was more apparent at the elevated temperature, but even then it did not amount to a complete loss of respiratory growth.

As no difference between the intron-containing and intronless mtDNA could be observed in any of the deletants, subsequent experiments were performed using only the intron-containing strains.

Effect of PPR motif deletions on the stability of the mitochondrial genome

Deletions of large segments of the N-terminal extension result in a loss of mitochondrial genome stability [33], manifesting itself in a complete loss [ρ^0] or extensive deletions [ρ^-] of mtDNA. These so-called cytoplasmic *petite* colonies arise also in wild-type yeast cultures, albeit at a low rate of about 1-5%. We measured the *petite* frequencies in dPPR1, dPPR2 and dPPR3 mutants (Figure 3) using the tetrazolium test [57] and by crossing to a [ρ^0] tester strain. The dPPR1_{int} and dPPR3_{int} deletants show a moderate, but significant ($p < 0.01$), increase in *petite* generation (17-18%), that is apparent at both 30°C and 37°C. Interestingly, the effect is slightly more pronounced at 30°C. The dPPR2 deletant also shows a similar increase in *petite* generation at 30°C (~22%), but a much stronger (up to ~50%) effect at 37°C that is consistent with a visible respiratory growth impairment at this temperature (Figure 2). In the wild-type strain there is no statistically significant difference in mtDNA stability in cells grown at the normal and elevated temperature (2.6% and 2.9%).

Effect of PPR motif deletions on the steady state levels of mitochondrial transcripts

Mutations in the N-terminal extension domain of Rpo41p can affect steady-state levels of specific mitochondrial RNAs [34, 45]. In order to verify, whether deletions of PPR motifs could also specifically influence mitochondrial transcripts we performed a series of Northern blots in deletant strains. Mitochondrial RNA from the wild-type, and the dPPR1, dPPR2, and dPPR3 strains grown at 30°C was hybridised to probes recognising some of the mitochondrially encoded mRNAs: *COX1*, and *COX2* encoding subunits of the cytochrome oxidase complex (Complex IV), *COB*, encoding the apocytochrome b (Complex III), and *ATP9*, and *ATP6-8*, encoding subunits of the ATP synthase (Complex V).

The results (Figure 4) show a moderate decrease in the levels of mature mRNAs encoding subunits of Complex IV, with each deletant following a different pattern. Deletion of the first motif (dPPR1) results in a marked decrease (to about 40% of wild-type) of *COX2* mRNA, with the remaining mRNAs mostly unaffected. Deletion of the second motif (dPPR2) does not show a clear effect on any mRNA. Deletion of the third motif (dPPR3) results in a visible decrease (to about 44% of wild-type level) of *COX1*. The mRNAs encoding apocytochrome b (*COB*), and the subunits of the ATP synthase (*ATP6/8* and *ATP9*) were not significantly affected in any of the deletants. Such a moderate change in the mature mRNA levels is likely to have little effect on respiratory growth, as yeast mutants with as little as 10% of wild-type mature mitochondrial transcript were found to be respiratory competent [25, 64]. The results are therefore consistent with the respiratory function assays that show mostly unaffected respiratory growth at normal temperature (30°C).

DISCUSSION

Of the five divergent PPR motifs that can be found in the sequence of the yeast mitochondrial RNA polymerase, three are located in the N-terminal extension (NTE) domain. Unlike the downstream N-terminal domain and the C-terminal domain, this region of the protein shows no homology to the T7 bacteriophage RNA polymerase, and is not conserved in evolution, with some regions of similarity shared only with other fungi [34]. Other organellar RNA polymerases, like the human POLRMT [29], also have an N-terminal extension, but no sequence conservation can be detected. This domain is not essential for the main catalytic activity of the protein, and even large deletions therein result only in a temperature sensitive respiratory deficiency phenotype [33].

