Allotopic RNA expression strategy to rescue an endogenous mitochondrial *ATP6[1]* mutation in *Drosophila*

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Allotopic RNA expression strategy to rescue an endogenous mitochondrial *ATP6[1]* mutation in *Drosophila*

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Mitochondria are essential organelles in the cell. One of their most critical functions is the generation of cellular energy in the form of ATP. The presence of DNA in the mitochondrial matrix makes this organelle semi-autonomous. However, it relies heavily on the nucleus and cytosol to import ~99% of its proteins and some RNA molecules for its normal functioning. Mutations in the mitochondrial DNA (mtDNA) cause several devastating disorders. Due to their complexity and our incomplete understanding of mitochondrial disease pathogenesis, these disorders are difficult to diagnose and currently no pharmacological treatment exists. Further, gene therapy for these devastating disorders is impeded due to lack of mitochondrial genome manipulation techniques. Understanding the mechanism of pathogenesis and developing mtDNA manipulation strategies are key to developing remedial therapies.

In my thesis, I investigated an RNA allotopic strategy of targeting RNA into the mitochondria *in vivo* in flies. In my first aim, I improved an *in vivo* mitochondrial-targeting tool (*mtTRES* vector) to manipulate proteins encoded by the mitochondrial DNA. This vector integrates into the nuclear genome and results in the transcription of a chimeric RNA consisting of a mitochondrial targeting signal sequence and a small non-coding antisense RNA.

Previous studies have attempted allotopic expression via both protein and RNA import with mixed results. Only a few of them, however, have been tested *in vivo* and none have been

examined for rescue in an animal model of mitochondrial disease. Since our lab has a well characterized mtDNA mutation fly model, *ATP6[1],* I had a unique opportunity to investigate rescue strategies in these models. In my second aim, I improved a unique set of *mtTRES*^{Pro} vectors for both flies and humans to target long coding RNAs into mitochondria. Once imported these long RNAs are designed to be endogenously translated in mitochondria. By targeting a wild type copy of the mutant *ATP6* gene, I explored the rescuing potential of allotopic RNA import *in vivo*. Our data suggest the *mtTRES* and *mtTRES*^{Pro} mitochondrial manipulation tools have genuine potential to be developed into a mitochondrial disease gene therapy.

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# of subunits coded by	Complex I	Complex II	Complex III	Complex IV	Complex V
mtDNA	7 (ND1, ND2, ND3, ND4, ND4L,ND5, ND6)	0	1 (Cyt b)	3 (COX1, COX2, COX3)	2 (ATP8, ATP6)
nuclear DNA	39	4	10	10	14
Total	46	4	11	13	16

Table 2. Variations of initiation and stop codons in human and fly mitochondria

Human mitochondria

Gene name	Start codon	Amino acid	Stop codon
1 *ND1*	*ATA**	*Met*	*UAA/TAA*
2 *ND2*	*ATT***	*Iso*	*UAG/TAG*
3 *COX1*	*ATG*	*Met*	*AGA*
4 *COX2*	*ATG*	*Met*	*UAG/TAG*
5 *ATP8*	*ATG*	*Met*	*UAG/TAG*
6 *ATP6*	*ATG*	*Met*	*UAA/TAA*
7 *COX3*	*ATG*	*Met*	*§§§*
8 *ND3*	*ATA**	*Met*	*§§§*
9 *ND4L*	*ATG*	*Met*	*UAA/TAA*
10 *ND4*	*ATG*	*Met*	*§§§*
11 *ND5*	*ATG*	*Met*	*UAA/TAA*
12 *ND6*	*ATG*	*Met*	*AGG*
13 *Cyt b*	*ATG*	*Met*	*§§§*

***Drosophila melanogaster* mitochondria**

Gene name	Start codon	Amino acid	Stop codon
1 *ND1*	*ATA**	*Met*	*UAG/TAG*
2 *ND2*	*ATT***	*Iso*	*UAA/TAA*
3 *COX1*	*ATA#*	*Met*	*§§§*
4 *COX2*	*ATG*	*Met*	*§§§*
5 *ATP8*	*ATT***	*Iso*	*UAA/TAA*
6 *ATP6*	*ATG*	*Met*	*UAA/TAA*
7 *COX3*	*ATG*	*Met*	*UAA/TAA*
8 *ND3*	*ATT***	*Iso*	*UAA/TAA*
9 *ND4L*	*ATG*	*Met*	*UAA/TAA*
10 *ND4*	*ATG*	*Met*	*§§§*
11 *ND5*	*ATG*	*Met*	*§§§*
12 *ND6*	*ATT***	*Iso*	*UAA/TAA*
13 *Cyt b*	*ATG*	*Met*	*UAA/TAA*

§§§ UAA/TAA stop codon is completed by the addition of 3' residues to the mRNA

Frameshift mechanism

* ATA used as an initiating methionine

** Initiation codon other than methionine

Source and credits: This table is compiled by Atif Towheed and Jessica Collins based on the gene annotation of mtDNA - http://mitomap.org/MITOMAP

PREFACE

Isaac Newton once wrote “If I have seen further than others, it is by standing upon the shoulders of giants.” My thesis dissertation and graduate career has been an incredible journey of looking beyond known scientific frontiers – of discovery, learning, and both professional and personal growth. This would not have been possible without the mentorship and support of some incredible people around me.

I am immensely grateful to my mentor and supervisor, Dr. Michael Palladino. He provided a perfect environment for ideas to sprout and develop. He gave me the opportunity and independence to solve scientific problems from multiple perspectives, thereby facilitating solid learning in the process. I would like to thank Dr. Michael Palladino and Dr. Alicia Celotto for initiating the allotopic RNA project. By teaching me most of the biochemical techniques such as blue native PAGE, histology, mitochondrial isolation, they set me up for success not only in my PhD but also for my scientific career. Michael and Alicia have been the constant source of positive energy and inspiration around me, not to mention unique scientific knowledge and insights.

I would like to thank my thesis committee members Drs. Guillermo Romero, Don DeFranco, Edward Burton and Yu Jiang for providing valuable perspectives on my project and guiding me from time to time.

I had the pleasure to work with and mentor enthusiastic group of undergraduate students in the Palladino lab including Desiree, Aaron C., Jessica, Kevin, Trey, and David. Desiree, Aaron C. and Jessica assisted in my thesis project, generated some of the valuable data and contributed to the manuscript, without which my thesis would have been incomplete.

The Molecular Pharmacology graduate training program and Department of Pharmacology and Chemical Biology has played a key role in supporting my research. I would like to thank Dr. Bruce Freeman, Dr. William Chet deGroat and Dr. Patrick Pagano for their generosity and continued support throughout my graduate study. I would like to acknowledge Dr. Sruti Shiva for helping me understand several mitochondrial physiology concepts.

Working at Pittsburgh Institute for Neurodegenerative Disease (PIND) has been intellectually rewarding as I got to interact with experts in the field of mitochondrial biology. I would like to especially acknowledge Dr. Timothy Greenamyre, Dr. Sarah Berman and Dr. Erik Hoffman.

I would like to specially thank my fellow graduate student, Bart, who has been an awesome friend and a peer mentor. Finally, I would like to express my gratitude for the support from my wife, Monica and friends, coworkers and family (in no particular order) Sascha, Sharmila, Daniel, Kenneth, Karen, Lucas, Amy, Courtney, Victor, Beth, Marsha, Steve, Stacy, Keri, Aaron T., Matt, Fareed, Sakshi, Deepti, Sheikhuma, Bhai, Nabila Bhabhi, Mom and Dad.

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1.0 GENERAL INTRODUCTION

1.1 Mitochondrial origin, structure and function

Mitochondria (*Greek* origin – *mitos* meaning thread-like and *khondros* meaning grain or granule) are essential organelles found in almost all cells having a nucleus. According to the endosymbiont theory, ~1.5-2 billion years ago, aerobic bacteria formed a permanent symbiosis with early eukaryotic cells that lacked the ability to use oxygen for metabolism [2,3]. This symbiotic relationship evolved into modern day eukaryotic cells that metabolize substrates using their mitochondria much more efficiently than their primitive counterparts.

Mitochondria are semi-autonomous organelles. They have their own DNA in the mitochondrial matrix. Mitochondrial DNA (mtDNA) is double stranded, circular and in humans contains 16,568 base pairs (**Figure 1)**. In all higher eukaryotes it encodes 37 genes: 2 rRNA (12*S* and 16*S*), 22 tRNA and 13 protein-coding genes. mtDNA are maternally inherited in contrast to nuclear DNA (nDNA), which is inherited from both parents.

Fly mitochondrial DNA shows remarkable similarity with the vertebrate mitochondrial DNA gene content. Just like the mammalian counterpart, it codes for 37 genes: 2 rRNAs (*lr*RNA and *sr*RNA), 22 tRNA and 13 protein-coding genes. However, the *Drosophila* mtDNA differs considerably from the mammalian mtDNA in size and the organization of genes (**Figure 1)** [4]. The mtDNA contains an A+T rich region, which ranges between 1-5 kb depending on the

species. The A+T region harbors regulatory sequences for mitochondrial replication and transcription and is thought to be functionally homologous to D-loop of vertebrate mtDNA [5-7].

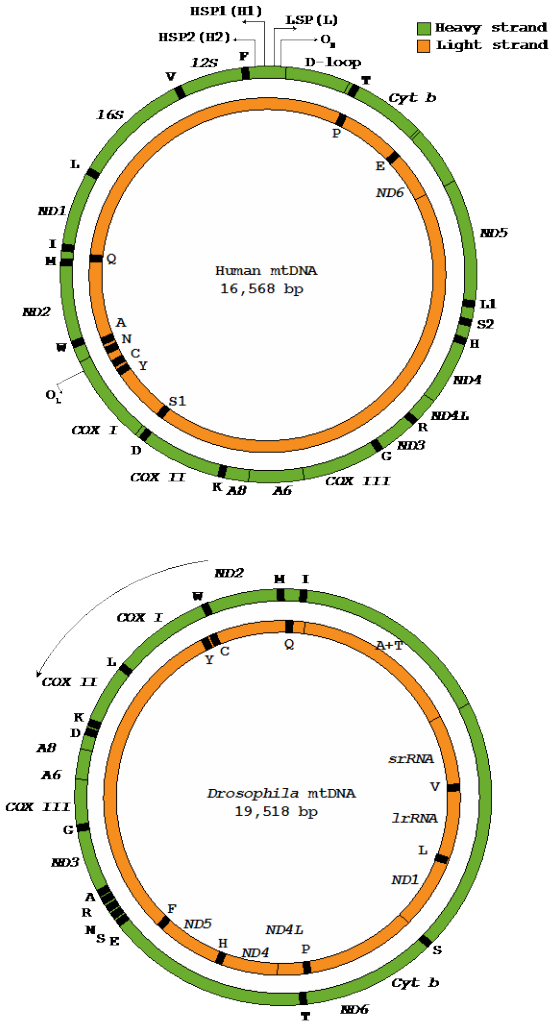

Figure 1. Map of human and *Drosophila melanogaster* mitochondrial DNA. Human mtDNA schematic adapted from: *DiMauro and Schon, NEMJ, 2003 and Fernandez-Silva et.al., Exp. Physiology, 2003)*. Fly mtDNA schematic adapted and modified from *Echevarria et.al., 2010*. lrRNA stands for large ribosomal RNA and srRNA stands for small ribosomal RNA.

Interestingly, it has recently been reported that the 16S region of mitochondrial genome can code a small peptide “humanin” [8]. These small peptides are termed as “mitochondrial-derived-peptides” and may have a role in mitochondrial stress response [9]. In contrast to the nuclear genes that are present in two copies, it is common to find more than one copy of mtDNA per mitochondrion. Depending upon the energy demands of a cell, it may have varying numbers of mitochondria. This means that there could be more than several hundred mitochondrial genomes in a single cell.

Structurally, the mitochondrion is a double membrane organelle with four distinct sub-compartments – **a**) outer mitochondrial membrane (OMM), **b**) inner mitochondrial membrane (IMM), **c**) inter-membrane space (IMS) and **d**) matrix. As other biological membranes, mitochondrial membranes are composed of phospholipids with embedded proteins. Phospholipid composition of mitochondrial membrane consists of phosphatidyl choline (40%), phosphatidyl ethanolamine (28.4%), phosphatidyl inositol (7%), phosphatidyl serine and phosphatidic acid (5%) [10,11]. Cardiolipin (22.5%) and phosphatidyl glycerol are found exclusively in the mitochondrial membranes [12,13]. There are numerous proteins in mitochondrial membranes such as the subunits of the respiratory chain complex or electron transport chain (ETC) that is discussed later. It is essential to note that lipids and proteins interact extensively within the mitochondrial membranes. Homeostasis of phospholipids in the mitochondrial membranes is key to maintaining mitochondrial structure and its normal function such as fission and fusion, import of macromolecules and optimal performance of the respiratory chain. Lipids also are known to stabilize the super-complexes within the IMM [14].

Contrary to general thinking, mitochondria in higher eukaryotes are not typically “sausage shaped or cylindrical”. In yeast, mitochondria are primarily tubular, whereas in humans it forms interconnected reticular networks. Studies focused on mitochondrial dynamics have

shown the existence of polymorphic mitochondria during the course of cell division from long filamentous network to fragmented and then back again [15]. Interestingly, mitochondria also appear to demonstrate “site-specific” functional differences as in the case of perinuclear versus synaptosomal mitochondria in neuronal cells [15]. This suggests that mitochondrial function may be linked to its location. Mitochondria are dynamic organelles and they continually undergo fusion and fission within a cell [16]. Consequently, this results in mixing and homogeneous distribution of mitochondrial content including mtDNA within the cell.

Mitochondria are involved in several cellular functions such as generation of energy in the form of ATP, generation and regulation of reactive oxygen species (ROS), buffering cytosolic calcium ion levels and regulating programmed cell death (apoptosis)[17]. The IMM folds and creates finger like projections known as “cristae”. The cristae provide greater surface area to accommodate the ETC complexes and other proteins such as adenine nucleotide translocase (ANT). ATP is generated by catalyzing high-energy substrates via oxidative phosphorylation (OXPHOS) and is the most important function of mitochondria.

OXPHOS is performed by proteins organized as complexes and super complexes termed the electron transport chain (ETC) which are located within the IMM. The ETC consists of 5 protein complexes – complexes I, II, III, IV and V (**Figure 2 and Table 1**). The components of the ETC are encoded by both the nuclear as well as mitochondrial genome. With the exception of Complex II, all other ETC complex have at least one subunit coded by the mtDNA (**Table 1**). Thus, OXPHOS is extensively regulated by both the genomes. These ETC complexes are arranged in the increasing order by their reducing potential. Reducing equivalents from various metabolic pathways such as the Kreb’s cycle, pyruvate oxidation, metabolism of fatty acids, amino acids and steroid are used to drive protons across the mitochondrial inner membrane. According to the classical Mitchell chemiosmotic hypothesis, this results in an electrochemical

proton gradient, which then drives the rotary component of Complex V to generate ATP (**Figure 3**). Complex V is a rotary complex in the IMM consisting of two domains F_1 (motor's stator, matrix exposed) and F_o (rotor, membrane embedded). As the protons flow through the F_o complex, it rotates the F_1 domain via a shaft. Rotation of F_1 is coupled with catalytic conversion of ADP and P_i to ATP that is then released in the matrix. ATP generated in the mitochondrial matrix is exported to the cytosol by adenine nucleotide translocator (ANT) to be used in various cellular processes.

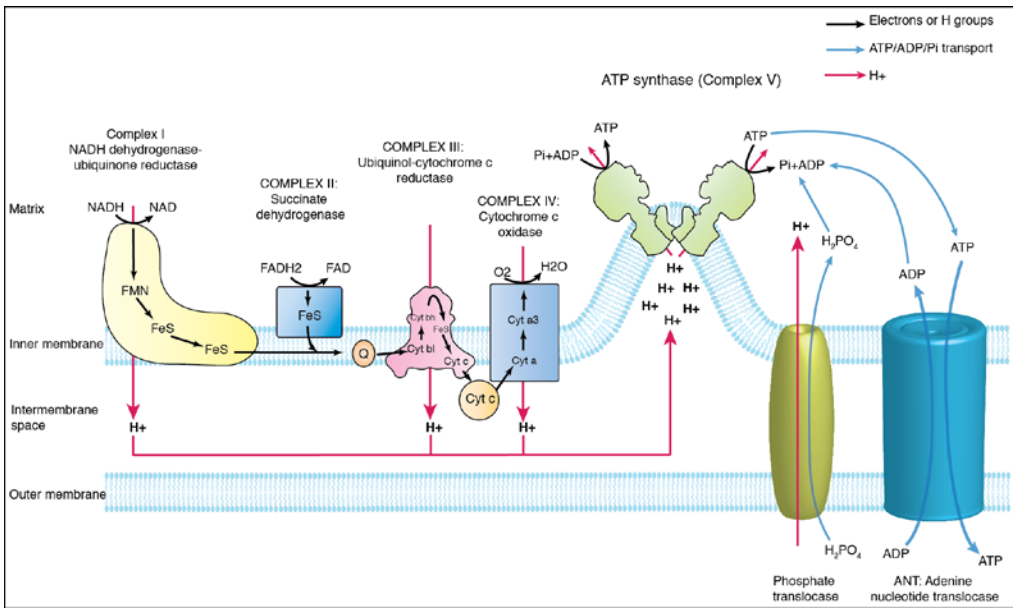

Figure 2. Schematic of mitochondrial electron transport chain showing electron and proton flow across various OXPHOS complexes. ***Adapted from Mourier and Larsson, 2011*** [18]**.**

1.1.1 Mitochondria and Reactive Oxygen Species

During the process of OXPHOS in the mitochondrial matrix, oxygen (O_2) is reduced to water (H_2O) by addition of 4 electrons (e^-) and 4 protons generated from the energy rich substrates. When only one or two electrons are transferred to O_2, the reaction yields highly reactive superoxide ($O_2^{\bullet -}$) or peroxide (O_2^{2-}) anions respectively. Superoxide dismutase converts superoxide to relatively inactive hydrogen peroxide (H_2O_2). However, H_2O_2 can be further reduced by a metal ion via a Fenton reaction to generate hydroxyl radical ($OH^{\bullet}$). $O_2^{\bullet -}$, O_2^{2-} and $OH^{\bullet}$ are called reactive oxygen species (ROS). ROS are a "double edged sword" and play important roles in cellular defense (neutrophils and macrophages), signaling, but, when in excess, result in DNA damage and pathological conditions [19].

The mitochondrial matrix is akin to a central processing unit. The machinery required for mtDNA replication, transcription and translation of 13 protein-coding genes of mtDNA lies in the matrix. All metabolic processes converge in the matrix ultimately resulting in the creation of reducing equivalents (protons), which are then funneled to the ETC. The enzymes for the Kreb's cycle, fatty acid metabolism, urea cycle, heme synthesis, and antioxidant defense mechanism all reside within the matrix. However, other than the 37 genes coded by the mtDNA everything else required for these mitochondrial processes is encoded by the nuclear DNA and imported into mitochondria. Given the impressive magnitude of protein and RNA traffic into the mitochondria, it is widely accepted that the mitochondrial import pathways play a critical role in determining mitochondrial homeostasis.

1.2 Mitochondrial genetics and inheritance

1.2.1 Maternal inheritance

During fertilization, embryonic mitochondria are derived from the oocyte. Males transmit only the nuclear genetic material but not the mtDNA to the embryo. Only a few mitochondria from the sperm enter oocyte through the process of fertilization and these are selectively eliminated by ubiquitin-mediated degradation or mitophagy. Why this is evolutionarily favored is not clear [20,21]. A study by Sharpley *et.al.,* in 2012, proposed that mixing of maternal and paternal mtDNAs would diminish the OXPHOS capacity and affects the cross signaling between the nuclear and mitochondrial genomes [22]. This could result in aberrant physiology and behavior, which might explain why there is uniparental inheritance of mtDNA. Mitochondrial genetic material is thus maternally inherited and follows a non-Mendelian pattern [23]. If there is a mutation in maternal mtDNA it can be transmitted by the mother to both sexes. Hence occurrence of disease in both sexes, dependent on maternal lineage is strongly indicative of mitochondrial mutation. Interestingly a study in 2002 reported that the paternal mtDNA was detectable in the muscle of the progeny [24]. Inconsistencies in the mtDNA sequences of blood and muscle of the subject led to the discovery that paternal inheritance may be possible although this is an exception rather than the rule. No other studies have since reported other cases of paternal inheritance in humans. Therefore, mtDNA is believed to be inherited exclusively from the mother.

1.2.2 Heteroplasmy and the threshold effect

As a consequence of mtDNA polyploidy, mitochondrial inheritance patterns of diseases are quite complex. When normal cells contain the same type of mitochondria, this condition is

termed as “homoplasmy”. Mutations in mtDNA can occur in just a few mtDNAs within a fraction of the mitochondria. The presence of a mutant mtDNA and wild type mtDNA is termed as “heteroplasmy” and is typically expressed as a ratio of mutant:wild type mtDNA [25]. The proportion of how much mutated mtDNA is present in a cell is used to calculate percentage heteroplasmy. PCR-SSCP is usually the method of choice to calculate the percent heteroplasmy levels, although experimental artifacts make it nearly impossible to predict the exact percentage. Early on during oogenesis, primordial germ cells divide to give rise to the primary oocyte. During this process, there is a reduction in the mitochondrial content that leads to a “mitochondrial genetic bottleneck” [26]. Since this process is random, it results in a “sampling effect” thus a range of resulting heteroplasmy levels. As a consequence, some oocytes receive higher levels of mutated mtDNA and others receive less or even none of the mutant mtDNA. This ultimately contributes to determining whether the offspring will be affected by the mutation or not. Studies of several mitochondrial disorders show that the presence of mtDNA mutation is not sufficient to result in a disease state; rather the percent heteroplasmy generally correlates with disease. The heteroplasmy level typically has to reach a “minimum critical mutation load” or “phenotypic threshold effect” for the mutation to manifest its symptoms [27]. For example individuals with mtDNA mutation T8993G who have ≤60% heteroplasmy will be asymptomatic. However, when the heteroplasmy level is 70% and 90% it results in NARP (Neuropathy, ataxia and retinitis pigmentosa) and it’s more severe form MILS (Maternally inherited Leigh syndrome), respectively [28-31]. Although the heteroplasmy levels and disease severity do not perfectly correlate, they are generally related and discrepancies likely are related to methods of assaying heteroplasmy and somatic differences in heteroplasmy observed within individuals.

1.2.3 Mitotic segregation

Mitochondrial disorders are characterized by their progressive nature; as the patient ages, the symptom worsens. During cell division the cellular contents are distributed to the daughter cells. This pattern of distribution for mitochondrial content however is not equal among the daughter cells. If the cell has a mtDNA mutation it may result in unequal transmission of the mutation. As a consequence, the mitochondrial mutant heteroplasmy may shift. This phenomenon is called "mitotic segregation" and could subsequently affect the phenotype [32]. The progressive nature of the mitochondrial disease could be caused, at least in part, by "mitotic segregation". Mitotic segregation was one of the technical challenges during earlier attempts to create transgenic mice models as discussed later in Chapter 1. Cytoplasm containing one type of mitochondria (donor-CAP^R mtDNA) can be fused to the cytoplasm of another cytoplasm (recipient) creating cybrids. However, initial experiments failed to identify transmission of CAP^R mtDNA in the cybrids [33]. This was due to loss or segregation of mitochondrial genomes.

1.3 Mitochondrial DNA replication

Mitochondrial genome has obvious differences from the nuclear genome. In addition to exclusive maternal inheritance, mtDNA also confers polyploidy of its genes, uses a different set of codons, undergoes higher rates of mutations and has a specific organization of genes lacking introns. Mitochondrial DNA size, expression mode and gene organization differs notably across phyla. Mitochondrial gene content and function however, is well conserved and mostly consists of protein coding genes for OXPHOS, tRNAs and rRNAs necessary for the mitochondrial translation [34]. mtDNA sequences from several other species can be found at

http://megasun.bch.umontreal.ca/gobase/. The referred sequence of the human mitochondrial DNA is the Cambridge reference sequence and the more recently revised Cambridge sequence [35,36].

Although mtDNA replication has been studied extensively and some of the key players involved in the process are known, a clear understanding of how it is regulated by nuclear genome is lacking [37,38]. How the mitochondrial DNA replication and transcription are coordinated in response to metabolic demands and developmental stages remains largely unexplored. Since the mtDNA structure and content are highly conserved in mammals, human mtDNA is considered as a model (**Figure 1**).

Human mtDNA is 16.6kB in size and is double stranded. The two strands can be separated in a density gradient owing to their distinct density due to the differences in their nucleoside composition (i.e. the heavier or H- strand and lighter or L- strand). There are two non-coding regions in the mtDNA. First is the displacement loop (D-loop). The D-loop is a triple stranded structure in the non-coding region of mtDNA and is the most variable region in term of sequence and size in different species [34,39]. It contains the major regulatory region of mitochondrial replication and transcription [37,40]. The D-loop may have a 'nascent H-strand' annealed to the L-strand in cells that are metabolically active. Origin of replication for the heavy strand (O_H), the heavy strand promoter (HSP) and the light strand promoter (LSP) for transcription are all located in the D-loop (**Figure 1**). In some species, the D-loop may also contain conserved sequence blocks (CSB) and termination associated sequences (TAS) [39]. The second non-coding region is the L-strand origin of replication (O_L).

