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The role of mammalian PPR domain proteins in the regulation of mitochondrial gene expression ☆

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ABSTRACT

Pentatricopeptide repeat (PPR) domain proteins are a large family of RNA-binding proteins that are involved in the maturation and translation of organelle transcripts in eukaryotes. They were first identified in plant organelles and their important role in mammalian mitochondrial gene regulation is now emerging. Mammalian PPR proteins, like their plant counterparts, have diverse roles in mitochondrial transcription, RNA metabolism and translation and consequently are important for mitochondrial function and cell health. Here we discuss the current knowledge about the seven mammalian PPR proteins identified to date and their roles in the regulation of mitochondrial gene expression. Furthermore we discuss the mitochondrial RNA targets of the mammalian PPR proteins and methods to investigate the RNA targets of these mitochondrial RNA-binding proteins. This article is part of a Special Issue entitled: Mitochondrial Gene Expression.

1. Introduction

The mitochondrial genome is a compact, circular, double-stranded genome encoding only 13 proteins that are all subunits of the electron transport chain, as well as 2 rRNAs and 22 tRNAs required for their translation [1]. Mitochondrial genes for proteins and tRNAs are located on both the heavy and light strands of the genome and are replicated by the mitochondrial polymerase γ (POLG) [2,3]. Mitochondrial genes are transcribed as large polycistronic transcripts covering almost the entire length of each strand [4–6]. A third transcript covering the start of the heavy strand and the two rRNA genes is also produced [7]. A comprehensive description of the current understanding of mitochondrial replication and transcription can be found in recent reviews [8–13], as well as in this special issue of BBA. The punctuation model of RNA processing has proposed that genes encoding for protein or rRNA are interspersed by one or more tRNAs, which can act as "punctuation" marks for cleavage [14], shown to involve the RNase P at the 5′ end of tRNAs [15], and the mitochondrial RNase Z, until recently hypothesized to be ELAC2, at the 3′ end of tRNAs [16]. Recently ELAC2 was shown to be the mitochondrial RNase Z that processes the 3′ end of mitochondrial tRNAs [17,18]. Following RNA processing, mitochondrial RNAs undergo maturation that involves the addition of a CCA triplet to the 3′ ends of tRNAs and modification of specific bases within both tRNAs and rRNAs [19,20], while mRNAs are generally polyadenylated at their 3′ ends [21,22]. Regulation of the processing of mitochondrial tRNAs can have profound effects on mitochondrial gene expression, by affecting the levels of mature species, the final processing of the different RNAs, and the overall level of translation and mitochondrial function [18].

Recently we provided the first comprehensive map of the human mitochondrial transcriptome and reveal its unexpected complexity by near-exhaustive deep sequencing of long and short RNA fractions from purified mitochondria [23,24]. Despite their common polycistronic origin, we observe wide variation between individual tRNAs, mRNAs, and rRNA amounts [23], indicating the importance of post-transcriptional processing and degradation mechanisms in the regulation of mitochondrial gene expression. The levels of the 13 individual mRNAs and their proteins must be controlled at a post-transcriptional level, which opens a new era for investigation of mitochondrial RNA-binding proteins and the mechanisms by which they govern mitochondrial gene expression.

In yeast, mitochondrial transcripts have 5′ untranslated regions that bind translational activators, however these are not conserved in mammals [25]. Because mammalian mitochondrial mRNAs generally begin at the start codon and lack 5′ untranslated regions or Shine-Dalgarno sequences [26], mitochondrial ribosomes must have evolved an alternative way to regulate translation initiation and to ensure accurate start codon recognition. Furthermore, the mammalian mitochondrial ribosome has the unusual property of frameshifting at the ends of the *CO1* and *ND6* mRNAs to enable the termination of protein synthesis [27]. Comparative studies of the

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mammalian mitochondrial ribosome with bacterial and cytoplasmic ribosomes of eukaryotes have identified large variations in the composition and number of mitochondrial ribosomal subunits [20,26,28–31] and the number of additional ribosomal subunits may increase to include previously uncharacterized proteins. Proteomic studies of bovine and yeast mitochondrial ribosomes suggest that they are composed of about 80 proteins [31–33]. Mitochondrial ribosomal RNA has considerably reduced in size during evolution and has been replaced by additional proteins. Consequently, mitochondrial ribosomes have acquired more proteins than their cytoplasmic and bacterial counterparts, some of which may have new functions in the translation and recognition of mitochondrial mRNAs. Our current knowledge about the function of these supernumerary proteins is limited to comparative genomic analysis [31] and their function is yet to be elucidated to determine how they contribute to mitochondrial gene expression.