The results obtained in this study are consistent with these previous findings. Deletion of either of the three PPR motifs located in the NTE domain does not affect respiratory growth at the normal temperature, and only deletion of the second motif (amino acids 112 to 146) gives

a partial respiratory deficiency at the elevated temperature. All three PPR motif deletions result in a decreased mitochondrial DNA stability, but not in a complete loss of functional mtDNA. By way of comparison, deletion of up to 212 amino acids in the NTE domain results in a complete respiratory deficiency at the elevated temperature with concomitant mtDNA destabilisation [34]. The phenotype observed for the PPR motif deletions in this study is therefore a milder, partial form of the deficiency observed for the NTE deletion. In fact, a single amino acid substitution (R129D) has a more pronounced effect on the function of the protein, compared to the PPR motif deletions [34]. It can thus be concluded that these motifs do not play a particularly significant role in the functioning of this domain.

The observed phenotypes, together with previously reported data obtained using large deletions in the N-terminal extension *in vivo* [33, 44, 45] and *in vitro* [46], also support the conclusion that this region of the protein is not involved in the main enzymatic activity. Deletions of PPR motifs studied here, as well as other mutations and deletions in the NTE region [44, 45], affect the steady state levels of some mitochondrial mRNAs (mainly *COX1* or *COX2*), while other transcripts remain unaffected. This suggests that the function of this region concerns RNA stability and translation rather than general transcription, and is likely regulatory.

These observations are consistent with a model suggesting that the N-terminal extension of the mitochondrial RNA polymerase is involved in coupling transcription with RNA processing and translation. This function is probably achieved through interactions with two known accessory proteins: Nam1 and Sls1 [34, 44, 45]. Sls1p interacts with the N-terminal part of Rpo41p, anchoring the RNA polymerase and the nascent transcript to the inner membrane complexes that include translational activators [44]. Nam1p is a putative RNA chaperone [65, 66] that interacts with Sls1p, NTE domain of Rpo41p, and the nascent RNA [44]. The PPR motifs appear to participate in these interactions, as their deletion results in a decrease of

mtDNA stability, changes in mitochondrial mRNA levels, and (in the case of the second motif), partial temperature-sensitive phenotype. They are not, however, essential, as the phenotype is relatively mild, compared to the larger N-terminal extension deletions.

In typical PPR proteins, the pentatricopeptide motifs form RNA binding surfaces and usually act as intermediates in interactions between organellar transcripts and other proteins [37-42]. Typical RNA-binding PPR proteins are, however, composed of multiple adjacent repeats, whereas in Rpo41p the motifs are few and dispersed. It is currently not clear whether they participate in RNA binding, or even whether the entire N-terminal extension interacts directly with RNA. Crystallographic studies of the human mitochondrial RNA polymerase revealed that the two PPR motifs, found at the boundary of the N-terminal extension and the conserved N-terminal domain, do not interact with RNA, but participate in intramolecular interactions between different domains of the protein [29]. In yeast Rpo41p, the fourth and fifth motif, also located near domain boundaries (see Figure 1), could arguably play a similar role, although without crystallographic data it remains only a speculation. It is also conceivable that the three PPR motifs in the NTE domain that were the subject of the present study also participate in interactions with the accessory proteins, consistent with the postulated function of the entire domain. The PPR motif shares evolutionary and structural relationship with other α - α repeat sequences, like TPR (tetratricopeptide), SEL1, and HAT motifs forming solenoid folds [42, 67, 68], many of which participate in protein-protein interactions.

Despite functional differences between the PPR motifs in the mitochondrial RNA polymerase and typical PPR proteins, they share a similar broad evolutionary role. The mitochondrial RNA polymerase is a descendant of the bacteriophage RNAP [5], which is a standalone enzyme that does not depend on any additional factors. In order to integrate the phage enzyme into the more complex organellar genetic system, interactions with accessory regulatory proteins had to evolve. The N-terminal extension of mtRNAP, which has no

