Dissecting the role of human PPR motif proteins in mitochondrial gene expression

by

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A Dissertation submitted to the Graduate Faculty in Biochemistry
in partial fulfillment of the requirements of the degree of Doctor of Philosophy,
The City University of New York
2010

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

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Pentatricopeptide repeat (PPR) motif proteins constitute a growing superfamily of proteins that are broadly defined by the presence of one or more copies of a conserved 35 amino acid sequence, the PPR motif. They are particularly abundant in plants, and those whose function has been characterized have been implicated in several aspects of RNA metabolism in mitochondria and chloroplasts. In humans, PPR motif proteins are fewer in number. They include LRPPRC (**L**eucine-**R**ich **P**PR-motif **-C**ontaining protein), an RNA-binding protein that is a component of nuclear ribonucleoprotein (RNP) complexes that contain spliced mRNAs. Most of the LRPPRC, however, localizes predominantly to mitochondria, where it binds polyadenylated RNAs. Mutations in the lrpprc gene cause cytochrome c oxidase deficiency in Leigh Syndrome (LSFC), which is accompanied by a decrease in COXI and COXIII mitochondrial mRNAs. Our hypothesis is that LRPPRC is an essential trans-acting factor in mitochondrial mRNA metabolism. In order to address the function of LRPPRC in mitochondria, we isolated LRPPRC-associated mitochondrial RNP complexes (mtRNPs). Analysis of isolated mtRNPs shows that the mitochondrially-encoded mRNAs associate with LRPPRC. A reduction in LRPPRC levels using RNAi causes a parallel reduction in steady-state levels of mitochondrially-encoded mRNAs, but not of nuclear-encoded mRNAs. Thus, LRPPRC is an important factor for mitochondrial

gene expression and is necessary for the accumulation of the mitochondrial mRNAs to which it binds. Using LRPPRC as a paradigm, we sought and analyzed other members of the PPR motif family in humans. Four other human PPR-motif proteins, PTCD1, PTCD2, PTCD3 and PTCD4, also localize in mitochondria. Moreover, some of these proteins also bind RNA and exist in the same complexes as LRPPRC. This indicates that the human PTCD proteins, as is the case with LRPPRC, are also involved in mitochondrial RNA metabolism, pointing to PPR motif proteins in humans as a novel family of trans-acting factors in mitochondrial gene expression. These findings open the way for an expanded and more detailed understanding of human mitochondrial gene expression, and for an exploration of the potential involvement of human PPR motif proteins in mitochondrial diseases, as has already been determined for LRPPRC.

Acknowledgements

I would like to express my gratitude to those whose contribution was pivotal to the completion of my PhD:

Dr Serafin Piñol_Roma, whose mentorship embodies guidance, understanding, patience and encouragement among other qualities, thank you for giving me the opportunity to be a member of your research team, for allowing me to explore and for knowing how to keep me focused. Most of all, thank you for your gift of friendship throughout this journey.

I am also grateful to the members of my dissertation committee for critiquing and providing much needed input to this work. I am especially thankful to Dr Horst Schulz who made my admission into the PhD program possible.

To the countless friends and colleagues whose advice, words of encouragement and moral support carried me through discouraging times, I express my love and appreciation.

This work was supported in part by funding from the UNCF • Merck Graduate Science Research Dissertation Fellowship.

This work is dedicated to the memory of my parents, Pierre Bangeranye and Marthe Nzalora, for a job well done.

To my children, Alice Uwamahoro and Albert Kamanzi, you are my *" raison d'être"*.

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ABBREVIATIONS

PPR: Pentatrico **P**eptide **R**epeat

LRPPRC: Leucine-**r**ich **PPR**-motif **c**ontaining

RNP: Ribo**n**ucleo**p**rotein

PTCD: Penta**tri**copeptide repeat **d**omain

OXPHOS: Oxidative **phos**phorylation

ETC: Electron **t**ransport **c**hain

ND: NADH **D**ehydrogenase (e.g. ND1- NADH Dehydrogenase complex subunit 1)

COX: Cytochrome c **ox**idase (e.g. COX1- Cytochrome c oxidase subunit 1)

ATPase: ATP synth**ase** (e.g. ATPase6- ATP synthase subunit 6)

Cytb: Cytochrome **b**

Cytc: Cytochrome **c**

LSFC: Leigh **S**yndrome **F**rench **C**anadian variant

nDNA: nuclear DNA

mtDNA: mitochondrial DNA

mtmRNA: mitochondrial mRNA

MRPP: Mitochondrial **R**Nase **P p**rotein

PolRMt: Mitochondrial **R**NA **Pol**ymerase

Chapter 1

Introduction

Mitochondria are membrane-bound organelles that are the site of aerobic oxidation of metabolic fuels. They lend their contribution to several metabolic processes including pyruvate and fatty acid oxidation, nitrogen metabolism, steroid synthesis and heme biosynthesis among others. Most notably, mitochondria contain the electron transport chain (ETC) and are the site of oxidative phosphorylation (OXPHOS) through which most of the cellular ATP is generated (Fig 1). The ETC is embedded in the lipid bilayer of the mitochondrial inner membrane (IMM) and is composed of five multiprotein enzyme complexes (I through V) and two additional electron carriers, coenzyme Q and cytochrome c. The main function of the system is the coordinated transport of electrons from NADH (and $FADH_2$), which are reducing equivalents generated from oxidation of nutrients such as glucose and fatty acids, to molecular oxygen as the final acceptor. This passage of electrons releases energy that is harnessed in the form of a proton gradient across the inner membrane and is used by the last OXPHOS complex (F1Fo-ATPase) to generate ATP from ADP and Pi. The complexes involved in ETC and OXPHOS are assembled from over 80 polypeptides encoded by both nuclear and mitochondrial DNAs (Saraste, 1999; Smeitink et al., 2001).

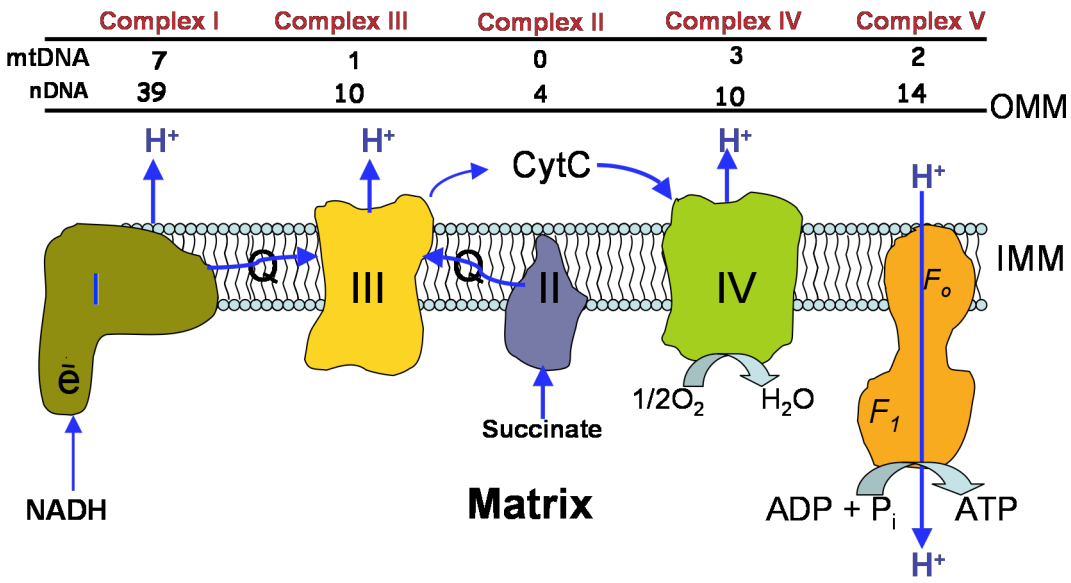

Fig.1: Summary of protein subunits of the five respiratory chain complexes encoded by nuclear (nDNA) and mitochondrial (mtDNA) DNA genes (adapted from Scarpulla, R.C.-2008).

There are features unique to mitochondria as compared with other cellular organelles. They exist in several copies in all eukaryotic cells that respire. Each mitochondrion can contain two to ten copies of the circular genome (mtDNA), which replicates autonomously from the nuclear genome. In humans, mtDNA is transcribed to generate mRNAs for 13 of the polypeptides required in the OXPHOS complexes as well as tRNAs and rRNAs that carry out translation of these mRNAs. Nonetheless mitochondria are not self-sufficient entities; they rely on the nucleus to code for most of the proteins required to carry out their various metabolic functions (Anderson et al., 1981; Scarpulla, 2008; Seidel-Rogol and Shadel, 2002; Taanman, 1999).

1.1 Mitochondrial genome organization in humans

Generally, the composition of the mitochondrial genome varies among species and its complexity and size decreases as we move from lower eukaryotes (e.g. yeast) to higher eukaryotes such as mammals (e.g. humans). Whereas for instance the mtDNA of *Schizosaccharomyces japonicus* is 80kb, and that of *Saccharomyces cerevisiae* is 76kb, the human mitochondrial (mt) genome is only 16.6 kb.

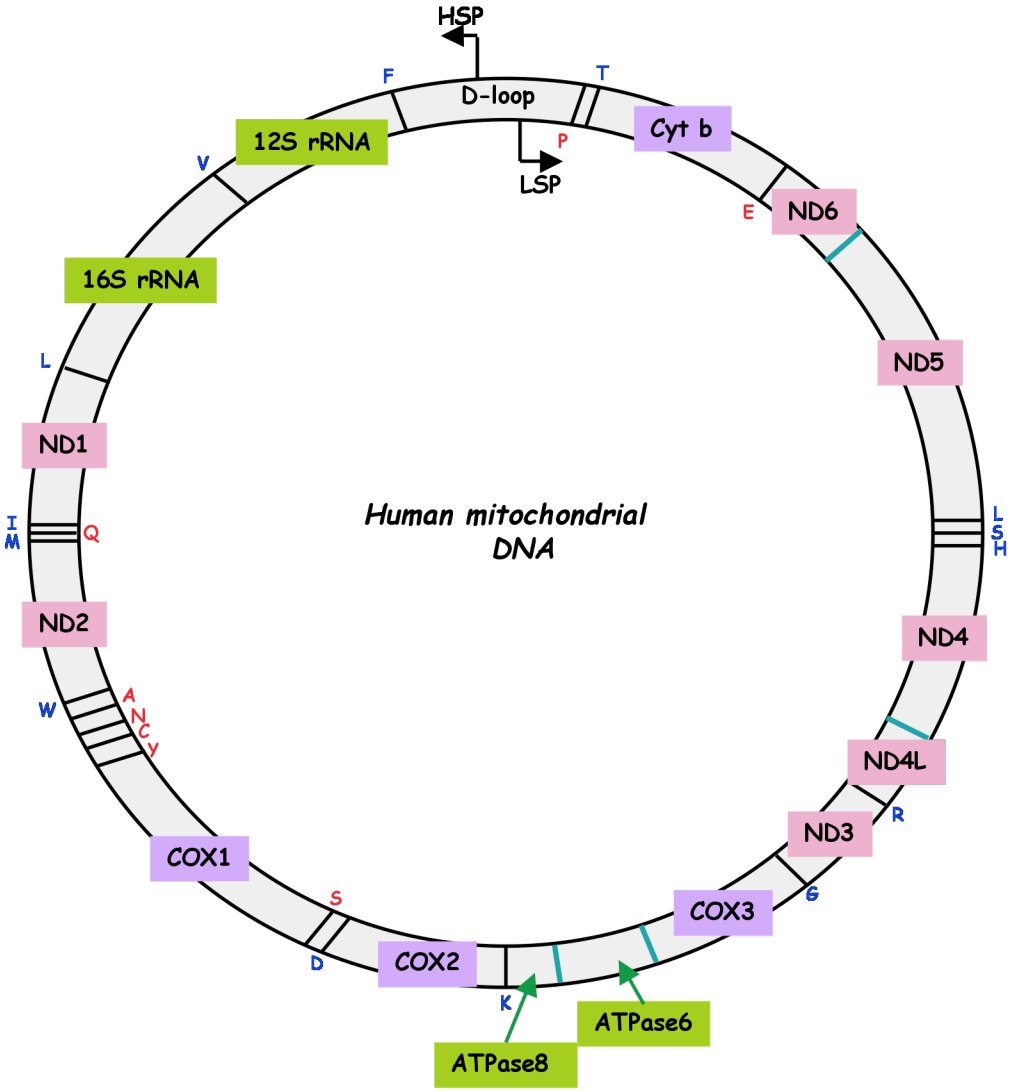

Fig.2 Organization of the human mitochondrial genome (adapted from Taanman, J.W.-1999)

Human mtDNA is a double-stranded circular DNA molecule, comprised of 37 genes that code for 2 ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs), and 13 mRNAs that encode polypeptides which are integral components of the enzyme complexes of the electron transport chain (ETC) and oxidative phosphorylation (OXPHOS). Genes for the 2 rRNAs and 12 of the polypeptides are on the heavy strand of the circular genome where they are found interspersed (or punctuated) by 16 of the 22 tRNA genes. The remaining genes coding for 1 polypeptide and 8 tRNAs are located on the light strand (fig 2) (Bullerwell et al., 2003; Scarpulla, 2008; Smeitink et al., 2001; Taanman, 1999). Thus, both strands of mtDNA are transcribed though the heavy strand contains the majority of the mitochondrial genes.

1.2. Human mitochondrial gene expression

Mammalian mitochondrial gene expression requires the coordination of conserved recognition sequences within the mitochondrial DNA (cis-elements) and nuclear-encoded trans –acting factors that recognize and act on these elements. Following is a brief summary of mitochondrial transcription and posttranscriptional events in humans.

1.2.1 Cis-elements in human mitochondrial gene expression

Transcription of the mtDNA genes is initiated in the displacement loop (D Loop) from the heavy strand promoter (HSP or IT_H) and from the light strand promoter (LSP or IT_L) by proteins encoded by nuclear genes, and generates a single polycistronic transcript from the L-strand and two different intronless transcripts (short and long)

from the H-strand (initiated from IT_{H1} and IT_{H2} respectively). The short transcript, which is more abundant, generates tRNAs for Phe and Val and the two rRNAs (the 12S and 16S) and is believed to terminate at the mTERM sequence (right after the 16S rRNA gene) via a process of attenuation mediated by the mTERF transcription factor. The long transcript generates all the gene products encoded on the H-strand. L-strand transcript initiated at IT_L not only yields 8 tRNAs and one mRNA, but also serves as primer for the replication of the (leading) H-strand (Fernandez-Silva et al., 2003; Ojala et al., 1981; Scarpulla, 2008; Shadel and Clayton, 1993; Taanman, 1999).

1. 2.2 Trans-acting factors in human mitochondrial gene expression

Nuclear-encoded trans-acting factors control the expression of mtDNA at the *transcriptional* and *post-transcriptional* level, including *processing* of pre-mRNAs, *stabilization* of transcripts, *and translation* of mRNAs. In addition, nuclear-encoded factors are necessary for *assembly* of translational products into functional holoenzymes of the ETC (Electron transport chain) and OXPHOS (Oxidative phosphorylation). *Transcription* initiation requires mitochondrial transcription factors A (TFAM) and either B1 (TFB1M) or B2 (TFB2M) to recruit a bacteriophage-like RNA polymerase (POLRMT) to the promoter for initiation of transcription. As mentioned above, both strands of mtDNA are transcribed to generate polycistronic pre-mRNAs (Fernandez-Silva et al., 2003; Ojala et al., 1981; Scarpulla, 2008; Shoubridge, 2002; Taanman, 1999). By contrast to many other organisms, these transcripts in humans are intronless and do not undergo splicing. Instead, *post-transcriptional processing* of the primary transcript begins with cleavage of tRNAs (which for the most

part are strategically interspersed between the 2 rRNAs and 11 of the other 13mRNAs) by RNase P at the *5'*-end followed by tRNase Z endonucleolysis at the 3'-end to release the individual gene transcripts. Maturation of tRNAs proceeds by addition of a CCA sequence at the *3'*-end by an ATP (CTP): tRNA nucleotidyl transferase (CCAse enzyme) (Fernandez-Silva et al., 2003; Holzmann and Rossmanith, 2009; Levinger et al., 2004; Ojala et al., 1981; Rossmanith et al., 1995; Taanman, 1999). The transcripts generated after cleavage of tRNAs are further processed by addition of a polyA tail (by mt-poly(A) polymerase) which serves to both stabilize mRNAs as well as complete the termination codon (UAA) for some of the mRNAs (Fernandez-Silva et al., 2003; Nagaike et al., 2005; Ojala et al., 1981). The *3'* ends of rRNAs are also modified by short polyA tail addition before they are assembled into mitoribosomes (Taanman, 1999).

Processing reactions and the processing factors involved in mitochondrial gene expression have been identified. However, whether these enzymes act singly or in concert with other additional factors is not known. In yeast, mitochondrial gene expression involves several trans-acting factors that act either generally or in a gene-specific manner. These factors control expression of mitochondrial genes at the post-transcriptional level, including processing of mitochondrial pre-mRNAs, stabilization of transcripts, translation of mRNAs or assembly of mitochondrial translational products into functional holoenzymes (Costanzo and Fox, 1990; Manthey and McEwen, 1995). Given that yeast mitochondrial DNA differs from human mitochondrial DNA in composition and has a higher complexity of organization, it therefore can be argued that yeast mitochondrial gene expression requires more post-transcriptional events. For example, while the yeast

primary transcript contains introns as well as untranslated regions, mammalian pre-mRNAs are intronless and have no 5' or 3' UTRs. Therefore, it is fair to assume that yeast requires functionally different trans-acting factors. The extent to which mammalian systems would equally require trans-acting factors (in addition to those already identified) for regulation of their post-transcriptional events including regulation of fidelity of processing, stability of mRNAs and regulation of their accumulation, and translation is not known. Indeed, emerging information as well as our own data (discussed in this thesis), show evidence of a previously unidentified set of trans-acting factors that participate in mitochondrial RNA metabolism. These factors collectively belong to the PPR-motif family of proteins detailed in the following section.

1.3. PPR motif proteins

1.3.1. General aspects

PPR motif proteins constitute an emerging family of proteins which by and large is significant to mitochondrial and chloroplast function. Proteins in this family bear as a signature one or several copies of a conserved 35 amino acid sequence, the PPR motif, which is related in primary structure to the 34 amino acids TPR (tetratricopeptide repeat) motif. While the TPR motif function was shown to mediate protein-protein interactions, the function of the PPR motif is not yet known although available data strongly suggests a role in RNA-binding (Small and Peeters, 2000). PPR motifs generally occur in tandem arrays of 2-26 or more copies. A definitive structure for the PPR motif has not yet been elucidated but the current model suggests that the motif is comprised of two anti-parallel

α-helices (see fig.3 below for consensus sequences of both the PPR and TPR motifs- also refer to table II). These helices are arranged in tandem arrays to form a solenoid-like structure with a hydrophilic cavity where the phosphate skeletons of RNA could potentially interact (Small and Peeters, 2000; Tavares-Carreon et al., 2008).

PPR motif consensus	.TYNALINAYAK.	G . .	EEA..LY..M…	G..PN.
	Helix A		Helix B	
TPR motif consensus	..AY…G..Y..	…	YE.A…Y.KAL.	LNPNN

Fig. 3: Alignment of PPR and TPR motifs (adapted from Small and Peters-2000)

Members of the PPR motif superfamily of proteins are more common in plants (> 400 predicted PPR proteins in *Arabidopsis thaliana*) than they are in vertebrates and fungi. They have been shown to preferentially localize to and function in mitochondria and chloroplasts. A common feature of those PPR proteins whose function has been studied to any extent is their involvement in one or several events of organelle gene expression through a function in RNA metabolism (Delannoy et al., 2007; Lurin et al., 2004; Mili and Pinol-Roma, 2003; Nakamura et al., 2003; Small and Peeters, 2000).

Notable examples of surmised or established functions for PPR motif proteins include but are not limited to roles in:

- **Transcription** – e.g. **p63** in *T. aestivum* enhances wheat transcription from

mitochondrial cox2 promoter (Ikeda and Gray, 1999).

- **RNA cleavage** – e.g. **Rf1a** (*O. sativa*) is essential for endonucleolytic cleavage of the mitochondrial dicistronic atp6-orf79 RNA while **CCR2** (*A.thaliana*) functions in the intergenic processing of chloroplast RNA between rsp7 and ndhB (Wang et al., 2006).
- **RNA processing and maturation** – e.g. **HCF152** (*A.thaliana*) is involved the processing and maturation of petB-containing transcripts (Meierhoff et al., 2003).
- **mRNA Stabilization** – e.g. **BSF** (Bicoid stability factor in *D.melanogaster*) is an RNA-binding PPR protein required for the stabilization of the bicoid mRNA during oogenesis (Mancebo et al., 2001).
- **RNA stabilization and/or translation**- e.g. **CRP1** (*Z.mays*) and **Pet309** (*S. cerevisiae*) are required for translation of both petA and psaC mRNAs and stabilization/translation of cox1 RNA respectively through an association of these proteins with the 5'UTR of their RNA ligand (s) (Manthey and McEwen, 1995; Schmitz-Linneweber et al., 2005). **Cya5** is required for the efficient ***translation*** of cox1 mRNA in *N. crassa* (Coffin et al., 1997), while **PGR3** functions in the ***stabilization and translation*** of the petL operon in *A. Thaliana* chloroplast (Yamazaki et al., 2004).

Other members of this family have been implicated in *RNA splicing* (PPR4) and *RNA editing* (CRR4) as well as *RNA degradation* (RF1b) (Delannoy et al., 2007). Additionally, those that have been identified as translational activators (e.g. Pet309 and Cya5), are known to interact with the mitochondrial inner membrane (IMM). This raises

the possibility of a role for these PPR motif proteins as chaperones that accompany the RNA to the site of translation, the IMM (Tavares-Carreon et al., 2008).

Of particular interest to the work presented in this thesis is the *S. cerevisiae* protein Pet309, the yeast putative homologue of the mammalian LRPPRC. Pet309 is an RNA-binding protein that contains an array of 7 predicted PPR motifs. Pet309 is known to function in the production (and/or stabilization) of the intron-containing COXI RNA and in the processing of COXI mRNA either by ensuring fidelity of splicing or by stabilizing the processing intermediates. Pet309 is also a translational initiation activator of the COXI coding region, a function mediated through binding of Pet309 to the 5'UTR of COXI mRNA (Manthey and McEwen, 1995). Recent studies delineating the possible function of the PPR motifs in Pet309 revealed that all the 7 motifs are necessary for translation but not for stability of the COXI mRNA (Manthey and McEwen, 1995; Tavares-Carreon et al., 2008). Questions therefore remain to be answered not only with regard to the exact function the PPR motif in general but also to the redundant occurrence of this motif within protein structures. What is clear however is that the PPR motif family of proteins is proving to comprise essential players in several aspects of organellar RNA metabolism and by extension in cellular energy metabolism.

1.3.2. LRP130 (LRPPRC) and other human PPR motif proteins

The first identified PPR motif protein in humans is LRP130 also known as LRPPRC (leucine-rich pentatricopeptide repeat cassette). It is an RNA-binding protein that is a component of hnRNP A1-containing nuclear RNP complexes associated with mature nuclear mRNA. It thus was proposed to be part of the shuttling mRNPs that

associate with mRNA from the nucleus to the cytosol (Mili and Pinol-Roma, 2003; Mili et al., 2001). The primary structure of this 130 KDa protein includes an array of 11 pentatricopeptide repeats (PPR) as well as a putative nuclear export sequence (NES), a nuclear localization sequence (NLS) and a cleavable mitochondrial targeting sequence (MTS) (Mili, S. Bangeranye C. and Piñol-Roma, S. –submitted) (Mili and Pinol-Roma, 2003). The proposed role in mRNA export was substantiated recently by findings that LRPPRC, together with eIF4E, mediate the export from the nucleus of a subset of mRNAs that contain an eIF4E sensitivity element (Topisirovic et al., 2009). Other studies have demonstrated a role for LRP130 in the transcription of some nuclear genes involved in glucose homeostasis in association with the transcription factor PGC1-α (Cooper et al., 2008) as well as the transcription of MDR (multidrug resistance) and MDR-related genes (Labialle et al., 2004). Possible functions in cytoskeletal organization, vesicular trafficking (Liu and McKeehan, 2002) and in the stability of minisatellite DNA (Tsuchiya et al., 2002) have also been suggested.

As mentioned above, LRPPRC is an RNA-binding protein with no recognizable *bona fide* RNA-binding domains. Rather, the RNA-binding activity of LRPPRC maps to the C-terminal region that contains 2 PPR motifs (Mili and Pinol-Roma, 2003). The relevance of PPR motifs to RNA binding remains to be clarified.