2. Pentatricopeptide repeat domain proteins

Pentatricopeptide repeat (PPR) domain proteins were discovered in *Arabidopsis thaliana* where they constitute a large family of mitochondrial and plastid proteins involved in transcript processing, stability, editing and translation [34–37]. PPR motifs comprise a set of related degenerate motifs of approximately 35 amino acids occurring as tandem arrays of 2–26 motifs per protein [35,36] (Fig. 1A). PPR motifs are related to tetratricopeptide repeat (TPR) motifs that mediate protein–protein interactions, but PPR domains appear to be mostly involved in RNA–protein interactions. Their RNA-binding affinity has been confirmed by *in vitro* and *in vivo* experiments, including gel shift, UV cross-linking and affinity assays [38]. However, the molecular mechanisms behind the specificity of PPR domain binding to RNA are unclear. Experimental and structural evidence is necessary to explain the mechanism of their interaction with RNA. Although studies are underway investigating the association of PPR proteins with RNA it is worth noting that these proteins are notorious for their insolubility, which has likely delayed determination of their atomic structure. In analogy to TPR motifs it has been thought that PPR motifs form two antiparallel α-helices (Fig. 1A) that can form a superhelix in tandem arrays with a central groove thought to be involved in ligand binding [36]. Unlike with TPR motifs it is predicted that the residues from the PPR motifs that project into the central groove are hydrophilic and the bottom of the groove is positively charged thereby enabling tandem PPR repeats to recognize and bind RNA in a sequence specific manner [38] akin to the Pumilio and FBF homology (PUF) family proteins [39,40], for which we have recently completed the recognition code [41,42]. Recently Prof Small and his team have described a predicted consensus model of the PPR structure that may give clues to the RNA recognition code of these proteins [43]. In this model amino acid residues at positions 1, 3 and 6 of each PPR motif are predicted to be on the internal face of the first helix of the motif similarly to PUF domain proteins [40,42], where this helix could form the RNA binding surface of the PPR motif. It is suggested that these residues may play a key role in the recognition of each individual base [43].

In mammals, to date, there are only seven identified mitochondrial PPR domain proteins, the mitochondrial RNA polymerase (POLRMT), the leucine-rich PPR cassette (LRPPRC) protein, PPR domain containing proteins (PTCD) 1, 2, and 3, mitochondrial RNase P protein 3 (MRPP3) and mitochondrial ribosomal protein of the small subunit 27 (MRPS27)

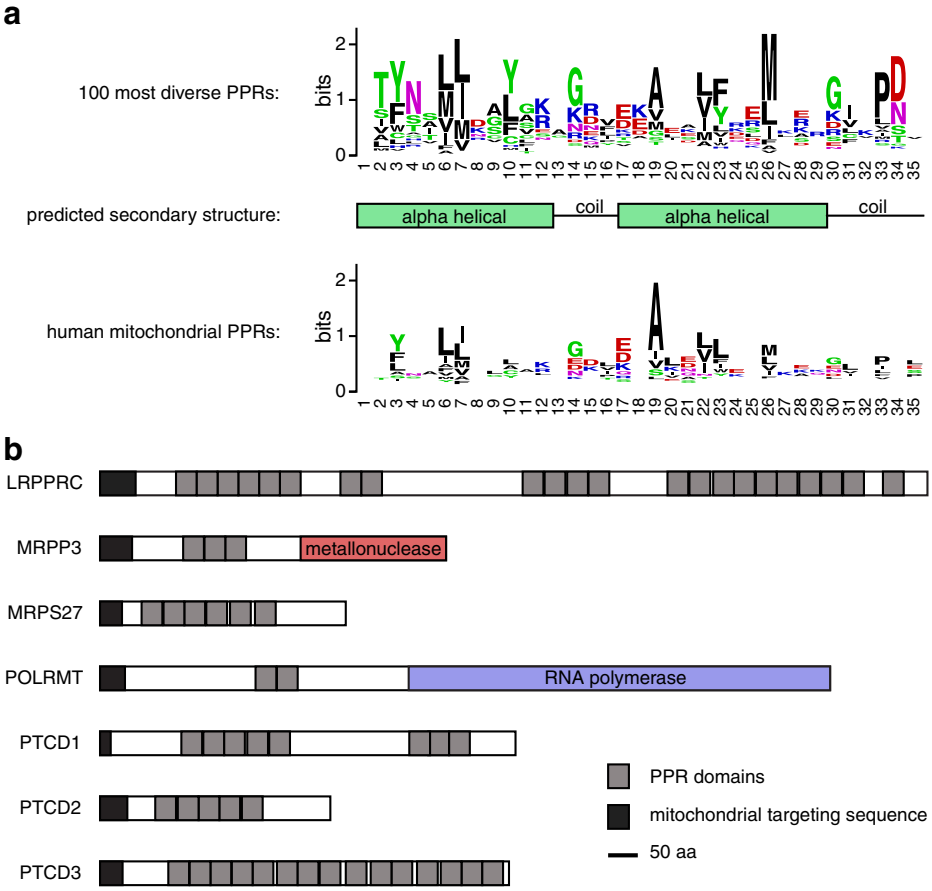

Fig. 1. Mammalian mitochondrial PPR proteins. (a) Characteristic sequence and structural motifs that define PPR motifs. Protein sequence logos were generated using either the 100 most diverse PPRs in the Pfam database (top panel) or from the entire set of human PPRs (bottom panel) using WebLogo [94]. (b) Schematic representation of the seven mammalian mitochondrial PPR domain proteins. PPR domains were identified using TPRpred [45] and N-terminal mitochondrial targeting sequences were predicted using MitoProt [95]. Human proteins were used as archetypes and the annotated sequences are provided as Supplementary Materials Online.

[15,44] (Fig. 1B). Here we discuss the role of each of these proteins in mitochondrial gene expression.