homology to the bacteriophage polymerase, arose to allow such interactions and coordinate the transcriptional activity with organellar RNA processing and translation. Similarly, nuclear-encoded PPR proteins evolved in the Eukaryotic host in order to provide activities necessary to maintain and regulate organellar gene expression, replacing prokaryotic mechanisms that were lost in the course of the evolution of the endosymbiont. Both the NTE domain of the mitochondrial RNA polymerase, and the PPR proteins in general, are thus examples of evolutionary adaptations in domesticating the endosymbiont, allowing to integrate it with the host system. Such adaptations often involve helical repeat sequences, as their modularity imparts great evolutionary plasticity. Indeed, the N-terminal extension sequences of mitochondrial RNA polymerases, as well as the entire PPR protein family show rapid divergent evolution that adapts them to the quickly changing evolutionary dynamics of the nucleo-organellar interaction.

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REFERENCES

1. Gray, M. W., Burger, G., and Lang, B. F. (1999). Mitochondrial evolution. *Science* **283**, 1476–1481.
2. Lang, B. F., Gray, M. W., and Burger, G. (1999). Mitochondrial genome evolution and the origin of eukaryotes. *Annu Rev Genet* **33**, 351–397.
3. Burger, G., Gray, M. W., Forget, L., and Lang, B. F. (2013). Strikingly bacteria-like and gene-rich mitochondrial genomes throughout jakobid protists. *Genome Biol Evol* **5**, 418–438.
4. Lang, B. F., Burger, G., O'Kelly, C. J., Cedergren, R., Golding, G. B., Lemieux, C., Sankoff, D., Turmel, M., and Gray, M. W. (1997). An ancestral mitochondrial DNA resembling a eubacterial genome in miniature. *Nature* **387**, 493–497.

5. Shutt, T. E., and Gray, M. W. (2006). Bacteriophage origins of mitochondrial replication and transcription proteins. *Trends Genet* **22**, 90–95.
6. Masters, B. S., Stohl, L. L., and Clayton, D. A. (1987). Yeast mitochondrial RNA polymerase is homologous to those encoded by bacteriophages T3 and T7. *Cell* **51**, 89–99.
7. Jang, S. H., and Jaehning, J. A. (1991). The yeast mitochondrial RNA polymerase specificity factor, MTF1, is similar to bacterial sigma factors. *J Biol Chem* **266**, 22671–22677.
8. Schubot, F. D., Chen, C. J., Rose, J. P., Dailey, T. A., Dailey, H. A., and Wang, B. C. (2001). Crystal structure of the transcription factor sc-mtTFB offers insights into mitochondrial transcription. *Protein Sci.* **10**, 1980–1988.
9. Matsunaga, M., Jang, S.-H., and Jaehning, J. A. (2004). Expression and purification of wild type and mutant forms of the yeast mitochondrial core RNA polymerase, Rpo41. *Protein Expr. Purif.* **35**, 126–130.
10. Levens, D., Lustig, A., and Rabinowitz, M. (1981). Purification of mitochondrial RNA polymerase from *Saccharomyces cerevisiae*. *J Biol Chem* **256**, 1474–1481.
11. Tracy, R. L., and Stern, D. B. (1995). Mitochondrial transcription initiation: promoter structures and RNA polymerases. *Curr Genet* **28**, 205–216.
12. Karlok, M. A., Jang, S.-H., and Jaehning, J. A. (2002). Mutations in the yeast mitochondrial RNA polymerase specificity factor, Mtf1, verify an essential role in promoter utilization. *J Biol Chem* **277**, 28143–28149.
13. Matsunaga, M., and Jaehning, J. A. (2004). Intrinsic promoter recognition by a “core” RNA polymerase. *J Biol Chem* **279**, 44239–44242.
14. Tang, G.-Q., Deshpande, A. P., and Patel, S. S. (2011). Transcription factor-dependent DNA bending governs promoter recognition by the mitochondrial RNA polymerase. *J Biol Chem* **286**, 38805–38813.
15. Paratkar, S., and Patel, S. S. (2010). Mitochondrial transcription factor Mtf1 traps the unwound non-template strand to facilitate open complex formation. *J Biol Chem* **285**, 3949–3956.
16. Cliften, P., Park, J. Y., Davis, B. P., Jang, S.-H., and Jaehning, J. A. (1997). Identification of three regions essential for interaction between a sigma-like factor and core RNA polymerase. *Genes Dev* **11**, 2897–2909.
17. Mangus, D. A., Jang, S. H., and Jaehning, J. A. (1994). Release of the yeast mitochondrial RNA polymerase specificity factor from transcription complexes. *J Biol Chem* **269**, 26568–26574.
18. Osinga, K. A., and Tabak, H. F. (1982). Initiation of transcription of genes for mitochondrial ribosomal RNA in yeast: comparison of the nucleotide sequence around the 5'-ends of both genes reveals a homologous stretch of 17 nucleotides. *Nucleic Acids Res* **10**, 3617–3626.

