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MITOCHONDRIAL RNA PROCESSING IN HEALTH AND DISEASE

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LIST OF SCIENTIFIC PAPERS

I. Clemente, P., Pajak, A., **Laine**, **I**., Wibom, R., Wedell, A., Freyer, C.*, Wredenberg, A.*. SUV3 helicase is required for correct processing of mitochondrial transcripts. *Nucleic Acid Research*. **43**, 7398–413 (2015).

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IV. **Laine**, **I**., Schober, FA., Clemente, P., Pajak, A., Haas, M., Filipovska, A., Wedell, A., Freyer, C., Wredenberg, A. Mitochondrial translation efficacy is dependent on RNA polyadenylation. Manuscript.

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ABSTRACT

Mitochondria are often described as the powerhouses of the cell, providing the main cellular energy source in the form of adenosine triphosphate. Five enzyme complexes, collectively termed the oxidative phosphorylation system, use the reducing power from nutrients to synthesise adenosine triphosphate via cellular respiration. This energy conversion is dependent on factors encoded by the nuclear and mitochondrial genome, with the latter encoding 13 subunits within four of the five oxidative phosphorylation system complexes. Adenosine triphosphate synthesis is therefore under dual genetic control, and this thesis addresses mechanisms that control and regulate mitochondrial gene expression. The mitochondrial genome is transcribed as long, polycistronic premature transcripts, which need to undergo cleavage and maturation before they can be used for correct translation on mitochondrial ribosomes. However, the mechanisms of this RNA processing, as well as the mechanisms underlying mitochondrial RNA homeostasis, are not fully understood. Here I used the fruit fly, *Drosophila melanogaster*, to study factors involved in mitochondrial gene expression. In two studies I addressed the functions of factors involved in the mitochondrial degradosome, responsible for RNA turnover. Additionally, I addressed the role of polyadenylation in RNA degradation, and studied how the polyadenylation machinery affects mitochondrial translation. Finally, defects of mitochondrial gene expression can have severe clinical consequences and form an important part of human pathology. One study of this thesis validated the pathogenicity of mutations in a tRNA aminoacyl transferase gene, identified in two siblings suffering from mitochondrial disease.

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LIST OF ABBREVIATIONS

ARS	Aminoacyl tRNA synthetases
ARS2	Mitochondrial aminoacyl tRNA synthetases
ATP	Adenosine triphosphate
BSF	Bicoid stability factor
CoQ_{10}	Coenzyme Q10/ubiquinone
Dm	*Drosophila melanogaster*
dsRNA	Double-stranded RNA
EM	Electron microscopy
GTPBP10	Guanosine triphosphate binding protein 10
HSP	Heavy-strand promoter
IEM	Inborn error of metabolism
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
iPSCs	Induced pluripotent stem cells
KD	Knock-down
KI	Knock-in
KO	Knock-out
LACTB2	Lactamase Beta 2
LRPPRC	Leucine rich pentatricopeptide repeat containing protein
LSP	Light-strand promoter
mtDNA	Mitochondrial DNA
MTPAP	Mitochondrial polyadenylic acid RNA polymerase
MTERF1	Mitochondrial transcription termination factor 1
mtLSU	Mitochondrial ribosomal large subunit
mtSSU	Mitochondrial ribosomal small subunit
MRPs	Mitochondrial ribosomal proteins
mS39	Mitochondrial small ribosomal protein 39
NESc	Neuro-epithelial stem cells
OE	Over expression

O_H	Heavy-strand origin
O_L	Light-strand origin
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation system
PNPase	Polynucleotide phosphorylase
POLRMT	Mitochondrial DNA-directed RNA polymerase
POLγ	Mitochondrial DNA polymerase gamma
RC	Respiratory chain
Redox	Reduction and oxidation
ROS	Reactive oxygen species
SLIRP	SRA stem-loop interacting RNA binding protein
TFAM	Mitochondrial transcription factor A
UTR	Untranslated region
WARS2	Tryptophanyl tRNA synthetase 2
WES	Whole exome sequencing
WGS	Whole genome sequencing

