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Quality Control of Mitochondrial Gene Expression

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Doctoral thesis

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Mitochondria are organelles found in all eukaryotic cells and essential for metabolic functions and cell fitness. Mammalian mitochondria contain their own genome and ribosomes to synthesize 13 proteins that are core components of oxidative phosphorylation (OXPHOS) complexes. The majority of the mitochondrial proteome is encoded by the nuclear genome and synthesized in the cytoplasm for transport into the organelle. Once imported into mitochondria, the 80 nuclear-encoded structural proteins assemble with the 13 mitochondrial-encoded counterparts to form multi-subunit complexes known as the OXPHOS system.

Mitochondrial gene expression is the process of translating the information encoded on the mitochondrial genome into proteins that make up the OXPHOS complexes. This process involves nuclear-encoded factors and therefore requires tight coordination between mitochondrial and nuclear genome. During mitochondrial protein synthesis, nascent chains emerging from the ribosomes are inserted into the inner membrane for assembly into OXPHOS complexes and those that failed to insert or assemble into complexes are targeted for degradation. The co-translational insertion and degradation of mitochondrial nascent chain are regulated by a dedicated set of quality control machinery.

Quality control of mitochondrial gene expression is a collective term for surveillance mechanisms that monitor molecular events leading to protein translation. In this dissertation, I have identified the chaperone protease complex composed of AFG3L2 subunits as the key component in co-translational quality control of mitochondrial nascent chains. AFG3L2 dysfunction triggers the activity of metalloprotease OMA1, which proteolytically process the dynamin-related GTPase OPA1 and remodels the mitochondrial membrane morphology. Failure to counteract this stress response in a timely manner affects mitochondrial membrane dynamics and generates negative feedback on mitochondrial gene expression. My research establishes that this stress response is due exclusively to defects in the quality control of mitochondrial nascent chains.

Co-translational insertion of mitochondrial nascent chain is mediated by OXA1L insertase. Here, I have shown how OXA1L cooperates with AFG3L2 in regulating the insertion and turnover of mitochondrial nascent chains. In the absence of OXA1L, MT-ATP6 nascent chains are not inserted
into the inner membrane and rapidly degraded by AFG3L2. These data have demonstrated how the activities of OXA1L and AFG3L2 determine the fate of a nascent chain. In addition, I have also found that translation of a specific pathogenic \textit{MT-ATP6} variant has a profound effect on mitochondrial gene expression. In the absence of AFG3L2 or OXA1L, this variant exhibits severe mitochondrial translation defect. Overall, these findings have provided new insights into the role of mitochondrial protein quality control mechanism in regulating mitochondrial gene expression.

The last part of the dissertation addresses the frequency of errors at the 3’ end of mitochondrial mRNAs. The human mitochondrial genome is transcribed into long polycistronic RNA transcripts that must be processed to release individual RNA (rRNAs, tRNAs and mRNAs). Errors in the RNA processing step will generate aberrant mRNAs that interfere with the translation of fully functional proteins. To determine the error rate and diversity of aberrant mitochondrial mRNAs, a novel next-generation sequencing approach was developed to provide comprehensive analysis of the 3’ end of mitochondrial transcripts. My results show the presence of aberrant mRNAs and variations in mitochondrial post-transcriptional modifications in health and disease. In addition, a significant proportion of these aberrant mRNAs lack a complete stop codon and are associated with translating mitochondrial ribosomes. Together, these observations demonstrate an inherent error rate in mitochondrial gene expression that requires quality control mechanisms to maintain functional protein synthesis.

In conclusion, my thesis has identified critical steps in co-translational quality control of mitochondrial nascent chain synthesis and investigated the functional consequences of defective quality control system on mitochondrial gene expression.
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LIST OF PUBLICATIONS

This dissertation is based on the following publications:


* Co-first author


# equal contribution

The publications are referred to in the text by their roman numerals.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAA</td>
<td>ATPase Associated with diverse cellular Activities</td>
</tr>
<tr>
<td>AFG3L2</td>
<td>AFG3 Like Matrix AAA Peptidase Subunit 2</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CDS</td>
<td>coding sequence</td>
</tr>
<tr>
<td>CL</td>
<td>cardiolipin</td>
</tr>
<tr>
<td>CM</td>
<td>cristae membrane</td>
</tr>
<tr>
<td>CJs</td>
<td>crista junctions</td>
</tr>
<tr>
<td>DRP1</td>
<td>dynamin-related protein 1</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>IM</td>
<td>inner membrane</td>
</tr>
<tr>
<td>IMS</td>
<td>intermembrane space</td>
</tr>
<tr>
<td>LRPPRC</td>
<td>Leucine-rich pentatricopeptide rich domain containing</td>
</tr>
<tr>
<td>MCU</td>
<td>mitochondrial calcium uniporter</td>
</tr>
<tr>
<td>MELAS</td>
<td>Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes</td>
</tr>
<tr>
<td>MERRF</td>
<td>Myoclonic epilepsy and ragged red fibers</td>
</tr>
<tr>
<td>MFN1</td>
<td>mitofusin 1</td>
</tr>
<tr>
<td>MFN2</td>
<td>mitofusin 2</td>
</tr>
<tr>
<td>MICOS</td>
<td>mitochondrial contact site and organizing system</td>
</tr>
<tr>
<td>MPP</td>
<td>mitochondrial processing peptidase</td>
</tr>
<tr>
<td>mtDNA</td>
<td>mitochondrial DNA</td>
</tr>
<tr>
<td>MTS</td>
<td>mitochondrial targeting sequence</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>OM</td>
<td>outer membrane</td>
</tr>
<tr>
<td>OMA1</td>
<td>overlapping activity with m-AAA 1</td>
</tr>
<tr>
<td>OPA1</td>
<td>optic atrophy 1</td>
</tr>
<tr>
<td>OXA1L</td>
<td>Oxidase assembly 1-like</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>oxidative phosphorylation system</td>
</tr>
<tr>
<td>PMCA</td>
<td>plasma membrane Ca(^{2+}) ATPase</td>
</tr>
<tr>
<td>POLRMT</td>
<td>mitochondrial RNA polymerase</td>
</tr>
<tr>
<td>PTC</td>
<td>peptidyl transferase center</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>SLIRP</td>
<td>SRA stem-loop interacting RNA binding protein</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
<tr>
<td>TFAM</td>
<td>Transcription factor A, mitochondrial</td>
</tr>
<tr>
<td>TIM</td>
<td>translocase of the inner membrane</td>
</tr>
<tr>
<td>TOM</td>
<td>translocase of the outer membrane</td>
</tr>
<tr>
<td>VDAC</td>
<td>voltage-dependent anion channel</td>
</tr>
<tr>
<td>YME1L</td>
<td>yeast mtDNA escape 1-like</td>
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1 INTRODUCTION

Mitochondria are unique and complex organelles pivotal for metabolic functions and cellular fitness. Failures to maintain mitochondrial homeostasis and integrity perturb cellular functions and contribute to a wide array of pathological diseases. Mitochondrial homeostasis is maintained at molecular and organelle level, which both require constant communication between mitochondrial and nuclear genomes. Mitochondrial gene expression translates proteins require for OXPHOS complexes. Multiple quality control processes have evolved to sense and counteract stress responses triggered by defective molecular steps in mitochondrial gene expression. These quality control mechanisms are integrated into the regulatory pathways of mitochondrial biogenesis and activated upon internal and external stress cues. Responsive quality control limits the accumulation of damaged mitochondria and protein components to prevent deleterious effects on mitochondrial gene expression.

Human mitochondria have compartmentalized gene expression system to synthesize 13 proteins that are core subunits of oxidative phosphorylation (OXPHOS) complexes\(^1\). These 13 proteins are synthesized by a dedicated set of translation machinery inside the mitochondrial matrix space. As the mitochondrial nascent chains emerge from the ribosomes, they are inserted into the inner membrane by OXA1L, a member of the Oxa1/YidC/Alb3 insertase family that is conserved across the three domains of life\(^2\). Members of the insertase family are shown to have critical roles in membrane insertion and assembly of protein complexes in eukaryotic organelles and bacterial systems\(^3\). Recent cryo-EM structure of mammalian mitochondrial ribosomes has revealed key interactions between OXA1L and large ribosomal subunit that are critical in facilitating membrane insertion of nascent chains\(^4\). OXA1L dysfunction is known to impair biogenesis of OXPHOS complexes and biallelic pathogenic mutations in OXA1L are presented with severe encephalopathy, hypotonia and developmental delay\(^5,6\).

To prevent aggregation of nascent chains at the inner membrane, mitochondria have evolved surveillance mechanisms to recognize and degrade aberrant polypeptides. One component of co-translational quality control is chaperone and protease AAA (ATPases associated with various cellular activities) complex formed by subunits of AFG3L2. In humans, AFG3L2 exists as homo- or hetero-hexameric complex and is known to be involved in quality control of mitochondrial protein synthesis and stability of respiratory chain biogenesis\(^7,8,9\). Pathogenic variants of *AFG3L2* are known to cause neurological diseases with heterogeneous clinical and pathological presentation\(^10,11\).
Molecular phenotypes associated with AFG3L2 dysfunction include loss of ribosomes, defective oxidative phosphorylation and fragmentation of the mitochondrial network\textsuperscript{12,13}.

Currently, studies related to mitochondrial protein quality control focus mainly at addressing post-translational events such as protein misfolding and aggregation\textsuperscript{14,15}. By contrast, critical gaps remain in the mechanistic understanding on how co-translation quality control components sense aberrant mRNAs and nascent chains. Unlike in the nucleus where nuclear membrane can act as a physical barrier to prevent the export of aberrant mRNAs into the cytoplasm, mitochondrial transcription and translation are not compartmentalized\textsuperscript{16,17}. There is currently no known mechanism to quality check the mRNAs prior their engagement with the mitochondrial ribosomes.

Aberrant mRNAs in mitochondria can arise from gene mutations, post-transcriptional modifications and errors in RNA processing events. Deletions or point mutations of mitochondrial DNA can generate truncated mRNAs with premature termination codons and non-stop mRNAs that lack in-frame stop codons. In eukaryotes, one of the surveillance mechanisms is the nonsense-mediated decay (NMD) pathway that recognizes and targets mRNAs with premature termination codon for degradation. The second mechanism is non-stop decay (NSD) that eliminates non-stop mRNAs during translation and rescues ribosomes that are stalled at the 3’ end of mRNAs\textsuperscript{18,19}. Both NMD and NSD are ribosome-associated quality control that degrade defective mRNAs and prevent translation of aberrant proteins. However, NMD and NSD pathways have not been discovered in mitochondria and we have limited understanding on mitochondrial ribosome-associated quality control mechanisms.

In this dissertation, I have identified key components critical to quality control of mitochondrial protein synthesis and ensuing organelle stress responses. By investigating the translation of truncated and non-stop mRNAs that arise from pathogenic mitochondrial DNA mutations, I have shown how absence of co-translation quality control affects downstream nascent chain synthesis. Lastly, a novel next-generation sequencing approach was employed to investigate the frequency and functional consequences of baseline errors in mitochondrial gene expression across different human cell types and tissues.
2 REVIEW OF THE LITERATURE

2.1 Mitochondrial biology

2.1.1 Mitochondrial origin and evolution

Mitochondria are double-membrane bound organelles that are best known for their role in generating ATP to meet cellular energy demands. However, research has shown that mitochondria perform diverse functions other than ATP production, and these functions can vary among eukaryotic lineages. Regardless of the diversity, mitochondria are known to have originated from an endosymbiotic event of alphaproteobacteria.

According to the endosymbiotic theory, mitochondria and plastids of green plant cells (chloroplasts) evolved from free-living bacteria that were taken up by eukaryotic cells via endosymbiosis\textsuperscript{20}. In 1883, German botanist Andreas Schimper observed that chloroplasts were reproduced by division that were independent from the nucleus and proposed that plants arose from symbiotic union of two organisms\textsuperscript{21}. In 1905, Russian biologist Constantin Mereschkowsky followed Schimper’s work and proposed that plastids were once free-living cyanobacteria that formed symbiotic relationship with a heterotrophic host\textsuperscript{22}. In his second paper published in 1910, Mereschkowsky concluded that plastids could divide independently from nucleus and should be regarded as symbionts\textsuperscript{23}. In 1920s, American biologist Ivan Wallin further extended the endosymbiotic theory from plastids to mitochondria\textsuperscript{24}. For the next 50 years, cell biologists dismissed the theory based on the assumption that plastids and mitochondria did not contain DNA, until the discovery of DNA in mitochondria by electron microscopy and biochemical assays\textsuperscript{25,26}. In the 1960s, Lynn Margulis revived the endosymbiotic theory and proposed that mitochondria and plastids originated from prokaryotic progenitors via symbiosis with eukaryotic host cells. The theory also states that mitochondria descended from proteobacteria and chloroplasts from cyanobacteria\textsuperscript{27}. Phylogenetic analyses of mitochondria and chloroplasts in the early 1980s confirmed the prokaryotic origin of these organelles, where their genes and proteins were found to be distinct from the eukaryotic lineage\textsuperscript{28,29,30}. The advent of modern technologies such as high-throughput sequencing and proteomics can potentially clarify the exact position of mitochondria in the tree of life. Phylogenomics analyses have revealed that the lineage of eukaryotic host is closely related to Asgard archaea, and mitochondria are indeed originated from alphaproteobacteria\textsuperscript{31,32}. 
The transition of mitochondria from endosymbiotic bacteria to fully integrated organelles involved major evolutionary changes such as reduction in gene content, where there is a significant loss of redundant genes as compared to their eubacteria relatives\(^{33}\). Mitochondrial gene content varies across eukaryotes, from 3 protein-encoding genes in strict parasitic protists (\textit{Plasmodium falciparum})\(^{34}\) to 67 in freshwater protist \textit{Reclinomonas americana}\(^{35}\). The difference in mitochondrial gene content is mainly due to endosymbiotic gene transfer to the nucleus, an on-going process that is observed in flowering plants\(^{36}\). Human mitochondrial genome has been reduced to 13 protein-encoding genes, with majority of the genes transferred to the nucleus. The tRNA punctuation model is likely to be the most recent evolution as it is only found in Bilateria\(^{37}\). In addition, the organization of animal mitochondrial genome is highly diverse in non-bilaterian lineages, varying in nucleotide substitution rate, presence of introns, genetic code and RNA editing\(^{38,39}\). Other important evolutionary changes include retargeting of proteins between mitochondria and other organelles, protein import machinery and the development of cristae\(^{40,41,42,43}\). These changes occurred gradually over time during the integration of mitochondria into the host cells. Although modern data have provided critical evidence into the evolutionary transition of mitochondria, controversy remains on the initial contact between the endosymbiont and host cells.

### 2.1.2 Mitochondrial architecture

#### 2.1.2.1 Mitochondrial compartments

Mitochondria are double-membrane organelles that are made up of four distinct subcompartments: outer membrane (OM), inner membrane (IM), intermembrane space (IMS) and the matrix space. The OM separates the organelle from the cytoplasm and surrounds the IM that separates the IMS from the matrix (\textbf{Figure 1}). Mitochondrial membranes are composed of proteins and phospholipids, including cardiolipin, a dimeric phospholipid that is exclusively found in mitochondria\(^{44}\). The outer membrane is lipid-rich and forms smooth envelope with high membrane fluidity\(^{45}\). In contrast, the inner membrane is highly folded and has high protein to lipid ratio (3:1)\(^{46,47}\). OXPHOS complexes that are critical in generating cellular energy are located in the inner membrane. The membrane-enclosed structure of mitochondria is thus essential in maintaining the electrochemical gradient generated by the OXPHOS complexes by controlling the influx of charged molecules across the inner membrane. In addition to energy production, the inner membrane is also a site for iron-sulphur biosynthesis, metabolite exchange and protein translocation into the matrix\(^{47,48}\). The IMS is small
aqueous subcompartment that functions in the exchange of metabolites, proteins and ions between matrix and cytoplasm\textsuperscript{49}.

The outer membrane allows diffusion of ions and small molecules such as NADH\textsuperscript{50} and ATP\textsuperscript{51} through a pore-forming membrane protein known as voltage-dependent anion channel (VDAC). By contrast, the inner membrane is less permeable to ions and metabolic substrates. The membrane potential generated across the inner membrane requires specific membrane carrier proteins for transport of charged metabolites into the matrix space\textsuperscript{52}. The inner membrane is divided into two distinct subdomains. The first subdomain is the inner boundary membrane (IBM) that interacts with the OM by forming contact sites\textsuperscript{53}. The best characterized function of IBM is the translocation of proteins via translocases of the outer and inner membrane. In addition, early studies have also proposed the transport of phospholipids through the contact sites\textsuperscript{46}.

The second subdomain is the cristae membrane (CM), formed by invaginations of IBM that extend into the matrix space. These invaginations originate from tubular or slot-like structures known as crista junctions (CJs), as discovered by electron microscopy (EM) tomography\textsuperscript{54}. CJs have a diameter between 12-40nm\textsuperscript{55,56} and have been proposed to form barriers between the intracistral space and IMS by limiting the diffusion of metabolites\textsuperscript{57,58}. Depending on the energy demand of the organisms and tissues, CMs can appear as disk-like lamellar sheets or cylindrical tubular cristae.\textsuperscript{59} Cristae are the main site for OXPHOS, creating large surface area to accommodate OXPHOS complexes and certain regulatory proteins involved in energy transfer\textsuperscript{60}. Thus, changes in cristae morphology can affect the stability of respiratory chain complexes and affect ATP production.

The biogenesis of cristae has been shown to be coordinated by mitochondria contact site and organizing system (MICOS), optic atrophy 1 (OPA1) and dimerized F\textsubscript{1}F\textsubscript{o}-ATP synthase\textsuperscript{61,62,63}. MICOS is critical in mediating the connection of inner and outer membranes and formation of CJs\textsuperscript{64} (Figure 1). In mammals, Mic60 of MICOS has been shown to interact with OPA1 to regulate the stability and number of CJs\textsuperscript{65}. OPA1 oligomerization is required to narrow the width of CJs to restrict the movement of pro-apoptotic factor cytochrome \textsubscript{c}\textsuperscript{66,67}, and to promote stabilization of ATP synthase\textsuperscript{68}. The dimerization of F\textsubscript{1}F\textsubscript{o}-ATP synthase plays an important role in cristae biogenesis. F\textsubscript{1}F\textsubscript{o}-ATP synthase dimers are formed through interactions between adjacent F\textsubscript{o} domains, which cause bending of CM and generate a positive curvature at the apex of cristae. The dimers are assembled into long ribbons along the curved cristae ridges and are ubiquitous feature of mitochondrial inner membranes\textsuperscript{69,70}.
The matrix is the innermost compartment of mitochondria surrounded by inner membrane. It is the second aqueous subcompartment of mitochondria and has a pH of 7.9-8.7, slightly higher than the pH of IMS. The difference in pH generates electrochemical gradient that is necessary to drive ATP production. The mitochondrial matrix is the site for major enzymatic reactions, including tricarboxylic acid (TCA) cycle. Mitochondrial DNA (mtDNA) replication, transcription and protein synthesis also take place inside the matrix.

Figure 1. Schematic illustration of mitochondrial subcompartments. ETC, electron transport chain; OXPHOS, oxidative phosphorylation; MICOS, mitochondria contact site and organizing system.

2.1.3 Mitochondrial functions

Mitochondria are not only important for energy production but also required for critical fundamental cellular processes such as metabolite biosynthesis, iron-sulphur cluster biogenesis and calcium homeostasis.

2.1.3.1 ATP production

Mitochondria generate ATP through OXPHOS, where chemical energy are released from nutrients through series of oxidation-reduction (redox) reactions. Glucose is the principal source of energy and catabolized through three successive processes: glycolysis, TCA cycle and OXPHOS.
Glycolysis occurs in the cytoplasm and is the first pathway of cellular respiration. It is an anaerobic process where one molecule of glucose is broken down to form 2 ATP, 2 NADH and 2 pyruvate molecules. The fate of pyruvate is depending on the micro-environment of the cells. Under aerobic conditions, pyruvate can enter TCA cycle in mitochondria and undergoes complete oxidation.

The TCA cycle, also known as Krebs cycle, occurs in mitochondrial matrix and involves eight enzymatic reactions. The first reaction is the oxidation of pyruvate to acetyl-CoA, an intermediate that is fed into TCA to complete a series of redox reactions. The completion of TCA cycle produces reduced electron carriers NADH and FADH$_2$ which will feed into respiratory complexes of ETC. OXPHOS consists of two parts: ETC and chemiosmosis. The first part involves the oxidation of NADH and FADH$_2$, where electrons are transferred across all four respiratory complexes (Complex I-IV) through redox reactions. The flow of electrons drives the pumping of protons (H$^+$) from the matrix into the IMS, thus generating electrochemical gradient across the inner membrane. The last step in ETC involves the transfer of electrons to molecular oxygen (O$_2$), which takes up H$^+$ and reduces to water. The second part is the production of ATP by Complex V (F$_1$F$_0$-ATP synthase). The electrochemical gradient generated by redox reactions causes accumulation of H$^+$ in the IMS. Due to the hydrophobicity of the inner membrane, F$_1$F$_0$-ATP synthase is the only channel where H$^+$ can be translocated back into the matrix. The movement of H$^+$ down the electrochemical gradient through F$_1$F$_0$-ATP synthase generates free energy difference and catalyzes the phosphorylation of ADP to ATP.

### 2.1.3.2 Calcium homeostasis

Calcium (Ca$^{2+}$) signalling is important in the regulation of various physiological functions such as cellular migration and skeletal muscle contraction. The signalling pathways are regulated through interactions between organelles and ion channels in the plasma membrane. Under resting conditions, Ca$^{2+}$ concentrations in the cytoplasm are kept low to prevent precipitation. Low level Ca$^{2+}$ is maintained by plasma membrane Ca$^{2+}$ ATPase (PMCA) and sodium/calcium (Na$^+$/Ca$^{2+}$) exchanger. Upon physiological stimulation, cytosolic Ca$^{2+}$ concentrations can be rapidly increased through influx of extracellular Ca$^{2+}$ via calcium channels in the plasma membrane or release of Ca$^{2+}$ from intracellular stores. The main intracellular Ca$^{2+}$ stores are the endoplasmic reticulum (ER), sarcoplasmic reticulum in muscle cells and mitochondria. The Ca$^{2+}$ stores maintain and stabilize the cytoplasmic Ca$^{2+}$ concentration by taking up excess Ca$^{2+}$. Although the ER is the main Ca$^{2+}$ store, mitochondria also play an important role in buffering cytoplasmic Ca$^{2+}$. The uptake of
Ca\textsuperscript{2+} into mitochondria is important for energy production as increase in cytosolic Ca\textsuperscript{2+} concentrations often signals an increased energy demand\textsuperscript{81}. A rise in cytosolic Ca\textsuperscript{2+} concentrations promotes uptake of Ca\textsuperscript{2+} into mitochondria, regulated by ion channels and exchangers localized on the outer and inner membranes. Due to the high permeability of mitochondrial outer membrane, Ca\textsuperscript{2+} can be transported freely into the IMS via VDAC\textsuperscript{82}. The transport of calcium from the cytoplasm into mitochondrial matrix is mediated by mitochondrial calcium uniporter (MCU), a transmembrane protein localized in the inner membrane. The influx of Ca\textsuperscript{2+} into mitochondrial matrix is dependent on transmembrane potential and concentration of cytosolic Ca\textsuperscript{2+}. MCU is a highly selective ion channel and has low affinity for cytosolic Ca\textsuperscript{2+} \textsuperscript{83}. MCU interacts with regulatory proteins mitochondrial Ca\textsuperscript{2+} uptake 1 (2) protein (MICU1, MICU2) to regulate the uptake of Ca\textsuperscript{2+} into the mitochondria. MICU1 has dual roles, it can act as an inhibitor to prevent channel opening of MCU at low cytosolic Ca\textsuperscript{2+} concentration, or enhances MCU’s activity at high cytosolic Ca\textsuperscript{2+} concentration\textsuperscript{84}. On the other hand, MICU2 prevents channel opening and limits Ca\textsuperscript{2+} uptake when Ca\textsuperscript{2+} concentration is low\textsuperscript{85}. The efflux of Ca\textsuperscript{2+} from mitochondrial matrix is mediated by Na\textsuperscript{+}/Ca\textsuperscript{2+}/Li\textsuperscript{+} exchanger (NCLX), which exchanges matrix Ca\textsuperscript{2+} for external Na\textsuperscript{+} or Li\textsuperscript{+} \textsuperscript{86}. In excitable tissues (heart and brain), Na\textsuperscript{+}/Ca\textsuperscript{2+} is the predominant antiporter for Ca\textsuperscript{2+} efflux is driven by electrochemical gradient of Na\textsuperscript{+} \textsuperscript{87}. In non-excitible tissues (liver and kidney), the extrusion of Ca\textsuperscript{2+} is mediated by H\textsuperscript{+}/Ca\textsuperscript{2+} exchanger\textsuperscript{88}. Mitochondria have Ca\textsuperscript{2+} buffering capacity in various cell types, sensing transient changes in Ca\textsuperscript{2+} and preventing severe Ca\textsuperscript{2+} overloads. Close interactions between mitochondria and ER generate microdomains that allow rapid uptake of bulk Ca\textsuperscript{2+} into mitochondria, which fine-tuned metabolism to meet cellular demands\textsuperscript{89}. Overall, mitochondria play pivotal role in modulating Ca\textsuperscript{2+} signalling that is important for aerobic metabolism and cell survival\textsuperscript{90}.

