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Many Facets of PNPase - Uncovering the Role of PNPase in the Mitochondria

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular Biology

by

Eriko Christine Shimada

2018

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ABSTRACT OF THE DISSERTATION

Many Facets of PNPase - Uncovering the Role of PNPase in the Mitochondria

by

Eriko Christine Shimada

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2018

Professor Michael Alan Teitell, Chair

PNPase is a conserved 3'-5' exoribonuclease localized in the mammalian mitochondria. Its expression is essential for embryonic development and mitochondrial homeostasis. PNPase has been recently implicated in regulating mitochondrial RNA import. Utilizing yeast mutants and knockdown systems in human cell lines, we show that porin or VDAC may function as a possible import channel in the mitochondria. The radiolabeled RNA that is imported into the purified mammalian mitochondria crosslinks specifically with VDAC1 suggesting that the imported RNA may go through this outer membrane channel.

In order to further study the role of PNPase, we attempted to create a knockout cell line by adjusting the culture conditions to allow cells to survive without functional mitochondria. Interestingly, the resulting cell lines did not have any mitochondrial DNA shown by the lack of signal from the PCR performed using probes designed to detect the mitochondrial DNA, and these cells also did not respire. Overall, PNPase knockout cells showed a transcriptional profile

that was similar to rho^{0} cells that lack mitochondrial DNA. Further, PNPase knockout cell lines showed changes in genes involved in cholesterol biosynthesis and regulating neuronal function. This study underscores the unexpected role of PNPase in affecting mitochondrial DNA maintenance.

Finally, we characterized endogenous RNA targets of PNPase, its effect on steady state levels of mitochondrial RNA when PNPase expression is knocked down, and its interaction partners. We find that PNPase binds all mitochondrial tRNAs, mRNAs and rRNAs but specific regions that include tRNAs are enriched in the PNPase CRAC assay. When PNPase expression is knocked down, the steady state levels of sense strands RNAs do not change significantly, but specific antisense strands RNA transcribed from the mitochondrial DNA accumulate at high levels. Using the proximity based BIOID2 biotin labeling system and mass spectrometry analysis, we identified that PNPase may interact with mitochondrial ribosome assembly factors such as FASTKD2, DDX28, and DHX30, and other members of the FASTKD family. Our research suggests that PNPase plays an essential role in the mitochondria where it may regulate mitochondrial DNA maintenance and mitochondrial RNA levels, and it interacts with specific mitochondrial ribosome assembly factors and RNA processing proteins.

The dissertation of Eriko Christine Shimada is approved.

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2018

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Chapter 1: Introduction – PNPase function in bacteria, plants and mammalian systems

Introduction

If one were to choose an organelle to study in the cell, the mitochondria would be the most curious one to select. Because of its proteobacterial origin, the mitochondria hold its own genetic material, transcribe and translate it and even possess the machinery to repair DNA damage (1). The organelle also undergoes fusion and fission, which allows itself to connect to other mitochondria in the cell to intermix its content or to sequester a piece of the organelle (2). Since the mitochondria do not encode enough material in its DNA to function self sufficiently, it must also coordinate with the gene expression and translation machinery to import the majority of its proteins into the mitochondria (3,4). In addition, the mitochondria regulates essential cellular processes such as, oxidative phosphorylation, metabolic regulation, heme synthesis (5), cellular apoptosis (6), and antiviral response (7). It is no wonder that researchers are drawn to study this organelle.

The Teitell Lab has been specifically interested in understanding the function of a protein called polynucleotide phosphorylase, which is an exoribonuclease localized in the mitochondria. Its biochemical activity and cellular function has been classically studied in the bacteria. It was initially mistaken to be the RNA polymerase and has been part of the research that was awarded a Nobel Prize. More than 50 years later from its initial discovery, the PNPase was found to strongly interact to an

oncoprotein T cell leukemia 1 (TCL1) in an immunoprecipitation experiment, though this interaction has not been confirmed to occur endogenously inside the cell. Regardless of this finding, PNPase proved to serve essential roles in the mitochondria and development processes and has captured more attention to be studied in mammalian systems in recent years.

Biochemical characterization of PNPase

PNPase is an evolutionarily conserved protein that is expressed in bacteria and most of eukarya but is not expressed in archae (1,2). It can catalyze an RNA degradation reaction, degrading its substrate from the 3' to 5' direction utilizing inorganic phosphate and polymerize RNA by adding any of the four available ribonucleotide diphosphates (3,4). In vitro, PNPase activity can be modulated by the amount of inorganic phosphate or ribonucleotide diphosphates and triphosphates (5,6) and the human PNPase has been shown to require significantly lower levels of inorganic phosphate to degrade RNA (7). *E. Coli* and chloroplast PNPase exhibit high binding affinities to polyadenylated RNA where as the human PNPase has not been shown to do the same (6). However, opposing results have been reported on the preference of PNPase on degrading polyadenylated RNA (7,8). Thus, human PNPase has been shown to degrade and polymerize RNA like its bacterial and chloroplast counterpart despite minor differences in cofactor requirement and binding affinities to polyadenylated RNA.

Structurally, PNPase contains 5 defined domains in the following order: the first RNase PH domain, α-helical domain, the second RNase PH domain, the KH and S1 RNA binding domains (2,6,9-11). The crystal structures of PNPase expressed in *E. coli, S.*

antibiotics, *C. crescentus*, and *H. sapiens* have been solved and all of these structures show a trimeric complex where the catalytic PH domains assemble into a doughnut shape (12-15). The KH and S1 domains reside on one side of the doughnut shape to allow threading of the single stranded RNA through its central cavity formed by the RNase PH domains (12-16). In bacteria and humans, the main catalytic activity is derived from the second RNase PH domain (7,14,16), whereas in spinach chloroplast, both the first and second RNase PH domains have been shown to participate in RNA degradation (6). In addition to the inorganic phosphates and nucleotides, several metal ions and citrate have been shown to modulate PNPase activity. In *E. coli*, the metal chelated citrate has been shown to inhibit enzymatic activity whereas lower concentration of metal-free citrate enhances polymerization and high concentration of citrate increases degradation activity (17). Magnesium chelated citrate also inhibits degradation activity of *H. Sapiens* and *S. Solfataricus* PNPase (18). Bacterial PNPase requires Mg^{2+} for RNA degradation and its activity can be inhibited by high amounts of Mn^{2+} (19), however the bacterial polymerization activity can be stimulated by Mn^{2+}, or Fe^{2+} and is inhibited by Mg^{2+} (20-23). In contrast, human PNPase degradation and polymerization occur in vitro in the presence of Mg^{2+} (7).

Interaction partners of PNPase have been studied most heavily in bacteria and limited information is available about proteins binding to chloroplast and human PNPases. In bacteria, PNPase exists in many different complexes. It can function as a homotrimeric complex on its own, interact with the RhlB helicase for degradation, or exist as a large canonical degradosome complex by interacting with RhlB, RNase E, and enolase (24-26). During coldshock, the RhlB helicase can be replaced by CsdA in the

RNA degradosome (27). In *D. Radiodurans,* PNPase associates with an Rho autoantigen ortholog Rsr through the scaffold created by a noncoding y RNA for degrading RNA to form the RYPER complex (28), which degrades structured RNAs like rRNA during stationary phase (29). In contrast, no interacting protein has been found for chloroplast PNPase and it has been proposed to exist as a homotrimeric or homo hexameric complex (30). The human PNPase is purified mostly as a homotrimeric complex in the blue-native gel (31), however, hSUV3, a mitochondrial localized RNA helicase, has been identified in several immunoprecipitation experiments as its major interacting protein (32,33). Upon treatment with glycolysis inhibitor 2-D- deoxyglucose and cytochrome c oxidase inhibitor, sodium azide, PNPase also interacts with hSUV3 and poly adenylation polymerase in the mitochondria (32). Also, recent studies on mitochondrial RNA processing and mitochondrial ribosome assembly factors have also shown preliminary evidence of interacting with PNPase (34).

Functions of PNPase in bacteria

Bacterial functions of PNPase are intriguingly diverse. The most well studied function of PNPase is its role in mRNA turnover (35,36). Of the 3 major exonucleases responsible for the bulk RNA decay in *E. Coli,* PNPase is thought to degrade the most RNA targets compared to RNase II and RNase R (36,37). In addition to RNA turnover, PNPase also functions in two other aspects of RNA metabolism. In *E. Coli*, it is implicated in rRNA and tRNA processing (38-40) and quality control (37,41,42). Also, PNPase is implicated in degradation of small noncoding RNA, sRNA (37,43,44) and maturation of sRNA from its precursors (45).

The enzymatic and RNA binding activity of PNPase is also involved in various aspects of *E. Coli* survival including stress responses, regulation of virulence, and biofilm formation. As mentioned above, PNPase plays an important role during cold shock response for both gram positive and negative bacteria (46-49). In addition, PNPase has also been suggested to function in ribosome assembly during cold shock (50). PNPase interaction with RhlB is necessary for degradation of the mRNA encoding the transcriptional activator of the cysteine biosynthesis operon in *E. Coli* for management of oxidative stress response (51). PNPase has recently been shown to be involved in nucleic acid stress response, and is implicated in damaged RNA removal through its binding to 8-hydroxyguanine residues (52-54). PNPase has also been shown to cause spontaneous mutations (55) and has been suggested to participate in DNA repair through binding to RecN and RecA proteins in various bacteria (56,57). The role of PNPase in regulating virulence in bacteria varies significantly and its mechanism of influence is quite unknown. In *Y. pseudotuberculosis* and *Y. pestis* the RNA binding of PNPase to an unidentified RNA is suggested to promote secretion of Yop effectors (58). In *S. aurelius*, the degradation activity of PNPase is important for regulating virulence (59). In several strains of *E. Coli*, PNPase has been implicated in negatively regulating the operon involved in extra-cellular polymer synthesis (60) and degrading RNAs encoding for adhesion molecules (37).

Function of PNPase in plants

In plants, PNPase is expressed in both the mitochondria and chloroplast and have been shown to have similar functions in both organelles. In plant mitochondria, PNPase

degrades rRNA, tRNA processing intermediates and noncoding RNAs (68,69). Plant mitochondria also requires PNPase for 3' termini removal of mRNA (70,71). In chloroplasts, PNPase has been suggested to be responsible for adding poly-A tails and non-poly A tails to RNAs in order to mark them for degradation (12,72). PNPase in chloroplast functions to process 3' ends of mRNA and rRNA (72-75), degrade tRNA leader sequences (75) and intronic sequences (72).

Function of PNPase in mammalian mitochondria

As a nuclease in the mitochondria, PNPase has been shown to affect levels of RNAs in the mitochondria and other RNAs traditionally known to exist outside of the mitochondria. Although the mechanism is not very clear, PNPase has been described to regulate CMYC RNA levels by interacting with EGFR (61). Curiously, it has also been observed that PNPase may play a role in the decrease of CMYC levels and mir-221, a regulator of cyclin dependent kinase inhibitor p27KIP1, upon interferon(r)β treatment of cultured cells (62,63). The role of PNPase regulating mitochondrial noncoding RNA has also been explored (64), and it seems that both SUV3 and PNPase both affect the accumulation of these noncoding RNAs in the mammalian mitochondria (65). Thus, in the context of mammalian cultured cells, hSUV3 and PNPase seems to exist mainly in the matrix to perform its functions. In addition, PNPase has also been implicated in regulating RNA import into mammalian mitochondria (31,66). PNPase's role in mitochondrial RNA degradation has become slowly established in recent years and it is very interesting to speculate how this function in the mitochondria affects the metabolic changes brought about by altering PNPase expression in different cellular contexts.

Recent studies have shown that the level of PNPase expression in induced pluripotent stem cells are shown to correlate with the extent of cellular respiration, where inhibition of PNPase expression is associated with decreased oxidative capacity and overexpression increases oxidative phosphorylation (67-69). In addition, overexpression of PNPase is associated with increased mtDNA content and ATP/AMP ratio (68). It is reasonable to assume that decreased PNPase expression may inhibit oxidative phosphorylation capacity of the cell by interfering with normal mitochondrial function. However, it is an interesting avenue to understand why overexpression of PNPase may lead to enhanced cellular respiration.

Finally, PNPase has been long been known to be an IFN induced gene. Its RNA levels are significantly increased upon IFNβ treatment in many cell types (69-71). However, the protein level changes do not necessary correlate with this increase and suggests another level of regulation for PNPase expression (72). Mitochondria has been recently associated with IFN signaling pathways through mitochondrial ROS production, the presence of the MAVS/Cardiff complex on the outer mitochondrial membrane and the mtDNA sensing mechanism that triggers the innate immune response (73,74). This area of study also warrants further work to elucidate the intriguing role of PNPase in the mitochondria.

PNPase mutations and human disease

Recent advancement of sequencing technology has quickly uncovered PNPase mutations that causes human diseases. Many of these diseases are associated with neurological disorders. It has been known that point mutations of PNPase cause encephalomyopathy and hearing loss (75,76). In addition recent studies have connected

delayed myelination, chorioretinal defects, gut disturbances and Leigh syndrome to defects in PNPase activity or expression (77-79). Therefore, understanding the basic cellular function of PNPase may reveal insights into how these processes impact human neurological health.

PNPase, is a 3'-5' exoribonuclease that initially became an interest to the Teitell Laboratory when it was discovered to be the most abundant protein that interacted with an oncoprotein TCL1 (T Cell Leukemia 1) in an immunoprecipitation experiment (8). This interaction has not been confirmed to occur in vivo but our lab continued to study PNPase because of its essential role in maintaining mitochondrial homeostasis (69). At the time of discovery for our lab, the mammalian PNPase had been identified in an overlapping pathway screen and has since been implicated in cellular senescence and terminal differentiation (70). Since then, we have sought to uncover the elusive role of PNPase in the mammalian mitochondria.

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Chapter 2: Porin/VDAC1 may be involved in mitochondrial RNA import

Introduction

Many macromolecules translocate into and out of mitochondria. In addition to metabolites, mitochondria import the majority of its proteins to sustain its function. Recent studies have also shown that RNA can be imported into the mitochondria (1-4). The most heavily studied area of RNA import pertains to the translocation of tRNAs(2,5). However, other noncoding RNAs have also been identified in the mitochondria(3,4,6-10). Up to date, there are roughly 5 categories of import pathways that have been characterized in eukaryotic mitochondria. For simplicity, these import pathways are described as separate processes in which different proteins factors contribute to the delivery and translocation of substrates. However, many translocation channels for these import pathways have not been characterized and it is possible that several of these pathways may share one or more import translocation channels.

Yeast mitochondria can import $tRNA^{Lys}{}_{CUU}$, utilizing the translocases of the protein import pathway. This import is membrane potential dependent (2,5), and the tRNA must associate with the precursor of mitochondrial lysyl-tRNA synthetase and be aminoacylated to allow for import into the mitochondria (5,11,12). The glycolytic enzyme, enolase helps deliver the $tRNA^{Lys}$ –lysyl tRNA synthetase complex to the import machinery (12). The structural or sequence motifs essential for the tRNA import has also been determined (13). In addition, the human mitochondria has been shown to import the

yeast $tRNA^{Lys}$ and artificial RNAs that contain tRNA import signatures in the presence of mammalian enolase, ENO2 and mitochondrial lysyl tRNA synthetase (pre-KARS2) (14). Interestingly, another tRNA import pathway that is independent of the protein import machinery has also been identified. In addition, the mammalian mitochondria has been shown to import its own endogenous $tRNA^{Gln}{}_{CUG}$ and $tRNA^{Gln}{}_{UUG}$ through a mechanism that requires ATP but no mitochondrial membrane potential or cytosolic protein factors (15).

The ancestral mitochondria had kept all of its ribosomal RNA, but it has been suggested that through evolution, mitochondria had gradually lost some its ribosomal RNA including the 5S rRNA (16). Thus 5S rRNA must be imported into the mitochondrial to allow mitochondrial ribosomes to function correctly. In the cytosol, 5S rRNA is known to be part of the ribosome complex but its role is still not clearly understood (17). Also, it has been known that the nuclear encoded 5S rRNA also exist inside the mitochondrial organelle (3,18). Recently, the mammalian matrix protein rhodanese has been shown to be important for the import of 5S rRNA(19). Structural element on the 5s rRNA that signals for import of the RNA has also been identified(20). In addition, a mitochondrial ribosomal protein, MRP (mitochondrial ribosomal protein)-L18 was shown to assist this import process, where the pre-MRPL18 binds to the import sequence of 5s rRNA and cause a conformational change that allows the RNA to be recognized by rhodanese (21). The 5S rRNA is thought to interact with MRPL18 in the matrix and potentially affect mitochondrial translation efficiency (22).

Aside from tRNAs and the 5S ribosomal RNA, RMRP and RNase P is another class of noncoding RNAs that are imported into the mitochondria. RMRP is the RNA

component of mitochondrial RNA-processing endoribonuclease complex and has been shown to function in the 5'end maturation of 5.8S rRNA for cytosolic ribosomes (23,24) and is also involved in cell cycle progression by affecting the degradation of cyclin B mRNA during mitosis (25). RMRP has been shown to interact with telomerase reverse transcriptase (hTERT) and synthesize double stranded RNA that is recognized by DICER1 and this can be further processed into short interfering RNA (26). In addition, the nuclear encoded RMRP has also been found in the mitochondria (1,27,28), and is known to be responsible for synthesis of RNA primers to allow for the replication of mitochondrial DNA(29,30). The import of this RNA is thought to be mediated by PNPase, and a specific stem loop structure that allows import of this substrate has been identified (1). Recent studies have also suggested that matrix residing mitochondrial RNA granule protein GRSF1 to be important in increasing the abundance of RMRP in the mitochondria (27). In addition to RMRP, RNase P is the RNA component that has been thought to be part of the RNase P complex functioning in 5' tRNA processing though the participation of RNA in this complex has been debated to be controversial(31-33). This RNA is also imported into the mitochondria with the help of PNPase, and the stem loop signature that is important for import has been identified (1).

micro RNAs (miRNAs) are a recent class of RNAs that have been identified to exist in the mitochondria **(34,35)**. miRNAs are ~ 22nt RNA that inhibit translation of mRNA or induce mRNA degradation (36,37). miRNAs are transcribed from the DNA as primary micro RNA (pri-miR), and is processed in the nucleus by DROSHA/DGCR8 complex to pre-miRNAs. The pre-miRs are then exported to the cytoplasm where they are further processed by DICER into mature miRNA and associates with the rna binding

protein argonaute 2 (AGO2) and is directed to its target mRNA (38-40). miRNAs typically have multiple targets (41) and its dysregulation has been associated with disease and cancer (42). Although, nothing is known about its import mechanism, pri-miRNA, pre-miRNA and mature miRNAs and AGO2 has been shown to exist in mitochondria (6,9,10,43-45). Only AGO2 of the Argonaute family proteins are known to be imported into the mitochondria (35), and it has been shown to interact with mitochondrial transcripts by RNA crosslinking assays (6).

Recent studies have been uncovering different biological aspects of miRNAs present mitochondria. Most miR found in the mitochondria are nuclear encoded but some have been found to come from mtDNA (43). miR-181c has been shown to regulate translation of mitochondrial encoded cytochrome c oxidase subunit 1 (COX1) in rodents, and the over-expression of miR-181C was shown to produce higher ROS production and resulted in lower exercise capacity and appearance of cardiac dysfunction (46). A carefully controlled studied using thoroughly purified mitochondria and mitoplasts and showed that miR1 increase COX1 and ND1 translation but it differentially regulates translation of its targets in the cytoplasm and mitochondria during muscle differentiation (35). Interestingly, several miRNAs were found to be imported into rho^0 cells, such as, miR-181c-5p and miR-146a-5p (7,47). In diabetic heart and HL-1 cells, miRNAs including mir -378 translocates into the mitochondria following diabetic insult, and it was shown to downregulate the mitochondrially encoded F_0 component, ATP6 expression essential for cardiac function (48).

With regards to RNA import channels in mitochondria, the import of tRNAs are mediated through the voltage dependent anion channel on the outer membrane of plant

and trypanosome mitochondria (49,50). In addition, components of the protein import machinery such as TOM20 and TOM40, have been shown to be important for tRNA binding to the mitochondria and internalization (49,51). However, the exact translocation channel for these RNAs in the mitochondria has not been identified in mammalian mitochondria. This aspect of mitochondrial RNA import has long remained an interest to the field especially because it is intriguing to understand how such a large negatively charge molecule can be translocated across the charged inner membrane of the mitochondria. Thus we have attempted to answer this question by using yeast and mammalian model systems.

Results

Mitochondrial outer membrane proteins are required for PNPase dependent mitochondrial RNA import in yeast

To examine whether mitochondrial outer proteins are involved in mitochondrial RNA import, yeast mitochondria with or without PNPase were isolated and treated with Trypsin in an isotonic buffer. The treatment led to degradation of the outer membrane protein marker Tom70 without significantly affecting the level of mitochondrial intermembrane protein Mia40, suggesting the integrity of the mitochondria was not compromised. The treated or untreated mitochondria were then used for the RNA import assay (Figure 2-1A). As expected, expression of PNPase in untreated mitochondria resulted in a significant increase of protected RNA import substrate, *RNase P* RNA, after incubation and nuclease digestion (Figure 2-1B). However, in the trypsin treated

mitochondria, the amount of imported *RNase P* RNA was reduced to the basal level, suggesting one or more of mitochondrial outer membrane proteins are required for PNPase dependent mitochondrial RNA import in yeast.

Mitochondrial outer membrane protein, porin is required for PNPase dependent mitochondrial RNA import in yeast

To identify the outer membrane proteins that are involved in mitochondrial RNA import, the import assay was performed on mitochondria isolated from strains lifted from a yeast deletion collection. It has been shown that in plant Solanum tuberosum, outer membrane protein VDAC (voltage dependent anion channel) and two components of the TOM (translocase of the outer mitochondrial membrane) Tom20 and Tom40 are all involved in mitochondrial tRNA import (49). We examined the PNPase dependent RNA import into mitochondria isolated from a por1 (yeast VDAC) deletion strain and *tom70* deletion strain (Figure 2-2A). The import of *RNase P RNA* is severely reduced in the *Δpor1* PNPase mitochondria, but unaffected by deletion of tom70, suggesting that Porin channel but not the protein import channel is involved in PNPase dependent mitochondrial RNA import in yeast (Figure 2-2B).

VDAC is involved in mitochondrial RNA import in mammalian cells

To examine whether the results in yeast can be transferred to mammalian cells, shRNA was used to knock down VDAC1 expression in HeLa Cells. There are three VDAC proteins (VDAC1, 2, 3) in human. However, VDAC1 is the most abundant one in almost all cell types (52,53). A three-fold reduction in VDAC1 level was achieved using the shRNA (Figure 2-3A). Mitochondria were isolated from the wild-type and VDAC1 deficient cells and the in vitro import assay was performed using radiolabeled *RNase P* RNA. A ~2 fold reduction in the amount of imported RNA was observed in the VDAC1 deficient mitochondria (Figure 2-3B). The reduction, however, could be due to a general effect on the mitochondrial function by VDAC1 knockdown. To rule out the possibility, antibody-blocking approach was used in the import assay. Only wild-type mitochondria were used in the approach guaranteeing the consistence of mitochondrial functions. Mitochondria were incubated with VDAC1 antibody, Tom40 antibody or the buffer only. The in vitro import assay was then performed using the antibody treated or untreated mitochondria. Treatment of wild-type mitochondria using VDAC1 antibody led to a ~2 fold reduction of imported *RNase P* RNA compared to the control mitochondria, suggesting that human VDAC mediates mitochondrial RNA import in mammalian cells (Figure 2-3C). Tom40 antibody partially inhibited mitochondrial import of a protein precursor Su9-DHFR, but had no significant effect on mitochondrial RNA import, indicating that unlike in the plant mitochondria, the TOM translocase is not directly involved in mitochondrial RNA import (Figure 2-3C). To confirm that VDAC directly mediates in mitochondrial RNA import, a co-immunoprecipitation assay was performed using antibodies specific for either VDAC or TOM40. Radiolabeled *RNase P* RNA or a control $tRNA^{Lys}$ precursor was incubated with the wild-type mitochondria in the import

buffer, and the samples were UV-crosslinked (Figure 2-3D). After the incubation and UV-crosslinking, Mitochondria were lysed and VDAC or TOM40 was isolated from the lysates by immunoprecipitation. Only when *RNase P* RNA was used as the import substrate and VDAC antibody used for immunoprecipitation, the radiolabled RNA was copurified with the protein, suggesting a direct interaction of the import RNA substrate with the VDAC channel.

VDAC1 knockdown affects steady state levels of *RNase P* RNA in mitochondria and the processing of mitochondrial transcripts

Mitochondrial RNAs are transcribed as long polycistronic transcripts, which then undergo processing to generate the individual mRNAs, tRNAs and rRNA (54). One of the RNA processing steps is mediated by *RNase P* complex, and *RNase P* RNA is one of the essential components of the *RNase P* complex (33,55). If VDAC1 serves as the outer membrane RNA import channel in vivo, its deficiency would lead to reduction of *RNase P* RNA level in mitochondria and in turn lead to insufficient processing of the long transcripts. The mitochondrial *RNase P* RNA level in the wild-type mitochondria and VDAC1 deficient mitochondria was examined using RT-PCR. Significantly higher level of *RNase P* RNA is detected in the wild-type mitochondria compared to the VDAC1 deficient mitochondria (Figure 2-4A). The levels of partially processed RNA transcripts were next examined by RT-PCR using primers individually annealing to mRNAs separated by two or three tRNAs: *COX1* and *COX2* separated by $tRNA^{Ser}$ and $tRNA^{Asp}$,

and *ND4* and *ND5* separated by $tRNA^{His}$, $tRNA^{Ser}$ and $tRNA^{Leu}$. Significantly higher amount of partially or unprocessed RNA precursors were observed in the VDAC deficient mitochondria compared to the wild-type mitochondria (Figure 2-4B). The negative effect on the downstream processes of mitochondrial RNA import by VDAC knockdown further confirms the direct involvement of VDAC in the import.

VDAC1 knockdown negatively affects mitochondrial functions

Our previous studies showed that knockdown of an essential component of mitochondrial RNA import pathway PNPase reduced OXPHOS and disrupted the integrity of mitochondrial structure (1,56). These mitochondrial functions were also examined in the VDAC1 deficient cells. Like the PNPase deficient cells, a 1.5-2 fold decrease of the stable level of mitochondrion-encoded COX2 was observed in the VDAC1 deficient cells, while no significant difference of mitochondrial proteins encoded in the nucleus was detected. The lower stable protein level can be attributed to lower translation level, as in vivo translation of mitochondrion-encoded proteins showed a significant lower yield (1/2) in the VDAC1 deficient cells. Staining of mitochondria using MitoTracker green also showed similar fragmentation of mitochondrial structure in the VDAC1 deficient cells. Finally, the OXPHOS function of the VDAC1deficient cells was examined, and the cells showed a significantly lower (~70%) respiration rate compared to the wild-type cells.

Concluding Remarks

As the mitochondria regulates the charge balance within the organelle, in order to maintain the mitochondrial membrane potential, the question of how the mitochondrial membrane can accommodate the movement of large negative charge molecule like RNA is extremely intriguing. Using the yeast *in organello* import and RNA crosslinking and pull down assays in the mammalian VDAC knockdown system, we show that VDAC1 may possible be responsible for allowing *RNase P RNA* to be imported to the mitochondria. Since the RNA translocation process requires the channel to accommodate a large negatively charged molecule, a biophysical characterization of how this transport takes place through the outer mitochondrial membrane is warranted. In addition, the inner membrane channel that is responsible for RNA translocation and any other factors directing the RNA to the mitochondria for import still remains to be identified for many of the RNAs imported into the mitochondria.

Materials and Methods

***Mitochondrial RNA purification*.** Mitochondria were treated with 25 µg/mL S7 micrococcal nuclease at 27°C for 30 minutes in nuclease buffer (0.6 M Sorbitol, 20 mM $MgCl_2$, 5 mM $CaCl_2$, 20 mM Tris-pH 8.0). The reaction was stopped by addition of 20 mM EGTA. The mitochondria were then solubilized in SDS buffer (100 mM NaCl, 1 % SDS, 20 mM Tris-Cl pH 7.4) at 65°C for 5 minutes. RNA was extracted using TRIzol (Invitrogen) reagent, treated with DNase I (Roche) for 1hr at 37°C in the manufacturer supplied buffer and DNase I was heat inactivated at 65°C for 10 min.

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In Vitro Transcription and Import Assay. Import substrate RNA were transcribed from PCR templates by incubating 800 ng PCR template with SP6 RNA Polymerase (NEB) at 40°C for 15 minutes in SP6 transcription buffer (40 mM Tris-Cl pH 7.5 , 6 mM $MgCl_2$, 2 mM spermidine, 0.1 mg/mL BSA) with 0.5 mM rNTP without GTP, 5 mM methyl G-Cap (NEB), 10 mM DTT and trace amounts ^{32}P rCTP (MP Biomedicals). The rGTP was then added to the reaction to a final concentration of 0.5 mM and allowed to incubate for 90 minutes at 40°C. Import substrates for mammalian experiments were transcribed using Ambion Sp6 MEGAscript® Kit according to manufacturer's protocol.

All yeast strains were grown at stationary phase for 2 days in selection medium. Yeast and mammalian mitochondrial isolation procedure were previously described (56,57) . The import assay for yeast mitochondria was performed in 200 µL volume by incubating 100 µg of isolated mitochondria at room temperature for 5 minutes in import buffer (0.6 M Sorbitol, 2 mM KH_2PO_4, 50 mM KCl, 50 mM Hepes-KOH pH 7.1, 10 mM $MgCl_2$, 2.5 mM EDTA, and 1 mg/mL BSA 5 mM ATP, 2 mM DTT, 5 mM NADH) and subsequently adding 5 pmol of radiolabeled RNA and incubating for 5 more min at room temperature for import. Then, mitochondria were spun down at 11,000 *g*, and rinsed with wash buffer (0.6 M Sorbitol and 20 mM Tris-Cl pH 8.0), treated with 25 µg/mL S7 micrococcal nuclease at 27°C for 30 minutes. Total RNA was isolated as described above, analyzed by 8M Urea 5% Acrylamide gel electrophoresis, and visualized by autoradiography. Mammalian import assays were performed using buffer containing 0.225 M Mannitol plus 0.075 M Sucrose instead of 0.6 M Sorbitol, and 20 mM succinate instead of 5 mM NADH were used.

Western Blot. 25-50 μg of protein lysates were separated by SDS-PAGE, transferred to a nitrocellulose membrane and blocked by incubating for 1 hour with 5% milk in TBS-Tween-20 and primary antibodies incubated at room temperature for 1h or at 4°C overnight. The following dilutions were used for each antibody: PNPase (1:5000) (57,58), Porin (1:5000) (Abcam), VDAC1 (1:1000) (Abcam), TOM40 (1:2000) (ULAB4), COX2 (1:1000) (Santa Cruz Biotech.), and HRP-conjugated mouse and rabbit secondary antibody (1:10,000) (Thermo Fisher Scientific). The blots were visualized using the ECL reagent from Thermo Fisher Scientific.

Crosslinking and Immunoprecipitation Assay. Immediately after import, the imported RNA and mitochondrial proteins were UV crosslinked (1.8 J in Stratalinker). The mitochondria were then solubilized in lysis buffer (150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH 8.0) containing protease inhibitor (Roche). Insoluble materials were removed by centrifugation at 20,000 *g* for 30 min at 4°C. The lysates were pre-cleared with protein A-Sepharose beads (Pharmacia) by incubating for 1 hour at 4°C, and immunoprecipitated using 5 μg of antibody conjugated to protein A-Sepharose beads for 2 hours at 4°C. The beads were then washed 6 times with lysis buffer. The immunoprecipitates were boiled in SDS loading buffer, separated on SDS-PAGE and analyzed by autoradiography.

RT-PCR. Isolated mitochondrial RNA were subject to RT-PCR using the AccessQuick™ RT-PCR kit (Promega) and a specific reverse primer. The AMV reverse transcriptase

were denatured at 95°C for 5 min and PCR amplification were performed in the same tube after addition of the specific forward primer.

Plasmids. All the PNPase, Su9-DHFR and RNase P RNA plasmids were previously described (1,56,57). The shRNA vectors for VDAC1 silencing were purchase from Sigma.