19. Christianson, T. W., and Rabinowitz, M. (1983). Identification of multiple transcriptional initiation sites on the yeast mitochondrial genome by in vitro capping with guanylyltransferase. *J Biol Chem* **258**, 14025–14033.
20. Christianson, T. W., Edwards, J., Levens, D., Locker, J., and Rabinowitz, M. (1982). Transcriptional initiation and processing of the small ribosomal RNA of yeast mitochondria. *J Biol Chem* **257**, 6494–6500.
21. Turk, E. M., Das, V., Seibert, R. D., and Andrulis, E. D. (2013). The mitochondrial RNA landscape of *Saccharomyces cerevisiae*. *PLoS ONE* **8**, e78105.
22. Foury, F., Roganti, T., Lecrenier, N., and Purnelle, B. (1998). The complete sequence of the mitochondrial genome of *Saccharomyces cerevisiae*. *FEBS Lett* **440**, 325–331.
23. Amiott, E. A., and Jaehning, J. A. (2006). Mitochondrial transcription is regulated via an ATP “sensing” mechanism that couples RNA abundance to respiration. *Mol Cell* **22**, 329–338.
24. Herrmann, J. M., Woellhaf, M. W., and Bonnefoy, N. (2013). Control of protein synthesis in yeast mitochondria: the concept of translational activators. *Biochim Biophys Acta* **1833**, 286–294.
25. Rogowska, A. T., Puchta, O., Czarnecka, A. M., Kaniak, A., Stepień, P. P., and Golik, P. (2006). Balance between transcription and RNA degradation is vital for *Saccharomyces cerevisiae* mitochondria: reduced transcription rescues the phenotype of deficient RNA degradation. *Mol Biol Cell* **17**, 1184–1193.
26. Krause, K., and Dieckmann, C. L. (2004). Analysis of transcription asymmetries along the tRNA^{Ala}-COB operon: evidence for transcription attenuation and rapid RNA degradation between coding sequences. *Nucleic Acids Res* **32**, 6276–6283.
27. Sanchez-Sandoval, E., Diaz-Quezada, C., Velazquez, G., Arroyo-Navarro, L. F., Almanza-Martinez, N., Trasviña-Arenas, C. H., and Briebe, L. G. (2015). Yeast mitochondrial RNA polymerase primes mitochondrial DNA polymerase at origins of replication and promoter sequences. *Mitochondrion* **24**, 22–31.
28. Morozov, Y. I., Agaronyan, K., Cheung, A. C. M., Anikin, M., Cramer, P., and Temiakov, D. (2014). A novel intermediate in transcription initiation by human mitochondrial RNA polymerase. *Nucleic Acids Res* **42**, gkt1356–3893.
29. Ringel, R., Sologub, M., Morozov, Y. I., Litonin, D., Cramer, P., and Temiakov, D. (2011). Structure of human mitochondrial RNA polymerase. *Nature* **478**, 269–273.
30. Schwinghammer, K., Cheung, A. C. M., Morozov, Y. I., Agaronyan, K., Temiakov, D., and Cramer, P. (2013). Structure of human mitochondrial RNA polymerase elongation complex. *Nat Struct Mol Biol* **20**, 1298–1303.
31. Nayak, D., Guo, Q., and Sousa, R. (2009). A promoter recognition mechanism common to yeast mitochondrial and phage ϕ 7 RNA polymerases. *J Biol Chem* **284**, 13641–13647.
32. Yang, X., Chang, H. R., and Yin, Y. W. (2015). Yeast Mitochondrial Transcription

Factor Mtf1 Determines the Precision of Promoter-Directed Initiation of RNA Polymerase Rpo41. *PLoS ONE* **10**, e0136879.