\subsection*{2.1.4 Mitochondrial dynamics}

Mitochondria are highly dynamic and mobile organelles. In most cell types, mitochondria form an interconnected tubular network within the cell. The distribution of mitochondria within cytoplasm can be heterogeneous and they are often enriched at sites with high energy demand. The transport of mitochondria to the sites of cellular demand is dependent on their interactions with motor proteins along cytoskeletal tracks\textsuperscript{91}. The morphology and distribution of mitochondria are essential for both mitochondrial and cellular functions and regulated through fusion and fission processes.
These processes are intimately linked to the physiological conditions of the cells to respond accordingly to cellular demand\textsuperscript{92}. For instance, mitochondria fragment during mitosis and hyperfuse to form highly connected network in respond to cellular stress\textsuperscript{93,94}. Changes in mitochondrial dynamics can affect metabolism, calcium homeostasis and transport of mitochondria and negatively impart cellular functions.

2.1.4.1 Mitochondrial fusion

Mitochondrial membrane fusion enables mixing of mtDNA and proteins between healthy and damaged mitochondria, buffering to maintain the integrity of mitochondrial population within a cell\textsuperscript{95,96}. The fusion process is mediated by dynamin-related GTPases consisting of mitofusin 1 (MFN1), mitofusin 2 (MFN2) and optic atrophy 1 (OPA1)\textsuperscript{97}. Human MFN1 and MFN2 are highly homologous, with 62\% identity at amino acid level\textsuperscript{98}. Mitofusins are located in the outer membrane and consist of large N-terminal GTPase domain, two transmembrane segments and C-terminal heptad-repeat domain\textsuperscript{99}. Biochemical assay in cultured human cells has confirmed the N\textsubscript{out}-C\textsubscript{in} topology of mitofusins, with more than 10 residues of its C-terminal extending within the IMS\textsuperscript{100}. Upon binding to GTP, MFN1 and MFN2 are known to assemble in \textit{trans} as heterotypic and homotypic dimers tethered to adjacent outer membranes prior fusion. Subsequent GTP-hydrolysis induces conformational changes of mitofusins and facilitates the fusion of outer membranes\textsuperscript{101}. The loss of mitofusins induces mitochondrial fragmentation where the fragments appear as either short tubules or spheres. In addition, embryonic lethality has been observed in both MFN1 and MFN2 knockout mice\textsuperscript{102}. Pathogenic mutations in \textit{MFN2} are the cause for Charcot–Marie–Tooth neuropathy type 2A (CMT2A), a clinically heterogeneous group of inherited peripheral neuropathies characterized by muscle atrophy and degeneration of motor axons\textsuperscript{103}.

Fusion of mitochondrial inner membrane is mediated by dynamin-like GTPase OPA1 and membrane phospholipid cardiolipin (CL). The domain organization of OPA1 consists of a mitochondrial targeting sequence (MTS), a transmembrane domain, a coiled-coiled domain, followed by three conserved dynamin domains: GTP-binding domain, middle domain and GTPase effector domain (GED)\textsuperscript{104} (Figure 2). In human, \textit{OPA1} has 30 exons that generate eight mRNAs variants by alternative splicing of exon 4, 4b and 5b. The expression of these eight variants is ubiquitous but their abundance and expression patterns are tissue-specific\textsuperscript{105}. Precursor proteins translated from the eight mRNAs are targeted to mitochondria IMS via MTS. Cleavage of MTS generates long isoforms (L-OPA1) that are anchored to the inner membrane. Each long isoform may be subjected to further
proteolytic cleavage at the N-terminus to generate soluble short isoforms (S-OPA1) that are released into the IMS. L-OPA1 is constitutively processed by two proteases located at the inner membrane. Metallopeptidase OMA1 cleaves at S1 site in exon 5 and ATP-dependent metalloprotease YME1L cleaves at S2 site in exon 5b\textsuperscript{106,107}. At steady state, the activities of OMA1 and YME1L produce a mixture of long and short OPA1 isoforms. Mitochondrial fusion requires both L-OPA1 and S-OPA1 that assemble into an oligomeric ring to maintain cristae structure\textsuperscript{67,108}. However, interference to cell proliferation and stress conditions activate OMA1 to completely process L-OPA1 into S-OPA1\textsuperscript{108,109,110}. Stress-induced processing of L-OPA1 causes excessive accumulation of S-OPA1 and promotes mitochondrial fragmentation\textsuperscript{92}. Loss of OMA1 abolishes OPA1 processing at S1 but mitochondrial network remains elongated and reticulated\textsuperscript{109,111}. In contrast, loss of YME1L impairs OPA1 processing at S2 and causes mitochondrial fragmentation\textsuperscript{112,113}. Cells lacking both YME1L and OMA1 retain fusion ability, indicating OPA1 processing is dispensable for fusion\textsuperscript{111,114}. A reconstituted experiment using liposomes has provided a link between OPA1 processing and membrane fusion. Membrane tethering was established through heterotypic between recombinant L-OPA1 and liposomes containing phospholipid CL. This step primed the subsequent GTP-dependent membrane fusion and could be modulated by S-OPA1. In contrast, OPA1 homotypic interactions mediate membrane tethering but is not required for membrane fusion\textsuperscript{115}.

**Figure 2.** Schematic illustrating domain organization and proteolytic processing in OPA1. (Top) OPA1 consists of mitochondrial targeting sequence (MTS), transmembrane (TM) domain, coiled-coiled (CC) domain, GTPase domain, middle domain and a GTPase effector domain (GED) containing a coiled-coil region (CC3). These domains are found in

\[\text{IMS} \quad \text{IM} \quad \text{Matrix}\]
all OPA1 splice variants while TM2a, TM2b, and CC1 are only present in spliced exons 4b and 5b. Proteolytic cleavage sites for OMA1 (S1) and YME1L (S2) are indicated. (Bottom) Proteolytic cleavage at S1 and S2 sites of membrane-anchored L-OPA1 by OMA1 and YME1L to generate soluble S-OPA1.

Pathogenic mutations in OPA1 are the most common cause of autosomal dominant optic atrophy, a condition where patients suffer from progressive vision loss due to degeneration of retinal ganglion cells and optic nerve\textsuperscript{116}. Homozygous mutation of OPA1 in mice leads to early embryonic lethality while heterozygous mutation is characterized by degeneration of optic nerve and fragmented mitochondria\textsuperscript{117}. In both patient-derived fibroblasts and mouse models, loss of OPA1 leads to mitochondrial fragmentation, OXPHOS defects and mtDNA instability\textsuperscript{118,119}. Furthermore, the loss of nucleoids in fusion-deficient mammalian fibroblasts could be the cause of reduced respiratory activities\textsuperscript{95}. Although small amount of mtDNA is still present in nucleoid-deficient mitochondria, it is not sufficient to support mitochondrial function. Thus, mitochondrial fusion is important for maintenance and distribution of mtDNA, protecting the organelle from the deleterious effects of mtDNA mutations.

2.1.4.2 Mitochondrial fission

Mitochondrial fission is a division process that facilitates trafficking and distribution of mitochondria. The division of mitochondria is mediated by dynamin-related GTPase, dynamin-related protein 1 (DRP1) in mammals and dynamin 1 (Dnm1) in yeast\textsuperscript{120}. DRP1 is composed of the highly conserved N-terminal GTPase domain, middle domain, variable or B-insert domain and GED at the C-terminal. Unlike other members of the dynamin family, DRP1 lacks the pleckstrin homology (PH) domain and the proline and arginine rich domain (PRD) at the C-terminal\textsuperscript{121}. The GTPase domain and GED come together to form the structurally distinct module known as the bundle signalling elements (BSE). The BSE is composed of three alpha helices and connects GTPase domain to the stalk domain of DRP1, mediating membrane binding and subsequent oligomerization\textsuperscript{122,123}. DRP1 predominantly resides in the cytoplasm and is recruited to fission sites on the outer membrane by membrane-associated receptors\textsuperscript{124}, forming oligomeric complexes that cause narrowing of membrane\textsuperscript{125}. DRP1 oligomers are capable of translocating along mitochondrial tubule and upon GTP hydrolysis, induces membrane constriction that leads to mitochondrial division\textsuperscript{126}. 
In mammals, tail-anchored proteins mitochondrial fission factor (MFF) and mitochondrial dynamics proteins 49 and 51 (MiD49 and MiD51) have been identified as receptors for DRP1. These proteins can act independently to modulate the recruitment and activity of DRP1. For instance, overexpression of MFF leads to mitochondrial fragmentation but MFF-null cells have elongated mitochondria\textsuperscript{127}. MiD49 and MiD51 play a direct role in the recruitment of DRP1 and their knockdown abolish DRP1 oligomerization, resulting in mitochondrial elongation\textsuperscript{128,129}.

In contrast to the outer membrane, very little is known about the mechanism of inner membrane fission. The inner membrane protein MTP18 has been proposed as a potential fission factor. Overexpression of MTP18 induces fission while depletion leads to mitochondrial tubulation\textsuperscript{130}. Alternatively, outer membrane fission machinery may be sufficient to drive inner membrane fission. Clearly, further work is required to identify factors and mechanisms coordinating outer and inner membrane fission.

### 2.2 Human mitochondrial genome

The human mitochondrial genome consists of a 16 kb circular and double-stranded DNA which encodes for 2 rRNAs, 22 tRNAs and 13 mRNAs essential for the OXPHOS system (complexes I-IV) (Figure 3). One characteristic feature of human mitochondrial genome is the lack of introns.
2.2.1 Mitochondrial genetics

Uniparental inheritance, cellular polyploidy and deviation from standard genetic code are unique features of mitochondrial genetics. The multi-copy nature of mitochondrial genome gives rise to homoplasmy and heteroplasmy. In the homoplasmic state, a cell contains identical copies of mtDNA, whereas in a heteroplasmic state, there is a mixture of two or more mtDNA variants. The threshold level of heteroplasmy can vary between cells in the same tissue or organ and range between 60-80% for biochemical defect to be detected\textsuperscript{131,132}. Mitochondrial genetics do not follow the Mendelian patterns of inheritance. Although the number of mtDNA molecules inherited by each daughter cell is approximately equal, their distribution seems to be independent of genotype. Therefore, if polymorphisms are present within a cell, heteroplasmy level will differ among daughter cells after each mitosis, leading to a random genetic drift at each division through vegetative segregation\textsuperscript{133}. Vegetative segregation occurs as a result of random replication of mtDNA molecules, independent of the cell cycle. Through a process known as relaxed replication that is independent of cell cycle, mtDNA can replicate continuously while maintaining a relatively constant number of mtDNA molecules within the cell. If one type of mtDNA molecules is copied more frequently than another, the level of heteroplasmy can drift up or down within the cell\textsuperscript{134,135}. Heteroplasmic variant is transmitted through the female germline during oocyte maturation and the process can be random or based on germline selection\textsuperscript{136,137,138}. The segregation pattern can be tissue-specific and independent of the function of OXPHOS capacity, mtDNA replication efficiency or copy number\textsuperscript{139,140}. Studies using heteroplasmic mice models have shown that tissue-specific segregation can be modulated by mitochondrial membrane morphology\textsuperscript{141} and mtDNA haplotype differences\textsuperscript{142}.

In animals, mtDNA is almost exclusively maternally inherited but paternal mtDNA transmission has been reported in species such as mice\textsuperscript{143}, cattle\textsuperscript{144} and \textit{Drosophila}\textsuperscript{145}. The possibility of paternal mtDNA transmission in human remains controversial, as paternal mtDNA is undetectable at the blastocyst stage after fertilization\textsuperscript{146} as sperm mitochondria are marked with ubiquitin for degradation\textsuperscript{147}. The first case study on biparental mtDNA transmission in human was published in 2002\textsuperscript{148}, where Schwartz and Vissing reported the presence of paternal mtDNA in skeletal muscle of...
a male patient with mitochondrial myopathy. However, paternal mtDNA was not detected in other tissues studied from the same patient. Although there are other reports on biparental mtDNA transmission\textsuperscript{149,150}, recent studies on have discovered the existence of large nuclear-mitochondrial DNA that mimics paternal mtDNA inheritance\textsuperscript{151}.

MtDNA copy number ranges from ~100 copies in sperm to hundreds of thousands in an unfertilized oocyte and is tightly regulated and varies across cell types\textsuperscript{152,153}. The segregation of mutant mtDNA during development of embryos contributes to the variability of mutation load in tissues and organs\textsuperscript{154}. The rapid segregation of mtDNA variants between generations has created the genetic bottleneck theory where some progeny return to the state of homoplasmy\textsuperscript{155}. The mechanisms of the genetic bottleneck theory remains controversial and is assumed to occur in one of the restriction/amplification event during oocyte maturation, leading to random shift of mtDNA type and reduction of mtDNA copy number in primordial germ cells\textsuperscript{136,156,157}. Other studies have proposed that the bottleneck is the result of preferential replication of a mtDNA subgroup during oogenesis without reduction of mtDNA\textsuperscript{158,159}.

\subsection*{2.2.2 Pathogenic mitochondrial DNA}

Mitochondrial disorders are group of diseases characterized by bioenergetics defects. They are highly heterogeneous in terms of clinical presentation and genetic etiology\textsuperscript{160}. Due to their high energy demand, tissues and organs such as brain, heart and muscles are most affected by mitochondrial diseases. Mitochondrial diseases can develop at birth or at a later stage in life, affecting at least 1 in every 5000 individuals\textsuperscript{161,162}. Mitochondria protein synthesis is under the dual genetic control of nuclear and mitochondrial genomes and mutations in either mtDNA or nDNA may result in OXPHOS deficiencies. This poses a challenge in elucidating the pathogenic mechanisms linking to the wide spectrum of clinical phenotypes. Mitochondrial genome has mutation rate that is 20 to 100-fold higher than nuclear genome\textsuperscript{163}, possibly induced by oxidative damage, low fidelity of mitochondrial DNA polymerase gamma (POLG) and less extensive DNA repair mechanisms\textsuperscript{164,165}.

Pathogenic mtDNA mutations include large-scale rearrangements (deletions or insertions) that are mostly sporadic, or maternally inherited point mutations. The best describe mtDNA deletion is the 4977bp common deletion that spans from nucleotide 8469 to 13147 of the mitochondrial genome. The deletion removes a number of genes encoding for protein subunits of complex I, III, IV and V
and tRNAs, thus affecting protein synthesis and results in OXPHOS dysfunction\(^{166}\). The link between large-scale deletion and mitochondrial disease was first reported in 1988, giving rise to Kearns–Sayre syndrome (KSS) and \(^{167}\). KSS is a rare neuromuscular disorder with ragged red fibers and a typical onset before 20 years of age. The disease is characterized by cardiac conduction defects, pigmentary retinopathy and progressive external ophthalmoplegia (PEO). KSS usually arises from sporadic heteroplasmic mutations involving single and large-scale deletions (3 to 8.5 kb)\(^{168}\). The length and position of deletions vary between individuals. Except in leukocytes\(^{167}\), high proportion of the deletions are consistently found in affected tissues such as brain and muscles\(^{169}\). Deficiency in respiratory chain activities\(^{170,171,172}\) and absence of mitochondrial translated product\(^{173}\) are known to be associated with KSS.

MtDNA point mutations may occur within genes encoding for rRNAs, tRNAs or proteins. Nonetheless, most disease-causing point mutations are located in the tRNA-encoding genes\(^{174}\). MtDNA point mutations are mostly heteroplasmic and highly recessive, displaying significant clinical heterogeneity. However, an increasing number of homoplasmic point mutations have been reported, affecting single tissue and are characterized with reduced penetrance\(^{175,176,177}\).

German ophthalmologist Theodore Leber was the first to describe the Leber Hereditary Optic Neuropathy (LHON) disease in 1871\(^{178}\). Features in LHON pedigrees are gender bias (80% males) and incomplete penetrance\(^{179}\). Disease onset can range between 15 to 35 years of age and is characterized by bilateral central vision loss. The first pathogenic point mutation associated with LHON was reported in 1988, with a homoplasmic mutation in \(MT-ND4\) (m.1178G>A) that changes arginine to histidine (R340H)\(^{180}\). Subsequently, additional mutations have been identified in \(MT-ND1\) (m.3460G > A) and \(MT-ND6\) (m.14484T > C). All the three mutations affect subunits of complex I and are associated with complex I deficiency and mitochondrial dysfunction\(^{181,182,183}\).

Mitochondrial tRNA are hotspots for pathogenic mutations and about 300 mutations are strongly associated with mitochondrial diseases\(^{184}\). These mutations can disrupt maturation and folding of tRNAs, thus affecting their functional properties in aminocacylation and binding to protein factors during protein synthesis. Two well-characterized mitochondrial diseases associated with tRNA mutations are myoclonic epilepsy with ragged red fibers (MERRF) and mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS). MERRF is a chronic neurodegenerative disease that presents in childhood or early adulthood. The features of MERRF include myoclonus, ataxia, hearing loss and the presence of ragged red fibers (RRF) in muscle
biopsy\textsuperscript{185}. Biochemical defects include decreased activities of respiratory chain complexes in muscle fibers, especially COX deficiency\textsuperscript{186}. The m.8344A\textgreater{}G mutation in \textit{MT-TK} (tRNA\textsubscript{Lys}) was first reported in 1990 and accounted for 80\% of the mutations in MERRF\textsuperscript{187}. MELAS is one of the common maternally inherited mitochondrial disease and is characterized by seizures, myopathy, stroke-like episodes and are often seen in early childhood. The m.3243A\textgreater{}G mutation in \textit{MT-TL1} (tRNA\textsubscript{Leu}) is found in 80\% of MELAS patients\textsuperscript{188,189}. Other mutations associated with MELAS have been identified in \textit{MT-TV}\textsuperscript{190}, \textit{MT-CO3}\textsuperscript{191} and \textit{MT-ND5}\textsuperscript{192,193}. These mutations lead to instability of tRNA and result in complex I, III and IV deficiencies\textsuperscript{185,194}.

\subsection{2.2.3 Organization of mitochondrial DNA}

The structure and gene organization of mitochondrial genome is highly conserved in mammals. The two strands of the DNA duplex have different guanine and thymine composition and are named heavy (H) and light (L) strands based on their buoyant densities in caesium chloride density gradient\textsuperscript{195}. Majority of the genes are encoded on the H strand: 2 rRNAs, 14 tRNAs and 12 mRNAs. The L strand encodes for 8 tRNAs and 1 mRNA (Figure 3). Most of the genes encoding for tRNAs are interspersed among the coding sequence of rRNAs and mRNAs. This unique gene organization led to the development of punctuation model\textsuperscript{196}, where the tRNA serves as a ‘punctuation mark’ for endonucleolytic processing after transcription. The mitochondrial genome also has a stretch of non-coding region that is highly polymorphic\textsuperscript{197}. Within the non-coding region lies the displacement loop (D-loop) that harbours the heavy- and light-strand promoters (HSP and LSP), as well as the origin for H strand replication (OH).

Mitochondrial genome is packaged into discrete nucleoprotein complexes known as the nucleoid. Nucleoids are dynamic macromolecular structures distributed throughout mitochondrial network\textsuperscript{198,199}. They appear as discrete punctae under the microscope\textsuperscript{200,201} and vary in size with an average diameter of 100 nm\textsuperscript{202}. The packaging of mtDNA into nucleoid is mediated by transcription factor A, mitochondrial (TFAM), mitochondrial single-strand binding protein (mtSSB), POLG, and mitochondrial RNA polymerase (POLRMT)\textsuperscript{203}. TFAM induces a U-turn bends on mtDNA\textsuperscript{204} and tightly pack the DNA molecules into a spheroid structure\textsuperscript{205}. The amount of mtDNA molecules that can be compacted by TFAM has been a matter of debate because of the variations in the reported copy number\textsuperscript{206,207}. Next-generation imaging studies have revealed that each nucleoid contains about 1.4 mtDNA molecules\textsuperscript{202}. Nucleoids have diverse key functions including DNA segregation of mtDNA and organization of replication, transcription, and translation\textsuperscript{16}. As an organizing body of
mtDNA, nucleoids ensure efficient integration of mtDNA into cellular signalling networks to maintain metabolic homeostasis.

2.2.4 Transcription of mitochondrial DNA

Mitochondrial transcription is driven by POLRMT that is closely related to RNA polymerase found in T7 bacteriophage. Unlike T7 RNA polymerase, the catalytic domain of POLRMT is missing key promoter-recognition elements and therefore requires accessory factors for transcription initiation. POLRMT has been proposed to generate RNA primers required for DNA replication. Transcription initiates from two promoters that are closely located in the D-loop region and requires additional factors such as mitochondrial transcription factors A (TFAM) and B2 (TFB2M). TFAM is a member of the high mobility group (HMG)-box DNA–binding protein family and is essential for transcription activation and mtDNA packaging. TFAM has two positively charged HMG-box domains that interact with the negatively charged backbone of mtDNA and its c-terminal tail is essential for transcription activation.

To initiate transcription, TFAM first binds upstream of the initiation start sites and induces a stable U-turn bend on the DNA. Next, POLRMT is recruited to HSP and LSP by binding to TFAM and DNA backbone in the promoter region. The promoter is melted by TFB2M to induce conformational changes for transcription initiation. During elongation phase, transcription elongation factor TEFM binds to POLRMT and promotes processivity to produce long polycistronic transcript. TEFM also prevents formation of G-quadruplex structure that triggers transcription termination. In the absence of TEFM, POLRMT terminates at the conserved sequence block II region downstream of LSP and producing short transcripts that are thought to serve as primers for replication. Thus, TEFM has been suggested to act as a regulatory switch between mitochondrial replication and replication-primer formation.

The termination of transcription for H strand was originally suggested to be mediated by mitochondrial termination factor 1 (MTERF1) that bound to the termination site within tRNA. The crystal structure of human MTERF1 shows unwinding of DNA at the termination sequence and induces termination by unwinding DNA and base flipping. MTERF1 has been shown to prematurely terminates transcription originating from LSP at the 3’ end of mitochondrial rRNA coding sequence. This delays the progression of replication fork during mtDNA replication and prevents antisense transcription of mitochondrial rRNA. Structural studies further show the strong
polarity of MTERF1 towards terminating transcription initiated at LSD\textsuperscript{223}. To date, termination of H strand transcription remains unclear and no associating termination factors have been identified.

2.2.5 Mitochondrial RNA processing

Endonucleolytic processing of long polycistronic transcript is required to liberate individual mRNAs, tRNAs and rRNAs.