Yeast Strains. The wild type and mutant yeast strains were previously described (59,60). All the strains were grown and maintained in synthetic complete media minus the amino acid for marker selection.

Cell Lines. HeLa and HEK293 cells cultured in DMEM supplemented with 10% fetal bovine serum. Stable VDAC1 knockdown cell lines were established by transfection of HEK293 cells with VDAC1 shRNA construct, VSVg and Hit60 packaging vectors using BioT reagent (Bioland Scientific LLC). The harvested viruses were used to infect HeLa cells and cells were selected with 3 µg/mL puromycin.

Oxygen Consumption Measurement. Cells were seeded at 50,000 cells/well in a XF24 Extracellular Flux Analyzer cell culture plate (Seahorse Bioscience) and incubated in the 37°C incubator with 5% CO_2 for 24 hr. The oxygen consumption rate was measured using the XF24 Extracellular Flux Analyzer.

Figures

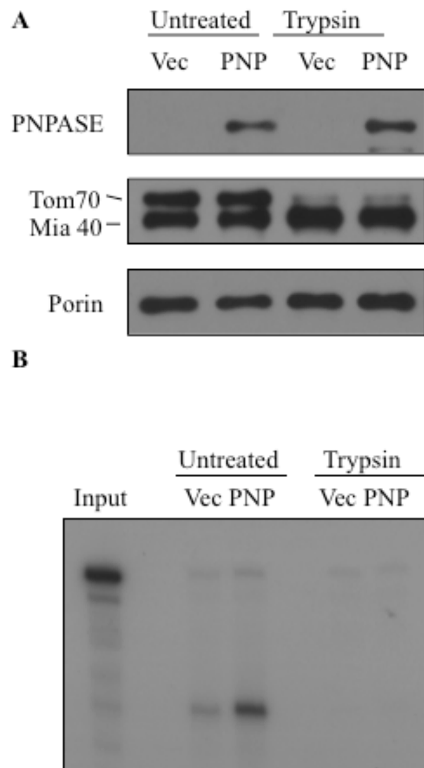


Figure 2-1. **PNPase mediated mitochondrial RNA import is sensitive to Trypsin shaving of the mitochondrial outer membrane proteins.** (A) Western blot show that the trypsin shaving was efficient and the outer mitochondrial protein, Tom70 is degraded. (B) Import of *RNase P* RNA into isolated mitochondria treated with Trypsin.

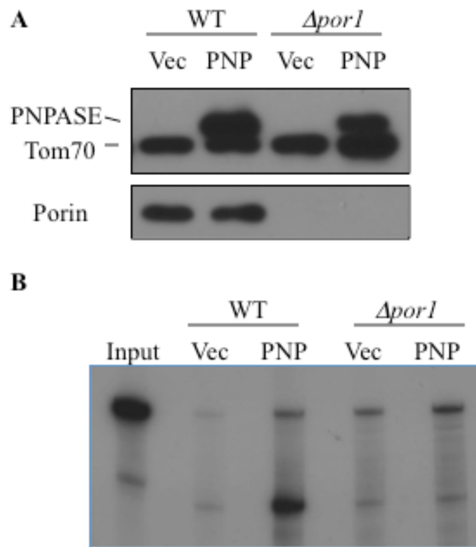


Figure 2-2. **Yeast mitochondrial outer membrane protein Por1 is required for PNPase-mediated mitochondrial RNA import.** (A) Western blot showing that the *Δpor1* strain does not express any porin. (B) Import of *RNase P* RNA into isolated mitochondria.

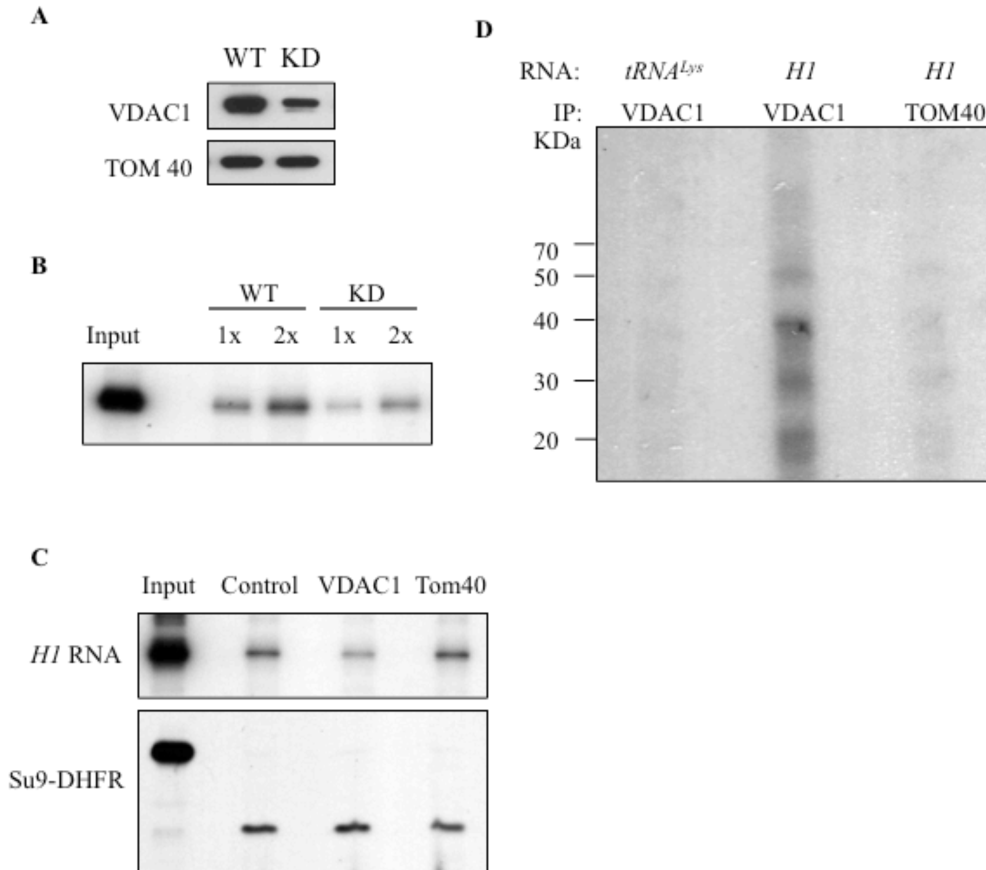
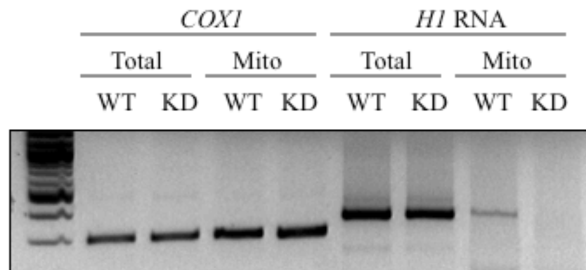


Figure 2-3. ***RNase P RNA* translocates through porin/VDAC in mammalian cells.** (A) Western showing VDAC1 knockdown. (B) Radiolabeled *RNase P* RNA was imported into isolated *VDAC1* knock down HeLa cell mitochondria and detected by autoradiography. (C) Antibody blocking assay showing import of *RNase P* RNA is partially blocked by VDAC1 antibody treatment, which has no effect on protein import. (D) Crosslinking of radiolabeled *RNase P* RNA and immunoprecipitation of porin/VDAC. $tRNA^{lys}$ was used as negative control for non-imported RNA and TOM40 was used as a negative control for immunoprecipitation.



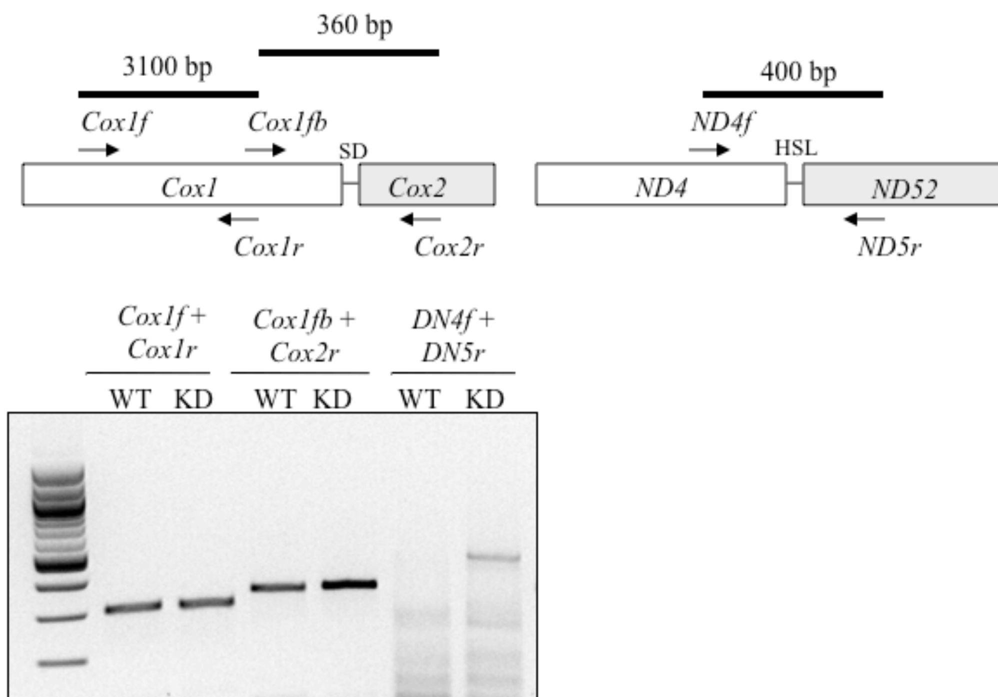


Figure 2-4. **VDAC1 knockdown affects steady state levels of *H1* RNA in the mitochondria and processing of mitochondrial transcripts.** (A) RT-PCR showing steady state levels of *H1* RNA. (B) RT-PCR showing that unprocessed mitochondrial transcripts accumulate in the VDAC knockdown cells.

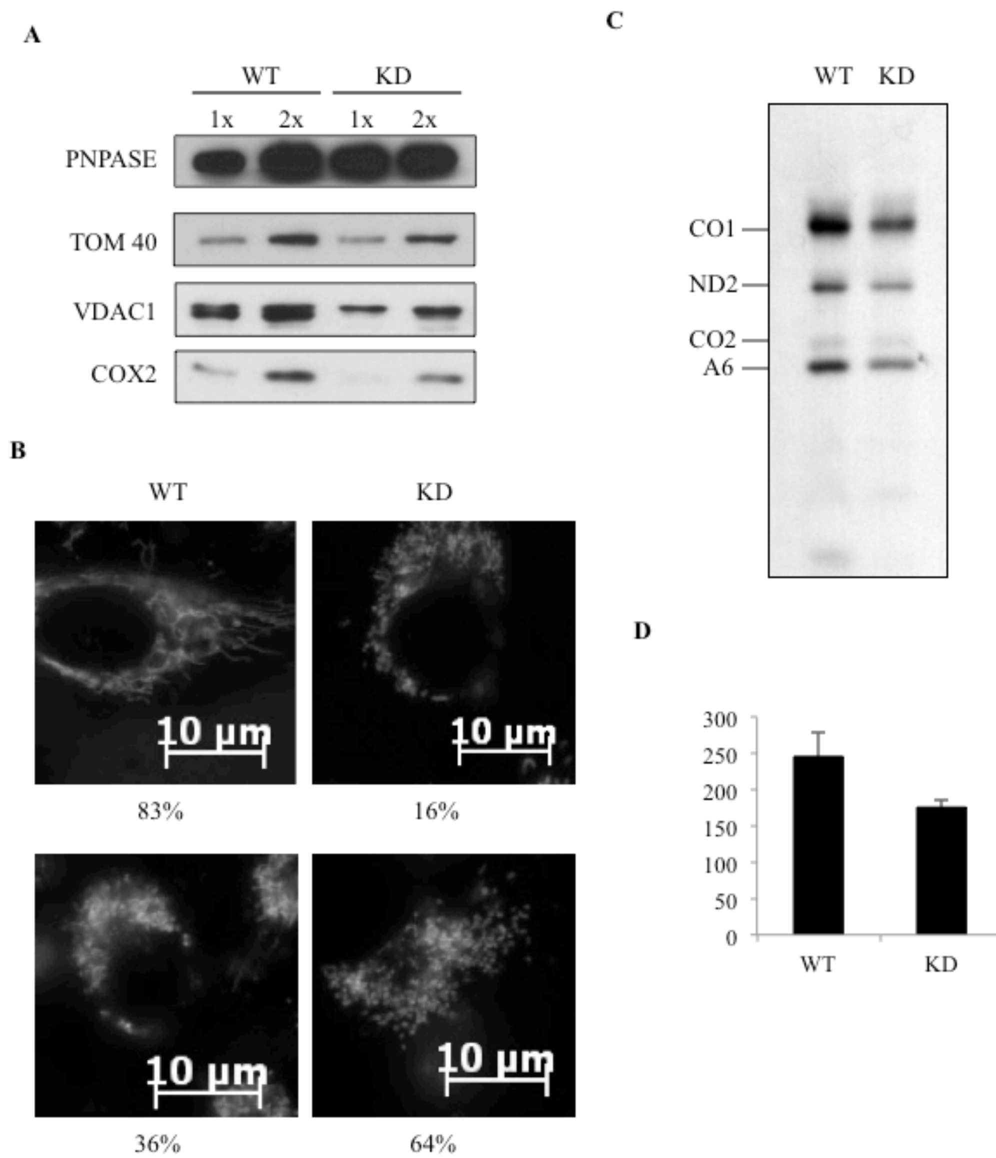


Figure 2-5. **VDAC1 knockdown shows similar effect on mitochondrial functions as PNPASE knockdown.** (A) Western blot showing that the steady state protein level of mitochondrion-encoded COX2 is lower in VDAC1 knockdown cells. (B) Immunofluorescence image demonstrates that the mitochondria are fragmented in

VDAC1 knockdown cells. 8 fields, over 160 cells were examined for each strain. (C) *In organello* translation reveals that defect in mitochondrial translation for VDAC1 knockdown cells. (D) Seahorse measurement of oxygen consumption demonstrates that VDAC1 knockdown cells do not respire as well as wildtype cells.

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Chapter 3: PNPase knockout results in mtDNA loss and an altered metabolic gene expression program

Abstract

Polynucleotide phosphorylase (PNPase) is an essential mitochondria-localized exoribonuclease implicated in multiple biological processes and human disorders. To reveal role(s) for PNPase in mitochondria, we established PNPase knockout (PKO) systems by first shifting culture conditions to enable cell growth with defective respiration. Interestingly, PKO established in mouse embryonic fibroblasts (MEFs) resulted in loss of mitochondrial DNA (mtDNA). The transcriptional profile of PKO cells were similar to rho^0 mtDNA deleted cells, with perturbations seen in cholesterol (FDR=6.35 x 10^{-13}), lipid (FDR=3.21 x 10^{-11}), and secondary alcohol (FDR=1.04x10^{-12}) metabolic pathways compared to wild type parental TM6 MEF cells. We found that processes related to axonogenesis (FDR=4.49 x 10^{-3}), axon development (FDR = 4.74 x 10^{-3}), and axonal guidance (FDR = 4.74 x 10^{-3}) were overrepresented in PKO cells. This was intriguing considering that mutations in PNPase cause delayed myelination, hearing loss, encephalomyopathy, and chorioretinal defects in humans. Because over representation analysis revealed alterations in metabolic pathways in both PKO and rho^0 cells, we assessed the correlation of genes implicated in cell cycle progression (KEGG: MMU04110) and total metabolic gene sets (KEGG: MMU01100) and observed a strong positive correlation between PKO cells and rho^0 MEFs compared to TM6 cells. As expected from the transcriptomic profiles, we observe the proliferation rate of PKO cells clones to be 1.7% (SD ±

2.0%) and 2.4% (SD ± 1.6%) of normalized cell biomass per hour, which was lower than TM6 cells at 3.3% (SD ± 3.5%). Furthermore, PKO in mouse inner ear hair cells cause progressive hearing loss that parallel human familial hearing loss previously linked to mutations in PNPase. Combined, our study reports a novel observation that a knockout of a mitochondrial nuclease results in mtDNA loss and suggest that mtDNA maintenance could provide a unifying connection for the large number of biological activities reported for PNPase.

Introduction

Polynucleotide phosphorylase (PNPase) is a conserved 3'-5' exoribonuclease that bacteria and most eukarya express, but is absent in archae (1,2). In addition to phosphorolytic RNA degrading activity, bacterial PNPase catalyzes template independent polymerization of RNA (3,4). The enzymatic features of bacterial PNPase have been well studied (4-10) and recent discoveries reveal bacterial PNPase involvement in modulating levels of multiple mRNAs and sRNAs (4,11-13), an etiology in cold-shock (14-16) and oxidative stress responses (17), biofilm formation (18-20), virulence (21), and even DNA recombination, repair and mutagenesis (22-25).

Similar to its bacterial counterpart, mammalian PNPase has several roles in RNA homeostasis, and it has also been found to function within mitochondria. Constitutive PNPase knockout (PKO) in mice is lethal at embryonic day 8, because PNPase is essential for maintaining mitochondrial homeostasis in all cell types (26,27). Mammalian PNPase exhibits enzymatic features that are similar to bacterial PNPase with a different optimal phosphate concentration for RNA degradation (28,29). In mitochondria, PNPase localizes to both the

intermembrane space (IMS) and matrix compartments as revealed in several model systems and by multiple localization methods (26,27,30,31). Knockdown studies have shown variable effects on the processing and polyadenylation of mitochondrial RNA (mtRNA) that is transcribed from mitochondrial DNA (mtDNA) (32-34). Recent studies have established that PNPase and the hSUV3 RNA helicase form a mtRNA degrading complex and degrade mirror-mtRNAs, which are noncoding mtRNAs that are antisense and complementary to coding mtRNAs (31,34,35).

Also similar to bacterial PNPase, mammalian PNPase participates in many physiologic pathways beyond mtRNA regulation. One study suggests that PNPase regulates c-Myc levels possibly through interactions with EGFR (36), whereas another study suggests PNPase controls an interferon-β (IFNβ)-induced reduction of c-Myc mRNA (37). PNPase modulates an IFNβ-induced decrease of mir-221 levels that results in growth inhibition in melanoma cells (38) and it regulates nucleus encoded small non-coding RNA import into mitochondria (27,39). PNPase is a type-I IFN induced gene (40) and over-expression at supra-physiologic levels affects reactive oxygen species (ROS) generation and NF-κB activation (41). Recently, PNPase was linked to metabolism control during somatic cell reprogramming to induced pluripotent stem cells (42,43). In humans, PNPase mutations genetically link to hereditary hearing loss, encephalomyopathy, and axonal and auditory neuropathy, gut disturbances, chorioretinal defects, Leigh syndrome, and delayed myelination (44-49). Combined, PNPase affects many essential cellular processes and pathways that regulate organism physiology and pathology without a unifying theme or underlying mechanism, which requires further investigation.

Almost all investigations to date elevate PNPase to supra-physiologic levels in gain-of-function studies or use knockdown approaches with incomplete loss of PNPase in loss-of-function work to gain insight into mammalian PNPase activities. A few studies examined

changes in gene expression profiles with gain and loss of PNPase function using these approaches to help explain the plethora of impacted physiologic systems (50,51). However, no study eliminated PNPase completely to evaluate the impact on global gene expression and cell function, likely due to the essential role of PNPase for cell growth and survival under usual conditions in vivo and in culture. Given its mitochondrial localization, roles in mtRNA regulation, and its activity in small nucleus-encoded RNA import into mitochondria to control respiration, we reasoned that reducing the dependence of cells on mitochondrial function first could generate a suitable internal cell environment for stable knockout of PNPase. Here, we established complete PKO cells by first reducing immortalized mouse embryonic fibroblast (MEF) dependence on respiration. The resulting PKO cell lines enabled studies of changes in gene expression and cell function directly due to PNPase loss. We also examined the effect of PNPase loss-of-function in inner ear hair cells in mice, as homozygous PNPase mutation or loss links with familial hearing loss in humans (44).

Results

PNPase knockout results in loss of mtDNA and inability to respire

Our previous attempts to generate a PKO MEF line failed because deletion of the PNPase-encoding gene, *Pnpt1*, ultimately caused cell death (27). Given its essential role in maintaining mitochondrial homeostasis and its mitochondrial localization, we considered that PKO might disrupt mitochondrial functions required for survival (26). To test this idea, and because cell lines are known to rely on mitochondria to varying extents, we sought to establish PKO MEFs using a two-step approach. First, we generated a cell line that lacked mtDNA (rho^0)

using *Pnpt1*$^{fl/fl}$ MEFs isolated from our *Pnpt1*$^{fl/fl}$ mouse colony. SV-40 large T-antigen immortalized *Pnpt1*$^{fl/fl}$ MEFs, designated TM6, were incubated in ditercalinium chloride supplemented with uridine for 3 weeks to eliminate the mtDNA (52). PCR validated, respiration defective rho^0 *Pnpt1*$^{fl/fl}$ MEFs were then infected with an adenovirus expressing Cre recombinase to delete the *loxP*-flanked portion of exon 2 in *Pnpt1,* generating non-functional PNPase-encoding alleles that translate out-of-frame with multiple stop codons in exons 3, 4, and 6 (27). Using this strategy, we obtained numerous independent rho^0 PKO MEF clones, indicating that eliminating mtDNA first provides a permissive internal cell environment for the complete loss of PNPase (data not shown).

rho^0 mammalian cells are pyrimidine auxotrophs that require uridine media supplementation to grow due to an inactive dihydroorotate dehydrogenase enzyme in the mitochondrial inner membrane, resulting from a non-functional electron transport chain (ETC) (53). Encouraged by generation of a PKO in rho^0 MEFs, we considered whether a simple pre-conditioning of mtDNA-containing MEFs with rho^0 permissive, uridine-supplemented media, rather than by chemical removal of mtDNA, would result in PKO MEF lines. Therefore, we incubated *Pnpt1*$^{fl/fl}$ TM6 MEFs in uridine-containing media for 3 weeks, followed by infection with an adenovirus expressing Cre recombinase. Multiple individual PKO MEF clones emerged from a background of dead and dying cells that were isolated, expanded, and 5 clones were PCR verified for complete loss of *loxP*-flanked exon 2 in the *Pnpt1* gene, as performed previously using internal and external PCR primer sets (Figure 3-1A) (27). We selected 3 PKO MEF clones, designated PKO-1, PKO-4, and PKO-6, for additional analyses. Immunoblotting revealed undetectable PNPase protein in these 3 PKO clone lines, in contrast with strong PNPase expression from *Pnpt1*$^{fl/fl}$ TM6 and rho^0 MEFs (Figure 3-1B). Thus, mtDNA-containing MEFs

conditioned to grow in uridine-supplemented media tolerate PKO, whereas MEFs in standard growth media are non-permissive for PNPase loss.

To examine the impact of PKO on cell function, we first determined the effects on mtDNA content because chemically induced loss of mtDNA enabled subsequent PKO. Surprisingly, PCR detection of the *Nd1* mitochondrial gene showed its absence in all 3 PKO clones (Figure 3-1C). This result suggested that PKO MEFs lost mtDNA, which we further examined by PicoGreen double-stranded DNA dye staining using fluorescence microscopy (Figure 3-1D). Whereas the TM6 MEF cells displayed green puncta in the cytosol marking abundant mtDNA, rho^0 and PKO MEFs lacked green cytosolic puncta, confirming the loss of mtDNA. All 3 PKO clones and rho^0 MEFs showed no respiration, which contrasted with TM6 MEFs, as measured by a Seahorse XF24 extracellular flux analyzer. This result was consistent with loss of mtDNA and resultant failure of the ETC (Figure 3-1E). Rather, these cells manufacture energy by substantially increasing glycolysis and show an elevated extracellular acidification rate (ECAR) that was ~two-fold higher than TM6 MEFs (Figure 3-1F). In cells with low or absent respiration, ECAR is a good approximation of glycolysis and lactate excretion. A plot of the ratio of OCR/ECAR provides a remarkable graphic display for the metabolic alteration caused by PKO (Figure 3-1G). Combined, these genetic, protein, and functional data indicate that MEFs that lose PNPase cannot respire or maintain the mitochondrial genome.

Transcriptional, growth and cell cycle profiles are similar for PKO and rho0 cells

To determine the effect of PNPase loss on gene expression and the potential to alter cell physiology, we performed RNA-Seq analyses on TM6 MEFs, rho^0 cells derived from TM6 MEFs and PKO-1 and PKO-6 MEFs. PKO-4 cells were excluded from analysis because of aneuploidy detected by cell cycle profiling (data not shown). Principal components analysis (PCA) indicated extensive transcriptional variance, denoted by the first principal component (PC1), between TM6 MEF cells and the remaining cell lines (Figure 3-2A). Isolated PKO cells clustered away from the parental TM6 cell line and showed variability in inter-clone gene expression with little intra-clone variation (Figure 3-2A). However, PKO-1 and PKO-6 MEFs displayed similar expression signatures to rho^0 TM6 MEFs (Figure 3-2A). A rank-rank hypergeometric overlap (RRHO) analysis calculating the significance between overlaps of averaged PKO and rho^0 expression changes with respect to TM6 indicates strong correlative overlap in expression profiles between PKO and rho^0 cells (maximum $-\log_{10}(P \text{ value}) > 4500$) (Figure 3-2B). We identified a significant overlap in up- and down-regulated transcripts within PKO and rho^0 signatures and additionally sought to identify differentially expressed genes (DEGs) in both expression profiles. Our analysis identified 1,629 DEGs between rho^0 and TM6 MEFs, with a false discovery rate (FDR) adjusted q value below 0.01 and an absolute $\log_2$ fold-change above 0.5, and 1,527 DEGs between PKO and TM6 MEFs using the same thresholds (Figure 3-2C). Of the DEGs expressed by rho^0 and PKO MEFs, 886 genes were in common, as anticipated due to significant overlaps in RRHO analysis and similar cellular physiology with the loss of mtDNA (Figure 3-2C).

We performed Gene Ontology (GO) gene set overrepresentation analysis (ORA) for each area represented in the Venn diagram (Figure 3-2C) and examined DEGs that are specific to rho^0 MEFs (cluster 1) and PKO MEFs (cluster 3) and those DEGs shared between rho^0 and PKO

MEFs (cluster 2). GO terms overrepresented in cluster 1 identified processes including regulation of cell morphogenesis (FDR = 4.45 x 10^{-4}), cell-cell junction assembly (FDR = 8.70 x 10^{-3}), mitochondrial DNA replication (FDR = 0.014), and regulation of protein stability (FDR = 0.026) (Figure 3-2D). Genes whose expression associates with mtDNA replication are elevated in rho^0 and reduced in TM6 MEFs (Figure 3-S1A). These genes include a subunit of ribonucleotide reductase that regulates cytosolic nucleotide pools (*Rrm2b*), an RNase removes an RNA primer in replicating mtDNA (*Rnaseh1*), and a pyrimidine transporter (*Slc25a33*) (Figure 3-S1A).

Amongst pathways overrepresented by PKO and rho^0 MEF DEGs (cluster 2) are cholesterol metabolic processes (FDR = 6.35 x 10^{-13}), sterol metabolic processes (FDR= 6.35 x 10^{-13}), lipid biosynthetic processes (FDR= 3.21 x 10^{-11}), and secondary alcohol synthesis (FDR = 1.04 x 10^{-12}), which were all reduced in both cell types (Figure 3-S1B). Cholesterol and sterol biosynthetic process repression is consistent with data from PNPase shRNA knockdown in melanoma cell lines, reported previously (51).

Pathways pertaining to neuronal function are overrepresented in PKO specific cluster 3 and include axonogenesis (FDR = 4.49 x 10^{-3}), axon development (FDR = 4.74 x 10^{-3}), and axon guidance (FDR = 4.74 x 10^{-3}). Many of these neuronal genes regulate cell migration and adhesion (Figure 3-S1C). Although this study uses MEFs, it is interesting that PNPase expression differentially affects genes that regulate neuronal function, especially since PNPase mutations link to delayed myelination, hearing loss, encephalomyopathy, gut disturbances, and chorioretinal defects in humans (44-49). Axonogenesis (FDR = 1.14 x 10^{-3}) and axon development (FDR = 2.00 x 10^{-4}) pathways are also overrepresented in genes shared between rho^0 and PKO MEF DEGs (cluster 2). However, these axonogenesis and axon development

pathway overrepresented genes (cluster 2) differ from genes in PKO MEF-specific cluster 3 for the same ontologies. Thus, both loss of PNPase and loss of mtDNA may contribute uniquely to neurologic pathologies in patients with PNPase mutations. s

Because of the concordance in gene expression profiles between PKO and rho^0 MEFs (Figure 3-2B), we examined cell growth, cell cycle progression, and metabolic features in these cell types. Genes implicated in mitotic progression (KEGG: MMU04110) show a strong positive correlation for rho^0 and PKO relative to TM6 MEFs (Pearson Correlation Coefficient (PCC) = 0.86, P value < 2.2×10^{-16}) (Figure 3-3A). Furthermore, given the importance of metabolism in cell growth and a key role for cholesterol biosynthesis in cell cycle progression, we assessed the correlation of metabolic gene expression between PKO and rho^0 MEFs (54,55). Total metabolic gene sets (KEGG: MMU01100) showed similar positive correlations for PKO and rho^0 expression profiles relative to TM6 (PCC = 0.79, P value < 2.2×10^{-16}) (Figure 3-3B and Figure 3-S2). We next assessed whether these correlated expression profiles show phenotypic similarity by measuring growth, or biomass accumulation, rates for TM6, rho^0, and PKO MEFs using a quantitative phase microscopy (QPM) technique called live cell interferometry (LCI) (56). As anticipated, rho^0 MEFs grew much slower than TM6 MEFs, and PKO clones accumulated biomass as slowly as rho^0 MEFs (Figure 3-3C). LCI quantification revealed a mean growth rate for TM6 and rho^0 MEFs of 3.3% (SD ± 3.5%) and 1.7% (SD ± 2.7%) of normalized cell biomass per hour, respectively, with a P value of 4.7×10^{-10} (Figure 3-3C). PKO-1 and PKO-6 showed a mean growth rate of 1.7% (SD ± 2.0%) and 2.4% (SD ± 1.6%) of normalized cell biomass per hour, respectively, and with P values compared to the TM6 growth rate of 6.49×10^{-12} and 5.54×10^{-5}, respectively (Figure 3-3C). Furthermore, cell proliferation studies supported LCI quantified growth rate profiling data in that rho^0, PKO-1 and PKO-6 cells replicated much slower than TM6

MEFs (Figure 3-3D). Finally, cell cycle analysis by flow cytometry revealed a trend in which rho^0 MEFs have a higher proportion of cells in S phase compared to TM6 MEFs (Figure 3-3E). This higher S phase trend persists for PKO-1 and PKO-6 MEFs, although the measurement standard deviation is too high for statistical significance (Figure 3-3E). The difference in cell cycling characteristics is easy to appreciate in the flow cytometry profiles of PKO-1 and PKO-6 MEFs, which resemble rho^0 MEFs, whereas the TM6 MEF flow profile has a distinct shape from the others (Figure 3-3F). Thus, integrated RNA-Seq, QPM, proliferation, and cell cycle analyses clearly show that PKO MEFs resemble rho^0 MEFs that do not respire.

PKO in inner ear hair cells causes progressive hearing loss

The PNPase E475G mutation results in hereditary hearing loss (44). We therefore examined PKO for physiological relevance by examining auditory effects in vivo using *Pnpt1$^{fl/fl}$ x Atoh1-Cre* expressing mice (Atoh1-Cre PKO mice). The auditory brainstem recordings of Atoh1-Cre PKO mice showed progressive hearing loss especially at higher frequencies. Up until 4 weeks of age both control and Atoh1-Cre PKO mice showed similar hearing capacities between 4 Hz and 16 Hz (Figure 3-4A). However, even at 3 weeks of age, the hearing capacity of Atoh1-Cre PKO mice above 16 Hz was significantly reduced compared to control mice (Figure 3-4A). By week 4, the lowest level of hearing for Atoh1-Cre PKO mice in the high frequency range at 32 kHz was above 78 db in contrast to 39 db for control mice (Figure 3-4A). Scanning electron microscopy (SEM) images of inner ear hair cell stereocilia show loss of cilia (yellow arrow) and stereocilia fusions (red arrow) in the middle turn and base of the cochlea that are responsible for hearing the middle and high frequencies, respectively (Figure 3-4B). Although the loss of PNPase in inner ear hair cells is likely to drive hearing loss, *Atoh1-Cre*

recombinase expression also causes loss of PNPase expression in spiral ganglion cells, and defects in these neurons may contribute to hearing loss in mice. Overall, hearing impairment observed in Atoh1-Cre PKO mice recapitulates the hearing loss observed in PNPase mutation harboring patients and emphasizes the association of PNPase with sensorineural defects.