33. Wang, Y., and Shadel, G. S. (1999). Stability of the mitochondrial genome requires an amino-terminal domain of yeast mitochondrial RNA polymerase. *Proc Natl Acad Sci USA* **96**, 8046–8051.
34. Rodeheffer, M. S., Boone, B. E., Bryan, A. C., and Shadel, G. S. (2001). Nam1p, a protein involved in RNA processing and translation, is coupled to transcription through an interaction with yeast mitochondrial RNA polymerase. *J Biol Chem* **276**, 8616–8622.
35. Lipinski, K. A., Puchta, O., Surendranath, V., Kudla, M., and Golik, P. (2011). Revisiting the yeast PPR proteins--application of an Iterative Hidden Markov Model algorithm reveals new members of the rapidly evolving family. *Mol Biol Evol* **28**, 2935–2948.
36. Lightowlers, R. N., and Chrzanowska-Lightowlers, Z. M. (2013). Human pentatricopeptide proteins. *RNA Biol* **10**, 1433–1438.
37. Filipovska, A., and Rackham, O. (2013). Pentatricopeptide repeats. *RNA Biol* **10**, 1426–1432.
38. Giegé, P. (2013). Pentatricopeptide repeat proteins. *RNA Biol* **10**, 1417–1418.
39. Herbert, C. J., Golik, P., and Bonnefoy, N. (2013). Yeast PPR proteins, watchdogs of mitochondrial gene expression. *RNA Biol* **10**, 1477–1494.
40. Schmitz-Linneweber, C., and Sluyter, F. (2008). Pentatricopeptide repeat proteins: a socket set for organelle gene expression. *Trends Plant Sci* **13**, 663–670.
41. Delannoy, E., Stanley, W. A., Bond, C. S., and Sluyter, F. (2007). Pentatricopeptide repeat (PPR) proteins as sequence-specificity factors in post-transcriptional processes in organelles. *Biochem Soc Trans* **35**, 1643–1647.
42. Small, I. D., and Peeters, N. (2000). The PPR motif - a TPR-related motif prevalent in plant organellar proteins. *Trends Biochem Sci* **25**, 46–47.
43. Kühl I, Dujeancourt L, Gaisne M, Herbert CJ, Bonnefoy N (2011) A genome wide study in fission yeast reveals nine PPR proteins that regulate mitochondrial gene expression. *Nucleic Acids Res* 39:8029–8041. doi: 10.1093/nar/gkr511
44. Rodeheffer, M. S., and Shadel, G. S. (2003). Multiple interactions involving the amino-terminal domain of yeast mtRNA polymerase determine the efficiency of mitochondrial protein synthesis. *J Biol Chem* **278**, 18695–18701.
45. Bryan, A. C., Rodeheffer, M. S., Wearn, C. M., and Shadel, G. S. (2002). Sls1p is a membrane-bound regulator of transcription-coupled processes involved in *Saccharomyces cerevisiae* mitochondrial gene expression. *Genetics* **160**, 75–82.
46. Paratkar, S., Deshpande, A. P., Tang, G.-Q., and Patel, S. S. (2011). The N-terminal domain of the yeast mitochondrial RNA polymerase regulates multiple steps of

transcription. *J Biol Chem* **286**, 16109–16120.