1 INTRODUCTION

When pursuing my postgraduate studies my goal was to become a good scientist, with acquired knowledge of how to conduct proper research and successfully lead a project forward. During my undergraduate studies I gained interest in metabolism and when I was given the opportunity to develop within the field of mitochondrial research I was happy to take on the challenge. I am intrigued by the mitochondrial system. For instance, it is the only metazoan organelle with its own DNA. Exclusively maternally inherited, the mitochondrial DNA (mtDNA) let us share something unique with the women on our mothers' side of the family. Thus, together with my mother and sister I share mtDNA with my grandmother and beyond. My postgraduate studies of mitochondrial gene expression revealed a system of local complexity globally affecting the cell and the living organism. Mitochondria are primarily known for being the main source of adenosine triphosphate (ATP) and thus function as the cells power supply. Nonetheless, the mitochondria also participate in the turnover of carbohydrates, lipids, amino acids, and steroids and are therefore central in the intermediary metabolism. Moreover, they are involved in urea synthesis, apoptosis, innate immunity[1], and non-shivering thermogenesis by the aid of uncoupling proteins[2], plus contributes in several signalling cascades by maintaining a major source of calcium and by producing reactive oxygen species (ROS)[3]. It is not surprising, that with such a repertoire of mechanisms any defect in mitochondrial function will affect the organism. Mitochondrial malfunction has been implicated in a number of common chronic diseases such as diabetes mellitus[4], Alzheimer's disease[5], heart failure[6] and the normal ageing process[7]. Inherited mitochondrial diseases also forms a large subgroup within inborn error of metabolism (IEM)[8]. Thus, deeper understanding of mitochondrial function, and how it operates in both health and disease, is important from a public health point of view. In my postgraduate studies I have tried to untangle some of the unsolved questions of mitochondrial RNA processing by studying a set of proteins suggested to be involved in these processes, with several also found mutated in patients with mitochondrial disease. I hope the reader of this thesis will find new knowledge and be intrigued by the complexity of the mitochondria.

2 LITERATURE REVIEW

2.1 OXIDATIVE PHOSPHORYLATION AND ATP PRODUCTION

The mitochondrion is a double-membrane organelle, with an outer and inner mitochondrial membrane (OMM respectively IMM) that encloses an intermembrane space (IMS) and protects an inner matrix. Pore-forming membrane proteins make the OMM highly permeable to ions and uncharged molecules up to 5 kDa in size[9,10]. In contrast, the IMM has one of the richest protein contents in the cell and is nearly impermeable. Electron microscopy (EM) tomography has shown that the IMM is highly invaginated and forms so called cristae structures. These structures increase the surface area of the IMM and create subcompartments that form different micro-environments within the matrix[11]. The four multi-subunit membrane-bound complexes (CI-CIV) of the respiratory chain (RC) and the F_1F_0 ATP synthase (CV) localises to these cristae. The RC creates an electrochemical gradient across the dense IMM, which is utilised by CV to generate ATP. The chain of reactions is referred to as oxidative phosphorylation system (OXPHOS)[12,13]. The matrix is the site of mitochondrial gene expression and numerous biochemical reactions. The required molecular components for these processes need to cross both mitochondrial membranes via an elaborate system of transporters and translocases, which are under profound regulation[14]. Together, mitochondria form a dynamic network that can fuse and divide to change their shape and size depending on the cell's energy requirements. These transitions are mostly controlled by nuclear-encoded proteins of the Dynamin family. For instance, recruitment of Dynamin-related GTPases OPA1 and mitofusins causes mitochondrial fusion that enhances OXPHOS activity[15]. Disruption of this machinery has been associated with Charcot-Marie Tooth disease and optic atrophy, which emphases the importance of mitochondrial morphology. However, much remains to be elucidated before changes in mitochondrial dynamics can be assigned to different pathological states[16].