Discussion

We report that loss of PNPase, an RNA degrading enzyme that localizes in mitochondria, results in unanticipated deletion of the mitochondrial genome. Transcriptome profiling of PKO MEFs correlates significantly with rho0 MEFs and exhibits reduced expression of cholesterol and lipid biosynthesis genes compared to control wild-type TM6 MEFs. Cell growth, proliferation, and cell cycle features are similar between PKO and rho0 MEFs, suggesting a role for PNPase in maintaining mtDNA. PNPase loss-related changes in gene expression programs pertaining to axon function suggest a link with reported mutant PNPase neuronal disease phenotypes in humans, including familial hearing loss. Modeling PNPase loss in mouse inner ear hair cells leads to progressive auditory loss, reinforcing a potential connection uncovered from mining gene expression profiling data presented here.

Since PNPase does not belong to any of the previously identified classes of genes associated with mtDNA maintenance defects, it seems surprising that PKO results in mtDNA loss. Deletions and mutations of genes involved in mtDNA replication and repair (POLG, TWNK, TFAM, RNASEH1, LIG3 and MGME1), cytosolic and mitochondrial nucleotide pool regulating and import genes (TK2, DGUOK, SUCLA2, SUCLG1, ABAT, TYMP, RRM2B, AGK, and MPV17), along with mitochondrial dynamics genes (OPA1 and FBXL4), all result in

mtDNA loss (Shokolenko et al., 2013; El-Hattab et al., 2017). Thus, it is intriguing to speculate on how PNPase may regulate the maintenance of mtDNA in a cell.

We hypothesize several potential reasons why lack of PNPase results in loss of mtDNA. Impaired import of small non-coding RNAs from the cytosol may reduce levels of nucleus-encoded MRP RNA that is required for mtDNA replication. Since accumulation of mirror mtRNAs occurs in PNPase knockdown cells and in cells expressing dominant negative hSUV3, the binding partner of PNPase, it is also possible that accumulation of mirror mtRNAs may somehow inhibit replication of mtDNA (Szczesny et al., 2010; Borowski et al., 2013). PNPase functions in DNA repair in bacterial systems (Cardenas et al., 2009) and mammalian PNPase may have a similar function in mitochondria. We also cannot exclude that the loss of mtDNA may be a result of long-term dysregulated mtRNA metabolism. Although the mechanism(s) for how PNPase maintains mtDNA warrants further investigation, it nevertheless is interesting that PKO causes cells to functionally assume a rho0 phenotype.

PNPase regulates oxidative phosphorylation during reprogramming of somatic cells to induced pluripotent stem cells and is an essential factor for maintaining mitochondrial homeostasis (Chen et al., 2006; Khaw et al., 2015; Nishimura et al., 2017). Understanding the specific functions of PNPase may reveal how RNA regulation may affect cellular metabolism. Here, we established PKO MEF lines utilizing uridine supplemented media and show that PKO cells lose their mtDNA. This brings a new perspective to PNPase activity and suggests that mtDNA levels are important to consider when dissecting PNPase functionality. Also, PNPase loss impairs neuron-relevant gene expression even in non-neuronal cell types and the Atoh1-Cre PKO mice show reduced hearing through auditory brainstem recording analysis. Altogether, our study describes an additional unsuspected function for PNPase in mtDNA maintenance, which

could provide an underlying or unifying connection for its large number of reported activities in multiple biological systems and contexts.

Materials and Methods

Cell Culture and Mice. Pnpt1fl/fl MEFs, named TM6 (Wang et al., 2010), were grown at 37°C and 5% CO2 in Dulbecco's Modified Eagle Medium supplemented with 10% Hyclone Fetal Bovine Serum (Thermo Scientific), 1X penicillin-streptomycin solution (Cellgro, Corning), and 50 μg/mL uridine (Sigma-Aldrich). MEF rho0 cells were established by culturing with 750 ng/mL ditercalinium dichloride dihydrochloride (NCI DCTD Developmental Therapeutics Program, NSC 335153, NIH), for 3 weeks followed by single cell colony isolation. PKO MEF lines were established by transduction of TM6 MEFs with a Cre-recombinase expressing adenovirus (SignaGen Laboratories cat # SL100707) in uridine- supplemented media, followed by isolation of single cell colonies. Atoh1-Cre PKO mice were established by crossing Pnpt1fl/fl C57BL/6J mice with Atoh1-Cre transgenic C57BL/6J mice (Perrin et al., 2010). All mice were housed in a pathogen-free animal facility at UCLA and IUPUI, with approval from the appropriate Institutional Animal Research Committees.

Characterization of PKO MEFs. Western blot was performed using standard molecular biology practices. PNPT 3370 antibody (Koehler/Teitell Labs) and β-tubulin MMS-410P (Covance) were used for immunoblotting. Assessments of mtDNA quantity were performed by PCR using primers for the nucleus-encoded control gene Lpl with forward primer: GATGGACGGTAAGAGTGATTC and reverse primer ATCCAAGGGTAGCAGACAGGT,

and mitochondria-encoded gene, Nd1 with forward primer CCCATTCGCGTTATTCTT and reverse primer AAGTTGATCGTAACGGAAGC. Immunofluorescence microscopy images were obtained from cells treated with Mitotracker Red CMXRos (Thermo Scientific) and PicoGreen dye (Thermo Scientific). OCR and ECAR measurements were performed as previously described (Zhang et al., 2012). The following primers were used to check for the presence of Pnpt1 exon 2 in the genome DNA: forward primer: TATCCTCTGGGAAACTGGCA, reverse primer: ATTCGTACTGCCCCAACAGG. The following primers were used to check for the deletion of exon 2 in Pnpt1 that spans the exon 2 region: forward primer: TCGGGCACTCAGCTATTTGC, reverse primer: CACCAACGGCATGAATTGGG. The following primers were used to check for the presence of the 3' end of Pnpt1: forward primer: ACCGCGACAATAACTGAAATC, reverse primer: GCAGCACTGCAGTCATGTTT..

RNA Extraction. TM6, rho0 MEFs, and PKO clones were grown in biological triplicates to 70-80% confluence and purified using TriZol Reagent (Life Technologies) or RNeasy Mini Kit (Qiagen). All samples used showed a A260/280 ratio > 2.00 (Nanodrop; Thermo Scientific).

RNA-Seq Library Preparation. Strand-specific ribosomal RNA (rRNA) depleted RNA-Seq libraries were prepared from 1 μg of total RNA using the KAPA Stranded RNA-Seq Kit with Ribo-Erase (Kapa Biosystems, Roche). Briefly, rRNA was depleted from total RNA samples, the remaining RNA was heat fragmented, and strand-specific cDNA was synthesized using a first strand random priming and second strand dUTP incorporation approach. Fragments were then A-tailed, adapters were ligated, and libraries were amplified using high-fidelity PCR. All libraries

were prepared in technical duplicates per sample (n = 12 samples, 24 libraries total), and resulting raw sequencing reads merged for downstream alignment and analysis. Libraries were paired-end sequenced at 2x125bp on an Illumina HiSeq.

RNA-Seq Pre-Processing. PKO clones 1 and 6, rho0, and TM6 MEFs were each sequenced in biological triplicates (n = 3, 12 total samples). Raw sequencing reads were converted into fastq files and filtered for low quality reads and Illumina sequencing adapter contamination using bcl2fastq (Illumina). Trimmed reads were then aligned to the Mus musculus transcriptome, generated using the NCBI mm10 (NCBI/mm10/GRCm38, December 2011) genome and RefSeq gene annotation, using RSEM 1.2.25 prepare-reference (command parameters --bowtie --bowtie2 --gtf $(BUILD)/genes.gtf $(GENOME).fa $(RSEM)) (Li and Dewey, 2011). Transcript counts were estimated using RSEM 1.2.25 calculate-expression, and collapsed to gene level counts using RSEM 1.2.25 tbam2gbam (Li and Dewey, 2011). Gene-level transcript counts were extracted from the results output using custom R/3.4.1 scripts.

Differential Gene Expression Analysis. The resulting sample gene count matrix was size factor normalized and analyzed for pairwise differential gene expression using R/3.4.2 Bioconductor 3.6 package DESeq2 v1.18.1. Expression changes were estimated using an empirical Bayes procedure to generate moderated fold change values (Love et al., 2014; Huber et al., 2015). Significance testing was performed using the Wald test, and resulting P values were adjusted for multiple testing using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). DEGs were filtered using an adjusted false discovery rate (FDR) q value < 0.01 and an absolute log2 transformed fold-change > 0.5. Variance stabilized transform (VST) values in the gene

count matrix were calculated and plotted for principal component analysis (PCA) using DESeq2 (Love et al., 2014; Huber et al., 2015). Scatterplots of gene expression fold-changes between TM6, rho0 and PKO MEFs were performed and Pearson/Spearman correlation coefficients calculated using R/3.4.1 package ggpubr v0.1.6 (https://cran.r-project.org/web/packages/ggpubr/index.html). Genes of interest were extracted and heat maps were prepared using R Bioconductor packages pheatmap v1.0.8 and gplots v3.0.1 (Kolde, 2015; Gregory Warnes, 2016).

Gene Set Overrepresentation Analysis (ORA). DEGs were extracted and analyzed for pathway/gene ontology (GO) term overrepresentation using the R/3.4.1 Bioconductor 3.6 package clusterProfiler v3.6.0 and ReactomePA v1.22.0, using a background gene set of all genes expressed with at least one read count in the sample gene count matrix (Yu et al., 2012a; Yu and He, 2016). Overrepresented Reactome/KEGG pathways and GO terms were identified using significance testing cutoffs of $P < 0.05$, and an adjusted $FDR < 0.25$.

Rank-Rank Hypergeometric Overlap Analysis (RRHO). To determine overlapping significance between PKO and rho0 expression patterns relative to TM6, rank-rank hypergeometric overlap was performed using the online web server (http://systems.crump.ucla.edu/rankrank/rankranksimple.php) (Plaisier et al., 2010). Separate gene ranking lists were constructed according to the signed log10 transformed Wald test q value in fold change comparisons between PKO/TM6 and rho0/TM6. Hypergeometric testing was then performed to determine the significance of overlap between ranks in both datasets. Heat map values were plotted as the signed log10 transformed hypergeometric P value of overlap between

ranks at the identified pixel; high values indicate enrichment of overlap, low values indicate reduced enrichment of overlap.

Live Cell Interferometry (LCI). Cells were plated into each well of an Ibidi 4 well Ph+ μ-slide (Ibidi, USA) at a density of 6 x103 cells/cm2. 24 hours later, Ibidi anti-evaporation oil (Ibidi, USA) was added to seal the liquid opening of each well and loaded onto the LCI stage. All 4 wells were imaged continuously, every 10 minutes for 24 hours with 15-20 locations per well. The LCI set up consists of a Zeiss Axio Observer Z1 inverted microscope, an on-stage incubation chamber (Zeiss) with temperature, CO2 and humidity modulations. QPM images were captured by a SID4BIO (Phasics) quadriwave lateral shearing interferometry camera. All cells were imaged using a 20x 0.4NA objective and a 660nm collimated LED (Thorlabs) light source.

Cell Cycle Flow Cytometric Analysis. Cells (~5 x 105) were collected, washed once with 500 μl FACS buffer and resuspended in 200 μl hypotonic propidium iodide staining solution containing 10 mg/ml RNAse, 10% Triton X-100, 85 mg/ml trisodium citrate and 2 mg/ml PI (Roche). Data was obtained on FACS BD LSRII and FACS BD Fortessa flow cytometers (BD Biosciences). Cell cycle analysis was performed with FlowJo software using the univariate model..

Auditory Brainstem Response (ABR). ABR waveforms were collected for frequencies between 4 kHz and 32 kHz at half-octave intervals, starting at supra-threshold levels and decreasing in 5 dB steps to a sub-threshold level. A Tucker-Davis Technologies System 3 was

used to generate symmetrically shaped tone bursts 1 ms in duration with 300 μs raised cosine ramps that were delivered to a calibrated magnetic speaker. Mice were anesthetized with Avertin and scalp potentials were recorded with subdermal electrodes, with signals amplified 20,000 times, bandpass filtered between 0.03 and 10 kHz, digitized using a 20,000 kHz sampling rate and subjected to artifact rejection. Stacked waveforms were compared and the lowest level of stimulation that evoked an unambiguous ABR waveform was designated as the threshold.

Scanning Electron Microscopy (SEM). Mouse cochlea were dissected and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer with 1 mM CaCl2¬ by perfusing dissected cochlea through the round and oval windows followed by incubation in the same solution at RT for 4 h. Following decalcification in 170 mM EDTA at 4ºC for 16 h, the organ of Corti was dissected and processed for SEM as described (Belyantseva et al., 2009). Briefly, tissues were successively incubated in 2% each arginine, glycine, glutamic acid and sucrose in water, 2% each tannic acid and guanidine-HCl in water and then 1% osmium tetroxide. Samples were critical point dried from CO¬2 and sputter coated with platinum before viewing on a cold field emission SEM (Hitachi S4700).

Accession Numbers. All raw RNA-Seq reads and processed gene count matrices are in submission to the NCBI Short Read Archive (SRA) and Gene Expression Omnibus (GEO), respectively. GEO accession numbers for these data will be provided upon completion.

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Figures

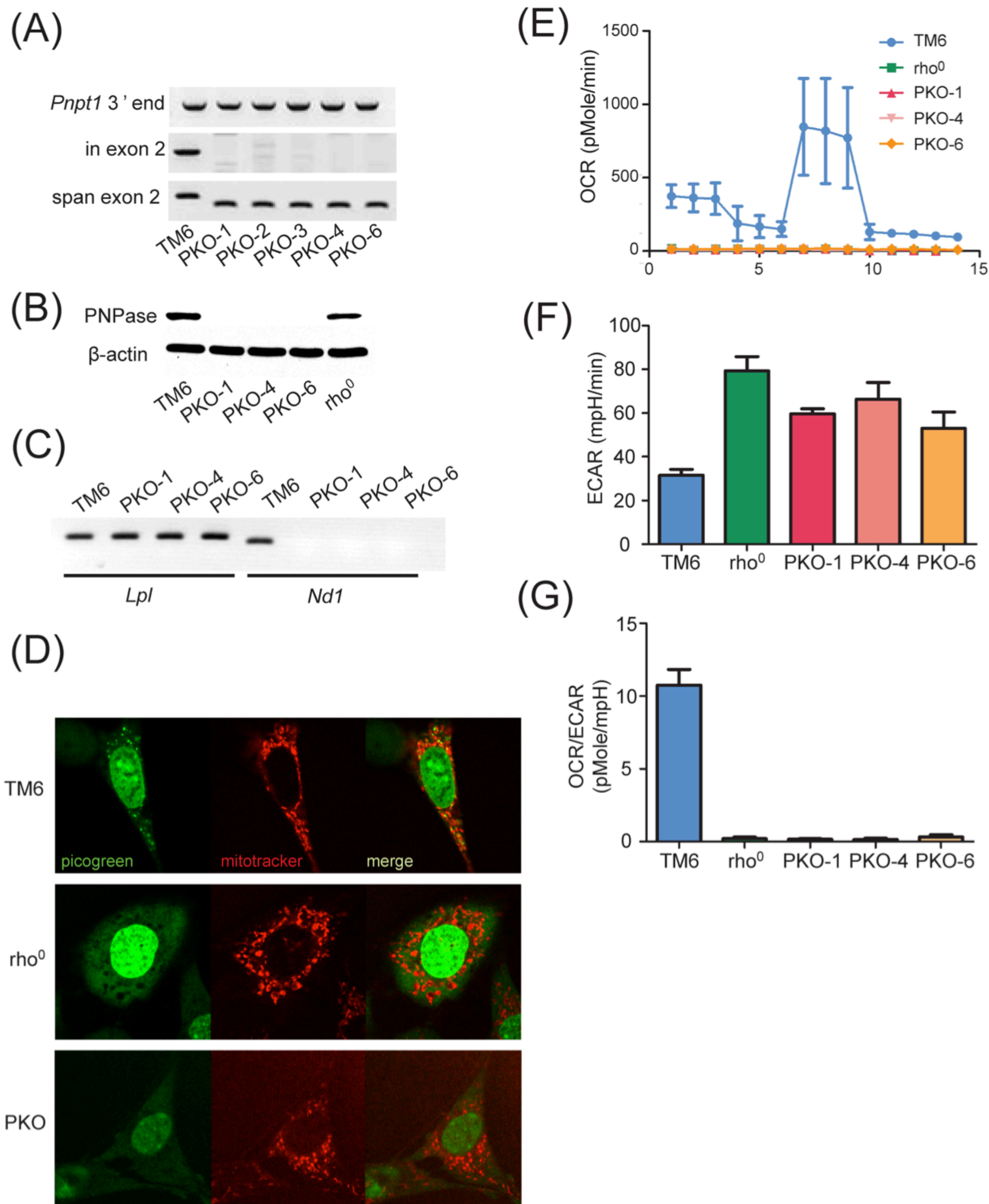


Figure 3-1. **PKO results in loss of mtDNA.** (A) PCR to evaluate Pnpt1 (encoding PNPase) exon 2 deletion in the parental MEF line, TM6, and 5 independent PKO lines (n = 5). Three primer sets include one to examine Pnpt1 exon 2 (in exon 2), a flanking set to capture deletion of

exon 2 (span exon 2), and a control set that amplified the 3’ end of Pnpt1 (PNPase 3’ end). (B) Representative immunoblot for PNPase protein in TM6 and 3 PKO clones. Total protein lysates were separated by SDS-PAGE and analyzed using polyclonal antibodies that target PNPase and β-tubulin. (C) PCR analysis of a control nucleus encoded Lpl gene and the mtDNA encoded Nd1 gene in TM6 and 3 PKO cell lines. (D) Fluorescence microscopy of TM6, rho0, and representative PKO (PKO-4) MEF cell lines with PicoGreen staining for double-stranded DNA (left), MitoTracker Red (center), and an overlay (right). (E-G) Respiration analysis using a Seahorse XF24 Extracellular Flux Analyzer on the cell lines in (B). Experiments were performed in biological triplicates ($n = 3, \pm$ SD) and normalized to protein content. (E) Oxygen consumption rate (OCR), (F) extracellular acidification rate (ECAR), and (G) OCR/ECAR ratios are shown ($n = 3, \pm$ SD).

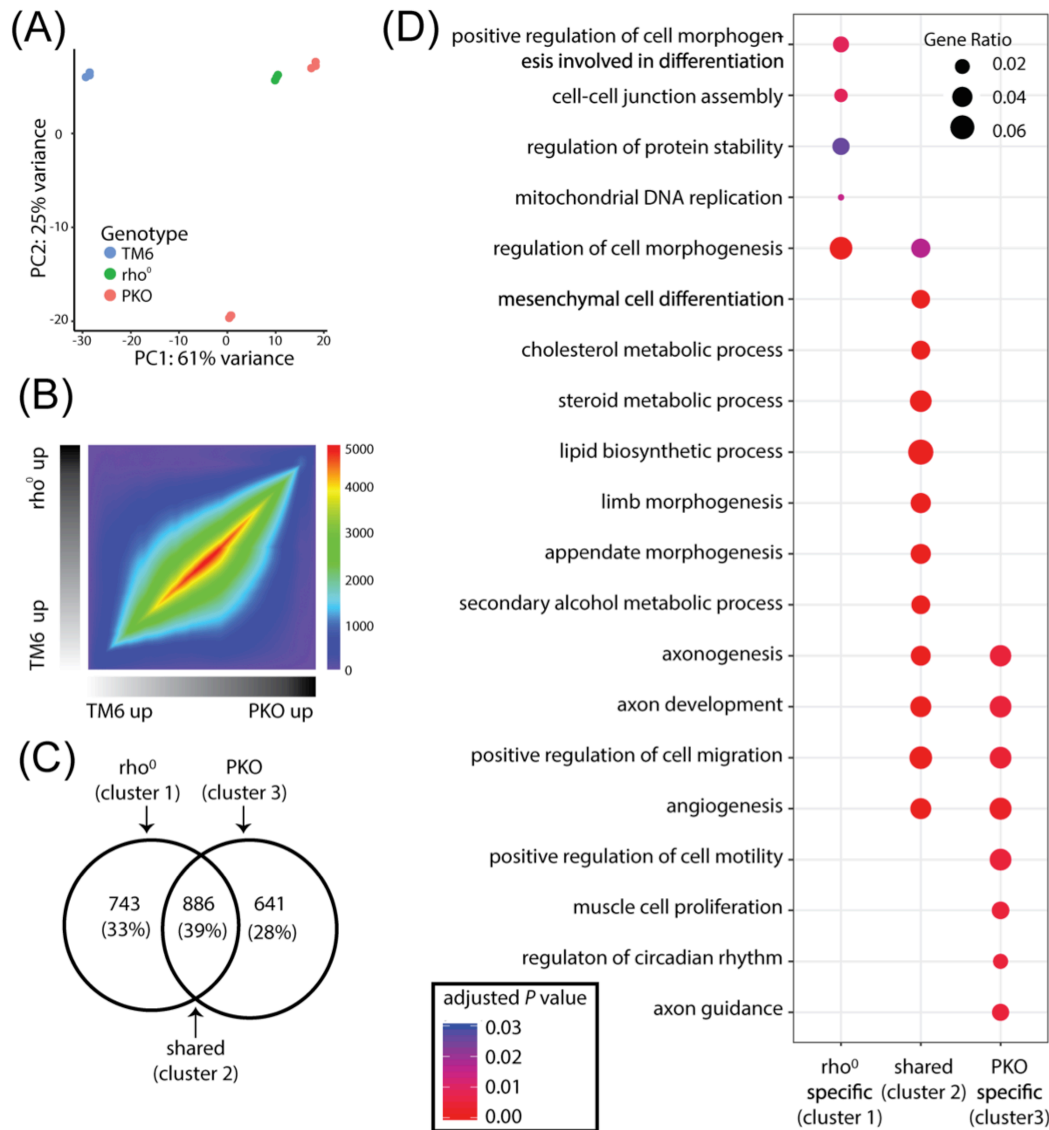


Figure 3-2. **PKO cell gene expression patterns converge and diverge with rho0 MEFs.** (A) Principal Component Analysis (PCA) of TM6, rho0, PKO-1, and PKO-6 MEFs (n = 3 biological replicates per line; 12 replicates total). Expression profiles for each sample were plotted for their PC1 and PC2 scores, providing primary and secondary sources of variance, respectively. Samples were color coded based on cell type. (B) Rank-rank hypergeometric overlap (RRHO) maps of the log2 fold-change expression profiles between PKO and rho0 MEFs with respect to

TM6 MEFs. Color coding represents –log10 transformed hypergeometric P values, with higher values indicating strength of overlapping enrichment between PKO and rho0 gene lists. (C) Venn diagram of differentially expressed genes (DEGs) between rho0 MEFs (left) and PKO clones (right) with respect to TM6 MEFs. DEGs were identified in each comparison using an absolute log2 fold-change threshold above 0.5 and a false discovery rate (FDR) adjusted Wald q value below 0.01. Clusters indicate rho0 specific (cluster 1), PKO specific (cluster 3), and shared DEGs (cluster 2). (D) Dotplot of Gene Ontology (GO) overrepresentation analysis (ORA) of the rho0 specific, shared, and PKO specific DEG clusters. Dot size indicates the ratio of genes from the selected ontology set in the DEG cluster, color indicates significance of gene set overrepresentation in the cluster (FDR adjusted P value) following hypergeometric significance testing.

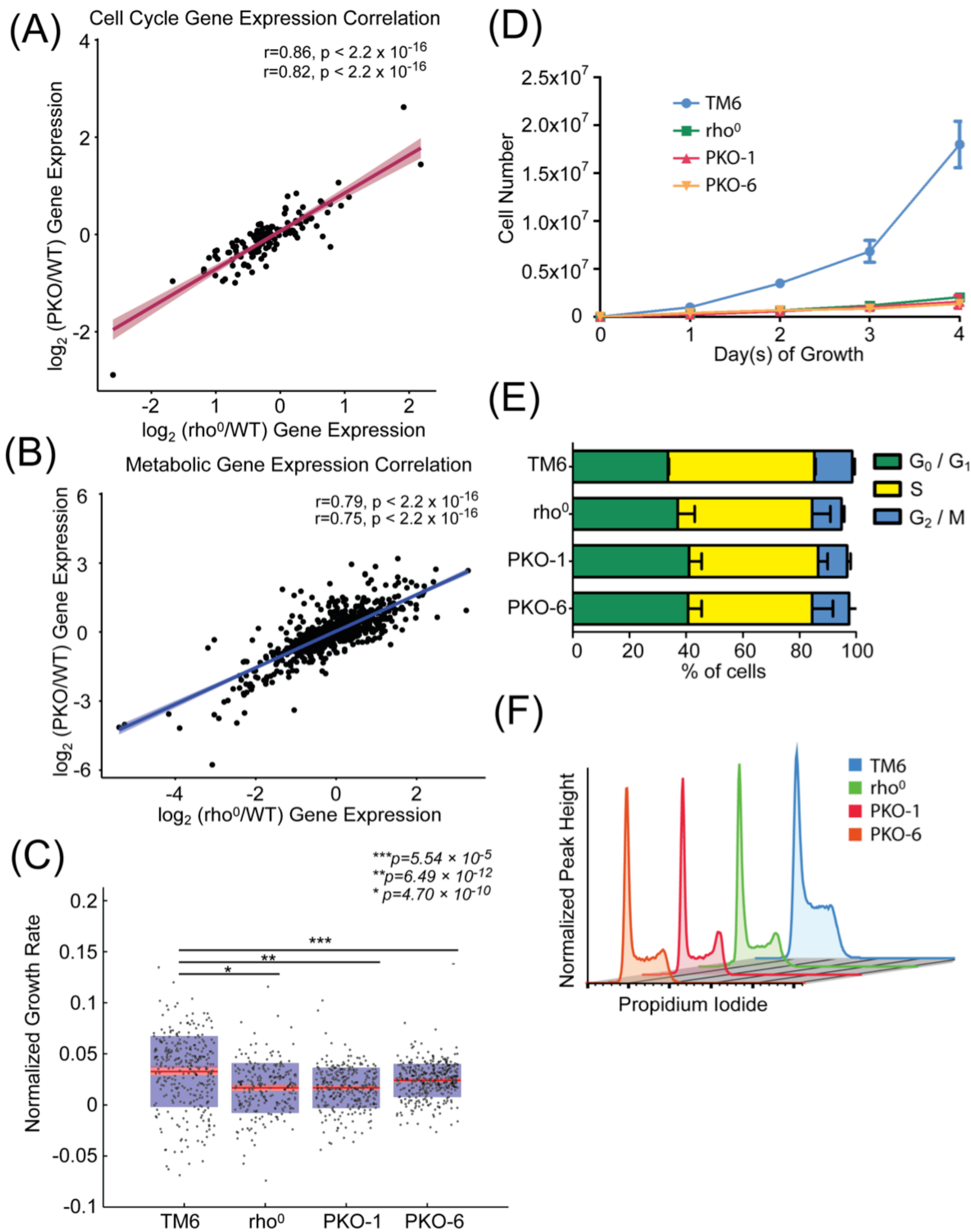


Figure 3-3. **PKO and rho0 MEFs have similar cell growth, cell cycle and metabolic gene expression profiles.** (A-B) Scatterplot of (A) mitotic progression and (B) metabolic gene expression changes between PKO (y-axis) and rho0 (x-axis) MEFs with respect to TM6 MEFs (calculated as log2 fold-change) (n = 3 biological replicates per line, 12 total). Linear regression lines were fit and Pearson (top value) and Spearman (bottom value) correlation coefficients were

calculated with accompanying P values calculated using two-tailed t significance tests. Gene sets were derived from KEGG database cell cycle ID MMU04110 (a) and metabolic ID MMU00100 (B). (C) Growth (biomass accumulation) rates for TM6, rho0, PKO-1, and PKO-6 cells were quantified by live cell interferometry (TM6 n=308, rho0: n=233, PKO-1: n=303 PKO-6: n =364). (Median=red stripe, 95% confidence interval=pink region ± SD = purple region. (D) Proliferation of TM6, rho0, PKO-1 and PKO-6 MEFs by manual cell counting (n = 3, ± SD). (E) Stacked barplots of phases of the cell cycle in percentage distributions for TM6, rho0, PKO-1, and PKO-6 MEFs (n = 3, ± SD). (F) Representative cell cycle analysis profiles for TM6, rho0, PKO-1, and PKO-6 MEFs. DNA was stained with propidium iodide and profiles assembled by f flow cytometry.

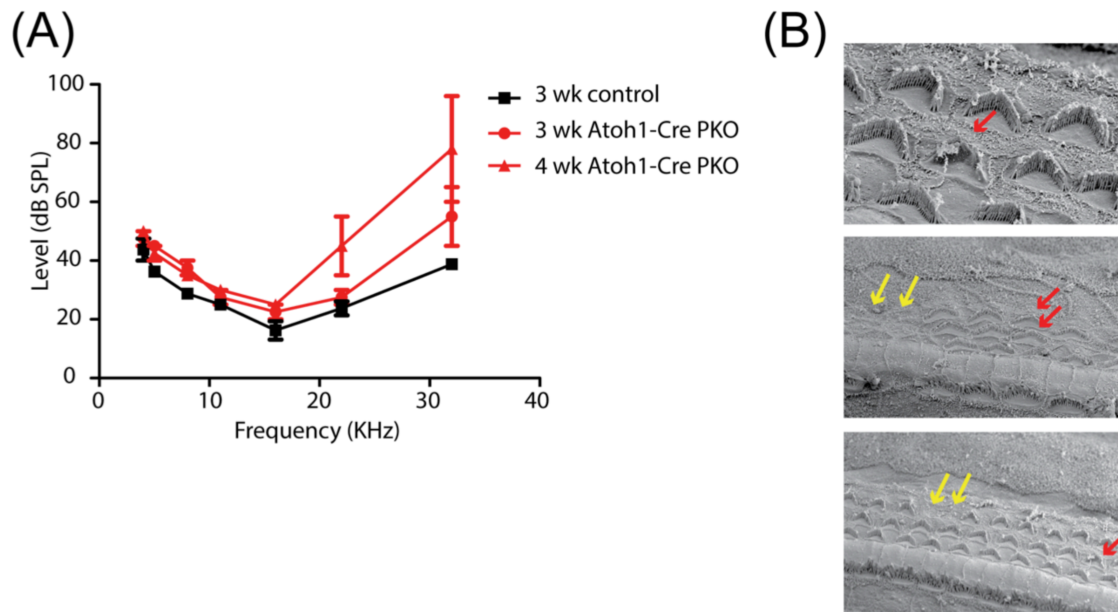
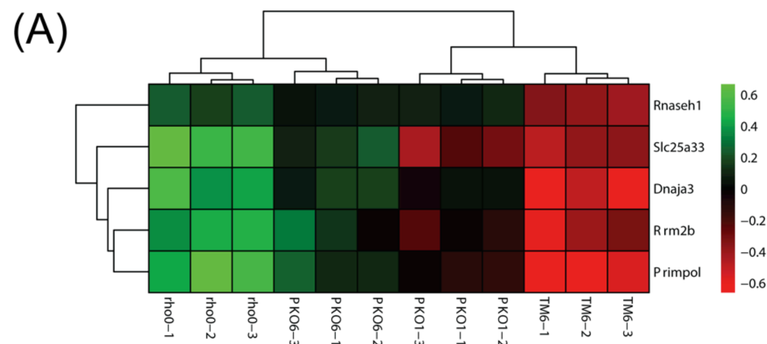
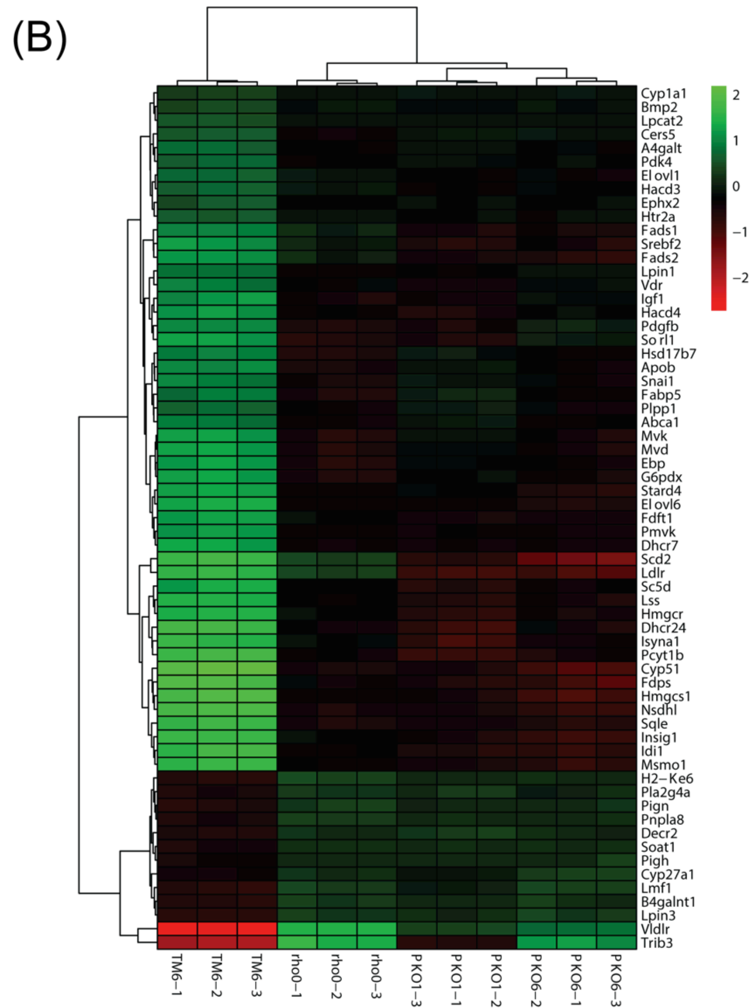


Figure 3-4. **Loss of PNPase results in hearing loss.** (A) Auditory brainstem response test for WT (black) and Atoh1-Cre PKO mice (red) at 3 weeks and 4 weeks (n = 2). (B) SEM analysis of hair cell stereocilia. Yellow arrows indicate regions that lack cilia, and red arrows indicate regions of stereocilia fusion.

