Schizosaccharomyces pombe Ppr10 and Mpa1 together mediate mitochondrial translational initiation

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Pentatricopeptide repeat (PPR) proteins are a large family of proteins that act primarily at different posttranscriptional steps of organellar gene expression. We have previously found that the *Schizosaccharomyces pombe* PPR protein mpal10 interacts with mitochondrial translational activator Mpa1, and both are essential for mitochondrial protein synthesis. However, it is unclear how these two proteins function in mitochondrial protein synthesis in *S. pombe*. In this study, we further investigated the role of Ppr10 and Mpa1 in mitochondrial protein synthesis. Mitochondrial translational initiation requires two initiation factors, Mti2 and Mti3, which bind to the small subunit of the mitochondrial ribosome (mt-SSU) during the formation of the mitochondrial translational initiation complex. Using sucrose gradient sedimentation analysis, we found that disruption of *ppr10*, *mpa1*, or the PPR motifs in Ppr10 impairs the association of Mti2 and Mti3 with the mt-SSU, suggesting that both Ppr10 and Mpa1 may be required for the interaction of Mti2 and Mti3 with the mt-SSU during the assembly of mitochondrial translational initiation complex. Loss of Ppr10 perturbs the association of mitochondrially encoded cytochrome *b* (*cob1*) and cytochrome *c* oxidase subunit 1 (*cox1*) mRNAs with assembled mitochondrial ribosomes. Proteomic analysis revealed that a fraction of Ppr10 and Mpa1 copurified with a subset of mitoribosomal proteins. The PPR motifs of Ppr10 are necessary for its interaction with Mpa1 and that disruption of these PPR motifs impairs mitochondrial protein synthesis. Our results suggest that Ppr10 and Mpa1 function together to mediate mitochondrial translational initiation.

Mitochondria provide the cellular energy currency ATP through oxidative phosphorylation (OXPHOS) (1). They are also involved in metabolism of nucleotides, amino acids, and lipids and synthesis of heme and iron–sulfur clusters, which are two iron-containing prosthetic groups involved in many important physiological processes (1, 2). In mammals, mitochondria also function as a signaling organelle that control cellular processes such as apoptosis and immune responses (1, 3).

Mitochondria carry their own genome (mtDNA) primarily encoding the key components of the OXPHOS system, as well as tRNAs and rRNAs required for mitochondrial protein synthesis. In *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, the mitochondrial genes *cob1* and *cox1* contain introns, some of which encode maturases required for their own splicing (4). Mitochondrial translation is necessary for the removal of mitochondrial introns.

Regulation of mtDNA expression occurs primarily at the posttranscriptional level (5, 6). Studies on *S. cerevisiae* and *S. pombe* reveal that mitochondrial translation is controlled by nuclearly encoded RNA-binding proteins (RBPs) (7–9), which are believed to act on the 5′-untranslated region (5′-UTR) of target transcripts. However, the exact mechanism by which these factors control mitochondrial translation remains to be determined. In contrast, mammalian mtDNA-encoded mRNAs (mt-mRNAs) do not have 5′-UTR, and thus it has been suggested that mammals use different mechanisms to regulate mitochondrial translation (10, 11). Indeed, in contrast to many translational activators in yeast, only two translational activators, TACO1 and leucine-rich pentatricopeptide repeat (PPR)-containing protein (LRPPRC), have so far been found in mammals. TACO1 is the *COX1*-specific translational activator that binds the *COX1* mRNA at multiple distinct regions (10). In contrast, LRPPRC and its binding partner, SLIRP function together as an RNA chaperone to enhance stabilization, polyadenylation, and translation efficiency of mt-mRNAs (12–16).

PPR proteins constitute a large family of proteins specialized to control nearly every stage of the posttranscriptional regulation of organellar genes, including RNA 5′-end maturation, intron splicing, RNA editing in plant organelles, RNA stabilization, and translational activation (17–22). PPR proteins are found only in eukaryotes and are most abundant in higher plants (23). Higher plants require large numbers of PPR proteins because their organellar RNAs undergo extensive posttranscriptional RNA editing, mostly C to U, to increase transcriptomic diversity (24).

The PPR motif is a degenerate ~35-amino acid (aa) sequence often arranged in tandem. Structural studies reveal that PPR motifs fold into solenoid structures containing a pair of antiparallel α-helices and consecutive repeats that are twisted into a right-handed superhelix (25–29). PPR motifs are involved in sequence-specific RNA-binding (26). In addition, they also participate in protein–protein interactions in humans (30).

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Ten PPR proteins have so far been identified in *S. pombe* (8, 9). There was no sequence homology between any of the proteins. *S. pombe* PPR proteins contain 2–18 PPR motifs. These PPR proteins are involved in mitochondrial RNA metabolism, including RNA stability and translation. Among them, Ppr2 and Ppr10 are general translational activators, as deletion of *ppr2* or *ppr10* dramatically impairs synthesis of all mtDNA-encoded OXPHOS subunits, resulting in defective mitochondrial respiration (8, 9).

PPR proteins have been found to interact with each or with non-PPR proteins. In humans and mice, LRPPRC and SLIRP form a complex and depend on each other for stability (31). However, stabilization of LRPPRC by deletion of the gene encoding the mitochondrial matrix protease LONP1, which is involved in the degradation of LRPPRC, cannot rescue mt-mRNA stability in the absence of SLIRP, suggesting that SLIRP is also required for the stability of mt-mRNAs (31). In *S. cerevisiae*, PPR protein Pet309, a *COX1* mRNA-specific translational activator, interacts with the DEAD-box helicase Mss116, and the Pet309-Mss116 interaction is required for Pet309 stability (32). In plants, PPR proteins are found to physically interact with each other to form a functional editosome (33, 34).

We have shown that in *S. pombe*, Ppr10 associates with Mpa1, and both act as mitochondrial translational activators (9). In this study, we further explore the roles of Ppr10 and Mpa1 in mitochondrial translation. We show that the PPR motifs in Ppr10 are essential for the mitochondrial function of *S. pombe*. We further show that the Ppr10-Mpa1 complex may facilitate the association of mitochondrial translational initiation factors Mti2 and Mti3 with the small subunit of mitochondrial ribosome (mt-SSU).