There are two models proposed for mtDNA replication: asynchronous 'strand-displacement' and synchronous 'coupled leading-lagging strand' [39,41,42]. The two different mechanisms have been proposed based on the use of different techniques, electron microscopy and mapping of 5' mtDNA ends for the asynchronous and 2D gel electrophoresis for the

synchronous or ‘strand-coupled’ model. The asynchronous strand displacement model is the orthodox model of mtDNA replication.

According to the strand-displacement model, mtDNA replication is initiated by priming at the O_H region. This initial primer is created by mtRNA polymerase (mtRNApol) while initiating transcription from the L-strand. This initial L-strand transcript is processed by mitochondrial RNAse MRP (mitochondrial RNA processing) to generate primers. The mitochondrial DNA polymerase (DNAPol ϒ) then extends the processed and mature RNA primer to replicate the H-strand. Once the replication unidirectionally proceeds to two-thirds distance from the O_H of the newly synthesized H-strand, the O_L region is exposed. The parental H-strand is rapidly cleaved by the S1 nuclease. Once the single stranded O_L region is exposed it acquires a stem-loop structure. This stem loop structure is then used by a specific primase to generate primers for the initiation of L-strand replication. During the process of replication, there are several proteins participating to unwind the mtDNA (helicase/Twinkle), single strand binding proteins that enhance the fidelity and activity of DNAPol ϒ, and mitochondrial associated topoisomerases to resolve the mtDNA supercoils [43-47]. A mitochondrial DNA ligase has been recently identified that may help seal the nicks in the mtDNA during replication [48,49]. Chaperones such as mtHsp90 and mtHsp70 are also required for mitochondrial nucleoid stability.

Interestingly, the majority of the proteins participating in mtDNA replication and transcription (discussed next) have also been identified and studied in *Drosophila,* mice and humans. This suggests that all these factors involved in mtDNA replication and transcription are well conserved among higher metazoans [7].

1.4 Mitochondrial transcription

Mitochondrial transcription produces eight tRNAs and one protein (ND6) from the L-strand and the rest from H-strand. mtRNApol, mtTFAM and mTERF are the three important components of the mammalian mitochondrial transcription process [50,51]. mtTFAM binds to the consensus region around mitochondrial promoters HSP1 (containing H1 initiation sequence) and LSP (containing L initiation sequence). Binding of mtTFAM allows transcription by mtRNApol. mtTFAM regulates the mitochondrial RNA transcription. The H-strand is transcribed as two separate units. The first transcription unit initiates at H1 and produces the two rRNAs $tRNA^{Phe}$ and $tRNA^{Val}$. It is terminated at the 3' end of the 16S rRNA sequence. mTERF is known to assist in termination of mature mitochondrial transcripts [52]. The second transcription units initiates at H2 at a much lower frequency. This second unit results in a polycistron covering almost the entire H-strand giving rise to twelve tRNAs and 12 protein-coding mRNAs after being processed. A single polycistron is generated by the transcription of L-strand that yields eight tRNA and one protein-coding mRNA. The termination mechanism or sequence elements for the H2 and L transcription units are not exactly well understood.

1.5 Mitochondrial mutation and associated disorders

The incidence of mtDNA disease is unknown. Several epidemiological studies have estimated the presence of specific mtDNA mutations and mitochondrial disease incidence within small populations. Based on estimates, the frequency of pathogenic mtDNA mutations in the population is approximately 1 in 200 [53,54]. However, most of these mutations will remain unnoticed because clinical symptoms of mitochondrial disorders manifests at very high heteroplasmy levels and in situations of stress. Based on clinical cases the incidence of mtDNA

disease is estimated to be 1 in 10000 [55]. According to a review in 2001, 4000 children were born with mtDNA disease every year in the United States [56]. This high incidence of mitochondrial disease underscores the need to invest time and resource in unraveling the mysteries of mitochondrial disorders and designing therapeutics targeted to cure mitochondrial disorders.

Due to its exclusive maternal inheritance and polyploid nature, mtDNA mutations follow the laws of population genetics [57]. This is a key difference from most nuclear genetic diseases that exhibit Mendelian inheritance patterns and contributes further to the complexity of mitochondrial disorders. The diversity of mitochondrial disorders has led researchers to classify them based on their pathogenicity or phenotype. One of the classifications is based on whether the mitochondrial disorder is due to mutations in the genes coding OXPHOS components and whether it is coded by the mtDNA or nDNA [58]. Nuclear or mitochondrial DNA encoded mutations that affects OXPHOS are typically classified as *Class I mitochondrial disorders*. Mutations in other genes that localize within the mitochondria but are not directly involved in the OXPHOS function are classified as *Class II mitochondrial disorders* [59]. Another form of classification groups mitochondrial disorders based on the clinical symptoms [2].

Since the discovery of the first mutations as the pathogenic cause of mitochondrial disorder, increasing numbers of mutations in a variety of genes have been discovered such as genes involved in translation of OXPHOS subunits, its assembly, mitochondrial dynamics (fission and fusion) and regulation of mtDNA [60,61]. Mitochondria have their own mtRNA processing and translation machinery. Mutations in factors facilitating mtRNA transcription, stability, processing including the addition of poly-A tails to mitochondrial mRNAs, amino-acyl tRNA synthetases and directly in the mitochondrial tRNAs have all been implicated in mitochondrial disorders [62-64]. One of the examples is a common point mutation in the mt-tRNA$^{Leu(UUR)}$ gene at position 3243 which is found in ~80% cases of MELAS (Mitochondrial encephalomyopathy, lactic acidosis and stroke like episodes) patients and also has been

correlated with diabetes mellitus [64,65].

Mutations in the mtDNA cause a large number of primary mitochondrial disorders with a wide spectrum of symptoms. Mitochondrial disorders often present with complex clinical symptoms and typically progressively worsen. Organs or tissues relying heavily on mitochondria for their general functioning are the most affected. Hence, the neuromuscular system, including the CNS, striated and cardiac muscles, is more affected than others in mitochondrial disorders. Additionally, mitochondrial disorders show quite complex heterogeneity between individuals of the same family. This is generally attributed to the heteroplasmy levels, although the heteroplasmy-severity correlation is not always perfect.

Numerous reviews and articles have been published describing various mitochondrial diseases in the past three decades. Hence, I have mentioned only a few here with descriptions limited to their typical symptoms related to major mutations of the primary gene affected. For convenience and due to the expansive nature of mitochondria related diseases, my thesis will focus largely on primary mitochondrial disorders: disorders caused by mtDNA mutations.

1.5.1 Ragged Red Fiber Mitochondrial Syndromes

“Ragged Red Fibers" is caused due to the accumulation of defective mitochondria in the sarcolemma region of the muscle fiber. When the affected muscle tissue is stained with ‘modified Gomori trichrome stain’, it reveals a distinct appearance of "ragged red fibers" and therefore, this is a histopathological classification for “Ragged Red Fiber mitochondrial syndromes“. These include MELAS (Mitochondrial encephalomyopathy, lactic acidosis and stroke like episodes), MERRF (Myoclonic epilepsy with ragged-red fibers) and KSS (Kearns-Sayre Syndrome).

1.5.1.1 Mitochondrial encephalomyopathy, lactic acidosis and stroke like episodes (MELAS)

First identified by Pavlakis *et.al.,* this is characterized by stroke, migrainous headaches, seizures, vomiting and ragged red-fibers [66]. It can clinically present at late childhood, early adult life or sometimes even in infancy. The majority of the MELAS cases (~80%) have an A3243G transition in the $tRNA^{Leu(UUR)}$ gene [67]. Approximately 10% of the cases are caused due to T3271C affecting $tRNA^{Leu(UUR)}$.

1.5.1.2 Myoclonic epilepsy with ragged-red fibers (MERRF)

This disorder was first identified by Fukuhara *et.al.,* 1980 [68]. The majority (~80%) of the cases have a point mutation in the mtDNA at 8344 affecting $tRNA^{Lys}$ [69]. Clinical symptoms include 'myoclonic (involuntary) epilepsy', myopathy and progressive ataxia (uncoordinated movement of the body). Progressive onset of dementia and mental retardation is also observed.

1.5.1.3 Kearns-Sayre Syndrome (KSS)

First described by Kearns and Sayre in 1958, this is one of the most studied mitochondrial cytopathies and exhibits brain and muscle symptoms [70]. Several different point mutations in the mtDNA with multiple symptoms can result in KSS and therefore it is known as a "syndrome". DiMauro and colleagues categorized KSS symptoms as "progressive external ophthalmoplegia of all extraocular muscles, retinitis pigmentosa (pigmentary degeneration of retina) and possibly cardiac conduction blocks, cerebellar defects" [71].

1.5.2 Non-ragged Red Fiber Mitochondrial Syndromes

1.5.2.1 Leigh' syndrome

Leigh described this syndrome first in 1951 [72]. Leigh syndrome could be caused by mutations in either nuclear or mitochondrial DNA. Mutations in nuclear encoded respiratory chain subunits or pyruvate dehydrogenase can lead to Leigh syndrome. If the mutation is nuclear, it is transmitted in an autosomal recessive manner. If it is caused by mitochondrial DNA mutation it is inherited maternally and is termed as "Maternally Inherited Leigh Syndrome" (MILS). One of the mtDNA mutations that result in MILS is a point mutation in *ATP6* gene at position 8993. MILS manifests itself when the mutation load increases to more than 90% [28-30]. MILS patients present clinically at a much younger age: typically around 3 months post-partum. Aphenotypic at birth, it is characterized by "progressive lethargy, visual impairment, dyspaghia, hypotonia with weakness and paucity of movement" [73]. Additional features may include movement and other neurological disorders such as seizures, progressive neurologic deficits, calcified neurons and abnormal mitochondrial morphology [73].

1.5.2.2 Neuropathy, ataxia and retinitis pigmentosa (NARP)

NARP is caused by mutations in the mitochondrial *ATP6* gene and is closely related to MILS in that the same mutation can cause either conditions [74]. The NARP symptoms first occur in childhood, in contrast to infancy as in MILS, and individuals often live into their thirty's [31]. Generally, NARP patients have a mutant heteroplasmy between 70-90 percent.

1.5.2.3 Leber's hereditary optic neuropathy (LHON)

Mutations in a gene encoding a subunit of Complex I often lead to a mitochondrially inherited optic neuropathy called LHON. It occurs at a higher prevalence in males as compared to females (M:F ratio of 5:1). The majority of the LHON cases have a mutation in any of the genes, *ND1* G3460A, *ND4* G11778A and *ND6* T14484C or G14600A.

LHON symptoms involve degeneration of retinal ganglion cells, atrophy of optic nerve and loss of central vision. The frequency of LHON affecting males is 1 in 14000 with typical clinical symptom of painless vision loss in one eye generally followed by loss of vision in the other eye within a short period. Interestingly, small populations of LHON patients show an unexplained visual recovery and alleviation of symptoms over time. It is observed that the presence of a LHON mutation may not necessarily result in a disease phenotype. Incomplete penetrance of LHON is typical of mitochondrial disorders and is not well understood at the molecular level. Inexplicable recovery of symptoms appears to be unique to LHON.

With the increasing correlation of abnormal bioenergetics observed in several diseases such as neurodegeneration, cancer, diabetes and even aging, mitochondria is now beginning to be viewed as the hub of metabolic disorders.

1.6 Modeling human mitochondrial disease

1.6.1 Cellular models of mitochondrial disorders

Lack of manipulation techniques for the mitochondrial genetic material is a severe limitation in creating models of mitochondrial disorders of specific mtDNA mutations. However, advances in cell fusion and organelle transfusions have made it possible to create cellular models from naturally occurring mutations. It is possible to create cytoplasmic hybrids or cybrids containing the desired mtDNA mutation. Generally, a cell line (recipient) is depleted of its mitochondria such as by treating with ethidium bromide (EtBr) or rhodamine 6G dye [75,76]. EtBr intercalates between the DNA strands and inhibits replication. Once the mtDNA is depleted, isolated mitochondria from patients harboring the mutation are then transplanted in these mitochondrial devoid cells [77,78]. The nucleus from the donor cells is extruded using cytochalasin B. The donor and recipient cells are then fused to create a cybrid. Most of our understanding of the mitochondrial disorders has arisen from cell cybrid models over the past couple of decades [79-82]. Cell cybrids have been instrumental in studying the effect of specific mtDNA mutations on mitochondrial bioenergetics [83]. These include examples such as partial tandem duplication of the $tRNA^{Phe}$ gene in mitochondrial myopathy and A3243G transition affecting $tRNA^{Leu(UUR)}$, both of which result in respiratory defect and reduced oxygen consumption rates [84,85]. Nuclear genetic background can have significant impact on disease risk. As a method to generate cell cybrids mutant mitochondria are placed in a foreign nuclear background, which alters the normal dual control of the organelle. This can be confounding but also might be used to evaluate the role of nuclear genetic background in disease pathogenesis.

Despite their significance, cybrid models have several limitations in terms of recapturing features of tissue specificity. Various groups have developed techniques to make these cybrids

more relevant to the disease such as by combining cybrid and stem cell technologies to evaluate mitochondrial dysfunction in neurons. In one report mechanisms of neurodegeneration were studied in mouse embryonic stem cell cybrids with Complex I and Complex IV mutations after differentiating them into neurons [86]. An interesting approach of mtDNA depletion using nucleoside analogs was employed to create cybrids in human pluripotent stem cell derived neuronal progenitors (hNPs). It was found that a family of nucleosides that are commonly used as antiretroviral agents (zidovudine (AZT), didanosine (ddI) and zalcitabine (ddC)) blocks mtDNA polymerase, terminating mtDNA strand synthesis and consequently depleting mtDNA [87,88]. Using one such dideoxynucleoside analog, zalcitabine, Iyer *et.al.,* created hNP cybrids carrying *ND4* G11778A LHON mutation [89]. Patient derived cell cybrids in various cell types such as fibroblasts and induced pluripotent stem (iPS) cells have been engineered to recapitulate mitochondrial disorders. With the recent advancement in techniques, it has been possible to regulate the level of heteroplasmy in these cell cybrid models [85]. This is a huge step forward as the heteroplasmy level is critical in determining the outcome of mtDNA mutation/s. Not only have cybrids revolutionized the understanding of pathogenesis they have also provided a unique *in vitro* platform to screen drug candidates for mitochondrial disorders.

1.6.2 Animal models of mitochondrial disorders

Although cell cybrid models have been instrumental in helping us understand mtDNA mutations and their pathogenicity, they are unable to capture the composite characteristic of mitochondrial diseases. Mitochondrial disorders are progressive in nature, are heterogeneous affecting multiple organs and are tissue specific. These phenotypes are impossible to study in a cellular model. Therefore, animal models were developed to study mtDNA mutations and test therapeutic strategies in an *in vivo* system.

Various techniques have been employed to generate animal models of mitochondrial disorders such as nuclear transgenesis (of genes involved in OXPHOS and mtDNA regulation), chemical induction, allotopic expression and transmitochondrial approaches [25,90-92]. Standard nuclear transgenesis has been quite useful in cases of nuclear genes involved in OXPHOS. One such transgenic mice model is the NADH dehydrogenase (ubiquinone) iron-sulfur protein 4 (NDUFS4) mutation modeling Leigh syndrome model [93]. NDUFS4 is a nuclear encoded Complex I subunit. The Leigh syndrome mouse model has phenotypes observed in human patients such as progressive encephalomyopathy, loss of motor ability, respiratory abnormalities and aberrant mitochondrial morphology. Other well-known mouse models created using nuclear transgenesis are mutator mice (mutation in polymerase (Pol γ (gamma)), deletor mice (mutation in helicase Twinkle), *ANT1* knockout and *PINK1* knockout mice [43,94-97]. A LHON model was created by chemical injections of rotenone and the mice exhibits degeneration of retinal ganglion cells in as seen in LHON [98].

Animal models of mitochondrial disorders due to mutations in the genes encoded by the nuclear genome has been successful in elucidating key features of these fatal disorders. However, engineering animal models of mtDNA mutation is much more challenging and rare. Allotopic expression strategies have so far resulted in at least two mouse models e.g. L156R-ATP6 and R340H-ND4 [99-101]. Both these models however, have an endogenous mitochondrial encoded wild type copy of ATP6 and ND4 proteins and hence are not truly mitochondrial mutants. These and other allotopic expression studies will be discussed in detailed later in Chapter 2.

Another strategy for creating mtDNA mutation models is by altering mtDNA in embryonic stem (ES) cells. Even though the xenomitochondrial approach of injecting cybrid 129S6 ES cells

(*Mus terricolor*) into blastocyst of C57BL mice (*Mus musculus domesticus*) generated distinct nuclear and mtDNA background, the resulting mice remained largely aphenotypic [102]. Microarray data of the resulting mice displayed a gene expression profile that did not show any involvement in mitochondrial function. *In vitro* data using the same mtDNA-nuclear genome combination previously showed respiratory defects. Lack of clear phenotype has been attributed to various compensatory mechanisms as a consequence of gene manipulations. These compensatory mechanisms contribute to the complexity of diagnosis and treatment of mitochondrial disorders, and require further investigation [103].

Trans-mitochondrial engineering of mice employs two common methods – mitochondrial injection and ES cell transfer technology [104]. In the first method, isolated mitochondria are injected into the cytoplasm of pronuclear unicellular fertilized ova (zygote). The second method is a fusion technique of cytoplast containing mutant mitochondria with mtDNA depleted ES cells. Depletion of mtDNA in recipient cells is critical and is usually achieved by the use of R6G dye. If the recipient cells have their own mtDNA, it is possible to lose donor mtDNA via the process of mitotic segregation as discussed earlier. Trans-mitochondrial techniques have yielded mitochondrial deletion mutants ("mito-mice") and the *ND6* G14600A mutant mouse model [105,106]. Interestingly, the *ND6* G14600A mouse model is homoplasmic and has been instrumental in identifying ROS as the primary cause of LHON pathogenesis.

Other organisms such as *Drosophila* and *C. elegans* have also been used to create mitochondrial disorder models. One of the earliest fly models was the technical knockout (tko^{25t}) mutant as the "Drosophila model of mitochondrial deafness" [107,108]. The tko^{25t} mutant was rescued by a 3.1 kb DNA fragment that was later found to code for mitochondrial ribosomal protein S12 (MRPS12). This mutant shows bang sensitivity, mitochondrial translational defects

and sensory defect. Several other fly mitochondrial mutation models mostly involved in neurodegeneration exist and has been reviewed by Debattisti and Scorrano in 2013 [109].

Given their relevance, animal models of mitochondrial disorder capture the range of compound phenotypes and are critical in unraveling the mysteries of mtDNA mutations and its impact on whole organism.

1.6.3 Drosophila model of mitochondrial encephalomyopathy

In 2006, the Palladino lab identified and characterized a pathogenic mutation in *Drosophila* mtDNA encoded gene *ATP6* [110]. The *ATP6* gene (*726 bp*) codes for an essential 25kDa protein subunit of Complex V. The mutation *ATP6[1],* is a missense mutations that leads to substitution of glycine for glutamate at position 116 (G116E) of the ATP6 polypeptide chain. Flies harboring this mutation have ~98% mutant heteroplasmy level. The mutation results in significant reduction in longevity, sensitivity to mechanical stress and strobe light-induced convulsive seizures and paralysis as a function of age. *ATP6[1]* flies also exhibit progressive myodegeneration, neural dysfunction, abnormal mitochondrial morphology, progressive increase in mtROS, and reduced ATP synthase activity [110-112]. Based on the predicted partial crystal structure of ATP6, this substitution lies at the interface of ATP6 dimers. Among other phenotypic and biochemical features, *ATP6[1]* also affects dimerization of the ATP synthase complex.

Interestingly, the *ATP6[1]* mutation was identified as a maternally inherited enhancer of *sesB*^{1}. Importantly this mutation has a high mutant heteroplasmy but is not homoplasmic and it is surprisingly stable, especially when maintained as a *sesB*^{1} double mutant. Although the

reason why this mutation is stable in the *sesB*^{1} background is unknown, this is an important feature of this model. We have suggested that ANT dysfunction (encoded by *sesB* in flies) may have caused and or selected for the *ATP6[1]* mutation. Families suffering from adPEO (autosomal dominate progressive external ophthalmoplegia) -- a dominant disease with severe ocular phenotype -- have also been found to have a specific mitochondrial dysfunction where they accumulate mtDNA mutations. Several gene loci have been implicated in adPOE (4, 10 and 15). The 4p gene locus encodes *ANT1*.

Thus, the *ATP6[1]* mutant fly strain captures key features of mitochondrial disorder, is stable, amenable for progressive studies and is an excellent model to study disease pathogenesis and validate therapeutic strategies *in vivo*.

1.7 ATP synthase complex and associated mutations

Complex V or ATP synthase complex is a massive multi-subunit protein complex (~300kDa) residing in the IMM. Complex V is assembled as two domains F_1 and F_o from 10-16 subunits encoded by nuclear DNA and 2 subunits (ATPase 6 and ATPase 8) are encoded by the mtDNA (**Figure 1 and 3**) [113,114]. The F_1 domain is exposed in the matrix whereas the F_o is membrane bound.

F_1-F_o ATPase complex couples the proton gradient generated by the reducing equivalents passing through the ETC to produce ATP from ADP and P_i [115-117]. Protons are passed from the IMM space to the matrix through the ATP6 proton channel of the F_o complex [118,119]. This passage of protons results in a “rotary catalysis” mechanism that drives the central

stalk making it work like a rotor (subunit γ) [117,119]. ATP6, in conjunction with subunits b and δ (analogous to mitochondrial OSCP) constitute the stator – the stationary part of the rotating "molecular machine" P_i [115]. Subunit γ works like a "cam" that induces a conformational change in the subunits α-β of F_1 domain. 3 α-subunits and 3 β- subunits are arranged in a hexameric arrangement alternating with each other (**Figure 3**). This unique arrangement allows for repeated cycles of binding with ADP and P_i to generate ATP and then release of ATP [120]. Interestingly the direction of the rotation determines whether the ATP will be generated or hydrolyzed [121]. In other words, ATP synthase complex can also act as an ATPase – hydrolyzing ATP instead of generating ATP.

The ATP synthase complex is known to exist as a dimer [122]. There are several factors that affect the dimerization status of Complex V [123]. Lack of dimerization however, has been shown to result in decreased respiration and is usually observed in mutant/pathogenic conditions. Several groups have reported mutations affecting ATP synthase originating either in the nucleus or the mitochondria [30,74,124-130].

Of particular interest to my thesis is the subunit ATP6. This subunit interacts with subunit c and couples the proton flow to the rotation of the c-ring of F_o domain [118]. In humans, nucleotides *8527-9207* of the mitochondrial genome encode subunit 6 (ATP synthase a chain (*EC 3.6.3.14)* (ATPase protein 6 - Uniprot accession: *P00846*)) of the mitochondrial ATP synthase complex. It is obvious that mutations in such a critical subunit could result in pathogenic conditions. There are at least 9 pathogenic mutations associated with the ATP6 protein. Out of these, 7 are point mutations and 2 are deletions. Flies have homologous ATPase protein 6 that serves the same function (*D. melanogaster*- Uniprot accession: *P00850*). The ATP6 subunit is predicted to have 5 transmembrane domains and seems to be highly conserved throughout metazoan evolution.

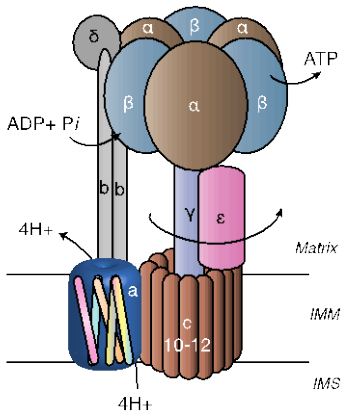

Figure 3. Subunits of bacterial ATP synthase complex – also known as Complex V. ATPa or ATP6 is shown dark blue. The five predicted TM domains of ATP6 are highlighted. Adapted and modified from *Rastogi and Girvin, Nature 1999*.

1.8 Management of mitochondrial disorders

The diversity of mitochondrial disorders not only makes them challenging to diagnose but even tougher to treat. In fact, current therapies for mitochondrial disorders are grossly inadequate. There is no known cure for mitochondrial disorders. In the absence of proper therapeutic options, the management of mitochondrial disorders is restricted to palliative care [131,132]. Patients with mitochondrial disorders are typically prescribed vitamins (such as riboflavin, thiamine, Vitamin C and E), cofactors and oxygen radical scavengers with a goal to delay or circumvent the damage to the respiratory chain especially during excessive physiological stress

[132]. Coenzyme Q_{10} (CoQ_{10}) supplements are used in cases of CoQ_{10} biosynthesis defects [133]. Drug cocktails with combination of vitamins, CoQ_{10} and other antioxidants (alpha lipoic acid) to act synergistically are commonly used although specific composition of these cocktails is universally not the same. Whether these cocktails vitamins and cofactors have any real benefits for patients with mitochondrial disorders remains debatable and data for their clinical trials is lacking [134]. Pharmacological interventions are unpredictable in mitochondrial disorders as the use of drugs could exacerbate physiological stress or mitochondrial toxicity in patients. In a review in 2009, Parikh *et.al.,* have listed and evaluated some of the drugs and their effects on mitochondrial toxicity [132].