There is some inconsistency about the number of PPR domain that each of the mammalian PPR proteins possess. The lack of structural and functional knowledge about PPR domains and how they contact RNA makes it challenging to accurately define functional PPR domains. We have used TPRpred [45] to analyze the primary structure of each protein and have shown the number of PPR repeats present in these proteins (Fig. 1B and Supplementary data). As there is some redundancy in the PPR domains there may be additional domains identified in these seven proteins as well as more PPR domain proteins in mammalian genomes. New methods for PPR prediction have been developed to identify PPR domains within yeast genomes, that in addition to previously known *Saccharomyces cerevisiae* PPR proteins, Pet309p, Dmr1p and Aep3p, have identified almost 200 potential new members across all yeast genomes [46], and some of these have recently been studied in *Schizosaccharomyces pombe* [47]. Identification of the code for RNA recognition by PPR proteins may facilitate the identification of heavily redundant PPR domains. The presence of PPR motifs in all of these proteins has enabled the prediction of their common role in RNA binding. In recent years the biological analysis of each individual mammalian PPR protein has revealed that in most part they have different and unrelated functions in mitochondrial RNA metabolism. This is not surprising as plant organelles have between 400 and 600 PPR proteins that have diverse functions in RNA processing, stability, editing, maturation and translation required for respiration, photosynthesis, development and fertility [35,37,48,49]. Furthermore, there is evidence that PPR proteins identified in *S. cerevisiae* and *Trypanosomes* play a role in mitochondrial RNA metabolism [50–54]. The task for the coming years is to characterize the role of each mammalian PPR protein beyond our current understanding (Fig. 2) and define their RNA targets that will give us further insight into their mode of binding and function as well as help reveal their code for RNA recognition.

2.1. Mitochondrial RNA polymerase (POLRMT)

POLRMT is an essential protein responsible for the transcription of the mitochondrial genome [9] (Fig. 2). POLRMT transcription of the mitochondrial genome requires the presence of the mitochondrial transcription factor A (TFAM) and one of the two mitochondrial transcription factor B paralogues (TFB1M and TFB2M) [55,56]. The human POLRMT is a single polypeptide subunit that is 139 kDa in size before it is imported inside mitochondria. It contains an N-terminal mitochondrial targeting sequence, an N-terminal region containing two PPR domains, whose function is not yet known, and a C-terminal RNA polymerase motif (Fig. 1B). In yeast the N-terminal region of the mitochondrial RNA polymerase has been suggested to play a role in coupling transcription to RNA processing and translation [57,58]. However, the exact roles of the PPR domains within the yeast or mammalian POLRMT are unclear. These PPR domains could be involved in binding nascent mitochondrial RNA transcripts to stabilize them during their synthesis. Identification of the binding targets of these two PPR domains of the POLRMT should shed some light on their role in transcription.

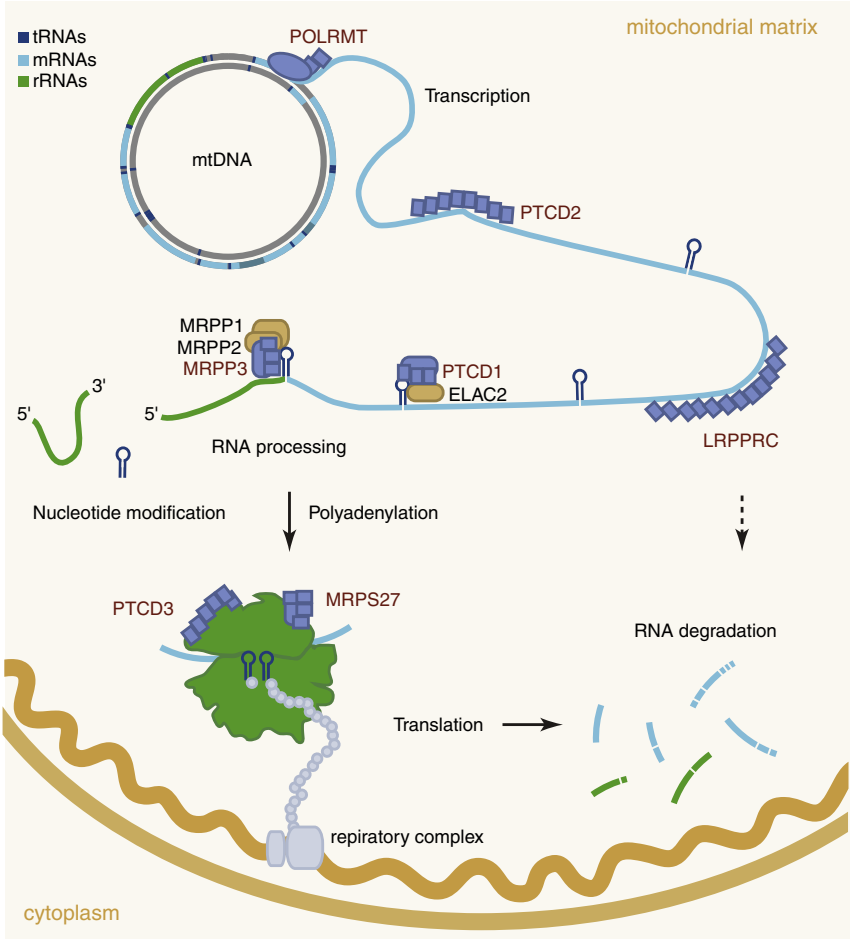

Fig. 2. The diverse roles of mammalian PPR domain proteins in mitochondrial gene expression. PPR domain-containing proteins are involved in transcription, RNA processing, stability and translation.