In addition to the above-mentioned nuclear functions, subsequent studies determined that the majority of LRPPRC is predominantly in mitochondria, and that it binds directly to mitochondrial polyadenylated mRNA both *in vitro* and *in vivo* (Mili and Pinol-Roma, 2003). Evidence that LRPPRC plays a crucial role in mitochondrial gene expression was provided by the discovery that a point mutation ($C^{1119} \rightarrow T$) in the lrpprc

gene (leading to an A354V substitution in the LRPPRC protein) is the defect that causes Leigh Syndrome French Canadian type (LSFC), a cytochrome c oxidase deficiency characterized by a reduction in cytochrome c oxidase subunit I (COX1) and subunit III (COX3) mRNAs (Mootha et al., 2003; Xu et al., 2004). Moreover, two putative homologues of LRPPRC, BSF in *D. melanogaster* and Pet 309 in *S. cerevisiae*, function in mRNA stability and both mRNA stabilization/translation respectively (Mancebo et al., 2001; Manthey and McEwen, 1995; Tavares-Carreon et al., 2008). Altogether, this evidence suggests an involvement of LRPPRC in mitochondrial gene expression. The biochemical findings in LSFC underscore an essential role of LRPPRC in cellular energy metabolism the nature of which is the purpose of our investigation.

As presented later in this work, the human family of PPR motif proteins includes LRPPRC and six other predicted members. These include the mitochondrial RNA polymerase (PolRMt) whose function in mitochondrial DNA transcription is well documented. They also include PPR Domains proteins (PTCD) 1, 2 and 3, KIAA0391 (PTCD4) or mitochondrial RNase P protein 3 (MRPP3) and the mitochondrial ribosomal protein of the small subunit 27 (MRPS27). Our own preliminary findings and emerging data show that all these proteins participate in varying stages of mitochondrial gene expression including **transcription**, **processing** of the precursor RNA, **tRNA excision**, **translation**, and **regulation** (detailed in chapter 4). While studies of these additional human PPR proteins are still in their nascence, a picture is emerging that they belong to a group of initially unidentified tans-acting factors required in several steps of mitochondrial RNA metabolism (Davies et al., 2009; Holzmann and Rossmanith, 2009; Rackham et al., 2009; Xu et al., 2008).

1.4. Mitochondrial proteome

It is estimated that over 1,000 proteins are required for the various mitochondrial metabolic functions including the citric acid cycle, fatty acid oxidation, apoptosis, ATP production/oxidative phosphorylation, amino acid metabolism, steroid metabolism, heme biosynthesis, etc. As stated above, only 13 of these proteins are encoded by mtDNA. All the additional proteins are nuclear-encoded and are imported into the mitochondria via a complex mechanism involving transporters and chaperones. Thus, mitochondrial function depends on two separate genomes located in two separate organelles. In fact, the enzyme complexes of the OXPHOS (with the exception of complex II) are assembled from both the 13 mitochondrial-encoded proteins and their nuclear-encoded binding partners. Complex I (NADH-ubiquinone oxidoreductase or NADH dehydrogenase complex) consists of a multimeric assembly of 7 mitochondria-encoded (ND1-ND6 and ND4L) and approximately 38 nuclear-encoded subunits (Bourges et al., 2004). Complex III (Ubiquinone-cytochrome C oxidoreductase) contains 1 mitochondrial-encoded subunit (cytB), and 10 nuclear-encoded subunits. Complex IV (CytC oxidase) is composed of 13 subunits of which 3 are mitochondria-encoded (COX I, II and III) and 10 are nuclear-encoded. Complex V (F0-F1 ATPase) has two mitochondria-encoded (ATPase 6 and 8) and 12 nuclear-encoded subunits (Smeitink et al., 2001). As such, the OXPHOS enzymes are under a complex regulation that falls under the dual genetic control involving interplay between mitochondrial and nuclear genomes. Mutations that disrupt any of these complexes can therefore have their origin in either or both genomes, with potential detrimental repercussions on cellular energy production. A thorough knowledge

of the biogenesis of the OXPHOs complexes and of the factors involved is vital to our understanding of defects that arise from their malfunction (Davies et al., 2009; Rackham et al., 2009; Xu et al., 2008).

1.5. Mitochondrial diseases and their origins

Because mitochondria produce most of the ATP required by the cell, they are of vital importance and any defects that impair energy production will therefore have repercussions on cellular function in general. The fact that mitochondria require a contribution from both the nuclear and mitochondrial genomes has important implications in determining the etiology of mitochondrial defects/diseases. Mitochondrial diseases are characterized by dysfunction of mitochondrial OXPHOS (the final system in which most of the ATP required for cellular function is produced from reducing equivalents (NADH and $FADH_2$)). Defects of the OXPHOS system manifest as multisystemic disease conditions of the organs/systems with the highest ATP turnover particularly muscle, heart, liver, brain and kidney. Some common clinical manifestations of OXPHOS deficiency include: psychomotor dysfunctions, mental retardation, peripheral neuropathy, myoclonus epilepsy and stroke-like episodes (CNS), muscle weakness, myopathies, myalgia (Muscle), cardiomyopathies, heart block (Heart), hepatomegally and hepatic failure (Liver), nephritis, nephritic syndrome, De Toni-Debre-Fanconi syndrome (Kidney), growth defects, hypoglycemia, Diabetes mellitus, hypothyroidism, hypoparathyroidism (Endocrine), deafness, ototoxicity (Ear), corneal opacity, optic atrophy, ophtalmoplegia, optic neuropathies (Eye), ketoacidotic coma, metabolic ketoacidosis, hypoglycemia, hyperlactemia (Metabolic*)* (Cohen and Gold,

2001; Smeitink et al., 2001). In view of the dual provenance of OXPHOS subunits, these disease conditions can be classified on the basis of their etiology as follows:

a) Mitochondrial diseases due to mtDNA mutations (maternally inherited). This group of diseases can be further classified into three sub-groups depending on their etiology:

- Diseases resulting from large-scale rearrangements (deletions/duplications) such as occur in Kearns-Sayre syndrome (KSS), progressive external ophthalmoplegia (PEO), and Pearson's syndrome (PS). A common feature of the deletions is that they involve a removal of large sequences that include at least one tRNA gene. Deletion of tRNA genes results in a defect in mitochondrial translation when the ratio of deleted to wild-type genomes exceed a threshold level of about 75% mutant DNA.
- Point mutations in tRNA and rRNA genes: disease-causing mutations in tRNA genes are the most frequently observed among mitochondrial mutations. They account for two-thirds of all mtDNA mutations. These mutations can lead to a defect in tRNA processing and/or defects in mitochondrial translation. Some diseases resulting from these mutations include mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS), and myoclonus epilepsy and ragged-red fibers (MERRF). One example is the U7445C substitution in the $tRNA^{ser}$ that results in lack of processing of the primary transcript at the 3'-end of $tRNA^{ser}$ characteristic of one type of non-syndromic deafness. The other type non-syndromic deafness arises from a mutation in the 12S rRNA gene.

- Point mutations and small deletions in protein-coding genes: These mutations lead to deficiencies in specific complexes of the OXPHOS. The most studied of these diseases include LHON (Leber's hereditary optic neuropathy) and NARP (neuropathy, ataxia, and retinitis pigmentosa). The most common mutations for these diseases are found in ND1, ND4 and ND6 for LHON and ATPase6 for NARP (Grossman and Shoubridge, 1996; Lenaz et al., 2004; Levinger et al., 2004; Morgan-Hughes and Hanna, 1999).

b) Mitochondrial diseases due to mutations of nuclear–encoded trans-acting factors that are targeted to mitochondria may arise as a result of mutations in either the nuclear-encoded OXPHOS subunits or other trans-acting factors directly or indirectly involved in OXPHOS function. They are of both paternal and maternal inheritance. Examples of ensuing conditions include Leigh syndrome (complex IV defect), Friedreich ataxia (mitochondrial superoxide dismutase defect), Wilson disease (complex V defect), deafness-dystonia (mitochondrial protein import machinery defect), etc., (Schon and Manfredi, 2003; Smeitink et al., 2001), and of particular significance to this thesis, the recently described Leigh syndrome French Canadian variant (LSFC).

LSFC (Leigh syndrome French Canadian) is an autosomal recessive cytochrome c oxidase deficiency characterized by developmental delay in children, hypotonia, mild facial dysmorphism, chronic well-compensated metabolic acidosis, and high mortality due to episodes of severe acidosis and coma. Cytochrome c oxidase activity is nearly

absent in the brain and liver of LSFC patients and is mildly diminished in muscle, fibroblasts and kidney, as a result of a failure to assemble an active complex IV. The underlying cause of LSFC is a point mutation in the lrpprc gene resulting in an A354V substitution in the protein. The consequences of this mutation in mitochondria are observed as a decrease in COXI and COXIII mRNA levels and a defect in complex IV activity (Mootha et al., 2003; Xu et al., 2004). Interestingly, studies done in our laboratory show that while the mutated LRPPRC localizes to mitochondria, it binds poorly to mitochondrial mRNAs (Mili and Piñol-Roma-unpublished). Since the mutation does not map to the LRPPRC RNA-binding region, it is unclear by what mechanism the point mutation affects the function of LRPPRC. Nonetheless, the RNA-binding activity of LRPPRC, the reduced binding of the mutant together with the implications of the mutation of the mutation for COXI and COXIII mRNAs in LSFC point to a role of LRPPRC in mitochondrial function in general and, in mitochondrial gene expression in particular. Whether or not this function is limited to only COXI and COXIII or includes the other mitochondrial mRNAs is not known.

The work presented in this thesis addresses the RNA-binding activity of LRPPRC and its functional significance to mitochondrial gene expression. We demonstrate that LRPPRC binds not only to COXI and COXIII mRNAs but also to all mitochondrial mRNAs as well as to unprocessed/partially processed RNAs. Importantly, functional studies reveal that LRPPRC plays an essential role in the maintenance of the steady-state levels of these mRNAs. Our data supports a function of LRPPRC in the processing of the primary transcript and/or in the stability of mitochondrial mRNAs. LRPPRC is therefore a novel trans-acting factor in mitochondrial RNA metabolism that is necessary in order to

assemble functional complexes of the OXPHOS. Moreover, using LRPPRC as a paradigm, we identify other PPR motif proteins in human for the purposes of defining their role (if any) in mitochondrial gene expression. Our data show that these proteins, termed PTCD1, 2, 3, and 4, localize to mitochondria, bind to RNA, and associate in complexes with LRPPRC. Thus, this work and that done by others shows these PPR motif proteins to be involved in various aspects of mitochondrial RNA metabolism including tRNA regulation, transcript processing, as well as translation (Davies et al., 2009; Rackham et al., 2009; Xu et al., 2008). While further studies are still needed in order to fully characterize the stage and mode of function of these proteins, the emerging picture is that PPR motif proteins in general may constitute a group of novel trans-acting factors in human mitochondrial function.

Chapter two

LRPPRC binds mitochondrial mRNAs *in vivo*

2.1 Introduction

LRPPRC was identified as an RNA-binding protein in the nucleus of human cells where it is a component of nuclear messenger ribonucleoprotein (nmRNP) complexes associated with shuttling protein hnRNP proteins (Mili et al., 2001). It was subsequently found, however, that most of the cellular LRPPRC is predominantly in mitochondria. UV-induced cross-linking coupled with oligo-dT selection, showed that LRPPRC is bound to mitochondrial poly (A) + mRNAs in living cells (Mili and Pinol-Roma, 2003). LRPPRC binds RNA *in vitro* in the absence of other proteins, indicating the presence of an RNA-binding domain. However, examination of the primary sequence of LRPPRC did not reveal any readily identifiable known RNA-binding motifs such as the RNP motif, the KH domain, or RGG box (Burd and Dreyfuss, 1994). Experiments done to delineate LRPPRC's RNA-binding domain demonstrated that it resides in a region within the protein that contains both the penultimate and ultimate PPR motifs of the c-terminal domain (Mili and Pinol-Roma, 2003; Mili et al., 2001). Whether the PPR motif itself is an RNA-binding domain is not known.

Evidence that LRPPRC has a role in mitochondria was provided by the discovery that an A354V substitution is the defect in LSFC and is characterized by a decrease in

COXI and COXIII mRNAs with the ensuing complex IV deficiency (Mootha et al., 2003; Xu et al., 2004). The RNA binding and the LSFC data suggest a function of LRPPRC in mitochondrial RNA metabolism. Whether this function is for all or a subset (COXI and COXIII mRNAs) of the mitochondrial polyadenylated RNAs is not known. Moreover, what is also not known is the functional implication of this binding. Identifying the RNA ligands of LRPPRC constitutes therefore a crucial first step towards determining the functional implications of this association for the metabolism of mitochondrial RNA.

2.2 Results

2.2.1 Strategy for identification of mitochondrial RNAs from mitochondrial RNP

LRPPRC is bound to mitochondrial RNAs. It therefore exists in ribonucleoprotein (RNP) complexes together with the bound RNAs and the other associated proteins. Isolation of ribonucleoprotein complexes by immunopurification has been successfully used before to determine the identity RNA-binding proteins associated with nuclear RNAs throughout the various stages of its remodeling (Choi and Dreyfuss, 1984; Pinol-Roma et al., 1988). We therefore utilized the same approach to isolate mitochondrial RNPs associated with LRPRC in order to identify the RNAs within these complexes. Immunopurifications were carried out using the previously described monoclonal antibody to LRPPRC (9C9) (Mili and Pinol-Roma, 2003) (and to hnRNP C proteins (4F4) (Choi and Dreyfuss, 1984) for controls). The RNAs were extracted and analyzed by northern blotting using biotin-labeled probes for mitochondrial mRNAs, as well as for nuclear-encoded mRNAs (see appendix). We tested the probes (both for sensitivity of detection and for binding specificity) by northern blotting using total cellular RNA

extract. Figures 4-A and 4-B show a typical result using a probe for COX2, a mitochondrial mRNA, and for Hsp60, a nuclear-encoded mRNA. These results show that the COXII probe binds a predominant band of ca. 680 nucleotides and the Hsp60 probe binds a single band of ca. 2,200 nucleotides. These bands are consistent with the full-length of the COXII and Hsp60 mRNAs respectively. The results also show that we can easily detect both COXII mRNA from 2μg of total RNA and therefore, we do not need to have to enrich for mitochondria in order to detect mitochondrial RNAs. We also tested the specificity with which our antibodies were able to pull down RNP complexes. For this, we performed immunopurifications of HeLa whole cell lysate using 9C9 (anti-LRPPRC) antibody and as a control, 4F4 (anti-hnRNPC1/C2) antibody, extracted the associated proteins and analyzed them by immunoblotting with the above-mentioned antibodies. Our observations (Results not shown) were that LRPPRC is pulled down by 9C9 (lane 9C9 + band of ca. 130KDa) and C proteins are pulled down by 4F4 confirming the specificity of the antibodies and of the immunopurification strategy. When we probe proteins that co-immunopurify with LRPPRC using the 4F4 antibody, we do not detect a band for hnRNP C proteins. The converse is true when we probe hnRNP C-associated proteins with 9C9, we do not detect a band for LRPPRC. The observation from this experiment is that LRPPRC and C proteins exist in different ribonucleoprotein complexes.

2.2.2 Identification of mitochondrial mRNAs associated with LRPPRC in mitochondrial ribonucleoprotein (mtRNP) complexes

Having ascertained the specificity of the antibodies and probes, we set out to identify the RNAs that are bound by LRPPRC. We isolated LRPPRC-associated RNPs by immunopurification of Hela whole cell lysate using the anti- LRPPRC (9C9) antibody. These immunopurifications were performed for relatively brief periods of time, at 4^{0} C, and under non-denaturing conditions in order to minimize disruption and/or rearrangement of the RNP complexes during their isolation (Choi and Dreyfuss, 1984; Pinol-Roma et al., 1988). Two sets of controls were included to ascertain the specificity of any observed interaction between LRPPRC and its RNA ligands and the specificity of immunopurifications. First, as already tested above, we performed parallel immunopurifications using 4F4 antibody to the hnRNP C1/C2 RNA-binding proteins. Second, we also performed these isolations using whole cell lysate rather than the mitochondrial fraction to expose LRPPRC to a broader spectrum of RNAs and so as to best determine if the association of LRPPRC to mitochondrial RNAs is specific or artifactual.

The RNAs associated with the immunopurified RNP complexes were extracted and analyzed by Northern blotting using the appropriate probes. The results (fig 5-A and fig 5-B) show that all the mitochondrial mRNAs that we analyzed are enriched in LRPPC-associated RNP complexes (fig 5A and 5B, “lanes anti-LRPPRC”). These RNAs include ND1, ND2, ND4, and ND5 for complex I of the OXPHOS, Cytb for complex III, COXI, COXII, and COXIII for complex IV, and ATPase 6 for complex V. By contrast, both nuclear-encoded β-actin and Hsp60 are barely detectable among RNAs that

associate with LRPPRC in RNP complexes (see figure 5-B for β-actin and fig 5-A for Hsp60, "lanes anti-LRPPRC"). The converse is observed when we analyze RNAs that are associated with hnRNP C1/C2, which do not show a significant association with the mitochondrial mRNAs (see full list above). In fact, results in figures 5-A and 5-B, lanes "anti-hnRNP C" show that only background amounts of mitochondrial mRNAs immunopurifiy with C proteins-associated RNP complexes whereas the nuclear-encoded β-actin and Hsp60 are enriched in these complexes.

These experiments were carried out under non-denaturing conditions that minimize disruptions of protein-protein as well as protein-RNA interactions (100mM NaCl, 0.5% Triton X-100 at 4^0 C and for relatively brief periods. Results from northern blot analyses of LRPPRC-associated RNAs showed an enrichment of mitochondrial RNAs. The lower signal for mitochondrial mRNAs detection in the hnRNP C-associated complexes that was observed to vary from experiment to experiment and among the various RNAs was attributed to background noise (example: compare ND1 and COXI in fig 5-A or ND4 and ND5 in fig 5-B "lanes anti-hnRNP C"). Our results indicate that this association of mitochondrial RNAs with LRPPRC is specific.

(*This work was undertaken in collaboration with Ji Fang in our laboratory).*

2.2.3. Binding of LRPPRC to mitochondrial mRNAs is direct and specific

Interpretation of data from the immunopurification experiments described above hinges on the assumption that the isolated RNP complexes remained unaltered throughout all the manipulations. However, recent findings have demonstrated that in some cases, such interactions might result from rearrangement of complexes generated

after cell lysis (Mili and Steitz, 2004). In order to ascertain that the observed association of mitochondrial mRNAs with LRPPRC reflects a *bona fide in vivo* binding and not re-association after cell lysis, we performed immunopurifications of RNP complexes under conditions that would deter any opportunistic association of complexes. To accomplish this, HeLa cells were first subjected to formaldehyde treatment to induce covalent cross-linking of proteins to their RNA ligands *in vivo* prior to cell lysis. Immunopurifications were then carried out as before using both 9C9 and 4F4 monoclonal antibodies. After binding of complexes to immobilized antibodies, high stringency washes (1M NaCl, 2M Urea, 1% NP40, 1% Deoxycholate) were performed in order to disrupt any RNA-protein associations that had not been covalently linked by formaldehyde treatment prior to cell lysis. The rationale for this approach is that only those complexes that existed *in vivo* would be covalently crosslinked and thus would survive the treatment. After reversal of cross-linking to release covalently linked RNAs from their protein ligands, these RNAs were extracted and used as templates for RT-PCR using primers that amplify both mitochondrial and nuclear mRNAs.

Results in fig 6 show amplification of products from mitochondrial RNA (COX2) in LRPRC-cross-linked complexes ("lane anti-LRPPRC RT-PCR), unambiguously confirming that the previously observed associations of mitochondrial mRNAs with LRPPRC represents a *bona fide in vivo* binding. By contrast, there is no detectable amplification of COX2 from hnRNPC proteins-associated RNP complexes, confirming that the previously observed small association of C proteins with mitochondrial RNAs (fig 5 A and B) was not true *in vivo*. PCR without RT shows that only mitochondrial mtRNA and not mtDNA is associated with LRPPRC (fig 6 "lane anti-LRPPRC panel:

PCR"). Finally, RT-PCR using primers that amplify the nuclear-encoded β-actin yields a product for hnRNP C-associated complexes but not for LRPPRC-associated complexes (fig 6), demonstrating that, under these conditions, there is little if any of this nuclear-encoded RNA that is associated with LRPPRC and underscoring once again the specificity of these associations.

2.3 Discussion

As mentioned earlier, previous experiments using UV-induced RNA-protein cross-linking had already revealed that LRPPRC binds polyadenylated RNAs in living cells. But these experiments did not reveal whether LRPPRC binds all or only a specific subset of mitochondrial mRNAs. As a part of this work, we set out to first determine the identity of the RNAs that are associated with LRPPRC in mitochondrial RNP complexes. RNA analysis from LRPPRC-specific RNP complexes revealed that all of the mitochondrial RNAs we analyzed are associated with LRPPRC. The fact that mitochondrial mRNAs but not nuclear-encoded Hsp60 and β-actin are enriched in LRPPRC-specific RNP complexes strongly supports our conclusion that the observed results reflect binding *in vivo* as opposed to adventitious binding during isolation of complexes. While these results by themselves do not conclusively tell us that LRPPRC is bound directly to the RNAs it co-isolates with, but together with the existing evidence from UV and formaldehyde cross-linking (Mili and Pinol-Roma, 2003) and fig 6, we can conclude that this association results from direct *in vivo* binding of LRPPRC on these mRNAs. It is worth noting that while LRPPRC is also a component of mRNP complexes in the nucleus, nuclear-encoded β-actin and Hsp60 mRNAs do not significantly co-

immunopurify with LRPPRC. This could be taken to mean that the nuclear LRPPRC-associated RNP complexes are less abundant especially in view of the fact that most of LRPPRC protein is found to localize to mitochondria (Mili and Pinol-Roma, 2003). Moreover, it is also possible that LRPPRC associates with only a subset of nuclear-encoded RNAs or that it only associates transiently with these mRNAs.

Conversely, hnRNP C1/C2 –associated complexes were abundant in the nuclear-encoded β-actin and Hsp60 mRNAs but only background levels of mitochondrial mRNAs as demonstrated by northern blotting (fig 5 A/B). Results in figure 6 confirm that the previously observed minimal association does not reflect an *in vivo* binding but is rather a result of re-association after cell lysis as it can be abrogated under denaturing conditions. The observed preferential association of nuclear-encoded but not mitochondrial-encoded mRNAs with hnRNP C proteins is consistent with the fact that C proteins are not known to localize to mitochondria and therefore would not exist in complex with mitochondrial RNAs *in vivo*. Additionally, the above results are in agreement with our previous observation that LPPRC and hnRNP C proteins exist in distinctly separate RNP complexes (Mili and Pinol-Roma, 2003). Altogether, the above findings show that LRPPRC binds mitochondrial mRNAs in general. These findings also point to an involvement of LRPPRC in mitochondrial gene expression beyond COXI and COXIII mRNAs as had been previously surmised from the existing LSFC data. This involvement would extend to complexes I, III, IV and V of the electron transport chain and oxidative phosphorylation all of which require a contribution to their enzyme complexes from the mitochondria genome. The specific stage and mechanism of this

involvement however still remains to be dissected and is the focus of our subsequent work.

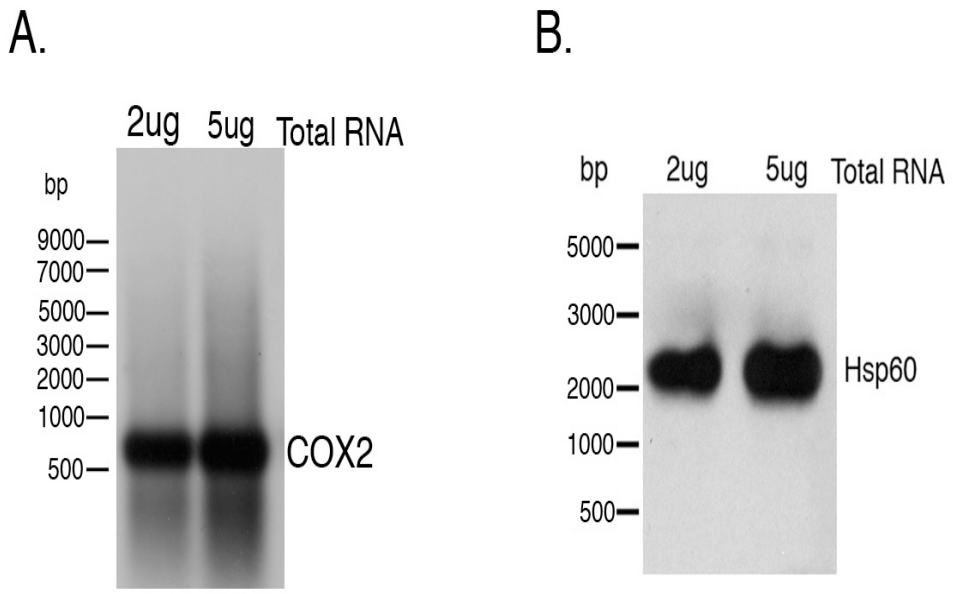

Figure 4A and B: Specificity of mitochondrial and nuclear probes
Total RNA was extracted from Hela cells. 2 or 5 μg were resolved by formaldehyde-agarose gel electrophoresis, and specific RNAs were identified by Northern blot analysis using biotin-N_4-dCTP labeled probes for mitochondrially (e.g. COX2-fig. 4A) or nuclear-encoded RNAs (e.g. Hsp60- fig. 4B).