Using exercise as a therapy ("aerobic conditioning") in mitochondrial myopathy has proven effective by enhancing normal mitochondria, and restoring/repairing muscle functions[135]. Exercise remains an integral part of the quest for clinical management of these disorders [136-138]. Some of the emerging therapies such as Sirtuin (SIRT-1) and PPAR agonist (bezafibrate) targeting multiple metabolic enzymes have recently been shown to improve mitochondrial performance [139]. However, none of these have been tested in clinical trial for their safety and efficacy in primary mitochondrial disorders. In the absence of definitive therapies to treat these devastating disorders, novel strategies such as hematopoietic stem cell transplant, nuclear transfer and mitochondrial gene therapy seems like a viable option [140-142].

2.0 ALLOTOPIC EXPRESSION AND MITOCHONDRIAL RNA IMPORT

Genetic material exchange between the nucleus and mitochondrion has taken place since the earliest endosymbionts. During the course of evolution thousands of mitochondrial genes have been transferred to the nucleus. More than 600 mtDNA-derived fragments are thought to have migrated to the nuclear DNA [143]. Interestingly, some of the genes from the nucleus acquired the capability to be targeted to the mitochondria. Most of the molecules targeted to the mitochondria are either proteins or RNA. Depending upon the organism, most metazoans are thought to target 900-1100 proteins to mitochondria [35,141]. These imported proteins participate in various mitochondrial functions. As a result, mutations in these proteins or RNA could lead to mitochondrial dysfunction. In fact many mitochondrial disorders have mutations in mitochondrial-targeted protein or RNA as the underlying cause of pathogenesis [144,145].

Why have only a few protein coding genes and tRNA genes remained in the mtDNA and others have migrated to the nucleus? Although this is not known, there are theories as to why this is the case. Studies of protein composition of the 13 human mitochondrial coded proteins revealed that they are extremely hydrophobic. Translation of very hydrophobic proteins in the cytosol can lead to inefficient import and cytosolic aggregation. Aggregated proteins may be cleared by the cell or worse – could lead to cytotoxicity. It is possible there was a selective advantage to retain the genes encoding these hydrophobic proteins within the mtDNA. These could then be translated in the mitochondrial matrix in close proximity to IMM where they are

likely co-translationally integrated into the IMM. Some species such as the algae *Chlamydomonas reiinhardtii* and some legumes have evolved a less hydrophobic protein that is now within the nucleus, consistent with the hydrophobicity hypothesis [146].

Gene therapy intended to replace defective or missing genes for nuclear encoded proteins are well established and have demonstrated significant benefit in several fatal disorders such as immunodeficiencies, thalassemia and leukodystrophies [147-150]. Discovery of mitochondrial disorders and their associated mutations sparked an interest in manipulation strategies to fix these diseases. There are several strategies to introduce foreign DNA into the mitochondria and a host of manipulation techniques for mitochondrial DNA have been proposed [151] . Although functional in cellular or *in vitro* models, none of them have been successful as a gene therapy [152,153]. As a consequence, currently, there is no technique to treat these devastating disorders.

2.1 Allotopic expression strategies

Allotopic expression is “the expression of a gene in a different cellular compartment to its target location. In the context described here, it is the recoding of a mitochondrial gene to allow it to be expressed in the nucleus. The subsequent conjugation of a mitochondrial-targeting sequence promotes import and localization of the gene product to the organelle” [144,151]. In general there are two strategies of allotopic expression – protein and RNA allotopic expression. Protein allotopic expression has been studied in detail using several mitochondrial genes [154,155]. Much is known about protein import and hence it makes it a bit easier to exploit this pathway for mitochondrial protein targeting. On the other hand, RNA allotopic expression has recently gained interest. The discovery of mitochondrial RNA import pathways and identification of

various RNA carrier proteins in the cytoplasm is an attractive avenue for a viable gene therapy. However, vigorous data to support this strategy is lacking.

2.1.1 Allotopic protein expression

Several proteins have been targeted to the mitochondria using nuclear allotopic protein expression strategies. A few examples involve heterologous protein, restriction enzymes, zinc finger nucleases and DNA repair factors. Using protein allotopic expression, a few mtDNA-encoded proteins have been allotopically expressed to complement and rescue the mutant counterparts such as ATP8[155], ATP6[156-158], ND4[152,156] and ND6[153].

This method was first demonstrated in yeast by Nagley *et al.,* 1988 [155]. In this paper, the authors used a normal copy of the ATP8 gene recoded for nuclear expression and supplemented with a mitochondrial targeting sequence to facilitate mitochondrial import. The normal copy of *ATP8* gene could rescue respiratory deficient mutants of *S. cerevisiae* that do not have endogenous subunit of ATP8. Allotopic expression was also attempted in human cells. As mentioned earlier, NARP and MILS are caused by a mtDNA point mutation at locus *T8993G* of ATP6 subunit. Manfredi *et al.,* 2002, successfully expressed and incorporated a normal copy of ATP6 in the ATP synthase complex of T8993G mutant cells by using cell cybrid models. Guy *et al.,* 2002 used a similar strategy for a mtDNA point mutation of G11778A, responsible for LHON that codes for ND4 subunit of Complex I. In this study, the rescued cybrids showed significantly enhanced ATP synthase activity. In contrast, in a 2006 study, Bokori-Brown *et al.,* found no evidence of improvement of mitochondrial function or assembly of normal ATP synthase subunits.

In a variation of this strategy, the “nuclear derived transcripts”, coding mtDNA-encoded proteins, are targeted to the OMM using specific sequences such as *3’UTRs*. Once the transcript is targeted to the OMM, it is believed to be either co-translationally imported or translated and then imported in the mitochondria [159]. One obvious advantage of this alternative approach is that this would address the aggregation of hydrophobic protein hurdle associated with protein allotopic expression [100,160,161]. Other studies have reported the use of non-human mitochondrial proteins to complement respiratory chain defects[162-165]. In the case of protein allotopic expression, there are a few hurdles that make it quite challenging to rescue a mutant phenotype.

2.1.2 Allotopic RNA expression

We, and others have proposed RNA allotopic expression as a possible therapy strategy. This involves targeting protein-coding transcripts to the mitochondrial matrix that will endogenously be translated in the mitochondria. Another application of this strategy is to target normal copies of the defective mitochondrial tRNAs into the mitochondria. A 2011 study by Karicheva *et.al.,* targeted ‘specifically designed transgenic tRNA’ to rescue MELAS syndrome due to a $tRNA^{Leu(UUR)}$ gene mutation A3243G in the mtDNA [166]. This imported tRNA was able to improve mitochondrial translation and rescue mitochondrial respiration defect. However, the absence of the mechanistic knowledge of RNA import into the mitochondria such as lack of clear understanding of RNA import pathways, the proteins involved and the identification of sequences that are recognized by import proteins currently makes this approach an extremely challenging task.

2.2 Mitochondrial import of macromolecules – proteins and RNA

2.2.1 Mitochondrial Protein Import

Out of approximately 900-1100 mitochondrial proteins, only 0.01% (13 proteins) are coded by the mammalian mtDNA [35,167]. The rest of the proteins are encoded by the nuclear genome and imported by the mitochondria. These imported proteins are involved in several mitochondrial functions.

Mitochondrial imported proteins may end up in the OMM, IMM, IMS or the mitochondrial matrix. The mitochondrial protein import machinery recognizes signals associated with the pre-protein that is being imported. In most cases these signals are N-terminal associated peptide sequences and are commonly referred to as “mitochondrial targeting sequences (MTS)”. Examples of MTS are $COX8^{MTS}$ of cytochrome oxidase subunit 8, $P1^{MTS}$, $P2^{MTS}$ or $P3^{MTS}$ of ATPase 9/c. These MTS have been identified and exploited to target non-mitochondrial proteins into the mitochondria such as EGFP, dsRED, GAPDH and other cytosolic proteins.

“Hetero oligomeric membrane complexes” translocase of outer membrane (TOM) and translocase of inner membrane (TIM) sort out incoming proteins with the assistance of several other proteins that constitute the mitochondrial protein import machinery. It is believed that once the pre-protein with the MTS enters the mitochondria, a peptidase cleaves the MTS thus releasing the mature protein. TOM is the major translocation complex akin to a “gatekeeper” through which most of the proteins have to pass for mitochondrial import [168]. Tom20, 22 and 70 subunits recognize pre-proteins and transfer it to the central channel Tom40 [169]. Other subunits of TOM such as Tom5, 6 and 7 assist in the assembly of TOM complex [170]. Sorting and assembly machinery (SAM), mitochondrial inner membrane assembly (MIA) and pre-sequence

associated motor (PAM) work in conjunction with TOM-TIM complexes to target some of the proteins to different mitochondrial compartments [171]. SAM, MIA and TIM (carrier translocase of inner membrane) are thought to target the proteins to OMM, IMS and IMM respectively [170]. Oxa1p is an insertase/export machinery of inner membrane that also interacts with mitochondrial ribosomes [172]. Oxa1p thus facilitates the insertion of mitochondrial-translated proteins in a way that the N-termini lie in the IMS.

Mia40, a conserved IMS resident oxidoreductase, has an interesting role in facilitating the IMS protein import. Proteins translated in the cytosol and destined to end up in the IMS are unfolded prior to mitochondrial import. Mia40 has an essential disulfide center in its Cys-Pro-Cys signature domain [173]. This redox-active disulfide bond oxidizes the unfolded proteins approaching from the cytosol and "locks" them in a stable folded state via a 'mitochondrial disulfide relay system' [174,175]. Thus, the IMS targeted protein remains in the IMS via Mia40 dependent oxidation process.

In addition, the protein import machinery also consists of mitochondrial chaperones or heat shock proteins (mtHSP) that are required for proper folding of the mitochondrial imported proteins. Mutations in the protein import machinery components or MTS have been associated with disorders such as deafness dystonia syndrome (DDS), PDHC deficiency and methylmalonic acidemia [176-178].

2.2.2 Mitochondrial RNA import

In contrast, mitochondria import only a few RNAs. All mitochondrial imported RNAs are non-coding and there is no evidence as of now that a coding RNA is naturally imported into

mitochondria. As discussed in the previous section, the mitochondrial protein import has a dedicated import pathway the components of which seem to be conserved across species. RNA import, however, is quite diverse across various species. Not only are there different types of RNAs imported in diverse species, divergent mechanisms exist to import these RNA into the mitochondria [179-182]. This adds to the complexity of mitochondrial RNA import and its pathways. Unsurprisingly, RNA import pathways of mitochondria are largely unknown and remain less well characterized in contrast to protein import.

Why only a few RNAs are imported into the mitochondrial? In a review in 2008, Salinas *et.al.,* have discussed few of the factors such as selectivity, targeting, translocation and regulation that could affect the import of tRNAs into the mitochondria [180]. Although it helps in understanding some aspects of RNA import, it remains to be validated whether these parameters are a general rule or specific to certain species.

2.2.2.1 RNAs imported into the mitochondria

a. RNase MRP

RNase MRP is a site-specific endoribonuclease, which is coded by the nucleus. In order to initiate mtDNA replication, primer processing is necessary. RNase MRP processes mtRNA transcripts to generate primers that facilitate mtDNA replication. RNase MRP was first identified in mouse mitochondria and is 275 nt long [183]. It has a 'decamer sequence' that is complementary to the mtRNA substrate. RNase MRP was identified having features of RNA polymerase III (RNAPIII) unit such as a region similar to Box A sequence (5'-RRYNNARYGG-3') of RNAPIII and a 3' tetra-T (RNAPIII termination signal) [184]. RNase MRP has nucleo-

cytoplasmic function as well and is mostly found in the nucleus [185,186]. It is required for the correct processing of the ribosomal 5.8*S* rRNA in yeast (*S. cerevisiae*) [187-189]. RNase MRP has also been shown to be critical for cleaving a B-type cyclin (CLB2) for cell cycle progression post mitosis [190,191].

b. RNase P

RNase P is a nuclear encoded endoribonuclease that is imported into the mitochondria and processes the 5' ends of tRNAs [192,193]. The mitochondrial genome is compact and often has tRNA 'punctuating' the coding regions [35,194]. The heavy and light strands generate polycistronic mtRNA that requires processing. In contrast to their cytoplasmic counterparts mitochondrial tRNA or mRNAs do not possess significant 5' or 3' pre-sequences or UTRs [195]. The polycistronic mtRNAs are processed by the RNase P to yield tRNAs and other RNAs in the mitochondria. There are two forms of RNase P – one that has a 340 nt long catalytic RNA component [193] and the other which is an RNA free mitochondrial RNase P. The second type of RNase P was described recently and is composed of three protein subunits (mitochondrial RNase P protein 1, 2 and 3) [196,197]. Both RNase Ps have been proposed to cleave the 5' end of the tRNAs. Interestingly, the RNA free RNase P activity has only been shown *in vitro* [196]. While investigating mitochondrial RNA import, Wang *et.al.,* 2010 developed an '*in vitro* tRNA processing assay' using mitoplast lysates. When the lysate was pre-treated with nuclease, the tRNA processing was lost. This result was consistent with the previous findings by Puranam *et.al.*, that also demonstrated nuclease sensitivity of the mitochondrial localized component of RNase P [193]. The same report also estimated that there are ~ 33-175 molecules of RNase P per HeLa cell. Although merely 0.1 to 0.5% of the nuclear pool, these numbers might be sufficient for tRNA processing in transcriptionally active cells.

c. 5*S* rRNA

5*S* rRNA is a nuclear encoded fragment of RNA that is most abundantly imported into the mitochondria (~1% of the nuclear fraction) [198]. That it is imported into mitochondria was first discovered in rabbit, chicken and bovine mitochondria [199]. In plants, the 5*S* rRNA is transcribed from the mtDNA and gets incorporated into the mitochondrial ribosomes [200]. Yeast is not known to import 5*S* rRNA into its mitochondria [198]. The 5*S* rRNA is a component of mammalian cytoplasmic ribosomes. However, the mitochondrial ribosome lacks the L5 subunit that is predicted to interact with the 5*S* rRNA [201]. Hence, the function of imported 5*S* rRNA in the mammalian mitochondria is a matter of debate. Several groups have studied the import of 5*S* rRNA into the mitochondria. The import of 5*S* rRNA is reliant on the mitochondrial membrane potential, requires ATP and other soluble factors [198]. A conclusive mitochondrial function of 5*S* rRNA remains elusive.

d. tRNAs

Unlike human mtDNA, mtDNA of several other species do not encode for an entire set of tRNA. For example, the mitochondrial genomes of *Spizellomyces punctatus* codes for eight tRNAs, *Arabidopsis thaliana* for 14 tRNAs (lacking 6 tRNA) and *Chlamydomonas reinhardtii* codes for only 3 tRNAs. There are organisms such as *Trypanosoma brucei* and *Leishmania tarentolae* that completely lack tRNA genes in their mtDNA. Since tRNAs are essential for mitochondrial translation and given that mitochondria from most species do not have a complete set, the mitochondria of many organisms are widely believed to import nuclear tRNAs from the cytoplasm. It is possible to predict how many tRNAs are being imported into the mitochondria by cross referring the available mtDNA sequences and comparing them with the tRNA genes and

their respective codons [202]. Importing a corresponding tRNA from the cytoplasm compensates the lack of tRNA gene encoded by the mitochondria. While the imported tRNA is only a fraction of what remains in the cytosol, it is sufficient to meet the demands of mitochondrial translation.

Even simple organisms like *Saccharomyces cerevisiae* import at least one tRNA from the cytosol despite having a complete set of mitochondrial encoded tRNAs [203-205]. Until 2008, it was assumed that tRNA import in human mitochondria did not occur. We now know that there are at least two tRNA ($tRNA^{Gly(CUG)}$ and $tRNA^{Gly(UUG)}$) imported into human mitochondria [206]. In this study, Rubio *et.al.,* demonstrated that the two out of four nucleus-encoded $tRNA^{Gly}$ isoacceptors can be imported from the cytoplasm into the mitochondria *in vitro*. As a result, both the mitochondrial and nucleus-encoded $tRNA^{Gly(UUG)}$ isoacceptors coexist in mammalian mitochondria. However, it is not clear why this redundancy occurs in nature. It is possible that this is an evolutionary burden or a prospective evolutionary maneuver directed towards losing analogous mitochondrial genes. Additionally, it may also be feasible that these redundant tRNA have a completely novel function other than aiding mitochondrial translation.

It was not until 2007 that the function of nuclear encoded $tRNA^{Lys(CUU)}$ in yeast was elucidated using an elegant set of experiments [207]. When grown at standard temperature of 30°C, the $tRNA^{Lys(UUU)}$ has the potential to recognize two codons, AAA and AAG. Kamenski *et.al.,* demonstrated that when the temperature is increased to 37°C, $tRNA^{Lys(UUU)}$ fails to recognize AAG codon due to inadequate modification of its first anticodon. Under this condition, the imported $tRNA^{Lys(CUU)}$ is able to read the AAG codon thus compensating the translation insufficiency at elevated temperatures. Likewise there can be numerous other possibilities for the apparent redundancy of tRNAs imported into the mitochondria including that some are non-selectively imported.

e. miRNAs

Recently, there are a number of reports that miRNAs are imported into the mitochondria [208,209]. These 'mitomiRs' play a significant role in mitochondrial homeostasis by responding dynamically to the changes in the cellular milieu. While majority of the cytosolic microRNA are known to alter the level of a protein expression via translational repression or degradation of the target transcript and gene silencing, there are reports of posttranscriptional upregulation as well [210,211]. mitomiRs are the newest addition to the growing list of imported RNAs. In 2012, Das *et.al.,* demonstrated that a mitochondrial miRNA, miR-181c could repress mitochondrial translation [212]. However, this repression led to an overall increase in the mitochondrial activities. In contrast, Zhang *et.al.,* in 2014, reported that muscle mitochondria import miR-1 (mitomiR) that regulated mitochondrial translation of at least two mtDNA encoded proteins, ND1 and COX1 [213]. The mitochondrial function of miR-1 is opposite to that of its cytoplasmic function where it downregulates its two targets HDAC4 and ELL2 [214]. The mechanism of mitomiR import is currently not known but it is possible that it relies on PNPase, porin or some other protein for mitochondrial import [208].

2.2.3 Mitochondrial RNA import mechanisms

Due to its polyphyletic evolutionary origin, it is challenging to classify RNA import pathways but it can broadly fall into two different categories [179,180]. These are 'protein import dependent pathway' and 'protein import independent pathway' (**Figure 4)**. It is evident from tRNA import studies that this is a selective process based on the sequence motif or structure of tRNA being imported [180]. In general RNA import is an active process and sensitive to protease treatment of the OMM [215]. The requirement of ATP for RNA import however may have a few exceptions [216].

The ‘protein import dependent pathway’ requires an intact mitochondrial membrane potential. This pathway uses the TOM-TIM complex similar to protein import. $tRNA^{Lys(CUU)}$ in yeast uses this pathway for import into the mitochondria and involves cytosolic factors such as pre-MSK, mitochondrial lysyl-tRNA synthetase precursor (LysRS) and the enolase enzyme [205,217]. Aminoacylation of cytosolic LysRS is thought to induce a conformational change that drives the interaction of $tRNA^{Lys(CUU)}$ with pre-MSK. This complex is then targeted to the protein import machinery by enolase [218]. The tRNA import pathway in yeast has been hypothesized to be conserved in mammals as yeast $tRNA^{Lys}$ derivatives can rescue a human $tRNA^{Lys}$ mutation [219].

Several studies have reported that aminoacyl-tRNA synthetases are involved in RNA import in plants. Porin or voltage dependent anion channel (VDAC) is critical for RNA import in plants *in vitro* [220]. In trypanosomatids, cytosolic factors such as eEF1a or dedicated RNA Import Complex (RIC) have been reported to play a role in mitochondrial RNA import [216,221,222].

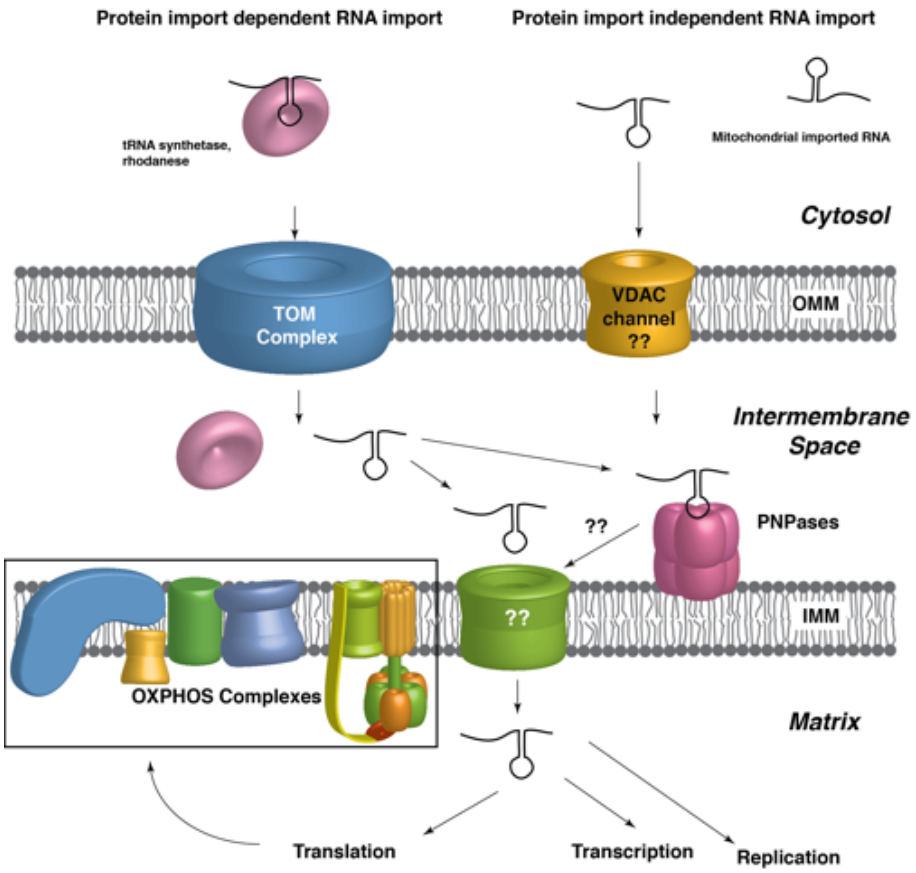

Figure 4. RNA import mechanisms. There are two proposed mechanisms of RNA import into the mitochondria, protein import dependent and protein import independent. Adapted from *Wang et.al., Biochimica et Biophysica Acta, 2011*.

2.2.3.1 PNPases and the mechanism of RNA trafficking

The PNPase (~78 kDa, polyribonucleotide nucleotidyltransferase) is a highly conserved nucleus-encoded mitochondrial 3'-5' exoribonuclease with distinct RNA-processing and RNA-import activities [223]. The PNPase protein has 4 major domains – N-term RNase PH1 and RNase PH2 domains that catalyze RNA degradation, alpha helical domain in between the two RNAse PH domains and C-term KH-S1 domain that binds to RNA [224]. Crystal structure of the PNPase from *Streptomyces antibioticus* (Uniprot accession: Q53597 and PDB entry: 1E3H) and *Escherichia coli* (Uniprot accession: A7ZS61 and PDB entry: 3GCM) reveal that the 3 monomers interact via its catalytic RNase PH domain forming a donut-shaped configuration [224]. This creates a central RNA binding substrate that can guide it through the trimeric complex. How the PNPase distinguishes the RNA for either of the activities is not yet completely understood. However, it seems that the stem loop structure is a recognition substrate for the PNPase mediated RNA import into mitochondria.

According to the reported crystal structures (PDB entries 1E3H and 3GCM), the neck region of the central channel of PNPase trimer has at least two critical arginine residues (R102 and R103) contributed by each monomer [225,226]. Both these residues are conserved in human and flies. Arginine is a positive amino acid and it appears to play an important role in grasping the single-stranded RNA targets. Mutation of the R102/R103 results in both decreased binding and cleavage efficiency of the target RNA which in this case was a 20-mer RNA. Interestingly, mutation in R106 residue affects the size of the neck and it loses its efficiency for cleaving smaller RNA molecules. These results suggest that charge and size of the channel are absolutely critical in identifying and capturing RNA substrates.

The N-terminal of PNPase contains a "putative mitochondrial-targeting signal". Although debated, there is convincing evidence that PNPase localizes to the IMM. shRNA mediated

knock down PNPase has been associated with morphological changes in the mitochondrial network from filamentous to fragmented, decreased membrane potential and reduced respiratory chain complex activities. These and other ultrastructure and functional data suggest PNPase plays an important role in the maintenance of mitochondrial homeostasis [227]. Studies in liver specific conditional *Pnpt1* knockout – HepKO liver cells, showed a significant reduction in mature mRNA transcripts and encoded proteins suggesting impaired mRNA processing [223]. It was further shown that PNPase deficiency led to a decreased import of *RNase P* RNA, which is a component of *RNase P* mtRNA processing complex [223].

2.3 Mitochondrial mRNA, tRNA and mitochondrial RNA processing

Only the mitochondrial protein ND6 is transcribed from the L-strand. Twelve of the thirteen mRNAs are transcribed as a single polycistron from the H-strand. This polycistron is processed resulting in 8 monocistronic mRNAs and 2 bicistronic mRNA (*ATP8/ATP6* and *ND4L/ND4*).