47. Kaliszewska, M., Kruszewski, J., Kierdaszuk, B., Kostera-Pruszczyk, A., Nojszewska, M., Łusakowska, A., Vizueta, J., Sabat, D., Lutyk, D., Lower, M., et al. (2015). Yeast model analysis of novel polymerase gamma variants found in patients with autosomal recessive mitochondrial disease. *Hum Genet* **134**, 951–966.
48. Burke, D., Dawson, D., and Stearns, T. (2000). Methods in yeast genetics: a Cold Spring Harbor Laboratory course manual.
49. Dujardin, G., Pajot, P., Groudinsky, O., and Slonimski, P. P. (1980). Long range control circuits within mitochondria and between nucleus and mitochondria. I. Methodology and phenomenology of suppressors. *Mol Gen Genet* **179**, 469–482.
50. Gietz, R. D., and Woods, R. A. (2002). Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Meth Enzymol* **350**, 87–96.
51. Cliften, P., Jang, S.-H., and Jaehning, J. A. (2000). Identifying a core RNA polymerase surface critical for interactions with a sigma-like specificity factor. *Mol Cell Biol* **20**, 7013–7023.
52. Chiu, J., March, P. E., Lee, R., and Tillett, D. (2004). Site-directed, Ligase-Independent Mutagenesis (SLIM): a single-tube methodology approaching 100% efficiency in 4 h. *Nucleic Acids Res* **32**, e174–e174.
53. Chiron, S., Suleau, A., and Bonnefoy, N. (2005). Mitochondrial translation: elongation factor tu is essential in fission yeast and depends on an exchange factor conserved in humans but not in budding yeast. *Genetics* **169**, 1891–1901.
54. Saint-Georges, Y., Bonnefoy, N., di Rago, J. P., Chiron, S., and Dujardin, G. (2002). A pathogenic cytochrome b mutation reveals new interactions between subunits of the mitochondrial bc1 complex. *J Biol Chem* **277**, 49397–49402.
55. Sikorski, R. S., and Boeke, J. D. (1991). In vitro mutagenesis and plasmid shuffling: from cloned gene to mutant yeast. *Meth Enzymol* **194**, 302–318.
56. Groudinsky, O., Dujardin, G., and Slonimski, P. P. (1981). Long range control circuits within mitochondria and between nucleus and mitochondria. II. Genetic and biochemical analyses of suppressors which selectively alleviate the mitochondrial intron mutations. *Mol Gen Genet* **184**, 493–503.
57. Ogur, M., St John, R., and Nagai, S. (1957). Tetrazolium overlay technique for population studies of respiration deficiency in yeast. *Science* **125**, 928–929.
58. Malecki, M., Jedrzejczak, R., Puchta, O., Stepień, P. P., and Golik, P. (2008). In vivo and in vitro approaches for studying the yeast mitochondrial RNA degradosome complex. *Meth Enzymol* **447**, 463–488.
59. Tomecki, R., Dmochowska, A., Gewartowski, K., Dziembowski, A., and Stepień, P. P. (2004). Identification of a novel human nuclear-encoded mitochondrial poly(A) polymerase. *Nucleic Acids Res* **32**, 6001–6014.