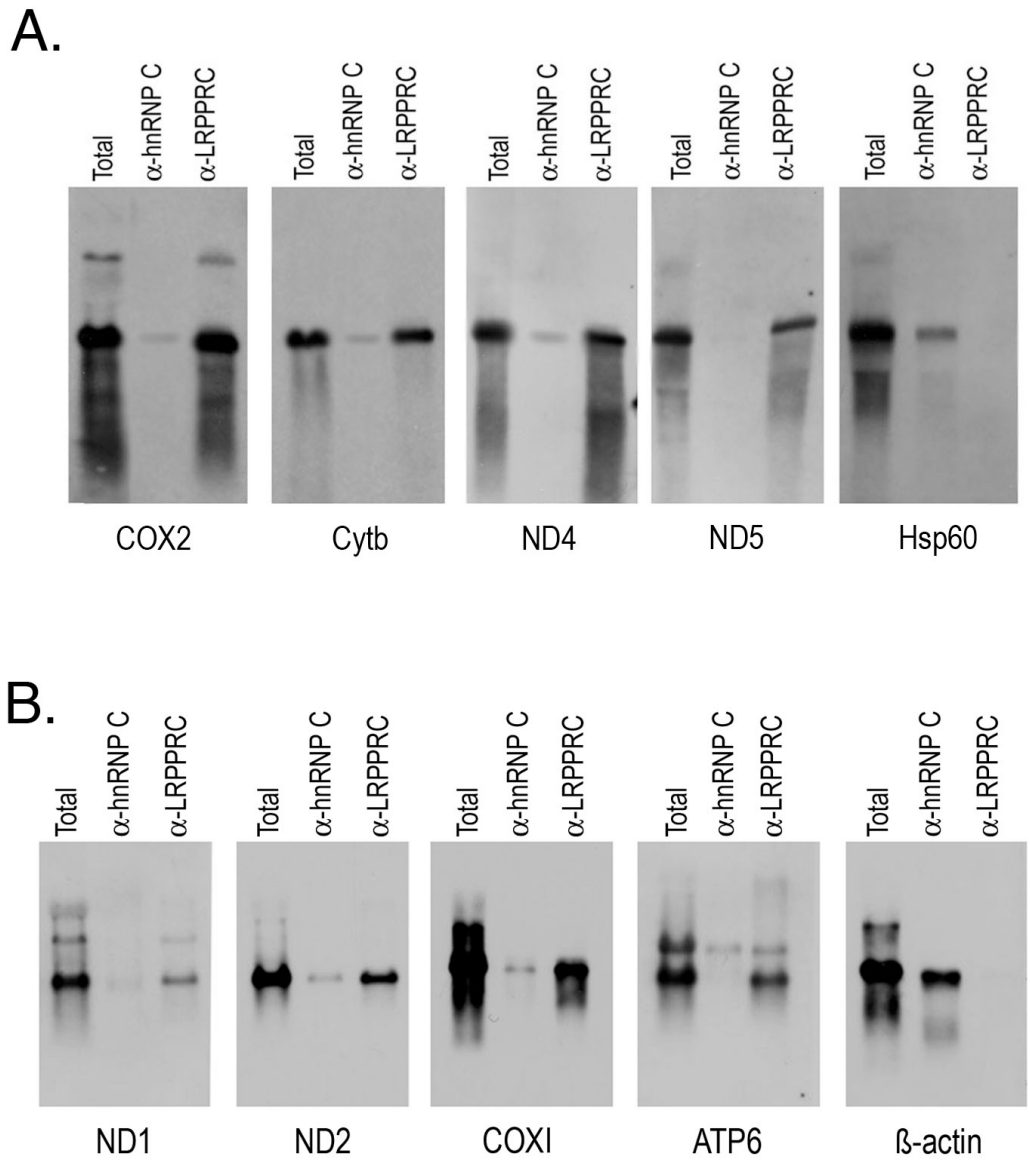

Figure 5A and B: Identification of LRPPRC-associated RNAs by Northern blotting. LRPPRC-associated complexes were isolated by immunopurification from HeLa whole-cell lysates, using the monoclonal antibody 9C9. As control, hnRNP complexes were isolated from the same lysates using the anti-hnRNP C1/C2 monoclonal antibody 4F4, as indicated. RNA was extracted from the isolated complexes, resolved by formaldehyde-agarose gel electrophoresis, and specific RNAs were identified by Northern blot analysis using probes for mitochondrially-encoded (ND1, ND2, ND4, ND5, Cytb, COXI, COX2 and ATP6) and nuclear-encoded (ß-actin and Hsp60) mRNAs, as indicated in the Figure. (*Figure 5-B. work done by Ji Fang)*

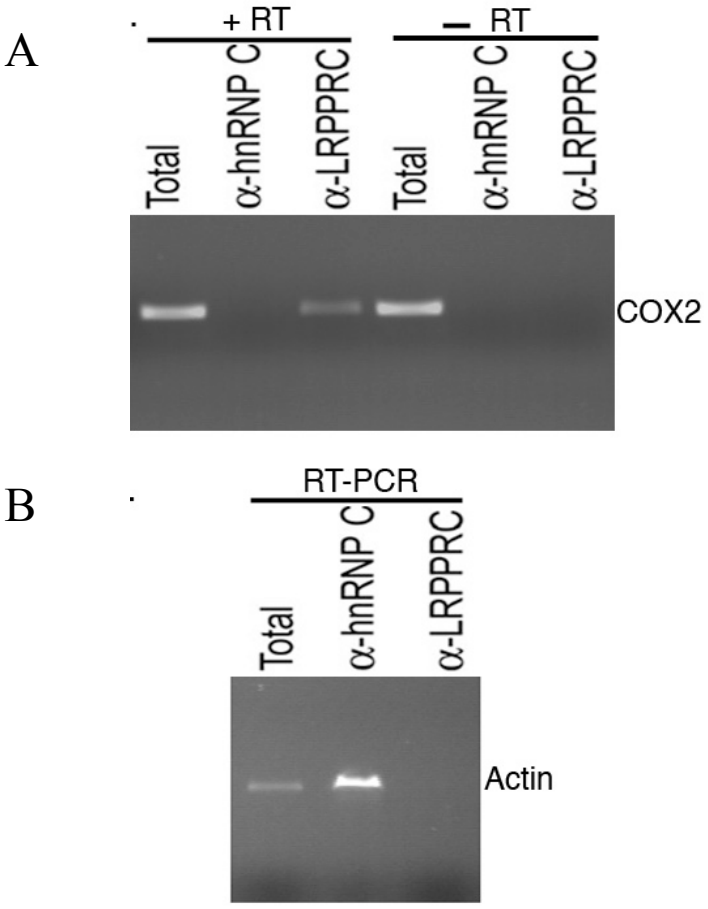

Figure 6: **Binding of LRPPRC to mitochondrial RNAs is specific**
LRPPRC-associated complexes were isolated from formaldehyde cross-linked HeLa cells using the anti-LRPPRC antibody 9C9. RT-PCR analysis of the associated RNAs was carried out using primers specific to a mitochondrial RNA (COX2) (panel A), or to a nuclear mRNA (β-Actin) (panel B). As a control, hnRNPC proteins-associated RNAs were similarly analyzed by RT-PCR using the same primers.

Chapter three

LRPPRC is necessary for the maintenance of steady-state levels of mitochondrial mRNAs

3.1 Introduction

In the preceding chapter, we demonstrated that LRPPRC is bound to all of the mitochondrial mRNAs we analyzed from the four complexes that require a contribution to their proteins from the mitochondrial genome. The functional significance of this binding however was not determined. Available information from Leigh syndrome French Canadian variant (LSFC) supports a role of LRPPRC in the maintenance of steady-state levels of only a subset of the mitochondrially-encoded mRNAs. In LSFC, an A354V substitution in the LRPPRC protein results in a decrease in COXI and COXIII mitochondrial mRNAs and a subsequent mitochondrial OXPHOS complex IV deficiency (Xu et al., 2004). The specific mechanism for the COXI and COXIII mRNAs reduction as well as the function of LRPPRC for these and the other mitochondrial mRNAs is not known. In this regard, knowledge of similar proteins in other organisms is useful and informative in providing a framework for formulating a hypothesis as to the function of LRPPRC for mitochondrial RNAs.

The consistent common link for PPR motif proteins is that almost all those for which functional studies have been carried out are involved in mitochondria or chloroplast gene expression through a function in RNA metabolism (Small and Peeters,

2000). Specifically, PPR motif proteins that bear a significant amino acid sequence similarity to LRPPRC have been characterized in the yeast *S.cerevisiae* (Pet309), *N.crassa* (Cya5), and *Z. mays* (CRP1). Crp1 plays a dual function in that it is involved in petD RNA processing and it activates both petA and psaC translation (Fisk et al., 1999). Both Cya5 and Pet 309 are required for translation of COXI mRNA (Coffin et al., 1997; Manthey and McEwen, 1995). The activation of translation for all these proteins is mediated by an interaction with the 5'UTR of their RNA ligand. In addition to a role in translation, Pet309 is involved in the production or stabilization of intron-containing COXI mRNA (Manthey and McEwen, 1995; Tavares-Carreon et al., 2008). Based on this collective information, we hypothesize that LRPPRC could function at any of the various stages of mitochondrial RNA metabolism including processing of the primary transcript, stabilization of RNA, translation of mRNAs or a combination of these roles. Furthermore, the RNA-binding findings described in the previous chapter suggest strongly that a function for LRPPRC might expand beyond the previously described involvement in complex IV (LSFC) to include complexes I, III, and V. The work described in this chapter aims to determine whether and, if so, which mitochondrial mRNAs are impacted by LRPPRC.

3.2 Results

3.2.1 SiRNA design and optimization

In order to carry out functional studies of LRPPRC for the mitochondrial RNAs to which it binds, our overall strategy relied on RNA interference-mediated 'knock down' of LRPPRC so as to reduce the steady-state levels of the protein. The downstream effects

(if any), of the 'knock-down' on the mitochondrial mRNAs could then be analyzed by northern blotting of these mRNAs, and a function of LRPPRC can be inferred from any observed changes. To this end, we made use of available software (Ambion (www.ambion.com/techlib/misc/siRNA_finder.html), Invitrogen (https://rnaidesigner.invitrogen.com/rnaiexpress/), and Dharmacon (www.dharmacon.com/designcenter/designcenterpage.aspx) to design siRNA sequence candidates. We chose the following two sequences based on their assigned score and according to siRNA design guidelines (Elbashir et al., 2001). Their target regions within the coding sequence of the LRPPRC mRNA are depicted below:

Target sequence 1: CCUAUAAGAGAUGUCCUAA

SiRNA duplex #1: 5' CCUAUAAGAGAUGUCCUAAtt 3'

3' ttGGAUAUUCUCUACAGGAUU 5'

Target sequence *2*: **CUAAGAAAAUUGAGGGAAA**

SiRNA duplex # 2: 5' CUAAGAAAAUUGAGGGAAAuu **3'**

3' uuGAUUCUUUUAACUCCCUUU **5'**

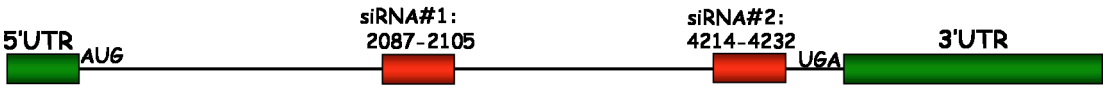

Figure 7. Schematic representation showing positions targeted by siRNAs within the LRPPRC mRNA

We tested the above sequences by transfecting them into HeLa cells using either Lipofectamine-2000 or Lipofectamine RNAiMAX (Invitrogen) at concentrations ranging from 10nM to 150nM. At these concentrations, no noticeable changes in cell viability were observed. Cells were incubated for 24 hours after transfection prior to performing the appropriate assays. Based on optimization studies, longer (36, 40 or 48 hours) incubation times necessitated a re-transfection of the siRNA 24 hours after the initial transfection. Results from immunoblot analyses of cells transfected with siRNA at 130nM show that we can achieve a moderate but detectable reduction of LRPPRC at 24 hours after siRNA transfection. Maximum reduction however is consistently observed with re-transfection samples at and after 36 hours time points (fig 9 compare lanes 'mock' and 'siRNA').

In order to ascertain that the observed reductions of cellular levels of LRPPRC were solely mediated by the siRNA itself and not by non-specific effects of the transfection reagents, we similarly transfected a control siRNA sequence (Ambion) with no known mRNA target in humans. Results in fig 9 show no detectable difference in the levels of LRPPRC in cells transfected with the control siRNA as compared to untransfected cells (compare lanes 'mock' and 'control').

The observed reduction of steady-state levels of LRPPRC in cells transfected with the siRNA to LRPPRC can therefore be attributed to a direct siRNA-induced knockdown of the LRPPRC mRNA. RNAi did not affect any other cellular proteins that we tested.

3.2.2 Decreasing cellular levels of LRPPRC results in decreased levels of COX2 (Cytochrome C oxidase subunit 2) protein.

Using the above standardized siRNA system; we undertook to delineate the function of LRPPRC for mRNAs in general and for mitochondrial RNAs in particular. As mentioned above, available data from LSFC support a role for LRPPRC in the maintenance of only a subset of mitochondrially-encoded mRNAs (COX1 and COX3) relevant for only complex IV of the OXPHOS. However, since LRPPRC binds other mitochondrial RNAs, our inclination is that LRPPRC might play a role for more than just COXI and COXIII RNA metabolism. We therefore began our functional study of LRPPRC by looking at effects (if any) of LRPPRC knockdown on COXII, the only other mitochondrially-encoded protein of complex IV that appeared to not be significantly affected in LSFC. Immunoblot analysis of samples from cells transfected with siRNA using anti-COXII antibody shows that when we decrease LRPPRC levels we induce a corresponding decrease in the COXII protein with no detectable decrease in the levels of hnRNP A1 (which was included here as a non-mitochondrial control) (fig.10). This novel finding is a departure from the LSFC data and points to a potential broader function of LRPPRC in mitochondrial gene expression.

3.2.3 LRPPRC is necessary for the maintenance of steady-state levels of mitochondrial mRNAs.

Having established that LRPPRC has a role in determining the levels of at least one of the mitochondrially-encoded proteins, COXII, we wanted to know if this role occurs, as hypothesized, at the mRNA level. Using a similar strategy, we performed

siRNA-mediated knockdown of LRPPRC in HeLa cells so as to reduce the cellular levels of the LRPPRC protein. As shown in fig 11A, immunoblot analysis with 9C9 antibody shows a "knock-down" consistent with our previous observations (compare lanes siRNA with mock). The steady-state levels of hnRNP A1 as detected using the 4B10 antibody, remained unchanged in both transfected and untransfected samples at all time points (compare mock and siRNA lanes in fig 11A).

To determine whether levels of mitochondrial mRNAs were affected, we performed northern blot analysis of total RNA extracted from siRNA-transfected and untransfected cells using probes specific to mitochondrial mRNAs (described in the previous chapter). The results show readily detectable reduction (fig 11B, lanes siRNA) in all mitochondrial mRNAs we analyzed. This reduction parallels and seems to be commensurate with that observed with the LRPPRC protein albeit with some slight variations between the RNA species. The mitochondrial RNAs analyzed include: ND1, ND2, ND4, and ND5 for complex I of the oxidative phosphorylation, Cytb for complex III, COXI, COXII, and COXIII for complex IV and ATPase 6 for complex V. These observations point to an essential function of LRPPRC in maintaining the levels of all mitochondrial mRNAs. By contrast to mitochondrially-encoded mRNAs, none of the nuclear-encoded mRNAs tested were visibly reduced upon reduction of LRPPRC cellular levels. The nuclear RNAs tested include NDUFS5 (complex I) and UQCRB (complex III) (see fig 12 top panel) as well as Hsp60 and β-actin (fig 12 bottom panel). Thus, LRPPRC is essential for maintaining the steady-state levels of mitochondrially-encoded mRNAs without affecting the nuclear-encoded mRNAs that we analyzed. The mechanism by which this function is achieved as well as the exact time of recruitment of

LRPPRC onto these RNAs remain to be elucidated in order for us to state its definitive function. Nonetheless, as stated in the introduction, we know that PPR motif proteins that closely resemble LRPPRC in their amino acid sequences have been implicated in activation of translation of organelle mRNAs (Pet309, Cya5 and Crp1), mRNA stability (Pet309) and processing (Crp1, Pet309) (Coffin et al., 1997; Fisk et al., 1999; Manthey and McEwen, 1995; Tavares-Carreon et al., 2008). Similarly, it is possible that LRPPRC serves in the processing and/or stabilization of most (if not all) the mitochondrial mRNAs to which it binds although we cannot rule out other possible functions on RNA metabolism.

3.2.4 LRPPRC is bound to mitochondrial unprocessed or partially processed precursor

The above-described RNAi experiments show that a reduction of steady-state levels of LRPPRC leads to a parallel reduction of mitochondrial mRNAs. This effect could be a consequence either of degradation of these mRNAs and/or a lack of processing of the primary transcript in the absence of LRPPRC. This in turn points to a role for LRPPRC at the level of processing of the primary transcript, stabilization of mRNAs and/or translation of mitochondrial mRNAs. Precedents exist for a role of PPR motif proteins in the processing of the primary transcripts during organelle gene expression. The maize PPR protein, CRP1, functions in the processing of petD mRNA in the intergenic region between petB and petD and in petA translation (Fisk et al., 1999). HCF152, an Arabidopsis PPR motif protein, also functions in intergenic RNA processing between psbH and petB and in petB splicing (Meierhoff et al., 2003). CRR2, another Arabidopsis PPR motif protein, functions in the intergenic processing of chloroplast RNA

between rsp7 and ndhb, and is essential for ndhb translation (Hashimoto et al., 2003). Thus a role of PPR motif proteins as co-factors in organellar precursor processing is well established.

Since processing of the primary transcript essentially involves excision of tRNAs to release the protein-coding RNAs as well as the rRNAs, the presence of tRNAs in the intergenic region of mitochondrial transcripts is therefore indicative of an unprocessed/partially processed mitochondrial pre-mRNA. Therefore, in order to determine whether LRPPRC is associated with the unprocessed RNA precursor and may have a function in the processing of the mitochondrial primary transcript, we designed sets of primers that would amplify fragments on the primary transcript that span the intergenic region including the intervening tRNA as depicted in the schematic example below:

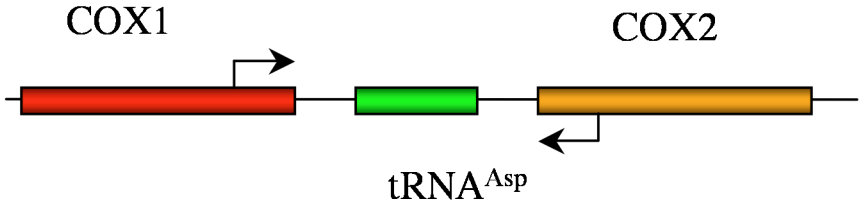

Figure 8:Position of primers designed to amplify a fragment including the intergenic region between COX1 and COX2 RNAs on the mitochondrial primary (unprocessed) transcript.

Only intact pre-mRNA substrate or partially processed intermediates can be amplified using these primers and amplification would in turn be indicative of unprocessed transcripts.

In this experiment, we used reversible formaldehyde cross-linking on intact Hela cells in order to induce covalent binding of proteins to their associated RNA ligands *in vivo*. The HeLa cell lysate was then used for immunopurification of LRPPRC and hnRNP C-associated RNP complexes as described earlier (chapter 2 and materials and methods). After rigorous washes and reversal of cross-linking, RNA was extracted from the isolated complexes and used as substrate for RT-PRC amplification with the primary transcript-specific primers for COX2/ATPase 8 and COX3/ND3 regions. Results shown in figure 13 indicate amplification of fragments of the expected sizes (ca. 390bp and 350bp respectively). These results confirm that LRPPRC is associated with the unprocessed/partially-processed transcript *in vivo* (figure 13, lanes anti-LRPPRC/+RT). This association is by direct binding since the RNA could be directly cross-linked to LRPPRC. No fragment amplification is observed from the anti-hnRNP C samples, indicating that mitochondrial precursor is not cross-linked and does not associate with hnRNP C proteins (fig 23, lane anti-hnRNP C +RT). As a control, parallel amplification was performed in which the reverse transcriptase was omitted (–RT) in order to ascertain that the observed amplification in the +RT samples is from RNA and not mtDNA. As shown in figure 13, (panel, –RT), no amplification is observed either from anti-LRPPRC and anti-hnRNPC samples, indicating that RNA and not DNA is associating with both proteins (see ‘lanes anti-LRPPRC and anti-hnRNPC, -RT). Total lysate (input) however shows amplification in the presence and absence of RT (see ‘lanes input, + and – RT).

This is an indication that mitochondrial DNA was a contaminant in the total lysate used for immunopurification of complexes. We also performed RT-PCR amplifications using primers specific for the nuclear-encoded β-actin mRNA to show that the absence of an amplicon using mitochondrial-specific primers is evidence that hnRNPC is associated with nuclear but not mitochondrial RNAs. Once more, results in figure 13 shown amplification of a fragment with the expected size for β-actin (ca.1250bp) from hnRNPC but not from RNA LRPPRC-associated complexes. These results demonstrate that LRPPRC is associated with the immature transcript *in vivo.*

3.2.5 Fate of the mitochondrial primary transcript in LRPPRC-depleted cells

The above experiments using *in vivo* formaldehyde cross-linking of RNA-protein followed by RT-PCR, demonstrate that LRPPRC is recruited onto the RNA prior to its full processing, possibly while still a primary transcript. As mentioned earlier, analysis of RNA from LRPPRC-depleted cells consistently showed a decrease in the steady-state levels of mitochondrial mRNAs. Moreover, this mRNA reduction was always accompanied by a reduction in other RNA species that would be consistent with the processing intermediates of the mitochondrial primary transcript (figure 14, panel A- see bands marked "*"). In addition to these reductions, a slower migrating high molecular weight nucleic acid species was often observed to accumulated more in the siRNA-transfected than in the untransfected samples (figure 14 panel A- see bands marked "**"). Together, these observations led us to hypothesize that LRPPRC is a necessary factor for the processing of the primary transcript and that therefore, a consequence of its knock-down resulted in the observed decrease in mitochondrial mRNAs and of the processing

intermediates as well as a possible accumulation of the primary transcript. To test this hypothesis, we sought to determine whether the observed band was RNA or mtDNA contaminant. To this end, total RNA from siRNA-transfected and untransfected Hela cells and mitochondrial DNA were each treated with DNase (Turbo DNase-Ambion) and RNase A (Qiagen) for 30 minutes at 37^0C then analyzed by northern blotting using a probe specific for COX2. Results in figure 14B show that both the upper band and the mitochondrial DNA are completely digested during DNase-treatment (fig 14B, lanes "m", "si" and "mtDNA" + DNase). Results from the RNase-treated RNA and mtDNA samples show that the slower migrating band as well as all RNAs are digested while mtDNA was undigested (fig 14B, lanes "m", "si" and "mtDNA" + RNase). Even though in both RNase and DNase treatments we loose the slower- migrating band for our RNA samples, it can nonetheless be inferred from the observed results of mitochondrial DNA digestions (fig 14B- lanes "mtDNA") that this slower-migrating band specie is RNA and not mitochondrial DNA otherwise it would have similarly survived the RNase digestion. The disappearance of this band in both treatments is likely due to either the lengthy manipulations or the presence of contaminant RNases irrespective of what enzyme was used. Had the band been DNA, we know from the control experiment (fig 14B, 'lane mtDNA + RNase) that it would have survived such manipulations

3.3. Discussion

The work described in this chapter addresses the functional implications of LRPPRC in mitochondrial RNA metabolism. This function was tested by three approaches: First, we used RNAi-mediated 'knock-down' of LRPPRC and analyzed the

effects on steady-state levels of mitochondrial mRNAs. Loss of LRPPRC was accompanied by a reduction in mitochondrial mRNAs that code for proteins for of complexes I, III, IV and V, revealing a more general function of LRPPRC in mitochondrial gene expression than had been previously reported from studies of LSFC point mutation in LRPPRC. Second, using *in vivo* cross-linking, immunopurification of RNP complexes and RT-PCR, we were able to determine that LRPPRC is bound to and is therefore recruited onto the mitochondrial precursor transcript. This piece of evidence, together with the observation that loss of LRPPCR is accompanied not only by a reduction in mitochondrial mRNAs but also by the accumulation of the primary transcript processing intermediates, led us to hypothesize a role for LRPPRC in the processing of the mitochondrial polycistronic pre-mRNA. Loss of LRPPRC would therefore result in an accumulation of the primary transcript due to a lack of processing. An accumulation in the RNAi-treated as compared to untransfected samples was from time to time observed as a slower migrating band in northern blot analyses. Using DNase and RNase digestions, we tested the identity of this upper band to determine whether it is RNA or mitochondrial DNA. Northern analysis of these digested samples point to (though not unambiguously) this material as being RNA.