As mentioned earlier, there are two forms of RNase P: one with a nuclear encoded RNA and the other that is RNA free and contains only proteins. This RNA free RNAse P complex consists of three subunits (i.e. mitochondrial RNAse P protein 1 (MRPP1), mitochondrial RNAse P protein 2 (MRPP2) and mitochondrial RNAse P protein 3 (MRPP3)) [196]. The RNAse P complex catalyzes cleavage at 5' end of the tRNA [192]. This has been studied extensively in bacteria and yeast. In humans, it has been suggested to act as a transcription factors in addition to being an endoribonuclease [228]. The 3' end of mitochondrial tRNA is cleaved by the nuclease ELAC2 [229,230]. Brzezniak *et.al.,* demonstrated that human ELAC2 gene is necessary for 3' end processing of several mitochondrial tRNAs coded by either of the mtDNA strands, including $tRNA^{Val}$, $tRNA^{Lys}$, $tRNA^{Arg}$, $tRNA^{Gly}$, $tRNA^{Leu(UUR)}$ and $tRNA^{Glu}$ [230]. After the cleavage of the

polycistronic transcript, mtRNA poly-A polymerase (MTPAP) polyadenylates mitochondrial mRNAs. This process of adenylation can add up to ~50 poly-A at the 3' end of the mt-mRNA.

2.4 Mitochondrial translation

In mammals, mtDNA codes for 13 critical protein subunits of OXPHOS. These polypeptides are translated in the mitochondrial matrix by the mitochondrial ribosomes and the endogenous mitochondrial translational machinery.

The 55*S* mitochondrial ribosomes or mitoribosomes consist of two subunits 39*S* and 28*S* consisting of 16*S* rRNA and 12*S* rRNA respectively. Mitochondrial ribosomes are larger in size than their cytoplasmic (80*S*) or prokaryotic (70*S*) counterpart**.** However, since mitoribosomes have lower RNA content their sedimentation coefficient is relatively lower (55*S*) [231]. The 16*S* and 12*S* rRNA of the large and small ribosomal subunits is coded by the mtDNA. There are at least 81 other proteins coded by the nuclear DNA that are required to assemble the mitochondrial ribosome. Interestingly, 5*S* rRNA typically found in cytosolic ribosomes have not been found to be associated with the mitochondrial ribosomes. It may be possible that the function of 5*S* rRNA in the mitoribosomes have been taken over by either of its two constitutive rRNAs. This hypothesis stems from the fact that a 23-bp region at the end of 16*S* rRNA displays a 68% sequence identity to a fragment of *Bacillus subtilis* 5*S* rRNA. A quantitative study of rRNA steady state levels in the rat liver estimated approximately 100 mitoribosomes per mitochondria [232,233].

Mitochondrial initiation factors mtIF1 and mtIF3 initiate mitochondrial translation. mtIF3 dissociates the mitoribosomes leading to the assembly of an initiation complex. The small

subunit of the ribosomes binds to the mt-mRNA aligning the start codon at its P-site. A number of nuclear encoded proteins are involved in the peptide elongation such as mitochondrial elongation factor Tu (mtEFTu) and mitochondrial elongation factor G1 (mtEFG1). These two elongation factors are primarily responsible for tRNA binding to the ribosome and facilitating the addition of new amino acids for peptide elongation. Once the elongation complex encounters a stop codon (UAG or UAA), the translational termination release factor 1a (mtRF1a) induces the release of nascent peptide from the terminal tRNA.

The 13 proteins coded by the mtDNA are incorporated into the IMM to function in a multisubunit OXPHOS complexes I though V. With the exception of complex II, all other complexes have one or more subunits that are endogenously translated in the mitochondrial matrix (**Table 1**). Since these mtDNA encoded proteins are extremely hydrophobic, it is hypothesized that they get incorporated into the IMM co-translationally. To support this hypothesis, Greber *et.al.,* proposed a model where the 39S large subunit of porcine mitoribosomes are tethered to the IMM via MRPL45 that acts as an anchor [234]. This structure-based finding reinforces the results of Liu and Spremulli that almost 40-50% of the mitoribosomes are attached to the IMM [235]. Thus, mitochondria have evolved a strategy to effectively translate and incorporate hydrophobic proteins within its matrix and IMM, respectively.

2.5 Challenges associated with mitochondrial genome manipulation and gene therapy

There are several challenges associated with manipulating mitochondrial genomes such as targeting multiple mitochondria genomes per cell, lack of effective technique to penetrate the

mitochondrial double membrane, no mtDNA recombination and overcoming selective pressure against mutant mtDNA that are deleterious to OXPHOS [104,236-238]. Artificial transfection methods utilizing lipofection or other lipid-mediated vehicles have been limited to the delivery of alien nucleic acids to the cytoplasm via plasma membrane fusion. This has been successful largely because the lipid composition of plasma membrane is very well known. Unfortunately, even though mitochondrial lipid architecture is known to a certain extent, the lack of exact lipid composition of the outer and inner membrane has made mitochondrial transfection strategies ineffective. How the lipids get incorporated into the IMM and OMM is key to developing mitochondrial transfection methods. Compatible lipid composition between the target and the vehicle liposome carrying passenger nucleic acid will ensure their effective fusion and delivery of the cargo.

Even after overcoming these biological and technical obstacles, there are other obstacles that make mitochondrial gene therapy a formidable task. First is the hydrophobicity of mitochondrial proteins. The mitochondrial DNA encoded proteins are membrane bound and are extremely hydrophobic. They need an optimal environment for proper folding and co-translational insertion that may only be possible in the mitochondrial matrix. The relative hydrophilic property of nuclear encoded mitochondrial-targeted proteins enables its translocation through the TIM-TOM complex. Therefore, adding a hydrophilic component to the allotopically expressed protein could be sufficient to allow its translocation through the TIM-TOM complex. However, strategies of adding a hydrophilic tail such as that of EGFP to allotopically expressed mtDNA encoded proteins, ND4 and apocytochrome-b, has failed to overcome the issue of effective import into the mitochondria as studied in COS-7 and HeLa cell lines [152]. Interestingly, the nuclear encoded ATP6 protein from *Chlamydomonas reinhardtii* was able to rescue the ATPase function in human cells harboring mtDNA NARP mutation [157]. Unfortunately, in most of the cases it leads to toxicity. Even if the proteins are expressed by using tags or

fusion proteins that reduce the hydrophobicity of these engineered mitochondrial proteins, these may end up in aggregating in the cytosol. This leads to cytotoxicity and is a major challenge in the field of genetic expression of mitochondrial proteins [152]. The third major obstacle is the endogenous mutant competition. Oca-Cossio and Moraes hypothesized that since the majority of the pathogenic mtDNA mutations are missense mutations, they can still be translated from the mitoribosomes creating a hurdle for effective mitochondrial gene therapy [152]. Therefore, even if the allotopic expression strategies become feasible, the endogenous competition from the mutant protein could present an obstacle to genetic rescue.

3.0 "SMALL MITOCHONDRIAL-TARGETED RNAS MODULATE ENDOGENOUS MITOCHONDRIAL PROTEIN EXPRESSION *IN VIVO"* BY ATIF TOWHEED *ET.AL.*, 2014

3.1 Abstract

Endogenous mitochondrial genes encode critical oxidative phosphorylation components and their mutation results in a set of disorders known collectively as mitochondrial encephalomyopathies. There is intensive interest in modulating mitochondrial function as organelle dysfunction has been associated with numerous disease states. Proteins encoded by the mitochondrial genome cannot be genetically manipulated by current techniques. Here we report the development of a *mitochondrial-targeted RNA expression system* (*mtTRES*) utilizing distinct non-coding leader sequences (NCLs) and enabling *in vivo* expression of small mitochondrial-targeted RNAs. *mtTRES* expressing small chimeric antisense RNAs were used as translational inhibitors (TLIs) to target endogenous mitochondrial protein expression *in vivo*. By utilizing chimeric antisense RNA we successfully modulate expression of two mitochondrially-encoded proteins, ATP6 and COXII, and demonstrate the utility of this system *in vivo* and in human cells. This technique has important and obvious research and clinical implications.

3.2 Introduction

Mutations in the mitochondrial genome cause a set of devastating disease conditions categorized as primary respiratory chain diseases, also known as mitochondrial encephalomyopathies (MEs) [2]. Mitochondrial gene therapy has been proposed as a treatment for ME, however, this approach remains controversial as there are limited preclinical data demonstrating efficacy and evidence suggesting this approach may have significant limitations [153,156,158].

Endogenously encoded mitochondrial proteins function within large well-characterized respiratory complexes that perform oxidative phosphorylation (OXPHOS). The mitochondrial genome is known to harbor hundreds of pathogenic mutations, including ones affecting all of the tRNA genes and over 260 distinct coding mutations. The vast majority of protein-coding gene mutations associated with human mitochondrial disease are missense mutations, accounting for ~ 225 of the pathogenic mitochondrial mutations (www.mitomap.org), implying that mutant protein is usually capable of being expressed in the disease state. We previously discovered and characterized a *Drosophila* model of ME with an endogenous missense mutation in the *ATP6* gene affecting the F_1F_o-ATPsynthase (complex V) [110,239]. Twenty-one distinct human missense mutations exist within the *ATP6* gene, fourteen of which have been shown to cause human MEs including Familial Bilateral Striatal Necrosis (FBSN), Neuropathy, Ataxia, and Retinitis Pigmentosa (NARP), or Maternally Inherited Leigh's Syndrome (MILS) [240-242]. *ATP6[1]* mutant flies contain a missense mutation with high mutant heteroplasmy and exhibit phenotypes analogous to human symptoms including locomotor and progressive neural dysfunction, seizures, myodegeneration, and reduced longevity [239].

Competition with mutant protein for incorporation into mature respiratory complexes is likely a major obstacle to a viable mitochondrial gene therapy: a fact that has largely been ignored. This competition may explain the controversial allotopic expression results and remains a formidable obstacle to the treatment of MEs resulting from any endogenous mitochondrial missense mutation. A method to specifically reduce expression of mitochondrial-encoded genes is not known.

Several RNAs are naturally imported into the mitochondria from the cytoplasm and detailed studies have provided critical insight into the import process and import substrates [179,181,202]. Although the exact mechanism of RNA import into mitochondria is unknown, several pathways have been suggested to mediate mitochondrial RNA import [223,243,244]. We have identified a nuclear encoded mitochondrial *5S rRNA* isoform and engineered a novel vector to express small RNAs *in vivo*. We developed a mitochondrial-targeted translational inhibition (TLI) approach using small chimeric RNAs to regulate endogenous mitochondrial protein expression. Here we demonstrate the efficacy of mitochondrial-targeted TLIs by targeting two distinct loci encoding essential proteins of two different OXPHOS complexes, one *in vivo* and the other *in vitro*. The ability to selectively modulate mitochondrial protein expression in animals represents an important technological advance with obvious research and clinical applications.

3.3 Material and methods

3.3.1 Engineering *mtTRES* and *mtTRES-TLI* constructs

The *mtTRES* vector was created using the available *pUAST-attB* vector as a backbone [245]. A StuI site was added by site directed mutagenesis 5' to the *attP* integration site using Quick

Change Lightning (Invitrogen, USA). The *5S rRNA RNAPIII* promoter (AE013599.4) and termination (AE013599.4) sequences were PCR amplified from wild type *Drosophila* genomic DNA and directionally inserted using standard cloning methods and the HindIII-EcoRI and StuI-KpnI cloning sites, respectively. For the mammalian *mtTRES* vector the human *U6* promoter (NT_010194.17) was PCR amplified from p*Silencer* 2.1 (Invitrogen, USA), purified and inserted in place of the fly *RNAP III* promoter. The EcoRI-EagI cloning sites were used to insert NCLs. The *5S rRNA*^{mt} variant was identified as the most abundant mitochondrial isoform by clonal analyses (88%) from three independent cloning events and sequence analysis of 135 clones. The *5S rRNA*^{mt} was the major mitochondrial isoform in all three independent clonal populations (Supplementary material: **Figure 22** and GenBank: CR33451). The *5S rRNA*^{mt} sequence was synthesized with flanking EcoRI-EagI cloning sites (GeneWiz, South Plainfield NJ, USA). The *MRP* and *RNAseP (RNP)* oligonucleotides were annealed and directionally cloned into EcoRI-EagI cloning sites using published sequences [215]. TLI complementary sequences were synthesized as oligonucleotides, annealed and directionally cloned into EagI-KpnI cloning sites. *TLI-5S*^{mt}*::ATP6(a*) is 25 bases long, whereas *TLI-5S*^{mt}*::ATP6(b*) is 26 nucleotides in length and the complementary region is shifted 3 nucleotides 5'. All oligonucleotides were commercially synthesized by IDT (Coralville IA, USA). The final constructs were sequence verified (GeneWiz, South Plainfield NJ, USA).

3.3.2 Drosophila transgenesis, longevity and locomotor assays

mtTRES vectors allow site-directed PhiC31-mediated *attP/B* transgenesis. We used the *VK00027 attP* insertion site and flies bearing the *VK27 attP* chromosome are the control for all transgenic experiments. DNA injections were performed by Genetic Services (Cambridge MA,

USA) and successful transgenesis events were identified using *white*$^{mc+}$. Homozygous transgenic strains were tested. Previously established methods were used to test longevity [246] and locomotor assays [247,248].

3.3.3 Western blotting and antisera production

Standard methods were used for western blot analyses [249]. Briefly, flies were carbon dioxide anesthetized and snap frozen in liquid nitrogen. Thoraces from 8 flies were dissected and homogenized in sample buffer (125 µl), heated at 95 °C for 5 min, loaded into the wells of an SDS-PAGE gel. Antisera was generated to fly ATP6 protein using purified HKEFKTLLGPSGHNGS peptide (hc17), immunized New Zealand rabbits and antigen affinity purification (NeoBioSci, Cambridge MA, USA). Anti-ATP6 antibody recognition specificity of hc17 peptide was confirmed by Southern Blot and ELISA (by NeoBioSci, Cambridge MA, USA). Western blotting identifies a single ~25 kDa protein that enriches with mitochondria. Competitive ELISA (kit by Cell Biolabs Inc., USA) using fly lysates and increasing concentrations of hc17 peptide was used to further validate the specificity of the anti-ATP6 antibody (Supplementary material, **Figure 23**). ATP6 antisera is used 1:2000. Anti-COXII antibodies (Proteintech, Chicago IL, USA) and anti-SOD2 antibodies (LSBio, Seattle WA, USA) were used at 1:2500 and 1:2000, respectively. Anti-ATP-alpha (a5-c antibody, Developmental Studies Hybridoma Bank, University of Iowa, USA) was used as a loading control. ATPalpha is a nuclear encoded plasma membrane protein (the catalytic subunit of the Na^+/K^+ ATPase). For HeLa cells, 1x 10^6 cells were electroporated and harvested after ~48-72 hr for western blot. GAPDH (1:3000) (Abcam, USA) was used as a loading control. Secondary detection was performed using anti-rabbit (1:4000) (Biorad, USA) and anti-mouse (1:10000) (Biorad, USA) HRP conjugated antibodies. For all Western blots sub-saturation images have been quantified. In some cases, darker exposures of the quantified images are used in the figures.

3.3.4 RNA isolation and Quantitative RT–PCR

RNA was extracted from 12 whole flies, using 250 µl Trizol (Invitrogen, San Diego, USA) and the RNeasy mini kit (Qiagen, Valencia, USA). RNA was eluted in 100 µl dH2O and quantified. 5 µg RNA was used to perform a reverse transcription reaction (Superscript RT, Invitrogen). Quantitative Real-Time PCR [Mx3000P QPCR System, Stratagene] was performed using standard techniques with normalization to *RP49* expression [250]. Only DNA-free cDNA samples were used. In a total reaction of 25 µl, 12.5 µl 2X-SYBR Green Supermix (Qiagen, Valencia, USA), 2 µl of cDNA and 400 nM each of forward and reverse primers (*ATP6*, *COXII*) were used. Fold change (FC) was determined using the equation, $FC=2^{-\Delta(\Delta Ct)}$. All QPCR experiments were performed with four biological replicates and the data were normalized to mRNA expression levels of *RP49*.

3.3.5 Isolation of mitochondria from HeLa cells

Mitochondria were isolated using standard differential centrifugation procedure. In short, 24 million cells were trypsinized and homogenized by Dounce homogenizer. Nuclear fraction was pelleted at 1000*g* for 15 min. The supernatant was then centrifuged at 10000*g* for 15min. The pellet contained the enriched mitochondria.

3.3.6 *In vitro* transcription and radiolabeling

Primers were designed to amplify *5S^{mt}* and *TLI-5S^{mt}::COXII* sequences from previously engineered *mtTRES-5S^{mt}* and *mtTRES-5S^{mt}-TLI::COXII* plasmids. *T7+5s_For* (TAATACGACTCACTATAGGGGCCAACGACCATACCACGCTGAATAC) and *5s_Rev*

(AGGCCAACAACACGCGGT GTTC) primers were used for 5S DNA amplification. For *TLI-5S^{mt}::COXII*, *T7+5s_For* and *COX2_Rev*(TCCAAAAAATCTTAATGGCACATGC

AGC) primers were used. Using Thermo Scientific TranscriptAid T7 High Yield Transcription Kit and [α-32P] adenosine 5'-triphosphate (MP Biomedicals) *in vitro* transcription was performed, as per the manufacturer's instructions. Unincorporated [α-32P] ATP was removed using NucAway™ Spin Columns (Ambion Inc. Austin, Texas). Specific activities of radiolabeled RNA products were quantified by LS6500 Multi-Purpose Scintillation Counter (Beckman Coulter) and equal amounts used in the mitochondrial import assay.

3.3.7 Mitochondrial RNA import assay

Mitochondrial RNA import assay was modified from [215,251,252]. In short, mitochondrial pellets were suspended with RNA probes in the import buffer (200 µl final volume) containing 0.25M sucrose, 2 mM KH_2PO_4, 50 mM KCl, 10 mM $MgCl_2$, 2.5 mM EDTA, 5 mM L-methionine, 1 mg/ml BSA, 5 mM ATP, 2 mM DTT, 20 mM succinate, 50 mM HEPES, [pH 7.1]. The mixture was incubated for 20-30 minutes at room temperature. Mitochondria were spun at 11000*g* for 5 min and washed once with wash buffer (0.6 M sorbitol, 20 mM Tris, [pH 8.0]). To remove RNA that was not imported in the mitochondria, the pellet was spun again and resuspended in 200 µl nuclease buffer containing 25 µg/ml of micrococcal nuclease (New England Biolabs Inc.) and incubated for 30 min at 27ºC. Mitochondria were collected, solubilized in SDS buffer at 65ºC for 5 min, RNA was purified using TRIzol® reagent (Life Technologies) and resolved by denaturing polyacrylamide urea gel (National Diagnostics). Autoradiography was performed using phosphor imager and gel was scanned using Image Quant software.

3.4 Results

3.4.1 Generation of *mtTRES* for TLI *in vivo*

The metazoan mitochondrial genome contains 37 genes that are critical for electron transport chain function. These include 13 protein-coding genes and RNAs to facilitate their expression: 22 tRNAs and 2 rRNAs (12S and 16S) [35,195]. All other proteins and RNAs functioning within the mitochondrion are imported from the cytoplasm. It has previously been described that 5S rRNAs are expressed from large nuclear gene arrays (~100-200 genes) and that *5S* rRNAs can readily be found within mitochondria from flies to humans [198,199,252-256]. Although these gene arrays encode many *5S rRNA* isoforms, we identified a single common mitochondrial *5S rRNA* variant (*5S rRNA*^{mt}) representing the majority of *5S rRNAs* within *Drosophila* mitochondria. To enable studies *in vivo*, we developed the *mtTRES* (mitochondrial Targeted RNA Expression System) vector using *5S rRNA*^{mt} as a non-coding leader sequence (NCL) and employing *RNAP III* promoter and termination elements (**Figure 5**). The RNAPIII promoter was selected due to its ability to direct transcription of rRNAs, tRNAs and other small non-coding RNAs [257].

We asked whether we might utilize this mitochondrial RNA targeting system to modulate expression of endogenous mitochondrial genes *in vivo*. To test this we generated transgenic *mtTRES* animals capable of expressing chimeric RNAs consisting of an NCL and a sequence complementary to a mitochondrial mRNA, specifically targeting the known translational start site (**Figure 5B, C**). Translational inhibition/repression has been demonstrated to be functional within the cytosol by antagonizing small ribosomal subunit docking and lowering translational efficiency but has never been demonstrated in mitochondria [258].

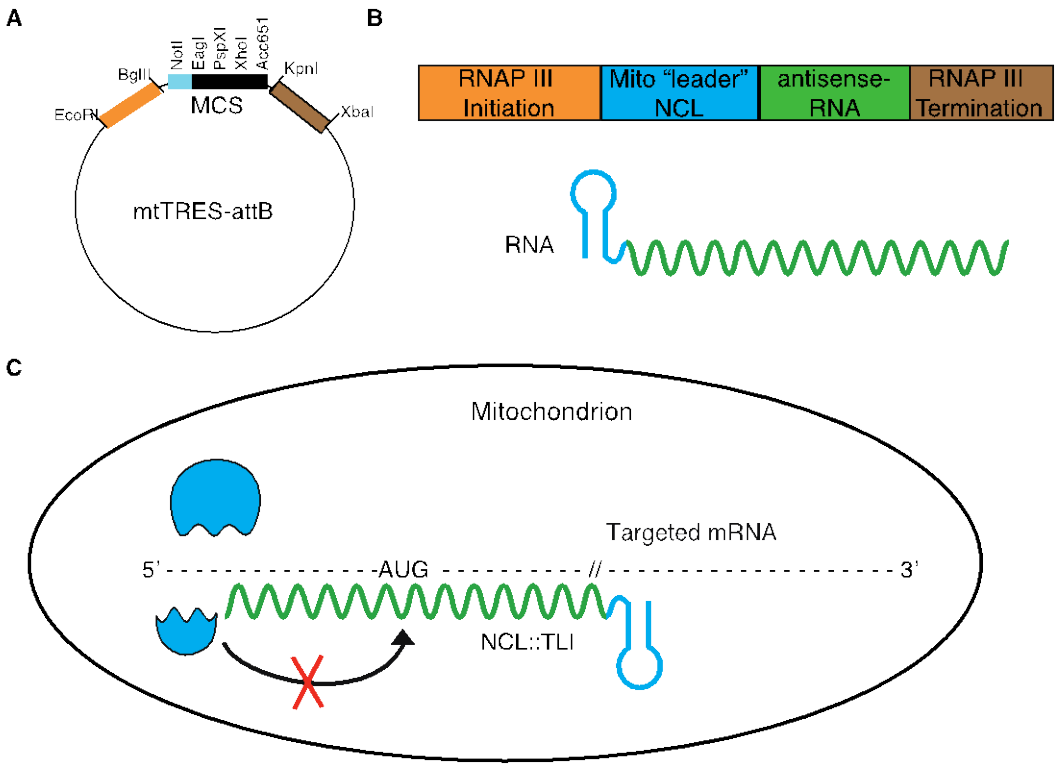

Figure 5. Design of the *mtTRES attB* transgenesis vector. (A) Restriction enzyme map of the *mtTRES-attB* vector. (B) Cartoon describing the linear 5' to 3' order of required components for allotopic RNA expression: RNAP III specific initiation and termination (orange and brown, respectively), non-coding leader sequence (NCL) RNA (blue) and the antisense RNA (TLI) (green). The subsequent RNA transcribed will be a chimeric NCL-TLI RNA. (C) Cartoon demonstrating the proposed mechanism of translational inhibition. The complementary sequence competes with the small subunit of the ribosome for binding thus inhibiting docking to the target RNA at the start codon (*AUG*).

3.4.2 *mtTRES ATP6* TLIs phenocopy *ATP6[1]* longevity and locomotor dysfunction

Numerous *ATP6* missense mutations are associated with human disease and our detailed understanding of *ATP6[1]* mutant phenotypes prompted us to initially ask whether we could functionally knock down ATP6 expression *in vivo*. We utilized *mtTRES* to generate two independent transgenic *ATP6* TLIs designated as *TLI-5S^{mt}::ATP6(a)* and *TLI-5S^{mt}::ATP6(b)*. Lifespan assays were performed to test whether *TLI-5S^{mt}::ATP6* TLIs affect the longevity of flies. We observed a significant decrease in the longevity of flies expressing *TLI-5S^{mt}::ATP6(a)* or *TLI-5S^{mt}::ATP6(b)* compared to wild type control flies (**Figure 6A**). These data demonstrate that *mtTRES* TLIs targeting *ATP6* reduce longevity consistent with a loss of ATP6 function *in vivo*.

TLI-5S^{mt}::ATP6(a) and *TLI-5S^{mt}::ATP6(b)* flies were tested for conditional locomotor function in response to sensory hyperstimulation (bang sensitivity), a progressive seizure-related phenotype resulting from loss of ATP6 function *in vivo* [110]. Young *TLI-5S^{mt}::ATP6(a)* and *TLI-5S^{mt}::ATP6(b)* animals (day 5) were aphenotypic; however, aged animals (day 50) exhibited conditional locomotor impairment compared to wild type control animals (**Figure 6B**). Strikingly, both *TLI-5S^{mt}::ATP6* transgenic strains phenocopy the conditional locomotor dysfunction observed in *ATP6[1]*, including the progressive nature of this mitochondrial seizure-related phenotype. Importantly, *ATP6[1]* is of extremely high mutant heteroplasmy (98%) and results in severe locomotor and longevity phenotypes, whereas, *TLI-5S^{mt}::ATP6* results in a ~50% knockdown and the observed phenotypes are qualitatively similar but less severe, as would be expected.