Results

The PPR motifs of Ppr10 are essential for the binding of Ppr10 to Mpa1

The *S. pombe* Ppr10 contains two highly degenerate PPR motifs (9). We first tested potential interaction of Ppr10 lacking PPR motifs (Ppr10ΔPPR) and Mpa1 (Fig. 1A) by coimmunoprecipitation from whole-cell extracts. We did not examine the interaction *in vitro* due to difficulties in obtaining recombinant full-length and mutant Ppr10 proteins. To do so, we generated strains expressing C-terminal c-Myc-tagged full-length Ppr10 or Ppr10ΔPPR under the control of its own promoter. A C-terminal c-Myc tag does not affect the function of Ppr10, as assayed by growth on YES medium containing either glucose or glycerol (9). The level of Ppr10ΔPPR-Myc is reduced compared with that of WT protein (Fig. 1B). We found that only the full-length Ppr10-Myc coimmunoprecipitated with Mpa1 from whole-cell extract, whereas the mutant Ppr10 did not (Fig. 1C).

Next we examined the effect of disruption of the Ppr10-Mpa1 interaction on production of mtDNA-encoded proteins. We determined the steady-state levels of mtDNA-encoded proteins in wild-type (WT), Δ*ppr10* and *ppr10*ΔPPR strains grown to exponential phase. Similar to the results seen in the Δ*ppr10* strain, deletion of PPR motifs in Ppr10 severely reduced the steady-state levels of Cob1 (subunit of the cytochrome *bc* 1 complex), the core subunits of cytochrome c oxidase (Cox1, Cox2, Cox3), and Atp6 (subunit of ATP synthase) (Fig. 1D).

The WT, Δ*ppr10*, and *ppr10*ΔPPR strains were examined for their ability to grow on YES-rich media containing glucose as fermentable carbon source or glycerol as nonfermentable carbon source. On nonfermentable source, *S. pombe* cells rely on the capacity of mitochondria to generate energy. Deletion of the PPR motifs in Ppr10 moderately affected the growth of *S. pombe* cells on glucose-containing media but severely impaired the growth on glycerol-containing media (Fig. 1E), similar to the *ppr10* knockout phenotypes. Taken together, these data reveal that the PPR motifs in Ppr10 are critical for the Ppr10-Mpa1 interaction and that disruption of the PPR motifs in Ppr10 impairs mitochondrial protein synthesis and, consequently, respiratory growth of *S. pombe* cells.

Ppr10, Mpa1, and the PPR motifs of Ppr10 are required for association of Mti2 and Mti3 with the mt-SSU

Because Ppr10 and Mpa1 function as activators of mitochondrial translation (9), we tested whether disruption of *ppr10*, *mpa1*, or the PPR motifs in Ppr10 would affect mitoribosome assembly by using sucrose gradient sedimentation followed by western blotting. Mitochondrial extracts from WT, Δ*ppr10*, Δ*mpa1*, *ppr10*ΔPPR cells were sedimented through sucrose gradient under conditions that maintained the intact mitoribosomes. The sedimentation profile of the small and large mitoribosomal subunits and the fully assembled mitoribosomes were determined by measuring the levels of the mt-SSU and the large mitoribosomal subunit (mt-LSU) proteins. For reasons that are not clear, mitochondrial polysomes cannot be separated from monosomes under the experimental conditions used here. Similar observations have been made in other species including *S. cerevisiae* (35, 36) and humans (37–39). Thus, we did not consider polysomes in our analysis. Disruption of *ppr10*, *mpa1*, or the PPR motifs of Ppr10 did not impair the assembly of mitoribosomes or their subunits (Fig. 2, A–D) and did not affect the steady-state levels of the protein subunits of the mt-SSU and mt-LSU (Fig. 2E).

Next, we examined whether disruption of *ppr10*, *mpa1*, or the PPR motifs of Ppr10 would affect the association of mitochondrial translational initiation factors with mitoribosomes. In the WT cells, the majority of Ppr10 remains at the upper fractions, comigrating with Mpa1 and a small fraction of Ppr10 and Mpa1 are associated with assembled mitoribosomes (Fig. 2A). In contrast, most of Mti2 and Mti3 were found associated with the mt-SSU and a minor fraction of these two proteins cosedimented with assembled mitoribosomes (Fig. 2A). We consistently found that disruption of *ppr10*, *mpa1*, or the PPR motifs of Ppr10 resulted in dissociation of Mti2 and Mti3 from the mt-SSU and reduced the association of Mti2 and Mti3 with mitoribosomes (Fig. 2, B–D).

Loss of Ppr10 perturbs association of cob1 *and* cox1 *mRNAs with assembled mitoribosomes*

We examined whether loss of *ppr10* affects the association of mitoribosomes with *cob1* and *cox1* mRNAs encoding the most energetically central components of OXPHOS III and OXPHOS IV, respectively. To do so, we performed sucrose gradient sedimentation of mitochondrial extracts from the P3 strain (WT[Δi]) devoid of mtDNA introns (40) and its isogenic Δ*ppr10* mutant (Δ*ppr10*[Δi]). We chose these two strains because we have previously shown that the levels of the mature