What is the function of LRPPRC? Our results clearly show that LRPPRC is necessary for the maintenance of steady-state levels of mitochondrially-encoded mRNAs, a function that depends on the direct binding of LRPPRC to these mRNAs and their precursors. The functional relevance of this binding can be the stabilization of these RNAs (Pet309 (Manthey and McEwen, 1995), BSF (Mancebo et al., 2001), CRP1 (Schmitz-Linneweber et al., 2005), processing of the primary transcript (HCF152

(Meierhoff et al., 2003)), or translation of mitochondrial mRNAs (Pet309, Cya5, Crp1 and PGR3 (Coffin et al., 1997; Manthey and McEwen, 1995; Schmitz-Linneweber et al., 2005; Yamazaki et al., 2004)) or a combination of these functions. Moreover, a role for LRPPRC in the transcription of the mitochondrial DNA cannot be ruled out although, RT-PCR using immunopurified RNP complexes did not show evidence for the presence of mtDNA in these complexes.

Our findings support a general role of LRPPRC in the processing of the mitochondrial RNA precursor. Loss of LRPPRC would have as a consequence, a defect in processing resulting in a decrease not only of mtmRNAs but also of any processing RNAs intermediates. This, in fact, is our observation in RNAi –mediated 'knock-down' experiments (see above). Based on *in vivo* binding to poly (A) + mRNAs (Mili and Pinol-Roma, 2003) and our results described in chapter one, LRPPRC stays bound to mature mitochondrial mRNAs. A possible implication for this binding is that LRPPRC is a stabilizing factor for these mRNAs.

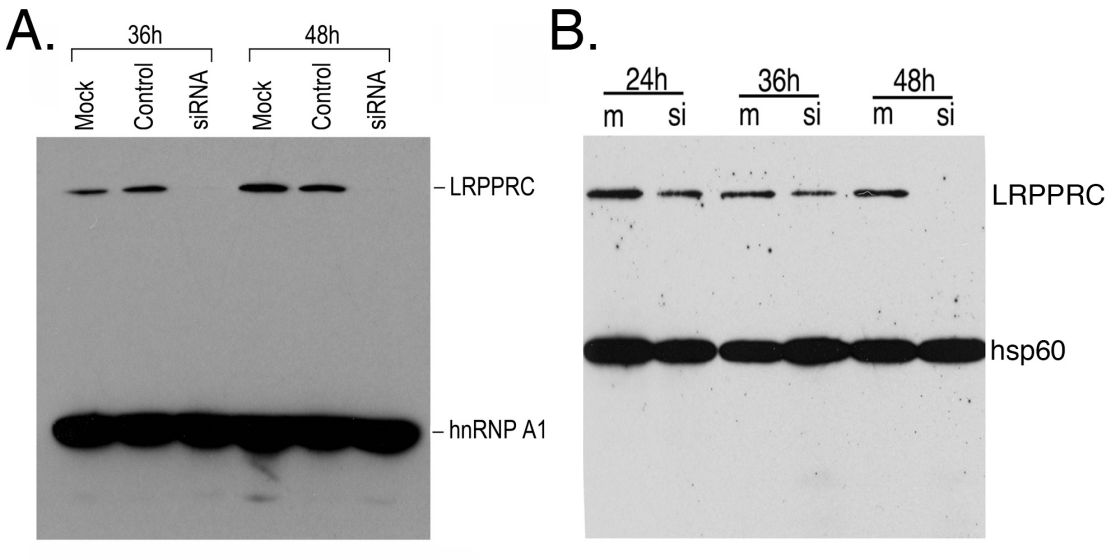

Figure 9: Specific 'knock-down' of steady-state levels of LRPPRC by RNA interference (RNAi)

HeLa cells transfected with siRNA duplexes targeting the LRPPRC mRNA were grown for the indicated periods of time. Immunoblot analysis of transfected cells using anti-LRPPRC and anti-hnRNP A1 (panel A) or anti-Hsp60 (panel B) were subsequently carried out. As a control, cells transfected with a control siRNA (control) (panel A) were similarly analyzed by immunoblotting.

"m" indicates mock-transfected, "control" indicate samples transfected with a control siRNA, "si" indicate samples transfected with an siRNA targeting LRPPRC mRNA.

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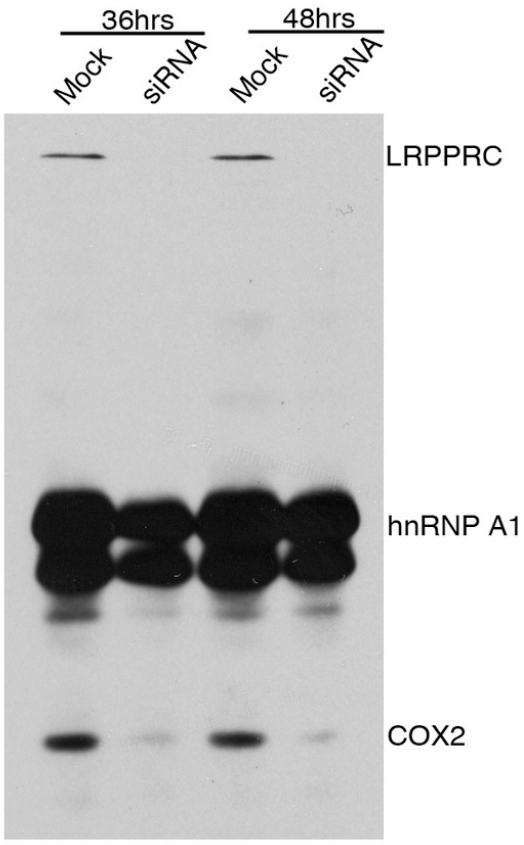

Figure 10: Steady-state levels of COX2 protein are reduced with reduced levels of LRPPRC

HeLa cells transfected with siRNA targeting LRPPRC were grown for the indicated time periods and analyzed by immunoblotting using anti-LRPPRC, anti-hnRNP A1, and anti-COX2 antibodies.

"mock," indicates mock-transfected, "siRNA," indicate samples transfected with an siRNA targeting LRPPRC mRNA.

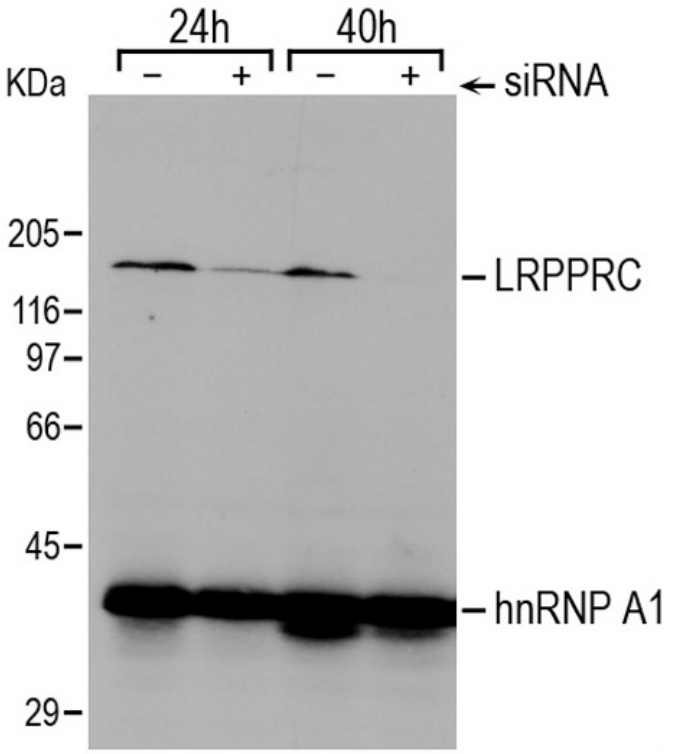

Figure 11A. Cells transfected with siRNA targeting LRPPRC mRNA (for either 24 or 40 hrs) were assayed for LRPPRC "knock-down" by immunoblotting (compare "+" siRNA to "-" siRNA lanes). RNA extracted from these cells was assayed by northern blotting (see below **figures 11B** and **C**).

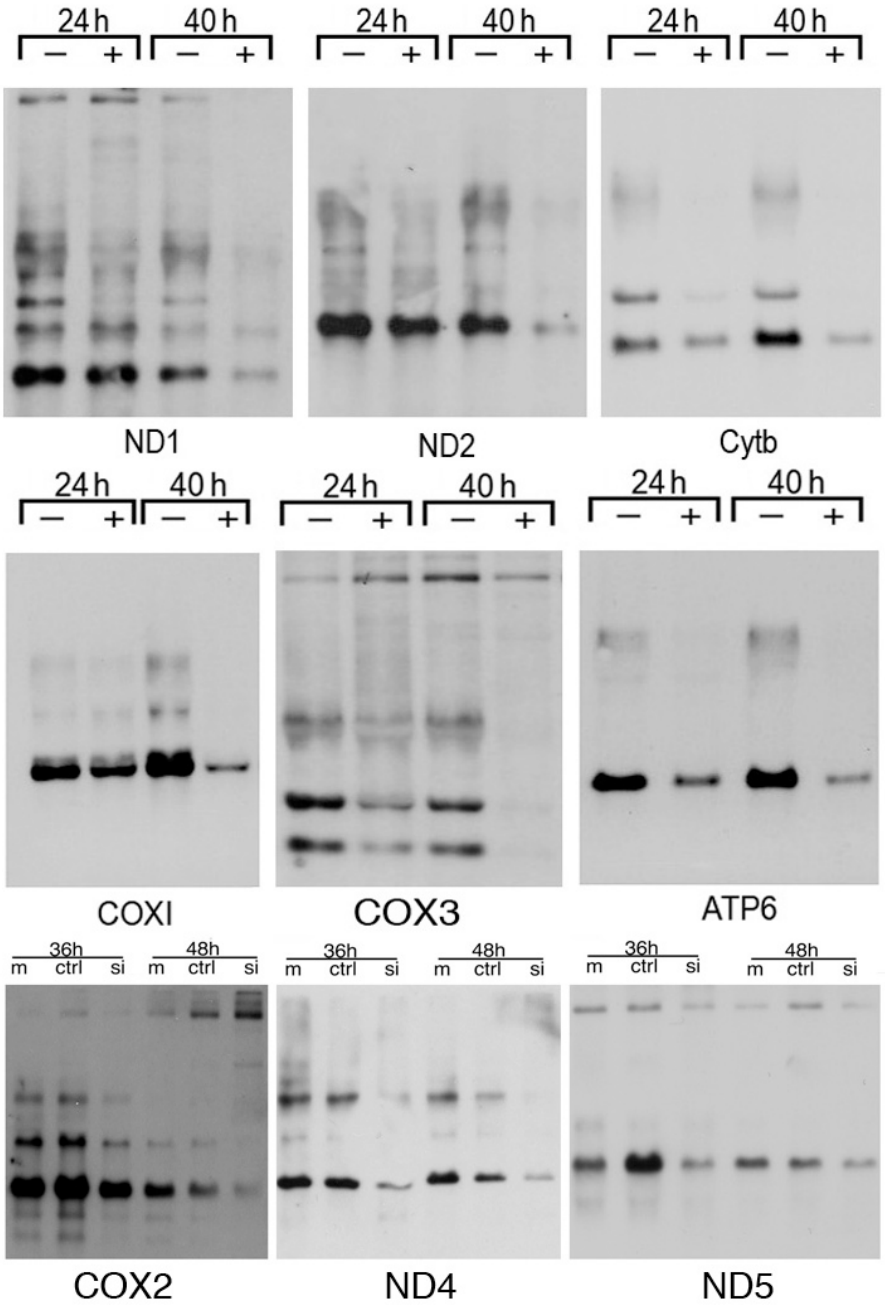

Figure 11B: Steady-state levels of mitochondrially-encoded RNAs are reduced in cells with reduced levels of LRPPRC. Total RNA extracts from HeLa cells transfected with siRNA targeting LRPPRC mRNA were analyzed by Northern blotting using probes specific for mitochondrially-encoded RNAs as indicated above. "+" and "-" indicate siRNA-transfected and untransfected samples. "**m**" indicates mock-transfected, "**ctrl**" indicate samples transfected with a control siRNA, "**si**" indicate samples transfected with an siRNA targeting LRPPRC mRNA.

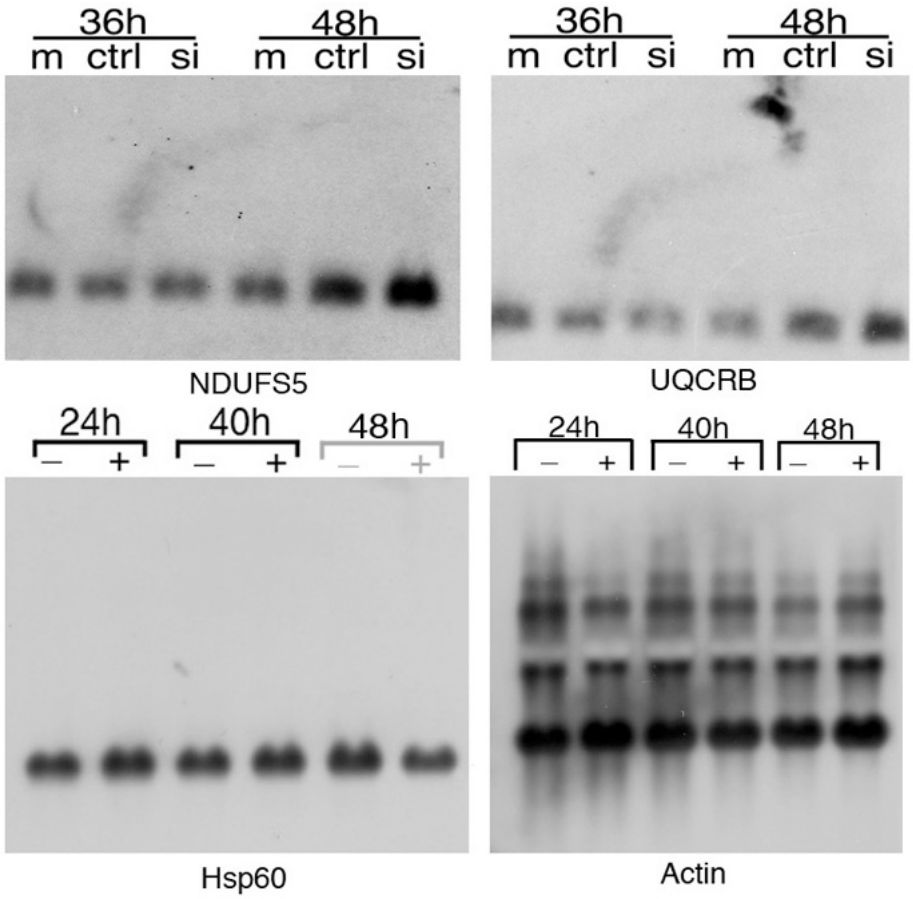

Figure 12: Steady-state levels of the nuclear-encoded RNAs analyzed are not affected by reduced levels of LRPPRC. Total RNA extracts from HeLa cells transfected with siRNA targeting LRPPRC mRNA were analyzed by Northern blotting using probes specific for cytosolic mRNAs that code for OXPHOS proteins (top panel) and mRNAs for hsp60 and beta-actin (bottom panel).
"+" and "-" indicate siRNA-transfected and untransfected samples.
"m" indicates mock-transfected, "ctrl" indicate samples transfected with a control siRNA, "si" indicate samples transfected with an siRNA targeting LRPPRC mRNA.

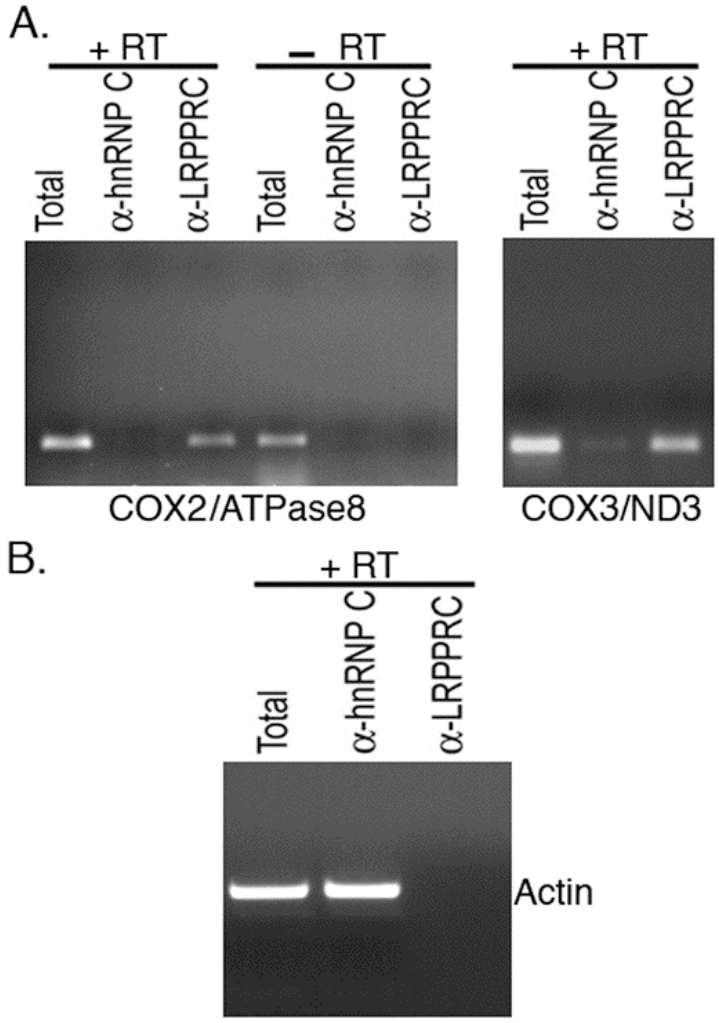

Figure 13. LRPPRC is bound to the unprocessed mitochondrial transcript. Formaldehyde-induced cross-linking of HeLa followed by immunopurification of LRPPRC or hnRP C-associated complexes using their cognate monoclonal antibodies. RNA extracted from these complexes was analyzed by RT-PCR using primers that amplify mitochondrial unprocessed/partially processed precursor RNA. ("Total" refers to starting material)
Panel A: amplification using mitochondrial precursor-specific primers
Panel B: amplification using primers specific for beta-Actin

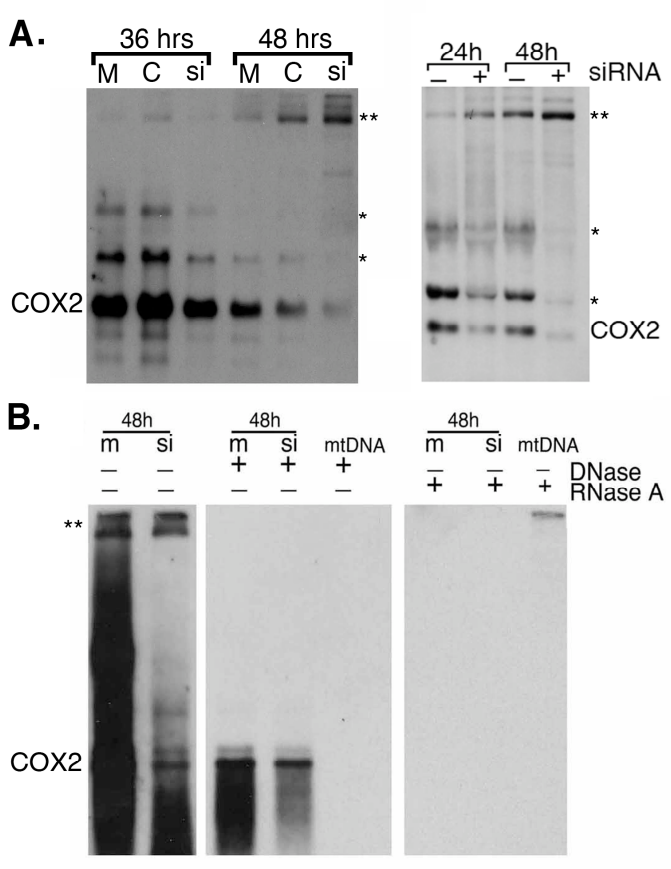

Figure 14: Fate of the mitochondrial unprocessed precursor in the absence of LRPPRC

Loss of LRPPRC is associated with loss of precursor processing intermediates (panel A "*") and an accumulation of the unprocessed precursor panel A"**"). RNA extracted from Hela cells transfected (or untransfected) with siRNA against LRPPRC mRNA was analyzed by northern blotting using a COX2-specific probe (panel A). To determine the fate of the higher molecular weight band, these RNAs were digested with DNase or RNase (panel B- lanes "m" and "si") prior to northern analysis using the COX2 probe. As a control, mtDNA was also digested with DNase or RNase (panel B-lanes mtDNA).
'm" = mock, "si" = siRNA-transfected, "C"= control siRNA, "+" and "-" refer to transfected and untransfected respectively.

Chapter four

Members of the human family of PPR motif proteins and their role in mitochondrial function

4. 1 Introduction

4.1.1 Overview of PPR motif proteins

As previously stated, the PPR-motif family of proteins is widespread across all eukaryotes. While the function of the PPR motif itself is not known, members of this family of proteins mostly function in organellar energy homeostasis for those species that have them. Inroads in identifying members of this family have been facilitated by the presence of complete genome sequences for various species as well as the availability of bioinformatics tools necessary to navigate them. Genome-wide analyses show this motif to be present in all eukaryotes albeit with a wide disproportion between plant and non-plant organisms. There are for instance, about 450 predicted PPR motif genes in *Arabidopsis Thaliana* and only five in the yeast *Saccharomyces cerevisiae* (Lurin et al., 2004; Small and Peeters, 2000). The human PPR-motif protein, LRPPRC, was previously described as an RNA-binding protein with a function in both the nucleus and mitochondria. Clinical findings in patients' fibroblasts revealed that an A → V substitution in the LRPPRC protein was the defect in Leigh syndrome French Canadian variant (LSFC) characterized by a complex IV deficiency (Mili and Pinol-Roma, 2003; Xu et al., 2004). Functional studies for LRPPRC led to the discovery that it is a novel

trans-acting factor that binds to all mitochondrial mRNAs and is essential for their steady-state accumulation. Based primarily on the yeast model of mitochondrial gene expression, our findings (and those of others) as to the role of LRPPRC in mitochondrial gene expression and based on the co-isolation of several proteins with LRPPRC (Mili and Pinol-Roma, 2003), we initially proposed that there may be yet unidentified trans-acting factors for human mitochondrial gene expression beyond those already identified and characterized. The discovery that LRPPRC was a bona fide general trans-acting factor that participates in the metabolism of all the mitochondrial mRNAs led us to ask whether there were additional potential human PPR motif proteins and if so, whether these proteins also play a role in mitochondrial gene expression.