2.2. The leucine-rich penatricopeptide repeat cassette (LRPPRC) protein

The leucine-rich penatricopeptide repeat cassette (LRPPRC) protein is a predominantly mitochondrial matrix protein [59] that contains 22 predicted PPR domains (Fig. 1B) and is 130 kDa in size [60–62]. Its gene was first identified when a C to T transition mutation at 1119 bp of the LRPPRC open reading frame was shown to change a highly conserved alanine to a valine at position 354 and cause the rare French-Canadian variant of the debilitating neurodegenerative condition Leigh syndrome [61]. A study of Canadian French Leigh Syndrome (LSCF) fibroblast cell lines that contain the A354V mutation has reported low levels of LRPPRC protein abundance in mitochondria that may specifically decrease cytochrome c oxidase (COX) 1 and 3 mRNAs [62]. A recent study has shown that replacement of the C-terminus of LRPPRC in mice with a β-galactosidase and neomycin phosphotransferase II fusion protein is embryo lethal and investigation of fibroblasts derived from these embryos has confirmed the role of LRPPRC in the production of cytochrome oxidase and its importance for cell health [63]. Sasarman et al. used LSFC fibroblasts as well as control cells where LRPPRC was knocked down by siRNAs and showed that decreased LRPPRC in both systems caused a reduction of all 13 mRNAs suggesting that LRPPRC has a global effect on mitochondrial RNA abundance [64]. This global effect of LRPPRC decrease on all 13 mRNAs has been shown by another study which further shows that nuclear gene expression of mitochondrial proteins is not affected severely [65], suggesting there are alternate mechanisms that can compensate for LRPPRC decrease in mitochondrial disease. Although RNA processing is not affected by decreased LRPPRC abundance, the synthesis of protein subunits required for cytochrome c oxidase assembly has been decreased specifically in the LSFC cells causing COX deficiency that is typically observed in Leigh Syndrome patients [64]. Further reduction of LRPPRC abundance using siRNAs leads to a more general decrease in respiratory complex assembly likely caused by reduction of the 13 mRNAs [64]. Recently we have observed similar effects with the PPR protein MRPS27 that we have discussed below. However, it is still not entirely clear which aspect of mitochondrial RNA metabolism and gene expression is affected by the reduction of LRPPRC and it has been shown that LRPPRC may play a role in the regulation of mitochondrial transcription [66]. Identification of the minimal RNA binding target of LRPPRC should shed insight into its mechanism as a mitochondrial RNA-binding protein and help explain how LRPPRC regulates the expression of mitochondrial mRNAs. Recently, progress has been made towards this end by showing that LRPPRC binds a short fragment of the *CO1* mRNA [63].

Interestingly, LSFC causes cytochrome c oxidase deficiency only in the brain and liver and consequently the patients do not suffer from cardiomyopathies, renal disease or other myopathies, as is the case with Leigh syndrome patients [67,68]. A recent study has suggested that LRPPRC mutations cause a different form of Leigh syndrome with cytochrome c oxidase deficiency that may be affected by other genetic or environmental factors [69]. It may be that there are tissue specific factors associated with LRPPRC that modulate the severity of mitochondrial dysfunction upon loss of LRPPRC function. LRPPRC has been found associated with a number of different proteins to date. It has been identified to associate with another mitochondrial RNA-binding protein, SLIRP [70], with which it has been shown to exist in a larger complex that may mediate mitochondrial RNA processing and stability [64]. Several different immunoprecipitation studies using LRPPRC have shown that it is associated with proteins located in cellular compartments outside of mitochondria. In the nucleus it has been shown that LRPPRC associates with the peroxisome proliferator-activated receptor coactivator 1-α (PGC-1α) and acts to regulate the expression of phosphoenolpyruvate carboxykinase (PEPCK), glucose 6-phosphate (G6P) and mitochondrial mRNAs [71], whereas in brown fat cells decreased LRPPRC abundance affects the expression of mitochondrial proteins such as uncoupling protein 1 [72]. The effects of LRPPRC knockdown on the expression of some nuclear encoded mitochondrial proteins have been observed recently [65], suggesting that changes in mitochondrial RNA metabolism may affect nuclear gene expression. LRPPRC was initially identified to associate with nuclear messenger ribonuclear proteins in 2001 [60] and more recently it was identified to associate with the eukaryotic initiation factor 4E (eIF4E) where it is suggested to play a role in its mRNA export [73]. There may be several possibilities for the observed association of LRPRC with nuclear and cytoplasmic proteins. LRPPRC could have dual localization within mitochondria and the nucleus, although Sterky et al. have shown that it is an exclusively mitochondrial protein in cultured cells [59]. An alternative explanation could be that under altered cell conditions [59] or in different cell types LRPPRC could also have nuclear localization. Nevertheless, all the studies to date indicate that LRPPRC plays a role in the regulation of mitochondrial mRNA expression and is important for mitochondrial RNA metabolism.

2.3. Pentatricopeptide repeat domain protein 1 (PTCD1)

Studies in our group have shown that PTCD1 is a mitochondrial matrix protein that contains eight PPR domains (Fig. 1B) [74]. We have shown that unlike most of the other PPR domain proteins, PTCD1 is a low abundance protein that is predominantly found in muscle and heart. Initial characterization of this protein indicated that it associated with leucine tRNAs and precursor RNAs that contain leucine tRNAs [74].