Supplementary Figures





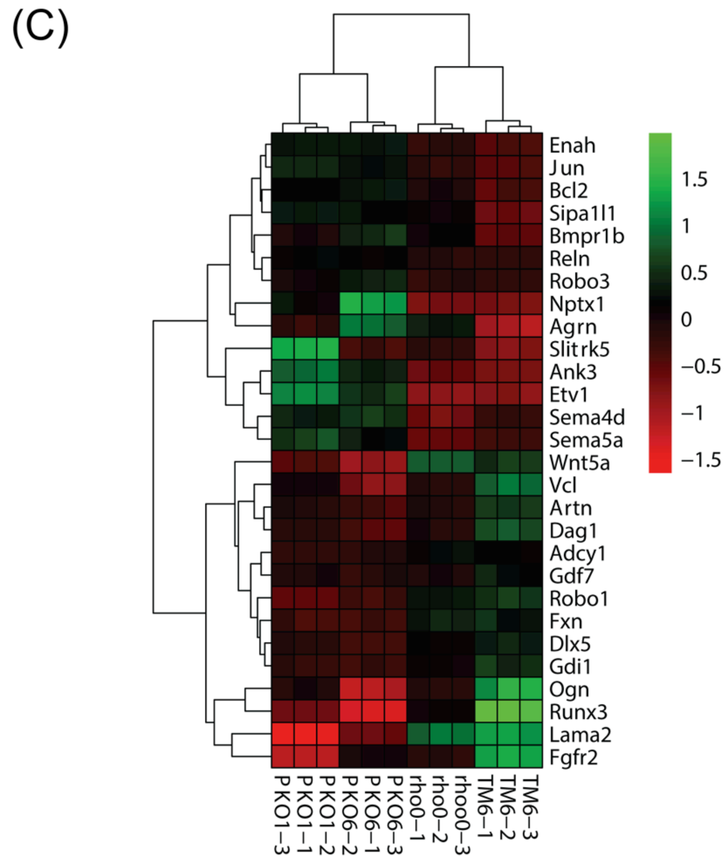


Figure S3-1. Heatmaps of selected overrepresented pathways in DEG clusters (related to Figure 3-2D). (A) Heat map of mtDNA replication genes (GO:0006264) overrepresented in cluster 1. (B) Cholesterol metabolic (GO:0008203), sterol metabolic (GO:0016125), lipid biosynthetic (GO:0008610), and secondary alcohol synthetic (GO:1902652) processes overrepresented in cluster 2. (C) Axonogenesis (GO:0007409), axon guidance (GO:0007411), and axon development (GO:0061564) genes overrepresented in cluster 3. Heat maps are hierarchically clustered based on Euclidean distance and Ward linkage. Heat map values are plotted as the variance stabilized transform (VST) subtracted by the gene row average mean of samples. (n = 3 biological replicates, 12 total).

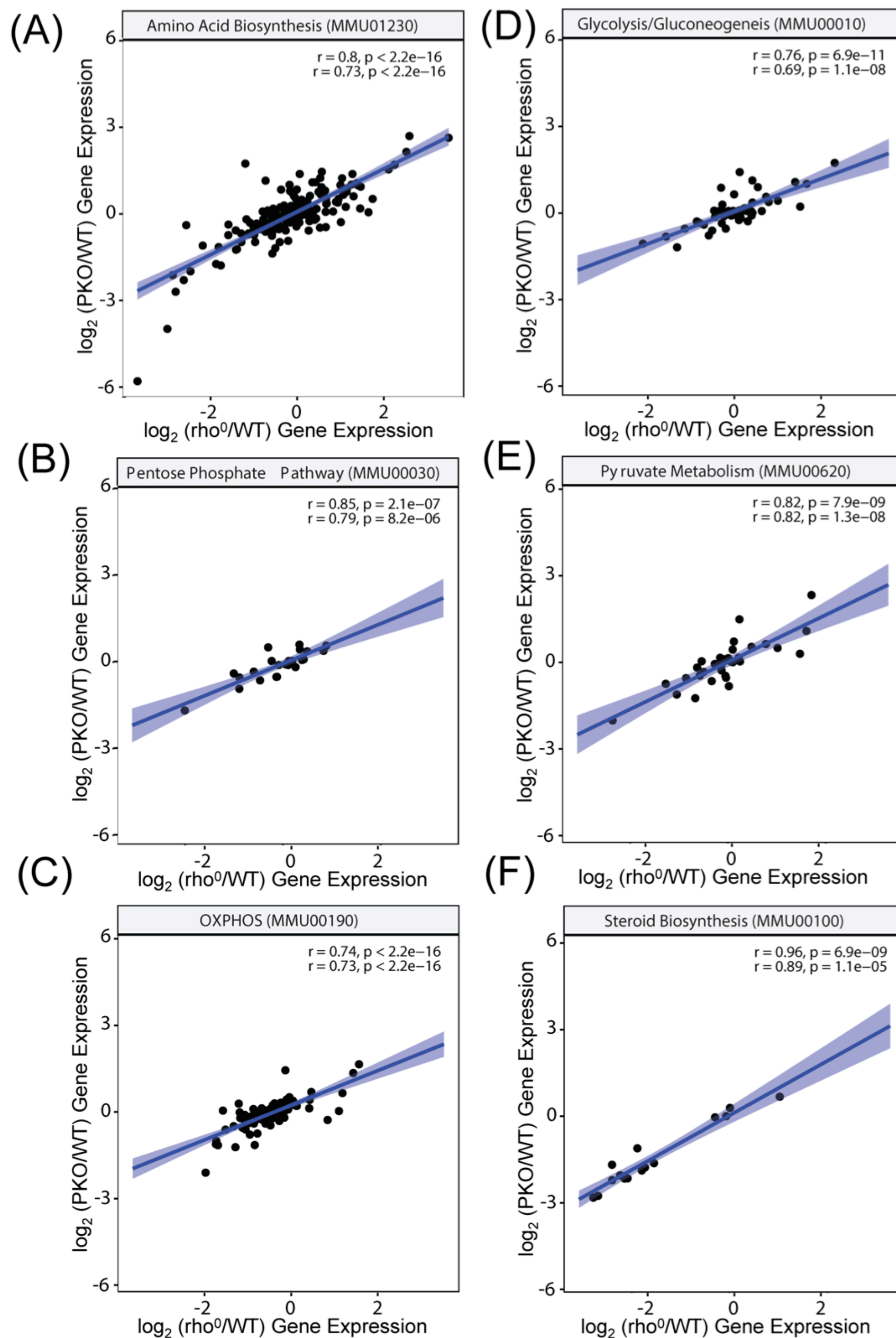


Figure S3-2. PKO and rho0 MEFs show highly correlated metabolic gene expression profiles (related to Figure 3-3B). Scatterplot of metabolic gene expression values between PKO (y-axis) and rho0 (x-axis) MEFs with respect to WT TM6 MEFs (calculated as log2 fold-

change). Linear regression lines were fit and Pearson (top value) and Spearman (bottom value) correlation coefficients were calculated with accompanying significance P values calculated using two-tailed t significance tests. Gene sets were derived from the KEGG database under the identification numbers indicated above each plot.

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Chapter 4: PNPase binds mitochondrial tRNAs, affects accumulation of antisense RNAs and binds mitochondrial ribosome assembly and RNA processing factors

Introduction

The mitochondrial DNA (mtDNA) carries genetic materials necessary for mitochondrial function and is uniquely organized with a compact design. The human mitochondrial DNA is a double stranded, circular molecule that is 16.5kb in length and encodes 22 tRNAs, 13 proteins and 2 rRNAs (1). Genes are encoded on both strands of the DNA, which is referred to as the heavy and light strand according to its buoyancy observed in denaturing cesium chloride gradients (2). The mammalian mitochondria DNA is very compact; some termination codons and 3' ends of tRNAs are not encoded in the DNA, it contains no intronic sequences and very few non-coding nucleotides between genes (1). The noncoding region called the control region located between tRNA phenylalanine and tRNA threonine encodes the origin of replication for the heavy strand and the promoter regions for both the heavy and light strand transcription (3). This noncoding region also contains the binding sites for transcription regulating factors (3). Knowing that the mtDNA is much smaller and structured differently than the nuclear DNA, it is understandable that it has an RNA transcription system that is vastly distinct from its nuclear counterpart.

The mitochondria produce polycistronic transcripts and process the individual RNAs from the precursor transcripts. The 3 polycistronic transcripts observed in the mitochondrial are:

H1 transcript (tRNA Phe to end of 16s rRNA), H2 transcript, which includes most of the heavy strand starting at 2 nt upstream of 12 srRNA and L strand transcript, which is transcribed from the opposite strand that encodes 8 tRNAs and ND6 (4,5). These polycistronic transcripts are mostly processed by the excision of tRNAs that flank coding or ribosomal genes, which is commonly referred to as the "tRNA punctuation" model (4,6). Many of the details in RNA processing and degradation for mitochondrial transcripts have not been heavily studied and have only recently become an active area of interest.

Despite efforts in studying nucleases and RNA degradation pathways in the mitochondria, currently there is no clear understanding of the RNA degradation mechanism in mammalian mitochondria. Mammalian mitochondrial RNA is believed to be stabilized by poly-A tails (7,8). However, unstable polyadenylation at 3' ends of truncated transcripts have also been reported and suggests a possible parallel to the polyadenylation assissted RNA decay pathway in the mitochondria (9). Thus the mammalian mitochondrial is thought to contain degradation pathways that are regulated by differential roles of polyadenylation, which can stabilize and trigger degradation through polyadenylation assisted RNA degradation as seen in the nuclear and cytosolic systems (10-12). In addition to endonucleases such as the RNase P complex and RNase Z, which are primarily involved in tRNA processing at 5' and 3' end respectively, there are several nucleases that have been identified in the mitochondria (13). Endonuclease G is a nonspecific DNA/RNA nuclease that is localized in the IMS and has a role during apoptosis (14,15). Rexo2 is in the IMS and matrix and is implicated in degrading oligoribonucleotides (16). The stable poly-A tails of mitochondrial RNAs have been shown to be degraded by PDE12, which is a 2' and 3' phosphodiesterase (17,18). In addition, the knockdown of LACTB2, which cleaves single stranded RNA in vitro, was shown to have a modest effect in

mitochondrial mRNA levels, but nevertheless induced mitochondrial dysfunction and apoptosis (19). The aforementioned nucleases have minor modifying roles for RNA degradation and the field has been interested in identifying the ultimate RNA degradation machinery that is responsible for bulk mitochondrial RNA degradation. Drawing from the DSS1 exoribonuclease/SUV3 RNA helicase RNA degradation complex identified in yeast mitochondria, the human homolog, PNPase exoribonuclease/hSUV3 RNA helicase has been studied for its potential role as a RNA degradation machinery in the metazoan mitochondria.

Contrary to expectations, demonstrating PNPase/hSUV3 as the primary RNA degradation machinery has not been a straightforward story. Stable knockdown of PNPase has shown variable effects on the nature of poly-A tail and steady state levels of mitochondrial coding RNAs(20,21) . Specifically, the poly-A tail of COX1 was abolished, COX3 poly adenylation did not seem to be affected, and ND3 and ND5 poly-A tails extended upon knockdown of PNPase (20). Steady state levels of ND2 levels were mildly increased upon PNPase knockdown, COX2, and CytB levels did not seem to change, and COX1 and 12S rRNA levels decreased slightly (21). Because mitochondria polycistronicaly transcribes its RNA through both strands of its DNA including regions that do not contain annotated genes, the mitochondrial matrix also contain many antisense RNAs that do not code for tRNA, rRNA or structural genes. Interestingly, PNPase and hSUV3 have been shown to participate in degrading these RNAs that are located on the antisense strand with respect to the annotated mitochondrial gene, which from here on will be referred to as mitochondrial antisense RNA (AS RNA) (21) (22). Thus, in contrast to coding RNAs, the AS RNAs of ND2, COX1, COX2, CYTB showed a stark increase upon PNPase knockdown while mitochondrial tRNA levels were shown to be mildly decreased (21).Similar increases of select mitochondrial AS RNA have been shown when hSUV3 function

has been suppressed (22).

Since the effect of PNPase on mitochondrial RNA levels was assayed on select mitochondrial transcripts, we sought to characterize the role of PNPase in the mitochondria with a multi-faceted approach. This study aims to further characterize the endogenous RNA targets of PNPase, characterize whether PNPase is responsible for degradation of other AS RNA species in the mitochondria, and identify its interaction partners.

PNPase binds to regions of mtRNA that contain tRNAs

Since PNPase is an RNA degrading and polymerizing enzyme in vitro, we performed UV crosslinking and analysis of cDNA (CRAC) to identify binding targets of PNPase using the WT PNPase and S484A PNPase, which is a catalytically inactive mutant that cannot degrade RNAs in vitro. At the time of this experiment, we strongly believed that PNPase was localized to the IMS of mitochondria based on previous studies (23,24). Therefore, we were surprised to find that all mitochondrial mRNAs and tRNAs were identified to bind PNPase (Figure 4-1A-B and data not shown). The target binding patterns for both the WT and S484A mutant PNPase were similar and there were minor difference between the peaks identified for WT and S484A PNPase samples. Though PNPase bound to all annotated mitochondrial transcripts on the sense strand, we observed that PNPase bound regions that include tRNAs at a high level. Specifically, PNPase bound regions include MT-TV, MT-TI, MT-TG, MT-TH, MT-TY and MT-TE. Both mitochondrial ribosomal RNAs were also seen to bind PNPase. In addition, 7S RNA transcribed from the noncoding region of the mitochondrial genome was also highly enriched in both of the PNPase CRAC samples (Figure 4-1A-B).

Knockdown of PNPase results in mild changes in mitochondrial RNA of coding genes, but specific antisense RNAs accumulate in the mitochondria

Because PNPase seemed to interact with mitochondrial matrix localized RNAs, we next wondered whether knockdown of PNPase affects mitochondrial RNA levels significantly. It has already been shown by Borowski et al that mitochondrial antisense RNAs are degraded by PNPase, and mitochondrial mRNA levels are not affected drastically upon knockdown of PNPase (21). However, we wanted to verify the effect of PNPase in all other transcripts that were not detailed in this previous study. We first started with checking the sense strand transcripts of ND1, ND2, ND3, ND4, and ND6. As shown in (Figure 4-2A), ND1 and ND2 levels seemed to increase slightly upon knockdown of PNPase (si58 and si59) compared to the negative control sample that was transfected with non-specific siRNA (si+). For ND3, the transcript level did not change significantly but the size of the transcript shifted slightly. For ND4, knockdown of PNPase did not affect the transcript levels, and for ND6, the level seemed to decrease slightly. When we looked at non-ND transcripts, we observed decreases in the mRNA levels of COX1, COX2, COX3, CYTB, and ATP6 levels compared to the control siRNA (si+) transfected samples (Figure 4-3A). These results were consistent with Borowski et al, where they showed that knockdown of PNPase resulted in mild changes for sense strand of mRNAs and increase in mRNA levels for selected mitochondrial transcripts. In addition, 12S rRNA and 16 S rRNA levels also decreased upon PNPase knockdown (Figure 4-4A).

In contrast, the accumulation of AS RNA complementary to the coding mRNAs showed drastic changes for select transcripts. AS ND1 and ND2 RNA levels were higher when PNPase was knocked down (Figure 4-5A). The band size for the ND1 AS transcript was slightly larger than sense ND1 RNA, but the AS ND2 is comparable in size to the sense ND2 mRNA. In addition, knockdown of PNPase also resulted in accumulation of AS RNA for ND5 and ND6 transcripts, however, there was also a drastic change in band pattern of AS RNA, suggesting that PNPase knockdown may perturb various processing steps of AS RNA degradation. In contrast to these observations, AS ND4 transcript levels were not affected by knockdown of PNPase, which is very interesting because the sense RNA levels were also not affected by PNPase knockdown (Figure 4-5A). For non-ND transcripts, AS COX1 transcripts accumulated upon PNPase knockdown the molecular weight of this transcript was similar in size to the sense COX1 RNA. AS ATP6 and CYTB also accumulated where there seemed to be an accumulation of large molecular weight transcripts (Figure 4-6A). Also, the AS RNAs for 12S and 16S ribosomal RNAs seemed to be decreased when PNPase was knocked down, similar to what we observed for the sense strand of 12S rRNA and 16S rRNAs (Figure 4-6A).

PNPase binds to ribosome assembly and RNA processing factors in the mitochondria

In order to better understand the function of PNPase in the mitochondria, we sought to understand the specific protein factors that interact with PNPase. Previous attempts to identify PNPase interaction partners by immunoprecipitation in native conditions did not reveal any reasonable candidates to study further (data not shown) and we reasoned that PNPase interaction

partners may interact weakly or transiently. Thus, we used the approach to label proteins in the proximity of the protein of our interest by expressing a fusion protein consisting of PNPase fused to a biotin ligase called BIOID2 (25)25). These constructs were expressed in HEK-293T FlpIn cell lines. We verified the expression of this fusion protein and checked that the BioID2 fusion to the PNPase still permits biotinylation by the fused BIOID2 protein (Figure 4-7A-B). As a control cell line, we expressed eGFP fused to BIOID2 with a PNPase mitochondrial targeting sequence (amino acid 1-82) at its N-terminus (PNP-GFP-B2) in the same HEK-293T FlpIn cell line. We further performed a sub-mitochondrial fractionation experiment and showed that the full length (FL) PNPase-BIOID2 (FL PNP-B2) were present mostly in the mitochondrial matrix (Figure 4-7C).

We then cultured the cell lines expressing the fusion protein of our interest in the presence of biotin and performed the pull down experiments to identify all proteins that are biotinylated in each cell line. When we subtracted the proteins identified in the FL PNP-B2 pull down assay from those identified in the PNP-GFP-B2 pull down assay, we identified that PNPase most strongly interacted with hSUV3 (Table 4-1), agreeing with published studies (22,26,27). We also identified, LRPPRC, which has a role in maintaining the stability of mitochondrial RNAs (28), GRSF1, which is required for mitochondrial ribosome assembly and RNA recruitment (29,30), and MRPP1, which catalyze the 5' processing of mitochondrial tRNAs (Table 4-1). Interestingly, we also identified DHX30, DDX28, FASTKD2, FASTKD5, and possibly FASTKD3 as potential interaction partners for PNPase (Table 4-1). DHX30, DDX28, FASTKD2, FASTKD3, and FASTKD5 have all been reported to interact with PNPase (31,32). In addition, hSUV3 has also been reported to interact with DHX30, DDX28, FASTKD2, and FASTKD5 (31). DHX30 and DDX 28 are mitochondria localized putative RNA helicases

implicated in mitochondrial ribosome assembly (31). FASTKD2, FASTKD3, and FASTKD5 are members of the Fas-activated serine/threonine (FAST) kinase domain containing protein family. FASTKD2 and FASTKD5 have been shown to be important for mitochondrial RNA processing, and FASTKD2 is also implicated in mitochondrial ribosome assembly (31,33). In contrast, the specific function of FASTKD3 in the mitochondria has not been studied thoroughly (32). While most of PNPase function has been studied with regards to RNA processing and degradation functions in the mitochondria, its interactions with other RNA processing factors and mitochondrial ribosome assembly factors suggests a possible intersection of these processes in the mitochondria.

Discussion

PNPase seems to bind all mitochondrial mRNAs, tRNAs, rRNAs, and even the 7S RNA. However, specific tRNA segments bound PNPase at high levels. When steady state levels of mitochondrial RNAs upon PNPase knockdown were assess, mitochondrial coding RNAs showed marginal changes in steady state levels. In contrast, on specific mitochondrial transcripts, there were increased accumulations of antisense noncoding RNAs upon knockdown of PNPase. Further, when we looked for PNPase interaction partners using the proximity labeling pull down method, we identified FASKD2, FASKD3, FASKD5, DHX30, and DDX28 to interact with PNPase. Thus, this study identified RNAs and protein factors that interact with PNPase and further characterized the effect of PNPase knockdown on mitochondrial RNA transcripts.

If PNPase is directly involved in the degradation of specific mitochondrial RNAs, it is interesting to consider how PNPase may recognize its specific targets. We have performed in

vitro systematic evolution of ligands by exponential enrichment (SELEX) analysis to identify motifs that are preferentially bound by PNPase. When we looked for the presence of these motifs (CTACCCC, CAACCCC, CTAGCCC) in the mitochondria RNAs, we identified that RNR1, ND1, ND2, COX1, ND3, COX3, and ND6 contained these motifs. However, we were not able to see a consistent pattern of steady state RNA level changes upon knockdown of PNPase for the aforementioned transcripts. Alternatively, RNAs that are polyadenylated or oligoadenylated may be a way to mark RNAs for degradation in the mitochondria. It is also conceivable that proteins that are attached to the RNAs may act as a signal for degradation.

The CRAC and BIOID2 analysis suggests that PNPase may localize in the mitochondrial matrix. Different experimental approaches have shown PNPase to be localized in the IMS and matrix of the mitochondria (21,23,34). Our experiments showed that PNPase interacts with mitochondrial RNAs and matrix localized proteins. Thus, there is a high likelihood of PNPase being localized to the matrix. In addition, the PNPase-BIOID2 fusion protein was localized into the matrix, again supporting that endogenous PNPase may localize in the matrix compartment.

Though PNPase binds to all mitochondrial tRNAs, rRNAs and mRNAs, high amount of specific tRNA containing regions interact with PNPase endogenously. The interaction of PNPase with other RNA processing enzymes and ribosome assembly factors suggest a possibly coordinated regulation of RNA processing and mitochondrial translation. In light of the recently discovered function of PNPase in regulating the metabolic shift during induced pluripotent stem cell differentiation, how expression levels of PNPase can affect the cellular reliance on oxidative phosphorylation and how its specific role in the mitochondria may relate to this metabolic shift is an interesting question to address in the future.

Materials and Methods

Construction of Cell Lines. All tissue culture cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% Fetal Bovine Serum (Omega Scientific) and 1x penicillin-streptomycin under 5% CO2 at 37°C. 293T FlpIn cell lines were constructed by transfecting the host cell line with pOG44 Flp-Recombinase Expression Vector (thermofisher) and pCDN5 FRT/TO vector expressing the fusion protein of interest (see supplemental). Specifically, approximately 1 million 293T FlpIn cells were plated in a 6 well plate the night before transfection. The next day, at 60-80% cellular confluency, the cells were transfected using 0.2μg of pCDNA 5 FRT/TO vector expressing the fusion protein of interest and 1.8μg of pOG44 vector using the BioT transfection agent (Bioland Scientific) following the manufacturer's protocol. After 24hours, the media was replaced, and the cells were split out of the 6 well plate after 48 hours into a 10cm plate and plated in selection media containing 10μg/mL blasticidin and 200μg/mL hygromycin. The cells were cultured until the individual colonies started to form on the 10cm, and all colonies were pooled together to establish a polyclonal cell line.

UV crosslinking and analysis of cDNA (CRAC) pull down. CRAC experiments were performed as described in Helwak et al (35). First set of CRAC experiments were performed in 293T cells transduced with retrovirus overexpressing S484A PNPase –histidine tag- protein C tag, Tim23-histidine tag-protein C tag, and WT 293T cells. Second set of CRAC experiments were performed in 293T FlpIn cells expressing a single copy of WT PNPase-histidine tag-protein C tag, S484A PNPase–histidine tag- protein C tag, and Tim23–histidine tag- protein C tag and the WT 293T FlpIn cells. Cell lysates were prepared as shown in step 1-7 of the

published CLASH nature protocol (35), however, the expression of fusion proteins were induced with doxycycline for 24 hours

CRAC Analysis. The single end reads were adaptor-trimmed using cutadapt with default parameters. The trimmed reads were converted from fastq to fasta format and the duplicated reads were collapsed with customized perl script. The non-redundancy reads were aligned to human genome hg38 using novoalign (version 2.4.0 with parameters "-t 85 -F FA -l 15 -s 1 -o Native -r None"). Next, mapping reads were converted to a bed file, then they were further converted to bed graph and finally the tags were clustered using perl script in CLIP Tool Kit (CTK). The clusters with tags more than 10 reads were defined as PNPase binding peaks. Genome features of PNPase binding sites were annotated including 8 categories: promoter-TSS (transcription start site), 5' UTR, exon, intron, 3' UTR, TTS (transcription termination site), non-coding RNAs and intergenic regions using annotatePeaks, and motif were found using homer finding findMotifsGenome in Homer (36). Peaks in Mitochondria were annotated by overlapping with Mitochondria annotation files using bedtools (37).

siRNA Knockdown. siRNA knockdown of PNPase was performed in 293T cell lines. Briefly, 1.6 million 293T cells were plated into 6 well plates and reverse transfected using RNAiMAX (thermofisher) following the manufacturer's protocols. However, the siRNA concentration was kept at 20nM to follow the protocol used by Borowski et al (21). After 24-48 hours, cells were split out to 10cm plate as necessary, and cells were harvested after 72 hours post transfection.

Northern Blotting. Most experiments were based on n=2 or more, except for sense strand of

ND1 and antisense strand of ND1, ND4, ND5, Cox1, 12S, 16S rRNA which were assessed with n=1. RNA samples were prepared using Trizol Reagent and precipitated with isopropranol. Samples were glyoxylated by incubating in glyoxylation buffer (60% DMSO (v/v), 20% (v/v) deionized glyoxal 1.2X BPTE buffer, 4.8% glycerol, .2mg/mL ethydium bromide) at a 1:4 (RNA to gyloxylate mix) at 55°C for 1hr, cool on ice for 10 minutes and stored in -20°C. The 10X BPTE buffer was prepared as 100mM PIPES, 300mM Bis-Tris 10mM EDTA (pH8.0) and diluted as needed. Glyoxylated RNA samples were separated on 1X BPTE agarose gel in 1X BPTE. The RNA samples were transferred onto a hybond N+ nylon membrane using standard capillary transfer method in 10X SSPE buffer 0.01M EDTA, 1.49M NaCl and 0.1M phosphate buffer pH 7.4. The membranes were crosslinked and blocked in church buffer (1% BSA, 1 mM EDTA, 0.5 M NaPO4 pH 7.2, 7% SDS) at 65 °C for 1 hour. P32 labeled RNA probes were prepared using T7 MAXIscript kit using the manufacturer's protocol and were stored in -20C until use. The membranes were then hybridized with the appropriate probes overnight at 65 °C, washed with 2X SSPE + 0.1% SDS, 1X SSPE + 0.1% SDS, and 0.1X SSPE + 0.1% SDS and signals were detected by phosphor imaging autoradiograph.

Proximity dependent biotinylation for identification of interacting proteins. BIOID2 sequence was PCR amplified out of myc-BIOID2-MCS (addgene plasmid #74223) and inserted to the pcDNA5 FRT/TO vector, thus all BIOID2 cell lines constructed from this plasmid contains a myc tag. PNP-GFP construct was designed to contain the first 82 amino acids of PNPase so that this segment will contain the mitochondrial targeting sequence and a long enough flanking region to allow for the cleavage of the targeting sequence. The PNPase-BIOID2 cell line expresses an inducible construct consisting of full length PNPase followed by myc tag and

BIOID2.

10 x 15cm were cultured per cell line, and grown to ~80% confluency. 24 hours before harvesting cells were the tissue culture medium was supplemented with 50mM biotin and 10nM doxycycline. Cells were subsequently harvested by scraping, and mitochondria was isolated by douncing in mitochondria isolation buffer consisting of 20mM HEPES ph 7.5, 70mM sucrose, 220mM mannitol and 0.5mM PMSF. The amount of purified mitochondria were normalized to equal protein mass among each sample, and were lysed in lysis buffer (8 M urea, 2% SDS, 100 mM Tris, pH 8) at room temperature fore 30 min. The samples were centrifuged and the supernatant was incubated with alkylated streptavidin beads for 2 hours at room temperature. The beads were then washed three times each with lysis buffer, wash II buffer (8 M urea, 2% SDS, 500 mM NaCl, 100 mM Tris, pH 8), wash III buffer (8 M urea, 0.2% SDS, 500 mM NaCl, 5% ethanol, 5% isopropanol, 100 mM Tris, pH 8), and digestion buffer (8 M urea, 100 mM Tris, pH 8.5). The beads were then spun down, and resuspended in 50uL digestion buffer. The samples were processed and analyzed by mass spectrometry by the Wohlschlegel lab. The BIOID2 analysis was performed with 3 biological replicate samples where each sample set contained 2 technical replicates.