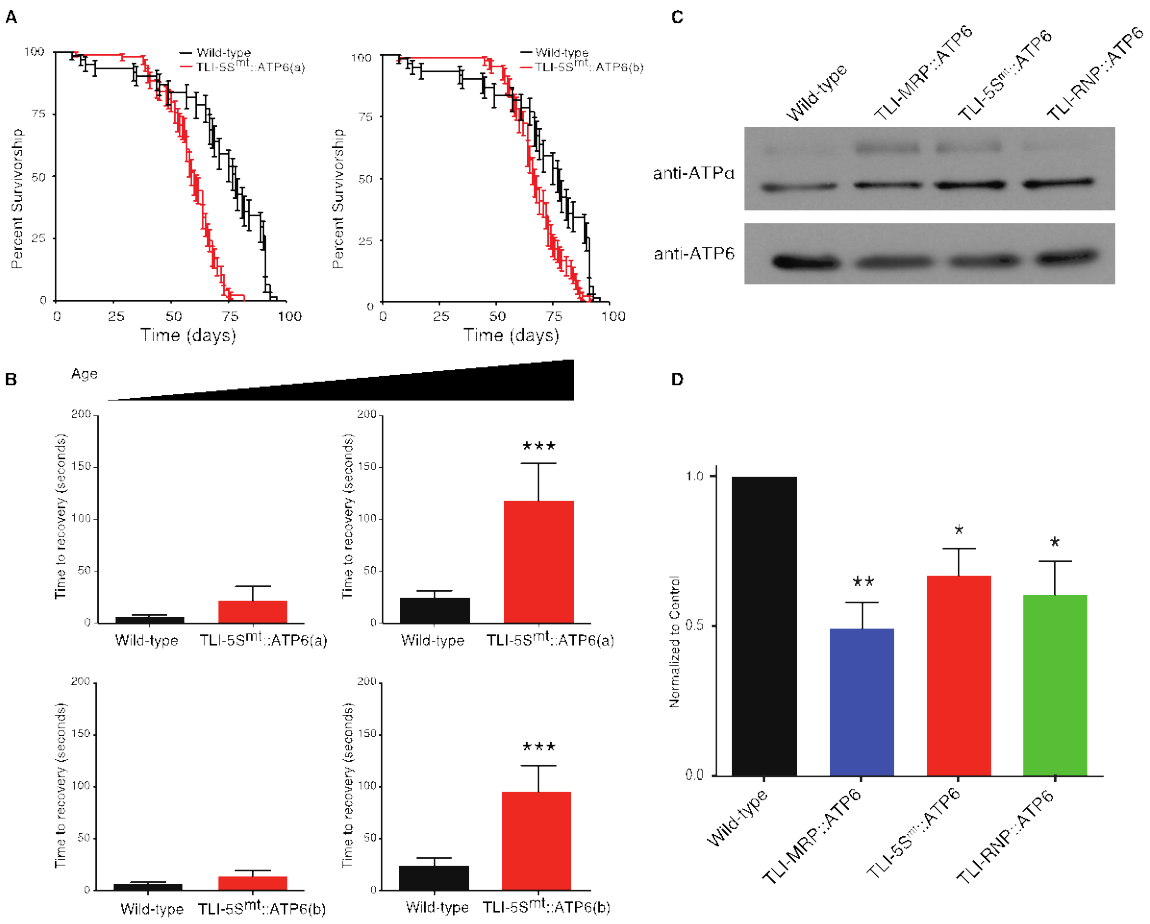

Figure 6. Translational inhibitors exhibit reduced longevity, mechanical stress sensitivity and lower steady state protein expression *in vivo.* (A) *TLI-5S^{mt}::ATP6(a)* (red) has a 24% reduction in survival as compared to the wild-type animals (black) (n=95, p<0.0001). *TLI-5S^{mt}::ATP6(b)* (red) displays a 15% reduction in survival as compared to wild-type animals (in black) (n=83, p<0.0001). (B) *TLI-5S^{mt}::ATP6(a)* animals exhibit a progressive increase in mechanical stress sensitivity (day 5 and day 50 shown). (C) *TLI-NCL::ATP6(a)* fly extracts were probed with anti-ATP6 antibody to examine steady state protein levels. The expression levels were normalized to ATPalpha, the plasma membrane Na/K ATPase catalytic subunit (upper panel). (D) Quantitation of western blots show 50% decrease in *TLI-MRP::ATP6(a)*, 34% decrease in *TLI-5S^{mt}::ATP6(a)* and 40%

decrease in *TLI-RNP::ATP6(a)*. Unpaired t-test was used as statistical test; *p<0.01, **p<0.001, ***=p< 0.0001; mean ± SEM, n=3-4.

3.4.3 Mitochondrial TLIs modulate protein levels

To more directly test the ability of *mtTRES* TLI's to modulate protein expression we performed western blotting with *TLI-5S^{mt}::ATP6(a)* transgenic fly lysates. Western blotting demonstrated a 34% reduction in ATP6 levels compared to lysates from wild type control animal (**Figure 6C, D**). These data demonstrate the *mtTRES* TLI approach is capable of endogenous mitochondrial protein modulation *in vivo*.

3.4.4 *mtTRES* TLI using distinct NCLs *in vivo*

Previously two small RNAs, *MRP* and *RNP,* were shown to be actively imported into mammalian mitochondria *in vitro* [223], suggesting their utility as NCLs. We generated two additional transgenesis vectors for *in vivo* animal studies, *mtTRES^{MRP}* and *mtTRES^{RNP}*. As an additional test of the functionality of the *mtTRES* system we generated *TLI-MRP::ATP6* and *TLI-RNP::ATP6* that express chimeric RNAs targeting *ATP6* mRNAs for TLI using *mtTRES^{MRP}* and *mtTRES^{RNP}*, respectively. ATP6 protein levels were examined in *TLI-MRP::ATP6* and *TLI-RNP::ATP6* animal extracts and were shown to be significantly reduced similar to *TLI-5S^{mt}::ATP6* (**Figure 6C, D**). Together these data demonstrate the ability to reduce steady state ATP6 protein levels using several independent constructs *in vivo*. Importantly these experiments utilize three distinct NCL sequences, including two discovered in mammals [183,193,259].

3.4.5 *mtTRES* TLIs modulate expression independent of RNA stability

mtTRES TLIs are designed to function by antagonizing translation and reducing steady state protein levels by an RNA-stability independent mechanism. To test whether these chimeric mitochondrial targeted RNAs are modulating protein levels by regulating RNA stability, we performed qRT-PCR analyses on total RNA from *TLI-5S^{mt}::ATP6(a), TLI-5S^{mt}::ATP6(b),* and wild type control animals to determine whether RNA levels of the targeted gene were altered. No changes in *ATP6* RNA levels were observed (**Figure 7A, B**). We also examined whether *TLI-5S^{mt}::ATP6(a)* or *TLI-5S^{mt}::ATP6(b)* altered the RNA levels of another mitochondrial expressed gene, *COXII*, and found no significant changes in *COXII* transcript levels with either of the TLIs (**Figure 7C, D**). Together these data are consistent with a translational inhibition/repression mechanism of action that is independent of alterations in RNA stability.

3.4.6 Mitochondrial TLIs specifically knockdown target proteins

The chimeric RNAs in the present study are predicted to knockdown mitochondrial protein expression levels by specifically inhibiting the docking of the small subunit of mitoribosomes on target mRNA akin to the cytosolic mechanism of action [258]. To test the specificity of *TLI-NCL::ATP6* chimeric RNAs, we examined COXII protein levels by western blot (**Figure 8**). *TLI-NCL::ATP6* chimeric RNAs were able to modulate ATP6 protein levels, however, COXII protein was not altered (**Figure 8A, B**). These data suggest that *TLI-NCL::ATP6* chimeric RNAs do not globally alter translation and modulate target mitochondrial gene expression specifically.

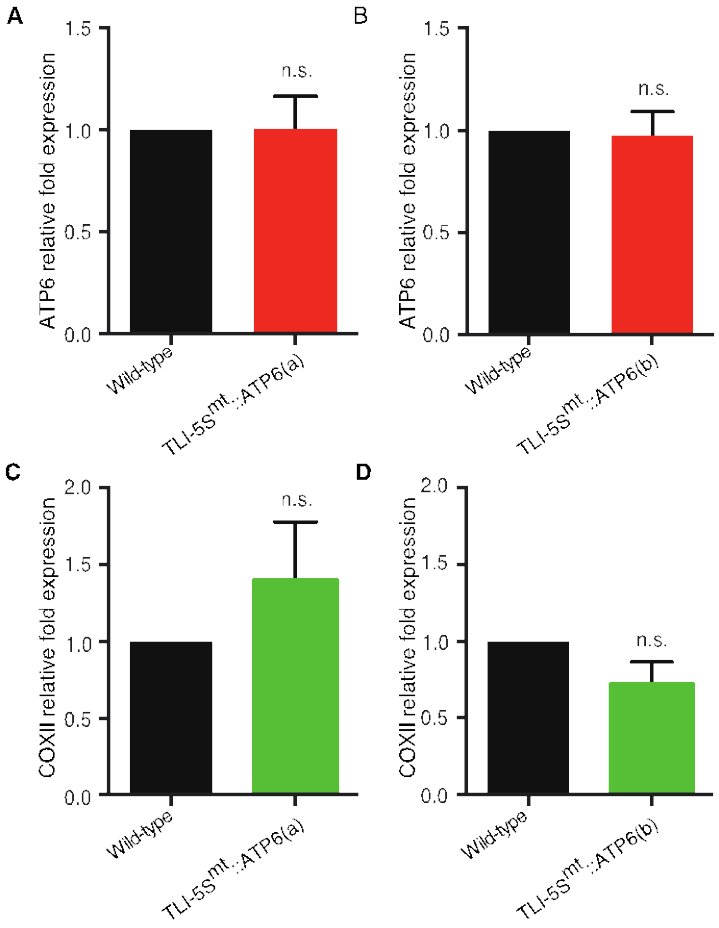

Figure 7. TLIs function by an RNA stability independent mechanism. Fold change in transcript levels determined by qRT-PCR. Fold change mRNA expression of *ATP6* (red) and *COXII* (green) is shown relative to wild type controls (black). (A and C) *TLI-5S^{mt}::ATP6(a)*. (B and D) *TLI-5S^{mt}::ATP6(b)*. All transcript levels were normalized to *RP49* expression. One-way ANOVA was performed to test significance; n.s. is p > 0.05; mean ± SEM, n=9 (3 biological and 3 technical repeats of each sample).

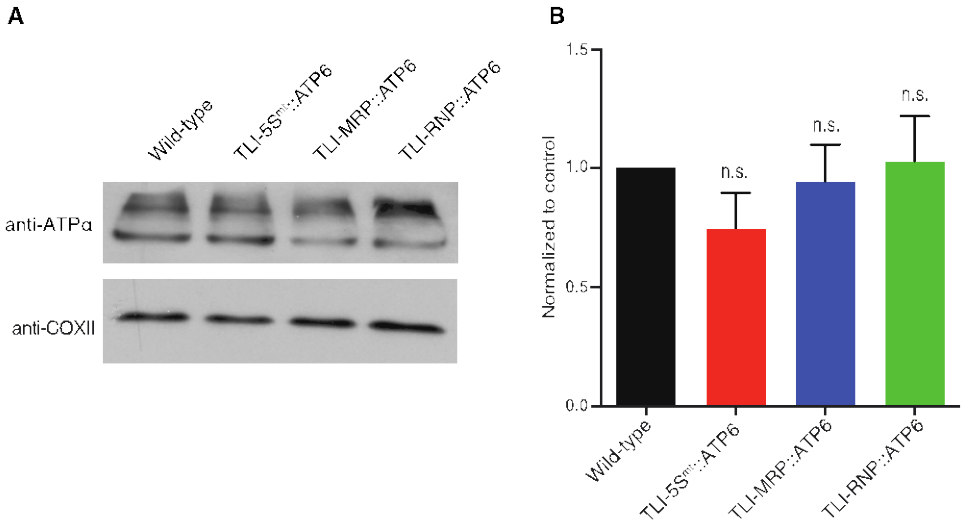

Figure 8. Translational inhibitors knockdown target protein specifically. (A) *TLI-NCL::ATP6(a)* fly extracts were probed with anti-COXII antibody to examine steady state protein levels. The expression levels were normalized to ATPα (upper panel). (B) Quantitation of western blots showing no significant change in the COXII expression levels in *NCL::ATP6(a)*. One-way ANOVA with multiple comparisons was used to test significance; n.s. is $p > 0.05$; mean ± SEM, n=3.

3.4.7 Mitochondrial TLIs *in vitro*, import assay and scrambled control

We developed a mammalian version of the *mtTRES* vector to examine efficacy of *mtTRES-TLI* constructs in human cells. We created a series of *TLI-NCL::COXII* constructs designed to target human *COXII* mRNAs. All of the *TLI-NCL::COXII* constructs significantly decreased COXII protein levels (**Figure 9**). These data demonstrate the ability of *mtTRES* TLI constructs to modulate protein levels in human cells using multiple NCL targeting signals, although the *TLI-5S^{mt}::COXII* reliably gave the most significant knockdown.

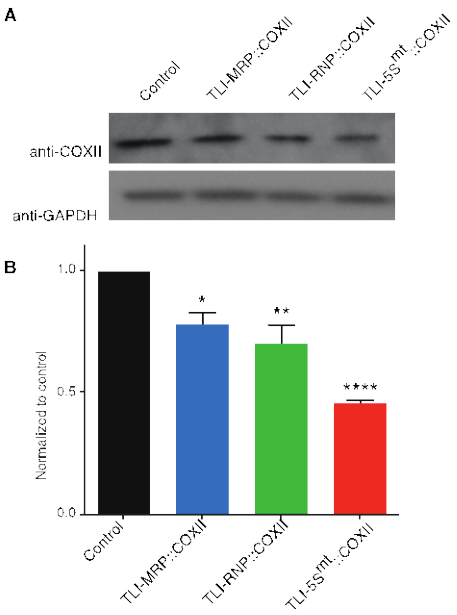

Figure 9. Translational inhibitors decrease steady state protein levels in HeLa cells. (A) HeLa cells were transfected with *mtTRES* plasmids expressing TLIs directed to human mitochondrial *COXII* RNAs. The cells were harvested at ~48-72 hrs and analyzed by western blot. (B) Quantification of steady-state COXII shown relative to the control plasmid (black) in cells transfected with mammalian *mtTRES* plasmids *TLI-MRP::COXII, TLI-RNP::COXII* and *TLI-5S^{mt}::COXII* revealed reduced expression of 22%, 30% and 55%,

respectively. GAPDH was the loading control. One-way ANOVA with multiple comparisons was performed to test significance; * is p < 0.03, ** is p < 0.0005; ** is p < 0.0001; mean ± SEM, n=3.**

For chimeric TLI RNAs to regulate expression of endogenous mitochondrial proteins via translational repression they must be efficiently imported into mitochondria. We directly examined mitochondrial import of *5S^{mt} rRNA* and chimeric *TLI-5S^{mt}::COXII* RNAs using an established import assay (**Figure 10**). RNA import was dependent on mitochondrial membrane potential as evidenced by a lack of import when mitochondria were treated with FCCP (Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) prior to adding the RNA probes. These data demonstrate robust import of the *5S^{mt} rRNA* used as an NCL and the chimeric *TLI-5S^{mt}::COXII* RNAs.

To further test the specificity of the mitochondrial TLI we generated a vector with a scrambled *COXII* complementary sequence (*TLI-5S^{mt}::scCOXII*) and repeated our analysis with this additional control. Western blot data shows that *TLI-5S^{mt}::COXII* reduced COXII protein, as it had previously, however, *TLI-5S^{mt}::sc-COXII* (scrambled) does not alter the target protein (**Figure 11**). Together these data demonstrate the utility of *mtTRES-TLI* constructs in human cells.

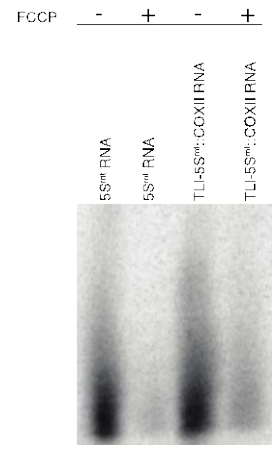

Figure 10. *In vitro* import of radiolabeled RNA into mitochondria. [α-32P] labeled *5S^{mt}* RNA and *TLI-5S^{mt}::COXII* RNA were transcribed *in vitro* and incubated with equal amounts of mitochondria isolated from HeLa cells in presence or absence of FCCP (+ or -). The pellet was treated with nuclease to digest non-imported RNAs and SDS treatment was performed to produce mitoplasts. Extracted RNAs were resolved using urea polyacrylamide gels and analyzed using Image quant (Storm 860 Molecular Imager). The experiment was repeated 3 times with a representative image shown.

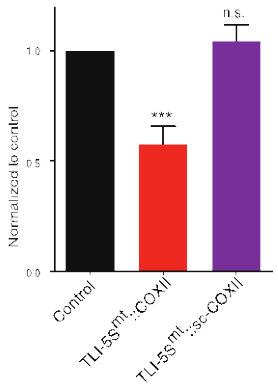

Figure 11. TLI with scrambled complementary sequence do not alter COXII protein level. ***TLI-5S^{mt}::COXII*** **reduced COXII protein expression in transfected HeLa cells, as previously.** ***TLI-5S^{mt}::scCOXII*** **with an intact 5S^{mt} NCL but a scrambled complementary sequence did not alter COXII protein levels. Quantitation of western blots showing no significant change in the expression levels of COXII in HeLa cells. One-way ANOVA with multiple comparisons was used to test significance; n.s. is p > 0.05, *** is p < 0.0004; mean ± SEM, n=3-6.**

3.5 Conclusions/discussion

Mitochondrial dysfunction has been associated with the pathogenesis of numerous significant disease conditions [32,260-263]. The study of ME has been severely hampered by a limited number of animal models, especially those affecting endogenous mitochondrial genes [239]. Researchers have developed innovative methods to alter heteroplasmy using mitochondrial-targeted restriction enzymes and reported manipulating mitochondrial DNA [264] or general effects on mitochondrial translation via RNA import strategies [166,215,219,265-267], however, the ability to directly manipulate the expression of mitochondrial-encoded proteins has remained elusive and has obvious basic and clinical applications. The identification of NCL sequences that direct RNAs to mitochondria enables a TLI approach using chimeric RNAs with a complementary element. The development of the *mtTRES* expression system enables expression of these chimeric RNAs *in vivo*. *mtTRES* utilizes RNAPIII promoter and termination elements such that NCLs and chimeric RNAs containing NCLs resemble natural substrates for RNA import. The demonstration that the *mtTRES* system is functional *in vivo* now enables the manipulation of mitochondrial genome expression, which opens up numerous avenues of investigation and is of immense value to the mitochondrial research and clinical communities.

These data demonstrate the general applicability of the approach by targeting two loci encoding proteins for which antibodies were available to verify functionality. *TLI-NCL::ATP6* RNAs achieved ~ 40-50% reduction in steady state protein levels *in vivo*. In human cells *TLI-NCL::COXII* achieved 20-50 % reduction in protein levels, dependent upon the NCL employed. Differences in reduction between the various NCLs and TLIs could be due to sequence selective effects or differences in the stability of chimeric RNA secondary structures affecting import or availability of the complementary RNA sequence for targeting.

Although these data demonstrate general applicability, there is notable constraint in the design of TLIs that has the potential to restrict its application. There are data suggesting antisense targeting for translational repression, at least in the cytosol, must be directed to the start codon to be effective, restricting construct design options. RNAP III is known to terminate within stretches of poly T, potentially restricting the use for some genes in some organisms where a poly A exists proximal to the start codon. Lastly, our data demonstrate differing levels of functionality within the NCL. Although we cannot fully explain these differences at this time, such sequence selectivity could reflect the fact that within the chimeric RNA the NCL must be recognized and the complementary sequence must still be accessible in its native structure. Certain NCL- complementary sequence combinations will form stable secondary structures that abrogate one of these functions in a manner that may not be fully predictable. Since the *5S^{mt}* NCL is larger and more highly structured with a lower delta G, it is predicted that this NCL will be more reliable but additional studies will be needed to fully test this prediction.

Mitochondrial-targeted TLIs were designed to function by antagonizing ribosome docking and lowering translational efficiency. Antisense RNAs are commonly employed to reduce expression of nuclear genes through a well-understood RNA interference mechanism that leads to target RNA degradation. We investigated this possibility by examining RNA levels of *ATP6* and *COXII* and the data demonstrate normal RNA levels *in vivo*, arguing against an RNA-destabilizing mechanism of action. To demonstrate that the 5S^{mt} RNA and *TLI-5S^{mt}::COXII* chimeric RNA are being imported into the mitochondria with similar efficiency we performed a direct *in vitro* RNA import assay. Earlier studies have suggested that RNA import is dependent on mitochondrial membrane potential. We observed that the RNA import into the mitochondria was almost negligible in presence of FCCP, which uncouples and depolarizes mitochondria.

The data presented here demonstrate the utility and general applicability of the *mtTRES* system as well as the ability to engineer TLI chimeric RNAs to modulate endogenous mitochondrial gene expression. The capability to modulate mitochondrial gene expression will enable detailed studies of mitochondrial function and organelle dysfunction *in vivo*. Efforts to develop a mitochondrial gene therapy face a formidable challenge of competing with endogenous mutant protein expression. The *mtTRES* TLI system described here has the potential to accelerate the realization of an effective gene therapy for mitochondrial diseases.

4.0 PROTEIN CODING MITOCHONDRIAL-TARGETED RNA RESCUE MITOCHONDRIAL DISEASE MODEL *IN VIVO*

4.1 Abstract

Mitochondrial encephalomyopathies (MEs) are a set of disorders resulting from mutations in genes encoded by the mtDNA that are critical for OXPHOS. Mutations affecting the 13 endogenous mitochondrial encoded protein-coding genes result in devastating and incurable ME. Currently, we do not have an effective technique to manipulate mitochondrial genome or mitochondrial protein expression, limiting gene therapy options. Here, we report the development of a system of vectors capable of expressing proteins in the mitochondria *mitochondrial-targeted RNA expression (mtTRES^{Pro})*. We have evaluated the translatability of nuclear transcribed and mitochondrial-targeted *mtTRES^{Pro}* RNAs coding EGFP *in vivo*. Additionally we tested the potential of *mtTRES^{Pro}* constructs encoding wild type ATP6 to rescue a previously characterized *ATP6[1] Drosophila* model of ME. In order to reduce expression of the endogenous mutant protein, genetic rescue is tested with or without expression of *TLI-NCL::ATP6* RNAs that function as translational inhibitors. The data demonstrate that when combined with a method to prevent competition for incorporation of the mutant subunit into the complex, *mtTRES^{Pro}*-r*ATP6* is sufficient to rescue a severe and established animal model of ME. These data demonstrate the importance of competition for the development of a gene therapy and suggest that the mitochondrial-targeted protein coding RNAs may be expressing functional proteins within mitochondria.

4.2 Introduction

Abnormal mitochondrial function has been implicated in several disease including neurodegenerative diseases, autoimmune disorders and cancer [2,32]. A number of respiratory diseases known as mitochondrial encephalomyopathies (ME) are caused by mutations in mitochondrial DNA (mtDNA). MEs are challenging to treat due to their progressive nature, variability and complexity in clinical symptoms [2]. At present, efficacious clinical treatments for these fatal disorders do not exist. Allotopic protein expression utilizing mitochondrial-targeted proteins have shown promise in recent years as potential gene therapies. However, the efficacy of this method is controversial and clinical data are not currently available [152,153]. One of the challenges that the current allotopic expression approaches face is an inherent issue of competition by endogenous mutant proteins. Mitochondrial oxidative phosphorylation complexes are assembled from numerous protein subunits encoded by mtDNA as well as the nuclear genome. Thus, allotopically expressed mitochondrial proteins will typically need to compete with mutant protein to assemble into a functional complex, a complication that has not yet been addressed.

As mentioned in the previous chapter, we devised a method to mitigate competition by modulation of mitochondrial-encoded proteins using small chimeric RNAs that function as translational inhibitors (TLI) [268]. These small chimeric RNAs have a mitochondrial targeting sequence attached to a complementary region of the target mitochondrial mRNA. This antisense or complementary region hybridizes with the target mRNA and obstructs mitochondrial translational initiation of the target mRNA knocking down protein levels. We have also shown that this works in a gene specific and RNA degradation independent manner.

We have reengineered these vectors to express mitochondrial-targeted protein coding RNAs. In the present study, we demonstrate the effectiveness of these protein-coding constructs when concomitantly expressed with *TLI-NCL::ATP6* to rescue a previously established *ATP6[1] Drosophila* ME model. This strategy of suppressing endogenous mutant protein expression supplemented with wild-type copy of the target protein has an obvious therapeutic application.