2.2.5.1 Mitochondrial tRNA processing

Mitochondrial tRNA processing is coordinated at the 5’ and 3’ of pre-tRNAs by RNase P complex and RNase Z (ELAC2), respectively\textsuperscript{224,225} (Figure 4). The human mitochondrial RNase P complex is composed of three protein subunits (MRPP1, 2 and 3) and evolutionarily unique in lacking a RNA moiety\textsuperscript{224,226}. The MRPP1 (\textit{TRMT10C}) subunit is a methyltransferase that methylates the nitrogen-1 (N1) atom of purine base at position \textit{9}\textsuperscript{225,227}. The N1 methylation is critical for folding of mitochondrial tRNAs and is found in 19 of the 22 tRNAs\textsuperscript{228}. MRPP2 (\textit{HSD17B10, SDR5C1}) subunit belongs to the short-chain dehydrogenase/reductase (SDR) superfamily\textsuperscript{229}. MRPP3 (\textit{PRORP}) subunit is a metallonuclease that cleaves the 5’ end of tRNAs \textit{82,224}. Recent cryo-EM structure of reconstituted RNase P complex reveal the detailed mechanism of pre-tRNA processing and methylation\textsuperscript{230}. MRPP1 first forms a subcomplex with MRPP2 and participates in methylation by binding to all four arms of pre-tRNA. MRPP2 is required for efficient methylation of tRNA as it stabilizes the anticodon MRPP1. Following methylation, MRPP3 is recruited to the 5’ end of pre-tRNA and forms an arch-like structure above the MRPP1/2 complex (Figure 4B). MRPP3 is stabilized by MRPP1/2 complex and catalyses 5’ end cleavage of pre-tRNA through possible rearrangements. In another study, the binding of MRPP1/2 to pre-tRNA has been shown to induce conformational changes that enhance substrate recognition and cleavage efficiency of MRPP3\textsuperscript{231,232}. All three subunits of RNase P complex are essential and the knockout of any of these proteins causes an accumulation of the RNA precursor molecules, reducing the steady-state levels of the mature form of mitochondrial tRNAs and some mRNAs\textsuperscript{225}. Pathogenic mutations in any of the genes encoding for RNase P complex lead to diverse clinical presentations which are associated with mitochondrial disorders\textsuperscript{233,234,235}.

RNAse Z is encoded by \textit{ELAC2}, the human homologue of \textit{E. coli} RNAse Z \textit{elacC}. In human, RNAse Z exists as short (RNAse Z\textsuperscript{S}) and long (RNAse Z\textsuperscript{L}) forms. RNAse Z\textsuperscript{S} is encoded by \textit{ELAC1} and RNAseZ\textsuperscript{L} by \textit{ELAC2} which is a zinc phosphodiesterase that has endonucleolytic activity at the 3’ end
of mitochondrial tRNA precursors (Figure 4). ELAC1 (RNAse Z\textsuperscript{5}) is primarily found in vertebrates and plants\textsuperscript{236,237} and required for repair of nuclear-encoded tRNAs during ribosome-associated quality control\textsuperscript{238}. ELAC2 (RNAse Z\textsuperscript{1}) is restricted to eukaryotes and suggested to have evolved from ELAC1 by gene duplication\textsuperscript{239}. An immunofluorescence assay revealed the distribution of ELAC2 in nucleus and mitochondria, processing both nuclear and mitochondrial tRNA precursors\textsuperscript{240}. Loss of ELAC2 impairs mitochondrial tRNA processing, causing accumulation of tRNA precursors and leads to hypertrophic cardiomyopathy\textsuperscript{241,242}.

**Figure 4.** Protein subunits involved in mitochondrial tRNA processing. (A) Domain organization of RNase P subunits and ELAC2. (B) Schematic illustrating endonucleolytic processing of mitochondrial pre-tRNA. MTS, mitochondrial targeting signal; SDR, short-chain dehydrogenase/reductase; PPR, pentatricopeptide repeat.
2.2.5.2 Mitochondrial mRNA processing

Endonucleolytic processing of mitochondrial tRNA by RNAse P and ELAC2 does not account for the cleavage of mRNAs that are not flanked by tRNAs. These include MT-ATP8/6/C03, MT-ND4L/ND4, 5’ end of MT-CO1 and MT-CYTB, and the 3’ end of MT-ND6. RNA-binding protein pentatricopeptide repeat domain protein 2 (PTCD2) has been reported to be involved in endonucleolytic cleavage of MT-ND5 and MT-CYTB junction. PTCD2 knockout mice show accumulation of MT-ND5-CYTB precursors and defects in the assembly of complex III. The Fas-activated serine/threonine kinase (FASTK) family proteins have been shown to be required for processing of RNA transcript at the non-canonical sites. Depletion of FASTKD2 leads to accumulation of MT-RNR2 and MT-ND6 precursors and affects cellular respiration. FASTKD5 is required to process mRNA precursors that are not flanked by tRNAs. Loss of FASTKD5 shows reduced level of mature MT-COI mRNA and affect the assembly of complex IV.

2.2.6 Mitochondrial RNA maturation and stability

2.2.6.1 mRNA modifications

After RNA processing of primary transcript, mitochondrial mRNAs undergo further post-transcriptional modifications. Mitochondrial mRNAs undergo polyadenylation at their 3’ ends, generating shorter poly(A) tails (~50 nucleotides) as compared to nuclear-encoded mRNAs. In human, polyadenylation is not detected in MT-ND6 and MT-ND5 is oligoadenylated. Mitochondrial polyadenylation is catalysed by homodimeric polyadenylic acid RNA polymerase (mtPAP) that transfer 40 to 50 adenines to the processed mRNAs. One proposed function of polyadenylation is to generate a complete UAA stop as 6 out of the 13 processed mRNAs have incomplete stop codon at their 3’ ends. Apart from this, functional role of polyadenylation of mitochondrial mRNA remains unclear. Some studies proposed that polyadenylation is required for stability of mRNA and mitochondrial translation. A study in Drosophila shows that polyadenylation is not required for stability of mRNAs and mitochondrial translation, but it is essential for preserving the 3’ end of the transcript. Polyadenylation of mitochondrial mRNAs in plants generally promotes degradation and turnover, which is similar to their bacterial ancestors. In contrast, poly(A) tails are necessary for nuclear export and the stability of cytoplasmic mRNAs.

The presence of m1A methylation in mitochondrial mRNAs has been reported by two independent studies. Both studies mapped the modification at single nucleotide resolution using next-
sequencing approach that misincorporates nucleotide during reverse transcription. Although there are differences in terms on the number of modification sites and stoichiometry, both studies have reported that 

\(MT-ND5\) is the most frequently modified mitochondrial mRNA. Li et al reported that the m'\(A\) modification in \(MT-COI, MT-CO2, MT-CO3, MT-CYB\) and \(MT-ND4L\) is dependent upon TRMT61C, a methyltransferase for m'\(A\) methylation of mitochondrial tRNAs. While these studies have provided a map on mitochondrial mRNA modifications, further research will be required to study the functional role of m'\(A\) methylation and the regulation of modification.

RNA-binding protein leucine-rich pentatricopeptide rich domain containing (LRPPRC) is required for the stability of mitochondrial transcripts. LRPPRC is a large (130 kD) and highly abundant protein that predominantly localized in the mitochondrial matrix. Loss of LRPPRC severely depletes mitochondrial mRNAs but has not effect on rRNAs and tRNAs. This leads to translation defects and impairs assembly of OXPHOS subunits. SRA stem-loop interacting RNA binding protein (SLIRP) forms complex with LRPPRC to regulate the stability of mRNAs and mitochondrial translation. LRPPRC-SLIRP complex has been reported to play a role in mitochondrial polyadenylation where loss of either LRPPRC or SLIRP results in a reduction of polyadenylated mRNAs. Another RNA-binding protein G-rich sequence factor 1 (GRSF1) has been reported to co-localize with processed mRNAs in mitochondrial RNA granules. Knockdown of GRSF1 affects processing of primary transcript, results in mitochondrial translation defects and assembly of OXPHOS. Recent study has reported tissue- and transcript-specific deadenylation of mitochondrial mRNAs in that pathogenic variant of LRPPRC, suggesting that LRPPRC may be involved in 3' end processing of a subset of mRNAs.

2.2.6.2 rRNA modifications

Mitochondrial rRNAs (12S and 16S) undergo post-transcriptional modifications for correct folding, stability and ribosome assembly. These modifications include 2'-O-ribose methylations, pseudouridylation and base methylations. In 16S rRNA, RNA pseudouridine Synthase D4 (RPUSD4) pseudouridylates position U1397 and mitochondrial rRNA methyltransferase (MRMs) methylates 2'-O-ribose at the aminoacyl and peptidyl sites of peptidyl transferase center (PTC). Methylation at the A loop region of 16S rRNA are important for facilitating interactions with aminoacylated tRNAs. In addition, tRNA methyltransferase TRMT61B introduces m'\(A\) methylation at position A947 of 16S and may contribute to stabilizing the assembled 55S monosomes during translation.
Five base modifications have been identified in 12S rRNA, two of which are dimethylations at m6A936 and m6A 937. The enzymes involved are S-adenosylmethionine (SAM)-dependent methyltransferases TFB1M and TFB2M, which were originally characterized as mitochondrial transcription factors269,270. A subsequent in vivo assay has revealed TFB1M as the main enzyme responsible for base modifications of 12S rRNA, while TFB2M functions as transcription factor271. Knockout of TFB1M in murine model leads to embryonic lethality, loss of reduces stability of small subunit and impairs mitochondrial translation272.

2.2.6.3 tRNA modifications

This section focuses on core modifications of mitochondrial tRNAs that contribute to their structural stability and folding. Disruptions to some of these modifications have been shown to impair mitochondrial translation. Following transcription and processing, mitochondrial tRNAs are matured by the addition of CCA sequence to their 3’ end. This maturation step is essential for aminoacylation and is catalysed by tRNA-nucleotidyltransferase 1 (TRNT1)273. Following aminoacylation, a subset of tRNAMet destined to act in translation initiation rather than elongation undergoes formylation by the mitochondrial methionyl-tRNA formyltransferase (MTFMT) to produce mt-tRNA^fMet 274. Formylation of tRNA^Met facilitates recruitment of mitochondrial initiation factor 2 (MTIF2) to initiate translation275. Different types of base modification have been found at the wobble position of mitochondrial tRNA. NOP2/Sun RNA Methyltransferase 3 (NSUN3) and demethylase ALKB1H introduce 5-formylcytosine at the wobble position (f^5C34)276. Mitochondrial tRNA translation optimization 1 (MTO1) and GTP Binding Protein 3 (GTPBP3) convert uridine at position 34 to tm^5U 277,278 and 2-thiouridylase MTU1 (TRMU) catalyzes 2-thiolation of tm^5U to tm^5s^2U279. Base methylations are important modifications that can influence the structure of tRNAs. The presence of m^1A^9 in 19 out of the 22 mitochondrial tRNAs implies the important of methylation at this position228. Pairing of unmodified A9 of mitochondrial tRNA^Lys with U64 leads to misfolding, causing the tRNA unrecognizable by the cognate lysyl-tRNA synthetase280,281,282. MRPP1 and MRPP2 play a dual functional role in 5’ end processing of tRNA and methylation of position A9. Pathogenic mutations in HSD17B10 encoding MRPP2 show reduced dehydrogenase activity and decreased m^1A9/G9 modifications229. The modification at position A9 is sufficient to induce formation of clover leaf structure by disrupting Watson-Crick base pairing with U64 in the extended hairpin281. The tertiary structure of tRNA is further enhanced by base methylation at position 58 on the T loop, catalysed by TRMT61B283. Modification at m^1A58 in tRNA^Lys is found to be absent in patients diagnosed with MERRF, affecting mitochondrial nascent chain synthesis and stability284. Over-expression of
TRMT61B restores modification and increases mitochondrial nascent chain synthesis, thus demonstrating the importance of m\(^1\)A58 modification in regulating mitochondrial translation.

### 2.3 Mitochondrial protein synthesis

#### 2.3.1 The mitochondrial translation machinery

**2.3.1.1 Mitochondrial ribosomes**

Mitochondria are equipped with their own set of mitochondrial ribosomes to synthesize 13 proteins that are core components of OXPHOS complexes. Mammalian mitochondrial ribosomes were first identified and isolated from rat liver\(^{285,286}\). Mitochondrial ribosomes resemble bacterial ribosomes in terms of their susceptibility to antibiotics, which has inhibitory effect on translation\(^{287}\). However, their structure and physical-chemical properties are distinct from bacterial ribosomes. For instance, mammalian mitochondrial ribosomes have low RNA: protein ratio of 1:2, as opposed to the 2:1 ratio found in bacterial ribosomes.

Mammalian mitochondrial ribosomes sediment as 55S particles and are composed of 39S large (mt-LSU) and 28S small (mt-SSU) subunits\(^{286,288}\). The rRNA components (12S and 16S) of these two subunits are encoded by mitochondrial genome. Mammalian mt-LSU lacks 5S rRNA and is replaced by either mtDNA-encoded tRNA\(^{\text{Val}}\) or tRNA\(^{\text{Phe}}\), which acts as a scaffold to bind MRPs\(^{289,290}\). Despite their low RNA content, mammalian mitochondrial ribosomes are larger than bacterial ribosomes, based on particle mass and physical dimensions\(^{291}\). The increased size is due to the extra mitochondrial ribosomal proteins (MRPs) that are either homologues of bacterial ribosomal proteins or completely new and unique. Another distinctive feature of mitochondrial ribosomes is the acquisition of an intrinsic GTPase activity through GTP-binding protein mS29. It is located on the subunit interface and involves in coordinating two mitochondrial intersubunit bridges (mB1a and mB1b)\(^{292}\).

Human mt-SSU is composed of 12S rRNA and 30 structural proteins, of which, 14 are mitochondrial-specific\(^{292,293}\). The entrance of the mRNA channel has been remodelled and widened by uS3m and uS5m\(^{292}\). However, the diameter of the entrance is less than a duplex RNA. Therefore, only single-stranded mRNA can enter the channel. A pentatricopeptide-repeat (PPR) protein mS39\(^{292}\) is found in
close proximity to the entrance and bound to the solvent side of the head domain. The RNA-binding feature and location of mS39 suggest its involvement in recruiting mRNA to the monosome.

Human mt-LSU is composed of 16S rRNA and 48 proteins, of which, 21 are mitochondrial-specific\(^{292}\). The bacterial LSU contains 23S and 5S rRNA that is located in the central protuberance\(^{294}\). However, cryo-EM structures of human and porcine mt-LSU confirmed the absence of 5S rRNA. Instead, the region is occupied by mitochondrial tRNA\(^{\text{Val}}\) (human)\(^{289}\) and tRNA\(^{\text{Phe}}\) (porcine)\(^{295}\). Interestingly, these two tRNAs are located adjacent to 12S and 16S rRNA. Analysis of mt-LSU from multiple tissues collected from different mammalian species, only tRNA\(^{\text{Val}}\) and tRNA\(^{\text{Phe}}\) are incorporated into the ribosomes. Furthermore, the selection is not tissue-specific and human mitochondrial ribosomes can switch to incorporate tRNA\(^{\text{Phe}}\) when tRNA\(^{\text{Val}}\) is severely depleted\(^{290}\).

Due to the absence of 5S rRNA and the incorporation of tRNA\(^{\text{Val}}\) and associated proteins (uL5, bL25 and bL31)\(^{289}\), the central protuberance of the human mt-LSU is significantly different from other ribosomes. Despite the remodelling, the functions of central protuberance are preserved to interact with the head domain of mt-SSU and ribosome-bound tRNAs. These interactions are important for the formation of intersubunit bridges and fidelity of translation\(^{296}\) and are mediated by mitochondrial-specific cluster\(^{289}\).

The PTC of the ribosome is highly conserved and catalyzes peptide bond formation and hydrolysis during protein synthesis. The rRNA elements in PTC mammalian ribosome is similar to those found in bacteria and cytoplasmic ribosomes. The aminoacyl (A), peptidyl (P) and exit (E) sites in mammalian ribosomes have remain conserved throughout the evolution. The A-site is important for binding to the charged tRNA during protein synthesis through its finger element. Although the A-site finger has been lost during evolution, mt-LSU has the ability to maintain contact between tRNAs and 16S rRNA\(^{297,295}\). The P-site is highly preserved but stronger interactions with tRNAs have been observed as compared to the bacterial ribosomes\(^{298}\). This is due to the interactions between P-site finger and the T loop of the bound tRNAs, that may contribute in maintaining the orientation of tRNAs during peptide bond formation. The polypeptide exit tunnel (PES) consists of a ring of conserved proteins and additional mitochondrial-specific elements critical for processing and folding of nascent polypeptides. mL39, mL44 and mL45 form a layer of proteins on top of the conserved proteins surrounding the exit tunnel. Both mL39 and mL44 show homology to RNA-binding proteins and associate with 39S via protein-protein interactions\(^{295}\). The position and orientation of mL45 at
the PES support the binding of mitochondrial ribosomes to the inner membrane for co-translational insertion of nascent polypeptide by OXA1L insertase.  

2.3.2 Mitochondrial translation cycle

Mitochondrial translation comprises of four phases: initiation, elongation, termination and recycling of ribosomes.

2.3.2.1 Translation initiation

During initiation, mitochondrial mRNA is bound by mitochondrial initiation factor mt-IF3 to prevent premature association with mt-LSU. Mt-IF3 interacts with mS37 to keep the mt-SSU in a conformation favourable for subsequent accommodation of mtIF2. The mtIF2 is a multi-domain GTPase that promotes binding of initiator tRNA. Unlike in bacteria, human mitochondrial tRNA\textsuperscript{Met} can act as initiator and elongator tRNA\textsuperscript{Met}. A subset of tRNA\textsuperscript{Met} is formylated that leads to an increase in affinity to mtIF2. The fMet-tRNA\textsuperscript{Met} has decreased affinity to elongation factor mtEF-Tu, a GTPase translation factor that delivers elongator tRNA\textsuperscript{Met} during elongation phase. This ensures that only fMet-tRNA\textsuperscript{Met} is recruited to the P-site during initiation. Once mitochondrial mRNA is recruited to mt-SSU, the mtIF2-bound GTP is hydrolysed and initiation factors are released. The mt-SSU can now associate with mt-LSU, forming the monosome.

2.3.2.2 Translation elongation

The elongation of the nascent chain begins once the monosome is formed. This first step of elongation phase is the formation of ternary complex, composing of mtEF-Tu, GTP and a charged tRNA. The ternary complex promotes binding of the charged tRNA to the A-site. Once the correct codon-anticodon pair is formed, GTP is hydrolyzed and leads to the release of mtEF-Tu-GDP complex. This complex is dissociated by nucleotide exchange factor mtEF-Ts to restore the active GTP-mtEF-Tu complex. The charged tRNA at the A-site changes its conformation to catalyze peptide bond formation. This facilitates the transfer of polypeptide chain from the peptidyl-tRNA at the P-site to the A-site tRNA. Next, elongation factor mtEFG1 promotes the translocation of tRNA and mRNA by one codon on the ribosome. This re-positions the deacylated P-site tRNA to E-site which is then released from the ribosome. The A-site is now ready to accept the next charged tRNA and the cycle continues until a stop codon is presented at the A-site.
2.3.2.3 Translation termination and recycling of ribosomes

The nascent chain needs to be released from the mitochondrial ribosomes once a stop codon enters the A-site. In human mitochondria, mitochondrial release factor 1a (mtRF1a) is thought to be sufficient to terminate all 13 open reading frames (ORFs)\(^{303}\). Similar to bacterial release factors, mtRF1a possesses the GGQ catalytic domain required for peptide release\(^{304}\). mtRF1a is a class 1 release factor that recognizes UAA and UAG stop codons via its codon recognition domain\(^{305}\). Mitochondria deviate from the universal genetic codes that are highly conserved among organisms. For instance, UGA has been re-assigned to code for tryptophan instead of a stop codon\(^{306}\). Furthermore, the ORFs of \textit{MT-CO1} and \textit{MT-ND6} end with AGA and AGG, respectively. These two codons code for arginine but have been reassigned as alternative stop codons due to the absence of mtDNA-encoded cognate tRNAs\(^{243}\).

In contrast to mtRF1a, ICT1 is a release factor that lacks a codon recognition domain and possesses a positively-charged C-terminal extension\(^{307}\). ICT1 is homologous to bacterial rescue factor ArfB and catalyzes the release of nascent chain from stalled ribosomes in a codon-independent manner\(^{305}\). It is a structural component (mL62) of mt-LSU and has no rescue activity\(^{308}\). However, purified ICT1 has been shown to possess peptide-release activity when the mRNA is less than 14 nucleotides pass the A-site\(^{309}\). Recent cryo-EM structure shows ICT1 specifically bound to ribosomes with an empty A-site\(^{305}\). Although EM density shows the binding of ICT1 to ribosome engaged with mRNA with an alternative AGA codon, most of the ICT-bound ribosomes do not harbour mRNA. Similar to ICT1, release factor MTRFR (C12orf65) has been proposed to rescue stalled mitochondrial ribosomes\(^{305}\). MTRFR does not engage with the ribosomes under conditions where mRNA is lacking stop codon or ends with non-canonical stop codon\(^{305}\). However, under the conditions where aminoacylated tRNAs are limiting, MTRFR cooperates with RNA-binding protein MTRES1 (C6orf293) to release nascent chain from the stalled ribosome\(^{310}\). In addition, MTRFR has been shown to act exclusively on mt-LSU and not 55S monosome\(^{310}\). Collectively, these cryo-EM data suggest MTRFR may execute different mode of mechanisms, depending on the physiological conditions of the stalled ribosomes.

After the release of nascent chain, the mRNA and deacylated tRNA remain bound to the ribosome, forming what is known as the post-termination complex. To initiate new round of translation, the two ribosomal subunits must be split to release the bound ligands. The dissociation of the subunits is catalysed by mitochondrial recycling factor mtRRF and elongation factor mtEFG2. The binding of mtRRF to mt-LSU locks the 55S ribosome in a fully rotated state\(^{311}\). The rotation conformation
promotes binding of mtEFG2 and catalyse the dissociation of subunits by destabilizing intersubunit bridges in the unrotated 55S ribosome.  

2.3.3 Co-translational membrane-insertion of nascent chain

In human, co-translation insertion is mediated by OXA1L, a member of the conserved YidC/Oxa1/Alb3 family of insertases. Members of this family function as insertases and chaperones for transmembrane proteins that are localized in bacterial cytoplasmic membrane, mitochondrial inner membrane and chloroplast thylakoid membrane. The YidC/Oxa1/Alb3 family proteins span the membrane five times, with the exception of YidC in gram-negative bacteria where it spans the membrane six times.

A recent cryo-EM structure of human mitochondrial ribosome during protein synthesis provides the first mechanistic evidence of OXA1L-mediated co-translational insertion of nascent chain. OXA1L binds to mitochondrial large subunit at three distinct sites and induces conformational changes of mL45 in the exit tunnel, resulting in the formation of two mitochondrial-specific constriction sites. The constrictions limit helix formation of nascent chain, restricting premature folding while emerging from the exit tunnel. mL45-mediated positioning of the exit tunnel maintains a gap between ribosome surface and OXA1L, facilitating co-translational processing of nascent chain prior to membrane insertion.

Oxa1 was first discovered in *Saccharomyces cerevisiae*, where Oxa1 mutant showed defects in the assembly of cytochrome c oxidase (complex IV) and was respiratory deficient. In addition, Oxa1 is important in the assembly of the F$_o$ sector of F$_1$F$_o$ ATP synthase by physically interacting with Atp9 subunit in a post-translational manner. Although Atp9 forms an oligomeric complex with F$_1$ subunits, its assembly with Atp6 is perturbed in the absence of Oxa1. The role of Oxa1 as an insertase was based on the analysis of isolated mitochondria from yeast Oxa1 mutant. The insertion of both mtDNA- and nuclear-encoded proteins into the inner membrane was impaired in the absence of Oxa1. Among the mtDNA-encoded proteins, insertion of Cox2 is strictly dependent on Oxa1.

In yeast, Cox2 is a polytopic protein that is synthesized as a precursor protein with a cleavable pre-sequence at the N-terminal. Cox2 spans the inner membrane twice, with its N and C terminus exposed to the intermembrane space. The export of Cox2 N-terminal from the matrix to intermembrane space is mediated by Oxa1. The loss of Oxa1 results in an accumulation of Cox2 precursor proteins in the
matrix and failure in membrane insertion\textsuperscript{319,320}. Export of Cox2 C-terminal requires membrane potential\textsuperscript{319} and is mediated by inner membrane proteins Cox18, Pnt1 and Mss2\textsuperscript{321,322}.

Human OXA1L shares 33\% sequence identity with yeast Oxa1 and was identified through a functional complementation experiment\textsuperscript{323}. Expression of human \textit{OXAIL} in yeast \textit{Oxa1} mutant partially rescued respiration defects and restored assembly of complex IV. Previously, knockdown of human \textit{OXAIL} was reported to impair assembly of respiratory complex I and V but has no effect on the activity of complex IV\textsuperscript{6}. However, biallelic \textit{OXAIL} pathogenic mutations identified by whole exome sequencing show depletion of OXA1L affects the assembly of complex IV. Clinical presentations include childhood onset encephalopathy, hypotonia and developmental delay\textsuperscript{5}.

### 2.4 Mitochondrial protein quality control

Owing to their endosymbiotic origin, mitochondrial chaperones and proteases are closely related to their bacterial counterparts. These quality control machineries are distributed across different subcompartments, monitoring mitochondrial protein synthesis, protein import, folding and turnover (Figure 5).