4.1.2 Other PPR-motif proteins in humans

Genome-wide screens for PPR motif proteins in several organisms had been carried out and the predicted PPR motif proteins compiled in a database (http://www.evry.inra.fr/public/projects/ppr/ppr.html). In order to identify members of the human PPR motif family, we carried out a search of this database and identified several potential candidates based. Further screenings were done to select for only candidates that corresponded to full-length proteins (http://blast.ncbi.nlm.nih.gov/blast.cgi). Using motif search programs, we ascertained the presence and the number of PPR motifs in these sequences (**Smart**- *http://smart.embl-heidelberg.de/* and **Motif Scan**- *http://myhits.isb-sib.ch/cgi-bin/motif_scan*) by matching them against Prosite or Pfam (local and global) protein databases. Each predicted PPR motif sequence was later compared to the consensus PPR sequence using an alignment

software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The results from these predictions (see tables I and II below) yielded 6 PPR motif proteins in addition to LRPPRC in humans. These are: PolRMT (Mitochondrial RNA polymerase), Pentatricopeptide Domain proteins (PTCD1, PTCD2 and PTCD3), PTCD4 (KIAA0391) also now known as MRPP3 (mitochondrial RNase P protein 3), and MRSP27 (KIAA0264 mitochondrial ribosomal protein S27). The number of PPR motif (s) contained in their structures varied slightly from one software program to another most probably due to variations in the stringency intrinsic to their parameters. For example, PTCD4 has one PPR motif predicted using Motif Scan and no predictable PPR motif when using the Smart program, suggesting that biological evidence is necessary to confirm the validity of this motif. We next used **MITOPROT** (*http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html*) and **TargetP** (*http://www.cbs.dtu.dk/services/TargetP*) to predict whether, like LRPPRC, these proteins may localize to mitochondria. These predictions revealed with over 90% probability that all the proteins with PPR motif in humans localize to mitochondria and contain potentially cleavable mitochondrial targeting sequence (MTS). In addition, PolRMT, PTCD1 and PTCD2 sequences contain a predicted bipartite nuclear localization sequence (NLS). In agreement with these predictions, PolRMt has been shown to localize and to function in mitochondria and the nucleus (Kravchenko et al., 2005). The validity of the mitochondrial and nuclear localizations for the other PPR proteins remains to be tested experimentally. A dual localization for PTCD1 and PTCD2, if true, would be reminiscent of LRPPRC. Following is a summary of information obtained from the above predictions (tables I and II, figure 15):

TABLE I – Predicted human PPR Motif Proteins

Accession number	Name	Location	# of PPR motifs	Subcellular localization
NP_573566.2	LRPPRC	2p21	11	Mitochondria/Nucleus
NP_056360.2	PTCD1	7q22.1	7	Mitochondrial (Nuc?)
NP_079030.3	PTCD2	5q13.2	1	Mitochondrial (Nuc?)
NP_060422.4	PTCD3	2p11.2	4	Mitochondrial
NP_055487.2	MRPP3 (PTCD4)	14q13.2	2	Mitochondrial
NP_055899.2	MRPS27	5q13.2	1	Mitochondrial
NP_005026.3	PolRMt	19p13.3	2	Mitochondrial (Nuc?)

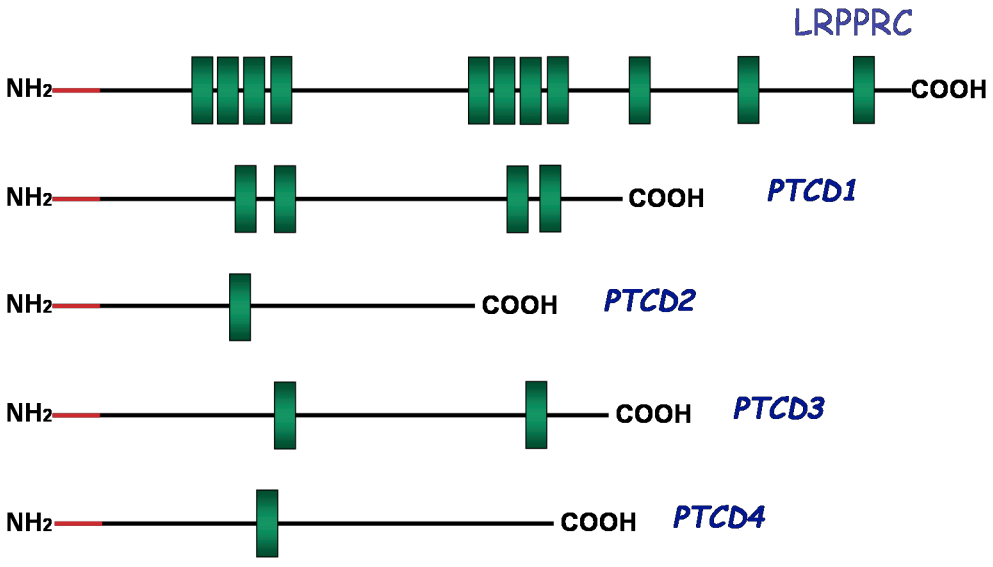

Figure 15. Schematic representation of human proteins with their PPR motifs

TABLE II – Comparative alignment of the human PPR motifs to the consensus sequence

PPR motif consensus -TYNALINAYAK-G—EEA—LY—M---G—PN-

PTCD1: NP_056360

PTCD1	Location	Sequence	E-value
PTCD1_PPR1	174-208	SNYTVLIGGCGRVGYLKKAFNLYNQMKKRDLEPSD	5.50E-10
PTCD1_PPR2	248-282	KTYHALLKMAAKCADLRMCLDVFKEIIHKGHVVTE	0.0023
PTCD1_PPR3	521-555	TFFNTLVRKKSKLGDLEGAKALLPVLAKRGLVPNL	0.0043
PTCD1_PPR4	588-622	HIYSALINAAIRKLNYTYLISILKDMKQNRVPVNE	0.0041

Alignment:

```
PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
PTCD1_PPR1          SNYTVLIGGCGRVGYLKKAFNLYNQMKKRDLEPSD 35
                     .*..**.. .: *  ::*  **  *   .  *.

PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
PTCD1_PPR2          KTYHALLKMAAKCADLRMCLDVFKEIIHKGHVVTE 35
                     **:**::  ** .  . .  ::  :   *   .

PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
PTCD1_PPR3          TFFNTLVRKKSKLGDLEGAKALLPVLAKRGLVPNL 35
                      :*:*:.  :* *  * *  *   :   *  **

PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
PTCD1_PPR4          HIYSALINAAIRKLNYTYLISILKDMKQNRVPVNE 35
                     *.*****  :         :   *       *
```

PTCD2: NP_079030

PTCD2	Location	Sequence	E-value
PTCD2_PPR	166-200	SNYTVLIGGCGRVGYLKKAFNLYNQMKKRDLEPSD	2.20E-07

Alignment:

```
PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
PTCD2_PPR           TSFNILMDMLFIKGKYKSALQVLIEMKNQDVKFTK 35
                     ::* *::      *   :.*  :   *   .   .
```

PTCD3: NP_060422

PTCD3	Location	Sequence	E-value
PTCD3_PPR1	257-291	HSYCTMIRGMVKHRAYEQALNLYTELLNNRLHADV	5.70E-06
PTCD3_PPR2	574-608	TSLNCIAILFLRAGRTQEAWKMLGLFRKHNKIPRS	0.039

Alignment:

```
PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
PTCD3_PPR1          HSYCTMIRGMVKHRAYEQALNLYTELLNNRLHADV 35
                     :* ::*.. .*    *:*  **  :       .:

PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
PTCD3_PPR2          TSLNCIAILFLRAGRTQEAWKMLGLFRKHNKIPRS 35
                     : *.:   : : *  :**  :   :   .  *.
```

PTCD4 (MRPP3): NP_055487

PTCD4	Location	Sequence	E-value
PTCD4_PPR1	211-244	RGYSLLIRGLIHSDRWREALLLLEDIKKVITPSK	3.10E-02
PTCD4_PPR2	245-279	KNYNDCIQGALLHQDVNTAWNLYQELLGHDIVPML	0.056

Alignment:

```
PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
PTCD4_PPR1          RGYSLLIRGLIHSDRWREALLLLEDIKKV-ITPSK 34
                      *. **..  : .  .**  *   :      *.

PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
PTCD4_PPR2          KNYNDCIQGALLHQDVNTAWNLYQELLGHDIVPML 35
                     .**  *:.       : *  **  :   .  *
```

LRPPRC: NP_573566

LRPPRC	Location	Sequence	E-value
LRP_PPR1	163-197	SHYNALLKVYLQNEYKFSPTDFLAKMEEANIQPNR	4.60E-03
LRP_PPR2	198-232	VTYQRLIASYCNVGDIEGASKILGFMKTKDLPVTE	1.00E-05
LRP_PPR3	233-267	AVFSALVTGHARAGDMENAENILTVMRDAGIEPGP	9.70E-07
LRP_PPR4	268-302	DTYLALLNAYAEKGDIDHVKQTLEKVEKSELHLMD	3.10E-03
LRP_PPR5	440-474	HYFWPLLVGRRKEKNVQGIIEILKGMQELGVHPDQ	1.10E-01
LRP_PPR6	712-746	GGYAALINLCCRHDKVEDALNLKEEFDRLDSSAVL	2.60E-02
LRP_PPR7	749-783	GKYVGLVRVLAKHGKLQDAINILKEMKEKDVLIKD	2.90E-03
LRP_PPR8	956-990	QMYYNLLKLYKINGDWQRADAVWNKIQEENVIPRE	7.70E-03
LRP_PPR9	1319-1353	EAYNSLMKSYVSEKDVTSAKALYEHLTAKNTKLDD	1.70E-04

Alignment:

```
PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
LRP_PPR1            SHYNALLKVYLQNEYKFSPTDFLAKMEEANIQPNR 35
                      ****::.* :     ..  :   *   .  **

PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
LRP_PPR2            VTYQRLIASYCNVGDIEGASKILGFMKTKDLPVTE 35
                     **: ** :*.: *  * *  :   *   .  .

PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
LRP_PPR3            AVFSALVTGHARAGDMENAENILTVMRDAGIEPGP 35
                     .:.**:..:*: *  *:*  :   *   *  *.

PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
LRP_PPR4            DTYLALLNAYAEKGDIDHVKQTLEKVEKSELHLMD 35
                     ** **:****: *  :..      :
```

```
PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
LRP_PPR5            HYFWPLLVGRRKEKNVQGIIEILKGMQELGVHPDQ 35
                      : .*: .  *    :    :   *   *  *:

PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
LRP_PPR5            HYFWPLLVGRRKEKNVQGIIEILKGMQELGVHPDQ 35
                      : .*: .  *    :    :   *   *  *:

PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
LRP_PPR7            GKYVGLVRVLAKHGKLQDAINILKEMKEKDVLIKD 35
                     .* .*:.. ** *  ::*  :   *   .   :

PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
LRP_PPR8            QMYYNLLKLYKINGDWQRADAVWNKIQEENVIPRE 35
                      *  *:: *   *  :.*  ::  :   .  *.
PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
LRP_PPR9            EAYNSLMKSYVSEKDVTSAKALYEHLTAKNTKLDD 35
                     :**:*:::*..     .*  **  :   .   :
```

PolRMt: NP_ 005026.3

PolRMt	Location	Sequence	E-value
Pol_PPR	262-296	DMYNAVMLGWARQGAFKELVYVLFMVKDAGLTPDL	3.50E-03

Alignment:

```
PPR_consensus       ZTYNALINAYAKZGZZEEAZZLYZZMZZZGZZPNZ 35
PolRMT_PPR          DMYNAVMLGWARQGAFKELVYVLFMVKDAGLTPDL 35
                      ***:: .:*: *  :*   :   :   *  *:
```

MRPS27: NP_ 055899

MRPS27	Location	Sequence	E-value

MRP_PPR	142-175	FTFNLLMDSFIKKENYKDALSVVFEVMMQEAFEV	4.00E-02

Alignment:

```
PPR_consensus       ZTYNALINAYAKZGZZEEAZ-ZLYZZMZZZGZZPNZ 35
MRPS27_PPR          FTFNLLMDSFIKKENYKDALSVVFEVMMQEAFEV-- 34
                     *:* *:::: *    ::*   ::  *   .
```

Legend: "*" denotes true conservation

"**:**" denotes conserved substitutions

"**.**" denotes semi-conserved substitutions

"**z**" represents any amino acid

E-values represent the probability of the identified PPR motifs occurring by chance (e.g. an e-value of 3.50E-03 for the predicted PolRMt PPR motif signifies that there is a 0.0035 chance to 1 (or 0.35%) that this prediction is a false positive).

4.2 Results

4.2.1 PTCD proteins (PTCD1, 2, 3, and 4) localize predominantly to mitochondria

Of the 7 human PPR motif proteins, two had been characterized at the start of this work, LRPPRC and PolRMt (mitochondrial RNA polymerase). The function of PolRMt in mitochondrial transcription and in synthesizing RNA primers for mtDNA replication has been documented (Ojala et al., 1981; Wanrooij et al., 2008). In addition to the mitochondrial function, PolRMt (devoid of the mitochondrial targeting sequence) has been shown to function in the transcription of some nuclear mRNAs (Kravchenko et al., 2005). LRPPRC has been the subject of this dissertation thus far. This chapter will

therefore focus on the remaining newly identified candidates of the human PPR motif family. We first wanted to ascertain the cellular localization and site of function for these proteins. In the absence of antibodies specific for any of them, we cloned their full-length coding sequences into either pcDNA3.1 or pCMV-HA for the purpose of expression into HeLa cells. Proteins to be expressed were tagged with a Myc sequence at their C-terminus, to enable detection with available anti-myc antibodies. In order to determine whether these proteins localize to mitochondria, Hela cells transfected with plasmids expressing PTCD1, 2, 3, or 4 were grown in the presence of the mitochondria-specific dye Mitotracker CMXRos. Cells were subsequently stained with the anti-myc antibody (9E10). Immunofluorescence microscopy revealed a staining whose distribution in the cytosol overlaps with that of mitotracker (fig 16 see FITC in green, mitotracker in red, and the overlay). This distribution indicates to us that PTCD1, 2, 3, and 4 co-localize in mitochondria. This mitochondrial localization was further confirmed by subcellular fractionation that separates mitochondria from nuclei and soluble cytosol by differential centrifugation. Immunoblot analysis of the three cellular fractions reveals that these proteins co-fractionate with the LRPPRC protein as indicated by their respective bands in the mitochondria-enriched fractions (Figure 17- see lanes mito). Their cytosolic presence is transient as indicated by very faint bands in the digitonin-soluble cytosolic fractions (fig 17-lanes cyto). The appearance of bands in the nuclear fractions for PTCD1, 2, 3, and 4 indicates that intact mitochondria/or cells were a significant presence in the nuclear fraction (see fig 17- lanes nuc) although a *bona-fide* nucleoplasmic localization cannot be ruled out at present. These results indicate that PTCD1, 2, 3, and 4 proteins much like

LRPPRC also reside in mitochondria. Whether they also function in RNA metabolism remains to be determined.

4.2.2 PTCD1, 2, and 3 bind RNA in vitro

The function of PPR motif proteins in RNA metabolism is linked to their RNA-binding. Since the human PTCD proteins were shown to contain predicted PPR motifs, we set out to determine whether they too, like LRPPRC, have RNA-binding properties. To this end, we used *in vitro* immobilized RNA homopolymers, an approach that was used before to determine the RNA binding preferences of LRPPRC. In this assay, LRPRC was found to bind preferentially to polyU than to polyG and polyC (Mili and Pinol-Roma, 2003). We carried out similar experiments using total cell lysate from Hela cells expressing my-tagged PTCD1, 2, and 3. These lysates were incubated either with polyC, polyG, or polyU at 4^0 C for 30 minutes. After several washes, bound proteins were eluted at increasing NaCl concentrations and analyzed by western blotting (figure 18).

4.2.3 PTCD1, PTCD2 and PTCD3 are components of LRPPRC-associated complexes

Analysis of LRPPRC-associated complexes shows that most of the proteins that co-purify with LRPPRC are distinct from those that are found in nuclear mRNPs [work done by Mili S., see Bangeranye et al.- manuscript submitted]. Most of the cellular LRPPRC resides in mitochondria where it is in association with mitochondrial RNA [The present work, (Mili and Pinol-Roma, 2003). It follows that LRPPRC-associated complexes are mostly mitochondrial mRNPs and therefore, LRPPRC-associated proteins include trans-acting factors involved in mitochondrial RNA metabolism. Whether these

proteins include all or a subset of the human PPR motif proteins is not known.

To test whether the PTCD proteins are among those associated with LRPPRC in mitochondrial RNP complexes, we transfected Hela cells with plasmids expressing PTCD1, 2, or 3 fused to a myc-tag. We then isolated RNP complexes using 9E10 (anti-myc) antibody. Isolated proteins from immunopurified complexes were analyzed by western blotting using both the 9E10 and 9C9 antibodies in order to detect the myc-tagged proteins and LRPPRC respectively. Figure 19 shows that PTCD1, 2, and 3 were successfully overexpressed in Hela cells (see fig. 19 'lanes input'). Figure 19, panels A, B and C, lanes (+ lysate) show bands that are consistent with LRPPRC and either of the transfected PTCD protein. These bands are absent in the controls (lanes - lysate) panels A, B and C as well as in the hnRNP C proteins –associated complexes (panel D, ip 4F4 lanes "+ " and "-" lysate). Our conclusions from these experiments are that PTCD1, 2, and 3 are components of LRPPRC-associated RNP complexes. Whether this association is through a direct binding of these proteins to LRPPRC-associated RNAs or through protein-protein interactions is not yet known.

4.2.4 PTCD1 is not cross-linked to polyadenylated mRNAs in vivo

As shown above, PTCD1, 2, and 3 bind to RNA homopolymers *in vitro,* suggesting that they may have an intrinsic RNA-binding domain and do not require the coordination of other factors for their RNA-binding. Whether this in vitro binding holds true in vivo remains to be ascertained.

Characterization of in vivo RNA-binding for the PTCD proteins is still on going and results for PTCD1 are described in this section. We used the already described UV-induced crosslinking approach on HeLa cells expressing a myc-tagged PTCD1 protein

followed by oligo-dT selection of poly (A) + RNAs and their covalently bound proteins. Immunoblot analysis of the bound proteins using an anti-myc antibody shows that PTCD1 is not cross-linked to polyadenylated mRNAs (figure 20, see lane + UV). Incubation with 9C9 (anti-LRPPRC) shows that LRPPRC is cross linked to poly (A) + mRNAs (figure 20, lane +UV) (and as previously demonstrated (Mili and Pinol-Roma, 2003)). This result suggests that PTCD1 does not associate with mRNAs in vivo.

4.3 Discussion

Using available software, we identified members of the PPR motif family in humans. These proteins were all predicted with high probability to localize to mitochondria. Our preliminary experimental results are in agreement with predictions. PTCD1, 2, 3and 4 localize to mitochondria as demonstrated by immunofluorescence microscopy and subcellular localization experiments. Preliminary in vitro RNA-binding assays show that PTCD1, 2, and PTCD3, all bind preferentially RNA homopolymers, suggesting the presence of RNA-binding domains within their sequences. Unlike LRPPRC which binds polyU, polyG, and weakly to polyC, the PTCD proteins were found to show specificity for polyG (figure 18). In fact, even PTCD2, which has only one PPR motif, binds homopolymers, an observation that as mentioned above, does not fit the originally proposed minimal requirement of 2 PPR motifs for all characterized PPR proteins (Small and Peeters, 2000). This *in vitro* RNA-binding of course, while predictive, remains to be confirmed by *in vivo* assays. The fact that PTCD1 cannot be cross-linked to polyadenylated RNAs may suggest a function for PTCD1 involving non-polyadenylated RNAs.

In addition, PTCD 1, 2, and 3 were found to associate with LRPPRC in mitochondrial ribonucleoprotein complexes. As mentioned earlier, several proteins were found to co-isolate with LRPPRC in mitochondrial RNP complexes. Their identity was as yet to be determined. In this work, we identify three candidates, PTCD1, 2, and 3 and we show that they are components of LRPPRC-associated complexes.

Altogether, these results point to PTCD1, 2 and 3 as potential trans-acting factors that are involved in some aspects of mitochondrial RNA metabolism. Indeed, while this work was ongoing, literature supporting our hypothesis has began to emerge about these proteins. Specifically, PTCD1 is thought to regulate the levels of the leucine tRNA in mitochondria through binding to the tRNA-containing precursor (Rackham et al., 2009). Mice expressing a defective PTCD2 were reported to have a decreased Complex I and Complex III activities, resulting from a lack of processing of the mitochondrial ND5-Cytb precursor. This finding implicates PTCD2 in processing of the precursor (Xu et al., 2008). The fact that both PTCD1 and PTCD2 are involved in precursor metabolism and were found to be associated with LRPPRC in RNP complexes supports our previous findings that LRPPRC is recruited on the mitochondrial precursor RNA. PTCD3 on the other was found to co-sediment with the small subunit of the mitochondrial ribosome and to function in translation (Davies et al., 2009), whereas MRPP3 (PTCD4) is a newly identified member of the mitochondrial RNase P multi-protein complex, implicating it in mitochondrial tRNA excision.

The picture that emerges, based on data described in this thesis, and based on the available literature, points to the human PPR motif family of proteins as essential trans-acting factors in mitochondrial gene expression. Further work is being carried out to

determine the exact manner in which these proteins exert their functions so as to better understand their implication on cellular energy metabolism in health and disease.

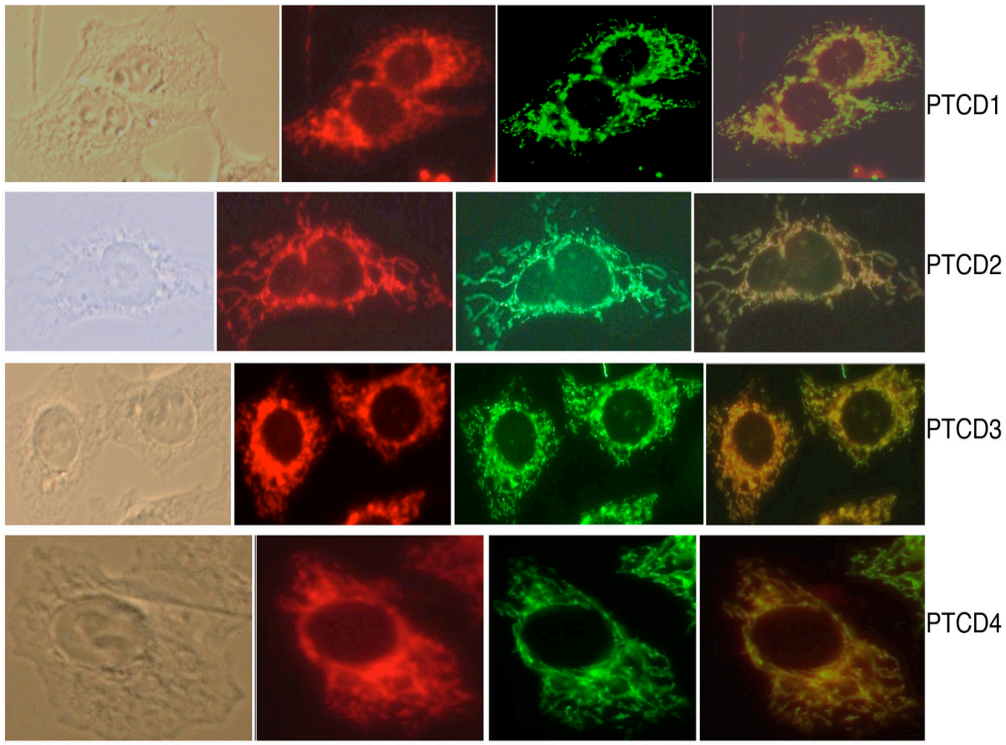

Figure 16: PTCD1, 2, 3, and 4 localize predominantly to mitochondria
Intracellular location of PTCD1, 2, 3 and 4 by immunofluorescence microscopy. Left to right: phase contrast, followed by the localization of a mitochondria-specific dye (mitotracker-Red), then detection of the PTCDs (green) and finally an overlay of the PTCDs localization over mitotracker.

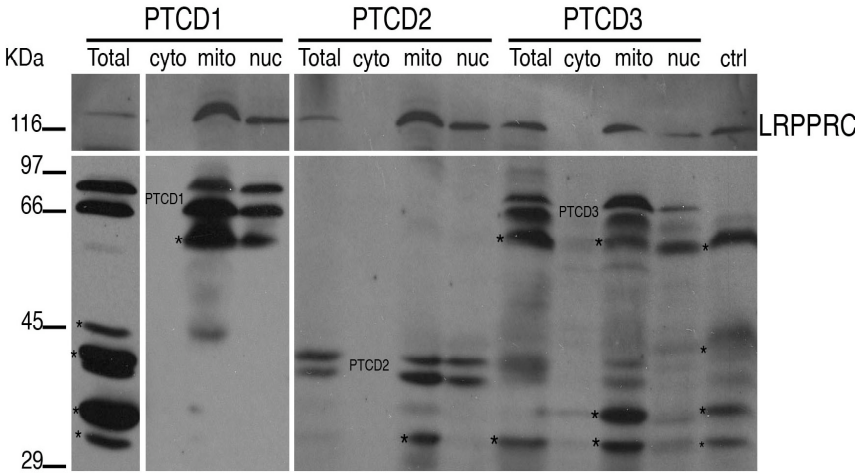

Figure 17. Subcellular localization of PTCDs. Cytosolic, triton faction and nuclear fractions were separated in cells expressing myc-tagged PTCD1, 2 or 3. Fractions were analyzed by immunoblotting using anti-myc antibody. As a control for localization of the PTCDs, blots were also probed using the 9C9 monoclonal antibody to LRPPRC.