cob1 and *cox1* mRNAs are dramatically reduced in Δ*ppr10* cells containing mtDNA introns but not in Δ*ppr10* cells lacking mtDNA introns (4). This is due to the fact that Δ*ppr10* cells containing mtDNA introns are defective in splicing of *cox1* and *cob1* introns, which is most likely caused by impaired mitochondrial translation (4). After sucrose gradient fractionation, one-third of each fraction was used for RNA extraction, and the presence of the small subunit and large subunit mitoribosomal RNAs (*rns* and *rnl*) and *cob1* and *cox1* mRNAs was analyzed by qRT-PCR, and the remaining two-thirds were analyzed by western blotting for levels of
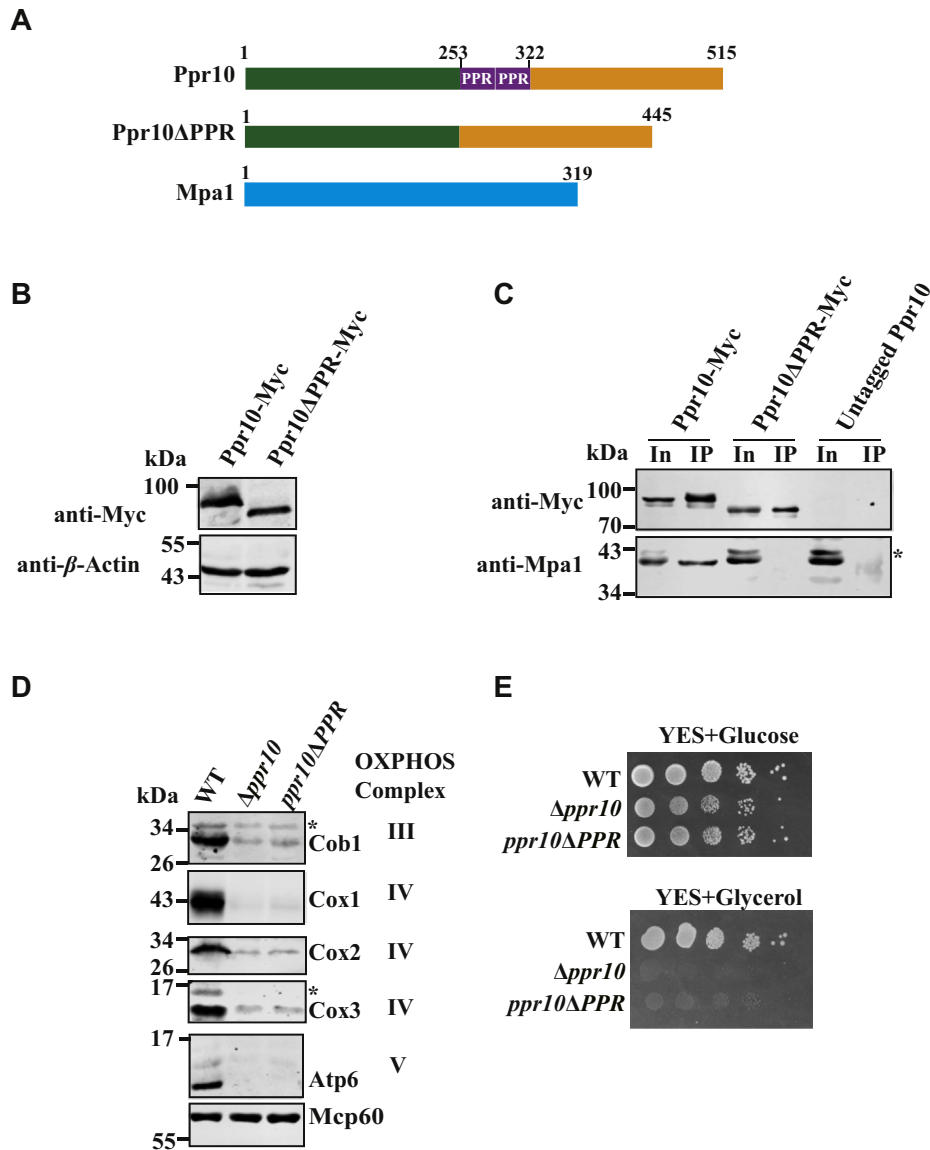
Figure 1. The PPR motifs of Ppr10 are critical for the mitochondrial protein synthesis and the respiratory growth of *S. pombe* cells. *A*, a schematic of full-length Ppr10, Ppr10 lacking PPR motifs (Ppr10ΔPPR), and Mpa1. PPR motifs are indicated. Numbers above the bar indicate the number of amino acids in each protein. *B*, western blotting of full-length Ppr10 and Ppr10 lacking PPR motifs in *S. pombe* extracts. Crude cell extracts from cells expressing c-Myc-tagged full-length Ppr10 (Ppr10-Myc) or Ppr10 lacking PPR motifs (Ppr10ΔPPR-Myc) were prepared by alkaline extraction and proteins were analyzed by western blotting using anti-c-Myc Ab. *S. pombe* Act1 detected by anti-β-actin Ab serves as loading control. *C*, deletion of PPR motifs in Ppr10 abolished the Ppr10-Mpa1 interaction. Crude cell extracts were prepared by glass beads lysis and were incubated with anti-c-Myc beads. The input (In) and immuno-precipitated (IP) proteins were analyzed by western blotting. *D*, *ppr10* deletion affects mitochondrial