\figure{5}{A simplified diagram illustrating the distribution of chaperones and proteases in mitochondria.}
2.4.1 Protein import and sorting pathways

Mammalian mitochondria contain ~1500 proteins and 99% are encoded by nuclear genes\(^{324}\). The nuclear-encoded mitochondrial proteins are synthesized on cytosolic ribosomes and imported into mitochondria as precursor proteins\(^{325,326}\). Depending on the final localization within the organelle, precursor proteins can carry different types of presequences that are non-cleavable and remain as part of the mature protein\(^{325,327}\). The precursor proteins contain mitochondrial targeting signal (MTS) presequence that direct them to mitochondria and into the correct subcompartment. MTS are categorized as cleavable and non-cleavable\(^{325,327}\). About 60% of precursor proteins are synthesized with cleavable MTS localized at their N-terminal\(^{328}\). These MTS are positively charged and consist of 15 to 50 amino acids. They are proteolytically removed by mitochondrial processing peptidase (MPP) and other proteases after import into mitochondria\(^{329,330}\).

The translocase of the outer membrane (TOM) complex is the common entry gate for majority of the nuclear-encoded mitochondrial proteins. After passing through the outer membrane, precursor proteins are sorted through different pathways into the subcompartments\(^{331}\). Precursor proteins with cleavable MTS interact with receptors Tom20 and Tom22 and translocate across outer membrane through the Tom40 channel. Presequence translocase of the inner membrane (TIM23) complex translocate precursor proteins into the inner membrane in a membrane potential-dependent manner. While the membrane potential is sufficient to translocate precursor proteins into the inner membrane, translocation of proteins destined for matrix requires presequence translocase-associated motor (PAM)\(^{332}\). The central subunit of PAM is ATP-dependent mitochondrial heat-shock protein 70 (mtHsp70) that facilitates the movement of polypeptide into the matrix through cycles of binding and release of substrate\(^{333}\).

Upon import into the matrix, the presequences are cleaved by heterodimer enzyme MPP. Several precursor proteins undergo a second sequential cleavage step in the matrix by one of the two processing enzymes, intermediate cleaving peptidase (Icp55) and octapeptidyl aminopeptidase (Oct1). The mitochondrial intermediate peptidase Oct1 removes N-terminal octapeptide after cleavage by MPP. This converts unstable intermediates formed by MPP into stable mature proteins\(^{334}\). The same function has been proposed for Icp55 where it removes one amino acid residue after processing by MPP\(^{328}\). After proteolytic processing, matrix proteins are folded into their mature and active forms by Hsp60/Hsp10 chaperonin complex. Members of the J-protein family sequester and deliver unfolded substrates to mtHsp70 and stimulates ATP hydrolysis to stabilize protein interaction\(^{335}\).
Lateral sorting of precursor proteins into the inner membrane is driven by the membrane potential. In addition to presequences, the inner membrane-sorted precursor proteins possess at least one hydrophobic sorting signal that arrests translocation in the TIM23 complex and mediates the lateral release of polypeptide into the inner membrane. For some precursor proteins, the sorting signal is removed by inner membrane peptidase (IMP) and leads to the release of polypeptides into the IMS. The TIM23 complex switches between two forms. One form associates with PAM for protein translocation into the matrix. The second form does not contain the motor and mediates lateral insertion of proteins into the inner membrane. Subunit Tim21 is only present in the sorting form of TIM23 and exists in two modular states. During the early import stage, Tim21 transiently binds to TOM complex and facilitates transfer of precursor proteins from TOM to TIM23. Tim21 physically interacts with supercomplex of complex III (\(bc_1\) complex) and complex IV (cytochrome c oxidase). This interaction stimulates membrane potential-dependent protein sorting and promotes insertion of sorted precursor proteins into the inner membrane.

The carrier import pathway transports large hydrophobic proteins that are synthesized without cleavable presequences. These proteins are mainly metabolite carriers that are integrated into the inner membrane via \(\alpha\)-helical transmembrane segments. The carrier proteins contain three to six non-contiguous internal targeting elements that are assumed to cooperate in binding of precursor proteins to surface receptor Tom70 and translocation across the outer membrane. Similar to the presequence pathway, the carrier pathway uses TOM complex as the entry gate into mitochondria. After synthesis on cytosolic ribosomes, the carrier precursor proteins are bound to chaperones HSP70 and HSP90 in the aqueous environment. The ATP-dependent chaperones deliver precursor proteins to mitochondria and interacts directly with Tom70. Receptor Tom70 has two distinct binding sites for precursor proteins and chaperones. Precursor proteins are bound by several Tom70 molecules, transferred to receptor Tom20 and inserted into Tom40 channel. Unlike cleavable precursor proteins that translocate as linear polypeptides, carrier proteins are inserted into Tom40 in a loop formation. TOM40 recruits small hexameric chaperone complex Tim9-Tim10 and bind to precursor proteins to transport them from intermembrane space to inner membrane.

Most of the intermembrane space proteins do not have cleavable presequences but possess signature cysteine motifs that form intramolecular disulphide bond. The precursor proteins are translocated across the outer membrane in reduced and unfolded state. Oxidoreductase Mia40 has been identified as the core component of the oxidative protein folding machinery and catalyzes disulphide bond formation in imported proteins. The disulphide bond promotes stabilization and assembly of
intermembrane space proteins. The sulphydryl oxidase Erv1 oxidizes Mia40 by transferring a disulphide bond to it. Oxidized Mia40 transfers disulphide bond to incoming precursor proteins. Mia40 may function in disulphide relay by shuttling between Erv1 and precursor proteins\(^{345}\), or forming a transient ternary complex with Erv1 and precursor proteins to transfer multiple disulphides\(^{346}\).

Mitochondrial outer membrane possesses two different types of integral proteins, α-helical and β-barrel proteins. Precursor β-barrel proteins are first translocated across outer membrane by TOM complex into the IMS. From the IMS side, these precursor proteins are inserted into the outer membrane by sorting and assembly machinery (SAM). The SAM complex consists of outer membrane-embedded Sam50 and two peripheral proteins Sam35 and Sam37\(^{347}\). Sam50 is homologous to bacterial β-barrel assembly machinery (BAM) and forms the β-barrel channel that is composed of 16 β-strands\(^{348,349}\). The transfer of β-barrel precursor proteins from TOM complex to SAM complex is facilitated by chaperones Tim9-Tim10 and Tim8-Tim13 in the intermembrane space\(^{350,351}\). The C-terminal of most β-barrel precursor proteins functions as a sorting signal that directs membrane insertion and binds to Sam50-Sam35\(^{352}\). Receptor Tom22 and Sam37 promotes transfer of β-barrel precursor proteins to SAM complex by transient formation of TOM-SAM supercomplex\(^{353}\). The β-barrel proteins are folded and released into the lipid phase of outer membrane.

The location of the transmembrane segment of α-helical proteins functions as a targeting signal for import routes. N-terminally anchored proteins use mitochondrial import 1 (Mim1) for membrane insertion. Precursor proteins with internal targeting signals use TOM complex for mitochondrial targeting and SAM complex for membrane insertion\(^{354}\). Although the mechanisms of sorting and insertion of α-helical proteins remain poorly understood, it is clear that these proteins do not follow the classical route through Tom40 channel.

### 2.4.2 Chaperone systems

Molecular chaperones or heat shock proteins (Hsp) were first identified by upregulation of protein expression in heat-stressed *Drosophila melanogaster*\(^{355}\). Further experimentation revealed their involvement in normal cellular activities such as protein stabilization, folding and degradation. Chaperones are classified into five groups according to their molecular weight: Hsp60, Hsp70, Hsp90, Hsp100 and small Hsp\(^{356}\). Client proteins of Hsp are mainly hydrophobic motifs that are exposed to aqueous environment. The chaperone activities of Hsp are often assisted by co-chaperones, which are non-client binding partners of Hsp70 and Hsp90. Co-chaperones may not interact directly with client
proteins. Instead, they stimulate ATPase activity in their associated chaperones and coordinate the cycle of substrate binding and release\textsuperscript{357}. Except for small Hsp, members of the Hsp60, Hsp70 and Hsp100 families have been identified in mitochondria\textsuperscript{358}. Hsp70 and Hsp60 are the two main chaperone systems that mediate the import and folding of nuclear-encoded precursor proteins destined for mitochondrial matrix.

2.4.2.1 Hsp60

Hsp60 is a ubiquitous and highly conserved molecular chaperone belonging to Chaperonin of Group I\textsuperscript{359}. Depending on its location, Hsp60 plays functional role in cellular processing including immune responses and cell proliferation\textsuperscript{360}. Mitochondrial Hsp60 is located in the matrix and assist in protein folding by directly interacting with newly imported precursor proteins\textsuperscript{361}. Hsp60 and its co-chaperone Hsp10 are essential for organelle function by regulating mitochondrial protein biogenesis. Despite its essential function, proteomic studies have shown that only 30\% of the imported matrix precursor proteins require folding by Hsp60\textsuperscript{362}. Few substrates are able to fold without Hsp10 and many do not follow the folding pathways of Hsp60-Hsp10 system\textsuperscript{363}. In contrast to its bacterial homologue GroEL, Hsp60 mainly exists in the form of single heptameric ring\textsuperscript{364}. The Hsp60/Hsp10 complex is composed of two stacked heptameric rings of Hsp60. The binding of Hsp10 to the apical domains of Hsp60 functions as a lid structure to cover the opening of the rings. The inner cavity of each ring is lined with hydrophobic amino acids that interact with unfolded polypeptides. Unlike GroEL, oligomerization and formation of double-ring complex of Hsp60 is dependent on ATP hydrolysis\textsuperscript{365}. Conformational changes of Hsp60/Hsp10 complex are regulated through repetitive cycles of ATP hydrolysis, eventually forming a more hydrophilic environment where substrate proteins can fold into their native conformation\textsuperscript{366}. The importance of Hsp60/Hsp10 complex has been demonstrated in bacteria and yeast, where the loss of either Hsp60 or Hsp10 leads to lethal phenotypes\textsuperscript{361,367,368}. In humans, two monogenic disorders are associated with missense mutations in \textit{HSPD1}. One is a dominant form of hereditary spastic paraplegia (SPG13) and the second is recessive inherited hypomyelinating leukodystrophy (HDL4)\textsuperscript{369,370}. These diseases are rare, as deleterious effects of mutations in genes encoding for Hsp60 and Hsp10 are incompatible with life\textsuperscript{371}.

2.4.2.2 Hsp70

Members of the Hsp70 family are ubiquitously expressed and are characterized by their high structural conservation. There are 10 to 15 different types of Hsp70 localized in the cytoplasm, nucleus, mitochondria, ER and chloroplasts\textsuperscript{372}. Hsp70 is composed of three domains: N-terminal
nucleotide binding domain, substrate binding domain and C-terminal domain that provides a lid-like structure to cover substrate binding site. One key feature of Hsp70 chaperones is their ATP-regulated substrate binding affinity. In ATP-bound state, Hsp70 has low affinity for substrate. The ATPase activity of Hsp70 increases when the substrate domain recognizes and interacts with hydrophobic amino acids. Hydrolysis of ATP increases substrate affinity and results in closing of the substrate binding domain and tight binding of substrate\(^{373}\). The efficiency of ATP hydrolysis is enhanced by co-chaperone Hsp40 (J-domain protein) and nucleotide-exchange factors (NEFs). J-domain proteins can bind and transfer unfolded substrates to Hsp70, thereby stimulating ATP hydrolysis. Subsequent substrate release and dissociation of ADP is stimulated by NEFs, through distortion of the ATP-binding domain of Hsp70\(^{374}\). Hsp70 functions in a myriad of cellular processes, modulating protein folding and translocation across membranes. In many cases, the functional diversity of Hsp70 is determined by the type of J-domain protein partner\(^{375}\).

The main mtHsp70 is encoded by \textit{SSC1} in yeast and is critical for cellular survival under all conditions\(^{376}\). mtHsp70 is indispensable for the import and folding of nuclear-encoded mitochondrial precursor proteins\(^{377}\). During the import process, mtHsp70 uses ATP hydrolysis to drive the translocation of precursor proteins into the matrix. Subsequently, as part of a chaperone complex with Hsp40 (DNAJA3) and NEFs (Mge1 in yeast and GrpEL1 in humans), mtHsp70 promotes folding of precursor proteins into their native conformation\(^{378,379}\). The mammalian mtHsp70, also known as mortalin, was first identified as mortality factor for its activity in senescence and cell death in mouse embryonic fibroblast\(^{380}\). Overexpression of mortalin has been shown to suppress pro-apoptotic effects and increases malignancy of cancer cells\(^{381,382}\). Mortalin is also implicated in the pathogenesis of neurodegenerative diseases, where mortalin expression is lower in the neuronal cells of Parkinson’s disease (PD) patients\(^{383}\). However, it remains to be determined whether the decrease in mortalin expression level is a direct consequence of the disease development.

### 2.4.3 Protease systems

Majority of mitochondrial proteases belong to the AAA+ superfamily of ATPases, also known as ‘chambered proteases’, these enzymes form large oligomeric complexes and degrade polypeptides in an internal cavity. Three proteases have been identified in the matrix compartment: serine peptidase LON, caesinolytic peptidase ClpXP and AFG3L2 metalloprotease. LON and ClpXP are responsible for degrading soluble substrates in the matrix\(^{384}\). Membrane-integrated AFG3L2 protease has its catalytic domain exposed to the matrix side and mainly degrades proteins in the
inner membrane. Another membrane-integrated protease, YME1L has inverted topology with its catalytic domain protruding into the IMS\textsuperscript{385}. Both membrane-tethered mitochondrial proteases are homologs of the bacterial FtsH protease\textsuperscript{386}. Together, these proteases are important for mitochondrial maintenance and adaptations to different physiological conditions.

\subsection*{2.4.3.1 LONP1}

Human Lon protease is encoded by \textit{LONP1} and expressed ubiquitously in tissues and organs, with high level in heart, brain and skeletal muscle\textsuperscript{387}. LONP1 is located in the matrix to degrade misfolded and oxidised proteins, with a small fraction known to regulate mitochondrial nucleoid dynamics\textsuperscript{388,389}. LONP1 consists of an N-terminal domain for substrate recognition, an ATPase domain and a protease domain (P-domain) that contains a highly conserved serine-lysine catalytic dyad\textsuperscript{387}. Subunits of LON protease oligomerize into homohexamers and form an internal degradation chamber. The substrates are unfolded and translocated through the protelytic chamber for degradation in ATP-dependent reactions. Known substrates of LONP1 include oxidized aconitase\textsuperscript{390}, mitochondrial processing peptidase alpha (MPPα)\textsuperscript{391} and TFAM\textsuperscript{392}. A recent cryo-EM structure of LONP1 has revealed underlying mechanisms in substrate proteolysis. Unlike in the bacterial Lon where substrate binding to ATP channel is sufficient to activate the P-domain\textsuperscript{393}, human LONP1 requires additional substrate binding within the protease active site\textsuperscript{394}.

Several studies in yeast and mammals have shown that LON expression is upregulated in response to cellular or environmental stress\textsuperscript{395,396}. The production of reactive oxygen species (ROS) from oxidative phosphorylation can modify surrounding mitochondrial matrix proteins. In response to oxidative stress, LON protease degrades proteins that are sensitive to oxidative damage and prone to aggregation\textsuperscript{396}. In mammals, LON dysfunction is known to cause an accumulation of oxidized proteins\textsuperscript{390,397,398}.

The first human disease linked to mutations in LONP1 was cerebral, ocular, dental, auricular, skeletal (CODAS) syndrome. It is an autosomal recessive inherited disorder characterized by hearing loss, hypotonia, developmental delays, skeletal and dental abnormalities\textsuperscript{399,400}. Majority of the mutations are located in the ATPase domain and result in amino acid substitution. Biochemical defects associated with LONP1 mutations include decreased activity of complex I and IV, loss of ribosomal proteins and pyruvate dehydrogenase deficiency\textsuperscript{401,402}. Recently, an \textit{in vitro} studies has proposed cooperation between LONP1 and mtHsp70 in stabilizing nascent chains of OXA1L\textsuperscript{403}. 
2.4.3.2 ClpXP

The human CLPXP protease complex is formed by chaperone CLPX and peptidase CLPP. CLPX is a member of AAA+ protein superfamily and is the only ATPase component that forms a complex with mammalian CLPP\textsuperscript{404}. The first step in the assembly of CLPXP involves formation of CLPP tetradecamer between two CLPP heptamers. Next, either end of the CLPP tetradecamer is capped with CLPX hexameric rings in the presence of ATP. This triggers a conformational change in CLPP active site and leads to formation of an inner proteolytic chamber for substrate degradation\textsuperscript{404,405}. Substrates are first recognized and unfolded by CLPX, followed by translocation of the unfolded polypeptide into the proteolytic chamber for degradation\textsuperscript{406}. Unlike CLPX which requires ATP to unfold substrate, proteolytic processing of substrate by CLPP is ATP-independent\textsuperscript{407}. Conformational changes in the N-terminal domain of CLPP has been shown to facilitate processive degradation of unfolded substrate\textsuperscript{408,409}. In contrast to \textit{E.coli} ClpP, which forms a stable tetradecamer in the absence of ClpX\textsuperscript{410}, human CLPP remains as an inactive heptameric ring without CLPX\textsuperscript{405,411}. Proximity labelling of CLPXP in mammalian cells has identified components of ETC as interacting partners\textsuperscript{412}. To date, only the GTPase nitric oxide-associated protein (NOA1) has been identified as an endogenous substrate for mitochondrial CLPXP\textsuperscript{413}.

In bacteria, ClpXP plays species-specific role for cell viability, stress tolerance and virulence\textsuperscript{414,415}. Several inhibitors of bacterial ClpP have been developed and exhibit antimicrobial activity\textsuperscript{416,417}. In human, pathogenic mutations in \textit{CLPP} are associated with Perrault syndrome, characterized by hearing loss and premature ovarian failure\textsuperscript{418}. Recently, upregulation of CLPP has been shown in primary and metastatic human tumors\textsuperscript{419,420}. Overexpression of CLPP supports tumor cell proliferation and desensitizes cells to chemotherapy drug cisplatin\textsuperscript{421}. However, transcription factors that lead to dysregulation of CLPP expression in cancer cells have not been fully identified. Thus, future studies would be required to determine how CLPP contributes to the progression of malignancy.

2.4.3.3 AFG3L2

The human AFG3L2 subunit can either forms homo-oligomers or hetero-oligomers with SPG7 (paraplegin)\textsuperscript{422,423,424}. In mouse, a third paralogue AFG3L1 is expressed and forms either homo-oligomers or assembles with AFG3L2 and SPG7\textsuperscript{425}. Each AFG3L2 subunit consists of two N-terminal transmembrane domains, ATPase domain and metalloprotease domain\textsuperscript{426}. A single chain of AFG3L2 has two transmembrane helices with its N-terminus and catalytic domain exposed to the matrix side of the inner membrane\textsuperscript{427}. Substrate recruitment and engagement by N- and C-termini of
AFG3L2 is the first step for processing of membrane-associated substrates\textsuperscript{428}. This is followed by substrate translocation through the central pore-like ATPase channel and the transfer of unfolded polypeptide to proteolytic chamber\textsuperscript{429}. Both ATPase and protease domains can function independently and has been shown to be important for maintaining the integrity of the inner membrane and mitochondrial protein synthesis\textsuperscript{110}.

Recent studies have shown how AFG3L2 dysfunction impairs mitochondrial protein synthesis in specific mitochondrial pathogenic variant\textsuperscript{430} and activation of ribosomal decay pathway upon heat stress\textsuperscript{110}. In addition, AFG3L2 is essential for axonal development in mammals. Loss or reduction of wild type AFG3L2 leads to pleiotropic phenotypes including impaired mitochondrial transport, mitochondrial fragmentation and reduced mitochondrial membrane potential\textsuperscript{12,13,431,432}. Pathogenic point mutations in \textit{AFG3L2} or \textit{SPG7} are linked to multiple neurodegenerative disorders in human that are characterized by ataxia and paraplegia\textsuperscript{10,424,433,434,435}. Cryo-EM structure of AFG3L2 mapping the location of pathogenic mutations have revealed that autosomal mutations are localized in the interface of hexameric subunit while recessive mutations are found in the protease active sites\textsuperscript{429}.

\textbf{2.4.3.4 YME1L}

Human YME1L contains a short N-terminal domain, a single transmembrane segment, ATPase domain and a zinc metalloprotease domain belonging to the M41 peptidase family\textsuperscript{436,437}. Insertion of the insoluble transmembrane segment into the inner membrane exposes the soluble ATPase and protease domains into the aqueous IMS. YME1L mainly degrades inner membrane polypeptides but is also capable of degrading soluble proteins of the IMS\textsuperscript{438}. A biochemical analysis of YME1L activity revealed the dual nature of the AAA\textsuperscript{+} proteases. The N-terminal domain preceding the AAA domain is responsible for the recognition and binding of substrate polypeptides. When expressed alone, this domain exhibits chaperone-like activity\textsuperscript{439}. Analysis of folding and aggregation reactions in the IMS further supported the role of YME1L as an IMS chaperone\textsuperscript{440}.

Known substrates of YME1L and its homologues include OPA1. Loss of YME1L1 accelerates OMA1-dependent L-OPA1 cleavage, resulting in S-OPA1 accumulation and mitochondrial network fragmentation\textsuperscript{111,441}. However, the mechanism of Yme1L in the regulation of mitochondrial morphology remains obscure. Other substrates of YME1L include soluble IMS proteins\textsuperscript{438} and integral membrane components of the respiratory chain complexes\textsuperscript{442}.
YME1L is essential for embryonic development in mice, whereas YME1L deficiency in adult cardiomyocytes causes dilated cardiomyopathy and heart failure\textsuperscript{111,441}. The loss of YME1L in the nervous system impairs mitochondrial morphology and proteostasis throughout the nervous system but results in striking cell-type-specific neurological defects in mice\textsuperscript{443}. Homozygous recessive mutations in human \textit{YME1L} triggers mitochondrial fragmentation, causes neuromuscular disorder with intellectual disability, developmental delay, optic atrophy as well as ataxia and movement deficiencies\textsuperscript{444}.

\subsection*{2.4.3.5 OMA1}

The overlapping with m-AAA protease 1 (OMA1) metalloprotease was first identified in yeast that demonstrated proteolytic activity for misfolded inner membrane proteins\textsuperscript{445}. OMA1 is localized in the inner membrane and consists of an N-terminal domain and a C-terminal M48 metalloprotease domain\textsuperscript{445}. The positively-charged N-terminal that is oriented towards the matrix side is important for sensing changes in membrane potential\textsuperscript{107}. The C-terminal domain is facing towards the IMS and is important for OMA1 proteolytic function. OMA1 constitutively cleave the membrane-anchored L-OPA1 at S1 site and the processing is enhanced upon exposure to stress stimuli\textsuperscript{107}. Dissipation of membrane potential by CCCP triggers OMA1 to proteolytically process L-OPA1. Loss of OMA1 in mammalian cultured cells has been shown to inhibit CCCP-induced OPA1 processing\textsuperscript{109,445}. AFG3L2 dysfunction is known to activate OMA1 that is independent of membrane potential and results in mitochondrial fragmentation\textsuperscript{109,110,446}. Recent studies have established a molecular link between AFG3L2 dysfunction and OMA1 activation\textsuperscript{109,110}. AFG3L2 plays an important role in interacting with the N-terminal of mitochondrial nascent chains as they emerge from the ribosomes and disruption to this step results in OMA1 activation. One important finding is that blocking mitochondrial translation elongation completely inhibits OMA1 activation in the absence of AFG3L2\textsuperscript{110}. Although the stress has been identified to originate from mitochondria translation, the mechanism in which the aberrant nascent chain activates OMA1 remains to be elucidated.
3 AIMS OF THE STUDY

The objective of my thesis is to investigate the quality control mechanisms associated with mitochondrial gene expression, and to understand how disruptions in these pathways are integrated into cellular stress responses associated with human diseases.