"*" shows non-specific bands that are recognized by the anti-myc antibody in both the transfected and untransfected samples. "ctrl" lane was loaded with untransfected whole cell lysate.

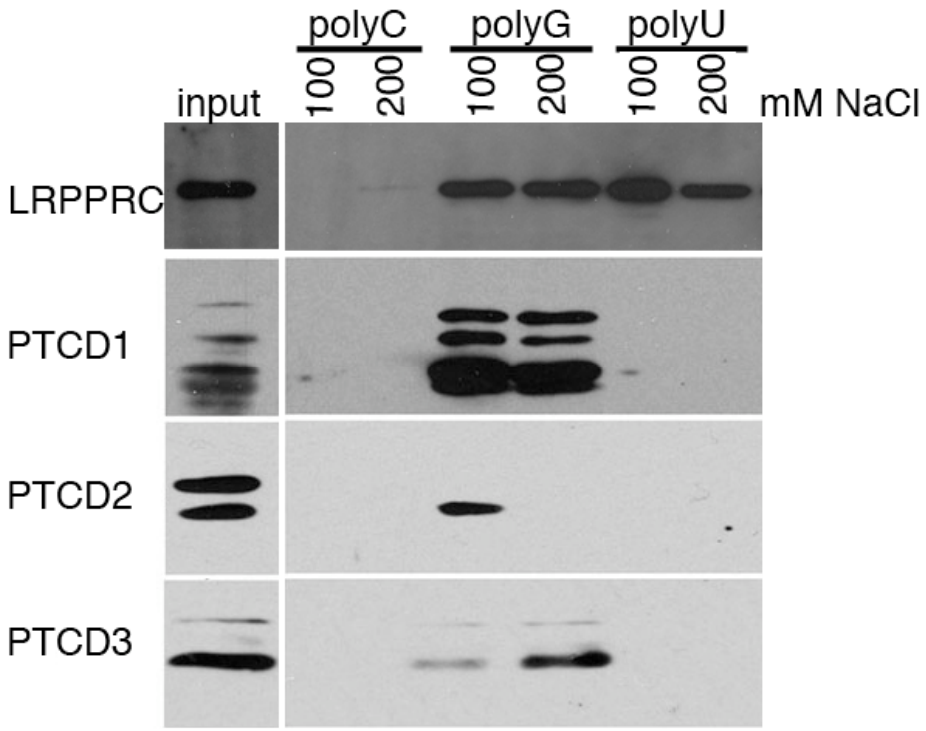

Figure 18: PTCD1, 2 and 3 bind RNA in vitro. Whole cell lysate from HeLa cells expressing myc-tagged PTCD or 2 or 3 was incubated with either polyU, C, or G. Proteins associated with bound RNAs were analyzed by immunoblotting using an anti-myc antibody. (Input refers to the whole cell lysate- NaCl concentrations indicate the concentrations at which the protein elutes from the column).

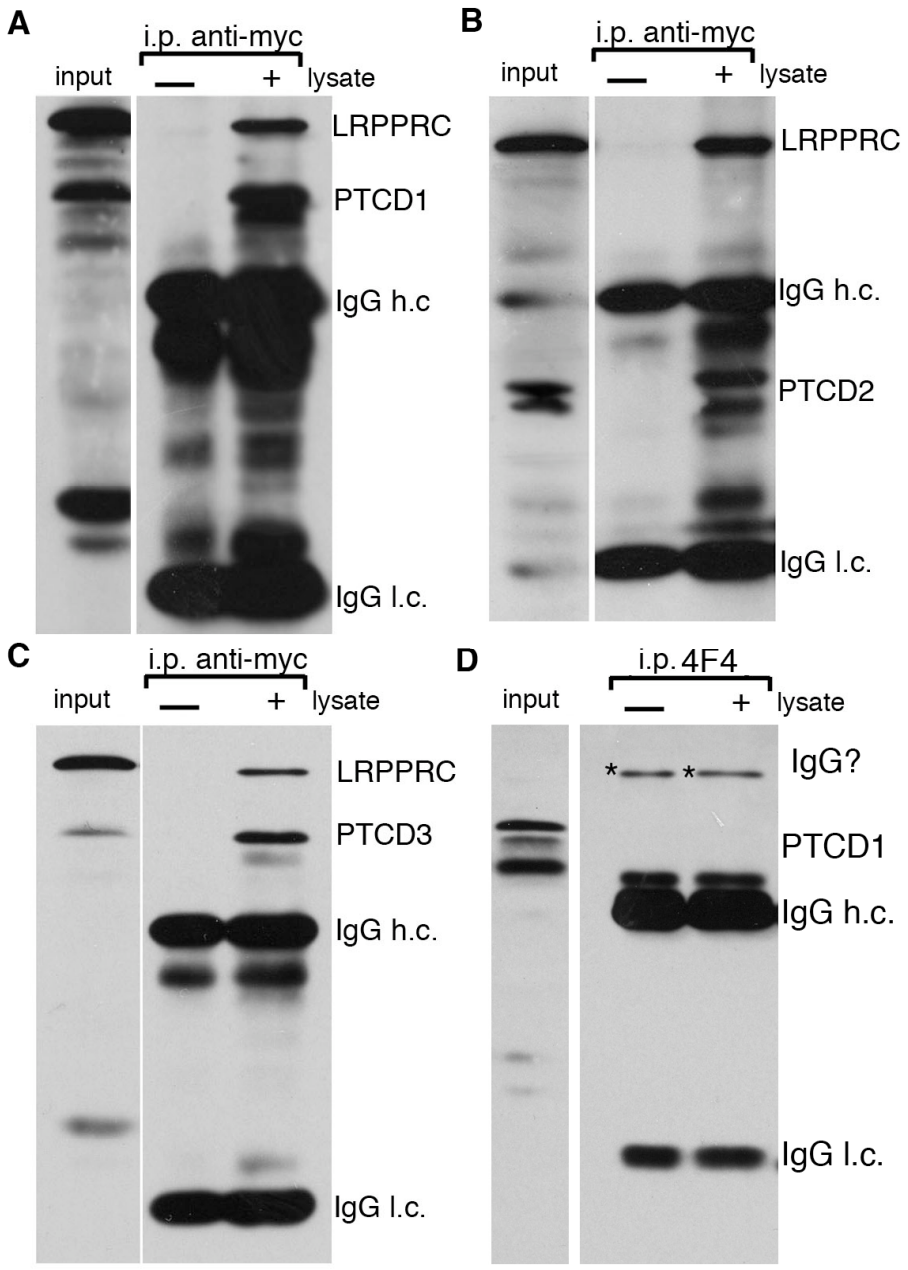

Figure 19: **PTCD1, 2 and 3 exist in complex with LRPPRC.** Whole cell lysate from HeLa cells expressing myc-tagged PTCD proteins was used for immunopurification with the anti-myc antibody- panels A, B, and C or with the anti-hnRNP C proteins antibody (4F4)- panel D (with (lanes "+") or without (lanes "-") cell lysate). Proteins within the PTCD proteins-associated complexes were analyzed by immunoblotting using both the anti-LRPPRC (9C9) and the anti-myc antibodies.
IgG hc an lc refer to the antibody heavy and light chains respectively.

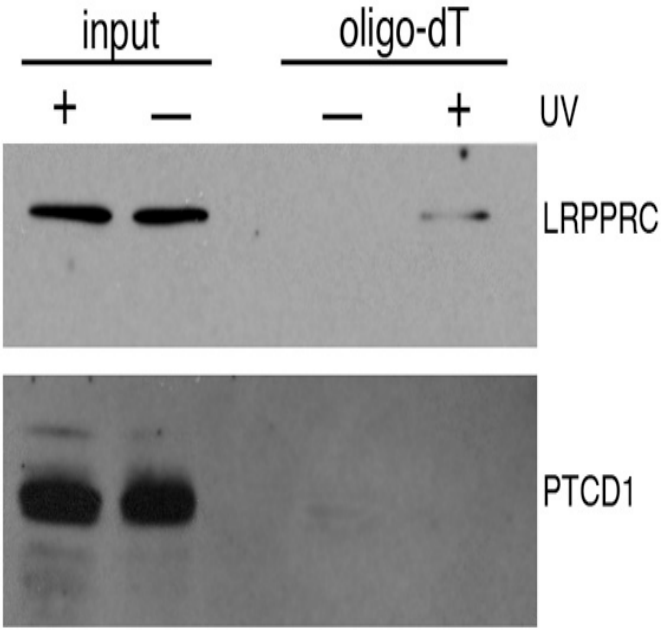

Figure 20: PTCD1 does not crosslink to polyadenylated mRNAs. UV-induced crosslinking of Hela cells expressing my-tagged PTCD1. mRNAs were selected by oligo-dT. Proteins associated with mRNAs were analyzed by immunoblotting using 9C9 and anti-myc antibodies. (+ UV are cross linked samples. Controls are not cross-linked -UV)

Chapter 5

CONCLUDING REMARKS

Mitochondria as the site of aerobic production of ATP are vital for cellular homeostasis. The machinery for the production of this ATP (OXPHOS) requires the coordinated transcription and translation of gene products from both nuclear and mitochondrial genomes. Indeed, expression of the mitochondrial genome requires the participation of several nuclear-encoded factors in order to produce the 13 polypeptides that are integral components of the enzyme complexes of the oxidative phosphorylation.

At the beginning of this work, we hypothesized that LRPPRC was a trans-acting factor with a possible function at the post-transcriptional stage of mitochondrial metabolism. This hypothesis was based on the mitochondrial location of LRPPRC, its overall RNA-binding as well as LSFC findings. In this work, we have isolated mitochondrial RNP complexes associated with LRPPRC, and established that LRPPRC binds all of the mitochondrial mRNAs. In delineating the functional significance of this binding, we discovered that loss of LRPPRC translates into a loss of all mitochondrial mRNAs. These findings thus establish LRPPRC as an essential trans-acting factor in mitochondrial gene expression. An unexpected finding is that, by contrast to the LSFC data in which only two mitochondrially-encoded mRNAs are affected, LRPPRC's role in mitochondrial RNA metabolism is more general as it impacts all mitochondrial mRNAs and not just COXI and COXIII mRNAs. This can in part be explained by the fact that in

the case of the mutation, LRPPRC is present but binds poorly to the RNA, whereas with RNAi-induced knockdown, there is loss of LRPPRC.

PPR motif proteins closely similar in sequence to LRPPRC are known to function in precursor processing (Crp1, Pet309), RNA stabilization (Pet 309), and translation of the RNAs to which they bind (Pet 309 and Cya5) (Coffin et al., 1997; Manthey and McEwen, 1995; Tavares-Carreon et al., 2008). Our findings favor a role in stability of mRNAs and/or in processing of the precursor RNA or in translation. In the case of processing, loss of LRPPRC would be expected to lead to a lack of processing of the primary transcript resulting in decreased levels of mRNAs observed with RNAi. On the other hand, loss of LRPRC could lead to degradation of mtmRNAs either due to the absence of a crucial stabilizing factor or due to impaired translation.

Further functional studies revealed that LRPPRC is bound to the unprocessed/partially- processed transcript. Together with existing evidence, the observation that loss of LRPPRC leads not only to loss of mature mitochondrial RNAs but also possibly to their processing intermediates with a possible accumulation of the precursor strongly supports a recruitment of LRPPRC on the primary transcript possibly co-transcriptionally. These observations also favor a role for LRPPRC in the processing of the primary transcript without discounting additional roles in stability and translation. Co-transcriptional recruitment raises the possibility of an interaction between LRPPRC and the mitochondrial RNA polymerase, another member of the human PPR motif family of proteins.

We expanded our work to look at other PPR motif proteins in humans. There are 7 PPR motif proteins in the human family of PPR motif proteins of which we focused on

the previously uncharacterized PTCD proteins. We demonstrate they all, like LRPPRC, localize to mitochondria. Moreover, they bind RNA *in vitro,* and exist in complexes with LRPPRC. This suggests an involvement of the PTCD proteins in mitochondrial metabolism and points to the PPR motif proteins as a new family of trans-acting factors in mitochondrial gene expression.

As described in chapter 4, recently emerging literature implicates PTCD1 in tRNA regulation through a binding to the unprocessed precursor (Rackham et al., 2009). A function for PTD2 in the processing of the ND5/cytb transcript has also been proposed (Xu et al., 2008). These functions for PTCD1 and PTCD2, together with their presence in LRPPRC-associated complexes, and our findings that LRPPRC is recruited onto the unprocessed transcript, point to the possibility that LRPPRC may be involved in processing. RNA-binding proteins have long been shown to be involved directly and indirectly in processing of RNAs. Besides those examples in the PPR motif family already mentioned in chapter 3 (CRP1, HCF152, and CCR2), nuclear RNA-binding proteins such as SR (serine/arginine) and hnRNP proteins, are involved in orchestrating the large number of splicing and alternative splicing decisions of nuclear pre-mRNAs. This function that is linked to where on the pre-mRNA these proteins bind, results in enhancement or silencing of splicing at a given splice site. These proteins are sometimes referred to as RNA chaperones that present the pre-mRNA as a suitable substrate for precise splicing as well as other processing reactions rather that having intrinsic enzymatic activities themselves (Fisette et al., 2010). Similar to nuclear pre-mRNA splicing, processing of the mitochondrial primary transcript requires precision in processing that conceptually should necessitate the coordination of RNA-binding proteins

to ensure proper folding of the RNA, to recruit the endonucleolytic enzymes involved in processing, and to regulate accumulation of mRNAs. Thus far, we have not identified a specific enzymatic activity associated with LRPPRC. Indeed, all the enzymatic activities associated with mitochondrial gene expression have been accounted for. By analogy to the function of hnRNP proteins, we propose a model in which LRPPRC is a chaperone that is recruited onto the precursor, possibly on the nascent transcript, and that remains bound to the mature mtmRNAs. This model favors a function of LRPPRC in processing, stabilization and possibly in translation of mtmRNAs. Moreover, some of the PTCD proteins exist in complex with LRPPRC. It is possible that these proteins may act in concert with LRPPRC in specific RNA remodeling events or may exert specific functions for specific genes (see figure 21 for this proposed model).

Proposed model for the participation of human PPR motif proteins in mitochondrial gene expression

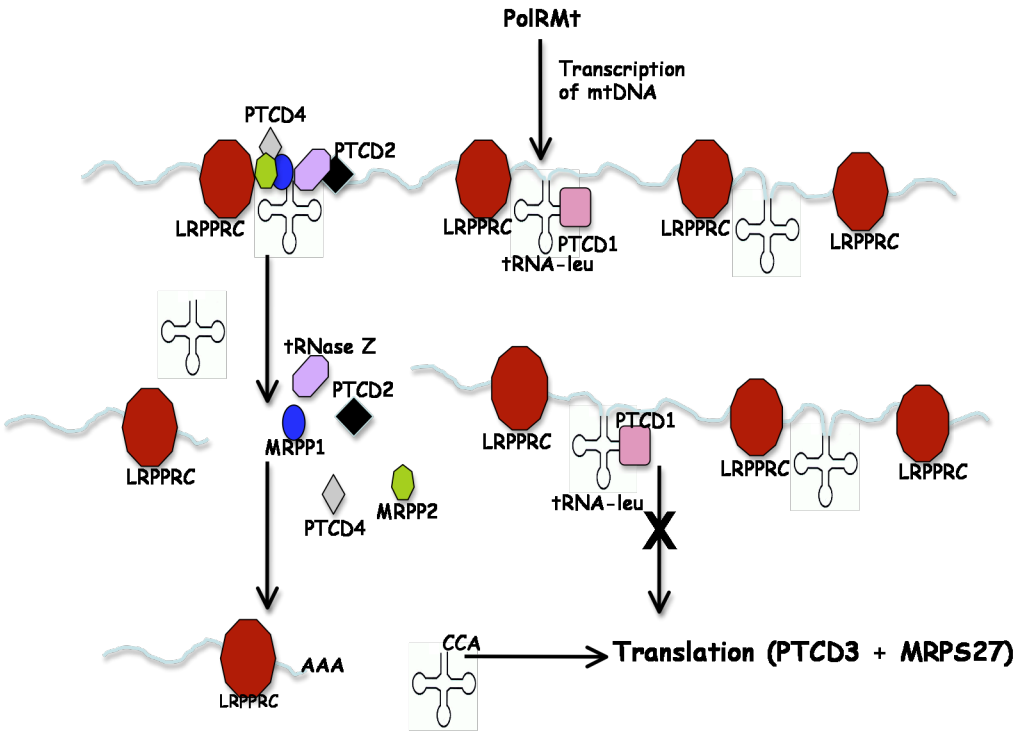

Figure21: Model- Human PPR motif proteins are proposed to participate in all stages of mitochondrial gene expression.

Legend:
MRPP1- Mitochondrial RNase P protein1 (blue)
MRPP2- Mitochondrial RNase P protein2 (green)
PTCD4= MRPP3 (grey), LRPPRC (red), PTCD1 (pink), PTCD2 (black), tRNase Z (purple)

Materials and Methods

Cell culture, transfection and immunofluorescence microscopy

HeLa cells were grown in monolayer to sub-confluent densities at 37^0C/5% CO_2 in DMEM (Dulbecco's Modified Eagle' Medium) supplemented with 10% FBS (fetal bovine serum) and 1% PS (penicillin and streptomycin. Transfections were performed using Lipofectamine-2000 (Invitrogen) or Lipofectamine-RNAiMAX (Invitrogen) according to the manufacturer's instructions. Briefly, cells were transfected in suspension at 60-70% confluency either with siRNA (130-150nM in culture) or plasmid (25-30 mg in a 15ml volume). SiRNA-transfected cells were harvested at 24 hours post-transfection or were re-transfected in order to achieve a longer knockdown. Immunofluorescence microscopy was carried out on transfected or untransfected cells as previously described (Mili and Pinol-Roma 2003).

Gel electrophoresis and Immunoblot analysis

SDS-PAGE and immunoblot analyses were carried out as described (Pinol-Roma et al., 1988) using the following antibodies: 9C9 (anti-LRPPRC), 4B10 (anti-hnRNP A1), 9E10 (ant-Myc tag), 4F4 (anti-hnRNP C1/C2), 7G2 (anti-nucleolin).

Subcellular fractionation and immunopurification of mitochondrial RNP complexes

Subcellular fractionation was essentially carried out as described (Mili and Pinol-Roma, 2003).

Immunopurification of mitochondrial RNP complexes was carried out using a triton extract. HeLa cells grown to a monolayer were washed three times with cold PBS and collected with a cell scraper in 0.75ml per 10cm dish of cold RSB-100 (Reticulocyte standard buffer) containing 0.5% Triton-X100. The cells were passed three times through a 251/2-gauge syringe, centrifuged first at 3,500g/5minutes then at 4,500g/8minutes at 4^0C. The supernatant was used for immunopurification of complexes as previously described (Choi and Dreyfuss, 1984; Pinol-Roma et al., 1988).

RNA homopolymer binding assays

HeLa cells transfected with a plasmid expressing our protein of interest were lysed 48 hours post-transfection as described above using RSB-100 containing 0.5% Triton-X100. The lysate was used for binding reactions to RNA homopolymers as described [4]. Bound proteins were eluted by boiling in SDS sample buffer and were resolved by SDS-PAGE.

RNA analysis

Total RNA was extracted from HeLa cells using Trizol reagent (Invitrogen) in accordance with the manufacturer's specifications. RNA was recovered from immunopurified complexes by heating in TE containing 1% SDS followed by extraction using Phenol-chloroform-Isoamyl alcohol (Invitrogen) according to the manufacturer's

instructions. In all cases, RNA was precipitated with Isopropanol and the pellet washed with 80% ethanol according to standard protocol.

5μg of total RNA per lane or RNA from immunopurified complexes were resolved on formaldehyde agarose gel and used in northern hybridization with biotin-labeled probes followed by chemiluminescence detection as per manufacturer's protocol (KPL).

Synthesis of probes for northern detection

Total RNA was used to synthesise a complete cDNA library using oligo-dT primers and AMV (Promega) according to manufacturer's instructions. Probes for nuclear-encoded RNAs were amplified by PCR using specific primers (see appendix). To synthesize mitochondria-specific probes, mitochondria DNA was used as a template for PCR (see appendix for complete list of primers). Mitochondria were enriched as described previously describe (Procaccio et al., 1999) and mitochondrial DNA was extracted following standard protocols of small scale isolation of plasmid DNA by alkali lysis (Sambrook, 1989). PCR products were cloned in TA vectors (Qiagen) for propagation and amplification. Probes were simultaneously amplified (using TA plasmids as templates) and biotin-labeled by incorporation of biotin-N4-dCTP according to manufacturer's protocol (KPL).

Cloning of PTCD 1, 2, 3, and 4

All the PTCD sequences were cloned in such a way as to tag them with a myc at the carboxy terminus.

PTCD1 and PTCD4 full-length cDNAs were cloned into pcDNA 3.1(+) A vectors (Invitrogen).

The vector for subcloning both PTCD2 and PTCD3 cDNAs was prepared from the pCMV-130-GFP plasmid by digesting it first with NotI at the 3'end, followed by T4 DNA polymerase blunting. The linearized plasmid was then digested with XhoI at the 5' end. The ca. 3,792 bp band corresponding to the vector was purified from agarose gel (Qiagen). For vector details, refer to pCMV-HA (Clontech) and Mili S. notes (cloning of pCMV-130-GFP).

Myc-tagged PTCD2 cDNA was excised out of pCMV-Entry-PTCD2 (Origene) using SmaI (c-terminus) and SalI (N-terminus) then re-cloned into the above vector to generate pCMV-intron-PTCD2 plasmid.

Myc-tagged PTCD3 cDNA was obtained from pCMV-Entry-PTCD3 (Origene) using PmeI and SalI, and then sub-cloned into the above-prepared vector to generate pCMV-intron-PTCD3 plasmid.