protein synthesis. Mitochondrial extracts from WT, Δ*ppr10*, and *ppr10ΔPPR* cells expressing c-Myc-tagged Ppr10 lacking PPR motifs were prepared from spheroplasts. mtDNA-encoded proteins were analyzed by western blotting using the indicated Abs. Mitochondrial heat shock protein Mcp60 identified by anti-HSP60 Ab serves as a loading control. The *asterisk* indicates a nonspecific band. *E*, *ppr10* deletion impairs the respiratory growth. Cells were grown in YES to stationary phase and serial 10-fold dilutions were spotted onto rich media containing 3% glucose (YES+Glucose) or 3% glycerol and 0.1% glucose.
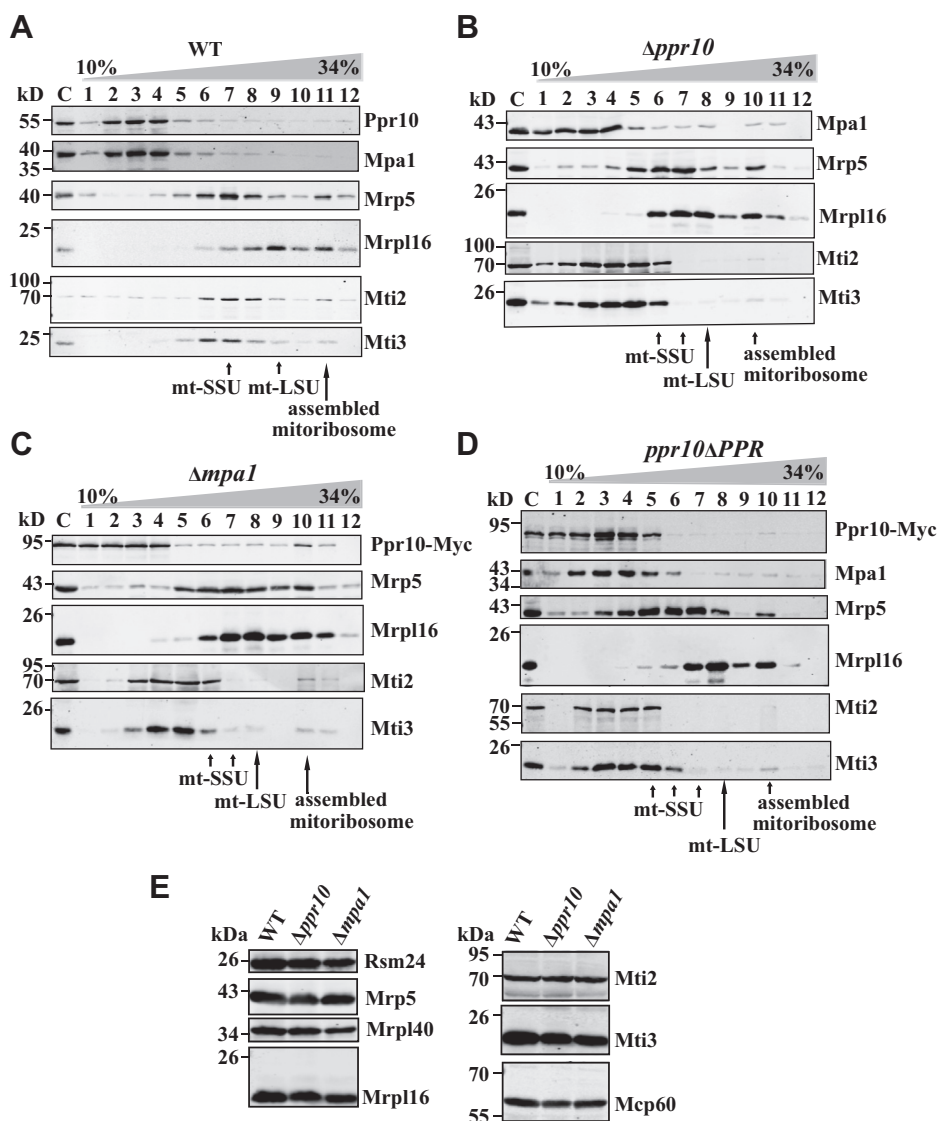
Figure 2. Disruption of *ppr10*, *mpa1*, or the PPR motifs of Ppr10 affects the association of Mit2 and Mti3 with the mt-SSU. *A–D*, sucrose gradient analyses of Ppr10, Mpa1, mitoribosomal subunits, fully assembled mitoribosomes, and mitochondrial translational initiation factors in WT (*A*), Δ*ppr10* (*B*), Δ*mpa1* (*C*), and Δ*ppr10* cells expressing Ppr10 lacking PPR motifs (*ppr10ΔPPR*) (*D*). Mitochondrial extracts were prepared from spheroplasts and were fractionated by sucrose gradient sedimentation. The input (*C*) and fractions were analyzed by western blotting for the various proteins as indicated. The small (Mrp5) and large (Mrpl16) mitoribosomal proteins mark the positions of the mt-SSU and mt-LSU, respectively. Positions of the mt-SSU, mt-LSU, and mitoribosome are indicated. *E*, disruption of *ppr10* and *mpa1* does not affect the steady-state levels of mitoribosomal proteins. Crude mitochondrial extracts were prepared and analyzed by western blotting with antibodies. Mcp60 detected by anti-HSP60 Ab serves as a loading control.