Knockdown of PTCD1 in 143B osteosarcoma cells did not change mitochondrial mRNA levels, however, it increased the abundance of leucine tRNAs and precursor transcripts containing these tRNAs, whereas PTCD1 overexpression led to a reduction of these tRNAs [74]. Lowering PTCD1 in cells increased levels of several mitochondria-encoded proteins and Complex IV activity both in 143B and HeLa cells [18,74], suggesting that it acted as a negative regulator of leucine tRNA levels and consequently affected the abundance of mitochondria encoded proteins. Our most recent studies in HeLa cells have indicated that PTCD1 has a more general role in the processing of mitochondrial tRNAs from their precursor transcripts [18]. In this study we investigated the roles of ELAC2, mitochondrial RNase P proteins 1 and 3 (MRPP1 and MRPP3), and PTCD1 in the processing of mitochondrial polycistronic transcripts using deep sequencing. We found that MRPP1 and MRPP3 process the 5′ ends of tRNAs and the 5′ unconventional, non tRNA containing site of the *CO1* transcript, and ELAC2 and PTCD1 affected the 3′ end processing of tRNAs [18].

We observed an association between ELAC2 and PTCD1 suggesting they may co-exist in an RNA processing complex within mitochondria. The role of PTCD1 in such an enzyme complex is unclear, nor whether the association with ELAC2 is via RNA or other additional proteins. Although we have observed that PTCD1 is a negative regulator of leucine tRNAs [74] and that it affects 3′ processing of mitochondrial tRNAs, it is still not clear how these two effects are related. Elucidating the minimal RNA target of PTCD1 should give clues to its function in tRNA metabolism. Many PPR domain proteins in plants are thought to exert their effects by binding to specific RNA sequences in order to stabilize particular RNA structures [35], recruit other factors to the site or have their own domains with RNA modifying activities [75]. PTCD1 does not contain any protein sequence regions with predicted nuclease activity. Whether PTCD1 can regulate the stability or facilitate the cleavage of tRNAs by regulating ELAC2 recruitment or facilitating the nuclease activity of ELAC2, by making favorable RNA substrates available, remain attractive models that we are currently investigating. To date it is difficult to ascertain the exact role of PTCD1 as the hierarchy of mitochondrial RNA processing is not as simple as previously thought and likely involves more proteins than previously identified [15,17,18,76,77].

2.4. Mitochondrial RNase P protein 3 (MRPP3)

MRPP3 is a 67 kDa mitochondria targeted protein that is composed of 3 PPR domains and a putative metallonuclease domain (Fig. 1B). MRPP3 has recently been identified as one of the three essential components of the mitochondria targeted RNase P in addition to mitochondrial RNase P protein 1 (MRPP1) and MRPP2 [15]. The mitochondrial RNase P differs from previously described RNase P enzymes, as it lacks an RNA component typically required for catalytic function [15,76,77]. The three protein subunits of the RNase P are necessary to give this complex an activity apparently identical to other members of the RNase P family, with individual proteins or pairs of the subunits unable to cleave tRNA precursors *in vitro* [15]. Interestingly, two of the subunits are proteins with additional functions and exist as a stable subcomplex, suggesting that recruitment into a mitochondrial RNase P complex represents an additional role for these proteins. MRPP1 was thought to be an m^1G_9 methyltransferase responsible for a common methylation modification of tRNAs and we have shown this function recently using RNA deep sequencing [18]. Furthermore it is possible that this domain may be involved in tRNA binding required for the RNase P complex. MRPP2 is a member of the short-chain dehydrogenase/reductase family [78], and has a NAD^+-binding domain that may be required for RNase P activity. MRPP2 does not contain RNA-binding domains and is located in the cytoplasm as well as in mitochondria.

Recently it has been shown that MRPP3 is necessary for the processing of mitochondrial tRNAs [15,18,76,77]. We observed that knockdown of MRPP3 in HeLa cells increases the presence of mitochondrial precursor transcripts and decreases the level of mRNAs and tRNAs [18]. However, the decrease in mitochondrial mRNAs and tRNAs caused by MRPP3 knockdown was not as significant as when MRPP1 was knocked down that consequently decreased mitochondrial translation, ribosome stability and respiration, suggesting that there may be additional functions for MRPP1 in mitochondrial gene expression. Furthermore the MRPP3 activity as part of the RNase P complex was noted to affect the processing of 3′ end transcripts in some cases, suggesting that the hierarchy of mitochondrial RNA processing is more complex than anticipated, particularly when several tRNAs are adjacent to each other.

The mechanism of MRPP3 activity along with the other two components of RNase P is not clear and needs additional detailed biochemical characterization. In the case of MRPP3 it could be that the PPR domains recognize and bind the substrate tRNA and the metallonuclease domain carries out the cleavage of the tRNA from the precursor transcript. For example in mitochondria and plastids of *A. thaliana* only an ortholog of MRPP3, named "proteinaceous RNase P" (PRORP1), is required for 5′ tRNA processing [79] and it would be of great interest to elucidate the role of MRPP1 and MRPP2 and their requirement for the RNase P activity in mammals.