The RC complexes conduct a series of reduction and oxidation (redox) reactions that transfer electrons from reducing agents to molecular oxygen, which is reduced to water (**Figure 1**). The reactions cause release of free energy that is used by CI, CIII and CIV to pump protons across the IMM. The main electron donor is the tricarboxylic acid (TCA) cycle that depends on several pathways of the intermediary metabolism including glycolysis, amino acid turnover and β-oxidation[12,13]. CI, or NADH:ubiquinone oxidoreductase, is the largest complex and consist of 45 subunits[17]. It takes up electrons from the reduced form of nicotinamide adenine dinucleotide (NADH) and transfer them to the mobile electron carrier ubiquinone (CoQ_{10}). CII, also known as succinate oxidoreductase, uses succinate to transfer electrons via a covalently bound flavin adenine nucleotide (FAD) to CoQ_{10}[18–20]. CIII, or ubiquinol-cytochrome *c* oxidoreductase, accepts electrons from the reduced form of CoQ_{10} (ubiquinol). In each transfer CIII takes up two electrons. One electron is transferred to the second mobile electron carrier cytochrome *c*. The other is re-cycled to CoQ_{10}[21]. The movement of electrons between CIII and CoQ_{10} is referred to as the Q-cycle[22]. The fourth

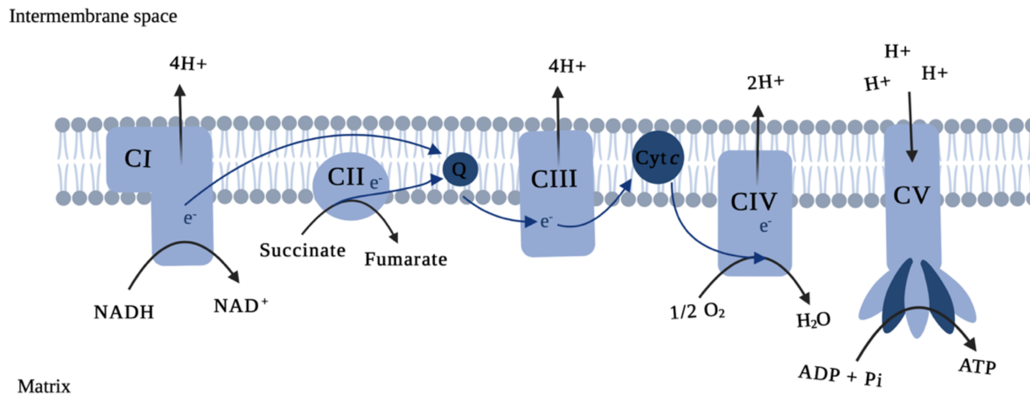

Figure 1. Oxidative phosphorylation. The RC consists of four complexes (CI-CIV) placed in the phospholipid bilayer of the IMM. The complexes transfer electrons from NADH and succinate to molecular oxygen via the mobile electron carriers CoQ_{10} (Q) and cytochrome *c* (Cyt*c*). The electron transfer is conducted via a number of redox reactions that releases energy. The energy is utilized to pump protons across the IMM that results in an electrochemical gradient used by CV to convert ADP and inorganic phosphate (Pi) to ATP. Created with BioRender.com

and final RC complex is cytochrome *c* oxidase (CIV). Electrons are donated by cytochrome *c* to haem and copper centres within CIV. An oxygen molecule reacts with the two reduced centres and create a peroxide bridge between them. This reaction enables the uptake of two protons, generating hydroxyl groups at each centre. The transport of electrons from cytochrome *c* and uptake of protons takes place twice to produce two molecules of water, one from the haem centre and the other from the copper centre. Hence, one oxygen molecule will generate two molecules of water. The protons pumped to the IMS by the RC complexes are allowed to re-enter back into the matrix via CV. The flow of protons down their electrochemical gradient drives the rotation of the F_0 subunit, resulting in the condensation of adenosine diphosphate (ADP) and inorganic phosphate to ATP within the F_1 subunit.

It has for long been debated how the RC complexes are organised in the IMM. The diligent use of high resolution cryo-EM have strengthened the idea that RC complexes form so called supercomplexes. In a supercomplex several RC complexes form a functional group, instead of each complex being free entities arranged in a row, as suggested previously. This arrangement perhaps provides a kinetic advantage, or it is necessary for the maintenance of the IMM morphology and packing of the high number of proteins. The organisation of the supercomplexes seems highly dynamic and dependent on energy demand however additional studies are required to reach a consensus on how they are formed[23–25].