mitoribosomal markers (Mrp5 and Mrpl16). The *cob1* and *cox1* mRNAs sedimented in two peaks, one comigrating with assembled mitoribosomes (fractions 9–11) and the other cosedimenting with the mt-SSU (fractions 4–8) (Fig. 3). The association of *cob1* and *cox1* mRNAs with assembled mitoribosomes was reduced by *ppr10* deletion, whereas their association with the mt-SSU was increased (Fig. 3). These results suggest that Ppr10 may have a role in mt-mRNA recruitment to the mitoribosome.

Ppr10 and Mpa1 copurify with a subset of mitoribosomal proteins

To identify additional proteins associated with Ppr10, we performed tandem affinity purification (TAP) using extracts from strains expressing untagged Ppr10 or TAP-tagged Ppr10 under the control of its own promoter. The TAP tag does not affect the function of Ppr10, as assayed by growth on YES medium containing either glucose or glycerol (9). Mass spectrometry (MS) analysis of Ppr10-copurified proteins revealed that some proteins were specifically detected in the Ppr10-TAP preparation, albeit at low levels. These proteins included mt-SSU proteins (Nam9, Mrps28, Mrp4, and Mrps9), mt-LSU proteins (Aco2-Mrpl49, Mrpl7, and Yml6), SPAC750.08c (a putative NAD-dependent malic enzyme), SPBP4H10.18c (a *Schizosaccharomyces* specific protein of unknown function), and Anc1 (a mitochondrial ADP/ATP carrier) (Fig. 4*A* and Table S2). The Aco2-Mrpl49 is a fusion protein of aconitase and a putative mitochondrial ribosomal (mitoribosomal)
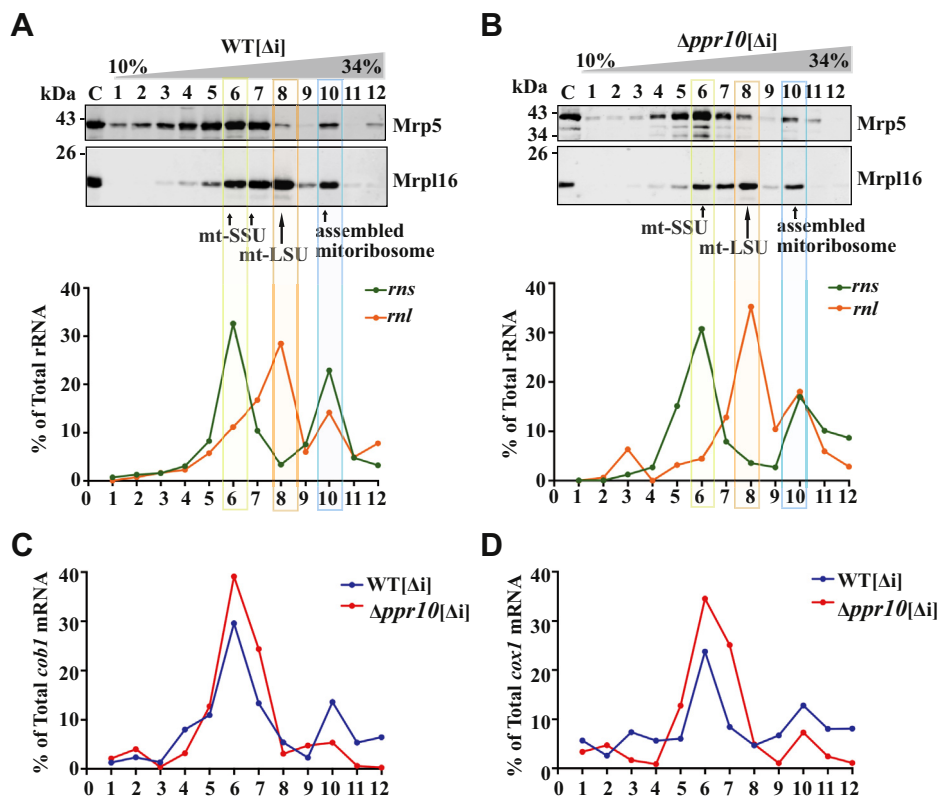
Figure 3. *ppr10* deletion affects the association of *cob*1 and *cox*1 mRNAs with mitoribosomes. *A* and *B*, sucrose gradient sedimentation profiles of mitoribosomal proteins and rRNAs from mtDNA intronless WT (*A*) and Δ*ppr10* (*B*) strains. Mitochondrial extracts were prepared from spheroplasts followed by centrifugation on 10–34% sucrose gradients. *Upper panel*, western blot analysis of the small (Mrp5) and large (Mrpl16) subunits of mitoribosome. *Lower panel*, qRT-PCR analysis of two mitoribosomal RNAs (rns and rnl). *Transparent yellow*, *orange*, and *blue* colors mark the peak fractions of mtSSU, mtLSU, and assembled mitoribosomes, respectively. *C* and *D*, sucrose gradient sedimentation profiles of the *cob*1 (*C*) and *cox*1 (*D*) mRNAs in mtDNA intronless WT and Δ*ppr10* strains. RNAs were analyzed by qRT-PCR using primers specific for rns, rnl, cob1, and cox1. The data are expressed as percentage of total specific RNA and are representative of three independent experiments.

protein Mrpl49, which is essential for mitochondrial translation (41).

To confirm the interaction of Ppr10 and Mpa1 with mitoribosomal proteins, we performed coimmunoprecipitation assays with extracts prepared from cells expressing FLAG-tagged Aco2-Mrpl49 and Myc-tagged Ppr10 or untagged Aco2-Mrpl49 and Myc-tagged Ppr10 from their native promoters. Proteins associated with anti-FLAG immunoprecipitates were analyzed by immunoblotting with the corresponding antibodies. Western blot analysis demonstrated that a fraction of Ppr10-Myc and Mpa1 was coimmunoprecipitated with Aco2-Mrpl49-FLAG, but not with untagged Aco2-Mrpl49 (Fig. 4*B*). As expected, two mitochondrial large ribosomal subunit proteins Mrpl40 and Mrpl16 were also coimmunoprecipitated with Aco2-Mrpl49-FLAG (Fig. 4*B*). We also performed reciprocal coimmunoprecipitation assays using extracts prepared from cells expressing FLAG-tagged Aco2-Mrpl49 and untagged Ppr10 or FLAG-tagged Aco2-Mrpl49 and Myc-tagged Ppr10 from their native promoters. As expected, Mpa1 is coimmunoprecipitated with Ppr10-Myc. Consistent with above immunoprecipitation results, we found that Aco2-Mrpl49-FLAG coimmunoprecipitated weakly with Ppr10-Myc (Fig. 4*C*). Altogether, these results suggest that a

fraction of Ppr10-Mpa1 interacts with a subset of mitoribosomal proteins.

Discussion

The data reported herein demonstrate that disruption of *ppr10*, *mpa1*, or the PPR motifs in Ppr10 affects the association of Mti2 and Mti3 with the mt-SSU. We propose that Ppr10 and Mpa1 function cooperatively to facilitate the association of Mti2-Mti3 with the mt-SSU during the initiation of mitochondrial translation. To our knowledge, this is the first report showing that the association of mitochondrial translational initiation factors can be affected by translational activators. Because we have shown previously that loss of Mti2 results in dissociation of Mti3 from the mt-SSU (42), dissociation of Mti3 from the mt-SSU in the absence of Ppr10, Mpa1, or the PPR motifs of Ppr10 could be caused by dissociation of Mti2 from the mt-SSU. Studies on *S. cerevisiae* (43), *S. pombe* (42), and cultured human cells (44) reveal that only mitochondrial translational initiation factor 2 (mtIF2), which assumes the role of both bacterial initiation factors IF1 and IF2, is absolutely required for mitochondrial protein synthesis, whereas mitochondrial translational initiation factor 3 (mtIF3) is dispensable for protein synthesis, indicating that mtIF2 plays a more important role than mtIF3 in mitochondrial