The levels of functional tRNAs available for protein synthesis could provide an important mechanism to regulate mitochondrial translation. Changes in the abundance of different tRNA processing proteins can dramatically affect the levels of tRNAs [17,18,76] that reveal a level of important physiological control [18]. Because of the organization of tRNA genes in the mitochondrial genome, the effect on some tRNAs could be quite different to others, depending on whether they are flanked by other tRNAs or mRNAs and rRNAs. It has been proposed that the 5′ ends of tRNAs with 5′ flanking tRNAs can be processed independently of RNase P [80], perhaps by RNase Z acting at the 3′ end of the flanking tRNA [76]. It appears that tRNA processing is not entirely predictable with our current understanding of human mitochondrial tRNA recognition by RNase P, ELAC2 and PTCD1. Although it has been suggested that cleavage by RNase P may precede that of RNase Z in some cases [81], it will be important to investigate the exact hierarchy of mitochondrial RNA processing by these two enzymes. This is particularly important since only knockdown of the components of RNase P causes the loss of mature mRNAs and decreased rRNA levels, ribosome instability, decreased mitochondrial protein synthesis and respiration [18].

2.5. Pentatricopeptide repeat domain protein 2 (PTCD2)

PTCD2 is a 44 kDa mitochondria targeted protein that contains 5 PPR domains (Fig. 1B) and its role has been investigated in mice homozygous for a PTCD2 gene disruption [82]. The gene disruption truncates the PTCD2 protein at the amino acid in position 269 [82], leaving the five annotated PPR domains intact, but removed the C-terminal 112 amino acids. Xu et al. have shown that truncated expression of PTCD2 results in the reduction of *Cyt b* and *ND5* mRNA expression and an increase in the precursor *ND5-Cyt b* transcript, indicating that PTCD2 may regulate *Cyt b* RNA processing in mice [82]. Interestingly, these processing defects of the *ND5-Cyt b* transcript have been observed in heart, muscle and liver. Identifying the RNA target of PTCD2 would give a greater insight into the exact role of PTCD2 in the processing of this transcript. The functional consequences of truncated PTCD2 expression in mice have been largely limited to the heart where they observed a defect in Complex III activity, although a reduced activity in Complex I and III was also found in the liver. This was most likely a consequence of the reduced abundance of assembled Complex III in the heart [82].

There are four exceptions to the "tRNA punctuation" processing model where there are no intervening tRNA genes that separate the mRNA coding regions. Recently our group and Brzezniak et al. have shown that RNase P is responsible for the processing of one of these sites, the region between *ND2* and *CO1* mRNAs [17,18]. This may not be that surprising as the strand complementary to this site encodes five tRNAs providing a strong secondary structure [23] that is likely to be recognized by RNase P at the 5′ end. The remaining three non tRNA containing regions of the mitochondrial genome are not processed by RNase P or ELAC2 [18], however it may be that PTCD2 has a role in the processing of the 5′ end of the *Cyt b* mRNA.

2.6. Pentatricopeptide repeat domain protein 3 (PTCD3)

PTCD3 contains 15 PPR domains and is 79 kDa in size (Fig. 3B). PTCD3 localizes specifically to mitochondria in cells and its expression is most abundant in skeletal muscle and heart tissue [83]. PTCD3 was initially hypothesized to be a respiratory complex assembly factor, however we investigated its function and showed that it is a mitochondrial protein that associates with the 12S rRNA and the small subunit of mitochondrial ribosomes [83]. PTCD3 knockdown and overexpression do not affect mitochondrial mRNA levels, suggesting that PTCD3 is not involved in RNA processing and stability. However, lowering PTCD3 in 143B osteosarcoma cells decreased mitochondrial translation of all 13 mitochondria encoded proteins, consequently decreasing mitochondrial respiration and the activity of Complex III and IV [83], suggesting that PTCD3 has an important role in mitochondrial protein synthesis. We found that PTCD3 is not required for ribosomal stability as decreasing its abundance does not affect the levels of other mitochondrial ribosomal proteins [83]. PTCD3 has not been annotated as a mitochondrial ribosomal protein, as is the case for the PPR protein MRPS27 [31,44], however it is tantalizing to consider if PTCD3 is one of the mitochondrial ribosomal "supernumerary" proteins or if it is a transiently associated small subunit translational regulator that may be involved in the recognition or decoding of mRNA transcripts, which we are currently investigating.

Recently PTCD3 was found associated with a transcription elongation factor of mitochondria along with POLRMT and a putative DEAD-box RNA helicase, DHX30, within the mitochondrial nucleoid [84]. This may lend some further support to the model that mitochondrial transcription is linked to translation [8]. Furthermore, it indicates that the mitochondrial nucleoid contains a greater number of proteins