2.2 THE MTDNA MOLECULE IN HUMAN AND FRUIT FLY

According to the endosymbiotic theory, the mitochondrion originated from an α - proteobacterium that was incorporated into a primitive eukaryotic cell about 1.5 billion years ago[26,27]. Over time, the number of mitochondrial genes has been extensively reduced, either

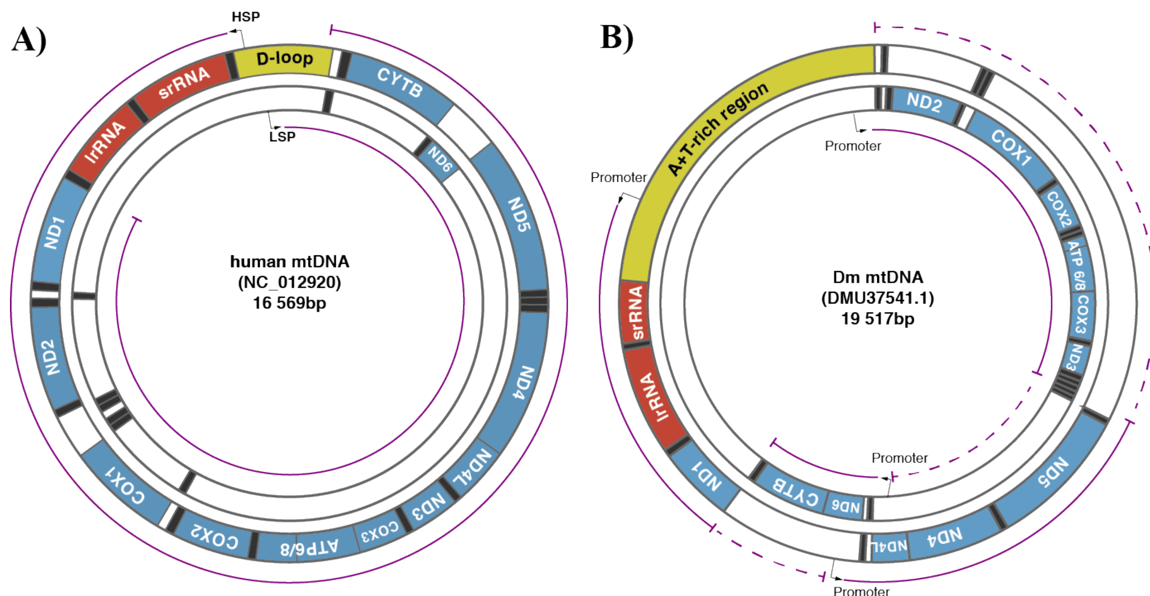

Figure 2. The mtDNA molecule. Mitochondrial mRNAs (blue), rRNAs (red) and tRNAs (grey) are marked. **A. Human mtDNA.** The human mtDNA is a compact 16,6 kB circular molecule that lack introns and only has one non-coding region where the D-loop can be found. The mtDNA is double-stranded with one light strand and one heavy strand, that encodes the majority of genes. Transcription starts from LSP or HSP and stops either at the control region or the antisense region of the rRNAs. This generates long polycistronic transcripts (purple solid lines). **B. *Dm* mtDNA.** The mtDNA is conserved between human and *Dm* however instead of the D-loop the *Dm* mtDNA has an AT-rich region and the genes are more evenly distributed between the two strands. The *Dm* mtDNA has been suggested to have additional promoter regions downstream of the AT-rich region. Transcription from these promoters would suggest the absence of several antisense RNAs (purple dashed lines). In **paper I** and **paper III** we have detected these antisense transcripts why our data speaks against the existence of additional promoters downstream of the AT-rich region. Figure reprinted from Freyer C *et al.*[28] with permission from Freyer C and Springer Nature.