The specific aims were:

1. To identify key regulatory steps in co-translation quality control of mitochondrial protein synthesis.
2. To study the functional consequences of defective quality control of mitochondrial protein synthesis.
3. To investigate the frequency of mRNA errors in mitochondrial gene expression.
4 MATERIALS AND METHODS

4.1 Cell culture

All cell lines (Table 1) were cultured at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (Lonza) with high glucose supplemented with 10% fetal bovine serum, 1x glutamax and 50 μg/ml uridine. All primary cells were immortalized by expressing E7 and hTERT unless previously immortalized by the donating laboratory. All cells were tested negative for mycoplasma infection (PromoKine). In heat shock experiments and chloramphenicol treatment, cells were treated with 400 μg/ml of chloramphenicol (Sigma) for 72 h before and during heat shock.

Table 1. List of cell lines used in this thesis

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Potential pathogenic variants</th>
<th>Organism</th>
<th>Reference or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type fibroblasts</td>
<td></td>
<td>human</td>
<td>Anu Wartiovaara, University of Helsinki</td>
</tr>
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<td>HEK293</td>
<td></td>
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<td>109</td>
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<tr>
<td>MT-ATP6 m.9205delTA fibroblasts</td>
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<td>human</td>
<td>448</td>
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<tr>
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<td></td>
<td>human</td>
<td>Anne Lombès, Institut Cochin, France</td>
</tr>
<tr>
<td>MT-ATP6 m.9176T&gt;C fibroblasts</td>
<td></td>
<td>human</td>
<td>Anne Lombès, Institut Cochin, France</td>
</tr>
<tr>
<td>MT-ATP6 m.8993T&gt;G fibroblasts</td>
<td></td>
<td>human</td>
<td>Anne Lombès, Institut Cochin, France</td>
</tr>
<tr>
<td>MT-ATP6 m.8993T&gt;C fibroblasts</td>
<td></td>
<td>human</td>
<td>Anne Lombès, Institut Cochin, France</td>
</tr>
<tr>
<td>TRMT10C c.[542G&gt;T];[814A&gt;G]</td>
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<td>234</td>
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<td>HSD17B10 c.277+3A&gt;G</td>
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<td>human</td>
<td>Robert Taylor, Newcastle University, United kingdom</td>
</tr>
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<td>p.Ala485Val</td>
<td>human</td>
<td>449</td>
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<tr>
<td>KIAA0391 c.[1334G&gt;A];[1197dupA]</td>
<td>p.[Arg445Gln];[Ser400IlefsX6]</td>
<td>human</td>
<td>Robert Taylor, Newcastle University, United kingdom</td>
</tr>
<tr>
<td>ELAC2 c.297-2_297delinsTG</td>
<td>p.Arg781His</td>
<td>human</td>
<td>242</td>
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<tr>
<td>ELAC2 c.460T&gt;C</td>
<td>p.Phe154Leu</td>
<td>human</td>
<td>241</td>
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<tr>
<td>COX10 deficient fibroblasts</td>
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<td>human</td>
<td>450</td>
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<tr>
<td>Opa1 knockout</td>
<td></td>
<td>mouse</td>
<td>Thomas Langer, University of Cologne, Germany</td>
</tr>
</tbody>
</table>
4.1.1 siRNA knockdown

Cells were seeded in 6 cm culture dish to obtain 30-50% confluency at the time of transfection. In an empty micro-centrifuge tube, 3.75 μL of siRNAs (25 nM) (Table 2) was mixed with 5 μL Lipofectamine™ RNAiMAX (Thermo Fisher) and 500 μL Opti-MEM™ reduced serum medium (Thermo Fisher) and incubated at room temperature for 10 min. siRNA mixture was added to the cells containing 2 mL of fresh media and incubated in 37°C incubator for 5 h. Following incubation, media was removed and replaced with fresh pre-warmed media. Cells were transfected on day 1 and day 3 and collected on either day 5 or day 8 for analysis. All siRNA knockdowns were evaluated by immunoblotting using specific antibodies.

Table 2. List of siRNAs (Thermo Fisher) used in this dissertation.

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Cat #</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC (medium GC content)</td>
<td>12935300</td>
<td>Scrambled control sequence</td>
</tr>
<tr>
<td>AFG3L2</td>
<td>HSS116886</td>
<td>GAG UAG UAG ACA GAU UGG AAG UCG U</td>
</tr>
<tr>
<td>ATP5B</td>
<td>HSS181745</td>
<td>CAU CUC CCA GAA CAG GCC UUC UAU A</td>
</tr>
<tr>
<td>OPA1</td>
<td>HSS107431</td>
<td>GGU CCU CAC CAU GUG GCC CUU UUA A</td>
</tr>
<tr>
<td>OXA1L</td>
<td>HSS107511</td>
<td>ACC ACU GGC AGU CAC UGC UAC AAU</td>
</tr>
<tr>
<td>TMEM70</td>
<td>HSS123635</td>
<td>CGA GUC UGA UUG GCC UUA CAU UUC U</td>
</tr>
</tbody>
</table>

4.1.2 Cloning

All cDNAs used in retroviral transduction experiments were generated by PCR using DNA isolated from wild-type fibroblasts as template. Primers (Table 3) were designed to contain attB1 sites at their 5’ end to facilitate Gateway® cloning into donor vector pDONR201. The PCR protocol was consisted of two parts and KAPA HiFi PCR kit (Roche) was used to generate the amplicons. In the first part, a 50 μL reaction was set up by mixing 10 μL of 5x KAPA HiFi buffer, 1.5 μL of 10 mM dNTPs, 0.5 μL of each primer (20 μM), 1 μL of DNA template (100 ng), 1 μL of KAPA HiFi DNA Polymerase and 36.5 μL of nuclease-free water. Amplifications were performed at 95 °C for 2 min, followed by 10 cycles of 98 °C for 20 sec, 55 °C for 30 sec and 68 °C for 2 min. The amplified products were stored at 4 °C. In the second part, 10 μL of PCR product from the first part was mixed with 8 μL 5x KAPA HiFi buffer, 1.5 μL of 10 mM dNTPs, 2 μL of 20 μM attB1 primer, 2 μL of 20 μM attB2 primer, 1 μL of KAPA HiFi DNA Polymerase and 25.5 μL water. Amplifications were performed at 95 °C for 2 min, 5 cycles of 98 °C for 20 sec, 45 °C for 30 sec and 68 °C for 2 min, followed by
followed by 15 cycles of 98 °C for 20 sec, 55 °C for 30 sec, 68 °C for 2 min and stored at 4 °C. The amplicons were analyzed on 1 % Tris-acetate-EDTA (TAE) agarose gel and gel purified with NucleoSpin Gel and PCR Clean-up kit (Machery Nagel).

Gateway entry clone was generated by setting up a 10 μL reaction consisting of 150 ng pDONR201, 150 ng attB-flanked purified PCR product, 1 μL Gateway BP clonase II (Thermo Fisher) and Tris-EDTA (TE) buffer, pH 8.0. The reaction mixture was incubated at room temperature for 1 h, followed by addition of 1 μL of 20 mg/mL proteinase K (Thermo Fisher) and incubated at 37 °C for 10 min. For transformation, 1 μL of the BP reaction mixture was added to 200 μL of chemically-competent DH10B and incubated on ice for 30 min. Heat shock was performed at 42 °C for 30 sec, kept on ice for 2 min, followed by addition of 500 μL of LB medium and put on shaking incubator for 1 h. Kanamycin-supplemented (50 mg/mL) LB agar plate was pre-warmed at 37 °C and 50 μL of the transformed DH10B was spread on the plate and incubated at 37 °C overnight. A number of clones were selected from the plate for plasmid isolation with NucleoSpin Plasmid Mini kit (Machery Nagel), digested with BsrGI restriction enzyme (NEB) for 1 h at 37 °C and separated on 1 % TAE agarose gel. Positive clones that had the correct fragment size were sent for Sanger sequencing for verification.

Gateway expression clone was generated by setting up a 10 μL reaction consisting of 150 ng of Gateway converted destination vectors (pBABE-puro or pMXs-IRES-Blasticidin), 150 ng of verified entry clones, 1 μL Gateway LR clonase II (Thermo Fisher) and TE buffer, pH 8.0. The reaction mixture was incubated at room temperature for 1 h, followed by addition of 1 μL of 20 mg/mL proteinase K (Thermo Fisher) and incubated at 37 °C for 10 min. For transformation, 1 μL of the LR reaction mixture was added to 200 μL of chemically-competent DH10B and incubated on ice for 30 min. Heat shock was performed at 42 °C for 30 sec, kept on ice for 2 min, followed by addition of 500 μL of LB medium and put on shaking incubator for 1 h. Ampicillin-supplemented (100 mg/mL) LB agar plate was pre-warmed at 37 °C and 50 μL of the transformed DH10B was spread on the plate and incubated at 37 °C overnight. A number of clones were selected from the plate for plasmid isolation with NucleoSpin Plasmid Mini kit (Machery Nagel), the presence of insert was verified by digestion with BsrGI restriction enzyme (NEB) for 1 h at 37 °C and separated on 1 % TAE agarose gel.
Table 3. List of primers used in cloning experiments. Underlined sequence indicates partial attB site.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene</th>
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<tbody>
<tr>
<td>attB-MRPP1 forward</td>
<td>TRMT10C</td>
<td>AAAAAGCGAGGGCTACCATGGCTGTTCCTCTCAA</td>
</tr>
<tr>
<td>attB-MRPP1 reverse</td>
<td>TRMT10C</td>
<td>AGAAAGCGCTGGTTTTAATAGCTTTGCTTTTTTA</td>
</tr>
<tr>
<td>attB-MRPP3 forward</td>
<td>KIAA0391</td>
<td>AAAAAGCGAGGGCTACCATGGACCTATTTGTTTGGTATT</td>
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<tr>
<td>attB-MRPP3 reverse</td>
<td>KIAA0391</td>
<td>AGAAAGCGAGGGCTTTAATGTCCTTTGTTGAGGC</td>
</tr>
</tbody>
</table>

4.1.3 Retroviral transduction

Retrovirus was generated by transient transfection of retroviral plasmids into the Phoenix amphotropic packaging line\textsuperscript{451}. 2 μg of DNA was mixed with 200 μL of jetPRIME\textsuperscript{®} (Polyplus-transfection), incubated at room temperature for 10 min and added to cells in a 6-well dish containing 2 mL media. Media was changed once after 24 h of transfection. On the third day, culture media containing the virus were filtered through 0.45 μM syringe filter. 1 μL of 10 mg/mL polybrene transfection reagent (Sigma) was mixed with 1 mL of filtered media and added to 6-well dish containing the recipient cells. Media was changed after 24 h of transduction and followed by three days of antibiotic selection with either puromycin or blasticidin. Following antibiotic selection, cells were allowed to continue growing in standard culture media and collected when they reached about 80 % confluency.

4.2 Immunoblotting

Cells were solubilized in 1x phosphate buffered saline (PBS), 1 % dodecyl-maltoside (DDM), 1 mM phenylmethylsulfonyl fluoride (PMSF), Pierce\textsuperscript{TM} Protease Inhibitor (Thermo Fisher) and incubated on ice for 20 min. Cell lysates were collected by centrifugation at 18 000 x g at 4 °C for 25 min, followed by measurement of protein concentration by Bradford assay (BioRad). All samples were loaded in equal amount of proteins. Samples were prepared by mixing with equal volume of 1x laemmli buffer (60 mM Tris-HCl pH 6.8, 2 % SDS, 10 % glycerol, 0.01 % bromophenol blue, 5 % β-mercaptoethanol), incubated at room temperature for 20 min, separated by Tris-Glycine SDS-PAGE and transferred to nitrocellulose membrane by semi-dry transfer. Membranes were blocked with 1 % milk in Tris-buffered saline/ 0.1 % Tween\textsuperscript{®} 20 (TBST) at room temperature for 1 h and washed twice with TBST. Membranes were incubated with primary antibodies (Table 4) diluted in
5 % BSA/TBST overnight at 4 °C. On the following day, membranes were washed 3x 20 min with TBST, incubation with HRP-conjugated secondary antibodies (Jackson ImmunoResearch) for 1 h at room temperature and washed 3x 20 min with TBST. Signal was detected with LumiGLO® and peroxide reagents (Cell Signaling Technology) on x-ray film or iBright imaging systems (Thermo Fisher). Representative data of independent experiments were cropped in Adobe Photoshop® with only linear corrections applied.

<table>
<thead>
<tr>
<th>Antigen</th>
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<th>Company</th>
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<td>ATP5B</td>
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<td>TOM40</td>
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<td>uL11m</td>
<td>15543-1-AP</td>
<td>Proteintech Group</td>
<td>1:20 000</td>
</tr>
</tbody>
</table>

### 4.3 Isokinetic sucrose gradients

Cells cultured on 150 mm plates were collected by rapidly transferred to ice where the media was removed and cells washed with cold PBS. Cells were lysed (50 mM Tris, pH 7.2, 10 mM Mg(Ac)$_2$, 40 mM NH$_4$Cl 100 mM KCl, 1 % DDM, 1 mM ATP, 400 μg/mL chloramphenicol, and 1 mM PMSF and incubated on ice for 20 min. Cell lysates were clarified following centrifugation for 20 min at 20 000 x g at 4 °C and measurement of protein concentrations by Bradford assay. From each cell lysate, a total of 1 mg of protein was loaded on top of a 16 mL linear 10-30% sucrose gradient (50 mM Tris-HCl, pH 7.2, 10 mM Mg(Ac)$_2$, 40 mM NH$_4$Cl 100 mM KCl, 1 mM ATP and 1 mM PMSF and
centrifuged for 15 h at 74 400 x g at 4 °C (Beckman SW 32.1 Ti). From the gradient, 24 equal volume fractions were collected for either protein or RNA isolation. Samples for protein analysis were precipitated with 13 % trichloroacetic acid (TCA), incubated on ice for 30 min and centrifuged for 30 min at 18 000 x g at 4 °C. Protein pellets were washed twice with cold acetone, air-dried for 5 min, and resuspended in 30 μL 1x laemmli buffer. Samples were heated at 95°C for 5 min, cooled at room temperature, separated on Tris-Glycine SDS-PAGE and transferred to nitrocellulose membrane by semi-dry transfer. Antibodies incubation and signal detection followed standard protocol for immunoblotting as described in the previous section.

4.4 Metabolic labelling of mitochondrial protein synthesis

Cells were washed twice with PBS and incubated in DMEM without cysteine and methionine (Sigma), supplemented with 1x glutamax, 10 % dialyzed FBS and 50 μg/mL uridine for 25 min in 37 °C incubator. This was followed by treating the cells with anisomycin (100 μg/mL) to inhibit cytoplasmic translation and pulse-labelled with 200 μCi/ml EasyTag™ 35S Met-Cys labelling mix (Perkin Elmer). In chase experiments, cells were pulse-labelled for 30 min followed by removal of media. Cells were washed twice with PBS before adding fresh media without the radioisotope and incubated for the indicated time. At the end of pulse-chase experiments, cells were washed twice with cold PBS, centrifuged and resuspended in appropriate amount of PBS for protein measurement by Bradford assay. Equal amounts of sample protein were treated with Benzonase® Nuclease (Sigma) for 30 min on ice and then resuspended in 1x gel loading buffer (186 mM Tris-Cl pH 6.7, 15 % glycerol, 2 % SDS, 0.5 mg/mL bromophenol blue, 6 % β-mercaptoethanol). Samples were left to equilibrate at room temperature for 2 h before separated on 12-20 % gradient Tris-Glycine SDS-PAGE. Gels were dried, exposed with a phosphor screen and scanned with a Typhoon 9400 (GE Healthcare) for quantification. Gels were rehydrated in water and stained with PageBlue™ Protein Staining Solution (Thermo Fisher) to confirm loading.

4.5 Southern blotting

Total DNA was isolated from cultured fibroblasts as described in QIAamp DNA Mini Kit (QIAGEN). DNA (5 μg) was digested with PvuII (NEB) overnight at 37 °C, separated on a 0.8% agarose gel and transferred to Hybond-XL membrane (GE Healthcare) in alkaline transfer buffer (0.4 M NaOH, 1 M NaCl). Two oligonucleotide probes for mtDNA (MT-CO1 5’-GGCTCCAGGGTGGGAGTAG
TTCCCTGC; *MT-ND6* 5'-CCTCCCGAATCAACCCTGACCCCTCC) and three for 18S rDNA (5'-GGCCGGAGGTATCTAGAGTCAC; 5'-TATTCCTAGCTGCGGTATCCAGG; and 5'-ACCATCCAATCGGTAGTGAGACG) were used. Oligonucleotide probes were labelled with radioisotope by mixing 5 μL of 10 mM primers, 2 μL of 10x T4 polynucleotide kinase buffer, 20 units of T4 polynucleotide kinase (NEB), 6 μL nuclease-free water, 50 μCi γ³²P ATP (Perkin Elmer) and incubated at 37 °C for 60 min. The labelling reaction was terminated by adding 2 μL of 0.5 M EDTA and purified with ProbeQuant™ G50 micro columns (GE Healthcare). Membranes were pre-hybridized (50 % formamide, 7 % SDS, 250 mM NaPO₄, pH 7.2, 10 mM EDTA pH 8.0, 240 mM NaCl₂) overnight at 37 °C. The following day, membranes were washed once with 2× SSC/0.1 %SDS for 60 min, followed by 0.5× SSC/0.1 % SDS for 60 min, and finally in 0.1× SSC/0.1 % SDS for 30 min. All washings were performed at 37 °C. Membranes were dried and exposed to a phosphor screen and scanned with Typhoon 9400 (GE Healthcare).

### 4.6 RNA analyses

#### 4.6.1 RNA isolation

Total RNA from cultured cells was isolated with TRIzol reagent or column purification by Monarch® total RNA Miniprep kit (NEB) or ReliaPrep™ RNA Cell Miniprep system (Promega) according to manufacturer’s instructions. For RNA isolation from fractions collected after isokinetic sucrose gradients, selected fractions were combined according to the established sedimentation profile of mitochondrial ribosomes and concentrated with Microsep™ centrifugal filter (MWCO 3kD) at 7 500 x g for 90 min at 4 °C. Every 250 μL of concentrated fraction was mixed with 750 μL TRIzol LS reagent (Thermo Fisher) and incubated at room temperature for 5 min. Chloroform (200 μL) was added to the mixture, incubated at room temperature for 3 min and centrifuged at 12 000 x g at 4 °C. Top aqueous phase was transferred to a new tube, equal volume of isopropanol was added and stored overnight at -20 °C. Sample was centrifuged for 30 min at 18 000 x g at 4 °C, pellet was washed once with 75 % ethanol, air-dried and resuspended in 20 μL nuclease-free water. The isolated RNA was further purified with 1/10 volume NaOAc and 2.5 volume of EtOH, incubated at -20 °C for 2 h and centrifuged for 30 min at 18 000 x g at 4 °C. The pellet was air-dried and resuspended in 20 μL nuclease-free water.
4.6.2 Northern blotting

For all samples, 5 \( \mu \)g of total RNA was separated through 1.2 % agarose-formaldehyde gel (1x MOPS, 20 % formaldehyde) and transferred to Hybond-N+ membrane (GE Healthcare) in 10x SSC buffer overnight. Membrane was washed once with 10x SSC for 5 min, UV cross-linked at 120 mJ and pre-hybridized (250 mM NaPO\(_4\), 250 mM NaCl, 25 % formamide, 7 % SDS, 10% BSA, 1 mM EDTA pH 8.0) for 1 h at 65°C. Membrane was then hybridized with \( ^\gamma P \)-labelled probes (Table 5) for 16–20 h at 37°C. Membranes were washed once with low stringency buffer (2× SSC/0.2 % SDS, 1mM EDTA) for 60 min, followed by second wash (0.5× SSC/0.2 % SDS, 1mM EDTA) for 60 min and final wash with high stringency buffer (0.2× SSC/0.1 % SDS, 1mM EDTA) for 30 min. Membrane was air-dried and exposure on a phosphor screen and scanned with Typhoon 9400 imaging system (GE Healthcare).

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>ACCATCCAATCGGTTAGTGACGACG</td>
</tr>
<tr>
<td>12S rRNA</td>
<td>GTTAATCGTGACC GGCGTGGCTGGC</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>GCTGTGGTATGCCCGCCTTTCAAGGG</td>
</tr>
<tr>
<td>MT-CO1</td>
<td>GGCTCCAGGGGTGGGAGTAGTTCCCTGC</td>
</tr>
<tr>
<td>MT-ATP8</td>
<td>TGGGTGATGAGGAATAGTGTAAGGAG</td>
</tr>
<tr>
<td>MT-ATP6/MT-CO3 junction</td>
<td>ATGGGTGGTTGATTATGTGTTGTC</td>
</tr>
<tr>
<td>MT-CO3</td>
<td>ATAGGCATGTGATTGGTGGGTCAT</td>
</tr>
</tbody>
</table>

4.6.3 RNA sequencing

4.6.3.1 Library preparation

Total RNA from culture fibroblasts was isolated with the Monarch Total RNA Miniprep Kit (NEB) or the Reliaprep RNA Miniprep system (Promega) and RNA for fractions collected from sucrose gradients was isolated with TRIzol LS reagent (Thermo Fisher). RNA (1 \( \mu \)g) was dephosphorylated with T4 PNK (NEB) for 30 min at 37°C and purified with RNAclean XP magnetic beads (Beckman Coulter). Template switching reverse transcription cDNA synthesis with TGIRT was performed by pre-annealing 1 \( \mu \)M each of Illumina sequencing primers (R2R DNA and 1 \( \mu \)M R2R RNA) in reaction.
buffer (10 mM Tris.HCl pH 7.5, 1 mM EDTA) for 2 min at 82°C in a thermocycler and cooled to 25°C with 3 % ramp. TGIRT cDNA synthesis was set up by adding 50 ng of dephosphorylated RNA to reaction buffer (450 mM NaCl, 5 mM MgCl₂, 20 mM Tris-HCl, pH 7.5), 100 nM of annealed R2 RNA/R2R DNA, 0.5 μM TGIRT enzyme and incubated at room temperature for 30 min. dNTPs (1 mM) was added to the reaction mix and incubated at 60 °C for 1h, followed by addition of 250 mM NaOH and incubation at 95 °C for 3 min. The reaction mix was cooled down, neutralized with 250 mM HCl and cleaned up with MinElute PCR Purification Kit (QIAGEN). In a 25 μL reaction, 10 μL of cDNAs was PCR amplified with Phusion High-Fidelity polymerase (Thermo Fisher), 0.2 μM dNTPs, 1.2 μL of gene specific primers (GSP) (Table 6) mixture (5.6 μM of each primer) and 1 μL of 10 μM R2R DNA primer at 98°C for 90 sec, followed by 12 cycles of 98 °C 10 sec, 64 °C 27 sec, 72 °C 27 sec, final extension at 72 °C 7 min and held at 4 °C. PCR products were bar-coded for sequencing on either one of the following Illumina platforms. For the cultured human fibroblast samples, three independent libraries were generated and sequenced on a miSEQ with 300 bp paired-end reads (total 1 to 2.4 million reads per sample). Two independent libraries were generated for the cultured human myoblasts and skeletal muscle biopsies and sequenced on a NextSeq 500 for 150 bp paired-end reads (total 5.1 to 7.9 million reads per sample).

Table 6. List of primers used in RNA sequencing.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene</th>
<th>Sequence (5'-3'); partial R1R primer sequence is underlined.</th>
</tr>
</thead>
<tbody>
<tr>
<td>UR159</td>
<td>MT-ATP6</td>
<td>AGAGTTCTACAGTCCGACGATCAGCCTACAAGCTACGTTTCACACCTTC</td>
</tr>
<tr>
<td>UR162</td>
<td>MT-CO1</td>
<td>AGAGTTCTACAGTCCGACGATCCTGGAGTGACTATATGGATGCCCC</td>
</tr>
<tr>
<td>UR172</td>
<td>MT-CO1 UTR</td>
<td>AGAGTTCTACAGTCCGACGATCAAGGAGGAAGGATCGAACCC</td>
</tr>
<tr>
<td>UR161</td>
<td>MT-CO2</td>
<td>AGAGTTCTACAGTCCGACGATCAGTCCACAGTTTCATGCCCCATCGTCC</td>
</tr>
<tr>
<td>UR158</td>
<td>MT-CO3</td>
<td>AGAGTTCTACAGTCCGACGATCCTGATACGCTATTGATAGTGGG</td>
</tr>
<tr>
<td>UR153</td>
<td>MT-CYTB</td>
<td>AGAGTTCTACAGTCCGACGATCTCCTAATCTCAATACCAACTATCTCCCC</td>
</tr>
<tr>
<td>UR164</td>
<td>MT-ND1</td>
<td>AGAGTTCTACAGTCCGACGATCACCCTAGTACTATTATATGATATGCTTCC</td>
</tr>
<tr>
<td>UR163</td>
<td>MT-ND2</td>
<td>AGAGTTCTACAGTCCGACGATCCTACGCCCTACCACGCTACTCC</td>
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<td>UR157</td>
<td>MT-ND3</td>
<td>AGAGTTCTACAGTCCGACGATCTCATCATCCTAGCCCTAAATGCTGG</td>
</tr>
<tr>
<td>UR155</td>
<td>MT-ND4</td>
<td>AGAGTTCTACAGTCCGACGATCCCTATTCTCCTCTATCCCTCA</td>
</tr>
</tbody>
</table>
4.6.3.2 Sequencing data analysis

Illumina sequencing reads were checked by FastQC (v0.11.8)\(^{452}\) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The paired and unpaired reads were separated using repair.sh function from BBQ (v38.79, https://sourceforge.net/projects/bbmap/). The adapters (for 3’ end sequencing including one extra nucleotide due to the variability at 5’ position (A, G, T, C) of the R2R primer), barcodes, low-quality reads, and low-quality bases are removed using bbduk.sh from the same bbmap tool (v38.79) (trimq=20, qin=auto, minlen=10, qout=auto, qtrim=r and qtrim=rl). For RNAseq libraries prepared for analysis of the whole mitochondrial transcriptome, processed paired-end reads were mapped to *Homo sapiens* mitochondrial genome (Accession number J01415.2) using bwa (v0.7.17)\(^{453}\).