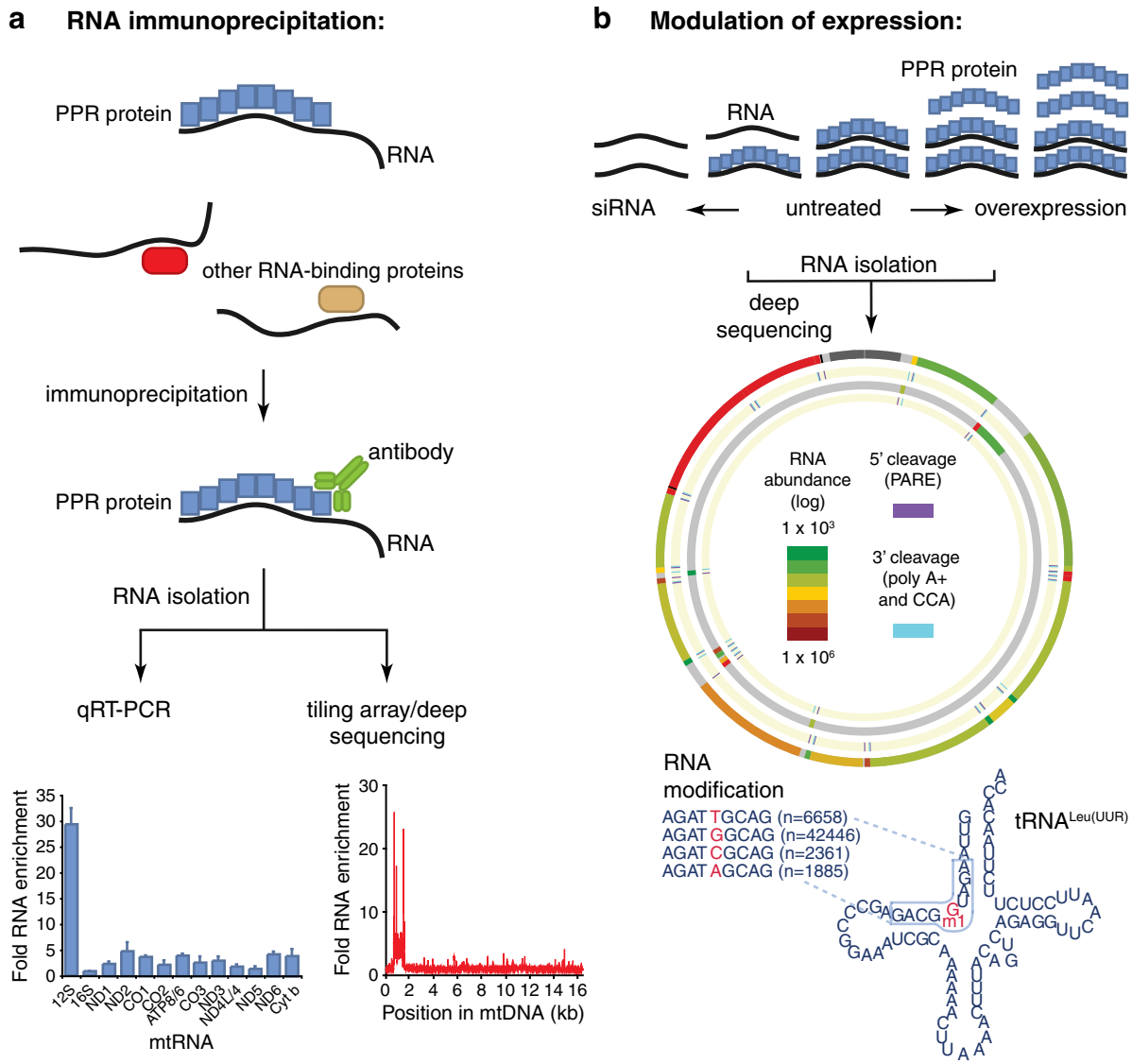

Fig. 3. Methods to study mitochondrial RNA-binding proteins. (a) RNA associated with immunoprecipitated RNA-binding proteins can be analyzed by qRT-PCR, using tiling arrays or by deep sequencing to identify their RNA targets in mitochondria. (b) The mitochondrial transcriptome following knockdown or overexpression of RNA-binding proteins can be analyzed by qRT-PCR, RNA sequencing (RNA-Seq), and parallel analysis of RNA ends (PARE) to reveal the roles of PPR proteins in mitochondrial RNA metabolism. An increased sequencing error rate in data sets from RNA-Seq can be used to identify potential base modifications in mitochondria encoded RNAs.

than previously anticipated. The next step will be to define the specificity and affinity of the interactions between the mitochondrial transcription and translation machinery to provide a direct mechanistic link.

2.7. Mitochondrial ribosomal protein of the small subunit 27 (MRPS27)

MRPS27 has been annotated as a mitochondrial ribosomal protein of the small subunit [31] and has six PPR domains (Fig. 1B). The MRPS27 gene is positioned adjacent to the PTCD2 gene and it is divergently transcribed from the same promoter. Some sequence similarity between these proteins has been reported beyond that in their PPR domains [82], most likely contributed by the other PPR domains that were previously unrecognized at the time, as the two proteins have different roles in RNA processing and translation. Contemporaneously with this review we have characterized the role of the PPR domain protein MRPS27 and show that it associates with the small subunit of the mitochondrial ribosome [85] as predicted by bioinformatic approaches and high throughput proteomics [29,31]. Interestingly, although MRPS27 associates with the mitochondrial 12S rRNA, it is not involved in the regulation of its abundance. MRPS27 does not affect mitochondrial RNA processing or stability. Instead, MRPS27 knockdown following three days affects the translation of the CO1 protein, however prolonged knockdown of MRPS27 for up to 9 days results in general decrease of all 13 polypeptides. Consequently MRPS27 knockdown in cells after 9 days results in decreased abundance of the respiratory complexes and decreased cytochrome oxidase activity [85]. Microarray analysis of the knockdown effects of MRPS27 compared to the mitochondrial translational activator PTCD3 reveals differences in cellular gene expression that may reflect specific roles of these PPR domain proteins within mitochondria [85]. The microarray analysis reveals that specific knockdown of MRPS27 or PTCD3 has a modest effect on global nuclear expression as it has been observed previously when LRPPRC was knocked down [65], suggesting that there are post-transcriptional mechanisms to compensate for their loss of function. Furthermore it is likely that retrograde gene expression in response to decreased PPR expression may depend on the stability of each PPR protein as well as on the protein turnover or

stability of the respiratory complexes that in turn may be the result of decreased processing efficiency, stability or translation of mitochondrial RNAs regulated by these PPR proteins.