4.3 Materials and methods

4.3.1 Engineering *mtTRES^{Pro}* vector, constructs

The *mtTRES ^{Pro}* expression vector was engineered from the previously described *mtTRES* vector [268]. Sequences 5' to known mitochondrial translational start codons were aligned but no sequence similarity were observed in the 13 mt-mRNA transcripts. We therefore chose the 5' ATP6 sequence as it is a bicistronic mRNA and named them as Translational initiation elements (*TIEs*). Three independent open reading frames (ORFs) each for *ATP6*, *EGFP*, and *rATP6* were cloned as *TIE::ORF* fusion products using *EagI-KpnI* cloning sites to generate *mtTRES^{Pro}* vectors. All ORFs were optimized for mitochondrial translation. Stretches of poly-T in the ORF were recoded to prevent premature RNAPIII termination. Early cytosolic stop codons, which encode tryptophan (TGA) in mitochondria, were introduced at position 58 in *EGFP* and position 20 in *ATP6/rATP6*. ORFs were commercially synthesized by Genewiz and sequence verified after introduction into *mtTRES^{Pro}* (South Plainfield NJ, USA). To ensure that *mtTRES^{Pro}-rATP6* was resistant to translation inhibition numerous nucleotide changes were engineered. A primer dimer PCR-based strategy was used to engineer *EagI-AvrII* DNA fragment bearing these changes from synthesized oligolucleotides with complementing 3' ends. Primers were

commercially synthesized by IDT (Coralville IA, USA). It was then and utilized in a template-free PCR reaction with 15-20 cycles of amplification at a 50ºC annealing temperature. The primer dimer DNA fragment was then purified and directionally cloned into *mtTRES^{Pro}-ATP6* using a unique *AvrII* site within the *ATP6* coding region using standard techniques. Five myc tags (3 epitope repeats) were engineered at the N-terminus, C-terminus, and positions 83, 133, and 179 of ATP6 gene. These genes were commercially synthesized by Genewiz (South Plainfield NJ, USA). All constructs were sequence verified by Genewiz (South Plainfield NJ, USA).

4.3.2 Fluorescence analysis of *mtTRES^{Pro}-EGFP*

Drosophila heads were removed, probosci dissected and fixed with Carnoy's fixative, as previously described [269]. Samples were paraffin processed, sectioned (5 um) and imaged using confocal microscopy. Fluorescence quantification of the neuropil region was performed using *ImageJ* software (NIH, Bethesda MD, USA).

4.3.3 *Drosophila* transgenesis, locomotor function, and longevity assays

The PhiC31-mediated *attB* site in the *mtTRES^{Pro}* vector allowed for site-directed transgenesis. We utilized the *attP18* insertion site for the *ATP6*, *rATP6*, *EGFP*, and myc-tagged *rATP6* constructs on the first chromosome. *mtTRES-TLI::ATP6* constructs were used as previously described and utilized the *VK00027 attP* (*VK27*) insertion site on chromosome 3. Control flies contained *attP18 and/or VK27 attP* chromosomes, as needed. Standard methods were used to assay longevity and locomotor function [269].

4.3.4 Western blotting

Western blots were performed with 8 adult thoraces homogenized in 125ul of sample buffer. In cases where concentrated mitochondria were required, western blots were performed on crude preps of mitochondria from 40 adult thoraces using the previously described protocol [268]. Mitochondrial pellet was suspended in a mixture of detergent and sample buffer. All samples were heated at 95° C for 5 minutes and resolved using either a 12%, 16% or 18% SDS-PAGE. Gradient gels (4-20%) (Life Technologies, New York) were also employed in some cases. Anti-myc antibody (Developmental Studies Hybridoma Bank, University of Iowa, USA) was tested at different concentrations- 1:800, 1:1000, 1:2000, 1:4000 and fly ATP6 antisera is used at 1:2500 (NeoBioSci, Cambridge MA, USA). Anti-COXII (Proteintech, Chicago IL, USA) was used as a loading control and is used at 1:2500. Anti-SOD2 (LifeSpan BioSciences, Inc., Seattle WA, USA) was used as a loading control at 1:2500. Secondary detection was performed using anti-rabbit and anti-mouse (Biorad, USA) HRP conjugated antibodies (1:4000). Standard methods were used for the analysis of the Western blots [249,268].

4.4 Results

4.4.1 Developing *mtTRES-protein coding* vectors from the *mtTRES* backbone

We previously developed a series of *mtTRES* (mitochondrial targeted RNA expression system) vectors that transcribe small chimeric mitochondrial-targeted RNAs in mammalian cells and *in vivo* [268]. These *mtTRES* vectors express antisense RNAs that act as translational inhibitors (TLIs) specifically decreasing the expression of endogenously encoded mitochondrial proteins. Utilizing the *mtTRES* system as a backbone we designed a novel series of vectors, *mtTRES*-

protein coding (*mtTRES^{Pro}*) vectors, to express mitochondrial-targeted protein coding RNAs. Since this was aimed at translation, we included a translation initiation sequence into *mtTRES^{Pro}* utilizing the sequence 5' of ATP6 translation initiation (**Figure 12A,B**). To test the import and translatability of the RNAs encoded by *mtTRES^{Pro}* vectors *in vivo,* we generated a series *mtTRES^{Pro}-EGFP* vectors using distinct non-coding leader sequences (NCLs). These vectors target the RNAs containing the coding region of the enhanced green fluorescent protein (EGFP) to mitochondria (**Figure 12**). To ensure that EGFP does not get translated in the cytoplasm, we recoded the tryptophan at position 58 of the EGFP gene as a TGA codon. The mitochondrial translation system recognizes the UGA codon as a tryptophan whereas for the cytosolic translation machinery, UGA is a signal for termination. Therefore full-length fluorescent protein should only be efficiently translated within mitochondria and not in the cytosolic compartment. Brain slices from transgenic *mtTRES^{Pro}-NCL::EGFP* flies exhibit fluorescence which is above background, indicating significant expression of EGFP *in vivo* (**Figure 12C,D**). Two independent transgenic strains utilizing either the MRP or RNP NCL resulted in significant and similar fluorescence expression, demonstrating translatability of *mtTRES^{Pro} -NCL::EGFP* RNAs *in vivo* (**Figure 12C,D**). We additionally, engineered a series of mammalian *mtTRES^{Pro}-NCL::EGFP* vectors that utilize a well established human U6 RNAPIII polymerase promoter sequence. Transfection of HeLa cells with *mtTRES^{Pro}-NCL::EGFP* resulted in significant mitochondrial fluorescence that co-localized with Mitotracker® (**Figure 12E**).

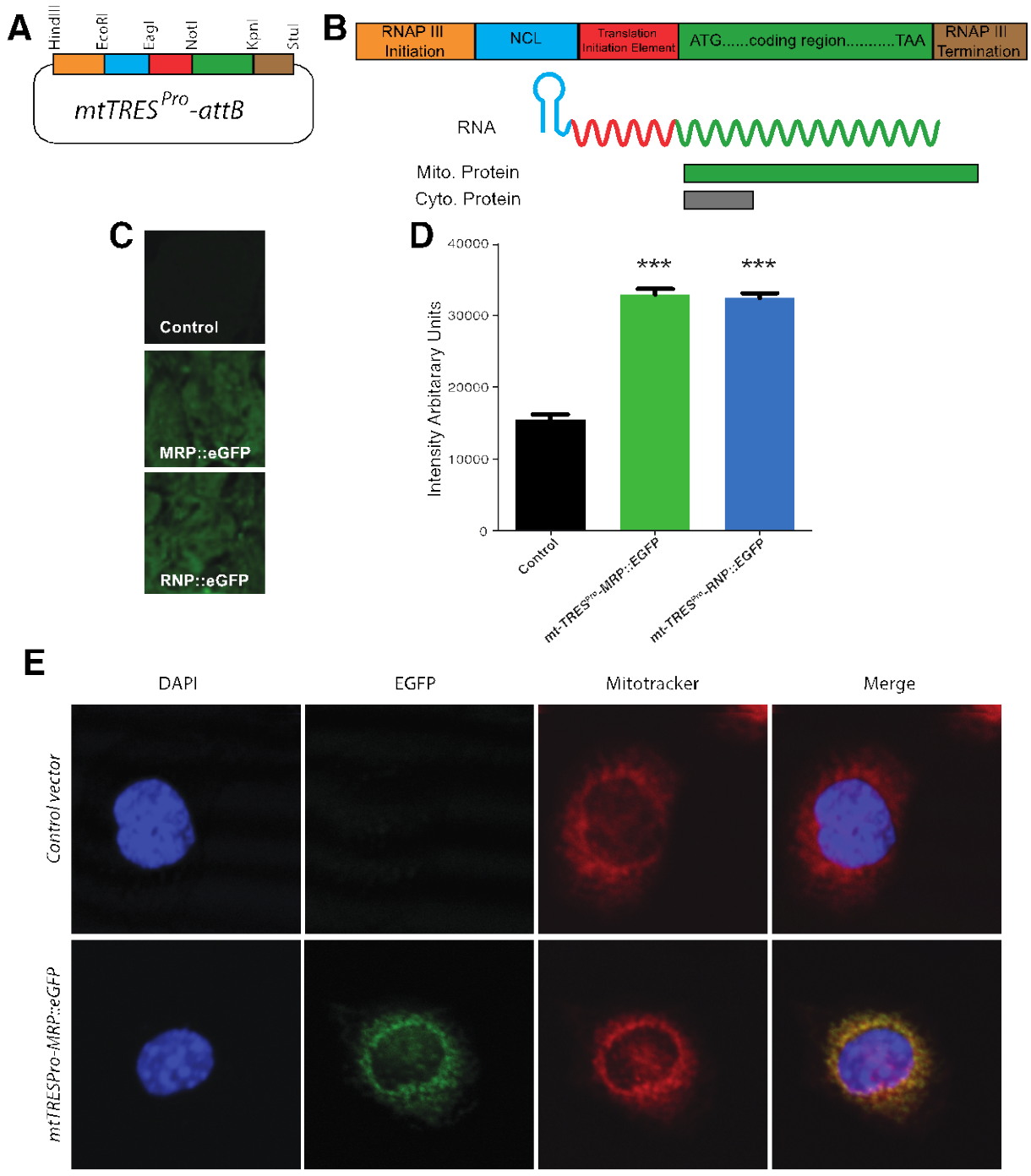

Figure 12. Translation of mitochondrial imported *mtTRES^{Pro}-EGFP* RNAs. (A & B) Design of *mtTRES^{Pro}-attB* vector showing location of RNAPIII initiation (orange), RNAPIII

termination (brown), non-coding leader sequence (NCL) (blue), translation initiation element (red) and protein coding region (green). Full-length protein will only be translated within mitochondria (green Mito. protein), whereas, cytoplasmic translation will be truncated and non-functional (grey Cyto. protein). (C) Imaging neuropil region of the brain to detect EGFP fluorescence. (D) Quantification of EGFP fluorescence intensities of brain slices (n > 85 for all genotypes). (E) HeLa cells transfected with *mtTRES^{Pro}-MRP::EGFP* plasmid and imaged by confocal microscopy (10X mag). Mitotracker® Red and DAPI were used as mitochondrial and nuclear markers respectively.

4.4.2 Is *mtTRES^{Pro}-NCL::ATP6* capable of rescuing *ATP6[1]* ?

ATP6[1] is a well-established animal model of mitochondrial encephalomyopathy with an endogenous mitochondrial loss-of-function mutation [110]. We asked whether *mtTRES^{Pro}-NCL::ATP6* could rescue the mitochondrial mutation disease in the animal model *ATP6[1]* flies by expressing wild-type *ATP6* mRNA targeted to the mitochondria. Three independent transgenic animal strains (*mtTRES^{Pro}-NCL::ATP6*) utilizing different known NCLs, *MRP, RNP* and *5S^{mt}* were tested. As previously described with the *mtTRES^{Pro}-NCL::EGFP* constructs, the ATP6 coding region was optimized for mitochondrial expression and contained a cytosolic stop codon at position 20. This was again engineered to ensure that ATP6 protein was expressed only from mitochondrial imported RNAs within the mitochondria and not in the cytosolic compartment. As an initial test of function *ATP6[1],mtTRES^{Pro}-NCL::ATP6* transgenic animals were examined for longevity, a well-characterized phenotype associated with the *ATP6[1]* mutation. All three characterized NCLs (*MRP*, *RNP* and *5S^{mt}*) were used. *ATP6[1],mtTRES^{Pro}-*

NCL::ATP6 transgenic animals did not significantly increase median age relative to *ATP6[1],attp18* control flies (**Figure 13**). Overall this experiment with the *mtTRES^{Pro}-NCL::ATP6* strains demonstrated that the constructs were unable to rescue the *ATP6[1]* longevity phenotype.

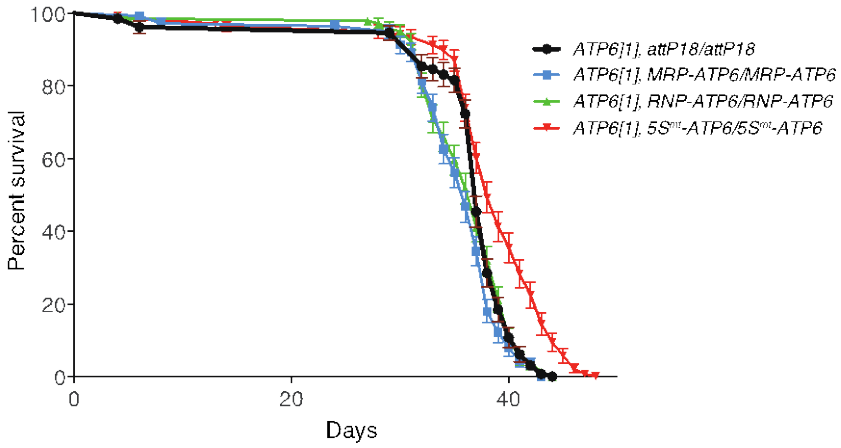

Figure 13. Allotopic RNA expression of mitochondrial–targeted ATP6 coding RNAs. Survival curves for *ATP6[1] attp18/attp18* (black), *ATP6[1];MRP-ATP6/MRP-ATP6* (blue), *ATP6[1];RNP-ATP6/RNP-ATP6* (green) and *ATP6[1];5S^{mt}-ATP6/5S^{mt}-ATP6* (red) (n > 150 for each genotype). Bars represent ±SEM.

4.4.3 Endogenous protein competition is a formidable challenge to genetic rescue

Our data with *mtTRES^{Pro}-NCL::ATP6* transgenic animals were disappointing, especially since we observed translatability of mitochondrial imported RNAs by *mtTRES^{Pro}-NCL::EGFP* RNAs

that are almost identical in size (**Figure 12**). We performed western blotting with ATP6 antisera asking a simple question about the endogenous ATP6 protein steady state levels in the mutant *ATP6[1]* flies. This experiment revealed that ATP6 protein levels were not altered in mutant *ATP6[1]* flies as compared to the wild type control animals (**Figure 14**). These data clearly demonstrate that the missense mutation in the mitochondrial encoded *ATP6* gene, *ATP6[1]*, does not significantly alter the steady state protein levels. This suggests that the mutant protein, even though non-functional and defective in dimerization is being translated and has comparable stability as wild-type protein. The mutant ATP6 protein may well be incorporated into the mature ~300kDa complex V. Thus, competition with the endogenous mutant protein for incorporation into the mature functional complex V presents an obstacle to achieving genetic rescue.

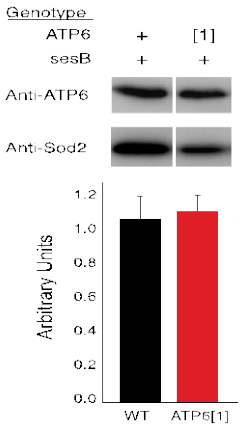

Figure 14. Competition with endogenous ATP6 protein expression. (A) Immunoblotting of fly lysates to measure ATP6 protein levels. Mitochondrial localized SOD2 protein used as a loading control. (B) ATP6 protein levels expressed relative to wild-type control flies (black) and *ATP6[1]* (red). N is greater or = to three. Bars represent ±SEM. One-way ANOVA.

4.4.4 Genetic rescue of *ATP6[1]* with allotopic RNA expression

Previously we have shown that *mtTRES* expressed chimeric RNAs are capable of decreasing protein expression *in vivo* via translational inhibition [268]. To directly test the hypothesis that our inability to rescue *ATP6[1]* flies with *mtTRES^{Pro}-NCL::ATP6* was at least in part due to the effect of mutant protein competition, we engineered a series of vectors expressing wild-type *ATP6* mRNA but resistant to TLI (*mtTRES^{Pro}-NCL::rATP6*). Silent changes were made to the target site of *NCL-TLI::ATP6* RNA complementary region to ensure the rATP6 construct is resistant to the TLI (**Figure 15**). Using three independent NCLs, we generated *mtTRES^{Pro}-NCL::rATP6* transgenic animals, co-expressed each with *TLI-RNP::ATP6* and examined their ability to rescue the *ATP6[1]* longevity phenotype. With concomitant expression of *TLI-RNP::ATP6* RNAs all *mtTRES^{Pro}-NCL::rATP6* constructs were able to significantly rescue the longevity phenotype of *ATP6[1]* control animals (**Figure 16A**). Additionally, the locomotor function was significantly rescued in these same genotypes and all combinations of *mtTRES^{Pro}-NCL::rATP6* and *TLI-RNP::ATP6* (**Figure 16B**). These data demonstrate a functional genetic rescue with three completely independent *mtTRES^{Pro}-NCL::rATP6* transgenes utilizing distinct NCL elements.

We have previously shown that TLIs with all three different NCLs are capable of knocking down ATP6 protein expression *in vivo* [268]. Therefore, we examined the efficacy of all three *mtTRES^{Pro}-NCL::rATP6* transgenes in combination with *TLI-MRP::ATP6* expression to test their ability to rescue the *ATP6[1]* longevity phenotype. Consistent with the previous results each of the *mtTRES^{Pro}-NCL::rATP6* transgenes, when expressed with *TLI-MRP::ATP6*, were able to significantly improve longevity over *ATP6[1]* controls (**Figure 17A**). We further examined locomotor phenotypes in all genotypes, and these combinations demonstrated a reduced mean recovery time. The *mtTRES^{Pro}-MRP::rATP6; MRP-TLI::ATP6* and *mtTRES^{Pro}-*

RNP::rATP6; MRP-TLI::ATP6 combinations showed significant improvement over the *ATP6[1]* control animals (**Figure 17B**). Taken together these data show that allotopic RNA expression of mitochondrial-targeted coding RNAs using *mtTRES*^{Pro} are capable of genetic rescue with concomitant expression of mitochondrial-targeted TLI RNAs.

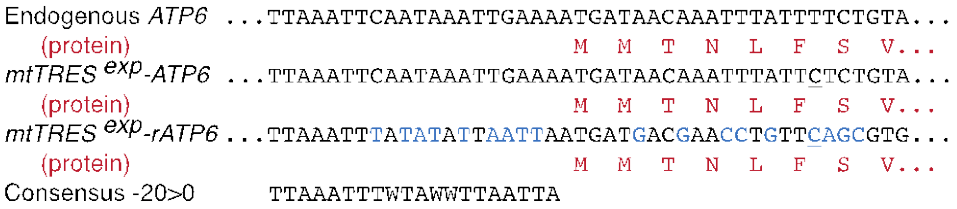

Figure 15. Mitochondrial translation initiation elements. The endogenous *ATP6* locus near the start of translation is shown with encoded protein below (red). *mtTRES*^{Pro}*-ATP6* sequence is shown. *mtTRES*^{Pro}*-rATP6* (TLI resistant) sequence is shown. Blue indicates changes from the endogenous locus designed to confer TLI resistance. Underlined is a silent change to avoid RNAPIII polymerase termination. Consensus translational initiation is from an analysis of nucleotides immediately 5 prime to the translation initiation of the 13 protein coding mitochondrial genes.

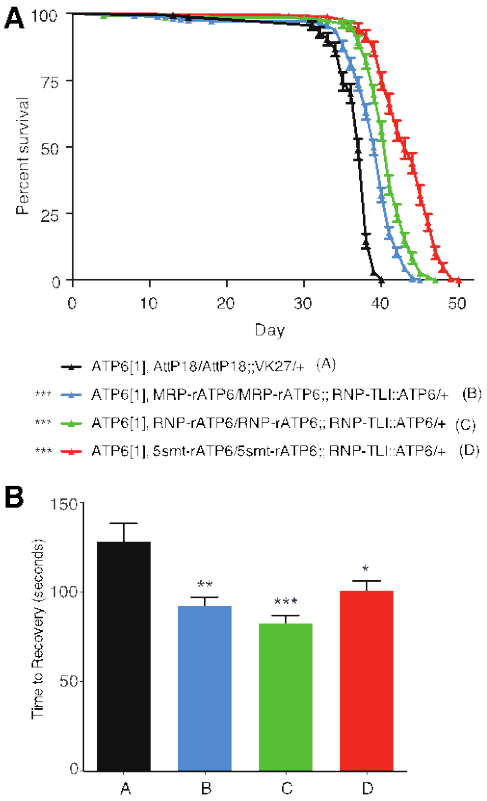

Figure 16. Allotopic RNA expression mitochondrial–targeted ATP6 coding RNAs with RNP::TLI. (A) Survival curve for flies *ATP6[1];attp18/attp18;;VK27/+* (black, A), *ATP6[1];MRP-rATP6/MRP-rATP6;;RNP-TLI::ATP6* (blue, B), *ATP6[1];RNP-rATP6/RNP-rATP6;;RNP-TLI::ATP6* (green, C) and *ATP6[1];5S^{mt}-rATP6/5S^{mt}-rATP6;;RNP-TLI::ATP6* (red, D), Log-rank (Mantel-Cox) test was done for statistical significance, bars represent ±SEM, n > 150 each genotype. (B) Mechanical stress sensitivity assay showing time to recovery for flies A, B, C and D (n>20, bars denote ±SEM, One-way ANOVA).

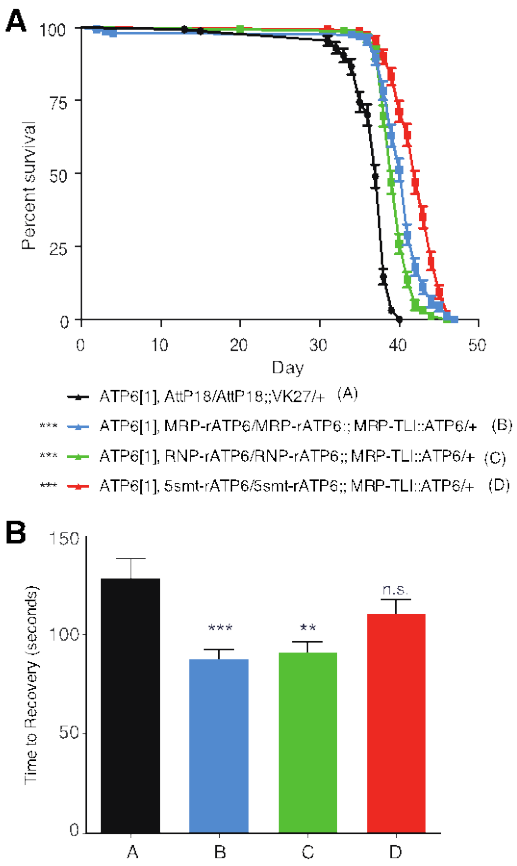

Figure 17. Allotopic RNA expression mitochondrial–targeted ATP6 coding RNAs with MRP::TLI. (A) Survival curve for flies *ATP6[1];attp18/attp18;;VK27/+* (black, A), *ATP6[1];MRP-rATP6/MRP-rATP6;;MRP-TLI::ATP6* (blue, B), *ATP6[1];RNP-rATP6/RNP-rATP6;;MRP-TLI::ATP6* (green, C) and *ATP6[1];5S^{mt}-rATP6/5S^{mt}-rATP6;;MRP-TLI::ATP6* (red, D) (Log-rank (Mantel-Cox) test was done for significance, bars represent ±SEM, n > 150 each genotype), bars represent ±SEM. (B) Mechanical stress sensitivity assay showing time to recovery for flies A, B, C and D (n>20 , bars denote ±SEM, One-way ANOVA).

4.4.5 Allotopic RNA expression constructs alter ATP6 protein expression

Only when the allotopically expressed *r*ATP6 (wild-type) constructs are co-expressed with the TLIs to specifically knock down endogenous mutant protein expression, we observe a significant genetic rescue. Hence, endogenous expression of mutant ATP6 protein is a barrier to genetic rescue. We performed western blot using ATP6 antisera as a biochemical test of our ability to genetically manipulate the ATP6 protein levels. *mtTRES^{Pro}-MRP::rATP6* constructs do not significantly change ATP6 levels *in vivo* (**Figure 18, lane 2**). However, as anticipated, flies expressing *TLI-MRP::ATP6* RNAs knock down endogenous mutant ATP6 levels (**Figure 18, lane 3**). When we simultaneously expressed *mtTRES^{Pro}-MRP::rATP6* and *TLI-MRP::ATP6* total ATP6 protein levels are significantly increased (**Figure 18, compare lanes 1 and 4**). These data demonstrate that the allotopic RNA expression constructs have the ability to genetically alter ATP6 protein expression levels *in vivo.* This further corroborates the conclusion that diminishing endogenous mutant protein expression is crucial for an efficacious genetic rescue expressing wild-type protein.