60. Kolondra, A., Labedzka-Dmoch, K., Wenda, J. M., Drzewicka, K., and Golik, P. (2015). The transcriptome of *Candida albicans* mitochondria and the evolution of organellar transcription units in yeasts. *BMC Genomics* **16**, 1.
61. Szczepanek, T., and Lazowska, J. (1996). Replacement of two non-adjacent amino acids in the *S.cerevisiae* bi2 intron-encoded RNA maturase is sufficient to gain a homing-endonuclease activity. *EMBO J* **15**, 3758–3767.
62. Mulero, J. J., and Fox, T. D. (1993). Alteration of the *Saccharomyces cerevisiae* COX2 mRNA 5'-untranslated leader by mitochondrial gene replacement and functional interaction with the translational activator protein PET111. *Mol Biol Cell* **4**, 1327–1335.
63. Greenleaf, A. L., Kelly, J. L., and Lehman, I. R. (1986). Yeast RPO41 gene product is required for transcription and maintenance of the mitochondrial genome. *Proc Natl Acad Sci USA* **83**, 3391–3394.
64. Schmidt, U., Maue, I., Lehmann, K., Belcher, S. M., Stahl, U., and Perlman, P. S. (1998). Mutant alleles of the MRS2 gene of yeast nuclear DNA suppress mutations in the catalytic core of a mitochondrial group II intron. *J Mol Biol* **282**, 525–541.
65. Manthey, G. M., Przybyla-Zawislak, B. D., and McEwen, J. E. (1998). The *Saccharomyces cerevisiae* Pet309 protein is embedded in the mitochondrial inner membrane. *Eur J Biochem* **255**, 156–161.
66. Wallis, M. G., Groudinsky, O., Slonimski, P. P., and Dujardin, G. (1994). The NAM1 protein (NAM1p), which is selectively required for *cox1*, *cytb* and *atp6* transcript processing/stabilisation, is located in the yeast mitochondrial matrix. *Eur J Biochem* **222**, 27–32.
67. Main, E. R. G., Lowe, A. R., Mochrie, S. G. J., Jackson, S. E., and Regan, L. (2005). A recurring theme in protein engineering: the design, stability and folding of repeat proteins. *Curr Opin Struct Biol* **15**, 464–471.
68. Kobe, B., and Kajava, A. V. (2000). When protein folding is simplified to protein coiling: the continuum of solenoid protein structures. *Trends Biochem Sci* **25**, 509–515.

FIGURE LEGENDS

Figure1. Schematic structure of the *S. cerevisiae* Rpo41p mitochondrial RNA polymerase. MTS – Mitochondrial Targeting Sequence, NTE – N-terminal Extension, NTD – N-terminal Domain, CTD – C-terminal (catalytic) Domain. Arrows indicate the positions of pentatricopeptide (PPR) motifs predicted *in silico* [35]. Numbers indicate amino acid positions, for the three named PPR motifs that were studied here, the position of the first amino acid is shown.

Figure 2. Respiratory growth of *S. cerevisiae* strains bearing the wild-type (W303) or deletant variants of *RPO1*. Strains dPPR1, dPPR2 and dPPR3 bear deletions of the first, second, and third PPR motif, respectively. Suffix “int” indicates strains with the intronless mitochondrial DNA. Fermentative growth was tested on YPD (YP with 2% glucose) plates at the normal (30°C) and elevated (37°C) temperature. Respiratory growth was tested on YPG (YP with 2% glycerol) plates at the normal (30°C) and elevated (37°C) temperature. Serial dilutions of appropriate cultures were spotted on plates as described in Materials and Methods. Only the dPPR2 and dPPR2int strains show slower respiratory growth, particularly at 37°C.

Figure 3. Mitochondrial genome stability in *S. cerevisiae* strains bearing the wild-type (W303) or deletant variants of *RPO1*. Frequencies of *petite* mutants in strains carrying the wild-type *RPO41* or alleles expressing variants with PPR motif deletions, grown at the normal (30°C) and elevated (37°C) temperature, were measured as described in Materials and Methods. All three deletants show significantly ($p < 0.01$) increased frequencies of *petite* cells relative to the wild-type. Differences between dPPR cultures grown at 30°C and 37°C are also significant ($p < 0.01$), while the elevated temperature does not significantly affect mtDNA stability of the strain carrying the wild-type *RPO41* gene.

Figure 4. Northern blot analysis of mitochondrial mRNAs in *S. cerevisiae* strains bearing the wild-type (W303) or deletant variants of *RPO1*. Mitochondrial RNA preparations from each strain, grown at 30°C, were separated in 1% agarose-formaldehyde gel, blotted onto nylon membrane, and hybridised to appropriate probes as described in Materials and Methods. With the exception of *COX1* in dPPR3 and *COX2* in dPPR1, all the mRNA levels are either unchanged, or slightly increased relative to wild-type. Blots were stained with methylene blue in order to normalise the amount of mitochondrial RNA preparations in each lane using rRNA bands (“loading”).