Deep sequencing libraries of the mitochondrial 3’ end were aligned to the *Homo sapiens* reference mitochondrial genome, using BLAST (v2.6.0, max_target_seqs: 1)\(^{454}\). Sequencing reads were further classified and sorted by custom R codes. Reads with an exact match to the 3’ CDS of each mRNA were classified as possessing the true 3’ end of the CDS and then further sorted into distinct bins based upon the potential intervening sequence up to 5’ of the R2R primer. These include no sequence extension (CDS), oligo and polyadenylation (CDS + polyA) and flanking UTR (CDS + UTR). Sequencing reads containing at least +3 nt of a flanking tRNA sequence were classified as CDS + tRNA. Truncated reads missing -3 nt from the 3’ of the CDS and possessing post-transcriptional adenylation were further retained for analysis. Sequencing reads generated from each independent RNAseq library were preprocessed and analysed individually. Summary figures presenting the data represent the pooled results of the replicates for each sample.
4.7 *In vitro* translation

The PURExpress ΔRF123 cell-free translation system (NEB) was used with the following modifications. DNA templates were generated by PCR using the supplied dihydrofolate reductase (DHFR) plasmid as a template with the respective primers (*Table 7, 8*). Each construct possessed an upstream T7 promoter sequence and a Shine-Delgarno sequence of the DHFR ORF. In a 20 µl reaction, 1 µl of 100 ng DHFR plasmid was amplified with 0.4 units of Phusion™ High-Fidelity DNA polymerase (Thermo Fisher) 4 µl of 5x Phusion™ HF buffer, 0.4 µl of 10 mM dNTPs, 1 µl of 10 µM primer (forward and reverse) at 95 °C for 5 min, followed by 30 cycles of 95 °C 30 sec, 55 °C 30 sec, 68 °C 30 sec, final extension at 68 °C 7 min and held at 4 °C. PCR product (3 µl) was analysed on 1.5 % agarose-TAE gel and remaining product was purified with NucleoSpin Gel and PCR Clean-up kit (Machery Nagel). All PCR-generated DNA templates were verified by Sanger sequencing. Equimolar reactions of each specific construct were prepared for *in vitro* transcription and translation reactions in a total volume of 12.5 µl containing 5 µl of PURExpress kit solution A, 3.75 µl of solution B, 0.25 µl each of the supplied release factor (RF1, RF2, and RF3), 8 U of RiboLock RNase inhibitor (Thermo Fisher), 3 ng DNA template, 5 µM anti-ssrA oligonucleotide (5'-TTAAGCTGCTAA AGCGTAGTTTTGCGA CTA)⁹, 500 µCi ³⁵S Met-Cys (EasyTag-Perkin Elmer) and incubated at 37 °C for 90 min. All samples were treated with Benzonase (Sigma) on ice for 30 minutes to hydrolyse any polypeptidyl-tRNAs. An equal volume of gel loading buffer (186 mM Tris-Cl pH6.7, 15% glycerol, 2% SDS, 0.5 mg/ml bromophenol blue) was added to the samples and incubated at room temperature for 60 minutes. Translation products were resolved on 12% NuPAGE Bis-Tris gels (Thermo Fisher) in MOPS running buffer (50 mM MOPS, 50 mM Tris, 1 mM EDTA and 0.1 % SDS). Gels were dried under vacuum then exposed with a phosphor screen and scanned with Typhoon 9400 (GE Healthcare) for quantification.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Name</th>
<th>Sequence (5’-3’)</th>
</tr>
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<tbody>
<tr>
<td>KY46</td>
<td>DHFR forward</td>
<td>GCGAATTAATACGACTCAGATATAGGG</td>
</tr>
<tr>
<td>KY54</td>
<td>DHFR non stop</td>
<td>AAACCCCTCCGGTGTGAGAGGAGGTTTGCTGATGATCCGCCGCTCCAGAATCTCA AAGCA</td>
</tr>
<tr>
<td>KY53</td>
<td>DHFR stop</td>
<td>TTAACCCCTCCGGTTTTAGAGAGGTTATGCTGATGCCGCCGCTCCAGAATCTCA AAAGCA</td>
</tr>
<tr>
<td>KY55</td>
<td>DHFR ND1 non stop</td>
<td>TAGGTTTGCCGCCGCTCCAGAATCTCAAGCA</td>
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<td>KY56</td>
<td>DHFR ND1 stop</td>
<td>TAGGTTTGCCGCCGCTCCAGAATCTCAAGCA</td>
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<td>KY49</td>
<td>DHFR ND1 partial tRNA</td>
<td>CTCTATTATATATCTATCAAGTAGTACTCTTTTATCAGACATATTTCTATCTGGTT TGCCGCCGCTCCAGAATCTCAAAAGCA</td>
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<tr>
<td>KY61</td>
<td>DHFR ND3 non stop</td>
<td>ATTCGGTGCCGCCGCTCCAGAATCTCAAGCA</td>
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<td>KY62</td>
<td>DHFR ND3 stop</td>
<td>TTATTCGGTGCCGCCGCTCCAGAATCTCAAGCA</td>
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<td>KY51</td>
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<td>KY82</td>
<td>bla reverse</td>
<td>TTACCAATGCTTAATCAGTG</td>
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<tr>
<td>KY83</td>
<td>WT MT-ATP/CO3</td>
<td>TGATTTGGTGGTGGTGTTATGTGGTGTTGTCGCTCCGCCGCTCCAGAATCTAAAGCA</td>
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<tr>
<td>KY85</td>
<td>+A MT-ATP/CO3</td>
<td>TGATTTGGTGGTGGTGTTATGTGGTGTTGTCGCTCCGCCGCTCCAGAATCTAAAGCA</td>
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<tr>
<td>KY86</td>
<td>ΔTA MT-ATP/CO3</td>
<td>TGATTTGGTGGTGGTGTTATGTGGTGTTGTCGCTCCGCCGCTCCAGAATCTAAAGCA</td>
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<tr>
<td>KY87</td>
<td>T7-RBS-bla</td>
<td>GCGAATTAATACGACTCAGATATAGGGCTTAAATGATTAAGGGGAGGAAAAATATGAGTATCCACATTCCCGTGTGCC</td>
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Table 8: List of primer pairs and their corresponding PCR product.

<table>
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<tr>
<th>Primer</th>
<th>PCR product</th>
</tr>
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<tr>
<td>KY46/KY53</td>
<td>DHFR stop control</td>
</tr>
<tr>
<td>KY46/KY54</td>
<td>DHFR non-stop control</td>
</tr>
<tr>
<td>KY46/KY55</td>
<td>DHFR-ND1 CDS (non-stop)</td>
</tr>
<tr>
<td>KY46/KY56</td>
<td>DHFR-ND1 + A (stop)</td>
</tr>
<tr>
<td>KY46/KY49</td>
<td>DHFR-ND1 + tRNA</td>
</tr>
<tr>
<td>KY46/KY61</td>
<td>DHFR-ND3 CDS (non-stop)</td>
</tr>
<tr>
<td>KY46/KY62</td>
<td>DHFR-ND3 + AA (stop)</td>
</tr>
<tr>
<td>KY46/KY51</td>
<td>DHFR-ND3 + tRNA</td>
</tr>
<tr>
<td>KY82/KY87</td>
<td><em>bla</em> control</td>
</tr>
<tr>
<td>KY46/KY83</td>
<td>WT MT-ATP6/CO3</td>
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<tr>
<td>KY46/KY85</td>
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</tr>
<tr>
<td>KY46/KY86</td>
<td>ΔTA MT-ATP6/CO3</td>
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</tbody>
</table>
5 RESULTS

5.1 Human pathogenic variants of MT-ATP6 reveal discrete steps in co-translational quality control of mitochondrial gene expression (I)

5.1.1 Co-translational quality control is required for stability of mitochondrial nascent chains

Mitochondrial nascent chains are co-translationally inserted into the inner membrane by OXA1L and subsequently assembled into OXPHOS complexes. The loss of OXA1L would result in an accumulation of uninserted nascent chains and require for prompt removal. Central to protein quality control of the matrix side of the inner membrane is the AFG3L2 complex, which unfolds and degrades non-native polypeptides. AFG3L2 has an important role in the turnover of mitochondrial nascent chains and its dysfunction triggers a cascade of stress responses. Despite the well-documented functions of OXA1L and AFG3L2, it remains unclear whether these two components work synergistically in co-translational quality control to maintain proteostasis during mitochondrial translation.

To investigate the cooperative functions of these factors in co-translational quality control, single and combined siRNA knockdown of AFG3L2 and OXA1L were performed in human cultured fibroblasts. The consequence of AFG3L2 and OXA1L deficiencies on the synthesis and turnover of mitochondrial nascent chains was determined by 35S methionine metabolic labelling. To estimate the turnover mitochondrial proteins, siRNA-transfected cells containing the pulse-labelled proteins were washed and cultured in non-labelling medium for 60 min and 180 min. In single siRNA knockdown, the loss of the OXA1L did not affect overall mitochondrial nascent chain synthesis. However, a slight reduction of MT-CO2/3 and MT-ATP6 synthesis were observed after 30 min of pulse-labelling (Figure 6). Following 1 h of cold chase, MT-ATP6 was rapidly degraded and nearly undetectable. Although not as rapid as MT-ATP6, the turnover of MT-CO2/3 and other mitochondrial nascent chains was moderately faster when compared to the siRNA control. In the single AFG3L2 knockdown, slight decrease in protein synthesis was observed across all mitochondrial nascent chains, with MT-CO2/3 being the most affected (Figure 6). In contrast to MT-CO2/3, the loss of AFG3L2 caused an increase in the abundance of synthesized MT-ATP6 and also affected its turnover. Similar to MT-
ATP6, the turnover of other mitochondrial nascent chains was affected by AFG3L2 dysfunction when compared to siRNA control. In the combined siRNA knockdown of AFG3L2 and OXA1L, the abundance of synthesized MT-CO2/3 was significantly reduced, this was in contrast to MT-ATP6 that appeared similar to the siRNA control (Figure 6). The turnovers of both MT-ATP6 and MT-CO2/3 were not significantly affected by the dual loss of AFG3L2 and OXA1L.

Taken together, my results have demonstrated the cooperative function of OXA1L and AFG3L2 in the quality control of mitochondrial nascent chain synthesis. Uninserted nascent chains caused by impaired OXA1L are rapidly degraded by AFG3L2. The loss of AFG3L2 has slightly impaired the turnover of mitochondrial nascent chains, demonstrating its essential role in proteolytic processing of excess nascent chains. Among all mitochondrial nascent chains, MT-ATP6 is most profoundly affected by the loss of OXA1L and AFG3L2. Therefore, it will be used as a model substrate to further investigate co-translational quality control of mitochondrial protein synthesis.

Figure 6. Loss of AFG3L2 and OXA1L affect synthesis and stability of mitochondrial nascent chains. (A) Immunoblotting of whole cell lysates treated with the indicated siRNAs. (B) A representative image of 35S
methionine/cysteine metabolic labelling of mitochondrial protein synthesis in human fibroblasts treated with the indicated siRNAs. Cells were pulse-labelled for 30 min, followed by 60 min and 180 min cold chase. (C) Quantification of $^{35}$S-incorporation into mitochondrial proteins in human fibroblasts treated with the indicated siRNAs. SC, scrambled control. Data represent mean ± SD from five independent experiments. a.u, arbitrary units.

5.1.2 Pathogenic $MT$-$ATP6$ variants as models to study co-translational quality control

Using MT-ATP6 as a model, I am testing whether translation of different forms of $MT$-$ATP6$ mRNAs could be modulated by co-translational quality control mechanism. Aberrant mRNAs can arise from genetic mutations, defects in RNA processing and post-transcriptional modifications of mitochondrial polycistronic transcripts. As it remains a challenge to modify the mitochondrial genome, two pathogenic variants of $MT$-$ATP6$ that generate aberrant mRNAs were selected to study how mutations affect the quality control of mitochondrial translation. One variant has a nonsense mutation that results in premature termination of protein synthesis (m.8611insC, L29PfsX36) and produces a polypeptide of 36 amino acids. The other variant has a 2-bp microdeletion that removes stop codon of $MT$-$ATP6$ (m.9205delTA) (Figure 7). Both variants are heteroplasmic (97% in m.9205delTA and 80% in m.8611insC) and produce stable mitochondrial proteins at steady state. \(^{110}\)

![Diagram of MT-ATP6 variants](image)

Figure 7. Schematic illustrating the types of $MT$-$ATP6$ pathogenic variants. Arrows indicate the position of AUG start codon. *stop codon of $MT$-$ATP8$.

The tricistronic mRNA encoding $MT$-$ATP8$, $MT$-$ATP6$ and $MT$-$CO3$ has no intervening tRNA and therefore cannot be processed by RNase P and ELAC2 (RNase Z) (Figure 8A). In $MT$-$ATP6$
m.9205delTA, the 2-bp microdeletion removes a stop codon in \textit{MT-ATP6} and maintains an open reading frame (ORF) with \textit{MT-CO3}. To determine whether mutations in \textit{MT-ATP6} affect the processing and stability of transcripts, total RNA was isolated from \textit{MT-ATP6} m.8611insC and m.9205delTA patient fibroblasts and analyzed by northern blotting. As expected, the \textit{MT-ATP8/6} bicistrionic mRNA transcript was only detected in wild-type and \textit{MT-ATP6} m.8611insC variant. Using an oligonucleotide probe that hybridized to the overlapping junction of \textit{MT-ATP6} and \textit{MT-CO3} (Figure 8B), the abundance of both unprocessed and processed mRNAs in patient fibroblasts were similar to the wild-type (Figure 8C). At the protein level, translation of aberrant \textit{MT-ATP6} mRNAs had no effect on the steady-state abundance of the other mtDNA-encoded proteins (Figure 8D).
**Figure 8.** Pathogenic *MT-ATP6* variants as models to study co-translational quality control. (A) Schematic illustrating RNA processing of mitochondrial polycistronic transcript. (B) Diagram illustrating the binding sites of oligonucleotide probes used in northern blot analysis. (C) Northern blotting of total RNA isolated from human fibroblasts with the indicated *MT-ATP6* genotypes and hybridized with the indicated oligonucleotide probes. (D) Human fibroblasts with the indicated *MT-ATP6* genotypes pulse-labelled with $^{35}$S methionine/ cysteine for 30 min. ΔTA, m.9205delTA variant; FS, m.8611insC variant. * indicates aberrant truncated product.

5.1.3 *MT-ATP8/6/-CO3* tricistronic mRNA co-migrates with mitochondrial ribosomes

At steady state, *MT-ATP6* mRNA consists of both unprocessed and processed forms. This raises a question on which transcript is used for mitochondrial translation. To address this, mitochondrial ribosomes were separated by sucrose gradient sedimentation. The migration profiles of mitochondrial ribosomal proteins revealed no difference between wild-type and *MT-ATP6* m.9205delTA variant (**Figure 9A**). Northern blot analysis of RNA isolated from selected gradient fractions revealed the unprocessed *MT-ATP8/6/-CO3* mRNA to be the dominant transcript co-migrating with the 55S monosomes (**Figure 9B, C**). Although there were presence of processed transcripts, they were mainly detected in the lighter fractions of the sucrose gradients (**Figure 9B**). These data have thus provided strong evidence that *MT-ATP8/6/-CO3* transcript in *MT-ATP6* m.9205delTA variant could be used in translation and thus would be a fusion ORF of *MT-ATP6* and *MT-CO3*.
Figure 9. *MT-ATP6* m.9205delTA non-stop mRNA associates with mitochondrial ribosomes. (A) Immunoblotting of sucrose density gradient fractions of human fibroblasts with the indicated *MT-ATP6* genotypes. (B) Northern blotting of RNA isolated from the indicated sucrose density gradient fractions and hybridized with the indicated oligonucleotide probes. Probe 1 and 3 binds to *MT-ATP8/6*-CO3 and *MT-CO3* respectively. (C) Quantification of unprocessed *MT-ATP6* and *MT-CO3* transcripts in (B).

5.1.4 *MT-ATP6* m.9205delTA generates fusion ORF

The observation of tricistronic *MT-ATP8/6*-CO3 mRNA associating with mitochondrial ribosomes prompted the development of an *in vitro* assay to test whether the *MT-ATP6* m.9205delTA variant transcript could generate a fusion ORF of *MT-ATP6* and *MT-CO3*. Due to the lack of a reconstituted translation system for mammalian mitochondria, the assay was developed using the PURExpress bacterial transcription/translation system. To avoid possible aggregation of full length mitochondrial protein, only five amino acids from the 3’ end of *MT-ATP6* and 5’ end of *MT-CO3* were used in the design of DNA template. The 10 amino acid sequence of *MT-ATP6/-CO3* was inserted between DHFR and bla by PCR (Figure 10A). The stop codon of DHFR was removed so that translation termination would depend upon the downstream stop codon of *MT-ATP6*. Apart from the wild-type *MT-ATP6/-CO3*, modifications were made at the junction of *MT-ATP6* and *MT-CO3* to generate two other constructs. One construct contained an additional adenine nucleotide (+A) to
produce distinct stop and start codons and the other had the 2-bp deletion found in $MT$-$ATP6$ m.9205delTA variant (ΔTA) (Figure 10A). Following in vitro translation, all reaction mixtures were treated with nuclease to hydrolyze any potential peptidyl-tRNA, which enabled me to assess the overall amount of synthesized proteins.

In both wild-type and +A constructs, a distinct band corresponding to DHFR was detected (Figure 10B). In ΔTA construct, a band corresponding to the predicted size of DHFR + bla (55 kD) was detected, which confirmed the possibility that the absence of stop codon for MT-ATP6 could generate the synthesis of a fusion protein (Figure 10B). Together, these data have provided evidence by which translation of $MT$-$ATP6$ m.9205delTA mRNA variant could lead to the synthesis of a fusion ORF.

Figure 10. An in vitro translation assay to investigate protein synthesis in MT-ATP6 variants. (A) Schematic illustrating design of templates. Control templates consist of full length DHFR and bla with stop codon. bla, gene encoding bacterial protein β-lactamase; +A, an additional adenine nucleotide was added to produce distinct start and stop codons; ΔTA, represent the MT-ATP6 m.9205delTA variant. (B) Autoradiogram of in vitro translation assay. Nascent chains translated from the indicated DNA templates were labelled with 35S methionine/cysteine for 90 min. All samples were treated with a nuclease prior to gel loading to hydrolyse any potential polypeptidyl tRNAs.
5.1.5 Defective co-translational quality control in pathogenic MT-ATP6 variants has differential consequences on mitochondrial protein synthesis

The MT-ATP6/-CO3 fusion ORF generated in MT-ATP6 m.9205delTA mRNA is predicted to disrupt nascent chain insertion and folding. Owing to the narrow exit tunnel of mitochondrial ribosomes, folding of MT-ATP6 nascent chain can only occur after it emerges from the ribosome surface. As the ribosomes continue to translate the fusion mRNA, the inability to terminate MT-ATP6 synthesis is likely to cause a problem in the folding of MT-CO3 nascent chain. To prevent accumulation of uninserted MT-ATP6/-CO3 would therefore require responsive proteolytic degradation. To study the mechanistic details of co-translation quality control during translation of aberrant mitochondrial mRNAs, MT-ATP6 m.9205delTA fibroblasts were transfected with single siRNA knockdown of AFG3L2 and OXA1L. The loss of co-translational quality control is predicted to negatively regulate mitochondrial protein synthesis. Consistent with the prediction, the loss of OXA1L severely affected mitochondrial nascent chains (Figure 11). A translation defect was also observed in the absence of AFG3L2. During the 60 min chase, there was no significant difference in the turnover of mitochondrial nascent chains between AFG3L2 and OXA1L siRNA knockdown. However, the turnover of nascent chains at the 180 min time point was markedly faster in the absence of OXA1L (Figure 11).
Figure 11. Defective co-translational quality control in MT-ATP6 m.9205delTA attenuates mitochondrial protein synthesis. (Top) A representative image of $^{35}$S methionine/cysteine metabolically labelled mitochondrial protein synthesis in MT-ATP6 m.9205delTA fibroblasts treated with the indicated siRNAs. Cells were pulse-labelled for 30 min, followed by 60 min and 180 min cold chase. SC, scrambled control. (Bottom) Quantification of pulse synthesis of $^{35}$S metabolically labelled MT-CO1 from five independent experiments. Data represent mean ± SD. a.u, arbitrary units.

Premature termination of translation elongation in the MT-ATP6 m.8611insC variant results in the synthesis of a truncated MT-ATP6 polypeptide (36 amino acids) that is predicted to be too short for interaction with OXA1L for insertion into the membrane. Previously, AFG3L2 has been shown to be involved in co-translational handling of mitochondrial nascent chains\textsuperscript{109}. Using AFG3L2 as a model, I investigated how absence of protease would affect the turnover of truncated MT-ATP6 nascent chain. During a 30 min pulse-labelling, the abundance of mitochondrial nascent chains in MT-ATP6 m.8611insC variant was similar to that of the wild-type (Figure 12). Although both MT-ATP6
m.9205delTA and *MT-ATP6* m.8611insC variants generate aberrant translated products, these data have demonstrated a differential dependence on AFG3L2 in co-translational quality control.

**Figure 12.** Dysfunctional AFG3L2 has no effect on mitochondrial protein synthesis in *MT-ATP6* m.8611insC. (Top) A representative image of $^{35}$S methionine/ cysteine metabolically labelled mitochondrial protein synthesis in *MT-ATP6* m.8611insC fibroblasts treated with the indicated siRNAs. Cells were pulse-labelled for 30 min, followed by 60 min and 180 min cold chase. SC, scrambled control. * indicates aberrant truncated product. (Bottom) Quantification of the $^{35}$S methionine/ cysteine metabolic labelling from three independent experiments with the mean ± SD. a.u, arbitrary units.
5.1.6 F\textsubscript{1}F\textsubscript{o}-ATP synthase assembly defects do not modulate translation of MT-ATP6 m.9205delTA variant

The F\textsubscript{1}F\textsubscript{o}-ATP synthase is composed of F\textsubscript{1} and F\textsubscript{o} domains. The F\textsubscript{1} domain is soluble and protrudes into the matrix space. It is composed of three copies each of α and β subunits, and one copy each of γ, δ and ε subunits. Together, these subunits make up the central stalk of ATP synthase\textsuperscript{457}. F\textsubscript{o} domain is insoluble and consists of eight copies of transmembrane c-ring subunit, and also one copy of subunit a and two copies of subunit b\textsuperscript{457}. To test whether assembly defects of F\textsubscript{1}F\textsubscript{o}-ATP synthase would affect translation of MT-ATP6 in the MT-ATP6 m.9205delTA patient fibroblasts, I transfected the cells with TMEM70 siRNA. TMEM70 is known to be the assembly factor for complex I and V\textsuperscript{458}. The synthesis and stability of mitochondrial nascent chains in TMEM70 siRNA-transfected MT-ATP6 m.9205delTA fibroblasts were assessed by \textsuperscript{35}S metabolic labelling. The loss of TMEM70 did not affect the rate of synthesis or turnover of mitochondrial proteins (Figure 13), indicating the translation defects observed in the absence of AFG3L2 and OXA1L was not a consequence of unassembled F\textsubscript{1}F\textsubscript{o}-ATP synthase.