Recently it was shown that MRPS27 associates with MRPS29 (DAP3), a GTP-binding protein of the ribosome known to act in apoptosis [86] and hNOA1, a mitochondrial GTPase which is the human homolog of a plant NO associated protein [87], and appears to act as a regulator of mitochondrial respiration and apoptosis. MRPS27 has also been found associated with the immature colon carcinoma transcript-1 (ICT1) mitochondrial translation factor [88] and the mitochondrial ribosome assembly factor Era G-protein-like 1 (ERAL1) [89], that are involved in mitochondrial translation further confirming its role as a mitochondrial ribosomal protein. It would be of great interest to elucidate the exact role of this protein along with PTCD3 in the mitochondrial small ribosomal subunit and to determine if they are involved in decoding of mitochondrial mRNAs.

3. Methods for identifying RNA targets of PPR proteins in mitochondria

Investigation of mitochondrial RNA-binding proteins such as PPR proteins can involve a range of molecular and cell biology techniques that have been commonly used for the study of cytoplasmic and nuclear RNA-binding proteins [90]. Furthermore, as next generation technologies expand and become more accessible these can be used to comprehensively investigate the regulation of mitochondrial gene expression [23,91]. We have found that fusing PPR proteins at the C-terminus to a tandem affinity purification (TAP) tag allows us to efficiently isolate PPR proteins from cell and mitochondrial lysates. The isolated proteins can be used to identify associated RNAs either by quantitative RT-PCR (qRT-PCR), using a re-sequencing array as a single nucleotide tiling array of the complete mitochondrial genome or by deep sequencing (Fig. 3A). A combination of these approaches coupled with RNA electrophoretic mobility shift assays (EMSAs) or the yeast three-hybrid system [42] should validate the RNA targets of mitochondrial RNA-binding proteins.

We have used a tiling array to investigate the RNA targets of PTCD3 (Fig. 3) and MRPS27 and show that they bind the 12S rRNA [83,85]. In addition, we have used RNA sequencing and parallel analyses of RNA ends to provide a complete map of the human mitochondrial transcriptome, RNA abundance in a range of cell lines and tissues, RNA processing and maturation sites and to identify new transcripts generated by the mitochondrial genome that include small RNAs (sRNAs) and non-coding RNAs (ncRNAs) [23,24]. Furthermore we have knocked down the levels of mitochondrial RNA-binding proteins including PPR proteins to investigate their role in the processing of mitochondrial transcripts by RNA sequencing (RNA-Seq) [18]. We have been able to analyze the abundance of mitochondrial transcripts as well as the changes in 5′ and 3′ ends of mitochondrial transcripts upon knockdown of each mitochondrial RNA-binding protein. Furthermore, we used an increase in the sequencing error rate at known modified nucleotides compared with non-modified nucleotides [92,93] as a marker for the presence of nucleotide modifications to suggest a role for MRPP1 in guanosine and adenine modification of tRNAs, supporting the existence of a functional guanine methyl-transferase domain within MRPP1 [18]. This method could be applied to other putative RNA-modifying enzymes to identify their nucleotide targets within mitochondrial transcripts (Fig. 3B). Next generation analyses could be applied to investigate the effects of knocking down or overexpressing mitochondrial RNA-binding proteins on the mitochondrial transcriptome (Fig. 3B) that when coupled with functional and *in vitro* studies can validate the RNA targets of mitochondrial RNA-binding proteins. *In vitro* approaches such as an RNA EMSA could give further insight into the affinity and specificity of the RNA–protein interactions.

4. Conclusions

PPRs have diverse roles in mitochondrial gene expression, mediated by RNA binding, thereby providing an entrée into understanding all aspects of mitochondrial gene regulation. The modular nature of these proteins that allows them to have versatile RNA regulatory functions coupled with next generation technologies opens the way to making exciting discoveries about the post-transcriptional regulation of the mitochondrial genome. Elucidating the RNA recognition code of PPR proteins would not only allow us to predict their RNA targets but perhaps engineer them as tools to selectively and specifically manipulate mammalian mitochondrial gene expression.

Note added in proof

Recently a study using conditional Lrpprc knockout mice has shown an important role of the LRPPRC protein in mitochondrial mRNA polyadenylation and translation [96]. Loss of LRPPRC from an RNA-dependent protein complex affects the poly(A) tail length of mitochondrial mRNAs and the maintenance of untranslated polyadenylated mRNAs [96]. An additional study has shown the bicoid stability factor of Drosophila melanogaster, that has some homology to the mammalian LRPPRC protein [59], also has a role in the control of mitochondrial polyadenylation and translation [97], suggesting that the function of these PPR proteins has been conserved through evolution.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbagrm.2011.10.007.

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