lost due to redundancy or transferred to the nucleus. Mitochondria contain about 1300 proteins[29–31] and, with the majority of genes encoded in the nucleus, they are strongly dependent on nuclear-mitochondrial crosstalk for proper function[32]. Nonetheless, the genes expressed by mtDNA produce core proteins of OXPHOS complexes and are absolute essential for subsequent ATP production[33]. Mitochondria harbour a multi-copy genome and, depending on species and tissue, the number of mtDNA molecules can vary between just a few to over half a million copies per cell. The mtDNA is packed in structures referred to as nucleoids[34,35] and interspersed in the matrix[36]. The mammalian mtDNA is a compact, double-stranded, circular molecule of 16,6 kb that encodes 11 mRNAs, of which two are bicistronic, 22 tRNAs and 2 rRNAs (**Figure 2A**). Of the mRNAs, seven encode for CI, one for CIII and three for CIV. CII is solely encoded by the nucleus. The two strands are referred to as the heavy and light strand because of their difference in cytosine and guanine content[37]. In humans, most genes are encoded on the heavy strand. The light strand only encodes *MT:ND6* and a few tRNAs and mainly produces antisense transcripts, complementary to the coding transcripts. The mtDNA lacks introns and only has one non-coding region, referred to as the control region. Herein, the two sole promoter regions are found, the heavy-strand promoter (HSP) and light-strand promoter (LSP), together with a regulatory sequence for mtDNA replication [38–40]. Although lost in many species, the human mtDNA has retained a displacement loop (D-loop) within the control region and the function is not entirely clear although it is likely involved in replication[41].

The mitochondrial genome is highly conserved between human and fruit fly (*Drosophila melanogaster* (*Dm*)), however, some differences exist (**Figure 2B)**. Instead of the D-loop, *Dm* has a long non-coding AT-rich region, that increases the mtDNA size to 19.5 kb. It is believed to have a similar function as the D-loop although no defined structures have been described[42]. As in humans, two promoters are suggested to localise to the region, resulting in transcription of each strand[43]. Conversely, the order of genes in *Dm* differs from human, by being more evenly distributed between the two strands[44,45]. This evoked a model suggesting the existence of additional promoters downstream of the AT-rich region[46]. However, we have detected antisense transcript upstream of the suggested promoters, which argues against this theory[47].

2.3 MTDNA INHERITANCE

Inheritance of mtDNA from one generation to another is regulated at several levels making it sometimes complex to understand. Unlike nuclear chromosomes, mtDNA is not inherited in Mendelian fashion, instead it is solely maternally inherited[48,49], although several attempts have been made to prove paternal contribution[50]. Upon spermatogenesis the number of mtDNA copies is significantly reduced[51] and several mechanisms that destroy mitochondria from the sperm after fertilisation have been reported[52–54]. It is not fully understood why there is an advantage of eliminating paternal mitochondria but one theory suggests that the high levels of ROS produced upon spermatogenesis and fertilisation might introduce deleterious damages on the mitochondria and its mtDNA[55]. Moreover, the mtDNA is found aside from the nucleus and does not participate in the meiotic recombination[56].

Before further discussing mtDNA inheritance an introduction of the genetic terms homoplasmy and heteroplasmy is of relevance. In mitochondria, homoplasmy refers to a cell, tissue or organism that has identical mtDNA copies, whereas heteroplasmy implies having mtDNA molecules encoding different gene variants[57]. An individual normally carries multiple mtDNA genotypes[58], hence heteroplasmy is normal under physiological conditions. However, variants are usually at low levels and a potentially deleterious mutation has to reach a certain threshold before having a negative impact on the cell[59]. The mtDNA has a much higher mutation rate compared to nuclear genes[60] and according to the Müller's ratchet effect the combination of mutation rate, asexual transmission of genetic material and no recombination, cause accumulation of pathogenic mutations and an eventual mutational meltdown[61]. Nature has found several ways to try keeping the numbers of pathogenic mtDNA mutations at bay, and thereby preventing a mutational meltdown of mtDNA. Firstly, a genetic bottleneck prevents a too high number of mtDNA molecules with pathogenic variants to segregate to the same oocyte. Briefly explained, the population of mtDNA molecules in the primordial germ cells of an individual are first distributed into different primary oocytes during oocyte development. Just a few copies of the maternal mtDNA molecules are transferred to each primary oocyte and the distribution between them seems to be random, although the mechanism is poorly understood[62,63]. Upon further oocyte maturation the mtDNA population will be randomly expanded, causing that fully matured oocytes might differ in their mtDNA population from one another. This phenomena explains why siblings can have different heteroplasmy levels, why a mother can be healthy but her child sick, and