![Figure 13](image-url)

**Figure 13.** Defects in assembly of F\textsubscript{1}F\textsubscript{o}-ATP synthase do not affect translation in pathogenic MT-ATP6 variant. (A) Immunoblotting of MT-ATP6 m.9205delTA treated with the indicated siRNAs. (B) Cultured fibroblasts metabolically...
labelled with $^{35}$S methionine/cysteine following siRNA knockdown with the indicated siRNAs. SC, scrambled control. Cells were pulse-labelled for 30 min, followed by 60 min and 180 min cold chase.

The stability of mitochondrial nascent chains determines whether they will be inserted into the membrane or targeted for degradation. Stable nascent chains are assembled into OXPHOS complexes together with their nuclear-encoded protein counterparts. MT-CO1 is the largest subunit of complex IV and consists of 12 transmembrane domain. It forms a core where other subunits build around it to assemble into subassemblies. Hemes $a$ and $a_3$ are two prosthetic groups essential for the enzymatic activity of complex IV. Heme $a$ is required for stability and folding of MT-CO1 nascent chains by interacting with helices 1, 11 and 12. Nuclear-encoded COX10 is an assembly factor required for the first reaction step in the biosynthetic pathway of the heme $a$ prosthetic group. Pathogenic mutations in COX10 leads to COX deficiency and are associated with clinical disorders. Therefore, I am using COX10 as a model to understand stabilization of MT-ATP6 nascent chain. My data showed that the loss of AFG3L2 in COX10 patient fibroblasts had no effect on the stability of nascent chains and did not exert negative feedback on mitochondrial translation (Figure 14). Together, the data demonstrated the specific role of AFG3L2 in co-translational degradation of mitochondrial nascent chains.

**Figure 14.** Loss of AFG3L2 in COX10 deficiency has no effect on the stability of mitochondrial nascent chains. Human fibroblasts with pathogenic COX10 mutation were retrovirally transduced with empty vector and wild type
In summary, study I has identified key mechanisms in co-translational quality control of mitochondrial nascent chain synthesis. Nascent chains emerging from mitochondrial ribosomes interact with OXA1L for membrane insertion and disruption to this step result in the degradation of uninserted nascent chains by AFG3L2. Furthermore, using pathogenic MT-ATP6 variants, my data have shown how OXA1L and AFG3L2 exert negative feedback on protein synthesis during translation of aberrant mitochondrial mRNAs.

5.2 Consequences of defective quality control of mitochondrial protein synthesis in health and disease (II)

5.2.1 Defective quality control of nascent chain synthesis triggers organelle stress response

In study I, I established distinct steps in co-translational quality control of mitochondrial translation and identified AFG3L2 as one of the key factors involved. Previously, we have also shown how the loss of AFG3L2 impairs turnover of mitochondrial nascent chains and triggers OMA1-dependent proteolytic processing of OPA1

To further investigate on how AFG3L2 dysfunction feedback on mitochondrial protein synthesis, AFG3L2-transfected cells were treated with chloramphenicol during the transfection period to block
mitochondrial translation elongation. Inhibiting mitochondrial protein synthesis with chloramphenicol suppressed proteolytic processing of long OPA1 isoform and loss of mitochondrial ribosomal proteins (Figure 15). This data suggested that the trigger for OMA1 activation originates from impaired quality control of mitochondrial nascent chain synthesis.

Figure 15. Inhibiting mitochondrial translation suppresses OPA1 processing and loss of mitochondrial ribosomal proteins. Immunoblotting of whole-cell lysates of human fibroblasts treated with the indicated siRNAs with (+) and without (-) chloramphenicol. * Non-specific band detected with AFG3L2 antibody. SC, scrambled control.

Loss of OPA1 is known to cause depletion of mtDNA copy number, affecting distribution of mtDNA in the nucleoid and genome stability\(^{464,465}\). To test whether the reduced abundance of mitochondrial ribosomal proteins was attributed to mtDNA instability, Southern blotting was used to determine mtDNA copy number (Figure 16). No difference in mtDNA copy number was observed between short- and long-term knockdown of AFG3L2 and the presence of chloramphenicol had no effect on the abundance of mtDNA. Together, these data suggest the loss of ribosomal proteins is a downstream response from impaired quality control of mitochondrial protein synthesis.
Prohibitin (PHB) complexes are large ring-like structure formed by prohibitin 1 (PHB1) and prohibitin 2 (PHB2) in the mitochondrial inner membrane (Figure 17). It is a highly conserved scaffold protein that has been implicated in the maintenance of mitochondrial morphology and stabilization of newly synthesized mitochondrial proteins. PHB forms supercomplex with AFG3L2 to regulate the proteolysis of unassembled respiratory subunits and its dissociation from PHB-AFG3L2 supercomplex leads to L-OPA1 processing and mitochondrial fragmentation. Furthermore, PHB interacts with OMA1 and inhibits proteolytic processing of OPA1. The absence of PHB is known to activate AFG3L2 and OMA1 and enhances proteolytic processing of L-OPA1. Wild-type fibroblasts were transfected with PHB2 siRNA to investigate whether PHB-dependent proteolytic processing of OPA1 could be modulated by mitochondrial protein synthesis.
As expected, the loss of PHB triggered proteolytic processing of L-OPA1 (Figure 18). However, L-OPA1 isoforms were restored in the presence of chloramphenicol, suggesting that the proteolytic activities of AFG3L2 and OMA1 were down-regulated by inhibiting mitochondrial protein synthesis. In addition, the abundance of mitochondrial ribosomal protein uL11m was not affected as opposed to AFG3L2 knockdown (Figure 15).
Figure 18. PHB-dependent OPA1 processing is suppressed by inhibiting mitochondrial translation. Immunoblotting of HEK293 whole cell lysates treated with the indicated siRNAs with (+) and without (-) chloramphenicol. PHB2-1 and PHB2-2 are two different siRNAs. SC, scrambled control.

5.2.2 Aberrant mitochondrial mRNA triggers translation-dependent stress response

In study I, I investigated how translation of two pathogenic MT-ATP6 variants in the absence of AFG3L2 have differential effects on mitochondrial translation. To further test on how defective quality control affect the translation of aberrant mRNAs, I have included three other pathogenic MT-ATP6 variants (>98% heteroplasmy) in this study. At steady-state, translation of these aberrant mRNAs did not trigger OPA1 processing or loss of mitochondrial ribosomal proteins (Figure 19). Except for MT-ATP6 m.9205delTA variant, all other patient fibroblasts produced stable full-length MT-ATP6 (Figure 19).

Figure 19. Biochemical analysis of pathogenic MT-ATP6 variants. (Top) Diagram illustrating sites of pathogenic mutations in MT-ATP6. The protein consists of six transmembrane domains (I-VI). (Bottom, left) Immunoblotting of whole-cell lysates from wild-type and patient fibroblasts with the indicated MT-ATP6 mutations. (Bottom, right) A 30 min pulse metabolic labelling of mitochondrial protein synthesis with 35S methionine/ cysteine in WT and MT-ATP6 patient fibroblasts.
Thus far, I have identified how impaired quality control during translation of an aberrant mRNA negatively feedback on mitochondrial translation and triggers membrane stress response. Apart from impaired quality control, mitochondrial stress response could be triggered by oxidative stress, extreme pH or heat. Under physicochemical stress conditions, proteins are prone to misfold, in particular nascent proteins that have yet to attain a stable native structure. To investigate how heat modulates mitochondrial protein synthesis in pathogenic MT-ATP6 variants, patient fibroblasts were subjected to heat shock at 45°C for 4 h. Among the tested MT-ATP6 variants, MT-ATP6 m.9205delTA was the only variant that displayed OPA1 processing and loss of mitochondrial ribosomal protein uL11m. Inhibiting mitochondrial protein synthesis with chloramphenicol completely blocked the stress response and restored L-OPA1 and uL11m (Figure 20A). Time-course analysis of heat shocked MT-ATP6 m.9205delTA revealed time-dependent reduction of uL11m that began after 1 h of heat shock (Figure 20B). These data suggest that translation of a specific class of aberrant mRNA triggers the same proteotoxic stress response as observed with AFG3L2 dysfunction.

Figure 20. Heat shock triggers proteolytic processing of L-OPA1 and ribosome decay pathway. (A) Immunoblotting of whole-cell lysates from wild-type and MT-ATP6 m.9205delTA fibroblasts heat shocked for 4 h at 45°C with and without chloramphenicol. (B, left) Immunoblotting of whole-cell lysates from MT-ATP6 m.9205delTA fibroblasts. (B, right) Quantification of immunoblotting from three independent experiments. Data represent mean ± SD. a.u, arbitrary units.
5.2.3 Heat shock attenuates protein synthesis and triggers ribosome decay pathway in *MT-ATP6* m.9205delTA

To further investigate the consequences of the heat-induced stress response, mitochondrial protein synthesis in *MT-ATP6* m.9205delTA was measured by metabolic labelling over a period of 4 h at 45°C (Figure 21A). In wild-type, a time-dependent and gradual attenuation of mitochondrial protein synthesis was observed upon heat stress. In contrast, translation of the aberrant *MT-ATP6* m.9205delTA mRNA resulted in an abrupt attenuation of protein synthesis after 30 min of heat shock (Figure 21B). This data showed that attenuation of protein synthesis preceded the loss of ribosomal protein uL11m, which was only observed after 60 min of heat shock (Figure 20B). This was in contrast to the wild-type where attenuation of protein synthesis did not lead to a loss of uL11m upon heat shock (Figure 20A).

**Figure 21.** Heat shock attenuates mitochondrial protein synthesis in *MT-ATP6* m.9205delTA. (A) Diagram illustrating the experimental workflow for 35S methionine/ cysteine metabolic labelling for mitochondrial protein synthesis during heat shock. Cells were pulse-labelled for 30 min following the pre-incubation times at 45°C. (B, left) A representative image of 35S methionine/ cysteine metabolic labelling for mitochondrial protein synthesis during heat shock in the...
indicated human fibroblasts. (B, right) quantification of $^{35}$S-incorporation into mitochondrial proteins during heat shock. Data represent mean ± SD from four independent experiments. a.u., arbitrary units.

Next, mitochondrial rRNAs of heat shocked $MT\text{-}ATP6$ m.9205delTA fibroblasts were assessed by northern blot to address the question on whether reduction of uL11m and attenuation of protein synthesis were caused by ribosomal decay. After 90 min of heat shock, abundance of 12S rRNA was reduced more rapidly as compared to 16S rRNA (Figure 22A, B). Importantly, the activation of mitochondrial ribosomal decay pathway was not caused by instability of mtDNA copy number (Figure 22C). Collectively, the data suggest the presence of a translation inhibition feedback loop to regulate mitochondrial gene expression.

**Figure 22.** Heat shock activates ribosome decay pathway in $MT\text{-}ATP6$ m9205delTA. (A) A representative image of Northern blotting showing the abundance of mitochondrial and cytoplasmic ribosomal RNA. (B) Quantification of Northern blotting from four independent experiments. Data represent mean ± SD. a.u, arbitrary units. (C) A representative image of Southern blotting analysis of mtDNA copy number in $MT\text{-}ATP6$ m.9205delTA fibroblasts.

### 5.2.4 Chaperone function of AFG3L2 is essential for maintaining L-OPA1 isoform

The yeast homologue of the AFG3L2-SPG7 hetero-oligomeric complex, Yta10-12 complex has been shown to exhibit chaperone-like activity that is independent of its proteolytic function. Respiratory function in yeast strain lacking $YTA10$ or $YTA12$ is restored upon expression of proteolitically inactive Yta10 or Yta12. To test the chaperone function of the AFG3L2 complex during heat shock,
glutamate 575 (E575) of HEXXH motif within the proteolytic domain was replaced by glutamine (E575Q) to generate proteolytically inactive AFG3L2. Proteolytic processing of OPA1 and loss of uL11m were suppressed in MT-ATP6 m9205delTA fibroblasts retrovirally transduced with AFG3L2 E575Q cDNA upon heat shock (Figure 23). However, AFG3L2 E575Q did not rescue translation defects of heat shocked MT-ATP6 m9205delTA fibroblasts (Figure 23), suggesting the proteolytic function of AFG3L2 is essential for processing of aberrant mitochondrial nascent chains.

Figure 23. Proteolytically inactive AFG3L2 suppresses L-OPA1 processing but does not rescue translation defects. (Top) Schematic illustrating domain structure of AFG3L2. MTS, mitochondrial targeting sequence; TM1 and TM2, transmembrane domains 1 and 2; PD, proteolytic domain; HEXXH, zinc-binding motif at protease catalytic site. (Bottom, left) MT-ATP6 m9205delTA fibroblasts stably transduced by retrovirus with an empty vector, wild-type AFG3L2 or E575Q AFG3L2 cDNA. Immunoblotting of whole-cell lysates from cells heat shocked for 4 h at 45°C. (Bottom, right) Quantification of 35S-incorporation into mitochondrial proteins in MT-ATP6 m.9205delTA fibroblasts retrovirally transduced with empty vector and E575Q AFG3L2 cDNA. Data represent mean ± SD from four independent experiments. a.u, arbitrary units.

5.2.5 OPA1 is essential for mitochondrial gene expression

OPA1 is known to mediate cristae remodelling and mitochondrial fusion, which is essential in the maintenance and distribution of mtDNA. Pathogenic OPA1 mutations have been shown to induce accumulation of mtDNA deletions in skeletal muscle. The activation of OMA1-dependent
processing of OPA1 in heat-shocked MT-ATP6 m9205delTA has revealed a link between regulation of OPA1 and mitochondrial protein synthesis. To further investigate the molecular link between mtDNA depletion and translation defects caused by OPA1 dysfunction, two wild-type OPA1 splicing isoforms (Sp1 and Sp7) were retrovirally transduced into OPA1 knockout MEFs. At steady state, absence of OPA1 caused a drastic reduction of MT-CO1 and uL11m and impaired mitochondrial protein synthesis (Figure 24). The presence of either Sp1 or Sp7 isoform completely restored the translation phenotype, in agreement with previous studies showing expression of any of the eight isoforms could restore mtDNA stability472.
Figure 24. OPA1 is essential for mitochondrial gene expression. (A) Schematic illustrating processing of OPA1 Sp1 and Sp7 isoforms by OMA1 (S1 site) and YME1 (S2 site). MTS, mitochondrial targeting sequence; TM, transmembrane; GED, GTPase effector domain. (B) Immunoblotting of whole-cell lysates from Opa1 knockout (KO) MEFs retrovirally transduced with wild-type Opal Sp1 and Sp7 cDNA isoforms or empty vector. (C) A 30 min pulse-
labelling with 35S methionine/ cysteine for mitochondrial protein synthesis in OPA1 KO MEFs retrovirally transduced with splicing isoforms SP1 and SP7 cDNAs.

Next, MT-ATP6 m.9205delTA fibroblasts were transfected with OPA1 siRNA to investigate how OPA1 dysfunction affect mitochondrial translation in this pathogenic variant. In both wild-type and MT-ATP6 m.9205delTA, the loss of OPA1 resulted in the reduction of MT-CO1 and uL11m (Figure 25A). Mitochondrial protein synthesis in OPA1 siRNA-transfected MT-ATP6 m.9205delTA was severely impaired (Figure 25B), recapitulating the translation phenotype observed in heat shock experiment (Figure 21). To determine whether the observed phenotypes were caused by direct effect of OPA1 on mtDNA or consequence of impaired protein synthesis, MT-ATP6 m.9205delTA fibroblasts were incubated with chloramphenicol to block mitochondrial translation elongation. The failure to restore mtDNA copy number indicated a direct role of OPA1 in sustaining mitochondrial gene expression (Figure 25C).

![Figure 25](image-url)

**Figure 25.** Loss of OPA1 impairs mitochondrial protein synthesis in MT-ATP6 m9205delTA. (A) Immunoblot of whole cell lysates of human fibroblasts treated with the indicated siRNAs. SC, scrambled control. (B) A 30 min pulse-labeling with 35S methionine/ cysteine for mitochondrial protein synthesis in MT-ATP6 m9205delTA. (C) Southern blot quantification of mtDNA copy number in MT-ATP6 m9205delTA fibroblasts treated with the indicated siRNAs. SC, scrambled control. Data obtained from two independent experiments. a.u, arbitrary units.

Collectively, study II studied the consequences of defective quality control of mitochondrial protein synthesis. In wild-type, progressive AFG3L2 dysfunction leads to loss of mitochondrial
ribosomal proteins. This phenotype is recapitulated by translating a specific class of aberrant mitochondrial mRNA under heat stress, which is first characterized by attenuation of protein synthesis. These data have shown how defective quality control and heat stress could exert negative feedback and modulate mitochondrial translation.

5.3 Frequency of mRNA errors in mitochondrial gene expression (III)

Study III is built upon study I and II which show consequences from translation of different types of pathogenic mitochondrial mRNAs. Aberrant mitochondrial mRNAs can arise from gene mutations, errors introduced during transcription and RNA processing events. Deletions or point mutations of mtDNA can generate fusion ORF and truncated mRNAs with premature termination codons and non-stop mRNAs that lack in-frame stop codons. Here, I investigated the baseline level of mitochondrial gene expression errors generated in health and disease.

5.3.1 Differential consequences of aberrant mitochondrial RNA processing

To study the effect of aberrant RNA processing on the abundance of mitochondrial mRNA, a collection of patient fibroblasts with pathogenic mutations in subunits of RNase P complex and ELAC2 were analyzed (Figure 26).
In study I and II, I showed how translation of an aberrant mRNA triggers a stress response that negatively affects mitochondrial protein synthesis. To test whether heat stress could affect RNA processing and generate more aberrant mRNAs, patient fibroblasts were subjected to heat shock for 4 h. Total RNA extracted from patient fibroblasts were analyzed by northern blotting, using an oligonucleotide probe that hybridized to MT-CO3. While there was no major difference in the abundance of mRNA and processing pattern between ELAC2 and HSD17B10 variants, high molecular weight RNA species were detected in heat shocked TRMT10C and PRORP variants (Figure 27). Notably, PRORP P2 variant displayed aberrations in RNA processing at steady state and generated multiple bands upon heat shock. As opposed to PRORP P2 variant, the abundance of RNA transcripts in PRORP P1 variant were severely reduced, which could be caused by destabilization of the subunit upon heat shock.
Figure 27. Northern blot analysis of total RNA extracted from human fibroblasts with pathogenic mutations in RNAse P subunits and ELAC2, at 37°C and after 4h of heat shock at 43°C. Arrows indicate aberrant mRNAs.

At steady state, TRMT10C variant had reduced abundance of MRPP1, whereas the level of MRPP3 in PRORP P2 variant was not affected (Figure 28A). Furthermore, MT-CO1 deficiency was only observed in PRORP P2 variant. Next, I investigated the effect of heat shock on the stability of subunits of RNAse P complex, using TRMT10C and PRORP P2 variants. After 4 h of heat shock, there was no change in the abundance of MRPP1 and MRPP3 in both variants, suggesting that heat shock had no effect on the stability of these subunits. Metabolic labelling of mitochondrial protein synthesis in these two variants revealed no differences in the abundance of mitochondrial nascent chains at steady state, including MT-CO1 (Figure 28B). However, it is likely that the newly synthesized MT-CO1 is unstable and rapidly degraded, which could explain the MT-CO1 defect in PRORP P2 variant (Figure 28A). Together, these results demonstrate how individual subunit of RNAse P complex could exert differential impact on mitochondrial protein synthesis.
Figure 28. Differential functional consequences of pathogenic mutations in RNAse P complex. (A) Immunoblotting of whole-cell lysates from wild-type and patient fibroblasts with the indicated mutations. Cells were heat shocked for 4 h at 43°C before collection. (B) A representative image of 30 min pulse-labelling with 35S methionine/ cysteine in TRMT10C and PRORP variants at steady state.

To test whether aberrant mitochondrial mRNAs generated by defective RNA processing are associated with translating ribosomes, mitochondrial ribosomes of wild-type, TRMT10C and PRORP P2 variants were separated by sucrose gradient sedimentation. The migration profile of mitochondrial ribosomal subunits between PRORP variant and wild-type was similar (Figure 29A), indicating the deficiency of MT-CO1 was not attributed to faulty ribosome biogenesis as observed with PRORP knockout mice. Next, RNA isolated from fractions collected from sucrose gradients were analyzed by northern blotting. In PRORP P2 variant, high molecular weight RNA species were detected in fractions containing the 55S monosome (Figure 29B). This further supports the absence of RNA quality control to prevent translation of aberrant mRNAs.
Figure 29. Aberrant mRNA co-migrates with mitochondrial ribosomes. (A) Sucrose density gradient separation of mitochondrial ribosomes from cultured human fibroblasts with the indicated genotypes. (B) Northern blotting of RNA isolated from the indicated sucrose density gradient fractions and hybridized with MT-CO3 oligonucleotide probe.

To further confirm the MT-CO1 deficiency is a consequence of pathogenic mutation in MRPP3 subunit, wild-type cDNA copies of TRMT10C (MRPP1 subunit) and PRORP (MRPP3 subunit) were retrovirally transduced into PRORP patient fibroblasts. Over-expression of wild-type PRORP led to a complete restoration of MT-CO1, a phenotype that was not observed with over-expressing
wild-type TRMT10C (Figure 30). Northern blot analysis of total RNA revealed suppression of the RNA processing defect when over-expressing wild-type PRORP. Together, these data demonstrate how aberrant mitochondrial RNA processing negatively affects the steady state abundance of synthesized proteins.

![Figure 30](image-url)

Figure 30. Functional rescue of aberrant processing in PRORP P2 variant by wild-type PRORP cDNA. (Left) Immunoblotting of whole-cell lysates from PRORP patient fibroblasts stably transduced with empty vector, TRMT10C and PRORP cDNAs. (Right) Northern blotting of total RNA hybridized with oligonucleotide probe against MT-ATP8/6-CO3.

### 5.3.2 Aberrant mitochondrial RNA processing generates non-stop mRNA

Mitochondrial RNA processing requires precise cleavage at the ends of tRNA to liberate mRNAs from the polycistronic transcript, of which six mRNAs require polyadenylation to complete a UAA stop codon (Table 9). This process can be impeded by errors in RNA processing, thus leaving tRNAs remain appended to the 5’ or 3’ end of mRNAs. These aberrant non-stop mRNAs can potentially form non-stop translation complex and adversely affect mitochondrial translation.
### Table 9. List of the 3’end of human mitochondrial mRNAs requiring adenylation to generate stop codon.

<table>
<thead>
<tr>
<th>Gene</th>
<th>3’ end</th>
<th>Additional adenine nucleotides to generate stop codon</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-ND1</td>
<td>UA</td>
<td>1</td>
</tr>
<tr>
<td>MT-ND2</td>
<td>U</td>
<td>2</td>
</tr>
<tr>
<td>MT-ND3</td>
<td>U</td>
<td>2</td>
</tr>
<tr>
<td>MT-ND4</td>
<td>U</td>
<td>2</td>
</tr>
<tr>
<td>MT-CO3</td>
<td>U</td>
<td>2</td>
</tr>
<tr>
<td>MT-CYTB</td>
<td>U</td>
<td>2</td>
</tr>
</tbody>
</table>

To investigate the consequences of impaired RNA processing, Illumina sequencing was used to characterize the 3’ ends of mitochondrial mRNAs. Thermostable group II intron reverse transcriptase (TGIRT) was in our RNA sequencing approach for cDNA library construction. TGIRT has higher processivity and fidelity as compared to other reverse transcriptases. In addition, TGIRT can function in high temperature (60–65°C) to melt stable secondary structure in RNAs, thus overcoming mitochondrial tRNA structure and provide greater coverage of mitochondrial transcriptome at single nucleotide resolution. TGIRT also has template-switching property that does not require RNA ligation and RNA-sequence adaptor is added simultaneously during cDNA synthesis step.