Figures

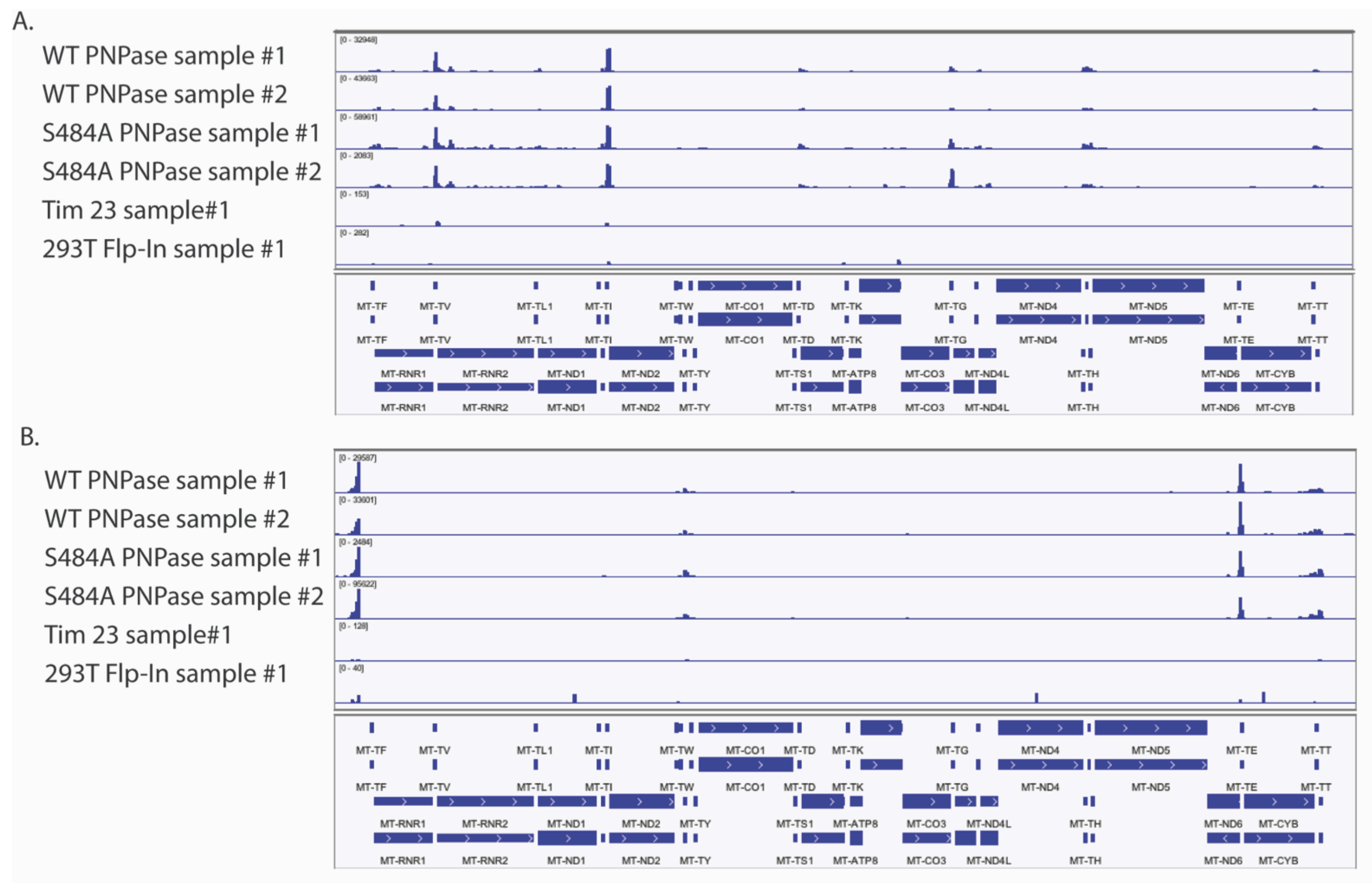
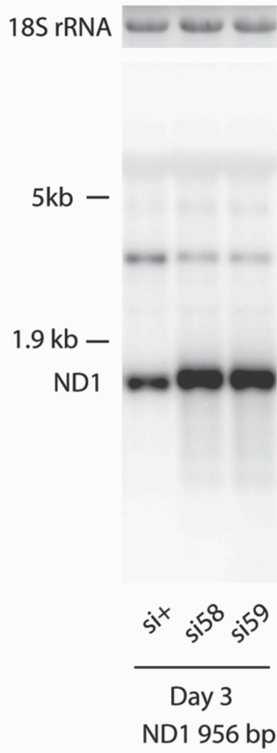
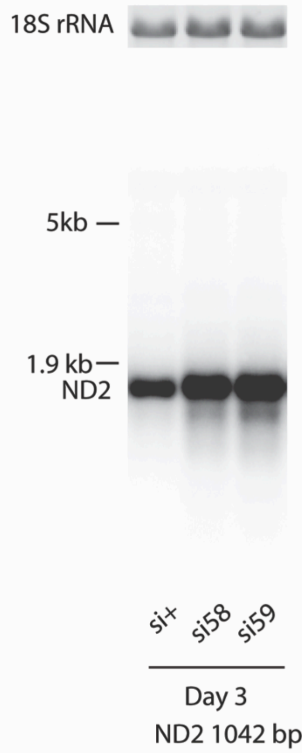
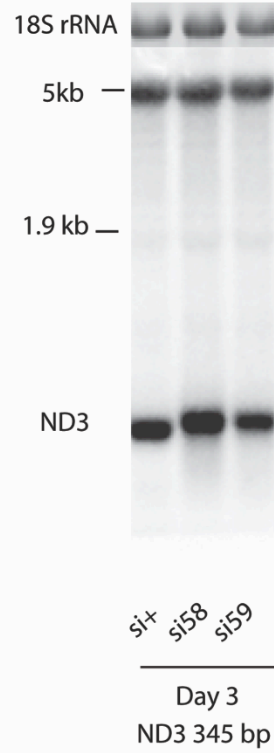
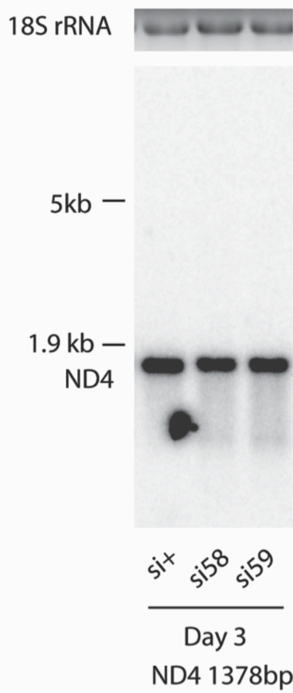


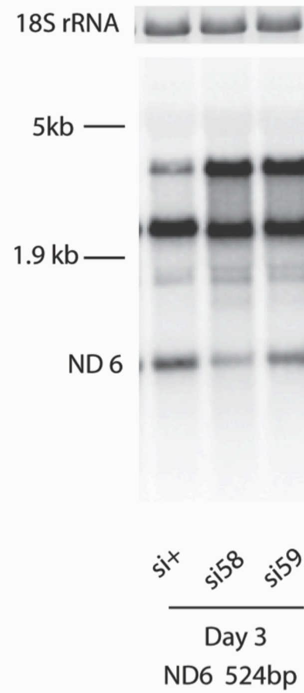
Figure 4-1. **PNPase binds to regions of mtRNA that contain tRNAs.** (A) PNPase binding targets aligning to the heavy strand of mitochondrial RNAs. (B) PNPase binding targets aligning to the light strand of mitochondrial RNAs. All samples were sequenced in duplicates All samples were sequenced in duplicates (duplicates of Tim23-HisPC and wild type cell line, 293T FlpIn cells are not shown).











B.

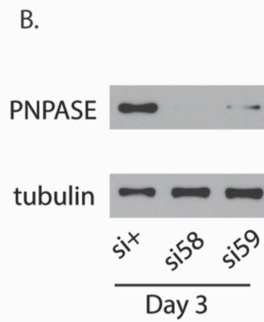
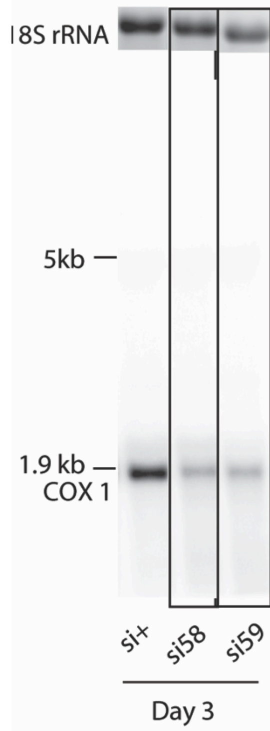
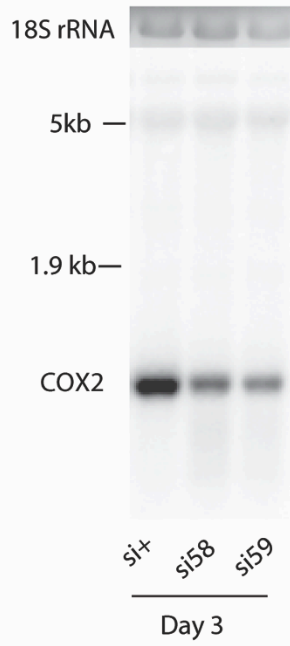


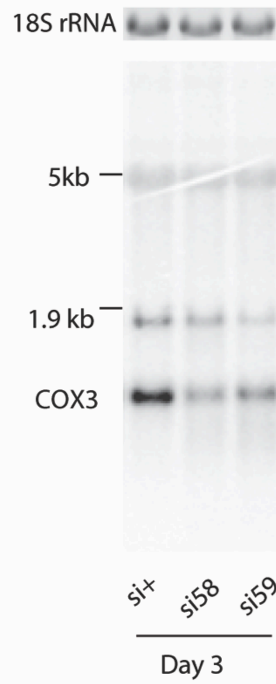
Figure 4-2. **Steady state level of sense RNAs encoding for ND-X transcripts do not change significantly upon knockdown of PNPase.** (A) Northern blots probing for *ND1, ND2, ND3, ND4*, and *ND6* are shown. *18S* rRNA is used as loading control (B) Western blot showing the extent of knockdown of PNPase in the samples transfected with si+ (negative control siRNA), si58 (siRNA #1 against PNPase) , and si59 (siRNA #2 against PNPase). These samples were used for subsequent northern blots.



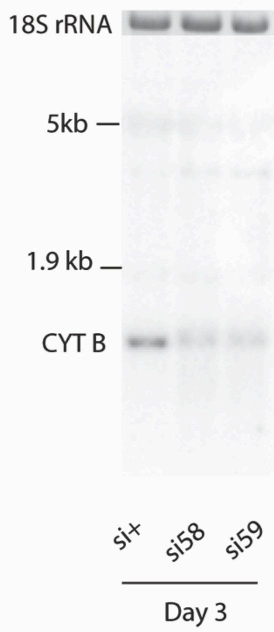
COX 1 1542 bp



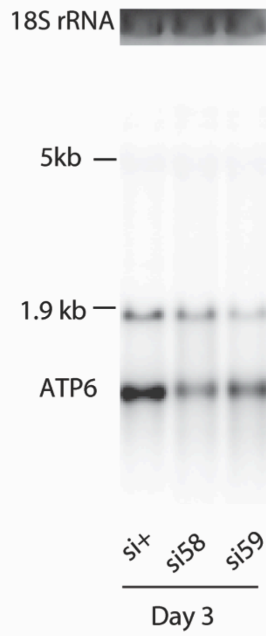
COX 2 684 bp



COX3 784 bp



CYTB 1141 bp



ATP6 681 bp

Figure 4-3. **Steady state level of sense RNAs encoding for non ND-X transcripts do not change significantly upon knockdown of PNPase.** (A) Northern blots probing for *COX1, COX2, COX3, CYTB,* and *ATP6* are shown. *18S* rRNA is used as loading control

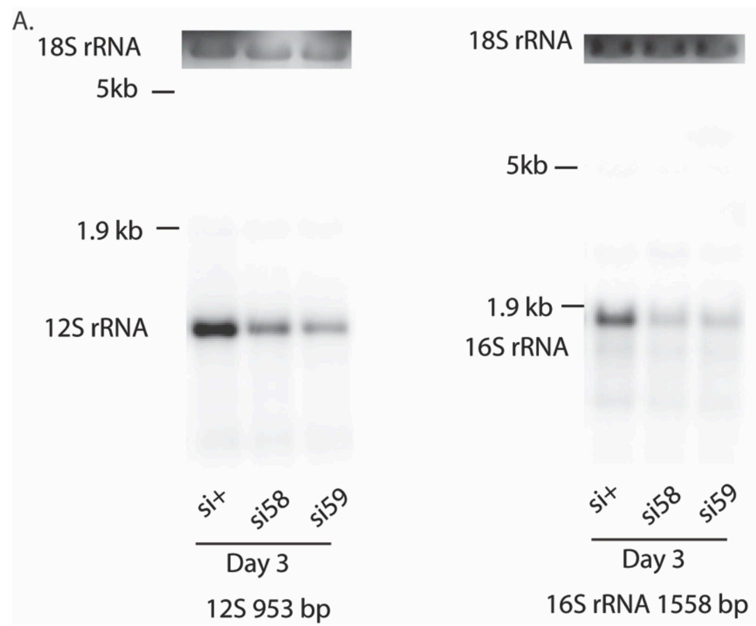


Figure 4-4. **Steady state level of *12S* rRNA and *16S* rRNA decrease upon knockdown of PNPase.** (A) Northern blots probing for *12S* rRNA and *16S* rRNA are shown. *18S* rRNA is used as loading control

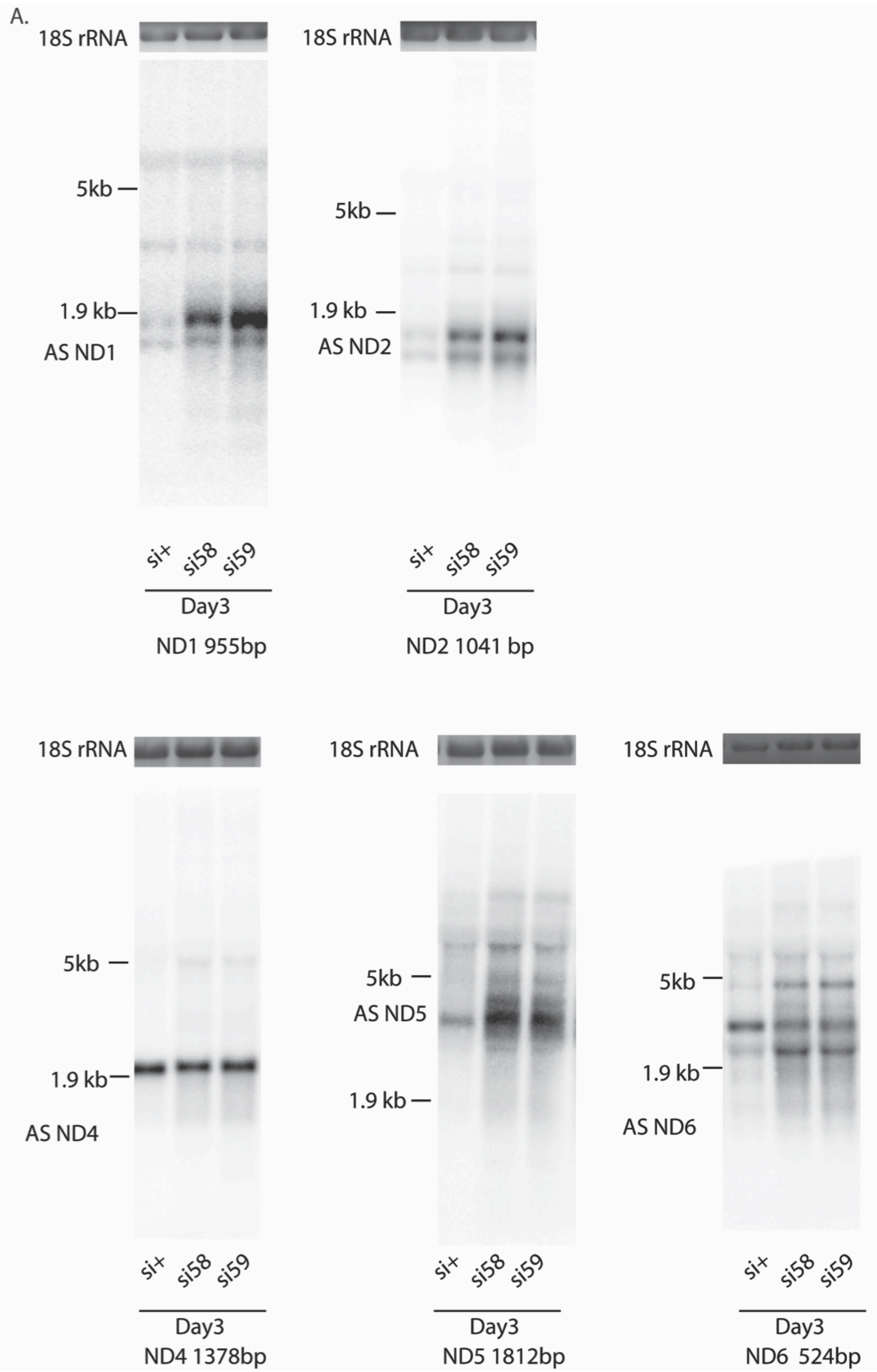
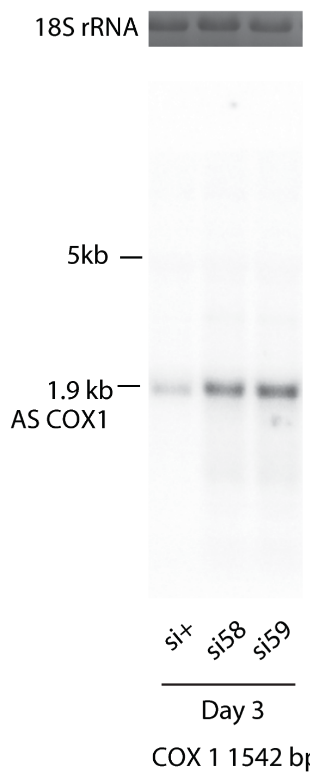
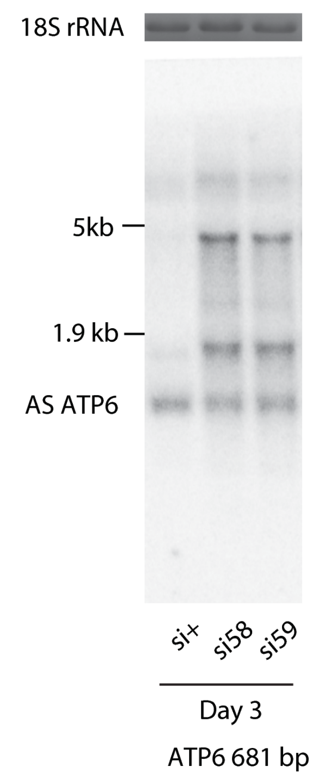
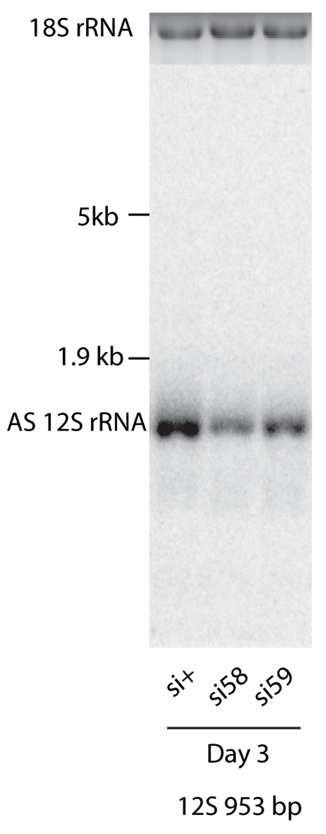

A.
18S rRNA
5kb
1.9 kb
AS ND1
si+
si58
si59
Day3
ND1 955bp
18S rRNA
5kb
1.9 kb
AS ND2
si+
si58
si59
Day3
ND2 1041 bp
18S rRNA
5kb
1.9 kb
AS ND4
si+
si58
si59
Day3
ND4 1378bp
18S rRNA
5kb
AS ND5
1.9 kb
si+
si58
si59
Day3
ND5 1812bp
18S rRNA
5kb
1.9 kb
AS ND6
si+
si58
si59
Day3
ND6 524bp

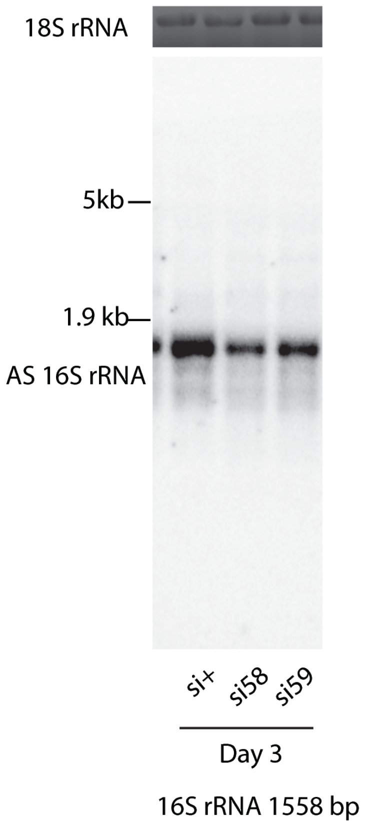
Figure 4-5. **Steady state level of specific antisense ND-X transcripts accumulate upon knockdown of PNPase.** (A) Northern blots probing for AS *ND1, ND2, ND4*, *ND5* and *ND6* are shown. *18S* rRNA is used as loading control

A.









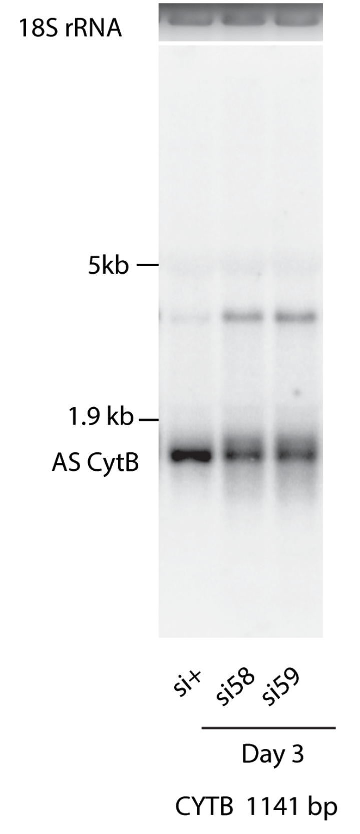


Figure 4-6. **Steady state level of specific antisense non ND-X transcripts accumulate upon knockdown of PNPase.** (A) Northern blots probing for AS *COX1, ATP6, 12S rRNA*, *16S rRNA* and *CYTB* are shown. *18S* rRNA is used as loading control

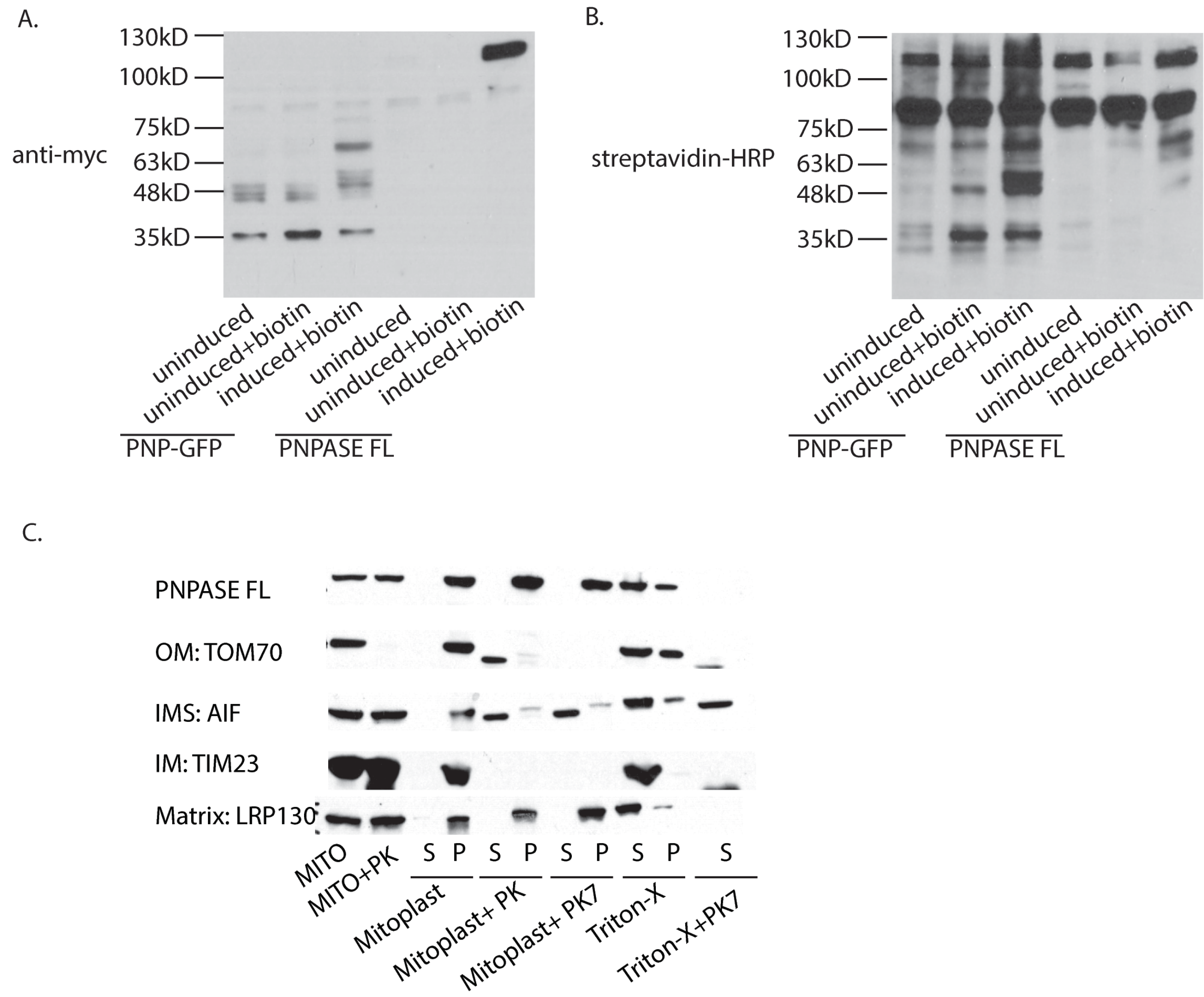


Figure 4-7. **PNPase interacts with ribosome assembly factors and RNA processing factors in the mitochondria.** (A) Western blot showing the levels of BIOID2 fusion proteins used in the proximity labeling assay. (B) Western blot showing the levels of total biotinylated proteins in the constructed cell lines. (C) Sub-mitochondrial fractionation of FL PNP-B2.

Target name		Function
Polyribonucleotide nucleotidyltransferase 1,	*PNPT1*	mtRNA degradation
ATP-dependent RNA helicase SUPV3L1	*SUPV3L1*	mtRNA degradation
Leucine-rich PPR motif-containing protein	*LRPPRC*	mtRNA processing and RNA loading to ribosmomes
Putative ATP-dependent RNA helicase DHX30	*DHX30*	mitochondrial ribosome assembly
Mitochondrial ribonuclease P protein 1	*MRPP1*	mt-tRNA processing
FAST kinase domain-containing protein 2	*FASTKD2*	mitochondrial ribosome assembly
G-rich sequence factor 1	*GRSF1*	mitochondrial ribosome assembly, recruitment of lncRNA and mRNA
FAST kinase domain-containing protein 5	*FASTKD5*	mtRNA processing of non canonical targets
FAST kinase domain-containing protein 3	*FASTKD3*	Unknown

Table 4-1. Potential PNPase interaction partners identified from the BIOID2 proximity labeling assay. PNPase interacting proteins identified from mass spectrometry analysis of streptavidin pull down elution of proteins biotinylated by FL PNPase-BIOID2 and EGFP-BIOID2 fusion proteins.

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Chapter 5: Future Directions

Role of PNPase in potentially regulating mitochondrial RNA levels

The CRAC assay has revealed that PNPase binds to regions containing tRNAs, and knockdown of PNPase results in accumulation of antisense RNAs in the mitochondria. Recently, processing intermediates of mitochondrial transcripts that contain tRNAs have been found in mouse cardiac mitochondria (1) . Thus, it is possible to hypothesize a model in which PNPase may be responsible for degrading mitochondrial RNA processing intermediates that may contain tRNAs. The accumulation of RNA processing intermediates may induced the aberrant increase of steady state antisense RNA levels in the mitochondria. In verifying this model, several issues must be addressed. First, the levels of tRNAs should be checked to see if it is affected by the knockdown of PNPase. Second, the RNA segments bound by PNPase must be verified to be RNA processing intermediates found in human mitochondria. Third, the steady state levels of these RNA processing intermediates should be assessed upon knockdown of PNPase and the direct involvement of PNPase in degrading these RNA processing intermediates must be verified. Lastly, it is important to check whether PNPase catalytic activity is directly involved with the antisense RNA accumulation phenomenon, since the involvement of catalytic activity affecting steady state mtRNA levels has only been adequately addressed on sense RNAs (2).

Though published data from Borowski et al., suggests that specific tRNA levels are lower in PNPase knockdown samples, steady state tRNA levels should be verified upon knockdown of PNPase (2). Acrylamide gel northern blots can be utilized to follow steady state levels of small

RNAs and it would be interesting to see whether there is a correlation between tRNAs whom their steady state levels are effected upon PNPase knockdown and tRNAs that are shown to bind PNPase in the CRAC assay. Although, it is apparent that lower levels of steady state mitochondrial coding RNAs may likely contribute to the decreased steady state levels of mitochondrial protein levels, it is also interesting to note whether decrease in tRNA levels may also contribute to this observation.

A recent study utilizing intramolecular ligation of RNA followed by sequencing of the circularized RNA revealed the presence of mitochondrial RNA processing intermediates, and it would be interesting to verify whether the RNA segments bound by PNPase are RNA processing intermediates (1). The study by Kuznetsova et al, purified RNA from mouse cardiac mitochondria and revealed a stable accumulation of RNA processing intermediate fragments around the region that contains mt-Tv, the region containing mt-Ti and mt-Tm, and the region containing mt-Th, mt-Ts2, and mt-Tl2. The patterns of these peaks are very similar to peaks identified from the PNPase CRAC samples. Furthermore, in their study they observed a remarkable increase of these intermediates in a system where one of the subunit of the 5' tRNA processing machinery, MRPP3 is knocked out (1). Thus, we can speculate that we would observe similar species of RNAs in the human cell lines, and PNPase may also bind these processing intermediates. Therefore, it would be interesting to identify these stable RNA intermediate species in human cell lines utilizing the same intermolecular ligation technique followed by RNA sequencing.

In parallel to verifying whether RNA processing intermediates of mtRNA accumulate in human cell lines, the accumulation of these tRNA containing regions upon knockdown of PNPase and the involvement of PNPase catalytic activity for the increase in these intermediates

should be assessed. To check for levels of RNA processing intermediates upon PNPase knockdown, the aforementioned RNA-seq method on RNA samples that have been intramolecularly circularized can be used to analyze RNA samples that have been purified from PNPase knock down cell lines. Alternatively, once the mtRNA processing intermediates are known, the accumulation of these species may be assessed using the traditional agarose or polyacrylamide gel northern approach. In order to verify whether PNPase catalytic activity is involved in the accumulation of RNA processing intermediates, RNAs purified from cell lines expressing the catalytically inactive mutant of PNPase, PNPase S484A PNPase or PNPase R445E, R446E may be utilized for RNA-seq or northern blot analysis.

We hypothesize that PNPase may be involved in degradation of RNA processing intermediates and the accumulation of these may indirectly result in increased steady state levels. Previous studies have suggested that PNPase may degrade antisense RNAs in the mitochondria, however the direct involvement of the catalytic activity of PNPase on the accumulation of mitochondrial antisense RNA has not been assessed, though its involvement on the accumulation of sense RNA was analyzed(2). Thus, it would be imperative to analyze whether the degradation capacity of PNPase is involved in the accumulation of antisense RNAs by repeating the agarose northern analysis using RNA samples extracted from cell lines expressing PNPase S484A or PNPase R445,446E. Though this assay will not be able to discern whether PNPase is directly or indirectly involved in the accumulation of antisense RNAs, nonetheless the involvement of PNPase catalytic activity on affecting the antisense RNA accumulation should be analyzed.

PNPase is a classically studied enzyme, but its role in the mammalian mitochondria remain to be elucidated. Though it is equally probable that PNPase may degrade both the RNA processing intermediates and the antisense RNAs, I hypothesize that the accumulation of

mitochondrial antisense RNA is a secondary effect to the possible accumulation of RNA processing intermediate. Because antisense RNAs were not found to be bound to PNPase in our CRAC assay, we hypothesize that antisense RNA may not be the direct target of PNPase in the mitochondria. From our work with the BIOID2 fusion protein mass spectrometry analysis, PNPase was found to transiently interact or be present in the vicinity of RNA helicases and other protein factors that are involved in mitochondrial ribosome assembly. This suggests that in addition to its potential role in affecting mitochondrial RNA metabolism, PNPase may also cross talk with the mitochondrial ribosome assembly processes. In addition to ribosomal protein factors, the mitochondrial ribosome is composed of ribosomal RNAs, and it is understandable that the assembly process may coordinate with the RNA regulatory proteins to maintain proper functioning of the translation machinery.