why the mtDNA genotype can vary greatly after some generations[62,64,65]. Secondly, by studying the offspring of mouse models carrying mtDNA mutations, Stewart and colleges were able to determine that pathogenic mtDNA mutations were selected against, and in many cases eliminated, in future generations, without the contribution of genetic drift[66]. Interestingly, it was later suggested that this genetic selection occurred during the pre-natal oocyte development[67]. This purifying selection mechanism has been observed previously for several types of mtDNA mutations but the mechanism remains unclear [66,68]. Another layer of complexity concerning mtDNA inheritance is that the level of heteroplasmy can vary in different tissues of an individual. After fertilisation, somatic segregation gives rise to different levels of heteroplasmy in different cells and tissues[69]. This phenomenon is likely one of the explanations of the variety of tissue specificities seen in mitochondrial diseases which is discussed later.

2.4 MTDNA REPLICATION AND TRANSCRIPTION

The minimal mtDNA replisome consists of mtDNA polymerase γ (POLγ)[70–72], the helicase TWINKLE[73] and mitochondrial single-stranded binding (mtSSB) protein[73,74]. Recently, several additional accessory proteins have been discovered such as primase polymerase (PrimPol) that aids upon unwanted stalled mtDNA replication[75–77]. Replication is conducted according to the strand displacement model, with a continuous synthesis of mtDNA along the entire length of the two strands[78]. Each strand contains an origin of replication, namely the heavy-strand origin (O_H) and light-strand origin (O_L). Replication seems to always be initiated at O_H and the replisome produces two thirds of the heavy strand before it passes O_L and start synthesising the light strand[38]. Primers for replication initiation are generated by mitochondrial DNA-directed RNA polymerase (POLRMT)[79,80]. The process of primer formation starts at LSP that is located some nucleotides upstream of the O_H region. Upon heavy-strand replication TWINKLE unwinds the double-stranded (ds) DNA and mtSSB protects the generated single-stranded (ss) DNA from transcription[73,74,81]. When O_L is reached, the replisome continues to replicate a few nucleotides downstream, and the generated ssDNA forms a stem-loop structure that prevents mtSSB to bind. This leaves ssDNA available for POLRMT that now produces primers at the light strand before soon being replaced with POLγ that starts light strand replication. In this way, replication of the light strand is highly dependent on heavy strand synthesis. Replication termination is less understood but is known to involve topoisomerases that separates the replicated mtDNA molecules. Termination probably occurs close to the O_H region[82]. The replisome is one of the major sites of pathogenic mitochondrial mutations[83,84] and has been described in both POLγ[85] and TWINKLE[86].

Replication is similar in *Dm*[45], although POLγ has a slightly different structure. Human POLγ has two accessory subunits (POLγB) accompanying the catalytic subunit (POLγA)[70,72], while *Dm* only has one accessory subunit[87]. Although less studied, transcription in *Dm* also seems to proceed in similar fashion to humans[88–95]. Mitochondrial transcription factor A (TFAM) plays a significant role in controlling the availability of mtDNA. It binds without sequence specificity, it is able to twist mtDNA in various fashions[96], and serves as the main scaffold

protein forming the nucleoids[36,97–99]. Nucleoids are suggested to be assembled in droplets via phase separation[100], which is a common assembly pathway for compartments lacking a membrane[101,102]. With help of STED super-resolution microscopy it was recently shown that the number of TFAM proteins that bind to a mtDNA molecule correlates with active replication and transcription[103,104]. A high amount of TFAM compacts the mtDNA and a low amount enables a looser structure that makes space for the transcription machinery and replisome[104]. In addition to form nucleoids, TFAM binds to the mtDNA a few nucleotides upstream of HSP and LSP and associates with POLRMT to initiate translation[105–107]. POLRMT also depends on structural changes of the promoter region and once bound mitochondrial transcription factor B2 (TFB2M) is recruited. TFB2M makes the final conformational changes that ultimately initiate translation. [106,108]. Elongation is promoted by transcription elongation factor (TEFM) that is found at the promoter region before initiation of transcription[109,110]. Transcription of each promoter will generate long polycistronic transcripts that cover the entire length of each strand[38,111,112]. Transcription from the heavy-strand has been suggested to also occur from a second promoter. Transcription from this promoter generates a shorter polycistronic transcript consisting of tRNA^{Phe}, 12S, tRNA^{Val}, 16S and tRNA^{Leu1}[111,113,114]. The steady-state levels of mitochondrial rRNAs are higher in comparison to those of mRNAs, strengthening the concept of a second promoter[38]. However, additional studies are needed to reach an agreement concerning the number of heavy-strand promoters.