Based on northern blotting analysis (Figure 27), defect in RNA processing was most prominent in PRORP P2 variant at steady state. Therefore, this variant was included as a positive control to estimate the frequency of errors in mitochondrial gene expression. As OXPHOS complexes are translationally regulated, I went on to investigate the population of mRNAs associating with translating ribosomes. Total RNA was extracted from monosome fractions collected from sucrose gradients. cDNAs libraries were generated from both total and ribosome-associated mRNAs and PCR was performed using mitochondrial gene-specific primers (Figure 31). Sequencing reads were mapped to the 3’ ends of all 13 mitochondrial mRNAs.
Figure 31. Schematic illustrating cDNA library preparation for RNA sequencing. TGIRT first binds to Illumina R2 RNA/R2 DNA oligonucleotide primers where R2R DNA primer has a 3’ overhang to direct TGIRT template switching. TGIRT extends the DNA primer to generate cDNA with R2R adaptor. After cDNA clean-up, PCR amplicons are generated using gene-specific primer that contains partial sequence of R1R sequencing primer and R2R primer. A second PCR amplification step is performed to add Illumina primer and barcode to the amplicons.

Although *MT-ATP6* and *MT-CO2* mRNAs encode a stop codon, RNA sequencing analysis of total whole cell RNA revealed a small proportion of truncated transcripts (non-stop mRNAs) in both wild-type and *PRORP* variant (Figure 32A). For ribosome-associated mRNAs, there was a low level of transcripts with aberrant tRNA processing detected in the wild-type (Figure 32B). Furthermore, there was also a small subset of non-stop mRNAs associating with the ribosomes (Figure 32C). Among the non-stop mRNAs, large proportion of transcripts were correctly processed and polyadenylated to generate a stop codon (Figure 32D).
Figure 32. Mitochondrial non-stop mRNAs are generated in health and disease. (A) The proportion of *MT-ATP6* and *MT-CO2* non-stop mRNAs deep sequenced from total RNA of wild-type and *PRORP* cultured fibroblasts. (B-D) The proportion of aberrant tRNA processing on the 3' end of mitochondrial mRNAs (*MT-CO3, CYTB, ND1, ND2, ND3* and *ND4*) associated with the mitochondrial ribosomes in the indicated cultured fibroblasts. CDS, coding sequence; CDS + poly A, polyadenylated CDS.

Next, to test whether the frequency of aberrant tRNA processing was dependent upon mitochondrial protein synthesis, wild-type fibroblasts were first treated with puromycin to terminate protein synthesis, followed by a 24 h incubation with chloramphenicol to inhibit translation elongation on mitochondrial ribosomes. Using the same sequencing approach described above, the analysis revealed no significant changes in the abundance of non-stop mRNAs and the proportion of the truncated polyadenylated transcripts (Figure 33).
Figure 33. Generation of mitochondrial non-stop mRNAs is independent of mitochondrial translation. (A) Workflow depicting the generating of RNA sequencing libraries from cultured wild type fibroblasts where mitochondrial protein synthesis was inhibited. (B-E) Deep sequencing of the 3’ end of MT-CO3, CYTB, ND1, ND2, ND3 and ND4 from cultured control human fibroblasts with (+) and without (-) mitochondrial translation. CDS, coding sequence; CDS + poly A, polyadenylated CDS; CDS + tRNA, CDS with tRNA sequence at the 3’ end; Trunc. CDS + poly A, truncated CDS that were polyadenylated.

5.3.3 Non-stop mRNAs impair translation termination

Next, I further analyzed the frequency of non-stop mRNAs in greater details, using MT-ND1 and MT-ND3 as examples. Both mRNAs do not encode a stop codon and are flanked by tRNAs at their 3’ end (Table 9, Figure 34). Analysis of mitochondrial ribosomes-associated MT-ND1 and MT-
ND3 mRNAs revealed low level of aberrant tRNA processing and truncated transcripts in the wild-type (Figure 34). In both wild-type and PRORP variant, there was a significant difference in the abundance of transcripts containing CDS between MT-ND1 and MT-ND3, suggesting differential post-transcriptional modification of mRNAs.

Figure 34. Types of non-stop mRNAs associated with the mitochondrial ribosomes. (A) Proportion of different types of non-stop mRNAs for transcripts MT-ND1 and ND3 mRNAs deep sequenced from mitochondrial ribosomes. CDS, coding sequence; CDS + tRNA, CDS with tRNA sequence at the 3’ end; Trunc. CDS + poly A, truncated CDS that were polyadenylated; (B) Frequency histograms indicating the mapped 3’ nucleotide position from mRNAs on the mitochondrial ribosome of non-stop mRNAs with a full-length CDS and not adenylated. CDS, coding sequence; nc, non-coding.

To understand how the nucleotide sequence at the 3’ end of mRNAs impact termination of protein synthesis, I developed an in vitro translation system to test the efficiency of peptide chain release in different types of mRNA templates. Since there is no established in vitro system to study mitochondrial translation, I adopted the bacterial in vitro PURE translation system as it contains translation factors that are similar to those found in mitochondria. To avoid possible protein aggregation from translation of full length MT-ND1 and MT-ND3, only last two codons at the 3’ end were included in the design of constructs. These two amino acids are important for translation termination, as it has been shown that the last three amino acids can affect the rate of peptide release. Three different variations were made for each construct, with each consisted of full length DHFR.
plasmid extended with either (i) CDS of *MT-ND1* and *MT-ND3*, (ii) CDS with additional adenine nucleotides or (iii) CDS with an adjacent tRNA (Figure 35B).

All constructs were *in vitro* translated with and without release factors (RF1-3) for quantification of released nascent chain. In the presence of release factors and stop codon, the signal intensity of released nascent chain increased between 2 to 4-fold (Figure 35C). The flanking tRNA sequence had differential consequences in translation termination. In *MT-ND1*, termination of protein synthesis was facilitated by the flanking *MT-TRNI* sequence because of an in-frame stop codon (Figure 35B, C). In contrast, *MT-ND3* with flanking *MT-TRNR* sequence had no in-frame stop codon and inhibited translation termination and release of the nascent chain (Figure 35B, C). Taken together, these data show how non-stop mRNAs impair translation termination and affect the abundance of translated proteins.

**Figure 35.** Mitochondrial non-stop mRNAs impair translation termination. (A) Schematic illustrating *in vitro* translation termination assay using DHFR as template. RF1-3, release factor 1-3. (B) Schematic illustrating the DHFR mitochondrial mRNA chimeric templates for *ND1* and *ND3*. The 3' sequence from the last two codons of *ND1* or *ND3* were added to the full length DHFR template. The CDS + tRNA constructs for *ND1* and *ND3* contained the sequence from the respective 3' flanking tRNA mapped in Figure 34B.
5.3.4 Resolving termination of non-stop mRNAs with MTRFR

In mitochondria, MTRFR (C12orf65) is identified as a class 1 release factor homologous to the bacterial ArfB protein\(^ {477}\). Substrates of ArfB are short mRNAs that are no longer than 9 nucleotides from the ribosomal decoding center\(^ {478,479}\). ArfB terminates protein synthesis by inserting its C-terminal extension into the ribosomal mRNA channel to re-position the GGQ motif to the PTC\(^ {304,478}\). Similar to ArfB, MTRFR possesses the highly conserved GGQ catalytic domain critical for peptidyl-tRNA hydrolysis\(^ {304}\). To investigate the types of non-stop mRNA substrates that require MTRFR function, the same deep sequencing approach was used in MTRFR human fibroblasts with R84X mutation that were retrovirally transduced with GSQ cDNA. The analysis focused on MT-CO1 and MT-CO3 transcripts that are lacking a stop codon (Table 9). MT-CO3 requires RNA processing of tRNA\(^ {\text{Gly}}\) at its 3’ end, followed by polyadenylation to generate a stop codon. In MTRFR patient fibroblasts (GSQ variant), there was an increase in the proportion of MT-CO3 reads with aberrant tRNA processing (Figure 36A). Among the sequenced cell types and tissues, there was no difference in the abundance of MT-CO3 transcripts that were correctly processed but lacking polyadenylation to generate a stop codon (Figure 36B).

MT-CO1 transcript encodes a long 3’ UTR sequence following the terminal AGA codon (Figure 36B). Thus, truncation of MT-CO1 UTR would be required for MTRFR to terminate translation in a mechanism similar to ArfB. To test this, I investigated the 3’ end of MT-CO1 in wild type fibroblasts and discovered a subset of reads with UTRs less than 12 nt (Figure 36C). However, in the MTRFR patient fibroblasts, an increase in truncated MT-CO1 transcripts was detected (Figure 36D).
Figure 36. The role of MTRFR in translation termination of non-stop mRNAs. (A-B) Deep sequencing of the 3' end of MT-CO3 mRNA from biological independent healthy controls taken from cultured fibroblasts, myoblasts and human skeletal muscle biopsies compared to MTRFR patient fibroblasts (GSQ variant). (C-D) Frequency histogram indicating the mapped 3' nucleotide position from deep sequencing of MT-CO1 mRNAs isolated from healthy control and MTRFR patient fibroblasts (GSQ variant).

Together, study III revealed low level of mitochondrial mRNAs errors that arise due to a lack of RNA surveillance mechanism. These errors generate non-stop mRNAs that are associated with mitochondrial ribosomes and have profound effect on mitochondrial translation termination.
6 DISCUSSION

Mitochondrial gene expression is a series of molecular events encompassing replication, transcription, RNA maturation, ribosome assembly and translation to produce proteins that make up the OXPHOS complexes. These processes are spatially and temporally regulated by nuclear-encoded factors across different subcompartments, including a network of evolutionary conserved proteases to remove surplus or dysfunctional proteins. Currently, we have limited knowledge on co-translational quality control and how this regulate mitochondrial gene expression. My data shows that the AFG3L2 protease complex has an indispensable role in safeguarding the integrity of the inner membrane from aberrations in defective protein quality control that affect mitochondrial membrane dynamics. Membrane integrity is required to maintain the membrane potential generated from OXPHOS for import of ions and factors essential for mitochondrial functions including protein synthesis. Furthermore, I have identified that progressive AFG3L2 dysfunction feedback on mitochondrial protein synthesis and leads to loss of mitochondrial ribosomal proteins.

AFG3L2 is a core-fitness gene and a complete knockout is lethal in model organisms. My research establishes the timeline of events ensuing from short- and long-term loss of AFG3L2 function. Both short- and long-term loss of AFG3L2 disrupt the handling of nascent chains and trigger proteolytic processing of L-OPA1, which tip the balance of long and short isoforms of OPA1 and leads to mitochondrial fragmentation. Based on these observations, I continued to test whether this initial response affect components of mitochondrial translation machinery. Short-term loss of AFG3L2 does not affect the abundance of mitochondrial ribosomal proteins, which is contrast to prolonged loss of AFG3L2. Importantly, in my experimental manipulations, AFG3L2 dysfunction did not directly affect the stability of mitochondrial genome. Translating the fusion ORF of MT-ATP6 mRNA recapitulates the phenotypes and leads to attenuation of mitochondrial protein synthesis and activates ribosomal decay pathway upon heat shock. These observations have identified distinct negative feedback mechanisms that buffer against the deleterious effect of stress response on mitochondrial gene expression. In this respect, the negative feedback onto gene expression may be activated to preserve mitochondrial genome until aberrations in co-translational quality control have been resolved.

Substrates of AFG3L2 are nascent chains that failed to be inserted into the inner membrane after emerging from the mitochondrial ribosomes. In study II, I have established how disruptions to the
insertion of mitochondrial nascent chains by dysfunctional OXA1L require AFG3L2 to resolve. Among the 13 mitochondrial nascent chains, MT-ATP6 is most profoundly affected by the loss of AFG3L2 and OXA1L. Although the molecular mechanism underlying the differential accumulation and turnover of MT-ATP6 is not known, it may be dependent on the assembly efficiency of OXPHOS complexes and the amount of synthesized subunits. Compared to other respiratory complexes, the assembly of F$_1$-F$_0$ ATP synthase is relatively efficient and MT-ATP6 is rapidly incorporated into the complex$^{484}$. All core subunits of F$_1$-F$_0$ ATP synthase are synthesized in minimal excess and unassembled components are generally degraded within 3 h. This is consistent with my findings on the rapid turnover of uninserted MT-ATP6 in the absence of OXA1L. Furthermore, absence of both AFG3L2 and OXA1L have not apparent impact on MT-ATP6 synthesis and turnover, this revealed an integrated network of co-translational quality control that work in concerted ways to maintain the balance of synthesized proteins.

### 6.1 Translation of fusion ORF impairs mitochondrial protein synthesis

The study of human mitochondrial gene expression using gene editing technologies remains a challenge, mainly due to a lack of robust tools for genome editing and absence of a reconstituted translation system. Therefore, a collection of MT-ATP6 pathogenic variants has enabled me to investigate how translation of different aberrant mRNAs can be affected by the loss of co-translational quality control. One discovery is the translation of a fusion ORF in MT-ATP6 m.9205delTA variant upon heat stress, which triggers OMA1 activation and followed by attenuation of mitochondrial protein synthesis and activation of mitochondrial rRNA decay pathway. The same phenotypes are also observed upon loss of AFG3L2 or OXA1L. The stress response is specific to the fusion MT-ATP6/-CO3 mRNA and is not triggered by the instability or an assembly defect of the nascent chain. One important finding is that the loss of co-translational quality control factors does not affect mitochondrial protein synthesis in the MT-ATP6 m.8611insC variant.

In MT-ATP6 m.8611insC, a premature termination codon generates a short polypeptide corresponding to 36 amino acids. The length of the ribosome exit tunnel is ~100 Å and spacing between membrane surface and ribosome surface is ~20 Å$^4$. Assuming the length of an amino acid is 3.5 Å, the total length of a linear truncated MT-ATP6 nascent chain would be 126 Å. This length is insufficient for OXA1L interactions and membrane insertion. Thus, unlike the fusion MT-ATP6/-
CO3 nascent chain which is likely to have a folding issue during the co-translation insertion step, the truncated MT-ATP6 does not pose a problem that would negatively feedback on mitochondrial protein synthesis.

Substrate processing by AFG3L2 involves recognition of unstructured solvent-exposed substrates that have a minimum length of 20 amino acid residues at its N-terminal, followed by pulling the substrate from the ATPase domain to proteolytic domain through cycles of ATP hydrolysis. The zinc-binding motif sequence HEXXH is essential for the activity of metallopeptidases such as AFG3L2. A glutamate (E) to glutamine (Q) amino acid substitution at position 575 in the motif abolishes proteolytic activity of AFG3L2, without affecting the chaperone activity of the unfoldase domain. My data shows over-expression of the proteolytically inactive AFG3L2 E575Q in MT-ATP6 m.9205delTA suppresses OPA1 processing and loss of mitochondrial ribosomal proteins during heat shock. Although there is no evidence to support misfolding of MT-ATP6 during heat shock, interaction between MT-ATP6 nascent chains and the chaperone domain of AFG3L2 E575Q is critical in maintaining inner membrane dynamics. Despite this, overexpressing AFG3L2 E575Q failed to restore mitochondrial protein synthesis in MT-ATP6 m.9205delTA. It is unclear on how the unfolded MT-ATP6 nascent chain is processed since the substrate will remain trapped in a proteolytically inactive homo-hexamer AFG3L2 E575Q. However, it is possible that the trapped MT-ATP6 is processed by SPG7 that retains its proteolytic function.

Deletion mutations can arise from errors in replication of mitochondrial genome, leading to OXPHOS deficiency and loss in muscle mass. These mutations contribute to sporadic diseases, which may start in early childhood such as Pearson and Kearns-Sayre syndromes. Expression of deleted mtDNA generates fusion ORFs that can be functionally dominant, although the transcript level from wild-type mtDNA is within normal range. Thus, mtDNA deletion can act as a negative regulator and interfere with the function of wild-type mtDNA. The two base pairs micro-deletion mutation in MT-ATP6 m.9205delTA is a fusion ORFs of MT-ATP6 and MT-CO3 and can exert dominant negative effect under stress conditions or absence of quality control. Fusion ORFs are common and widespread in plants where ATP6 fusion ORFs are generated and cause cytoplasmic male sterility that disrupts pollen development.

A conceptual model has been proposed to illustrate the co-translational quality control pathways using MT-ATP6 nascent chain synthesis as a substrate (Figure 36). In the wild-type scenario, functional AFG3L2 and OXA1L facilitate membrane insertion and housekeeping degradation of MT-ATP6...
nascent chains (Figure 36A). In the absence of OXA1L, uninserted MT-ATP6 is rapidly degraded by AFG3L2 without affecting the integrity of inner membrane (Figure 36B). However, short-term loss of AFG3L2 impairs degradation of excess MT-ATP6 nascent chains, triggering stress response on the inner membrane and leads to fragmentation. This stress response does not feedback on mitochondrial translation (Figure 36C). Long-term loss of AFG3L2 not only triggers stress response on the inner membrane, it also negatively feedback on mitochondrial gene expression and reduces the abundance of mitochondrial ribosomal proteins (Figure 36D). Lastly, translation of a fusion ORF mRNA recapitulates all the observed phenotypes and exacerbated with temperature stress (Figure 36E).

Figure 36. Model for co-translational quality control of MT-ATP6 nascent chain synthesis. (A) In wild-type, OXA1L assists in membrane insertion of stable nascent chain. Excess nascent chain is rapidly degraded by AFG3L2. (B) In the absence of OXA1L, uninserted nascent chain is degraded by AFG3L2. (C) Short-term loss of AFG3L2 results in accumulation of nascent chains and triggers proteotoxic stress response in the inner membrane. (D) Long-term loss of AFG3L2 triggers proteotoxic stress response and negatively feedback on mitochondrial gene expression, causing a reduction in mitochondrial ribosomal proteins. (E) Translation of a fusion ORF mRNA in the absence of AFG3L2 and/or OXA1L negatively affect mitochondrial gene expression, activating rRNA decay pathway upon heat stress.
6.2 Mitochondrial RNA processing errors generate non-stop mRNAs

At the mRNA level, the *MT-ATP6* transcript exists in two forms: unprocessed tricistronic *MT-ATP8/6-CO3* and the processed *MT-ATP8/6*. Sucrose gradient sedimentation has revealed the tricistronic mRNA as the predominant transcript associating with mitochondrial 55S monosome. It remains unclear on how translation is initiated on *MT-CO3*, which at the stable protein level lacks the N-terminal formyl group on the starter methionine residue. Furthermore, the 3’ end of *MT-ATP6* is not flanked by tRNA and thus the non-canonical processing of the transcript would require additional factors.

Currently, little is known about the frequency and types of errors during RNA processing of mitochondrial polycistronic transcripts. My RNA sequencing data revealed a low level of RNA processing errors in the wild type setting that generate non-stop mRNAs. The presence of these aberrant mRNAs may explain the need of AFG3L2 for clearance at steady state. The fidelity of mitochondrial RNA processing may be regulated by multiple factors and at different points between transcription and translation. My data shows no significant changes in the frequency of mRNA errors when mitochondrial protein synthesis is inhibited, suggesting either a lack of feedback mechanism or regulation of RNA processing that occurs upstream of translation.

In addition, the existence of non-stop mRNAs on mitochondrial ribosomes indicates the absence of mRNA surveillance mechanism to eliminate aberrant transcripts. Although the SUV3 helicase and polynucleotide phosphorylase (PNPase) complex has been proposed to play a role in mitochondrial RNA decay, there is no evidence yet to support their involvement in the quality control mechanisms of mitochondrial mRNAs established in my thesis. As discussed in the previous sections, patient fibroblasts generating fusion ORF mRNA exhibit normal mitochondrial translation at steady state. However, translation defect can be manifested under stress condition. Together, these observations have highlighted the importance of RNA quality control and how it can influence the pathogenesis of mitochondrial diseases under adverse cellular conditions.

6.3 Non-stop mRNAs inhibits translation termination

Stalling of mitochondrial ribosomes can occur during translation of fusion ORF or mRNA lacking an in-frame stop codon. Mitochondrial ribosome-associated quality control functions differently from bacterial rescue mechanisms and ubiquitination in eukaryotic cytosol. Despite the recent
cryo-EM structure of mitochondrial ribosome rescue by release factor mtRFR (C12orf65)\textsuperscript{310}, the quality control pathway linking ribosome rescue and translation of non-stop mRNAs remains to be determined.

Mutations in genes encoding for mitochondrial rRNAs, ribosomal proteins and assembly factors impede mitochondrial ribosome biogenesis and result in protein synthesis defects and respiratory chain dysfunction\textsuperscript{498,499}. Translation of non-stop mRNAs can have functional consequences on elongation termination and impact on ribosome recycling. Using genetic and biochemical approaches, I have shown how different types of non-stop mRNAs can affect the hydrolytic reaction of release factors. Most importantly, the hydrolysis of peptidyl-tRNA in mRNA with an in-frame stop codon is not hindered by the flanking tRNA sequence. Together, my data demonstrates how non-stop mRNAs that arise from errors in mitochondrial gene expression can negatively regulate protein synthesis.
7 CONCLUSIONS AND FUTURE PROSPECTS

In the last twenty decades, functional roles of mitochondrial protein quality components have been extensively studied. The comprehensive analysis of eukaryotic mitochondrial quality control pathways uses mainly *S. cerevisiae* as model organism. Furthermore, there has been little focus on linking the underlying molecular mechanisms of quality control to the pathogenesis of mitochondrial disease. Emerging structural studies have provided new mechanistic insights into the activity of mitochondrial protein quality control components. Despite the advancement in technology, we still have limited understanding on how quality control machineries are organized and operated.

In this dissertation, I have investigated the quality control of mitochondrial gene expression. My findings reveal that translation of an aberrant mitochondrial mRNA or compromised co-translational quality control can trigger a stress response on the inner membrane. Failure to counteract this stress response leads to the loss of mitochondrial ribosomes, mitochondrial RNA decay and remodelling of mitochondrial inner membrane. The source of insult on the organelle originates from mishandling of nascent chain which has no effect on the mitochondrial genome. This molecular event is tightly coupled to the enzymatic activity of AFG3L2 and the chaperone function of AFG3L2 has been found to be the key in maintaining quality control of newly synthesized polypeptides.

In addition to AFG3L2, OXA1L has been identified as a critical component for co-translational quality control. Using both genetic and biochemical approaches, I have identified discrete steps in the regulation of nascent chain membrane insertion and degradation. Translation of different types of aberrant mitochondrial mRNAs in the absence of co-translational quality control leads to differential consequences. The difference in phenotypes is likely to be attributable to the length and folding state of the nascent chain. Non-stop mRNAs arising from errors in mitochondrial tRNA processing constitutes the major source of aberrant mRNAs. These non-stop mRNAs are either lacking an in-frame stop codon or flanked by tRNA sequence at their 3’ end. Non-stop mRNAs can evade surveillance mechanisms and engaged with mitochondrial ribosomes, interfering with translation termination and ribosome recycling. Therefore, co-translational or ribosome-associated quality control is critical in resolving the errors to resume normal translation.

Outstanding questions for future research include identifying which of the 13 mtDNA-encoded proteins are substrates for AFG3L2 and how substrate sequence influence its proteolytic activity. It
is also important to systematically identify additional factors and putative insertases involve in co-
translation quality control. The cooperative function of AFG3L2 and OXA1L have suggested the
close proximity of these two factors. Proximity labelling approach would provide more details on
their interactions and may identify other neighbouring auxiliary factors. Finally, future studies to
investigate the physical interactions between nascent chain and quality control machineries will
undoubtedly advance our understanding on the molecular basis of mitochondrial diseases associated
with dysfunctional quality control.
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REFERENCES


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Cent. 25, 593–604 (1905).


44. Pangborn, M. C. ISOLATION AND PURIFICATION OF A SEROLOGICALLY ACTIVE


71. Frezza, C. *et al.* OPA1 Controls Apoptotic Cristae Remodeling Independently from


91. Anesti, V. & Scorrano, L. The relationship between mitochondrial shape and function and
129. Losón, O. C., Song, Z., Chen, H. & Chan, D. C. Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. Mol Biol Cell 24, 659–667 (2013).


Chevrollier, A. et al. Hereditary optic neuropathies share a common mitochondrial coupling


117


241. Haack, T. B. et al. ELAC2 Mutations Cause a Mitochondrial RNA Processing Defect


261. Sasarman, F. et al. LRPPRC and SLIRP Interact in a Ribonucleoprotein Complex That Regulates Posttranscriptional Gene Expression in Mitochondria. Mol Biol Cell 21, 1315–
1323 (2010).


303. Soleimanpour-Lichaei, H. R. *et al.* mtRF1a Is a Human Mitochondrial Translation Release


343. Gabriel, K. *et al.* Novel Mitochondrial Intermembrane Space Proteins as Substrates of the


385. Opalińska, M. & Jańska, H. AAA Proteases: Guardians of Mitochondrial Function and


468. Steglich, G., Neupert, W. & Langer, T. Prohibitins Regulate Membrane Protein Degradation