PNPase and possible formation of a separate complex under stress conditions

With regards to PNPase interacting proteins, it would be interesting to see whether PNPase complex formation follows a similar pattern to what is seen in *E. Coli*. During cold shock, PNPase present in these bacteria binds CsdA, which is a different RNA helicase than what is normally present in the degradosome complex bound to PNPase (3). Thus it may be possible to imagine when the cell is under stressed conditions, such as an antiviral response, cold shock response, metabolic stress, or DNA damage response, PNPase may form a different multimeric complex under these circumstances. Since PNPase is an interferon induced gene, it is possible that it may have a specific function during antiviral response. Drawing from the

bacterial PNPase example, it is conceivable that it may also have a specific function in mammalian cells under this condition. In addition, human PNPase has been co-crystalized with cysteine and regulation of bacterial PNPase activity by cysteine has been reported, thus conditions that alter the level of cysteine may affect PNPase activity and complex formation. Finally, since *E. Coli* and *B. Subtilis* PNPase has been implicated to have a role in DNA damage responses, it is possible that PNPase may form a different complex and function in this pathway also in the mammalian mitochondria (4,5). The involvement of PNPase in DNA damage repair is also interesting because our observation that knock down of PNPase results in mitochondrial DNA loss suggests a potential role of PNPase in mtDNA biosynthesis, maintenance or repair (6).

PNPase and mitochondrial RNA translocation

As PNPase has been suggested to regulate RNA import, we have been interested in identifying factors involved in this pathway. The BIOID2 mass spectrometry assay revealed that PNPase may also be in proximity of two channel proteins, prohibitin 1 and prohibitin 2 (data not shown), though much less protein spectra were identified for these factors compared to mitochondrial ribosome assembly factors. Thus, it is possible that these proteins may be involved in translocating RNAs in the mitochondria. During stressed conditions, such as when PNPase is knocked down, many antisense mitochondrial RNAs accumulate to levels that are higher than normal (2), it is conceivable that accumulated mitochondrial RNA may translocate through these channels to the cytosol.

Concluding Remarks

PNPase is an intriguing nuclease with many suggested roles in the mammalian cell. Despite a few studies that utilized cell lines that expressed PNPase at supra-physiologic levels, the advent of reliable knockdown and knock out methods have slowly revealed some interesting functions of PNPase in the mammalian mitochondria. Our study has revealed the effects of PNPase knockdown on mitochondrial RNA levels, RNAs binding to PNPase and protein factors that bind to PNPase. Further studies are warranted in understanding the role of PNPase in affecting mitochondrial RNA metabolism, how PNPase may cross talk with mitochondrial ribosome synthesis pathways, how PNPase complexes may change during stress conditions and possible roles under these settings, and what other protein factors maybe involved in the translocation of RNAs in the mitochondria. In lieu of the recent identification of PNPase as a factor that regulate the oxidative capacity in iPS cells during reprogramming, it would be very interesting to consider how the specific role of PNPase in the mitochondrial will contribute to this phenomenon (7).

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Appendix I: Correcting Human Mitochondrial Mutations with Targeted RNA Import

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Correcting human mitochondrial mutations with targeted RNA import

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Mutations in the human mitochondrial genome are implicated in neuromuscular diseases, metabolic defects, and aging. An efficient and simple mechanism for neutralizing deleterious mitochondrial DNA (mtDNA) alterations has unfortunately remained elusive. Here, we report that a 20-ribonucleotide stem-loop sequence from the *H1* RNA, the RNA component of the human RNase P enzyme, appended to a nonimported RNA directs the import of the resultant RNA fusion transcript into human mitochondria. The methodology is effective for both noncoding RNAs, such as tRNAs, and mRNAs. The RNA import component, polynucleotide phosphorylase (PNPASE), facilitates transfer of this hybrid RNA into the mitochondrial matrix. In addition, nucleus-encoded mRNAs for mitochondrial proteins, such as the mRNA of human mitochondrial ribosomal protein S12 (*MRPS12*), contain regulatory sequences in their 3′-untranslated region (UTR) that confers localization to the mitochondrial outer membrane, which is postulated to aid in protein translocation after translation. We show that for some mitochondrial-encoded transcripts, such as *COX2*, a 3′-UTR localization sequence is not required for mRNA import, whereas for corrective mitochondrial-encoded tRNAs, appending the 3′-UTR localization sequence was essential for efficient fusion-transcript translocation into mitochondria. In vivo, functional defects in mitochondrial RNA (mtRNA) translation and cell respiration were reversed in two human disease lines. Thus, this study indicates that a wide range of RNAs can be targeted to mitochondria by appending a targeting sequence that interacts with PNPASE, with or without a mitochondrial localization sequence, providing an exciting, general approach for overcoming mitochondrial genetic disorders.

The mtDNA of mammals encodes 13 proteins of the electron transport chain, 22 tRNAs, and 2 rRNAs (1, 2). The majority of mitochondrial proteins and some noncoding RNAs are encoded in the nucleus and imported into mitochondria (3, 4). Whereas the pathways of protein translocation into mitochondria have been well studied, the mechanisms for nucleus-encoded RNA import into mitochondria, however, are not well understood, even though mitochondrial RNA import is a universal process (5, 6). Recent detailed sequencing analysis of the mitochondrial transcriptome indicates that the collection of mitochondrial RNAs is more complex than previously thought, including an enrichment of several nucleus-encoded tRNAs and other noncoding RNAs (7), indicating that these RNAs are actively imported into mitochondria.

A subset of nucleus-encoded tRNAs is imported into the mitochondria of almost every organism (5, 8). The number of imported tRNAs ranges from one in yeast to all in trypanosomes. Recent mitochondrial transcriptome analysis suggests that this tRNA collection is broader in mammals than previously thought (7) as several unsuspected nucleus-encoded tRNAs are imported and processed to mature forms. In addition to in vitro evidence for a protein-only RNase P enzyme within mitochondria (9), the nucleus-encoded RNA components of RNase P and MRP enzymes are also imported into mitochondria and function in mitochondrial (mt)DNA replication and transcription (10–12). The 5S ribosomal RNA translocates into mitochondria, assembles with ribosomes (13, 14), and may function in mtRNA translation, although the exact function of the 5S ribosomal RNA within mitochondria has yet to be firmly established. Finally, specific nucleus-encoded microRNAs have been isolated from mitochondria (7, 15). Thus, a broad spectrum of nucleus-encoded RNAs localize to mitochondria, although the functions for many of these diverse RNAs in mitochondria have not yet been determined.

The nucleus-encoded RNAs in the mitochondrion have potentially diverse import pathways, but the details of these pathways and import mechanisms are still being revealed (16). tRNAs in every organism are imported by a variety of pathways, some of which require cytoplasmic chaperones (17). The 5S ribosomal RNA is imported from the cytosol with the assistance of two proteins, matrix-localized rhodanese and mitochondrial ribosomal protein L18 (14). The ribosomal protein L18 serves as a conduit or chaperone to facilitate the association of the imported 5S ribosomal RNA with mitochondrial ribosomes. And the mammalian polynucleotide phosphorylase (PNPASE) enzyme localizes to the mitochondrial intermembrane space (18–20) where it may function as an RNA receptor to augment translocation of RNAs into the mitochondrial matrix (6, 21).

Specific mutations in mtDNA have been implicated in muscular and neuronal diseases and in the decline of organ function with aging (22, 23). Despite a significant need, there are currently no effective treatments for deleterious mtDNA alterations. DNA import into mitochondria to repair mtDNA alterations has been difficult and low in efficiency, so approaches at mitochondrial repair have exploited import pathway mechanisms (24). For example, allotopic expression of a subset of mitochondrial genes (recoded mtDNA expressed from the nucleus) has been explored to partially correct the effects of deleterious mutations for some mitochondrial genes, including tRNAs (25–27). However, this approach has been limited because imported mitochondrial proteins fail to assemble correctly in respiratory complexes. The import of RNA has been restricted to tRNAs from different species and requires additional foreign factors. We recently identified a unique approach to target nucleus-encoded RNAs to mitochondria, using the 20-ribonucleotide stem-loop sequence of *H1* RNA, the RNA component of the RNase P enzyme that

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regulates its import (6). When appended to a nonimported RNA, the *H1* RNA import sequence, designated *RP*, enables the fusion transcript to be imported into isolated mitochondria (6).

Two longstanding models of human mtDNA disease are cytoplasmic hybrids (cybrids) derived from samples from patients with myoclonic epilepsy with ragged red fibers (MERRF) and mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) (28–31). These cybrid lines harbor an A8344G ($mt\text{-}tRNA_{AAA}^{Lys}$) mutation for MERRF and an A3243G ($mt\text{-}tRNA_{UUR}^{Leu}$) mutation for MELAS, both of which cause inefficient mtRNA translation, which results in defective cell respiration. We demonstrate here that the mitochondrial defects in these mutant cybrid cells can be partially rescued by targeted import of allotopically encoded wild-type tRNAs, using the RP import signal and, for corrective tRNAs, a mitochondrial localization signal, derived from the 3′-untranslated region (UTR) of human mitochondrial ribosomal protein S12 (*MRPS12*), which targets the mRNA to the mitochondrial outer membrane (32). We also show that the RP import sequence is also capable of importing much larger, mitochondrial protein-encoding mRNAs in vivo, broadening the approach to target defects in all mtDNA alterations.

Results

H1 RNA Import Sequence Regulates Mitochondrial Import of mt-tRNA Precursors. Initially, we determined whether corrective, in vitro synthesized mitochondrial tRNA (mt-tRNA) precursors could be imported into isolated mitochondria and, if so, whether they were processed into mature mt-tRNAs. The $mt\text{-}tRNA_{AAA}^{Lys}$ precursor contains 67 and 74 ribonucleotides, and the $mt\text{-}tRNA_{UUR}^{Leu}$ precursor contains 93 and 76 ribonucleotides that are cleaved from the 5′ and 3′ transcript ends, respectively, during mt-tRNA maturation (2). In engineering the imported mt-tRNAs, the 5′ end of each mt-tRNA precursor contained or lacked the 20-nt stem-loop sequence of *H1* RNA that directs the import of this RNA component of the RNase P enzyme (6); this sequence is designated the *RP* sequence (6). Engineered tRNAs were then added to import assays that used mouse liver mitochondria isolated from wild-type or a liver-specific "knockout" (designated HepKO) of *Pnpt1*, the gene encoding for PNPASE (6) (Fig. 1*A*). The abundance of PNPASE in the HepKO mitochondria was decreased to approximately one-third the level present in WT mitochondria. Residual PNPASE expression in the HepKO liver is from heterogeneous liver elements and from the Alb_{CRE} recombinase strain, which is hepatocyte specific but deletion incomplete (33). Only mt-tRNA precursors with the appended *RP* sequence were efficiently imported into isolated mitochondria, and import was markedly impaired to approximately one-sixth to one-eighth the level seen in WT mitochondria with reduced PNPASE expression (Fig. 1 *B* and *C*). Importantly, the 5′ and 3′ mt-tRNA precursor sequences were removed inside the mitochondria to yield mature 60- to 80-ribonucleotide mt-tRNAs (Fig. 1*B*). To examine whether the imported mt-tRNAs could rescue defective mtRNA translation, in vitro import was combined with mitochondrial in organello protein synthesis studies. The A8344G mutation ($mt\text{-}tRNA_{AAA}^{Lys}$) in MERRF and the A3243G mutation ($mt\text{-}tRNA_{UUR}^{Leu}$) in MELAS cause a substantial reduction in mtRNA translation (28, 29). A statistically significant increase in the steady-state abundance of total translated mitochondrial polypeptides was observed in both MERRF and MELAS cells following incubation with the mt-tRNA precursors containing the *RP* import sequence, but not with mt-tRNA precursors lacking *RP* (Fig. 2 *A* and *B* and Fig. S1). These data indicate that the *RP* sequence enabled PNPASE-dependent mt-tRNA precursor import into isolated mitochondria and that the imported mt-tRNA precursors were processed and functioned in at least partially correcting defective mtRNA translation.

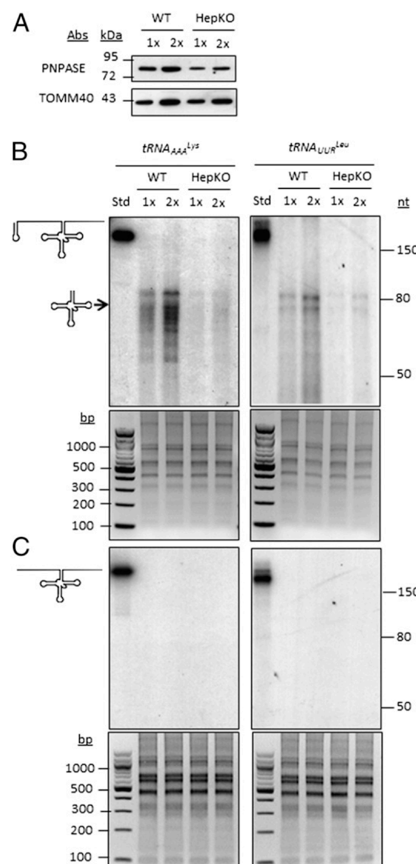

Fig. 1. *H1* RNA import sequence regulates mitochondrial import of mt-tRNA precursors. (*A*) Hepatocyte-specific *Pnpt1* knockout (HepKO) in 6-wk-old mice (6). Immunoblot from 6-wk-old WT and HepKO mouse livers shows ~50% reduction in PNPASE expression. (*B*) Radiolabeled mt-tRNA precursors with (*Upper*) a 5′ *H1* 20-ribonucleotide predicted stem-loop sequence (designated *RP,* marked with loop in schematic) were in vitro transcribed and incubated with WT or HepKO liver mitochondria. Nonimported RNA was digested with added nuclease, followed by RNA isolation, separation on a urea acrylamide gel, and autoradiography. Import reactions were repeated with 1× and 2× amounts of mt-tRNA. (*Lower*) Loading control showing equivalent amounts of mitochondria used in the imports, as revealed by total mitochondrial nucleic acids separated on an agarose gel. (*C*) As in *B*, but the mt-tRNA precursor lacks the 5′ *H1* 20-ribonucleotide predicted stem-loop sequence.

The RP Sequence Directs Import of mt-tRNAs into Mitochondria in Vivo. To determine whether the *RP* import sequence functions in vivo, a mouse cytochrome oxidase 2 (*mCOX2*) mtRNA was used for import into human cells, because the sequence of *mCOX2* differs significantly from that of human *COX2* (*hCOX2*) (1, 2). The *mCOX2* gene, with or without the added 5′ *RP* import sequence, was placed under the control of the *H1* promoter (Fig. 3*A*) and constructs were introduced into HeLa cells via transient transfection. Two days after transfection, mitochondria were isolated and subjected to digitonin treatment (100 μg/1 mg of mitochondrial protein) in the presence of nuclease to generate mitoplasts as a means to determine whether the *mCOX2* RNA

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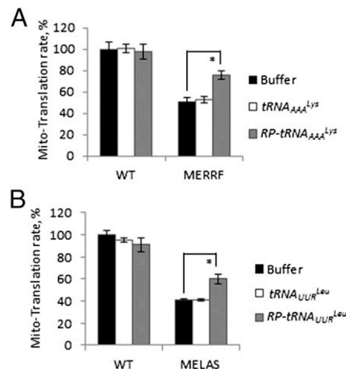

Fig. 2. Import mt-tRNA precursors with the *RP* sequence partially rescue the translation defect of isolated MERRF and MELAS mutant mitochondria. (*A*) mt-tRNA precursors with or without *RP* were imported into isolated WT or MERRF mitochondria from cybrid lines for 2 min at RT, followed by an additional 5 min with rNTP supplementation. Following RNase A digestion of the nonimported mt-tRNA, mitochondria were pelleted and resuspended in an in organello translation buffer with radiolabeled methionine and cysteine for 30 min at 37 °C. The autoradiograms are shown in Fig. S1. Individual lanes were quantified, and total radioactivity was calculated and normalized to total protein amounts. The WT control in the presence of assay buffer was set at 100%. $n = 3$ independent experiments. *$P < 0.01$ with Student's t test. (*B*) As in *A* with WT and MELAS cybrid cell lines. $n = 3$ independent experiments. *$P < 0.01$ with Student's t test.

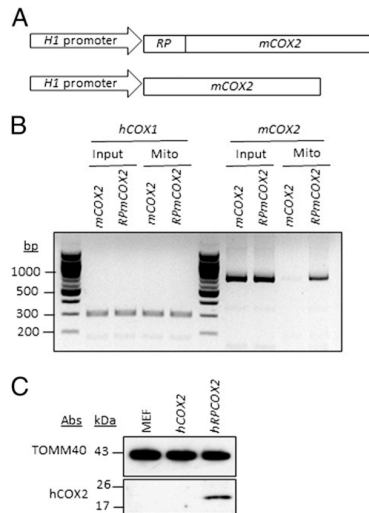

Fig. 3. In vivo import of mitochondrial-coded *COX2* into mitochondria, using the *RP* sequence. (*A*) Diagrams of *mCOX2* expression vectors. (*B*) Mitochondria were isolated from HeLa cells expressing *mCOX2* or *RP-mCOX2*. Mitoplasts were made with digitonin, followed by treatment with nuclease. RNA was then isolated from total cell lysates (Input) or from nuclease-treated mitoplasts (Mito) and analyzed by primer-specific RT-PCR. *hCOX1* is a control for total and mitochondria-isolated RNAs. (*C*) Mitochondria were isolated from mouse embryonic fibroblasts stably expressing *hCOX2* or *RP-hCOX2*. hCOX2-specific expression was analyzed by Western blot from isolated mitochondria.

was indeed imported into the mitochondrial matrix. The presence of the *mCOX2* RNA in the mitochondrial matrix was examined by RT-PCR. Only the mtRNA fusion transcript containing the *RP* import sequence directed the *mCOX2* transcript into the mitochondrial matrix with an import efficiency of ∼5–10%, indicating that the *RP* import sequence is required and functions in vivo (Fig. 3*B*). To examine whether the imported mtRNA is translated, *hCOX2* expression constructs, with or without the *RP* import sequence, were generated and stably introduced into mouse embryonic fibroblasts, because the monoclonal COX2 antibody is specific for human COX2 protein. Cells expressing *RP-hCOX2*, but not *hCOX2*, nucleus-encoded mtRNA showed mitochondrial transcript import (Fig. S2 *A* and *B*) and hCOX2 protein translation within mitochondria (Fig. 3*C*), indicating that the *RP* import sequence also is required and functions with coding mtRNAs in vivo. hCOX2 localization was also verified within mitochondria (Fig. S2 *B* and *C*). hCOX2 protein was trypsin resistant, similar to inner membrane protein TIMM23 (Fig. S2*B*), in contrast to outer membrane protein TOMM40. In contrast to soluble PNPASE, hCOX2 assembled in the membrane because the protein was recovered in the pellet fraction after carbonate extraction, similar to the control, TOMM40 (Fig. S2*C*). Thus, hCOX2 is imported and assembles into the mitochondrial inner membrane. The data also show that the *RP* sequence enables mitochondrial import and processing of RNAs much larger (683 ribonucleotides) than tRNAs (60–80 ribonucleotides), providing a broader therapeutic potential. Thus, the *RP* sequence can potentially be used in general strategies to target large RNAs for import into mitochondria.

Functional Rescue of Mitochondrial tRNA Mutants. The in vivo rescue of function in mitochondria with mt-tRNA mutations has proven challenging (5). The usual processing of nucleus-encoded tRNA precursors occurs inside the nucleus (34, 35). When stably expressed from inside the nucleus, mt-tRNA precursors with the fused *RP* sequence did not rescue the respiratory defect of MERRF or MELAS cells (Fig. 4 *A* and *B*). Instead, the *RP* and 5′ mt-tRNA presequences were cleaved inside the nucleus (Fig. S3*A*). Moving the *RP* import signal to the 3′ end of the mt-tRNA presequence was also ineffective as both 5′ and 3′ mt-tRNA presequences were cleaved in the nucleus (Fig. S3*B*). To stop the cleavage of mt-tRNA presequences with the *RP* sequence inside the nucleus, several ribonucleotides adjacent to the aminoacyl stem of the mt-tRNA were replaced, creating *LysA*, *RPLysA*, *LeuA*, and *RPLeuA* (Fig. 4*A*). The ribonucleotides adjacent to tRNA stems are usually unpaired; when the aminoacyl stem is extended by eliminating the mismatch caused by unpaired ribonucleotides, the cleavage rates of tRNA presequences are significantly reduced (36). When *LysA*, *RPLysA*, *LeuA*, and *RPLeuA* were expressed in mammalian cells, an increase in unprocessed mt-tRNA precursors was detected (Fig. S3*C*). However, these nucleus-encoded mt-tRNA precursors still failed to rescue the MERRF or MELAS respiration defect (Fig. 4*B*).

We reasoned that the mt-tRNA precursors might not localize near the mitochondria and, therefore, the *RP* sequence could not function as an import signal, as it does with isolated mitochondria in vitro. Consistent with this reasoning is the known requirement for regional localization of cellular mRNAs. It was originally assumed that mRNAs associated only with the endoplasmic reticulum, but now it has been shown that different mRNAs can be targeted to different subcellular locations where translation occurs locally (37). Mitochondrial mRNAs, mainly of prokaryotic origin, are targeted to the mitochondrion by a 3′-UTR (38, 39). As an example, the mRNA of human mitochondrial ribosomal protein S12 (*MRPS12*) is targeted to the proximity of mitochondria through the microtubule network (32). Targeting is mediated by elements within 154 ribonucleotides

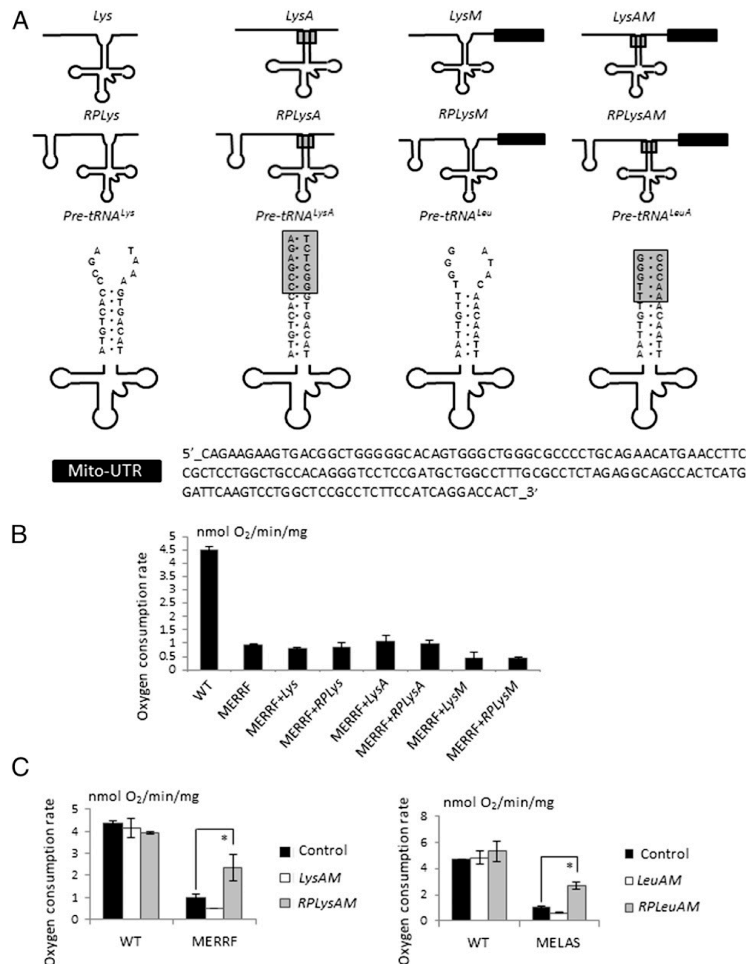

Fig. 4. Three elements are required for mt-tRNA precursors encoded in the nucleus to rescue mt-tRNA respiratory defects in vivo. (*A*) Schematic of the mt-tRNA precursors generated for the in vivo rescue assay. The single step loop at the 5′ of the second row structures is the *H1* RNA import sequence, *RP*. The shaded box indicates ribonucleotides that were changed to make tRNA precursors less susceptible to processing in the nucleus. The solid box is the 3′-UTR of *MRPS12* that localizes RNA to the vicinity of mitochondria (32). (*B*) *tRNALys* precursors lacking one or two of the three elements do not rescue the MERRF respiratory defect. (*C*) *tRNALys* or *tRNALeu* precursors with all three elements rescue respiration in MERRF and MELAS cells. $n = 3$ independent experiments. $^{*}P < 0.01$ with Student's *t* test.

of its 3′-UTR (32). To test whether the 3′-UTR of *MRPS12* assists in the import of mt-tRNA precursors into mitochondria in vivo, we fused the *MRPS12* 3′-UTR to the 3′ end of stabilized and *RP*-containing mt-tRNA precursors. In total, eight expression constructs were generated, including *LysM* (*mt-tRNA*$_{AAA}^{Lys}$ precursor with *MRPS12* 3′-UTR), *RPLysM* (*mt-tRNA*$_{AAA}^{Lys}$ precursor with *RP* and *MRPS12* 3′-UTR), *LysAM* (*mt-tRNA*$_{AAA}^{Lys}$ precursor with the extended stem and *MRPS12* 3′-UTR), *RPLysAM* (*mt-tRNA*$_{AAA}^{Lys}$ precursor with *RP*, the extended stem, and *MRPS12* 3′-UTR), *LeuM* (*mt-tRNA*$_{UUR}^{Leu}$ precursor with *MRPS12* 3′-UTR), *RPLeuM* (*mt-tRNA*$_{UUR}^{Leu}$ precursor with *RP* and *MRPS12* 3′-UTR), *LeuAM* (*mt-tRNA*$_{UUR}^{Leu}$ precursor with the extended stem and *MRPS12* 3′-UTR), and *RPLeuAM* (*mt-tRNA*$_{UUR}^{Leu}$ precursor with *RP*, the extended stem, and *MRPS12* 3′-UTR) (Fig. 4*A*).

Stable polyclonal transfectants with the various tRNA chimeras in MERRF and MELAS cells were made and cell respiration was measured with an XF24 Extracellular Flux Analyzer (Seahorse Biosciences). When all three elements (the extended stem, the *RP* sequence, and *MRPS12* 3′-UTR) were present, the mt-tRNA precursors rescued MELAS and MERFF respiration defects (~2.5-fold increase); otherwise, no rescue was detected (Fig. 4*C*). Expression of mt-tRNA precursors in vivo did not have a significant effect on the respiration of wild-type cybrid cells, suggesting little perturbation of other cellular functions (Fig. 4*C*). To evaluate whether the rescue of respiration is from a correction in mtRNA translation by imported wild-type mt-tRNAs, an in vivo mitochondrial translation assay was performed with MERRF and MELAS cells expressing different versions of the mt-tRNA precursors. Consistent with the respiration

results, MERRF and MELAS cells showed a substantial reduction in the synthesis of mitochondrion-encoded proteins compared with the wild-type cybrid cells. Only when the mt-tRNA precursors with all three elements were expressed, did mitochondrial protein synthesis recover (threefold to sixfold increase) (Fig. 5 *A* and *B*). A complete recovery was not expected, as the mutant mt-tRNAs were still present in the mitochondria and likely competed with the imported wild-type mt-tRNAs during mtRNA translation. Stable mitochondrial protein levels in MERRF and MELAS cells were also examined. Consistent with the increase in respiration and in vivo translation results in the mutant cells expressing the mt-tRNA precursors containing all three elements, the levels of mitochondrial-encoded COX2 and ND6 proteins were markedly increased (Fig. 5*C*). To assess the assembly and activity of the respiratory chain complexes, in-gel activity assays for complex I were performed, using blue-native gel electrophoresis. In the MELAS and MERRF cells expressing the mt-tRNA precursors containing all three elements, the activity of complex I increased to 30–40% of the WT level (Fig. S4). These results show that for nucleus-encoded mt-tRNA precursors to be imported into and adequately function within mitochondria, an extended stem is required for a subpopulation of these precursors to escape the nucleus without the presequences being processed. Also, a mitochondria localization sequence such as *MRPS12* 3′-UTR is required, probably for the precursor to traffic to the proximity of mitochondria. And finally, an RNA import sequence, in this case *RP*, is required for the mitochondria to internalize the mt-tRNA precursor.

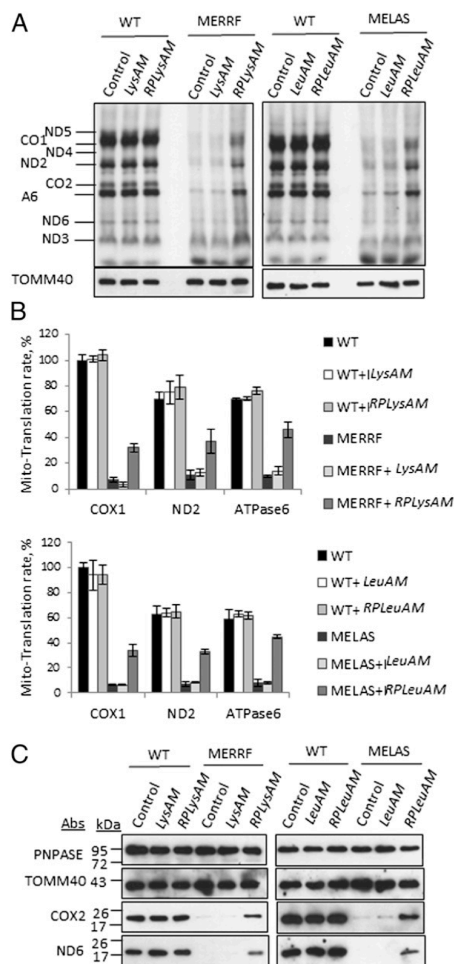

Fig. 5. Rescue of respiration is due to restoration of mitochondrial translation. (*A*) Analysis of mitochondrial translation in vivo with stable rescue cell lines. Mitochondrial translated proteins were separated by SDS/PAGE and visualized by autoradiography. (*B*) Quantification of specific bands on gels from *A*. Autoradiogram counts were normalized to protein amounts and expressed relative to WT control samples. (*C*) Steady-state levels of nucleus-encoded and mitochondrial-encoded proteins in WT, MERRF, and MELAS cells. TOMM40 and PNPASE also serve as loading controls.

Discussion

Defects in mtDNA are implicated in a plethora of human conditions, including neurodegenerative and cardiovascular diseases, muscular disorders, and the process of aging (22, 23). Despite a significant need, there are currently no effective treatments for deleterious mtDNA alterations. Our results show that these disorders can potentially be treated using targeted mitochondrial RNA import. We show that the RP import sequence mediates import of not only mitochondrial tRNA precursors but also much larger nucleus-expressed mRNAs into the mitochondrial matrix. Disease-causing mutations and deletions in the mitochondrial genome seem to be fairly equally distributed along the mitochondrial DNA (5, 22, 23) and this approach may generalize to rescue them all.

Up to now, experimental approaches using mitochondrial RNA import to rescue mitochondrial function have been restricted to correcting the defects caused by mitochondrial tRNA mutations. These efforts require the introduction of nonnative tRNAs with foreign protein factors or the transfer of a large multisubunit aggregate into cells, which is of low efficiency and difficult to reproduce in desirable disease-relevant settings (27, 40, 41). Our approach, however, has no such restriction; rationally engineered human mitochondrial tRNAs and mRNAs can both be efficiently targeted and functional. The fusion RNA presequences are encoded in the nuclear genome and can be imported into mitochondria where they are processed, restore translation, and are degraded via normal pathways in the mitochondrion. Importantly, these modified RNAs do not appear to have negative effects on other cellular processes, as wild-type cell metabolism is not affected. In vivo, the efficient import also depends on how well the RP import sequence is protected from processing in the nucleus and cytosol and whether the RNA precursors can gain access to the vicinity of mitochondria. We have shown that even for precursors that are normally processed in the nucleus and have no access to the vicinity of mitochondria, there are avenues to manipulate the processing and trafficking, although it is interesting that, at least for allotopically expressed *COX2* transcripts, a mitochondrial localization sequence is not required for *RP* sequence-PNPASE–mediated import.