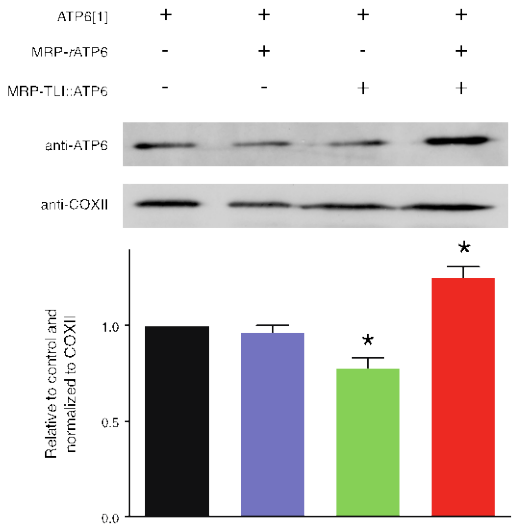

Figure 18. Allotopic RNA expression constructs alter ATP6 protein. (A) Western blot showing steady state level of ATP6 proteins in flies. *ATP6[1]* (lane 1), *ATP6[1];MRP-rATP6/MRP-rATP6* (lane 2), *ATP6[1];TLI-MRP::ATP6* (lane 3) and *ATP6[1];MRP-rATP6/MRP-rATP;TLI-MRP::ATP6* (lane 4). Mitochondrial protein COXII was used as a loading control. (B) Quantification of western blot data for ATP6 protein levels was normalized to the loading control. One-way ANOVA, n=3, ±S.E.M.

4.4.6 Can the import of allotopically-expressed *r*ATP6 biochemically demonstrated?

We identified five different locations on the ATP6 protein to engineer a myc-tag as an independent confirmation that allotopic *mtTRES^{Pro}-MRP::rATP6* derived protein is being expressed. We engineered a series of *mtTRES^{Pro} -MRP::rATP6^{MYC}* transgenic animals with 5 distinct tagged sites; two at the termini (N-term and C-term) and three internal sites (positions 83, 133 and 179) (**Figure 19A**). On performing initial tests of functionality to determine which

epitope tag locations abrogated functional rescue of *ATP6[1]* we found that *mtTRES^{Pro}-MRP::rATP6^{MYC}* with N-term myc tag completely abrogated functional rescue of *ATP6[1].* On the other hand, the C-term myc retained the ability to functionally rescue the *ATP6[1]* phenotype (**Figure 19B**). Similarly *mtTRES^{Pro}-MRP::rATP6^{MYC}* with internal tagging sites were examined. The internal myc-tagged constructs retained some functionality and partially rescued the *ATP6[1]* longevity phenotype. Myc-tagged construct with myc tag at position 179 demonstrated the best phenotypic rescue (**Figure 19C**).

We then asked whether we could detect myc-tagged rATP6 proteins by western blot using anti-myc antibodies. Unfortunately, we could not detect any myc signal at the anticipated molecular weight (~29 kDa) by western blot. Attempts to detect a double band at approximately ~29 kDa using anti-ATP6 antisera also did yield any bands at the desired location. We also tested several different myc-antibodies and used a positive control to confirm the specificity of the antibody. This yielded negative results as well. Since ATP6 is an extremely hydrophobic protein, we pursued additional experiments by optimizing western blot conditions for hydrophobic proteins. This too failed to reveal a band of interest. Taken together these data suggest the protein level even though capable of rescuing phenotypes, may not be translated at such high levels so as to be detected by conventional western blotting techniques. It may also be possible that the allotopically-expressed protein may have a very short half-life. Inefficient import and short half-life thus would not reach a sufficient enough steady state level to be detected by western blot.

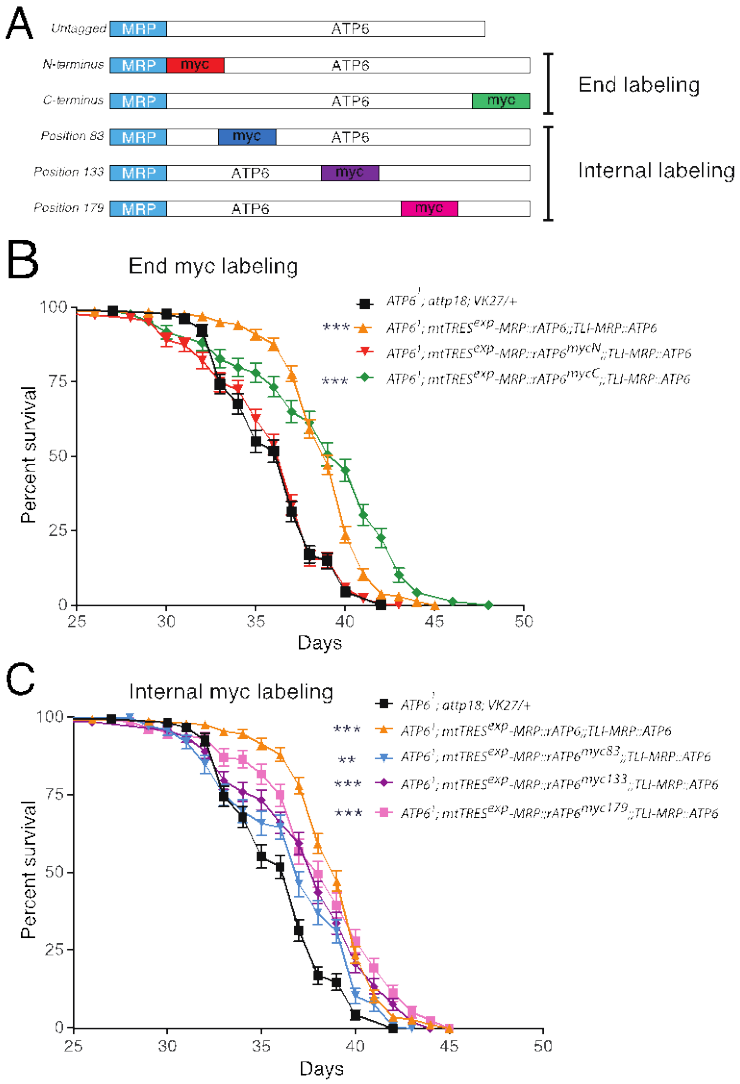

Figure 19. Myc-tagged allotopically expressed *r*ATP6 rescues mutant phenotype. (A) Illustration of myc-tagged constructs. Untagged *mtTRES^{Pro}-MRP::rATP6* (no color). N-term *mtTRES^{Pro}-MRP::rATP6^{MYC}* (red), C-term *mtTRES^{Pro}-MRP::rATP6^{MYC}* (green), 83 position- *mtTRES^{Pro}-MRP::rATP6^{MYC}* (blue), 133 position- *mtTRES^{Pro}-MRP::rATP6^{MYC}* (violet), 179 position- *mtTRES^{Pro}-MRP::rATP6^{MYC}* (magenta). (B) Survival curves for external myc-tagged flies. Untagged mutant control *ATP6[1], attp18;;VK27/+* (black),

mtTRES^{Pro}-MRP::rATP6 **control (orange), N-term *mtTRES^{Pro}-MRP::rATP6^{MYC}* (red) and C-term *mtTRES^{Pro}-MRP::rATP6^{MYC}* (green). (C) Survival curves for myc-tagged (internal) flies. Untagged mutant control *ATP6[1], attp18;;VK27*/+ (black), *mtTRES^{Pro}-MRP::rATP6* control (orange), 83 position- *mtTRES^{Pro}-MRP::rATP6^{MYC}* (blue), 133 position-*mtTRES^{Pro}-MRP::rATP6^{MYC}* (violet), 179 position- *mtTRES^{Pro}-MRP::rATP6^{MYC}* (magenta). (B and C). Log-rank (Mantel-Cox) test was done for significance, bars represent ±SEM, n > 150 each genotype.**

4.4.7 Amplification of chimeric RNA reveals polyadenylation

To further confirm that the chimeric transcript is being transcribed, total RNA was isolated from transfected HeLa cells and cDNA prepared using reverse transcriptase, using either poly-T oligos or gene specific primers for EGFP. Using nested primers and standard 35-cycle PCR amplification we found that the cDNA isolated using both the methods (poly-T or gene specific primer) yielded amplified products of the exact size of the chimeric EGFP transcript (**Figure 20**). This suggests that the chimeric EGFP is being actively transcribed in the transfected cells. Importantly, amplification of cDNA using poly-T oligos suggests that they are being polyadenylated. Since the *mtTRES^{Pro}* constructs do not contain a cytosolic polyadenylation signal, it is plausible that these chimeric transcripts get polyadenylated in the mitochondria after being imported.

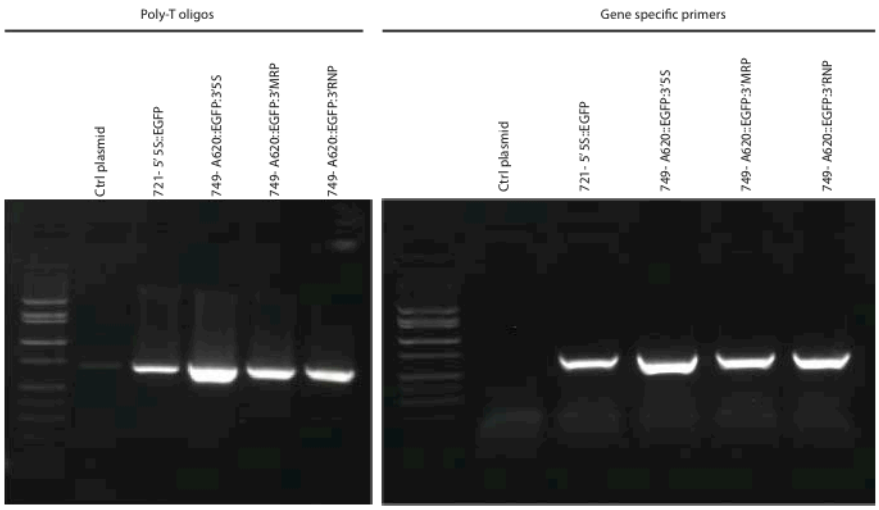

Figure 20. PCR amplification of chimeric EGFP transcript using two different methods of cDNA preparation – first, using poly-T oligos and second, using gene specific primers

4.5 Discussion

Allotopic protein expression gene therapies have been tested by a number of laboratories with different degrees of efficacy [152,153]. Noted throughout the literature are issues with competition of imported allotopic protein and endogenous protein for incorporation into OXPHOS complexes and hydrophobicity. Import of proteins with three or more transmembrane spanning regions has proved to be difficult [270]. ATP6 exemplifies both of these issues of allotopic protein expression because of its hydrophobicity (due to 5 transmembrane spanning regions) and competition through its incorporation into F_1F_o-ATP synthase (Complex V).

In order to resolve these issues, we designed constructs to import translatable RNAs using RNA allotopic strategy along with simultaneous repression of endogenous mutant proteins. How the RNA is imported into the mitochondria is not very well understood and is an active area of research. Mitochondria are known to import different types of RNAs. It could be possible that there are different mechanisms for their import in different species. A well-studied RNA import mechanism involves the PNPase dependent and TOM dependent pathways [215].

In our present report, we demonstrate that long RNAs can be imported and translated utilizing already known mitochondrial RNA targeting sequences as *NCL*s (*MRP, RNP* and *5S^{mt}*). Our *EGFP* data suggests that translation of *mtTRES^{Pro}*-coded transcript is occurring after being imported into the mitochondria. The signal is mitochondrial because we engineered an early stop codon in *EGFP* both *in vivo* and *in vitro* in mammalian cells. The controls used in these reporter assays were constructs that did not have EGFP sequence. However, a more appropriate control to test the EGFP import and mitochondrial expression would be an EGFP construct without NCL sequence. This construct will have EGFP but will not be imported into the mitochondria due to the lack of import sequence (NCL).

We were able to utilize this system to optimize tagging sites within ATP6 gene that would not disrupt the protein's functionality. The two external and three internal sites were tested. The internal sites were chosen based on the partial crystal structure of ATP6 [118]. Previously, tagging of ATP6 has been restricted to end tags (HA tag) only when ATP6 is being translated in the cytoplasm [152]. Internal tagging of ATP6 has never been reported, which also makes this a novel report. We show that the external (except N-term) and internal sites are capable of functionally rescuing the *ATP6[1]* mutant phenotype. Not only do these data reiterate that our RNA is both imported into the mitochondria and translatable, but also opens the field to a number of new optimal tagging sites for future study.

Although some modest rescue of *ATP6[1]* was observed with the rescue constructs, a more robust rescue was seen when the rATP6 was combined with TLI. Again, this points to the challenge of competition from mutant protein for incorporation into the OXPHOS complexes. This is a significant finding if allotopic gene therapies need to be considered as clinical therapeutic options in future. The mutant protein is being produced so that methods of endogenous protein repression can be employed to knockdown this competition. Future studies are needed to optimize translation of the rescue constructs as well as to optimize the TLI repression system.

5.0 DISCUSSION AND FUTURE WORK

5.1 Mitochondrial disorders and associated challenges

Mutations in mtDNA lead to primary mitochondrial disorders. Due to the complexity of mitochondrial inheritance, variability of symptoms and lack of distinct clinical biomarkers, mitochondrial disorders are difficult to diagnose and even more challenging to treat. Unfortunately, there is no known cure for mitochondrial disorders. Further, the lack of relevant mitochondrial disease model organisms have impeded the development and testing of novel therapeutic strategies for mitochondrial disorders.

Factors such as diversity of symptoms, external modulators and tissue specificity make it difficult to study pathogenesis of mitochondrial disorders. The same mitochondrial mutation in different individuals may result in diverse symptoms, many times manifesting at variable stages of life. While some symptoms appear quite early, some appear around 10-12 years of age, and others much later in life. For example, patients with MELAS or MERRF may remain symptom free until they reach their thirties [56]. This late appearance of symptoms is particularly challenging because delayed onset of symptoms around or beyond the reproductive age may result in transmission of mtDNA mutations to the next generation. Hence, individuals with a family history of mtDNA disorders need to undergo genetic screening and counseling early in life.

External modulators seem to have an impact on the manifestation of mitochondrial diseases symptoms. It is well known that the level of mutant mtDNA affects the severity of

mitochondrial disease. As illustrated by NARP and MILS (Maternally Inherited Leigh Syndrome), caused by a single pathogenic point mutation T8993G in *ATPase 6*, an individual may remain symptoms free or be affected when mutant heteroplasmy exceeds a threshold [28,30,31]. Other factors such as mitochondrial haplogroups, environmental factors and nuclear modifiers are also known to affect the severity of mitochondrial disease. Certain mutations occur at a higher prevalence in certain haplogroups such as *ND4* G11778A and *ND6* T14484C in haplogroup J. Specific symptoms associate with mutations and background haplotype as in the case of *ND1* G3460A in haplogroup K demonstrating enhanced risk of vision loss [271]. Some reports have suggested that haplotypes determine the quantity of mtDNA in a cell. Others indicate that levels of estrogen can contribute to the occurrence of mitochondrial disorders. This explains why mitochondrial disorders predominantly affect males because they have relatively lower estrogen levels as compared to females [272,273]. It is possible that mtDNA number and levels of estrogen affect the OXPHOS capacity and the rate of ATP production resulting in variable compensatory effects. However, the exact role of genetic background and external modulators remain less well understood and is an active area of research.

Tissue specificity is a hallmark of mitochondrial disorders. Tissues within the neuromuscular systems are generally more affected than other tissues in the body. One hypothesis is that very high-energy requirements of these tissues amplify the dysfunction. However, there are several unanswered questions of tissue selectivity in mitochondrial disorders. Previous studies have found preferential occurrence of mutations in specific tissues such as the brain. In an experiment using *in situ* hybridization, Tanji *et. al.,* showed that mtDNA deletion responsible for *KSS* abundantly accumulates in the choroid plexus of the brain [274]. The same group also found that 8344-*MERFF* mutation resulting in COXII depletion concentrated in olivary nucleus of the cerebellum [275]. In another report, Betts *et.al.,* found the 3243-MELAS mutation localizing in the cerebral artery [276]. These classical studies indicate preferential tissue

accumulation of mtDNA mutations. It is also interesting to note that certain tRNA mutations are associated with specific diseases. Mutations in $tRNA^{Ile}$, $tRNA^{Glu}$ and $tRNA^{Lys}$ genes are oftentimes connected with cardiomyopathy, diabetes and multiple lipomas respectively [32]. However, neither the mechanism of tissue selectivity nor their correlation with symptoms is clearly understood.

To sum up, the complexities of transmission, variations in symptoms, external modulators influencing severity of the disease such as genetic background, age and tissue type all seem to contribute a great deal in the pathogenicity of mitochondrial disease. As a result, the exact pathogenesis of mitochondrial disorders remains practically unknown. Despite such diversity, some key pathways tend to be universally affected such as ROS and ATP generation, calcium homeostasis, mPTP regulation, mitochondrial biogenesis and mitochondrial import. This opens up avenues for therapeutic strategies targeted at these common pathways.

5.2 Mitochondrial therapies and alternative strategies

In the past several years, many research groups have worked to develop therapies for mitochondrial disorders, most of them being targeted to nuclear genes. The development of mitochondrial gene therapy has been impeded by mitochondrial heteroplasmy, polyploidy of the mitochondrial genome, lack of mitochondrial transfection techniques and absence of amenable models for mitochondrial disorders. Various strategies have been suggested to rescue mitochondrial dysfunction such as mtDNA manipulation [166,265,266,277], RNA import [223], enzyme targeting [264], carrier mediated nucleic acid delivery (RIC complexes) [267] and protein targeting [152,153,157].

With the discovery of mitochondrial import components, it was an obvious scientific endeavor to target wild type proteins to rescue the defects caused due to mutation in protein coding regions of the mtDNA. Such an approach, known as allotopic expression, has had mixed outcomes both in cases of protein and RNA import strategies. Allotopic expression strategies have the potential as a therapeutic approach to overcome the challenges of mitochondrial mutations.

Protein import pathways are better understood than RNA import and its key players have been well characterized. Researchers have attempted to target wild-type proteins into the mitochondria using protein allotopic strategies. The gene of interest can be artificially coded by the nucleus, translated in the cytoplasm and then imported into the mitochondria. Since the mitochondria uses different set of codons, this approach requires recoding of the transcript to enable its cytosolic translation. In addition, these also carry a mitochondrial targeting sequence (MTS). Depending on the type of the MTS employed (discussed in Chapter 2), it acts as a “zip-code” that targets the proteins into specific mitochondrial compartments. A tag such as HA- or FLAG-tag can be used to detect whether the targeted protein localizes to mitochondria.

RNA allotopic expression strategies are an alternative approach to protein allotopic strategy. Several nuclear encoded RNAs are imported into the mitochondria such as RNase MRP, RNase P, 5*S* rRNA, tRNAs and miRNAs (discussed in Chapter 2). Several of these mitochondrial imported RNAs contain unique structural components or “stem-loop” structures that are believed to be critical for import. It is known at least in the case of PNPase-dependent import that stem loop structures in the imported RNAs are critical for their recognition and import into mitochondria. However, not all RNAs are thought to be recognized and imported by the PNPase-dependent pathway. Are there other proteins that work in an analogous manner to import RNAs into mitochondria? Can RNA be imported without any help from proteins?

Unfortunately, these pathways are largely unknown and their identification will open up avenues for allotopic gene therapy strategies.

The studies discussed earlier clearly exemplify the efforts aimed at developing gene therapies for mtDNA disorders. However, with only a few exceptions, almost all mitochondrial rescue strategies including allotopic protein expression have resulted in modest or no rescue of symptoms [152,153,270]. More importantly, none of these studies have evaluated allotopic expression approaches in an *in vivo* model of mitochondrial disease mutation. Irrespective of the techniques used, mitochondrial gene therapy faces several major obstacles in the form of overcoming hydrophobic toxicity and translocation barriers across the mitochondrial membranes, aggregation and competition from endogenous mutant proteins. Due to the presence of endogenous mutant protein, even if a wild-type copy of the mutant protein effectively reaches mitochondria, it may not get properly incorporated into a functional complex.

5.3 Development of *TLI* and its application

The first aim of my thesis was to use an *in vivo* approach to develop effective chimeric antisense approach- TLIs (translational inhibitors) that specifically knock down mitochondrial target proteins.

Several nuclear encoded factors play a role in regulating mitochondrial translation [278]. Manipulating any of the essential players could hypothetically have an impact on mitochondrial translation. In fact, research groups have used RNA*i* mechanism to knockdown mitochondrial translation elongation or initiation factors such as Initiation Factor 3 (IF3) and Elongation Factor Tu (EF-Tu) to inhibit mitochondrial translation [279]. There are a few chemicals that are commonly used to inhibit mitochondrial translation i.e., chloramphenicol, oxazolidinones (e.g., linezolid),

doxycyclin and recently reported tigecycline [279]. These drugs appear to interact with the large or small subunit of the mitochondrial ribosome. However, these methods cause a generic inhibition of the mitochondrial translational machinery blocking all 13 mitochondrial-encoded proteins. In other words, these approaches lack targeting specificity.

We developed a targeted translation inhibition approach using a novel series of *mtTRES* vectors. This vector is designed to integrate with the nuclear genome and transcribe a chimeric RNA that is targeted to the mitochondria. This chimeric RNA has a sequence, which we termed as non-coding leader (NCL) sequences consisting of a structured element. The NCL functions as a mitochondrial import determinant. We used three previously characterized NCLs (MRP, RNP and $5S^{mt}$) to direct the localization of a small non-coding antisense RNA (TLI) into the mitochondria. The TLIs are designed to hybridize to its target mRNA complementary sequence surrounding the start codon. Once hybridized, it is proposed to inhibit the docking of the small subunit of mitoribosomes and inhibit translation of that specific mRNA. Our results demonstrate that the TLIs work in a specific and sequence dependent manner. This specificity is unique as opposed to other generic translation inhibitors mentioned earlier. The TLI can be expressed concurrently with either the allotopic expression (RNA or protein) strategy or other techniques (for introducing wild-type copy of the mitochondrial gene) thus making it a widely applicable tool.

5.4 RNA allotopic expression and rescue

The second aim of my thesis was to combine TLIs against ATP6 transcript with mitochondrial targeted full length mRNA coding wild-type *ATP6*, and test them for their ability to rescue mutant *ATP6*.

To achieve this goal, we modified the *mtTRES-NCL::TLI* vector initially used to target small non-coding RNAs into mitochondria. We termed this new set of vectors as *mtTRES^{Pro}*. We modified and introduced several elements into this new vector in order to make its transcripts importable and translatable in mitochondria. We introduced open reading frames (*ORF*) after the RNAPIII promoter sequences. This *ORF* contains translational initiation elements (*TIE*) followed by the protein coding sequence. The *mtTRES^{Pro}-NCL* uses the same *RNAPIII* termination signal previously used in the *mtTRES-NCL::TLI* vectors.

During the engineering process, we realized that a reporter assay would tremendously benefit our endeavor to effectively import and translate using the *mtTRES^{Pro}-NCL.* Hence, we engineered another set of *mtTRES^{Pro}-NCL::EGFP* vectors specific for flies and mammalian cells. As an internal control, we introduced stop codons in both the mammalian and fly versions of the *mtTRES^{Pro}*. We exploited a difference in the mitochondria codon usage as compared to the cytosol. The *TGA* codon is read as a stop codon when a transcript is being translated in the cytoplasm. However, mitochondrial translation machinery recognizes *TGA* as a tryptophan and continues with the translation. Thus, we ensured that the allotopically expressed chimeric transcript is translated to a full-length protein only in mitochondria but not in the cytoplasm.

Using first generation *mtTRES^{Pro}-NCL::ATP6* constructs, we targeted longer protein-coding RNA transcripts into mitochondria in an attempt to rescue a fly mitochondrial mutant *ATP6[1].* Our initial data demonstrated lack of phenotypic rescue when assayed for longevity. This was surprising because both the transgenic flies and mammalian cells demonstrated EGFP fluorescent intensities above background as compared to the control. Although having very low intensity EGFP signal, these images suggested that the *mtTRES^{Pro}-NCL::EGFP* was transcribing a chimeric transcript that was being imported and translated in the mitochondria. However, western blot on both the transgenic EGFP fly and transfected mammalian cell

expressing using various anti-EGFP antibodies did not show band at the expected size (~27 kDa).

The choice of cell type could affect the outcome of transfection experiments. We previously used PC3 cell for reported assays. After repeated attempts of inefficient transfections, we switched to HeLa cells. HeLa cells are immortal cell line that relies primarily on glycolysis. The relative inactivity of mitochondria in HeLa cells could be one the factors for the poor efficiency observed in the reporter assay. Another factor is the efficiency of transfection. Since the reporter assay displayed EGFP expression in a few cells sporadically, it may be due to the fact that a certain copy number of the plasmid is required show EGFP fluorescence from the imported transcript but too many copies may lead to toxicity. Since there are multiple mitochondria in a single cell, it is possible that a specific number of chimeric transcripts are required to optimally translate EGFP. Too many molecules or too few molecules of the chimeric transcript may lead to toxicity or inefficient signal of the reporter. A study in 2001 by Puranam and Attardi calculated the presence of ~33-175 RNase P molecules per HeLa cell [193]. Even if there are ~20 transcriptionally active mitochondria per cell, it might end up having ~5 molecules (assuming ~100 RNase P/cell) of RNase P per mitochondrion. Is this sufficient for the fluorophore signal that we are trying to detect? This aspect of the reporter assay has presented a limitation because even though we are able to observe a select few cells with EGFP intensities higher than the background by confocal imaging, several attempts to detect it on the western blot have resulted in repeated failures.