A

B

C
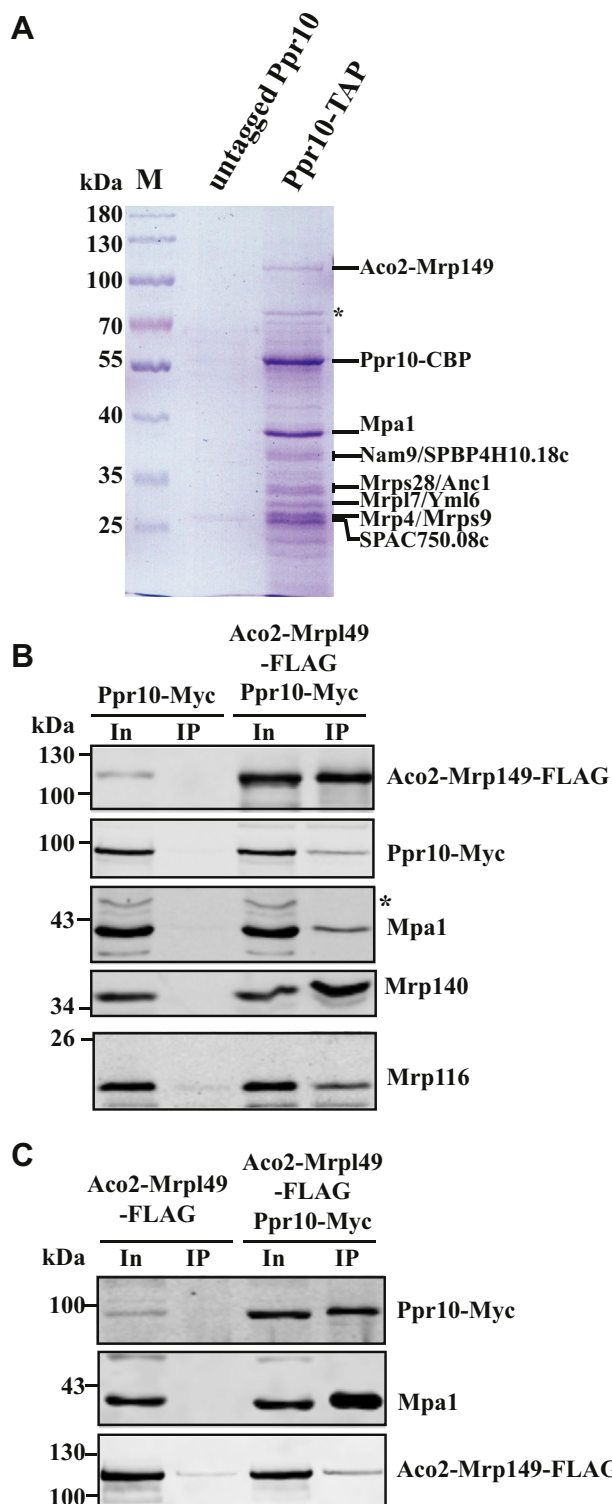
Figure 4. Ppr10 and Mpa1 associate with a subset of mitoribosomal proteins. *A*, TAP purification and MS analysis of proteins associated with Ppr10. TAP purification was performed by using whole-cell extracts. Proteins associated with Ppr10-TAP were TAP-purified, separated by SDS/PAGE, and stained with Coomassie blue. Protein bands that were only present in the Ppr10 purified fraction were excised and identified by MS. The molecular weight of the protein markers is indicated on the left. Mock purification was performed with extracts expressing untagged Ppr10. The band (indicated by the *asterisk*) was not identified. *B*, coimmunoprecipitation of Aco2-Mrpl49 with Ppr10-Myc and Mpa1. Whole-cell extracts (In) from cells expressing Ppr10-Myc (control) or Ppr10-Myc and Aco2-Mrpl49-FLAG were immunoprecipitated (IP) with anti-FLAG beads. Coimmunoprecipitated

translation. Because mitochondrial translational initiation factors function in mitochondrial translation *via* their association with the mt-SSU, the reduced association between Mti2-Mti3 and the mt-SSU may compromise the function of Mti2 and Mti3 and could explain impaired translation in cells lacking Ppr10, Mpa1, or the PPR motifs of Ppr10.

It remains unclear how Ppr10 and Mpa1 facilitate the association of Mti2 and Mti3 with the mt-SSU. One possible explanation is that Ppr10 and Mpa1 may exert their effect through their direct interaction with Mti2. This is supported by our previous finding that Ppr10 interacts weakly with Mti2 but not with Mti3 *in vivo* and *in vitro* (9).

Our results revealed that a very small fraction of Ppr10 and Mpa1 cosedimented with mitoribosome and that Ppr10 and Mpa1 copurified, albeit in substoichiometric amounts, with a subset of mitoribosomal proteins. Disruption of the Ppr10-Mpa1 interaction by deleting the PPR motifs of Ppr10 does not appear to affect the association of Ppr10 with mitoribosomal proteins (Fig. S1), suggesting that the Ppr10-Mpa1 interaction is not required for this association. These results also suggest that Ppr10-Mpa1 may have a role beyond the Mti2 and Mti3 association with the mt-SSU. It is likely that Ppr10-Mpa1 may also function through interaction with mitoribosome.

Disruption of *ppr10* perturbs the association of *cob1* and *cox1* with the assembled mitoribosomes and increases the association of *cob1* and *cox1* with the mt-SSU. One possible explanation is that the mt-mRNAs can bind to both the mt-SSU and the assembled mitoribosomes, and the absence of Ppr10 favors the binding of mt-mRNAs to the mt-SSU. This explanation is supported by the finding that in the absence of auxiliary initiation factors, mammalian mt-mRNAs can bind to the mt-SSU in a sequence-independent manner (45). Future studies will be required to elucidate the mechanism by which Ppr10 facilitates the binding of mt-mRNAs to assembled mitoribosomes.

Unexpectedly, we observed that the association of Mti2 and Mti3 with the mt-SSU is significantly affected in cells lacking Ppr10, Mpa1, or the PPR motifs of Ppr10, but the formation of assembled mitoribosomes does not appear to be significantly affected. One possible explanation for this is that the impaired interaction between Mti2-Mti3 and the mt-SSU in the absence of Ppr10, Mpa1, or the PPR motifs of Ppr10 leads to aberrant mitoribosome assembly and consequently, to defective translation.