The approach we have described here can be adapted for importing different kinds of RNA into mammalian mitochondria. The RNA sequences can be altered to inhibit processing steps in maturation in the nucleus without affecting the function of the mature RNAs in the mitochondria. For RNAs sequestered elsewhere in the cell, a mitochondria localization sequence from the 3′-UTR of MRPS12 can be appended to redirect the RNA precursor to the mitochondrion. Thus, this approach may generalize to mtDNA mutations in mt-tRNAs, mt-rRNAs, and protein-encoding mtRNAs as well as to heteroplasmic mtDNA populations, where ribozymes can be targeted (42). Thus, this rational transcript engineering approach may represent a unique therapeutic opportunity for a wide range of diseases caused by

mutations in the mitochondrial genome for which current effective therapies are lacking.

Materials and Methods

Cell Culture, Transfection, and Transduction. Mammalian cell lines were maintained in DMEM growth medium supplemented with 10% FBS and 2% L-glutamine. MERRF and MELAS cybrid lines (kindly provided by Carlos Moraes, University of Miami Miller School of Medicine) were maintained in DMEM growth medium supplemented with 10% FBS, 2% L-glutamine, and 0.5 mg/mL uridine. Transient transfections were performed using Bio-T reagent (Bioland Scientific). In transfections with *COX2* constructs, the calcium phosphate uptake method was used. Retroviral supernatants were produced by transient transfection of the 293T Phoenix packaging cell line, after which the cells were bulk infected and selected in puromycin.

Biochemical Assays with Mitochondria and Additional Methods. Detailed methods are listed in *SI Materials and Methods*.

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Appendix II: PNPase and RNA trafficking into mitochondria

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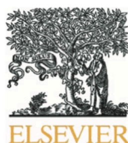

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Review

PNPASE and RNA trafficking into mitochondria☆

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ABSTRACT

The mitochondrial genome encodes a very small fraction of the macromolecular components that are required to generate functional mitochondria. Therefore, most components are encoded within the nuclear genome and are imported into mitochondria from the cytosol. Understanding how mitochondria are assembled, function, and dysfunction in diseases requires detailed knowledge of mitochondrial import mechanisms and pathways. The import of nucleus-encoded RNAs is required for mitochondrial biogenesis and function, but unlike pre-protein import, the pathways and cellular machineries of RNA import are poorly defined, especially in mammals. Recent studies have shown that mammalian polynucleotide phosphorylase (PNPASE) localizes in the mitochondrial intermembrane space (IMS) to regulate the import of RNA. The identification of PNPASE as the first component of the RNA import pathway, along with a growing list of nucleus-encoded RNAs that are imported and newly developed assay systems for RNA import studies, suggest a unique opportunity is emerging to identify the factors and mechanisms that regulate RNA import into mammalian mitochondria. Here we summarize what is known in this fascinating area of mitochondrial biogenesis, identify areas that require further investigation, and speculate on the impact unraveling RNA import mechanisms and pathways will have for the field going forward. This article is part of a Special Issue entitled: Mitochondrial Gene Expression.

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1. Introduction

Mitochondrial biogenesis requires the import of nucleus-encoded macromolecules including proteins and RNAs. Compared to the mitochondrial protein import pathways, which have been well characterized [1–4], the pathways importing RNAs into mammalian mitochondria, and the functions of imported RNAs, are just being discovered. Mitochondrial import of nucleus-encoded RNAs including tRNAs, *5S* rRNA, *RNase P* RNA, and *MRP* RNA is essential for mitochondrial DNA replication, transcription and translation [3,5–10]. The mitochondrial matrix localization of these small non-coding mammalian RNAs relies on a newly described RNA import regulator, polynucleotide phosphorylase (PNPASE) [11].

PNPASE is a highly conserved 3′–5′ exoribonuclease expressed in organisms that include bacteria, plants, flies, mice, and humans, but is absent in fungi, Trypanosoma, and Archaea [12–16]. PNPASE degrades RNA by phosphorolysis and can also function as a template independent polymerase [17–23]. Prokaryotic and plant PNPASE function in RNA quality control through its RNA polymerase and degradation activities [22–26]. However, the search for a specific function for mammalian PNPASE beyond its overall role in maintaining mitochondrial homeostasis has been confounding. This is because PNPASE was localized in the mitochondrial intermembrane space (IMS), which was believed to be devoid of RNA. The discovery that PNPASE regulates the import of selected nucleus-encoded small RNAs into mitochondria marks a turning point in understanding the function of PNPASE. This exciting discovery still leaves open many unanswered questions, including the mechanism(s) and pathway(s) of PNPASE-regulated RNA import.

2. *PNPT1* expression and PNPASE structure

PNPT1, the gene encoding for PNPASE in humans, was first reported in a screen for upregulated genes in both senescent progeroid fibroblasts and terminally differentiated melanoma cells [27]. The *PNPT1* gene is ~60 kb in length, contains 28 exons, and is located at 2p16.1 (UCSC Genome Browser, Assembly GRCh37/hg19, February, 2009). This genomic region shows deletions and amplifications in human B cell lymphoma and in several genetic disorders [28,29]. Relatively little is known about the transcriptional or post-transcriptional regulation of *PNPT1* expression. So far, the only known inducers of *PNPT1* transcription are

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the type I interferons (IFNs) [13]. Stimulation with IFNα or IFNβ was shown to activate the Janus-activated kinase (JAK)/signal transducer and activators of transcription (STAT) pathway, causing interferon-stimulated gene factor 3 (ISGF3) to bind to an interferon stimulated response element (ISRE) in the *PNPT1* promoter, inducing gene transcription [13]. The *PNPT1* promoter includes many additional regulatory protein-binding sites, such as a putative site for E2F transcription factor 3 (E2F3), which silences target gene expression during the G_1 to S cell cycle phase transition [13]. Since *PNPT1* expression was originally identified in senescent and terminally differentiated cells that have left the cell cycle, it would be interesting to know whether E2F3 acts as a *PNPT1* repressor and what its role could be in regulating *PNPT1* expression in cycling or senescent mammalian cells.

Whether PNPASE protein expression is regulated by type I IFN induction is still unresolved. From our own studies in mice, PNPASE is strongly expressed in all primary tissues examined including brain, heart, lung, liver, intestine, kidney, thymus, and skeletal muscle (data not shown). In one set of human cell line studies, PNPASE was not detectable without IFNβ induction [30,31]. By contrast, other studies showed abundant PNPASE expression in human cell lines under basal conditions [32–34] with IFNβ exposure failing to induce further expression in some of the lines examined [32,33]. These differing results may reflect species and cell line specific differences or differences in detection reagents and methods employed. Since *PNPT1* knockout is embryonic lethal and hepatocytes or mouse embryonic fibroblasts (MEFs) devoid of PNPASE expression cannot be propagated [11], it might be that all primary mammalian cell types require some level of PNPASE expression. This is sensible to consider, given the role of PNPASE in maintaining mitochondrial homeostasis and its underlying essential functions in RNA import and processing. What role type I IFN induction plays in PNPASE regulation of mitochondrial physiology is currently unknown. However, Type I IFNs are cytokines released by virally-infected cells that generally result in antiviral and growth inhibitory autocrine and paracrine responses [35]. The recent discovery of the mitochondrial antiviral signaling complex (MAVS, CARDIF) located on the mitochondrial outer membrane (OM), and its interaction with processed viral RNA fragments, suggests that juxtaposed PNPASE could have a RNA processing role in cellular antiviral responses, although there is no evidence for this possibility thus far [36].

PNPASE proteins from different species share five highly conserved sequence and structural motifs (Fig. 1). Two RNase PH domains, which are homologous to the *Escherichia coli* tRNA processing RNase PH enzyme, are located at the PNPASE amino-terminus [14]. An alpha-helical domain unique to PNPASE proteins is located between the two RNase PH domains [20,37]. And KH and S1 RNA binding domains, which are also present in other RNA binding proteins, are located at the PNPASE carboxy-terminus [37–39]. In *E. coli*, PNPASE can exist on its own, it can be bound to the RhlB RNA helicase, or it can participate in a degradosome complex with RNase E, RhlB, and enolase [15,21,40]. Crystallography studies using PNPASE isolated from *Streptomyces antibioticus* and from *E. coli* revealed that three PNPASE monomers form a doughnut-shaped structure through interactions of their catalytic RNase PH domains [37,41]. In such a configuration, the KH and S1 domains of each monomer localize to one face of the doughnut, where they can bind RNA substrates and guide them through the central enzymatic channel in the trimeric complex [37,41]. A cylindrical structure for PNPASE is also possible, in which two trimers stack into a hexameric configuration with an elongated central enzymatic channel. Plant genomes contain two distinct genes that encode for PNPASE, with one gene containing a chloroplast transit peptide and the other gene having a mitochondrial targeting pre-sequence (MTS) [38,42]. In contrast with its bacterial counterpart, chloroplast PNPASE is not known to interact with any other proteins and may instead form a homo-hexameric complex [43]. Isolated human PNPASE is ~260–280 kDa in native gels, also suggesting a homo-trimeric complex [11,32,37,41]. Interestingly, the archaeal and eukaryotic exosomes, which do not contain PNPASE but also function in RNA turnover and surveillance, have a similar trimeric doughnut-shaped quaternary structure [12,20].

3. Subcellular localization of PNPASE

Mammalian PNPASE has an amino-terminal MTS and has been localized to mitochondria in both immunofluorescence and cell-fractionation studies [32,44,45]. Since PNPASE processes RNA it was anticipated to reside in the mitochondrial matrix where mtRNA transcription, processing, and translation occur. However, sub-fractionation and protease-protection assays using isolated mouse liver mitochondria, or yeast mitochondria expressing exogenous human PNPASE, repeatedly showed an unexpected intermembrane space (IMS) localization [11,32]. This result was very surprising because RNAs are not known to reside in the mitochondrial IMS. Carbonate extraction studies further showed that PNPASE is a peripheral inner membrane (IM)-bound protein facing the IMS [32]. To reach its IMS location, PNPASE traffics through the translocase of the outer mitochondrial membrane (TOM) as a precursor, followed by engagement of the translocase of the inner mitochondrial membrane (TIM)-23 complex. These trafficking steps require an intact $\Delta\Psi$ and are followed by the amino-terminus of PNPASE extending through the TIM23 complex so that the matrix processing peptidase (MPP), consisting of a Mas1/Mas2 heterodimer, can cleave away the 37-residue MTS. Then, the IM-bound *i*-AAA (ATPases associated with several diverse cellular activities) protease, Yme1, mediates the release of the mature amino-terminal portion of PNPASE into the IMS and functions as a translocation motor to pull the carboxy-terminal portion of PNPASE through the TOM complex into the IMS [44] (Fig. 2). IMS-imported and processed PNPASE then assembles into a functional homo-oligomeric complex, consisting of a trimer or a dimer of trimers [44].

The IMS localization of mammalian PNPASE is highly reproducible. However, whether all PNPASE localizes exclusively in the IMS of mammalian mitochondria is controversial. Over-expressed PNPASE

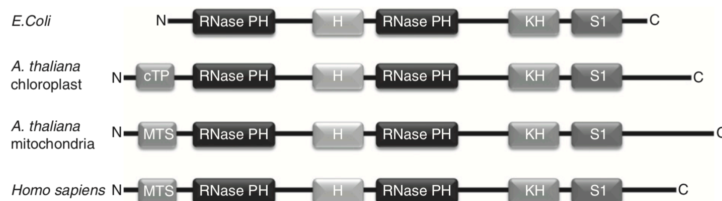

Fig. 1. PNPASE structural domains are conserved between species. PNPASEs contain two RNase PH domains that are homologous to the bacterial tRNA processing enzyme, RNase PH, and these domains catalyze RNA degradation. The alpha helical domain (H) is unique to PNPASE, and the KH and S1 domain at the C terminus bind RNA. In contrast to bacteria and other mammals, plants express two forms of PNPASE, one with a chloroplast transit peptide (cTP) and the other with a mitochondrial targeting sequence (MTS).
Adapted and reprinted with permission from Chen, et al., Trends in cell biology 17 (2007) 600–608.

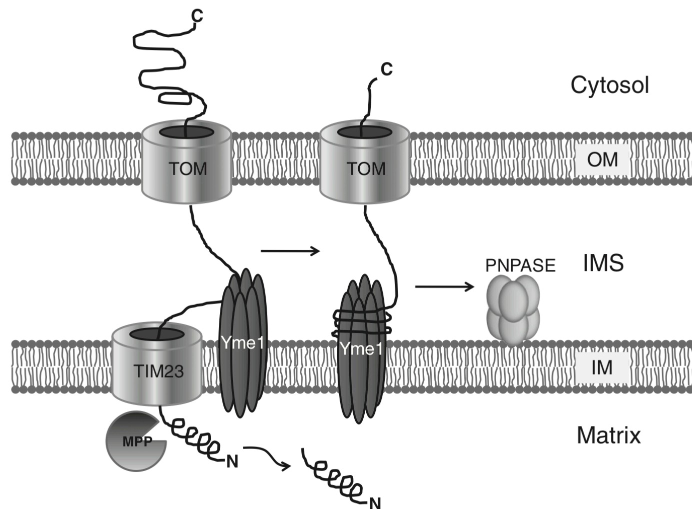

Fig. 2. Mammalian PNPASE is imported into the mitochondrial IMS via a Yme1-dependent mechanism. After passing through the TOM complex at the OM, the PNPASE amino-terminus extends through the TIM23 complex at the IM into the matrix, where the 37 amino-acid MTS is removed by the matrix-processing peptidase (MPP), Mas1/Mas2. The mature amino-terminus is then released into the IMS with the help of IM-localized Yme1 *i*-AAA protease, which also helps to reel the remaining portion of PNPASE into the IMS from the cytosol. Mature, processed PNPASE then assembles into a homo-oligomeric complex, consisting of a trimer or a dimer of trimers attached to the IM facing the IMS.

with a carboxy-terminal HA-tag was detected in the cytosol of the HO-1 melanoma cell line [31]. PNPASE has also been reported as an interacting partner for TCL1, a cytosol-localized nonenzymatic oncoprotein that promotes B- and T-cell malignancies, and as a weak binding partner for hSUV3, a mitochondrial matrix RNA helicase [46,47]. These extra-IMS locations, however, could be cell-type specific, condition-specific, or experimental artifact. For example, terminal differentiation, cellular senescence, or apoptosis could alter the distribution of PNPASE in a cell. Over-expression studies can also lead to the accumulation of proteins in locations that are normally devoid of them. Finally, the solubilization step used to identify interacting proteins can bring together proteins that normally reside in different compartments of a cell.

4. The maintenance of mitochondrial homeostasis by PNPASE

The effect of reduced PNPASE expression on mitochondrial structure and function has been well studied using shRNA approaches. In a variety of mammalian cell types PNPASE expression above a critical threshold is required for mitochondrial homeostasis. Conversely, a reduction in PNPASE expression that fails to reach a critical knockdown threshold results in no observable changes in mitochondrial morphology or function [11,32,48]. PNPASE reduction to ~20–30% or less of the wild-type expression level caused filamentous mitochondrial networks to fragment with a drop in $\Delta\Psi$ and a 2-fold or greater reduction in the activities of linked respiratory chain complexes I and III, II and III, or individual complexes IV and V [32]. These data indicate that PNPASE expression is required for efficient oxidative phosphorylation. PNPASE deficiency also leads to cellular changes secondary to mitochondrial dysfunction that include lactate accumulation, reduction in steady state ATP levels, and reduced cell proliferation [32].

Pnpt1 knockout mice have been generated. A whole-animal *Pnpt1* knockout was embryonic lethal, indicating an essential role for PNPASE in early mammalian development [11]. By contrast, a liver-specific conditional *Pnpt1* knockout (HepKO, for hepatocyte knockout) was viable. The HepKO mouse was generated using albumin-CRE recombinase to excise *loxP* recombination sites that were inserted flanking exon 2 of *Pnpt1*. A 2 to 4-fold PNPASE reduction in HepKO liver cells was achieved at ~6 to 8 weeks of age followed by a rebound in PNPASE expression thereafter, likely due to incomplete *Pnpt1* knockout from CRE recombinase-mediated excision and liver regeneration from hepatocytes still expressing *Pnpt1* [11]. Transmission electron microscopy of HepKO liver cells showed disordered, circular, and smooth IM cristae in contrast to the ordered, linear, stacked cristae with convolutions exhibited by wild-type control liver mitochondria. Oxygen consumption studies with a Clark-type electrode also revealed a 1.5 to 2-fold decrease in the activity of respiratory complex IV and complexes II + III + IV. These ultrastructure and functional results validate a role for PNPASE in maintaining mitochondrial homeostasis in a physiological, in vivo setting.

In addition to PNPASE loss-of-function studies, gain-of-function studies, despite the risk for aberrant PNPASE localization, also seem to support a role for PNPASE in mitochondrial homeostasis. PNPASE over-expression results in increased reactive oxygen species (ROS) accumulation over time, which subsequently leads to NF-κB activation and an increase in NF-κB-regulated pro-inflammatory cytokines, including IL-6, IL-8, RANTES, and MMP-3 [49,50]. The mechanism of ROS accumulation with PNPASE over-expression is unclear, although most ROS is produced within mitochondria as a result of respiratory chain activities [51–53]. PNPASE reduction inhibits respiratory chain production, respiratory complex activities, and oxygen consumption [11,32]. Excess PNPASE could have the opposite effect, with increased respiratory activity and mitochondrial ROS production, although this idea requires experimental validation.

5. PNPASE, mtRNA processing, and RNA import into mitochondria

A reduction in respiration and the synthesis of mitochondrion-encoded proteins of the respiratory chain in HepKO liver cells compared to wild-type controls led to studies of PNPASE in mtRNA transcription and translation. HepKO liver cells showed a surprising reduction in mature mtRNA transcripts and encoded proteins, directly related to deficient mtRNA processing [11]. All of the mammalian mtRNA processing molecules are encoded in the nuclear genome and synthesized either

in the nucleus (RNAs) or cytosol (proteins), followed by import into mitochondria [10,53–56]. PNPASE deficiency had no significant effect on the mitochondrial import of nucleus-encoded proteins [11]. By contrast, HepKO liver cells showed a significant reduction in the RNA component of the RNase P mtRNA processing complex. The reduction in *RNase P* RNA was not from increased degradation, indicating instead a defect in *RNase P* RNA import with PNPASE deficiency [11]. Studies using a variety of in vitro and in vivo experimental systems have now demonstrated a direct role for PNPASE in regulating the import of multiple non-coding RNAs into mitochondria. The RNA import function of PNPASE is separable from its RNA-processing function, as an engineered point mutation that cripples its RNA-processing activity has no effect on RNA import into mitochondria [11].

The molecular mechanism by which PNPASE distinguishes RNAs for processing versus those for import remains to be clarified. PNPASE recognizes an RNA stem–loop structure in some and perhaps all of the RNAs it helps to import [11]. It is not known whether mammalian PNPASE binds RNA stem–loop structures with its KH/S1 RNA binding domain in distinct ways to either trigger RNA import or processing. RNA structural elements regulate PNPASE functions in chloroplasts and prokaryotes and a stem–loop structure protects RNAs from degradation by chloroplast PNPASE [23,57,58]. It would be of great interest to determine whether mammalian PNPASE functions in a similar manner.

5.1. RNAs imported into mitochondria

Most non-coding RNAs that function in mitochondria are encoded within the mtDNA [59]. On one end of the spectrum, the mtDNA of land plants and protists, such as *Chlamydomonas*, *Paramecium*, and *Tetrahymena* lack one or a few tRNAs that are required to translate mtRNA. At the other extreme, the trypanosomatid protozoa lack all of the mitochondrial tRNA genes. And for *Saccharomyces cerevisiae* and *Marchantia polymorpha*, the mtDNA encodes a set of tRNAs that is sufficient for translating all mtRNA codons, but nucleus-encoded tRNAs are nevertheless imported for unknown reasons [60,61]. Mammalian mitochondria do not import tRNAs, except maybe the nucleus-encoded $tRNA_{CUG}^{Gln}$ and $tRNA_{UUG}^{Gln}$. Additional nucleus-encoded small RNA molecules are also present in mammalian mitochondria [54,62–65].

Early studies on tRNA import into mitochondria have shown that it is a selective process, with the selectivity determined by specific tRNA structural or sequence motifs [66]. RNA import is ATP-dependent and involves protease-sensitive receptors at the mitochondrial OM. Thus far, two main general mechanisms for tRNA import into mitochondria have been identified. One mechanism is similar to protein import and is used by yeast to import $tRNA_{CUU}^{Lys}$ (Fig. 3) [67,68]. This tRNA import mechanism requires an intact $\Delta\Psi$ and utilizes the protein import pathway. An association with the precursor mitochondrial tRNA synthetase and aminoacylation of the tRNA by the cognate cytosolic synthetase are also required for import. In addition, the metabolic enzyme enolase helps to deliver a tRNA/synthetase complex to the import machinery [69–71]. A second mechanism is independent of the protein import pathway and does not require cytosolic factors even though they may still have an influence when present (Fig. 3) [72]. This additional mechanism has been identified in most organisms that import tRNA into mitochondria including yeast [66,73], but this import pathway is less well defined. The OM voltage-dependent anion channel (VDAC) has an essential role for tRNA import in plants in vitro, but a homologous protein is dispensable for tRNA import in the protozoan, *Trypanosoma brucei* (Fig. 3) [74,75]. For *Trypanosoma brucei*, elongation factor 1a (eEF1a) mediates mitochondrial tRNA import (Fig. 3) [76].

RNase MRP is a site-specific endoribonuclease. It was originally isolated from mouse mitochondria and in vitro shows activity in processing mtRNA transcripts to form primers complementary to the mtDNA origin of replication [65]. RNase MRP has a nucleus-encoded RNA component that contains a decamer sequence complementary to a conserved region of mtRNA substrates [65]. Most RNase MRP, however, resides in the nucleus [77,78]. In *Saccharomyces cerevisiae*, nuclear RNase MRP processes rRNA precursors to generate the mature *5.8S* rRNA [79–82]. During mitosis in *S. cerevisiae*, nuclear RNase MRP is transiently relocated to temporal asymmetric MRP (TAM) bodies in the cytosol, where it cleaves the mRNA for a B-type

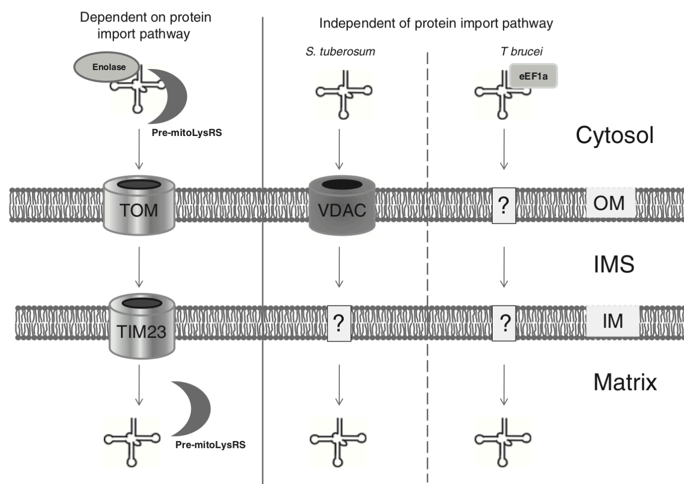

Fig. 3. Routes of mitochondrial tRNA import. Two mitochondrial tRNA import mechanisms have been reported. Shown on the far left is a tRNA import that utilizes the protein import pathway, including the TOM complex at the OM and the TIM23 complex at the IM. The imported tRNA is delivered to the mitochondrial OM bound to pre-mitochondrial lysyl tRNA synthetase (pre-mitoLysRS) and enolase after aminoacylation by its cognate cytosolic synthetase. Enolase dissociates at the surface of mitochondria and the remainder of the protein–tRNA complex is imported together into the matrix. Thus far, this mechanism has only been described for the import of $tRNA_{CUU}^{Lys}$ into yeast mitochondria. A second mechanism has been reported in organisms ranging from kinetoplastids to human that does not depend on the protein import pathway. Different factors have been identified in this pathway that are organism-specific, including the VDAC channel in the OM for plants and the translation elongation factor, eEF1a, for *T. brucei*.

cyclin, Clb2, to promote the end of mitosis [82,83]. The *S. cerevisiae* mitochondrial and nuclear RNase MRP have identical RNA components but contain distinct protein components and show differing enzymatic activities [84].

Another nucleus-encoded RNA that is imported into mammalian mitochondria is the RNA component of RNase P [10,54]. RNase P is an endoribonuclease that processes the 5′ end of mitochondrial tRNAs [10]. The mammalian mitochondrial genome consists of a circular chromosome with tightly packed genes. Large polycistronic RNA transcripts are generated from the heavy and light strand promoters. tRNAs often separate the coding regions for electron transport chain complex subunits and, unlike nucleus-encoded tRNA precursors, do not have individual 3′ or 5′ pre-sequences [53,85]. In most arrangements, multiple tRNAs are grouped together without an intervening pre-sequence in the polycistronic transcripts. The processing of polycistronic transcripts to individual mtRNAs and mature tRNAs requires RNase P enzymatic activities. Earlier studies showed that the RNA component of RNase P was imported into mammalian mitochondria and was identical to the RNA component of nuclear RNase P [10,54]. By contrast, the RNA component of mitochondrial RNase P in *S. cerevisiae* is encoded within its mtDNA [86]. Recently, a functional protein-only mitochondrial RNase P enzyme was isolated from human mitochondria. This alternative mitochondrial RNase P has three protein subunits and processes single tRNA precursors with artificial 5′ pre-sequences in vitro [56]. It remains to be determined whether the protein only mitochondrial RNase P can efficiently process physiological mitochondrial tRNA substrates, especially those that are sequentially linked in polycistronic transcripts with no intervening pre-sequences. Interestingly, a tRNA processing assay using mitoplast lysate with or without nuclease pre-treatment to eliminate RNA showed that an RNA component or components are required for efficient processing of abutting tRNA precursors, further confirming the existence of a RNA-containing mitochondrial RNase P complex [11]. Most likely, protein-only and RNA-containing RNase P complexes coexist in mammalian mitochondria.

The nucleus-encoded *5S* rRNA was localized within mammalian mitochondria [64,87]. The *5S* rRNA is the most abundant imported mammalian RNA and an essential ribosome component in almost all living organisms, providing regulatory interactions among most functional sites within the translating ribosomal machinery [88]. Although *5S* rRNA has been detected in most non-mammalian organisms, the exact function of *5S* rRNA inside mitochondria is still unresolved [89]. Recent studies suggest that *5S* rRNA is a component of the mitochondrial ribosome, but based on prior structural analysis of the mitochondrial ribosome its role could be very different from its cytosolic counterpart [7,89,90].

There are also recent reports suggesting that specific microRNAs (miRNAs) localize inside mitochondria [63]. miRNAs are a class of small (~22 ribonucleotide) non-coding RNAs that regulate gene expression post-transcriptionally by binding to imperfectly or perfectly matched complementary sequences at the 3′ end of target mRNAs, usually resulting in translational repression or transcript degradation and gene silencing [91,92]. They are present in almost all eukaryotes except fungi, algae, and marine plants, and are all nucleus-encoded [93]. The human nuclear genome is estimated to encode for over 1000 miRNAs. Fifteen miRNAs were identified in rat liver mitochondria and five of these were confirmed using specific probes [63]. Based on target analysis using sequence algorithms, such as Miranda and TargetScan, it appears that the miRNAs neither target mtRNA transcripts nor complement the nuclear RNAs that encode imported mitochondrial proteins, but instead seem to be involved in the regulation of genes associated with apoptosis, cell proliferation, and differentiation [63,94]. Hence, it was proposed that mitochondria might serve as reservoirs for specific miRNAs that modulate these cellular processes. Most recently, miRNAs along with Argonaute 2, a component of the RNA-induced silencing complex (RISC), were coimmunoprecipitated with the *COX3* mtRNA transcript, suggesting that miRNA-mediated translational regulation could also occur in mammalian mitochondria [95]. Whether miRNAs localize in mitochondria is still under debate. A recent study showed that the enrichment of some miRNAs in mitochondria disappeared once mitoplast procedures were performed [96], suggesting that these miRNAs could be low level contaminants, or more interestingly, could be associated with the outer membrane or localized inside the IMS.

A virally-encoded RNA was also suggested to be translocated into mammalian mitochondria. The 2.7-kb cytomegalovirus (CMV)-encoded *β2.7* RNA targets the Grim-19 (NDUFA13) protein of complex I in the electron transport chain [94]. CMV *β2.7* RNA associates with Grim-19 to maintain mitochondrial function in support of viral replication. Interestingly, the mitochondrial OM protein MAVS is activated by viral RNA binding and functions to protect cells from infection by inducing a type I IFN anti-viral response [36,97]. At present, whether viral RNAs are localized inside mitochondria has not been firmly established. Candidate viral RNAs could associate with the cytosolic precursor of proteins rather than the mature mitochondrion-localized proteins. If mitochondrial localization indeed occurs, nothing is known about the mechanism(s) of viral RNA import or the mechanism(s) for providing processed viral RNA fragments to MAVS. An interesting candidate for mechanistic studies is PNPASE, as it processes RNA, is localized in the IMS, and its expression may be regulated in concert with both Grim-19 and MAVS, as all 3 genes appear to be induced by type 1 interferons [13,32].

5.2. tRNA import into yeast and mammalian mitochondria

As already mentioned, nucleus-encoded $tRNA^{Lys}_{CUU}$ is imported into isolated yeast mitochondria in vitro [67,68] with tRNA synthetase binding, aminoacylation [69,70] and help from cytosolic enolase [71]. This import utilizes the TOM and TIM23 translocons and requires ATP and an intact $\Delta\Psi$, similar to protein translocation [98]. The yeast tRNA import pathway appears conserved in mammalian systems because isolated mitochondria from HeLa or HepG2 cells can be substituted for yeast mitochondria [68], and yeast cytosolic import factors remain essential for the process. However, studies addressing whether human cytosolic import factors were able to replace yeast cytosolic factors yielded conflicting results [68]. It is not known whether nucleus-encoded mammalian $tRNA^{Lys}_{CUU}$ can be imported into yeast or mammalian mitochondria under the same conditions. The in vitro transcribed mitochondrion-encoded $tRNA^{Lys}$, however, was imported into isolated human mitochondria. In this case, human cytosolic factors were essential for import and could not be replaced by yeast cytosolic factors [64]. In vivo, yeast $tRNA^{Lys}$ derivatives can also be imported into mammalian mitochondria and rescue defects caused by mitochondrial $tRNA^{Lys}$ mutations [99].

Yeast $tRNA^{Gln}$ is also imported into isolated yeast mitochondria without requiring added cytosolic factors [100]. Recently, mammalian mitochondria were shown to import nucleus-encoded mammalian $tRNA^{Gln}_{CUG}$ and $tRNA^{Gln}_{UUG}$ [62]. Both $tRNA^{Gln}_{CUG}$ and $tRNA^{Gln}_{UUG}$ were also identified in mitochondria isolated from human and rat liver using subcellular fractionation and RT-PCR [62], providing in vivo relevance. These tRNAs were also imported into isolated mammalian mitochondria independent of added cytosolic factors. Import required ATP but not an intact $\Delta\Psi$ [62].

5.3. RNA import mechanisms

In vitro and in vivo assay systems were developed to study the function of PNPASE in mitochondrial RNA import because the RNA component of RNase P was markedly decreased in PNPASE deficient cells [11]. Systems to test import included (1) liver mitochondria isolated from HepKO mice, (2) MEFs isolated from *Pnpt1 loxP*-flanked mice infected with a CMV-CRE recombinase-expressing retrovirus to

reduce PNPASE expression, and (3) several mammalian cell lines containing shRNAs targeting *Pnpt1* transcripts [11]. In these assay systems, the import of *RNase P* RNA into mitochondria correlated with PNPASE abundance. Additional nucleus-encoded RNAs, such as *MRP* RNA and *5S* rRNA, that also localize to mitochondria also showed PNPASE-dependent import in these assay systems. Yeast mitochondria were also tested since the RNA component of RNase P is encoded within the yeast mitochondrial genome. Heterologous expression of PNPASE supported the import of *RNase P* RNA into yeast mitochondria, suggesting that general mechanisms and/or features of the RNA import pathway are conserved in yeast, rodents, and humans [11].