5.5 Using allotopic RNA and *TLI* in combination

Mutations in the mtDNA (point mutations) protein-coding region can lead to a decrease in the protein level. An affected family discussed in this study demonstrated a typical variability in symptoms as observed in primary mitochondrial disorders [280]. The mutation was found in the start codon of the gene encoding COXII. As a result of "near-homoplasmic level" of the mutation, translation of COXII was significantly decreased in cells affected with the mutation. Interestingly, cells with this mutation were found to also have 2.3 fold decreased mRNA level of COXII as compared to the control.

We found that the mutant *ATP6[1]* flies expressed ATP6 protein levels at the same steady state level as the wild type flies. In a study Bokori-Brown *et.al.,* found that an allotopically expressed wild-type copy of the mitochondrial protein was not being integrated into a functional complex [158]. Could it be possible that the endogenous mutant protein that was stably expressed is competing with the allotopically expressed wild-type copy of the protein?

Based on this novel finding of the steady state level of mutant ATP6 protein, we hypothesized that we could combine the TLI against *ATP6* mRNA with concomitant expression of *mtTRES^{Pro}-NCL::ATP6*. As we demonstrated earlier, TLIs are capable of altering the levels of a specific target mitochondrial encoded protein; it would significantly reduce this endogenous competition. Therefore, we combined the strategies of specifically knocking down competition endogenous ATP6 subunit using *NCL-TLI::ATP6* and co-expressing wild-type of the *ATP6* gene via *mtTRES^{Pro}-NCL::ATP6*. This ultimately paves the way for the exogenously targeted wild-type copy of the gene to be successfully integrated into a functional complex.

To ensure that the *NCL-TLI::ATP6* would not inhibit the allotopically expressed wild-type copy of *ATP6* mRNA we recoded the wild-type *ATP6* mRNA sequence and changed 11 nucleotides to make it resistant to the *NCL-TLI::ATP6.* Interestingly, the initial sequence of endogenous *ATP6* also harbors a "tetra-T" sequence that is a signal for *RNAPIII* termination. To eliminate the risk of early termination, we replaced this last 'T' keeping the amino acid conserved as coded by the endogenous codon. The vector coding this new *TLI* resistant *ATP6* transcript was now termed as *mtTRES^{Pro}-NCL::rATP6,* where 'r' stands for resistant to *TLI.*

Transgenic flies created using these novel constructs simultaneously expressing *NCL-TLI::ATP6* and wild-type *rATP6* were evaluated for phenotypic rescue. Our results suggest that this strategy was able to partially but significantly rescue mutant ATP6 fly phenotypes. Both longevity and mechanical stress sensitivity demonstrated modest improvements as compared to the *ATP6[1]* control flies suggesting that the allotopic RNA expression strategy is working although inefficiently. Another interesting control could involve expressing mutant *ATP6[1]* chimeric RNA in mutant *ATP6[1]* flies. Mutant *ATP6[1]* chimeric RNA should not affect the phenotype of *ATP6[1]* flies.

Interestingly, the steady state level of ATP6 protein was found to be elevated in flies expressing both the *NCL-TLI::ATP6* and wild-type *rATP6* transgenes. To further quantitate this increase of ATP6 level, we needed to differentiate the allotopically expressed rATP6 protein from the endogenously mitochondrial expressed ATP6 subunit. Since the customized anti-ATP6 antisera that we use for western blots is unable to differentiate between mutant and wild-type ATP6 we engineered another set of constructs with *mtTRES^{Pro}-NCL::rATP6-myc* tag sequences.

Incomplete knowledge of ATP6 structure and lack of previous tagging information for ATP6 protein made it challenging to identify suitable tagging sites. We therefore, chose five sites based on the partial crystal structure of ATP6 subunit and its five predicted transmembrane (TM) domains. The sites were selected such that the triple myc-tags lay between the two adjacent TM domains or at the termini. However, this hypothesis was entirely based on a theoretical assumption that these tags would not interfere with protein assembly or function. When the chimeric *rATP6::myc* transcript is translated, it is possible that the tags obstruct the natural folding and integration of the ATP6 protein into the IMM. If this happens, it will stimulate a faster degradation and removal of the translated product due to mitochondrial unfolded protein response (UPR^{mt}). Recent studies of the UPR^{mt} suggest that normal functioning of mitochondria is monitored in terms of mitochondrial protein import efficiency [281]. The cell then responds to the abnormal mitochondrial function by transcriptionally regulating UPR^{mt}. It is possible that as a consequence of aggregation of unfolded proteins in the mitochondrial matrix, the mitochondrial protein-synthesis rates are adapted and mitochondrial autophagy gets activated to eliminate the defective mitochondrion.

All *mtTRES^{Pro}-NCL::rATP6-myc* constructs should express a protein of the expected ~ 29 kDa size. Contrary to our expectations, repeated attempts to detect the myc-tagged *r*ATP6 protein failed. There could be several reasons why the myc-tagged ATP6 protein was unable to be detected by western blot. Western blot is a technique that has a sensitivity of detection at the lowest range of 10 pg. For the *r*ATP6-myc (~29 kDa) protein, this translates to ~ 0.000344 pmole or roughly 180,000,000 molecules. It appears that the *NCL-mtTRES^{Pro}::rATP6-myc* constructs are capable of delivering the chimeric and translatable RNA coding sequences sufficient for a phenotypic rescue but not enough to be detected by western blotting. In a report in 2002, Manfredi *et.al.,* suggested that only a small number of “corrected functional complexes” would be sufficient to rescue a mutant mitochondrial phenotype [156].

Even if using crude mitochondrial preps could possibly concentrate such high numbers (~180,000,000 molecules) of our target protein molecules for western blot detection, there could be several other factors that might affect the detection of *r*ATP6-myc. Overwhelming the RNA import pathway resulting in limited import, lack of proper ribosomal docking and translation, the stability of the chimeric RNA and its half-life are just a few of these factors. Another issue of tagging hydrophobic protein, such as ATP6, is that the tags could remain hidden within the hydrophobic pockets making it inaccessible to conventional detection techniques. A counter argument is that a high-resolution technique such as mass spec might be able to detect the signal at such low levels. We attempted mass spec analysis using our fly *mtTRES^{Pro}-NCL::rATP6-myc* constructs. Preliminary data indicated two out of four predicted post-trypsinized peptide ion fragments. However, further attempts to concentrate the sample by using mitochondrial preps proved futile. At such extreme level of sensitivity the signal to noise ratio loses resolution to conclusively prove whether the protein is present in the sample.

The allotopic expression strategies used in our project appear to have worked but with poor efficiency. We established a modest rescue in *ATP6[1]* mutant flies when WT-rATP6 and NCL-*TLI::ATP6* were concurrently expressed. Our data demonstrate the importance of mutant protein competition in the development of genetic rescue and gene therapy approaches for mitochondrial disease resulting from a protein coding missense mutations. It is pertinent to note that the *ATP6[1]* model used in this study has 98% heteroplasmy level. This is by far the highest level of mutant heteroplasmy reported in any model organism for mitochondrial disorder. Partial recovery of longevity and a behavioral phenotype may be because RNA allotopic expression only modestly changes the ratio of wild-type to mutant proteins. Because the mutant will still express a significant amount of mutant protein in presence of allotopically expressed WT-*r*ATP6 and *NCL-TLI::ATP6*, this may contribute to a modest rescue effect. In actual disease

phenotypes such as NARP where the symptoms begin to appear above a threshold of ~60-70% heteroplasmy, a modest change in the ratio of mutant to wild-type protein has the potential for a significant rescue.

5.6 Mimicking endogenous mt-mRNA to improve import and translational efficiency

The canonical translation machinery requires several initiation factors, ribosomal binding site (such as Shine-Delgarno sequence in prokaryotes or IRES), modification of mRNA (such as 5' capping in eukaryotes) and long 3' UTRs. In contrast, only three out of eleven mitochondrial cistrons have 5′ UTRs. These three UTRs are extremely short and may only be a few nucleotides in length. The other eight mitochondrial mRNAs are leaderless and do not have a 5′ UTR [282]. This means that the mitochondrial ribosomes are capable of recognizing, initiating and translating these "compact no frills" mt-mRNAs. How does this process occur? In the absence of *in vitro* mitochondrial translation this process is largely unknown.

Although mt-mRNAs either lack or have extremely short 5'UTRs, it is believed that they form structured 5' ends and have their start codon embedded in one of these stem loop structures [283]. It is further hypothesized that mitochondrial ribosomes may have the capability of initiating translation from elements called "IRES" for internal ribosome entry sites. Cytosolic IRES are sequences of RNA that fold into special secondary structures that maybe recognized by the initiation factors required for translation initiation and something similar could happen in mitochondria. The initiation factors may help 'melt' the secondary structure making it accessible to mitoribosomes for initiation of translation [283].

Inspired by the divergent mt-mRNA features, we identified and proposed several modifications that could increase efficiency of mitochondrial translation. We therefore, focused

our efforts on creating second generation of *mtTRES^{Pro}-NCL::EGFP* vectors. These second generation vectors were designed to simulate endogenous mt-mRNA that would result in more efficient mitochondrial translation. Initially, we will validate the efficacy of *mtTRES^{Pro}-NCL::EGFP* constructs in cell culture systems. Hence, we decided to use HeLa cells to validate these second generation *mtTRES^{Pro}-NCL::EGFP* vectors.

As reported in earlier allotopic protein expression strategies, the targeted genes need to be recoded. The phenomenon of using specific tRNAs at a higher frequency relative to other tRNAs for the same amino acid is known as "codon bias" [284]. It is well known that certain codons are preferentially recognized in the cytoplasm by the tRNAs at a higher frequency than others. The actual reason for codon-bias is not well understood but it may have a translational advantage.

Analogous to this cytoplasmic phenomenon of codon bias, is there a preference for tRNA usage in proteins coded in the mitochondrial matrix? In other words, is there codon bias in mitochondrial proteins? We, and others, have calculated the percentage usage of ATG versus AGA for internal methionine in every human and flies mtDNA encoded protein (**Figure 21, Table 2**). The results were exciting. On further analysis of the rest of the codon usage by all mitochondrial proteins, it is evident that they have a codon bias as well. This led us to hypothesize that designing our engineered allotopic mRNA could be optimized for mitochondrial translation. Hence, we replaced five internal methionine in EGFP coded by ATG with the more frequently used ATA sequence to optimize it for mitochondrial translation. It remains to be tested whether codon optimization worked better than the previous non-optimized sequence.

As mentioned earlier, we exploited the non-canonical codon usage by mitochondrial translation machinery to ensure that the translation of the allotopically expressed chimeric RNA

does not occur in the cytoplasm. There are three stop codons in the canonical genetic code i.e., TAG *amber*, TGA *opal*, and TAA *ochre* codons. Since the earliest discovery of translation and the genetic code, there have been several reports of variations in the use of the universal genetic code [285]. In addition to commonly observed reassignments of stop codons in virus (bacteriophages) and bacteria, mitochondria use *opal* (TGA) to code for tryptophan instead of a stop codon. Variation from the standard genetic code in viruses provides a distinct advantage when the virus manipulates and takes over the host to fuel its own packaging and then lysis from the host [285]. Why do mitochondria have to use a non-canonical genetic code when it relies heavily on the "host" nucleo-cytoplasmic content? We know that there is abundant cross talk and retrograde signaling between the mitochondria and the nucleus [286]. Does this reassigned codon usage confer a regulatory role of mitochondria towards its host nucleus? A general search of the literature does not provide a definitive clue to this question. Since mitochondria evolved as an endosymbiont the presence of reassigned codons could serve an ulterior regulatory role or might just be a remnant of its prokaryotic evolutionary lineage.

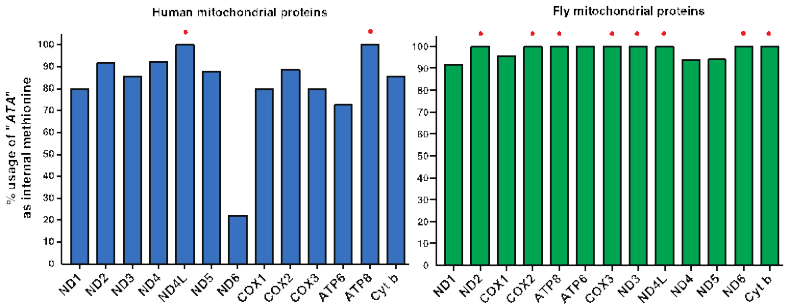

Figure 21. *ATA* codon usage for internal methionine in human and fly mitochondrial protein

Nevertheless, the usage of TGA to code for tryptophan in the mitochondria is intended to ensure that this recoded transcript does not yield a full-length protein in the cytoplasm. However, this strategy has a caveat. There is evidence that “stop codon readthrough” occurs at TGA when immediately followed by CTAG [287]. Although our constructs do not contain a CTAG sequence immediately succeeding TGA, “stop codon readthrough” might be a possibility in our constructs. There are 283 readthrough events predicted in *Drosophila* out of which 6 have been experimentally confirmed [288,289]. These may possibly be higher in mammalian cells and could lead to translation and aggregation and/or toxicity of the mitochondrial-targeted rATP6 or EGFP in the cytosol. Although this does not explain why we don’t observe cytoplasmic EGFP signal, it may explain the poor efficiency of rescue with the fly rATP6 constructs.

It is difficult to design allotopic expression chimeras due to the absence of complete crystal structures of ATP6 protein. The ATP8 and ATP6 subunits are predicted to have one and five transmembrane domains respectively [290]. This makes it impossible to predict the orientation of these proteins in terms of their N-and C-termini. We currently do not have any data to confirm whether the fusion protein that we engineered to be translated in the mitochondria ends up in the matrix, IMM or IMS. EGFP is sensitive to pH. Attaching C-term EGFP to ATP8 or ATP6 could result in EGFP in any of the three compartments i.e., matrix, IMM or IMS.

In a review in 1996, Stuart and Neupert highlighted that some yeast mitochondrial IMM proteins such as COX2 and ATPase Su9 insert and orient themselves in the IMM such that both their N- and C-terminal lie in the IMS [291]. In yeast, COX2 and ATPase Su9 is coded both by the mitochondrial and nuclear genomes. Generally, the IMM localizing proteins, either coded by mtDNA or nDNA, have sequence determinants known as ‘topogenic signals’ that determine their topological arrangements in the IMM [291]. These ‘topogenic signals’ are usually a hydrophobic sequence flanked by hydrophilic, charged amino acids. Some of these signals of

nuclear encoded proteins are known, but the 'topogenic signals' of mitochondrial-encoded proteins remain uncharacterized [291]. Based on the partial crystal structure of ATP6, it appears that the C-terminal domain faces the matrix and so should be the C-term fused EGFP. In case of ATP8, we do not have sufficient evidence of its N-term or C-term location. However, since ATP8 has a hydrophobic N-term domain, it is possible that it gets embedded in the IMM first and remains as such. This will keep the EGFP in the matrix but tethered to the IMM. Unless we have conclusive data, we can only predict it to be in the matrix.

We have engineered novel *mtTRES^{Pro}-NCL::EGFP* vectors with either just ATP6::EGFP or both ATP8/ATP6::EGFP sequences to create fusion proteins that will be translated in the mitochondrial matrix once the chimeric RNA is imported in the matrix. These novel designs were based on the fact that mtDNA encoded proteins are co-translationally inserted into the IMM. Our hypothesis is that attachingATP6 or ATP8/ATP6 sequences to the imported transcript will enable co-translational incorporation of EGFP into the IMM. The cryptic translation initiation sequences within ATP6 or ATP8/ATP6 sequences will enable a productive recognition and landing of mitoribosomes on the chimeric transcript. The fusion protein will then remain tethered to the IMM. This will reduce the risk of EGFP being degraded by the UPR^{mt} as mentioned earlier.

Although it remains to be verified how these second generation of vectors will perform in terms of import and translatability, these novel mt-mRNA mimicking strategies have the potential to be developed into more efficient RNA allotopic expressing chimeras.

5.7 Future work and scope of mitochondrial gene therapy

The complications discussed here need to be addressed in the future, but it is important to note that inefficient expression and cytotoxicity are not only associated with our vectors but also with the entire field of allotopic expression.

My project represents significant progress toward solving these problems. The first aim of the project helped alleviate one of the most important limitations in the field of mitochondrial genetic rescue, namely, competition by endogenous mutant protein expression. The second aim of genetic rescue by allotopic RNA expression has been more challenging but shows potential. My project has laid the groundwork for further improvement of these vectors targeting full-length protein coding RNAs, which will require further understanding of the molecular frameworks of mitochondrial RNA import, RNA processing and mitochondrial translation. These three components, in addition to a precise design of allotopically expressed chimeric RNA conducive to mitochondrial import and translation, are key to achieving effective and robust allotopic RNA rescue.

Despite all attempts to engineer a more favorable 'mitochondrial mRNA like' chimeric constructs, we face a severe limitation of testing actual mitochondrial translation due to the lack of a reliable *in vivo* mitochondrial translation assay. To circumvent this huge technical gap, we could use a different approach and evaluate whether the chimeric transcript is actually being translated by mitochondrial ribosomes. In a recent report, Rooijers *et.al.,* significantly improved the protocol for ribosomal profiling of 'mitochondrial ribosome protected fragments (RPF) [292]. Using similar strategy of identifying and isolating mitochondrial monosomes and their associated RPF, we could isolate mitochondrial ribosome associated RPFs and analyze by deep sequencing. In the absence of a mitochondrial *in vitro* translation system, this approach can be

instrumental in determining whether the chimeric transcripts associate and get translated by the mitoribosomes.

It has previously been shown that PNPases play a role in mitochondrial RNA import. One possibility to enhance the allotopic RNA import efficiency is to over-express PNPases. However, fly PNPases have not been characterized as well as the human homologues. In mammalian cells, over expression has been shown to enhance ROS production via increased respiratory chain activity. Additionally, PNPase overexpression also leads to activation of NFkB and related proinflammatory cytokines such as IL-6, IL-8, RANTES and MMP-3 [293]. It is not clear whether over expression of PNPase will upregulate RNA import into the mitochondria. It is possible that this might have an overall negative impact resulting in increased ROS production and activation of inflammatory signals. Thus, this approach requires further experimental validation.

Through the years of my PhD, I realized the importance of understanding the mechanism of the RNA import pathway. Since my project was directed towards developing tools, I had the opportunity to investigate molecular aspects of RNA import and processing. I examined whether the chimeric transcript is being transcribed by a simple PCR amplification of the target gene. Using two different strategies of isolating mRNA and preparing cDNA that is by using gene specific or poly-T oligos, I isolated the chimeric transcripts from the transfected cells. These data suggest that after transcription in the nucleus, the transcript traverses to the cytoplasm and then possibly to the mitochondria as it gets polyadenylated. Currently, we do not know how many “A”s are added to this chimeric transcript. Using “Topo-TA” cloning strategy followed by sequencing will determine the level of polyadenylation in these chimeric transcripts.

As mentioned earlier, there are several pathways to import RNA into the mitochondria. It appears that the type of RNA to be imported determines the pathway that will handle its delivery to the mitochondria. Based on most of the RNA import mechanism reports, interaction of RNA with the proteins is an essential step. Understanding the interactions of the target RNA with proteins in the cell might be crucial in determining the partners that interact with these RNA. RNA immunoprecipitation (RIP) is a powerful tool to study RNA-protein interactions [294]. Some of the interactions may be direct and others could be indirect. Using RIP *in vivo*, it is possible to not only identify the direct binding partners but also indirect protein associations to better elucidate RNA import pathways. Several other questions still remains to be answered such as whether the RNA import mechanism involves a dedicated pathway for specific RNA type, whether the importability is determined by a structure or is reliant on the sequence or both.

Mitochondrial gene therapy is a highly dynamic field as more laboratories around the globe are focusing on therapeutic strategies to treat mitochondrial disorders. Alternative strategies such as allotopic strategies have been used to target proteins and RNA into the mitochondria. Mitochondrial gene therapy, however, is far from reality. My thesis focused on manipulating endogenous mitochondrial proteins and expressing wild type of the mutant mitochondrial protein by exploiting the natural RNA import mechanism. Although inefficient in its present stage, this strategy has a strong potential to be developed into a practical gene therapy.

APPENDIX A

A.1 SUPPLEMENTARY MATERIAL

A.2 "SMALL MITOCHONDRIAL-TARGETED RNAS MODULATE ENDOGENOUS MITOCHONDRIAL PROTEIN EXPRESSION *IN VIVO*" BY ATIF TOWHEED *ET. AL.*, *2014*

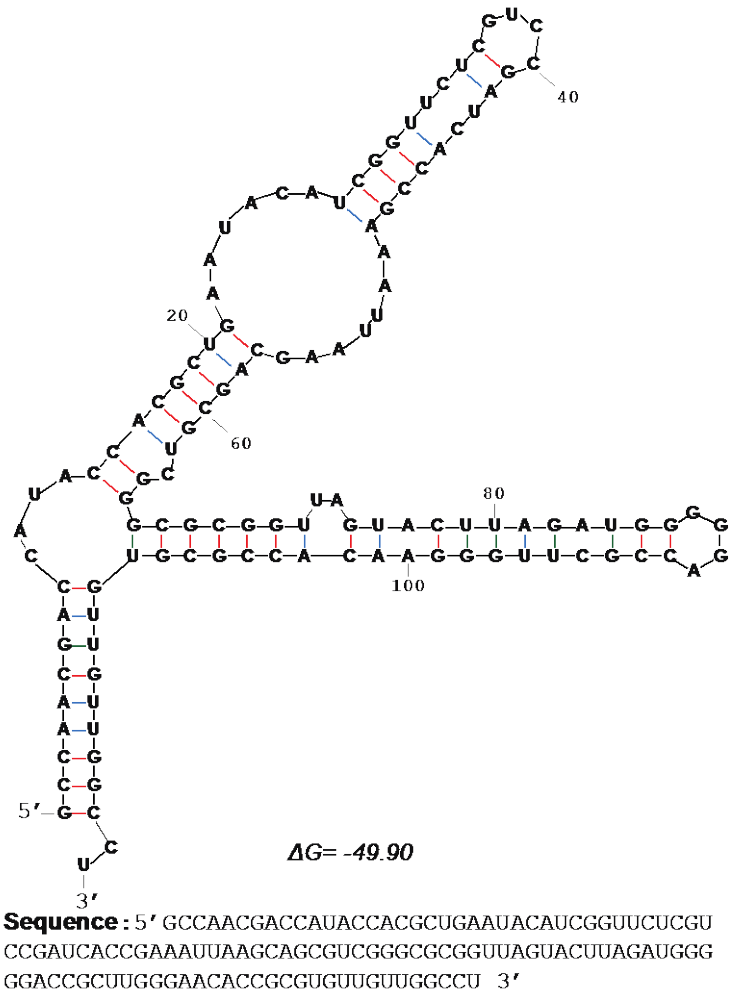

Figure 22. Predicted secondary structure of *5S^{mt} RNA*. Mfold (http://mfold.rna.albany.edu) was used to generate the secondary structure of the *5S^{mt} RNA* used in the present study. Sequence of *5S^{mt} RNA* (120 nucleotides) is shown at the bottom.

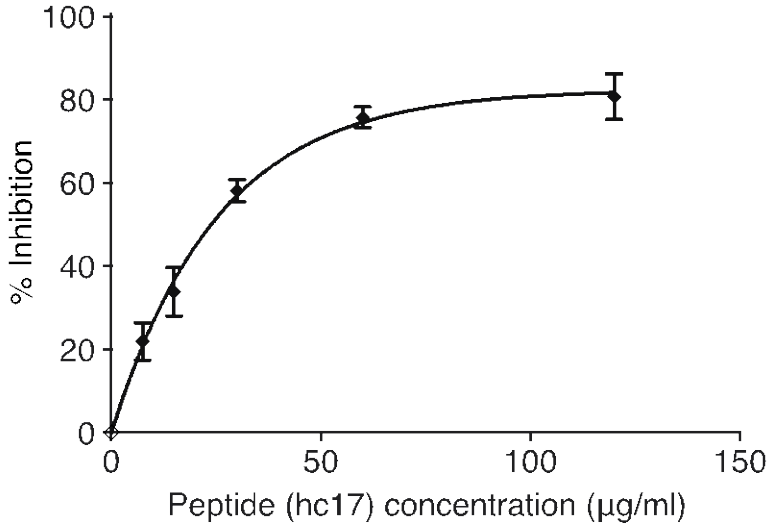

Figure 23. Competitive ELISA using hc17 peptide. Fly lysate (15 flies) was diluted to 1:50000 in 1X PBS (with protease inhibitor cocktail, Roche) and coated on ELISA plate wells overnight at 4ºC. Anti-ATP6 antibody (1:1000) and increasing concentrations of hc17 peptide (7.5, 15, 30, 60 and 120 µg/ml) was added and incubated overnight, washed extensively, anti-rabbit HRP conjugated secondary antibody (1:2000) was added and incubated for 2 hr at room temperature. Absorbance was quantified using ELISA plate reader at 450 nm after adding substrate and stop solution. The O.D. values are were plotted as a function of hc-17 peptide concentration and one phase decay curve fit applied using Prism (ver. 6) to extrapolate 0 µg/ml O.D. value. The O.D. values were then normalized to 0 µg/ml and plotted as percent inhibition (mean O.D., ± SEM, n >3).

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