Unlike bacterial translation initiation, mitochondrial translational initiation requires activators (46). The requirement for activators for mitochondrial translational initiation varies greatly among eukaryotic organisms (47). In *S. cerevisiae*,

proteins were detected by western blotting by using anti-c-Myc Ab (for Ppr10-Myc), and Abs against Mpa1 and mitochondrial large ribosomal subunit proteins Mrpl40 and Mrpl16. The *asterisk* indicates a nonspecific band. *C*, reciprocal coimmunoprecipitation of Ppr10-Myc and Mpa1 with Aco2-Mrpl49-FLAG. Whole-cell extracts (In) from cells expressing Aco2-Mrpl49-FLAG (control) or Aco2-Mrpl49-FLAG and Ppr10-Myc were immunoprecipitated (IP) with anti-c-Myc beads. The immunoprecipitates were analyzed by western blotting using the Abs indicated.

mitochondrial translational initiation is governed by mt-mRNA-specific translational activators, and in some cases, multiple activators are required for the synthesis of one protein (48). These translational activators function through interactions with 5′-UTRs of mt-mRNAs or with proteins of the mitoribosome. In *S. pombe*, mitochondrial translational initiation appears to require both specific and general translational activators (8, 9). Mammalian mitochondrial mRNAs generally lack 5′-UTRs, suggesting that different mechanisms to regulate mitochondrial translational initiation may be used in mammals (44). So far, TACO1 (10, 49) and LRPPRC (13, 16, 31) are the only two mitochondrial translational activators that have been identified in mammals. But their exact role in mitochondrial translational initiation has not been established. The general translational activators Ppr10 and Mpa1 described herein appear to be fission yeast-specific. Nevertheless, our findings suggest a possible mechanism for the global regulation of mitochondrial translational initiation, which has not been reported so far.

Our findings reveal that the PPR motifs in Ppr10 are required for the Ppr10-Mpa1 interaction. The involvement of PPR motifs in protein–protein interactions has been previously reported. Spahr *et al.* (30) reported that three neighbouring PPR motifs in LRPPRC contribute to the LRPPRC-SLIRP interaction. Our results also show that disruption of PPR motifs in Ppr10 results in phenotypes similar to those of Δ*ppr10* cells, indicating that PPR motifs in Ppr10 are essential for the function of the protein. It is very likely that the PPR motifs in Ppr10 exert their effects through mediating the Ppr10-Mpa1 interaction. However, we could not rule out the possibility that effects of disruption of the PPR motifs in Ppr10 may be independent of their role in mediating the Ppr10-Mpa1 interaction. Further study is needed to directly discern the impact of the Ppr10-Mpa1 interaction on mitochondrial translation. Nevertheless, the findings of this paper further support the notion that Ppr10 and Mpa1 function together in mitochondrial translational initiation by facilitating the association of Mti2 and Mti3 with the mt-SSU.

Experimental procedures

Yeast strains, plasmids, media, genetic manipulation, and primers

S. pombe strains used in this study are listed in Table 1. Strains deleted for *ppr10* or *mpa1* and strains expressing Ppr10 tagged with TAP (Ppr10-TAP) or c-Myc-tagged (Ppr10-Myc) from its endogenous promoter and native locus were constructed as described (4, 9). Strain yWQ1 expressing FLAG-tagged Aco2-Mrpl49 (Aco2-Mrpl49-FLAG) from its endogenous promoter and native locus was constructed in the same way as yYJ12 expressing FLAG-tagged Mti2 (9). Strain yWQ2 expressing Aco2-Mrpl49-FLAG and Ppr10-Myc was constructed by integrating the *aco2-mrpl49::[aco2-mrpl49-FLAG-ura4]* integration cassette into the endogenous *aco2-mrpl49* locus of strain yYJ1.

Strains expressing HA-tagged Aco2-Mrpl49 (Aco2-Mrpl49-HA) and *c*-Myc-tagged WT Ppr10 (Ppr10-Myc) or Ppr10

Table 1
Strains used in the present study

Strain	Genotype	Source
yHL6381	*h⁺ his3-D1 leu1-32 ura4-D18 ade6-M210*	H. Levin
yHH1	*h⁺ his3-D1 leul-32 ura4-D18 ade6-M210 Δppr10::kanMX6*	(9)
yZQ1	*h⁺ his3-D1 leul-32 ura4-D18 ade6-M210 Δmpa1::kanMX6*	(9)
P3	*h⁺ ade7-50 rho⁺* (no mitochondrial introns)	(45)
yXW1	*h⁺ ade7-50 rho⁺* (no mitochondrial introns) *Δppr10::kanMX6*	(4)
yZL1	*h⁺ his3-D1 leul-32 ura4-D18 ade6-M210 Δppr10::kanMX6 leu1-32::[ppr10–13Myc-leu1-hphMX6]*	This report
yZL2	*h⁺ his3-D1 leul-32 ura4-D18 ade6-M210 Δppr10::kanMX6 leu1-32::[ppr10ΔPPR-13Myc-leu1-hphMX6]*	This report
yYJ1	*h⁺ leu1-32 his3-D1 ura4-D18 ade6-M210 ppr10::[ppr10–13Myc-kanMX6]*	(9)
yWQ1	*h⁺ leu1-32 his3-D1 ura4-D18 ade6-M210 aco2-mrpl49::[aco2-mrpl49-2FLAG-ura4]*	This report
yWQ2	*h⁺ leu1-32 his3-D1 ura4-D18 ade6-M210 ppr10::[ppr10–13Myc-kanMX6] aco2-mrpl49::[aco2-mrpl49-2FLAG-ura4]*	This report
yWY1	*h⁺ leu1-32 his3-D1 ura4-D18 ade6-M210 Δppr10::kanMX6 leu1-32::[ppr10–13Myc-leu1-hphMX6] aco2-mrpl49::[aco2-mrpl49-3HA-natMX6]*	This report
yWY2	*h⁺ leu1-32 his3-D1 ura4-D18 ade6-M210 Δppr10::kanMX6 leu1-32::[ppr10ΔPPR-13Myc-leu1-hphMX6] aco2-mrpl49::[aco2-mrpl49-3HA-natMX6]*	This report

lacking PPR motifs (Ppr10ΔPPR-Myc) under the control of their respective endogenous promoters was constructed as follows. The DNA fragment for HA tagging at the C-terminus of Aco2-Mrpl49 was obtained by overlap PCR as follows. The first DNA fragment containing 547 bp of sequence upstream of the Aco2-Mrpl49 stop codon was amplified by PCR using the *S. pombe* genomic DNA as template. The second DNA fragment containing 3XHA epitopes, the *ADH1* transcriptional terminator, and the *natMX6* cassette conferring nourseo-thricin resistance was amplified from plasmid pZH1. The third DNA fragment containing 579 bp of sequence downstream of the Aco2-Mrpl49 stop was amplified by PCR using the *S. pombe* genomic DNA as template. These three fragments were then fused by PCR and the PCR product was transformed into strains yZL1 and yZL2, yielding the yWY1 and yWY2 strains, respectively.