A physical interaction between *RNase P* RNA with PNPASE adds support for a direct PNPASE import mechanism and suggests that PNPASE may function as a RNA import receptor in the IMS. *RNase P* RNA has mitochondrial targeting features. A systematic dissection identified a 20-ribonucleotide sequence that was essential for in vitro import of human *RNase P* RNA [11]. When appended to a non-mitochondrial RNA, this core sequence targeted the non-mitochondrial RNA to the mitochondrion and PNPASE bound directly to the fusion transcript. Interestingly, the identified import sequence is predicted to form a stem–loop structure. A ~20 ribonucleotide non-identical sequence was identified in *MRP* RNA that was predicted to form a stem–loop structure. This *MRP* RNA sequence could also direct non-mitochondrial RNA import when placed into chimeric fusion transcripts. Thus, a common theme for imported RNAs may be the formation of a stem–loop that facilitates PNPASE binding without activating its RNA processing functions. Currently, there are no reports on the mechanism(s) for miRNA import into mitochondria.

Components of the import pathway for *5S* rRNA into mammalian mitochondria have been described. Basic requirements include ATP, an intact $\Delta\Psi$, the protein translocases, and cytosolic factors [62]. The cytosolic factors include the cytosolic precursor of the mitochondrial ribosomal protein L-18 (MRP-L18) [7] and the mitochondrial thiosulfate sulfurtransferase, rhodanese [101]. MRP-L18 first binds to the *5S rRNA*, inducing a conformational change. Misfolded rhodanese then binds to the *5S* rRNA and the combination acts as reciprocal chaperones to facilitate import [101]. Inhibition of rhodanese expression decreased *5S* rRNA import and mitochondrial translation in vivo [101]. Two distinct structural elements of *5S* rRNA are required for its mitochondrial import. One is in the proximal part of helix 1 containing a conserved uncompensated G:U pair, and the second is associated with the loop E–helix IV region with several non-canonical structural features [87,102]. Whereas PNPASE-dependent import did not seem to require cytosolic proteins, the import efficiency seems more robust with cytosolic factors, suggesting that multiple import pathways may be used for *5S* rRNA import.

5.4. The mechanism of PNPASE-dependent RNA import into mitochondria

For the import of nucleus-encoded mammalian $tRNA^{Gln}_{CUG}$ and $tRNA\text{-}_{UUG}{}^{Gln}$, ATP is required, but unlike the import of yeast $tRNA^{Lys}$ and *5S* rRNA, import into mammalian mitochondria does not require an intact $\Delta\Psi$ or cytosolic factors [62]. Interestingly, PNPASE enhances RNA import in yeast, which does not have a PNPASE homolog, indicating that PNPASE can augment a distinct RNA import mechanism directly or independently [11]. An intact $\Delta\Psi$ is required for PNPASE-dependent import of *RNase P* RNA and no cytosolic factors are required (Fig. 4) [11]. In mammalian cells PNPASE expression is required for cell survival, so how essential PNPASE is for RNA import remains unclear. Also, it is not known whether there are PNPASE dependent and independent mitochondrial RNA import mechanisms in mammals, as may be the case in PNPASE-modified yeast. Identification of additional RNA import pathway components, including OM and IM channels, is essential to dissect mechanisms of RNA import into mammalian mitochondria.

Although PNPASE RNA degradation and mitochondrial RNA import activities are separable [11], it is not known whether PNPASE has a gatekeeper function for RNA import, degrading misplaced RNAs in the IMS. A stem–loop structure protects RNA from degradation by PNPASE in chloroplasts and stem–loop sequences in human *RNase P* and *MRP* RNAs could have a similar role, protecting these RNAs from PNPASE degradation during import [23,57]. This speculated gatekeeper function could be a differential property of mammalian PNPASE in comparison to its bacterial and chloroplast counterparts. Recombinant mammalian PNPASE does not preferentially bind poly(A) containing RNAs, consistent with data that PNPASE does not metabolize poly(A) mRNA tails [103]. Human PNPASE also has a

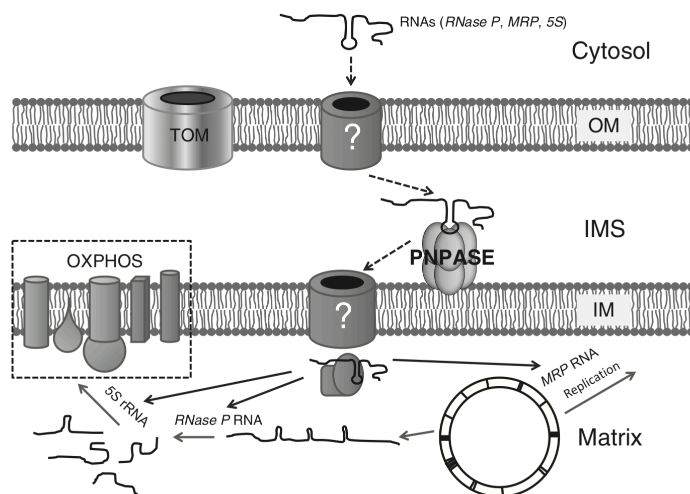

Fig. 4. PNPASE regulates the import of nucleus-encoded small RNAs including *RNase P* RNA, *MRP* RNA, and *5S* rRNA into the mitochondrial matrix. PNPASE promotes the import of RNAs from the cytosol into the matrix by binding to specific stem–loop motifs in the imported RNAs. Imported non-coding RNAs function in mitochondrial replication, transcription, and translation. The mitochondrial translation products are components of the oxidative phosphorylation (OXPHOS) complexes I–IV.

lower poly(A) polymerase activity than its *E. coli* counterpart. Poly(A) polymerization activity depends on the concentration of ADP, which is significantly lower in the IMS compared to the matrix [103]. It would be interesting to know whether PNPASE degradation activity is regulated by pH, as the mitochondrial IMS has a redox buffering system and lower pH than the matrix.

5.5. Mammalian PNPASE and mtRNA processing

In mammalian mitochondria, RNA editing is a critical step in gene expression. Large polycistronic RNA transcripts are generated from the heavy and light strand promoters [85]. RNA cleavage occurs at the 3′ and 5′ ends of the tRNAs that intervene between RNA coding genes, followed by polyadenylation of mtRNAs [104–106]. For several mitochondrial genes with a partial U or UA stop codon, the polyadenylation step completes the stop codon [104]. Whether polyadenylation also has an additional role in enhancing mtRNA stability is still debated. Detection and isolation of truncated, non-abundant, polyadenylated mitochondrial RNAs suggests that polyadenylation could occur with RNA transcripts destined for degradation, whereas another study shows deadenylation sometimes leads to quick decay of certain mutant transcripts [107,108].

In bacteria, organelles, and some eukaryotes, polyadenylation is catalyzed by one of the poly(A) polymerases (PAPs) or PNPASE [109]. Previously, it was proposed that mammalian PNPASE could act as a mtRNA poly(A) polymerase and also degrade polyadenylated mtRNAs because it exhibits both polymerization and RNA degradation biochemical activities [103]. However, the IMS location of mammalian PNPASE potentially excludes a direct role in these processes, although an indirect effect on mtRNA processing may be possible [32]. shRNA against *PNPT1* altered mtRNA polyadenylation without changing the mtRNA abundance [32,48,110]. In other studies, shRNA against *PNPT1* affected polyadenylation to different extents for different mtRNAs. For example, 5′ processing of *COX1* mtRNA was defective and its poly(A) tail was abolished [110]. By contrast, PNPASE knockdown had no effect on *COX3* polyadenylation and even led to longer poly(A) tail extensions for *ND5* and *ND3* mtRNAs [110]. Whether PNPASE regulates mtRNA polyadenylation by changing mitochondrial ATP concentrations is also unclear. Transient *PNPT1* shRNA knockdown caused a decrease in cellular ATP, which could alter mtRNA poly(A) tail lengths [48,110]. However, stable *PNPT1* shRNA knockdown showed inconsistent effects, with ATP levels decreased, slightly increased, or unaffected in different cell clones, all of which showed similar defects in mtRNA polyadenylation [110]. Also, the effect that *PNPT1* knockdown and ATP depletion had on mtRNA poly(A) tail lengths appears distinct, with the latter causing a decrease in poly(A) tail lengths in *ND3* and *ND5* mtRNA transcripts compared to an increase in poly(A) tail lengths by *PNPT1* knockdown [110]. Other work showed that the human mitochondrial matrix protein hSUV3 regulates mtRNA stability and the removal of non-coding processing intermediates. PNPASE was reported to interact with hSUV3, but this would place PNPASE in the mitochondrial matrix [47]. Even though it is impossible to rule out a small fraction of PNPASE in the matrix, an equally plausible explanation for this interaction between hSUV3 and PNPASE is that it occurs during the solubilization step in co-immunoprecipitation assays. In fact, human mtPAP has been shown to polyadenylate the mature 3′ ends of mtRNAs [48,111].

How mammalian PNPASE affects mtRNA processing is not resolved. PNPASE regulates the import of nucleus-encoded small RNAs including *RNase P* and *MRP* RNAs [11]. RNase P processes mitochondrial tRNAs and PNPASE knockdown results in the accumulation of partially processed polycistronic mtRNA transcripts, indicating a defect in cleaving polycistronic transcripts into individual coding RNAs, rRNAs, and tRNAs [11]. Interference of this early processing step could inhibit later processing steps, such as mature transcript polyadenylation and degradation. The polycistronic transcript cleavage sites are unique and could require different processing mechanisms, so that defective RNA import could cause variable defects in generating mature transcripts [85].

6. Future directions and concluding remarks

Thus far, PNPASE has been identified as an IMS localized protein that directly regulates RNA import into mammalian mitochondria. The remaining components of this and potentially additional mammalian RNA import pathways, however, remain unknown. The in vivo and in vitro import assay systems established for PNPASE studies provide tools for identifying and dissecting additional import components and mechanisms [11]. Importantly, even though *S. cerevisiae* does not have a PNPASE homolog, heterologous PNPASE expression enhances the import of RNA in vivo and in vitro, suggesting the import pathways in mammalian cells and *S. cerevisiae* are compatible, which may provide a system where yeast genetic approaches can be employed.

A second area of potential significance is in the identification of additional imported RNA species and the elucidation of their functions inside mitochondria. So far, mammalian mitochondria have been shown to import *RNase P* RNA, *MRP* RNA, *5S* rRNA, CMV *β2.7* viral RNA, several tRNAs, and a few miRNAs. It is likely that additional nucleus-encoded RNAs, and possibly more viral RNAs, will be identified inside mitochondria. Knowledge of the range of mitochondrial RNA import signals will provide clues as to which RNAs are potential candidates for import. New assay systems may also be required to unravel the functions of imported RNAs.

A fundamental question in considering RNA import into mammalian mitochondria also remains to be addressed. Are there distinct pathways, even PNPASE-dependent and independent pathways, or is there one major import pathway with different RNA substrates requiring similar or distinct accessory factors? In fact, a few features seem to converge in all systems studied to date. ATP seems to be universally required for RNA import and import appears to depend on one or more OM factor(s). So far, it is still unknown whether mammalian PNPASE is involved in tRNA import into mitochondria.

The function(s) of PNPASE in the mitochondrial IMS is still not fully understood. One remaining challenge is to understand a role for its biochemically demonstrated RNA degradation activity. PNPASE degradation and import activities are separable. More work is required to determine whether PNPASE RNA degradation activity plays a role in RNA import, such as a gate-keeper for import or a waste-disposal shunt. Also, what role, if any, PNPASE has in responding to infection and the localization of viral RNA in the IMS remains to be elucidated.

In addition, there are possible roles for mammalian PNPASE in cellular locations other than the IMS. For example, PNPASE has been shown to be released from the IMS into the cytosol late, hours after apoptosis induction and caspase activation [32]. Could PNPASE participate in degrading cytosolic RNAs in cells committed to death? Also, PNPASE may participate in antiviral responses; does type I IFN-induced *PNPT1* expression produce additional PNPASE and, if so, does all the increased PNPASE traffic to the mitochondrial IMS? With the nearby MAVS complex on the OM, does PNPASE process IMS localized viral RNAs and, if so, what is its role in delivery to MAVS? Finally, new approaches will be required to determine unequivocally whether small amounts of endogenous mammalian PNPASE localize into the mitochondrial matrix, and, if so, what may be the functional consequences.

In addition to potential significance in viral infection and cell death, the RNA import function of mammalian PNPASE may be manipulated to correct mitochondrial dysfunction. mtDNA mutations have been linked to multiple human neuromuscular diseases [112–114]. Aging is also associated with the accumulation of mtDNA

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mutations [115,116]. Currently, there is no efficient approach for overcoming mtDNA mutations in these morbid diseases. Existing therapies target symptoms instead of mending primary defects. Mitochondrial gene therapy provides an interesting approach to treating the root cause of these diseases. One practical application of studies on RNA import into mitochondria is to complement mtDNA alterations by introducing and importing corrective RNAs. Mitochondrial RNA import sequences could potentially be used to import RNAs that normally are not imported, such as those that could repair defective or inefficient electron transport chain mtRNAs. Another possible application is to manipulate the mitochondrial transcription or translation profile. For example, anti-sense RNAs could be imported to inhibit the expression of mutated mtRNA transcripts or to generate models of disease. Overall, the accumulating data indicates that RNAs, like proteins and lipids, traffic to the mitochondria and are required for proper mitochondrial function. Mammalian PNPASE is required for the import of some if not all of these small nucleus-encoded RNAs.

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Appendix III: Mitochondria-Targeted RNA Import

Chapter 11

Mitochondria-Targeted RNA Import

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Abstract

The import of a modest number of nucleus-encoded RNAs into mitochondria has been reported in species ranging from yeast to human. With the advent of high-throughput RNA sequencing, additional nucleus-encoded mitochondrial RNAs are being identified. Confirming the mitochondrial localization of candidate RNAs of interest (e.g., small noncoding RNAs, miRNAs, tRNAs, and possibly lncRNAs and viral RNAs) and understanding their function within the mitochondrion is assisted by in vitro and in vivo import assay systems. Here we describe these two systems for studying mitochondrial RNA import, processing, and functions.

Key words Mitochondria, RNA import, Polynucleotide phosphorylase (PNPASE)

1 Introduction

Mitochondria import a range of nucleus-encoded RNAs, with different species having different substrate specificities [1]. Some of these RNAs are processed within mitochondria and have functions different from their cytosolic or nuclear counterparts [2]. Studying nucleus-encoded mitochondrial RNAs emphasizes a few general approaches. First, RNA localization inside mitochondria can be confirmed using organelle fractionation after the substrate RNA is imported in vitro. Second, the processing or cleavage of imported RNAs can be studied using northern blotting or in vitro import of radiolabeled RNA substrates of known length. Specific RNA processing sites and the addition of nucleotides can be identified using RNA ligation, followed by semiquantitative RT-PCR and sequencing, as described previously [3]. Finally, the functions of imported RNAs can be studied using approaches that are specific to the RNA species of interest. In addition to mechanistic and functional studies, mitochondrial RNA import pathway(s)

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and signal sequence(s) can be co-opted to import RNAs of interest into mitochondria to rescue defects caused by mtDNA mutations or to change the mitochondrial genome expression profile [4, 5]. Here, we describe two mitochondrial RNA import systems for these studies. One system is an in vitro import assay that uses in vitro-transcribed RNA substrates and either isolated yeast or mammalian mitochondria. A second, more challenging, and currently less efficient and less well-understood system is an in vivo import assay that utilizes an RNA import signal sequence in exogenously expressed DNA to target nucleus-encoded RNAs for import into mitochondria. The RNA substrates for in vitro import can be either radiolabeled or unlabeled and detected using autoradiography or RT-PCR, respectively. For yeast mitochondria, exogenous expression of the mammalian RNA import protein, polynucleotide phosphorylase (PNPASE), generates a system with enhanced import efficiency for substrate RNAs [6]. The efficiency of the in vivo import system varies markedly depending on the RNA to be imported; unfortunately, the precise rules for efficient RNA import in vivo have not been fully elucidated. In vivo, pre-mitochondrial processing of target RNA in the nucleus and/or cytosol and trafficking of RNA to the mitochondrion needs to be carefully considered, as these factors seem to greatly affect RNA import efficiency [5].

2 Materials

All solutions should be prepared using RNase-free water and the reagents used should be analytical grade. Unless indicated otherwise, all reagents should be prepared and stored at room temperature.

2.1 *In Vitro* Mitochondrial RNA Import

2.1.1 *In Vitro* Transcription

1. MEGAscript® SP6 Kit from Ambion (catalog number: AM1330) is used for in vitro transcription. The kit contains an enzyme mix, 10× reaction buffer, and solutions of ATP, CTP, GTP, and UTP (*see* **Note 1**).
2. α-P^{32}-labeled CTP, 6,000 Ci/mmol, 10 mCi/mL (*see* **Note 2**).

2.1.2 RNA Isolation

1. RNase-free water. Store at 4 °C.
2. Trizol reagent (Invitrogen). Store at 4 °C.
3. Chloroform.
4. 75 % Ethanol (RNAse-free). Store at 4 °C.
5. Isopropyl alcohol.

2.1.3 *In Vitro* RNA Import

1. 2× Import buffer for yeast mitochondria: 1.2 M sorbitol, 100 mM KCl, 100 mM HEPES, 20 mM $MgCl_2$, pH 7.1. Store at −20 °C.

2. 2× Import buffer for mammalian mitochondria: 0.45 M mannitol, 0.15 M sucrose, 20 mM HEPES, 50 mM KCl, 10 mM $MgCl_2$, pH 7.4. Store at −20 °C (*see* **Note 3**).
3. 100 mM ATP. Store at −20 °C.
4. 100 mM DTT (prepare fresh).
5. 500 mM NADH. Store at −20 °C.
6. 500 mM sodium succinate (prepare fresh).
7. 10 mg/mL RNase A. Store at −20 °C.
8. 10 mg/mL proteinase K. Store at −20 °C.
9. SDS buffer: 1 % SDS, 100 mM NaCl, 10 mM Tris–HCl, pH 7.4.

2.1.4 Urea-Polyacrylamide Gel Electrophoresis (Urea-PAGE)

1. 5× Tris-borate-EDTA (TBE) buffer.
2. 40 % Acrylamide/bisacrylamide (29:1) solution. Store at 4 °C.
3. Ammonium persulfate: 10 % solution in water (prepare fresh).
4. *N,N,N,N*-tetramethyl-ethylenediamine (TEMED).
5. Formamide loading buffer: 95 % deionized formamide, 5 mM EDTA, 0.025 % (w/v) SDS, 0.025 % (w/v) bromophenol blue. Store at −20 °C (*see* **Note 4**).

2.2 In Vivo Mitochondrial RNA Import

2.2.1 Transient Transfection

1. 2× HEPES-buffered saline (HBS): 50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HP0_4, pH 7.1.
2. 1 M $CaCl_2$.

2.2.2 Mitochondria Isolation

1. Mitoprep buffer: 0.225 M mannitol, 0.075 M sucrose, 20 mM HEPES, pH 7.4. Store at 4 °C.
2. 0.5 M EDTA. Store at 4 °C.
3. 0.2 M PMSF. Store at −20 °C.

2.2.3 RNase A Treatment, RNA Isolation, and DNase Treatment

1. 10 mg/mL digitonin (prepare fresh).
2. 10 mg/mL RNase A. Store at −20 °C.
3. SDS buffer: 1 % SDS, 100 mM NaCl, 10 mM Tris–Cl, pH 7.4.
4. RNase-free water. Store at 4 °C.
5. Trizol reagent (Invitrogen). Store at 4 °C.
6. Chloroform.
7. 75 % RNase-free ethanol. Store at 4 °C.
8. Isopropyl alcohol.
9. DNase I, RNase-free (Thermo Scientific).

2.2.4 RT-PCR

1. One-Step RT-PCR Kit (e.g., Promega).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 In Vitro Mitochondrial RNA Import

3.1.1 In Vitro Transcription

1. Prepare a DNA template that contains the SP6 polymerase promoter site for in vitro transcription.
2. Perform in vitro transcription using the MEGAscript® Kit from Ambion (*see* **Note 5**). Mix all of the components at room temperature in a 0.5 mL Eppendorf tube. The following is an example of a 20 μL reaction (*see* **Note 6**):

ATP	1.5 μL
CTP	1.0 μL
GTP	1.5 μL
UTP	1.5 μL
10× Reaction buffer	2.0 μL
α-P^{32}-CTP	7.5 μL
Linear template DNA[a]	0.1–1.0 μg
Enzyme mix	2.0 μL
Nuclease-free water	to 20 μL

[a]Use 0.1–0.2 μg PCR-product template or ~1 μg linearized plasmid

3. Pipette the mixture up and down or flick the tube gently. Then centrifuge the tube briefly to collect the reaction mixture at the bottom of the tube. Incubate at 37 °C for 2–4 h. If the transcripts are less than 500 ribonucleotides (nt), a longer incubation time (up to 16 h) may be advantageous.

3.1.2 RNA Isolation

1. After incubation, add 400 μL Trizol reagent and 170 μL chloroform to the mixture and vortex for 1 min.
2. Centrifuge the sample at 12,400 × *g* for 5 min at room temperature.
3. Transfer the upper aqueous phase into a fresh Eppendorf tube carefully without disturbing the interphase. Add 300 μL isopropanol to the aqueous solution and mix by pipetting or inverting the tube.
4. Centrifuge the sample at 20,000 × *g* for 10 min at 4 °C (*see* **Note 7**).
5. Carefully remove the supernatant. Add 600 μL ice-cold 75 % ethanol to the tube and invert the tube ten times.
6. Centrifuge at 20,000 × *g* for 2 min at 4 °C. Remove the supernatant (*see* **Note 8**).

7. Dry the RNA pellet at 37 °C and resuspend the RNA in RNase-free water (*see* **Note 9**). The RNA can be used fresh or stored at −80 °C for later use.

3.1.3 *In Vitro* RNA Import Assay

For import into mammalian mitochondria, always use freshly isolated mitochondria. For import into yeast mitochondria, freshly isolated mitochondria or mitochondria that were previously isolated, flash-frozen, and stored at −80 °C can be used. For import, finding a good negative control RNA that is not imported is required, and it is recommended that at least two different concentrations of test and control RNA is used in each experiment.

1. Freshly prepare 500 mM sodium succinate and 100 mM DTT. Thaw the 2× RNA import buffer, 500 mM NADH, and 100 mM ATP at room temperature.
2. Assemble the import reaction at room temperature. The following amounts are for a single 200 μL reaction in a 1.5 mL Eppendorf tube:

   Yeast mitochondria import reaction:

2× Import buffer (yeast)	98 μL
RNase-free water	62 μL
100 mM ATP	10 μL
500 mM NADH	2.0 μL
100 mM DTT	4.0 μL
Mitochondria (100 μg)	10 μL
5 pmol RNA	10 μL

   Mammalian mitochondria import reaction:

2× Import buffer (mammals)	98 μL
RNase-free water	58 μL
100 mM ATP	10 μL
500 mM sodium succinate	6.0 μL
100 mM DTT	4.0 μL
Mitochondria (100 μg)	10 μL
5 pmol RNA	10 μL

3. Add together everything in the import reaction except the RNA and incubate at 30 °C for 5 min.
4. Add RNA to the import reaction and mix gently. Incubate at 30 °C for 10 min to import RNA (*see* **Note 10**).

5. After import, add 1 μL of 10 mg/mL RNase A to the import reaction. Incubate at 30 °C for 20 min (*see* **Note 11**).
6. Transfer the mixture to a new tube and incubate at 30 °C for an additional 10 min (*see* **Note 12**).
7. Centrifuge the mitochondria into a pellet at 11,000 × *g* for 4 min at room temperature. Proceed immediately to **step 2** in the next section.

3.1.4 RNA Purification

1. Add proteinase K to 1× SDS buffer at a final concentration of 25 μg/mL and heat the SDS buffer to 95 °C.
2. Add 100 μL of SDS buffer from the previous step to the mitochondrial pellet and incubate at 80 °C for 1 min.
3. Immediately isolate RNA according to the purification steps detailed in Subheading 3.2, above, and dry the RNA pellet.

3.1.5 5 % Urea-PAGE

1. Prepare a 5 % urea-polyacrylamide gel before starting the import assay and pre-run the gel for 30 min at 300 V.
2. Dissolve mitochondrial RNA in 30 μL of formamide loading buffer and incubate at 95 °C for 10 min.
3. Immediately load RNA samples onto the urea-polyacrylamide gel. Electrophorese at 300 V until the dye front reaches the bottom of the gel (*see* **Note 13**).

When unlabeled RNA is used, the imported RNA can be detected by semiquantitative RT-PCR. Since many more steps are required, it may potentially introduce additional inefficiencies and/or may result in increased RNA degradation. Also, it is important to note that if the RNA is processed upon import, the primers that detect the RNA precursor form may not detect the processed RNA version.

3.1.6 Film Exposure

1. Following electrophoresis, remove the gel and place it onto a Whatman filter paper and dry it on a vacuum gel dryer.
2. Expose the gel for 1–7 days on X-ray film (*see* **Note 14**).

3.2 In Vivo Mitochondrial RNA Import

The system described here is for adherent mammalian cells (e.g., HEK293, HeLa, mouse embryonic fibroblasts) only. Clone the RNA-encoding gene into a desired mammalian vector. Noncoding RNA promoters or mRNA promoters have both been used successfully. Since semiquantitative RT-PCR is used for detection, it is essential to design primers that specifically amplify the imported RNA and distinguish it from endogenous sequences. In vivo RNA import is generally more complicated than in vitro RNA import with many parameters that affect efficiency still to be defined and with RNA localization and processing issues to consider, so a mitochondrial RNA import signal appended to an RNA of interest alone rarely results in successful mitochondrial import without further expression vector modifications.

3.2.1 Mammalian Tissue Culture Cell Transformation

1. Split a fairly confluent (70–90 %) culture between 1:3 and 1:4 into 15 cm plates (*see* **Note 15**).
2. When the cells are 70 % confluent, transfect the cells with control and RNA import constructs of interest (the protocol below is one example method of many; *see* **Note 16**).
   (a) Replace cell media with fresh DMEM + 10 % FBS and supplements.
   (b) In a sterile tube, mix 100 μg of DNA with 128 μL 1 M $CaCl_2$, 500 μL 2× HBS, and enough ddH_2O to make up a total volume of 1 mL (*see* **Note 17**). Incubate the mixture at room temperature for 20 min.
   (c) Carefully add the mixture to the plates in a dropwise fashion. Mix by rocking back and forth.
   (d) Incubate the transfected cells in a 5 % CO_2 incubator for 48 h (*see* **Note 18**).

3.2.2 Mitochondria Isolation

1. Collect cells by centrifuging at 2,000 × *g* for 2 min at room temperature. Remove the supernatant. Wash the cells with 1 mL 1× PBS, pH 7.4.
2. Resuspend the cells in 1.5 mL mitoprep buffer with 1 mM EDTA and 0.5 mM PMSF and transfer to a 5 mL glass/Teflon homogenizer. Perform 30 dounce strokes (*see* **Note 19**).
3. Transfer homogenate into a 1.5 mL Eppendorf tube and centrifuge at 800 × *g* for 5 min at 4 °C. Transfer the supernatant to a new Eppendorf tube.
4. Resuspend the pellet in 1.5 mL mitoprep buffer. Perform 20 dounce strokes. Transfer homogenate into a 1.5 mL Eppendorf tube and centrifuge at 800 × *g* for 5 min at 4 °C. Transfer the supernatant to a new Eppendorf tube. Take out 25 μL from each supernatant and keep it on ice as a measure of assay input.
5. Spin down the supernatants from the first and second douncing at 800 × *g* for 5 min at 4 °C.
6. Transfer the supernatants to new Eppendorf tubes and centrifuge at 11,000 × *g* for 5 min at 4 °C. Combine the pellets and wash with 1 mL mitoprep buffer. Centrifuge at 11,000 × *g* for 5 min at 4 °C, remove the supernatant, and resuspend the pellet in 30 μL mitoprep buffer.

3.2.3 RNase A Treatment, RNA Isolation, and DNase I Treatment

1. RNase A treatment.
   (a) Add 200 μg fresh mitochondria into 200 μL mitoprep buffer.
   (b) Add 1 μL 10 mg/mL digitonin to the mitochondria. Vortex gently at a low speed (*see* **Note 20**).

(c) Add 0.5 μL RNase A to the mixture (*see* **Note 21**). Incubate the mixture at 27 °C for 20 min. After incubation, centrifuge at 11,000 × *g* for 5 min. Remove as much supernatant as possible.

2. Trizol RNA isolation.

(a) Resuspend the pellet from the previous step in 100 μL SDS buffer with 25 μg/mL proteinase K preheated to 95 °C. Quickly put the tubes in an 80 °C heat block for 5 min (*see* **Note 22**).

(b) Add 400 μL Trizol reagent and 170 μL chloroform to the lysate. Purify the RNA as indicated in Subheading 3.2, above, of the in vitro import protocol (*see* **Note 23**).

3. DNase I treatment.
Resuspend the nucleic acid mixture in 30 μL DNase buffer with 0.5 unit of DNase I. Incubate at 37 °C for 30 min. Transfer the tubes to 65 °C and incubate for 10 min to inactivate DNase I.

3.2.4 RT-PCR

Use 1 μL sample as template for a 20 μL reaction. Specific primers are used for the one-step RT-PCR reaction (*see* **Note 24**).

4 Notes

1. For in vitro transcription, different RNA polymerase promoters, such as SP6, T7, or T3, can be used.
2. The radiolabel can be on any of the four ribonucleosides, but it must be labeled at the α position. Do not use β- or γ-labeled ribonucleoside triphosphates. Make sure to use caution when working with radioactive materials and follow institutional handling, use, and waste management protocols.
3. Many different buffers are used for mammalian mitochondria isolation. However, we recommend the use of a mannitol-sucrose buffer for the isolation procedure, so the mitochondria are maintained in the same conditions as the import buffer.
4. Heating the RNA sample with formamide loading buffer denatures the RNA and nucleases.
5. It is essential to pick the kit for the specific promoter. It is not necessary to use a kit, and homemade transcription systems can be used instead. However, we have observed that the commercial kits generally have a higher yield and increased full-length RNA products.
6. Different genes can have very different yields even with the same promoter and the same kit, so it is important to adjust the volume and reaction time as needed.

7. In most cases, a pale white RNA pellet will form at the bottom of the tube.
8. Remove as much supernatant as possible. Otherwise the drying would take much longer.
9. Do not dry the RNA at temperatures higher than 37 °C. Drying at a higher temperature makes it more difficult for the pellet to dissolve in water. For unlabeled RNA that requires semiquantitative RT-PCR for detection, it is necessary to perform the DNase treatment before proceeding to the next step.
10. In general, RNA import is quick. 10 min is normally sufficient, but the import reaction time can be adjusted up to 20 min.
11. RNase A is chosen because it does not require a strict buffer condition, so a buffer exchange step is not required. It is important to handle RNase A carefully, so it will not contaminate the samples in later steps. Hold the stock tube with only one hand and change gloves right after adding the enzyme. Alternatively, 25 μg/mL of S7 nuclease may be used in a 200 μL reaction containing 50 mM Tris–Cl, pH 8.0, and 5 mM $CaCl_2$.
12. This step ensures thorough RNase A treatment because some RNA may stick on the upper wall of the tube and not mix with the enzyme.
13. The percentage of the gel should be decided according to the length of the precursor and mature RNA species of interest. For RNA lengths between 200 and 500 nt, a 5 % urea-polyacrylamide gel is sufficient. It is not recommended to run the dye front off of the gel. This will cause the buffer to become radioactive.
14. Often, the import efficiency is not high, so a long exposure time is needed.
15. This method works best for adherent cells. The transfection efficiency varies among cell lines.
16. A control construct should encode RNA that is not imported into mitochondria. An import construct encodes the RNA of interest or RNA with an import signal and other sequences that are required for import.
17. The amount of the construct used should be adjusted so that the control and the import RNAs are expressed at similar levels, if possible.
18. The incubation time after transfection should be determined according to the experimental need.
19. It is important to avoid bubbles. Perform dounce strokes slowly.
20. This step permeabilizes the mitochondrial outer membrane.
21. Do not add too much RNase A because it cannot be easily inactivated even by added SDS and heat.