Appendix I

Table III: Primers for amplification of probes specific for mitochondrial and nuclear RNAs

Mitochondrial genes	Product size (bp)	Primers	Annealing Temprature (0C)
ATPase 6	540	5' TATTGATCCCCACCTCCAAA	58
		3' TTAAGGCGACAGCGATTTCT	
ATPase 8	146	5' ATGGCCCACCATAATTACCC	58
		3' TCGTTCATTTTGGTTCTCAGG	
COXI	863	5' CTTAGGGGCCATCAATTTCA	58
		3' CATCGGGGTAGTCCGAGTAA	
COXII	462	5' TTCATGATCACGCCCTCATA	58
		3' CGGGAATTGCATCTGTTTTT	
COXIII	501	5' TCCACTCCATAACGCTCCTC	58
		3' GGAAGCCTGTGGCTACAAAA	
CytB	762	5'TGAAACTTCGGCTCACTCCT	58
		3' GACGGATCGGAGAATTGTT	
ND1	666	5' CCCTGGTCAACCTCAACCTA	58
		3'GGGGAATGCTGGAGATTGTA	
ND2	760	5' CCGGACAATGAACCATAACC	58
		3' GTGGTAAGGGCGATGAGTGT	
ND3	238	5' ACCACAACTCAACGGCTACA	58
		3' GGCCAGACTTAGGGCTAGGA	
ND4	909	5' TGAACGCAGGCACATACTTC	58
		3' GGAGAATGGGGGATAGGTGT	
ND4L	194	5' TCGCTCACACCTCATATCCTC	58
		3' GGCCATATGTGTTGGAGATTG	
ND5	744	5' AGCCCTACTCCACTCAAGCA	58
		3' GCGAGGGCTGTGAGTTTTAG	
ND6	328	5' TGATTGTTAGCGGTGTGGTC	58

		3’ CCAATAGGATCCTCCCGAAT	
12S rRNA	731	5’ CCACGATCAAAAGGGACAAG	55
		3’ GGCCCTGTTCAACTAAGCAC	
16S rRNA	1018	5’ GCTAAACCTAGCCCCAAACC	55
		3’ GGCAGGTCAATTTCACTGGT	
Nuclear genes	**Product size (bp)**	**Primers**	**Annealing Temprature (0C)**
ATPAF2	1096	5’ CATCACACAGGGTGAAGGTG	55
		3’ AACACGCCATGGGAGATAAG	
ATP5S	1567	5’ GTCGAGCGTCCACTAGAAGG	55
		3’ GTTCCAGAGAAGGCAGTGCT	
NDUFS4	451	5’ TACTGAGGCAGACGTTGTGG	55
		3’ GACTTGGACTTGGGTTTTGG	
NDUFS5	414	5’ TAGCCATGCCTTTCTTGGAC	55
		3’ TTTGACAAGGAGGTTTGTCG	
COX VI	262	5’ GTCAGGAAGGACGTTGGTGT	55
		3’ ACCAGCCTTCCTCATCTCCT	
COX VII	687	5’ CCTTCTGCGTTTCCTCACTC	55
		3’ AGGCCAGCGTTTATTGACAC	
UQCRB	727	5’ AGGCAACGCTTCTCTTTCTG	55
		3’ GCAAACTAGGGGGTGCATAC	
Rieske Fe-S	933	5’ AGCCTGTGTTGGACCTGAAG	55
		3’ TTTCGCGTGTATGACTGAGC	
Hsp60	1295	5’ CACAGTCTTTCGCCAGATGA	55
		3’ CAATGCCTTCTTCAACAGCA	
ß-Actin	1246	5’ AAATCTGGCACCACACCTTC	55
		3’ ATGCTATCACCTCCCCTGTG	

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Appendix II: Publication Manuscript

LRPPRC, a human PPR motif protein, is essential for accumulation of mitochondrially-encoded mRNAs

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Running title: LRPPRC function in mitochondrial mRNA accumulation

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LRPPRC (LRP130) is an RNA-binding protein that belongs to the pentatricopeptide repeat (PPR) motif family of proteins, which have been implicated in metabolism of mitochondrial and chloroplast RNAs. In humans, LRPPRC is bound *in vivo* to both mitochondrial and nuclear

polyadenylated RNAs. Mutations in LRPPRC cause cytochrome c oxidase deficiency associated with the French Canadian variant of Leigh Syndrome (LSFC), and are accompanied by a decrease in the mitochondrially-encoded COXI and COXIII mRNAs. In order to delineate in detail the function of LRPPRC in human cells and understand better its role in mitochondrial disease, we isolated LRPPRC-associated ribonucleoprotein (RNP) complexes, identified their RNA constituents, and determined the functional implications of LRPPRC for these RNAs. LRPPRC-associated RNPs contain several additional proteins that are distinct from those found in nuclear mRNPs. Identification of the RNAs in the isolated RNPs showed that most (if not all) mitochondrially-encoded mRNAs are bound directly or indirectly by LRPPRC. The functional relevance of this association is underscored by the finding that a reduction in LRPPRC levels using RNAi resulted in a reduction in steady-state levels of mitochondrially-encoded mRNAs, but not of nuclear-encoded mRNAs. Mutations in LRPPRC associated with cytochrome c oxidase deficiency in LSFC caused a notable reduction in the ability to crosslink LRPPRC to RNA *in vivo*. Altogether, these results point to an essential role of LRPPRC in mitochondrial gene expression, through a function in the accumulation of mitochondrially-encoded mRNAs mediated by the binding of LRPPRC to these RNAs.

INTRODUCTION

LRPPRC (LRP130) is an RNA-binding protein that associates with both nuclear and mitochondrial RNAs in humans (1). It belongs to the growing family of pentatricopeptide repeat (PPR) motif proteins that bear as a signature one or several copies of a conserved 35 amino acid sequence initially reported by Small and Peeters (2). This motif is found in over 500 predicted gene products described to date, and is particularly prominent in plants (3,4). While the specific function of the PPR motif is not known, a common feature of the PPR motif proteins for which functional information is available is that most have been implicated in one or several aspects of gene expression in mitochondria and in chloroplasts, through a function in RNA metabolism (5,6).

In mammalian cells, mitochondria are the organelles where most of the ATP necessary for cellular function is produced through the coupled processes of electron transport and oxidative phosphorylation (OXPHOS). This involves five multi-protein complexes (I through V), a unique aspect of which is that, with the exception of complex II, their constituents are encoded by both nuclear and mitochondrial genomes (nDNA and mtDNA, respectively; for a recent review, see 7). Thus, of the 80 or so proteins required for OXPHOS in humans, 13 are encoded by the mtDNA, while the remaining ones are encoded by the nuclear genome (7-9).

The mitochondrial genome consists of a circular, dsDNA molecule that in humans is 16,569 bp long. The genes for 2 rRNAs and 12 of the mitochondrially-encoded mRNAs are contained in the heavy strand of the circular DNA, where for the most part they are interspersed by the genes for 14 of the 22 tRNAs. The genes that encode the remaining mRNA and 8 tRNAs are located on the light strand (10,11). Both strands of the mtDNA are transcribed by a mitochondrial RNA polymerase (POLRMT), yielding polycistronic transcripts that are subsequently processed primarily through excision of the tRNAs, a process that releases in turn the adjoining rRNAs and mRNAs (9,12,13).

Excision of mitochondrial tRNAs from the primary transcripts involves endonucleolytic cleavage at both their 5' and 3' boundaries by RNase P and tRNase Z respectively. The tRNAs are subsequently modified by addition of a CCA [by an ATP (CTP): tRNA nucleotidyl transferase] at their 3' end (for reviews, see 14-16). The resulting mRNAs and, to a lesser extent, also the rRNAs, are further processed by addition of short oligoadenylylate tails (by a mitochondrial poly (A) polymerase) that serve to both modulate stability and, in the case of some of the mRNAs, to generate a termination codon (UAA) (10, 17-19). All of the known *trans*-acting factors required for processing of mitochondrial RNAs are encoded by nuclear DNA. However, the full repertoire of factors required for mitochondrial mRNA formation and maintenance is not known. Indeed, recent findings have implicated LRPPRC among additional

potential *trans*-acing factors in mitochondrial gene expression at the post-transcriptional level in humans.

Specific evidence that LRPPRC has a role in mitochondrial function and, more specifically, in mitochondrial gene expression, was provided by the discovery that a point mutation in LRPPRC underlies the French Canadian variant of Leigh Syndrome (LSFC), a cytochrome c oxidase deficiency (20). This defect was subsequently linked to a decrease in steady-state levels of mRNAs for the mtDNA-encoded COXI and COXIII subunits of the cytochrome oxidase complex in fibroblasts from patients with LSFC, although the specific mechanism by which this reduction occurs is not known (21). LRPPRC binds RNA *in vitro* and *in vivo*, as it can be cross linked directly to mitochondrial polyadenylated RNA in living cells, supporting a direct involvement in mitochondrial gene expression at the post-transcriptional level. The intrinsic RNA-binding activity of LRPPRC resides in a C-terminal portion that excludes most of the PPR motifs in LRPPRC (1). The specific identity of the RNAs bound by LRPPRC, however, is not known.

Besides its binding to mitochondrial RNA, LRPPRC also binds to nuclear-encoded RNA and appears to have functions in the nucleus, where it associates with nuclear RNPs that contain spliced mRNA (22). This suggests a possible role of LRPPRC in mRNA export, which is supported by the finding that overexpression of LRPPRC causes nuclear accumulation of mRNA (23).

LRPPRC also binds directly or indirectly to nuclear DNA (24), and participates in transcription of nuclear genes involved in blood glucose homeostasis in a complex with the transcription factor PGC1-α (25), as well as of genes involved in multidrug resistance (26). Additional possible functions of LRPPRC in linking mitochondria, the microtubular cytoskeleton, chromosome remodeling, and tumor suppressors have also been proposed (27). Whether and how these varied proposed roles of LRPPRC in nuclear and mitochondrial gene expression relate and are coordinated with one another is not known.

In this study, we set out to define specifically the place of LRPPRC in mitochondrial gene expression by determining RNA targets of LRPPRC in mitochondria, addressing the function of LRPPRC on these RNAs, and relating these questions to the defects in mitochondrial gene expression and function observed in LSFC. We find that LRPPRC associates with all the mitochondrial mRNAs that we examined, and that it has a general, essential function in the maintenance of steady-state levels of these mRNAs. The results support a model in which LRPPRC is a new *trans*-acting factor in mitochondrial mRNA metabolism, with a function in processing of the primary transcript and/or in stabilization of mitochondrially-encoded mRNAs.

EXPERIMENTAL PROCEDURES

Cell culture, labeling, and transfections - HeLa cells were grown in monolayer culture in DMEM (Dulbecco's modified Eagle medium) supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. Hybridoma cells were grown in serum-free medium (HyClone). Transfections of DNA constructs and siRNAs were carried out using Lipofectamine 2000 (Invitrogen), as per manufacturer's instructions. In the case of dsRNA, we used a final concentration of 130nM dsRNA, and the same plates of cells were transfected again 24 hrs after the initial transfection. Labeling of cells with ^{35}S-methionine was done for 16 – 20 hrs as previously described (22).

UV light-induced crosslinking of proteins to RNA in living cells - Crosslinking of proteins to RNA *in vivo* by UV light irradiation of cells was performed as previously described (22, 28). Briefly, HeLa cells grown in monolayer culture were exposed to UV light for 3 min to induce covalent RNA-protein crosslinks. Cells were then lysed under protein-denaturing conditions, and polyadenylated RNA (with crosslinked proteins) was selected by oligo (dT) chromatography. The crosslinked complexes were eluted from the columns and digested for 1 hr at 30°C with a mixture of RNase A at 25 μg/ml and micrococcal nuclease at 400 U/ml, for subsequent analysis of the released proteins by SDS-PAGE and immunoblotting with the indicated antibodies (22, 28).

Immunopurification of RNP complexes – HeLa cell fractions that are enriched in soluble nuclear RNPs as well as mitochondria ('Triton' fraction) were prepared

as previously described (22). For preparation of whole cell lysates, HeLa cells were lysed in RSB-100 [10 mM Tris-Cl pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$] containing 0.5% Triton X-100 and were sonicated twice for 5 sec on ice, using a microtip sonicator (model XL2015; Heat Systems, Farmingdale, NY) at setting 2.5. The sonicated material was layered onto a 30% sucrose cushion in RSB-100 and centrifuged at 4,000 g for 15 min. The supernatant was collected and clarified further by centrifugation for 5 min at 10,000 rpm in a microcentrifuge. RNP complexes were isolated from the supernatant fraction by immunopurification with the indicated antibodies, using protein A-Sepharose CL-4B (Pharmacia; 22). Briefly, the lysates were incubated with the antibody-coated beads for 20 minutes at 4°C. Following 5 washes in the lysis buffer containing 0.5% Triton X-100, the complexes were eluted from the beads using SDS-PAGE or NEPHGE sample buffer (for protein analysis), or by heating at 65°C for 3 min. in TE buffer containing 0.1% SDS, followed by phenol and chloroform extraction (for RNA analysis; 29).

Gel electrophoresis and immunoblot analysis - SDS-PAGE, two-dimensional gel electrophoresis, and immunoblotting were carried out as previously described (22,28). The following antibodies were used for immunoblot analysis: 4C12 and 9C9 (LRP130; 1), 4B10 (hnRNP A1; 30), 4F4 (hnRNP C1/C2; 31), and anti-hsp60 (StressGen). For preparation of whole cell lysates, HeLa cells grown in monolayer culture were rinsed with PBS and lysed using SDS-PAGE lysis buffer. Prior to loading the gel, the samples were sonicated, heated to 65°C for 5 minutes, and centrifuged to remove insoluble materials.

Preparation of probes and Northern blot analysis - Mitochondria were enriched essentially as previously described (32), and the mitochondrial DNA was isolated following standard protocols for small-scale isolation of plasmid DNA by alkali lysis (33). The DNA was used as template for amplification of specific regions to be used in preparation of probes by PCR. The primers used for each mRNA are listed under "Supplemental Materials". The resulting PCR products were subcloned into TA vectors (Qiagen) for propagation and amplification. Probes for each of the mitochondrial mRNAs were prepared from the corresponding plasmid by labeling of amplicons with biotin-N_4-dCTP using PCR, following the manufacturer's instructions (KPL). Probes for nuclear-encoded mRNAs were prepared essentially in the same way, except that the initial template was generated by reverse transcriptase – PCR using whole cell mRNA as template (see 'Supplemental materials' for sequence of primers used). All PCR products were sequenced to confirm their identity. Northern blots were performed according to standard protocols (33), using 5µg total RNA per lane, followed by chemiluminescence detection as per manufacturer's instructions (KPL). Total RNA was prepared using TriZol reagent (Invitrogen).

LRPPRC-GFP fusion protein constructs and fluorescence microscopy – As noted before, the LRPPRC cDNA that we used in previous studies lacked a region encoding an additional N-terminal amino acids of LRPPRC, as determined from extensive EST database searches as well as from the corresponding genomic sequence (1). The missing LRPPRC cDNA fragment was obtained by PCR amplification from an LRPPRC EST clone (Acc # BC026034; obtained from the

ATCC) that comprised the initial 1500 nucleotides of the LRPPRC cDNA. An XhoI site was introduced in the process at position 25 before the initial ATG and the fragment was ligated with the LRPPRC cDNA in the pcDNA3.1(-)/Myc-His vector that we previously described (1) to yield the full-length LRPPRC cDNA with an in-frame myc/His tag. The construct encoding the A354V mutation was generated using the Quickchange site-directed mutagenesis kit (Stratagene). To generate the GFP fusion proteins, the NheI-KpnI fragment from the pcDNA3.1 vector containing the corresponding LRPPRC cDNA was ligated into the pEGFP-N3 vector (Clontech) digested with NheI-SmaI, to yield the full-length LRPPRC sequence in frame with the EGFP reading frame. The sequence of all constructs was confirmed by DNA sequencing. Fluorescence microscopy was performed essentially as previously described (1).

Antibodies and siRNAs - Antibodies used in this work have been previously described as follows: 4F4, anti-hnRNP C (31); 9C9, anti-LRPPRC (1); 4B10, anti-hnRNP A1 (30). siRNAs used for RNA interference experiments were purchased from Ambion. The dsRNA used for the RNAi experiments to silence LRPPRC expression corresponded to the sequence 5'-AACCTATAAGAGATGTCCTAA-3' of the LRPPRC mRNA (positions 2085 – 2105 of the mRNA, or 2027 – 2047 of the ORF). The effectiveness of the siRNAs for knocking down LRPPRC was determined by western blotting with the anti-LRPPRC antibody 9C9, using whole cell lysates from RNAi-treated cells. Control siRNA, with no known specificity towards human mRNAs, was obtained from Ambion.

RESULTS

Isolation of LRPPRC-associated RNP complexes by immunopurification – Previous studies showed that human LRPPRC associates with nuclear mRNP complexes that can be immunopurified with antibodies to the pre-mRNA and mRNA-binding protein hnRNP A1 (22). Immunolocalization and subcellular fractionation experiments indicated that most of the cellular LRPPRC, however, resides in mitochondria where it is associated at least in part with mitochondrial RNPs (1). We therefore reasoned that immunopurification of LRPPRC from human (HeLa) cell fractions enriched in mitochondria under similar conditions should yield primarily mitochondrial RNA-protein complexes that contain LRPPRC.

In order to isolate RNP complexes associated with LRPPRC, we used a monoclonal antibody, 9C9 (1), for immunopurification experiments under non-denaturing conditions that minimize disruption of protein-protein as well as protein-RNA interactions (100mM NaCl, 0.5% Triton X-100), as done in the past to isolate hnRNP, nmRNP, and pre-rRNP complexes (22, 29, 34; see below). The RNP isolations were performed from HeLa cell fractions that are enriched in mitochondria as well as in nuclear soluble RNPs (22; see Materials and Methods). Following isolation of the complexes, their protein composition was analyzed by SDS-PAGE followed by fluorography. As a control for specificity, we performed identical isolations using immunoglobulins secreted by the non-immune parent

myeloma cell line SP2/0. As shown in Figure 1, immunopurification with 9C9 results in the isolation of a complex set of proteins in addition to LRPPRC itself (Fig. 1, lane 1). Notably, the co-immunopurification of most of these proteins is all but abolished if the samples are pre-digested with RNase (Fig. 1, lane 2), indicating that RNA is also co-isolating with LRPPRC and that it mediates the association with most of the other proteins (the prominent protein band at ca. 48kDa in the '+RNase' lane co-migrates with a 9C9-immunoreactive LRPPRC fragment, and therefore is most likely a degradation product of LRPPRC bound directly by the antibody). Thus, the isolated complexes represent LRPPRC-associated RNPs. No detectable proteins were isolated when identical immunopurifications were performed with non-immune antibodies (Fig. 1, lanes SP2/0), demonstrating that the observed isolations with 9C9 were due to specific antibody-antigen interactions.

Since some of the cellular LRPPRC proteins are located in the nucleus and associate in nmRNP complexes with hnRNP A1, we sought to determine which of the proteins that co-purify with LRPPRC are also in hnRNPA1-associated nmRNPs, and which were unique to LRPPRC. We did so by performing identical immunopurifications with the 9C9 antibody and the anti-hnRNP A1 antibody 4B10, and comparing the protein composition of the corresponding complexes by two-dimensional gel electrophoresis. This analysis, shown in Figure 1B, revealed that the most prominent proteins that co-purify with 9C9 are unique to LRPPRC-associated complexes, as they are not detected to any significant extent in complexes with hnRNP A1 (compare panels 'LRPPRC' and 'hnRNPA1' in Fig.

1B). Longer exposure of the gels to film showed that proteins with mobilities similar to those associated with hnRNP A1 can indeed be detected in complexes with LRPPRC, albeit at much lower levels (data not shown). These results indicate that, while some hnRNP A1 and LRPPRC co-exist in the same complexes, the majority of the LRPPRC in the cell is in distinct complexes. Given that the majority of LRPPRC appears to be located in mitochondria, the most plausible explanation of these results is that the most prominent proteins that co-purify with LRPPRC are mitochondrial, and are likely constituents of mitochondrial LRPPRC-associated RNPs. This is supported by the RNA analyses described below. The fact that the complexes isolated with the different antibodies have different protein composition underscores in turn the specificity of the immunopurifications. Identification of the LRPPRC-associated proteins is currently in progress. We note that attempts at isolating LRPPRC-associated complexes from mitochondrial fractions that were devoid of nuclear contaminants yielded very low recovery of complexes, most likely due to their disruption during the relatively lengthy process required for preparation of such fractions.

Identification of LRPPRC-bound RNAs - Previous studies using UV-induced RNA-protein crosslinking demonstrated that LRPPRC associates with mitochondrial polyadenylated RNAs *in vivo* (1). Those experiments, however, did not reveal the specific identity of the RNAs bound by LRPPRC. To identify specific RNAs associated with LRPPRC, RNPs were immunopurified with the 9C9 monoclonal antibody and the co-isolated RNA was analyzed by Northern

blotting using probes specific for mitochondrially-encoded as well as nuclear-encoded mRNAs (see "Materials and Methods"). Again, the immunopurifications were performed for relatively brief periods of time and at 4°C in order to minimize disruption and/or rearrangement of the RNP complexes during the isolation. We included two sets of controls to ascertain the specificity of the observed LRPPRC-RNA interactions. First, we performed identical isolations using a monoclonal antibody, 4F4, directed against the nuclear RNA-binding proteins hnRNP C1/C2, which are not known to bind to mitochondrial RNA *in vivo*. Second, we performed the isolations from whole-cell lysates (rather than fractions enriched for mitochondria) to expose LRPPRC and its associated complexes to non-mitochondrial RNAs, so that we would be able to determine the extent to which there were rearrangements of the complexes during the isolation. The rationale was that, if LRPPRC were associating artifactually with any RNAs to which is was exposed during the isolation, non-mitochondrial RNAs (such as ß-actin mRNA) would also be found in the LRPPRC-complexes.

Representative results from these experiments are shown in Figure 2. Northern blot analysis of LRPPRC-associated RNAs using probes corresponding to the mitochondrially-encoded mRNAs for the ND1 and ND2 proteins (complex I), Cytb (complex III), COXI (complex III), and ATPase6 (complex V) showed that each of these RNAs is enriched in complexes associated with LRPPRC, as compared with those associated with hnRNP C (Fig. 2, compare lanes 'a-LRPPRC' with 'a-hnRNPC', as well as with the 'ß-actin' panel). The lower

signal for these RNAs in the anti-hnRNP C immunopurifications varies among experiments and most likely reflects the background of the isolation. Similar results were observed with other mitochondrially-encoded mRNAs (data not shown). Altogether, these findings show that RNAs associated with LRPPRC include mitochondrial mRNAs coding for components of each of the complexes (I, III, IV, and V) that contain mitochondrially-encoded proteins. By contrast, analysis of the same samples with a probe for the nuclear-encoded ß-actin mRNA showed it to be enriched in hnRNPC-associated complexes, whereas it was present at only background levels in LRPPRC-associated RNPs (see panel 'ß-actin). The association of ß-actin mRNA with hnRNP C is in agreement with our previous findings (22). The preferential enrichment of mitochondrially-encoded mRNAs in association with LRPPRC and the converse preferential association of nuclear-encoded mRNAs with hnRNP C, together with previous studies using UV light-induced crosslinking *in vivo*, indicate that the observed interactions are specific and that mitochondrial mRNAs for proteins in each of the complexes with mtDNA-encoded components are bound, directly or indirectly, by LRPPRC.

Mutations in LRPPRC that cause Leigh Syndrome, French Canadian type, reduce the binding of LRPPRC to poly (A)$^+$ RNA in vivo - The finding that mitochondrially-encoded mRNAs for proteins in each of the OXPHOS complexes are in RNP complexes with LRPPRC suggests that LRPPRC may have a general function in mitochondrial RNA metabolism through its binding to these RNAs. Fibroblasts from patients with LSFC, in which LRPPRC protein carries a single-

amino acid substitution in which an Alanine at position 354 is replaced with a Valine, have decreased overall levels of COXI and COXIII mRNAs in cells (21). In order to determine whether this change impacted the RNA-binding activity of LRPPRC *in vivo*, we expressed both wild-type and mutant forms of LRPPRC in HeLa cells by transfection of the corresponding cDNAs, and evaluated the binding of the proteins to RNA *in vivo* using UV-induced protein-RNA crosslinking. Both constructs encoded the corresponding LRPPRC protein with green fluorescent protein (GFP) fused to the carboxyl terminus (to avoid interfering with the N-terminal mitochondrial targeting sequence of LRPPRC). The GFP-tagged proteins could be distinguished readily from endogenous LRPPRC by their slower migration by SDS-PAGE (Fig. 3B).

Fluorescence microscopy of transfected cells showed a similar subcellular location of both sets of proteins (Fig. 3A) that overlapped with that of mitochondrial markers (data not shown). To determine whether both proteins were able to bind to RNA similarly, we induced crosslinking of proteins to RNA *in vivo* by exposure of living transfected cells to ultraviolet light, followed by selection of crosslinked RNA-protein complexes by oligo(dT) chromatography. The crosslinked proteins were released from the complexes by RNase digestion, and analyzed by immunoblotting with anti-LRPPRC antibody. The results, shown in Figure 3B (panel 'input'), show that comparable levels of wild-type and mutant fusion proteins are expressed in the transfected cells. Again, the fusion proteins (denoted 'LRPPRC-GFP in Fig. 3B) are easily distinguished from the endogenous

LRPPRC (which in turn serves as a loading control) because of their slower migration in the gel. As we had reported before, endogenous LRPPRC can be crosslinked readily to poly(A) RNA (Fig. 3B, panel "oligo-dT selected"). Similarly, the wild-type LRPPRC-GFP fusion is crosslinked to poly $(A)^+$ RNA to a comparative extent, as illustrated by a comparison of total relative levels with endogenous LRPPRC in both 'total' and 'crosslinked' panels (Fig. 3B). By contrast, the relative levels of crosslinked mutant LRPPRC recovered by oligo(dT) chromatography are significantly reduced as compared with the wild-type protein (Fig. 3, compare lanes 'wt' and 'A354V' in the "oligo-dT selected" panel). This lane was overloaded purposely to reveal the low levels of crosslinked mutant LRPPRC that, while drastically reduced as compared with the wild-type protein, can nonetheless be detected in overexposed gels. These results show that the mutation found in LSFC causes an overall reduction in the ability of LRPPRC to bind to poly$(A)^+$ RNA *in vivo*. Taken together with previous studies (21), these findings show that mutations in LRPPRC that cause lowered levels of mitochondrially-encoded COX mRNAs correlate with its impaired RNA-binding activity.