Strains expressing C-terminally c-Myc-tagged WT Ppr10 or Ppr10ΔPPR under the control of the *ppr10* promoter and integrated at the *leu1-32* locus were constructed as follows. Because *S. pombe* cells lacking functional Ppr10 protein failed to grow in minimal medium, we first constructed a vector that contains the hygromycin resistance cassette (*hphMX6*) as a selectable marker, which allows selection of transformants in rich medium. To this end, an integrative plasmid carrying the c-Myc tag, the *ADH1* transcriptional terminator, and *hphMX6* was constructed by overlap PCR as follows. The first DNA fragment containing the 13 c-Myc epitopes followed by the *ADH1* transcriptional terminator was amplified from plasmid pFA6A-13Myc-kanMX6 (50). The second DNA fragment containing the *hphMX6* cassette was amplified from plasmid pFA6a-hphMX6 (9). These two fragments were then fused by PCR and the fused product was digested with Sma I and Sal I

and ligated into the Sma I and Sal I sites of pJK148 (51) to give plasmid pZL1. The promoter sequence and the coding sequence of *ppr10* were amplified by PCR using the *S. pombe* genomic DNA as template. The resulting PCR product was digested with BamH I and Sma I and ligated into the BamH I and Sma I sites of pZL1 to give pZL2. pZL2 is linearized with Nru I and was integrated at *leu1-32* in the Δ*ppr10* strain, generating strain yZL1 expressing full-length Ppr10 tagged with the c-Myc at C-terminus and under control of its own promoter. The sequences of gene encoding Ppr10ΔPPR and its promoter were generated by overlapping PCR using pZL2 as template and two sets of PCR primers including overlapping primer pairs spanning the deleted region. The PCR product was digested with BamH I and Sma I, and cloned into the BamH I and Sma I sites of pZL1. The resulting plasmid was integrated into the Δ*ppr10* strain, generating strain yZL2 expressing Ppr10ΔPPR tagged with the c-Myc at C-terminus and under control of its own promoter. PCR primer sequences are provided in Table S1.

S. pombe cells were grown in rich media (YES, 0.5% yeast extract, and supplements) supplemented with 3% glucose for fermentative growth or 3% glycerol and 0.1% glucose for respiratory growth (52, 53). Standard media and protocols for genetic manipulation of fission yeast were used as described previously (52).

Affinity purification, coimmunoprecipitation, and western blot analyses

Preparation of protein extracts, protein affinity purification, coimmunoprecipitation, and western blot analyses were performed as described previously (9). Antibodies (Abs) against Ppr10 and Mpa1, rabbit HSP60, and the c-Myc epitope (9) and Abs against Mrp5, Mrpl16, Mti2, and Mti3 were described previously (42). Other Abs were generated as follows: synthetic peptides corresponding to amino acid (aa) residues 239–258 of Rsm24 or 32–50 of Mrpl40 were used to immunize rabbits and antibodies were affinity purified on peptide columns (Bioworld). Anti-β-actin Ab was used to detect *S. pombe* actin (Act1) (Proteintech, 60008-1-Ig).

Sucrose density-gradient analysis of mt-RNAs

Mitochondrial extracts were prepared and fractionated by sucrose gradient sedimentation as described previously (42) except that 200 u/ul of RNaseOUT RNase inhibitor (Invitrogen) was included in the mitochondrial lysis buffer to prevent degradation of RNA. In brief, about 2 mg of mitochondrial proteins dissolved in 300 μl of lysis buffer was loaded onto a 10–34% linear sucrose gradient (4 ml total volume). Gradients were centrifuged for 3 h at 4°C in SW 60Ti (Beckman Coulter, USA). Twelve equal fractions were collected. Total RNA was purified from one-third of each fraction as described (54). After treatment with DNase I to remove genomic DNA, RNA in each fraction was reverse-transcribed into cDNA using HiScriptIIQ RT SuperMix (Vazyme, China). Quantitative Real-Time RT-PCR (qRT-PCR) was performed using AceQ qPCR SYBR Green Master Mix (Vazyme) with primers specific for *rns*, *rnl*, *cob1*, and *cox1*. PCR primer sequences were previously described (9). All reactions were performed in triplicate. Data analysis was performed by StepOne software. The abundance of *rns*, *rnl*, *cob1*, and *cox1* in each fraction was calculated by using the $2^{-\Delta Ct}$ method and expressed as percentage of the summed amount of each transcript in all fractions. The remaining two-thirds of each fraction were precipitated with trichloracetic acid, and proteins were separated by SDS-PAGE and immunoblotted with anti-Mrp5 and anti-Mrpl16 Abs.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE (55) partner repository with the dataset identifier PXD025862. All data that support the findings of this study are contained within the article and its supporting information.

Abbreviations—The abbreviations used are: aa, amino acids; Ab, antibody; Co-IP, co-immunoprecipitation; COX, cytochrome *c* oxidase; IP, immunoprecipitation; mitoribosome, mitochondrial ribosome; MS, mass spectrometry; mtDNA, mitochondrial DNA; mt-LSU, large subunit of the mitoribosome; mt-RNA, mtDNA-encoded RNA; mt-SSU, small subunit of the mitoribosome; OXPHOS, oxidative phosphorylation; PPR, pentatricopeptide repeat; qRT-PCR, quantitative reverse transcription-PCR; TAP, tandem affinity purification; WT, wild-type.

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