LRPPRC is essential for maintenance of steady-state levels of RNAs to which it binds - We showed in Figure 2 that LRPPRC binds to all mitochondrially-encoded mRNAs. On the other hand, the available information from LRPPRC with the A354V amino acid substitution supports a role of LRPPRC in the maintenance of steady-state levels of only a subset of these mitochondrially-

encoded mRNAs, namely COXI and COXIII (21). As described above, this point mutation impairs (but does not completely abolish) RNA-binding by LRPPRC, which could in principle explain why not all mitochondrially-encoded mRNAs were affected similarly. This could be due to different minimal requirements for LRPPRC RNA-binding activity among different RNAs, or to possible changes in mRNA binding specificity of LRPPRC due to this particular mutation. In order to determine more directly whether LRPPRC is essential for steady-state accumulation of mitochondrial mRNAs in general, we performed RNA interference-mediated "knock-down" of LRPPRC in HeLa cells so as to reduce overall levels of the protein. To do so, we transfected HeLa cells with double-stranded RNAs corresponding to nucleotides 2085 – 2105 in the LRPPRC mRNA. As shown in Figure 4A, immunoblotting with the 9C9 antibody shows that steady-state levels of LRPPRC decline slightly 24 hours after transfection, and are reduced to virtually undetectable levels by 40 hours. This effect is not general, as steady-state levels of the RNA-binding protein hnRNP A1 remain virtually unchanged at all times (Fig. 4A). Transfection with a non-specific dsRNA of the same length had no detectable effect on levels of LRPPRC (data not shown), indicating that the reduction of LRPPRC protein levels is not due to introduction of dsRNAs in general.

Northern blot analysis of mitochondrially-encoded mRNAs in the transfected cells showed a readily detectable reduction in each of these mRNAs that, in turn, paralleled the reduction in LRPPRC protein (Fig. 4B). Importantly, this reduction

affected all of the mitochondrially-encoded mRNAs that we tested, which included those shown here coding for proteins in each of the complexes with a mitochondrially-encoded constituent: ND1 and ND2 (complex I), Cytb (complex III), COX I (complex IV), and ATP 6 (complex V). This reduction is apparent to varying extents (depending on the mRNA) already 24 hours post-transfection, a time at which there remains some detectable LRPPRC (Fig. 4A), and is clearly evident for all of the mitochondrially-encoded mRNAs by 40 hours post transfection (Fig. 4B, lanes '40h'). By contrast to mitochondrially-encoded mRNAs, none of the nuclear-encoded mRNAs that we tested were visibly reduced upon reduction in LRPPRC levels (Fig. 4C). The RNAs tested included ß-actin and hsp60, as shown here, as well as nuclear-encoded mRNAs for constituents of OXPHOS complexes (data not shown). Thus, an overall reduction in steady-state levels of LRPPRC causes a specific reduction in steady-state levels of mitochondrially-encoded mRNAs.

DISCUSSION

This work aimed at dissecting the role and mechanism of action of LRPPRC in mitochondrial function. Using a monoclonal antibody, we have isolated human mitochondrial RNA-protein complexes associated with LRPPRC (Fig. 1). Analysis of the RNAs in the isolated RNP complexes revealed that LRPPRC binds, directly or indirectly, to mitochondrially-encoded mRNAs that code for proteins in each of the complexes (I, III, IV, and V) that have mtDNA-encoded

constituents (Fig. 2). Two lines of evidence provide a functional relevance of this association: first, the Ala354→Val mutation in LRPPRC that was previously shown to cause cytochrome oxidase deficiency (20) accompanied by reduced levels of COX I and COXIII mRNAs (21) results in a reduction in the binding of LRPPRC to mRNA *in vivo* (Fig. 3); second, a reduction in steady-state levels of LRPPRC causes a parallel reduction in steady-state levels of all the mitochondrially-encoded mRNAs that we analyzed, which in turn code for proteins in each of the OXPHOS complexes (I, III, IV, and V) that have mitochondrially-encoded constituents (Fig. 4). Altogether, these results point to LRPPRC as an essential *trans*-acting factor in the post-transcriptional portion of mitochondrial gene expression.

While LRPPRC associates with both nuclear and mitochondrial mRNP complexes (1,22), This work shows that most of the proteins that co-purify with LRPPRC are distinct from those that are found in nuclear mRNPs (or at least those associated with hnRNP A1), as evidenced by a comparison of both sets of complexes by two-dimensional gel electrophoresis (Fig. 1). The most plausible explanation for this observation is that the majority of LRPPRC in the cell is in complexes that are distinct from those that associate with nuclear-encoded mRNA. As discussed below, the identification of specific mitochondrially-encoded mRNAs in association with LRPPRC, together with our previous findings that LRPPRC binds mitochondrial mRNA *in vivo*, provide strong evidence that the complexes that we have isolated here represent by-and-large

bona fide mitochondrial mRNPs. This is also in agreement with our previous observations that most of the LRPPRC in the cell co-localizes and co-purifies with mitochondria (1). As is the case also with RNP complexes associated with nuclear-encoded hnRNAs and pre-rRNAs (29, 34), the association of the proteins in these complexes with one another is dependent at least in part on the presence of the RNAs, as the complexes dissociate upon digestion with RNase (Fig. 1). Thus, in the absence of RNA, protein-protein interactions (if any) are not sufficient to maintain the association of LRPPRC with the other proteins in the complexes. This suggests in turn that there are additional RNA-binding proteins in these complexes.

A central finding from this work centers on the identity of the RNAs that are bound by LRPPRC. UV-induced RNA-protein crosslinking *in vivo* had already revealed that LRPPRC binds to mitochondrial polyadenylated RNAs, but they did not provide the specific identity of those RNAs (1). Here, we have determined the identity of specific RNAs in the complexes. The results show that most (if not all) of the mitochondrially-encoded mRNAs are indeed bound with LRPPRC in RNP complexes (Figure 2), indicating that LRPPRC associates with (and therefore potentially acts on) mtDNA-encoded mRNAs in general. While these results do not allow us to determine unambiguously whether LRPPRC binds directly to each of these RNAs or whether it associates with them indirectly via a separate RNA-binding protein, the previous demonstration that LRPPRC has intrinsic RNA-binding activity and that binds RNA directly *in vivo* indicates

strongly that the binding observed here is direct. Furthermore, RNA binding reflected by the immunopurification results is specific, as evidenced by the absence of nuclear-encoded mRNAs in the complexes. As Figure 2 illustrates, the nuclear-encoded ß-actin was conspicuously absent from the LRPPRC-associated complexes. Similar results were observed also for the nuclear-encoded hsp60 mRNA (not shown). Conversely, immunopurification of hnRNP complexes with antibodies to hnRNP C showed readily detectable levels of associated ß-actin mRNA. By contrast, only background levels of mtDNA-encoded mRNAs co-purified with hnRNP C proteins. Thus, the observed association of mitochondrial mRNAs with LRPPRC is unlikely to result from gross rearrangements of the complexes and/or from spurious association with RNAs during the isolation, as had been observed for other RNA-binding proteins (35). Again, this specificity is in full agreement with our previous finding that LRPPRC binds mitochondrial polyadenylated RNA *in vivo* (1). We note that, while we had previously observed LRPPRC also in association with nuclear mRNP complexes (1,22), we observe only background levels of the nuclear-encoded mRNAs that we assayed here in association with LRPPRC. This could be due to a number of reasons, which include the possibility that the nuclear mRNP complexes associated with LRPPRC are less abundant (for example, due to what is probably a short transient association with the nmRNA and the likelihood that the complexes are thus short-lived) or less stable such that they dissociate during the isolation procedure. It is also possible that hsp60 and ß-actin mRNAs are not among the natural nuclear-encoded mRNAs that serve as substrates for LRPPRC.

Altogether the previously available information on LRPPRC and the findings presented here support our conclusion that the complexes isolated in association with LRPPRC represent primarily mitochondrial RNP complexes. This is supported by previous indications that most LRPPRC is mitochondrial (1), the finding here that most of the proteins that co-purify with LRPPRC are distinct from those found in nuclear mRNPs (22), and that the RNA associated with LRPPRC represents by-and-large mitochondrially-encoded RNA. To our knowledge, this is the first reported isolation of human RNP complexes stably associated with mitochondrially-encoded mRNAs, and it opens up the way for the identification of other proteins in these complexes that are, in turn, additional potential *trans*-acting factors in mitochondrial gene expression. It also offers some insights into the mechanism of action of LRPPRC and of its involvement in LSFC, as discussed below.

Functional implications of the association of LRPPRC with mitochondrial RNA were tested here by two different approaches. As stated above, previous reports had shown that LRPPRC binds mitochondrial RNA *in vivo* (1), and that a point mutation in LRPPRC causes a cytochrome c oxidase deficiency associated with Leigh Syndrome, French Canadian type (20). A possible mechanistic link between these two findings was indicated by the observation that fibroblasts derived from LSCF patients had reduced levels of the mitochondrially-encoded COXI and COXIII mRNAs (21). We have shown here, using ultraviolet light-

induced crosslinking or proteins to RNA *in vivo*, that LRPPRC protein that carries the mutation found in LSFC has impaired RNA-binding activity (Figure 3). This raises the likely possibility that it is the failure of LRPPRC to bind efficiently to RNA that results in the loss of the COXI and COXIII mRNAs. While Xu and co-workers (21) had indicated that the mutated protein is also impaired in its import into mitochondria, in our hands there is no discernible difference in subcellular location of the mutant protein as compared to its wild-type counterpart. However, this apparent discrepancy could be explained at least in part by the possibility that overexpression of LRPPRC used in our experiments masks such differences in localization, which were otherwise apparent when observing endogenous protein in human fibroblasts from patients (21).

The impact on mitochondrially-encoded mRNAs was more general when overall levels of endogenous LRPPRC (rather than its impairment by a point mutation seen in LSFC) were reduced using RNAi, as we showed in Figure 4. In this case, loss of LRPPRC was accompanied by a consistent reduction in steady-state levels of mitochondrially-encoded mRNAs for proteins in each of the complexes (I, III, IV, and IV) that rely on mitochondrial and well as nuclear genomes, indicating a more general function of LRPPRC on mitochondrially-encoded mRNAs than was suggested by the studies of LSFC patients. While this work was in progress, similar observations were reported for COXI, COXII, COXIII (complex IV), and Cytb (complex III) by Cooper and co-workers (25). Taken together, our findings show that LRPPRC is a general essential *trans*-

acting factor for the accumulation and maintenance of steady-state levels of mitochondrially-encoded mRNAs. Interestingly, the residual RNA-binding activity of the mutant LRPPRC provides a possible explanation of the apparent specific reduction of only subset of the mRNAs observed in LSFC patient fibroblasts, if the impact of the residual RNA-binding activity of the protein differs depending on the particular RNA.

What is the function of LRPPRC? Our results clearly show that LRPPRC is essential for the maintenance of steady-state levels of mitochondrially-encoded mRNAs, a function that most likely is effected through direct binding of LRPPRC to these RNAs or to their precursors. At this stage, however, we cannot pinpoint a specific stage in the metabolism of mitochondrially-encoded mRNAs as a specific target of LRPPRC. Nonetheless, knowledge of similar proteins in other organisms is informative in providing a framework for interpreting our results. PPR motif proteins with significant amino acid sequence similarity to LRPPRC have been characterized in the yeast *Saccharomyces cerevisiae* (Pet309p; 36), *Neurospora crassa* (cya5; 37), and *Zea mays* (crp1; 38). Both Pet309p and cya5 are required for translation of COXI mRNA (36,37). The activation of translation by Pet309 is mediated by an interaction with the 5' UTR of the COXI mRNA. Pet309p is also required for stability of the COX1 mRNA (36). These two activities are separable, however, in that the PPR motifs in Pet309 are required for its function in translation but not in stability (39). A similar action of a PPR motif protein on its target mRNAs by interaction with their 5' UTR has been described

for the maize Crp1 protein, which activates translation of the chloroplast petA and psaC mRNAs (38,40). This would point to similar roles of LRPPRC in mitochondrial gene expression in humans. In particular, a role of LRPPRC in determining RNA stability through binding to mitochondrial mRNAs would be in complete agreement with our findings except that, in the case of LRPPRC, its role would be more general in that it would impact all the mitochondrially-encoded mRNAs as opposed to only a subset. It is of interest in this regard that a likely homologue of LRPPRC in *Drosophila melanogaster*, BSF1, was also implicated in RNA stability, in this case for the nuclearly-encoded *bicoid* mRNA (41). Importantly, however, other possible functions need to be considered also for LRPPRC, as PPR motif proteins have also been implicated in all remaining stages of organellar gene expression (for a recent review, see ref. 5). In principle, LRPPRC could act similarly at one or several of the steps in mitochondrial gene expression, ranging from transcription of the mitochondrial DNA through processing of the primary transcripts to determining the stability of the resulting mRNAs. A participation of LRPPRC in transcription, perhaps by an interaction (direct or indirect) with the mitochondrial RNA polymerase, is an intriguing possibility that is raised also by the fact that both mitochondrial RNA polymerase and LRPPRC contain PPR motifs (see ref. 42). LRPPRC could also participate in the processing of the primary transcripts, in which case we would have expected an accumulation of the precursor. This possible role remains an open question, as levels of detectable primary transcripts vary quite dramatically from experiment to experiment in our hands, and therefore we could not rule in or out

unambiguously an effect of reducing LRPPRC protein levels on the relative levels of such RNAs. Current work is aimed at resolving this and the above-mentioned issues.

ACKNOWLEDGEMENTS

This work was supported by grants from the Muscular Dystrophy Association, the American Heart Association, and the NIH (NIGMS; GM067894) to S.P.R., and a United Negro College Fund (UNCF) Merck Graduate Science Research Dissertation Fellowship to Catherine Bangeranye.

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FOOTNOTES

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Abbreviations used are: COX, cytochrome c oxidase; LSCF, hnRNP, heterogeneous nuclear ribonucleoprotein; Leigh Syndrome, French Canadian; mtDNA, mitochondrial DNA; OXPHOS, Oxidative Phosphorylation; RNP, ribonucleoprotein.

FIGURE LEGENDS

FIGURE 1. ***Isolation of RNP complexes associated with LRPPRC and analysis of protein composition.*** LRPPRC-associated complexes were isolated from ^{35}S methionine-labeled HeLa cells, by immunopurification with the monoclonal antibody 9C9 (lanes 'a-LRPPRC/LRP130'), without (lane '-') or with (lane '+') prior digestion of the cellular lysate with RNase, as described in the text. The associated proteins were resolved by SDS-PAGE and detected by fluorography. Lanes 'SP2/0': identical immunopurifications with non-immune parent myeloma immunoglobulins. B. Comparison of LRPPRC-associated proteins and hnRNP A1-associated proteins isolated from a mitochondrially-enriched fraction using 9C9 [panel 'a-LRPPRC/LRP130'] and 4B10 [panel 'a-hnRNPA1 (nmRNP)] monoclonal antibody, respectively. The proteins were resolved by two-dimensional gel electrophoresis, using NEPHGE in the first dimension (left to right) and SDS-PAGE in the second dimension, and were subsequently detected by fluorography.

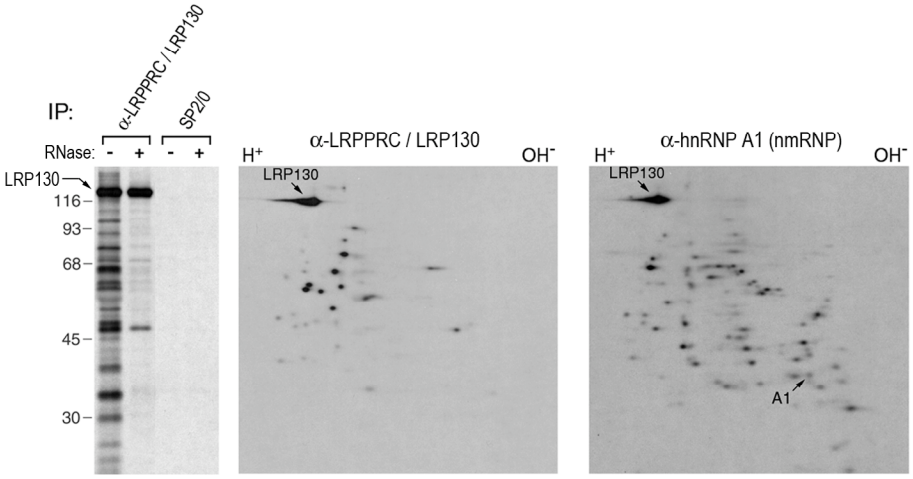
FIGURE 2. ***Northern blot analysis of LRPPRC-associated RNAs.*** LRPPRC-associated complexes were isolated by immunopurification from HeLa whole-cell lysates, using the monoclonal antibody 9C9 (lanes 'a-LRPPRC'). As control, hnRNP complexes were isolated from the same lysates using the anti-hnRNP C1/C2 monoclonal antibody 4F4, as indicated (lanes 'a-hnRNP C'). RNA was extracted from the isolated complexes, resolved by formaldehyde-agarose gel electrophoresis, and specific RNAs were identified by Northern blot analysis using probes for mitochondrially-encoded (ND1, ND2, Cytb,

COXI, and ATP6) and nuclear-encoded (ß-actin) mRNAs, as indicated. Lanes 'Total' contain an aliquot of the starting material used for immunopurification.

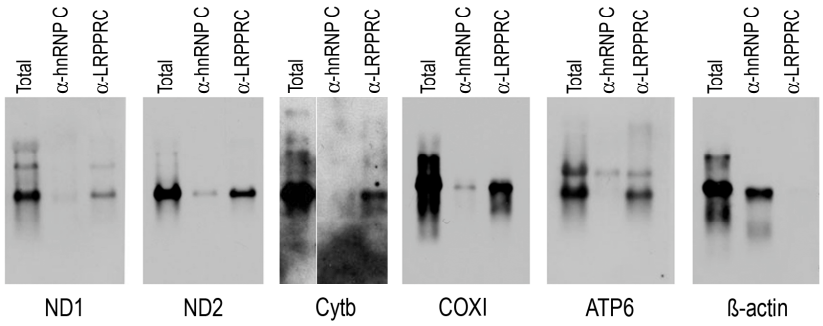
FIGURE 3. ***Subcellular localization and RNA-protein crosslinking of wild-type and mutant (A354V) LRPPRC.*** Wild-type or mutant (A354V) LRPPRC, fused to GFP, was expressed in human (HeLa) cells by transfection of the corresponding cDNAs. A. Intracellular location of the GFP-fused proteins, as detected by fluorescence microscopy; B. UV light-induced crosslinking of the wild-type (wt) and mutant (A354V) LRPPRC to poly(A)$^+$ RNA *in vivo*, followed by isolation of the crosslinked complexes by oligo(dT) chromatography and analysis of crosslinked proteins by immunoblotting with the anti-LRPPRC antibody 9C9. Note that both exogenous and endogenous (GFP-fused) proteins are detected by the antibody under these conditions, as indicated on the right. Lanes '-': mock-transfected cells; panel 'input': total protein prior to selection by oligo(dT) chromatography.

FIGURE 4. ***Steady-state levels of mitochondrially-encoded (but not of nuclear-encoded RNAs) are reduced in cells with reduced levels of LRPPRC.*** Cells were transfected with RNAi constructs targeting LRPPRC, incubated for 24 hours or 40 hours, as indicated, and subsequently analyzed by immunoblotting or Northern blotting. A. Immunoblot analysis of transfected cells, using anti-LRPPRC 9C9 and anti-hnRNP A1 antibodies. B. Northern blot analysis of the transfected cells, using probes for mitochondrially-encoded RNAs as indicated. C. Northern blot analysis of the same cells

using probes for the indicated nuclear-encoded mRNAs. Lanes '-': mock-transfected cells. Lanes '+': cells transfected with siRNA.



Bangeranye et al., Figure 1



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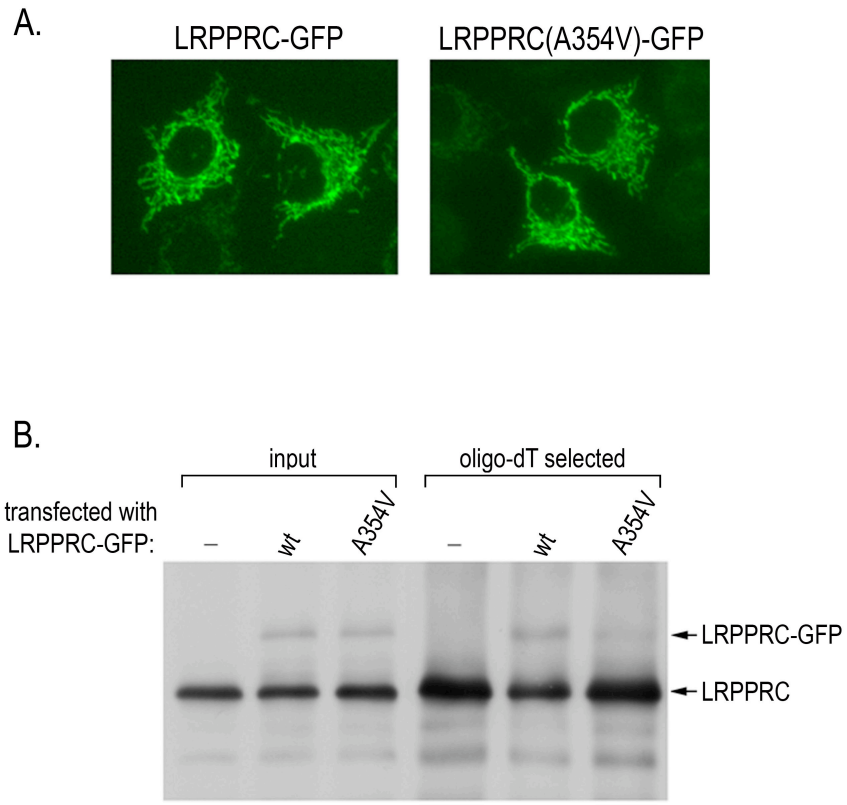

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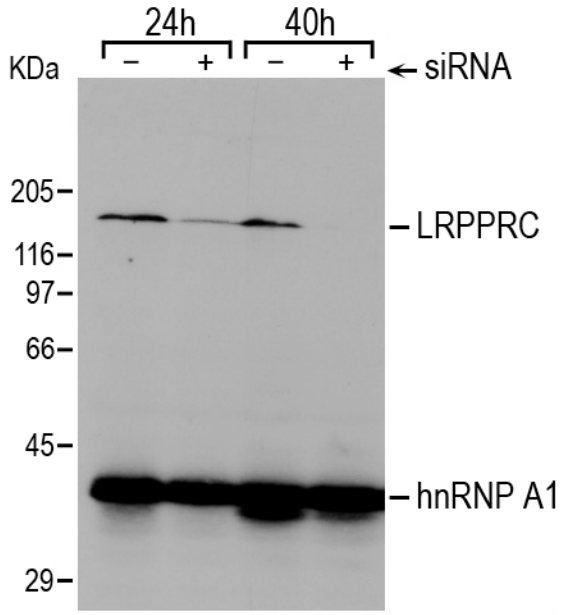
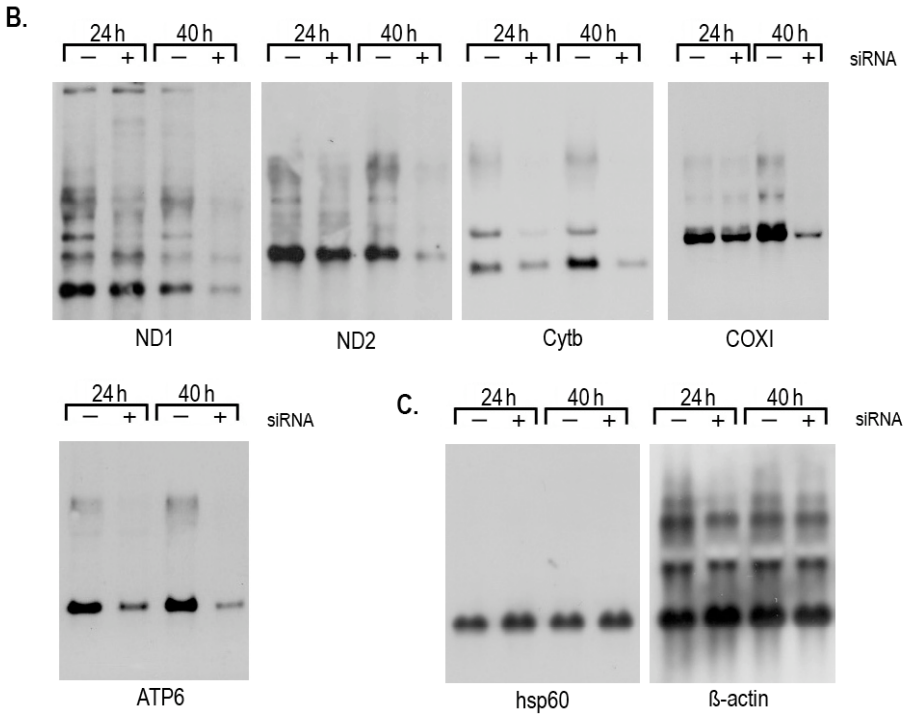


Bangeranye et al., Figure 4A



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