Transcription termination of the light strand likely involves mitochondrial transcription termination factor 1 (MTERF1)[115]. In contrast, recent *in vitro* and *in vivo* studies could not support the involvement of MTERF1 in the termination of heavy strand transcription[115,116]. Instead, specific termination associated sequences at the end of the D-loop might be involved[117,118].

2.5 MITOCHONDRIAL RNA PROCESSING

2.5.1 Definition of mitochondrial RNA processing

The often rapid changes in cellular energy demand requires a strict regulation of mitochondrial gene expression to control ATP synthesis. The rate of mitochondrial translation and subsequent OXPHOS function are highly dependent on the availability of mature mitochondrial RNA molecules. Processing of mitochondrial RNA is therefore important to meet the cell's energy requirements. Mitochondrial RNA processing starts upon transcription at the HSP and LSP that generates long polycistronic transcripts. To be used in the translation process each individual transcript has to be released from the polycistrone. An accumulation of unprocessed precursor transcripts can cause problems with translation[119]. Further post-transcriptional processing events ensure proper maturation of mRNAs, rRNAs and tRNAs so that they can be used in downstream processes (**Figure 3**). Nonetheless, the availability of mature transcripts is also controlled by degradation of unwanted products. The proteins involved and their molecular mechanisms are not fully understood. A number of

mitochondrial diseases have been linked to errors in mitochondrial RNA processing[84] which emphasises the urgency of understanding these processes and is further discussed below.

2.5.2 Release of mitochondrial transcripts

The polycistronic precursor transcripts produced from the heavy strand and light strand are processed by a machinery of proteins to release each individual transcript. The majority of mRNAs and rRNAs in the mtDNA are flanked by tRNAs and the excision of each transcript is explained by the tRNA punctuation model[120]. The secondary structure of the tRNAs is recognised by riboendonucleases that catalyse the release of the transcripts. The 5′ end is processed first by the mitochondrial RNase P, consisting of the protein subunits MRPP1-MRPP3[121–123]. The 3′ end is processed by ELAC2[124,125]. Non-canonical processing of the remaining RNAs is believed to be performed by members of the FASTK family of proteins, although the mechanism is not fully understood[126–129]. RNA processing is believed to occur co-transcriptionally in so called RNA granules. These granules are membraneless compartments in the matrix that consist of RNA and RNA binding proteins[130,131]. The RNA granules were first discovered in the early 21st century as punctuates of newly synthesised RNA by 5-bromouridine (BrU) labelling[132]. Over the years they have been associated with an increasing number of proteins, belonging to all parts of mitochondrial RNA processing and mitochondrial ribosome assembly[131,133,134].

2.5.3 The unique mitochondrial RNAs and their modifications

2.5.3.1 Mitochondrial mRNAs

The mitochondrial encoded mRNAs differ considerable from mRNAs found in the cytosol[135]. They lack introns but also base modifications and the 5′ 7-methylguanosine cap[136]. In contrast to the bacterial counterpart mitochondrial mRNAs also lack Shine-Dalgarno sequences. How mitochondrial mRNAs are recognised by the ribosome is still poorly known and discussed in a later section of this review. The loss of RNA characters is possibly a consequence of the progressive compaction of mtDNA during evolution. There is also a difference between mitochondrial mRNAs. The majority of mitochondrial mRNAs lack a 5′ untranslated region (UTR), and only short ones are found in *MT:ND1*, *MT:CO1* and *MT:ATP8/6*. In addition, only *MT-ND5*, *MT-ND6* and *MT-CO1* have a 3′ UTR. The majority of mitochondrial mRNAs are polyadenylated at their 3′ end but also here heterogeneity is found which is discussed in detail below[135]. Furthermore, mitochondrial transcripts use a non-conventional genetic code, where most open reading frames (ORFs) starts with AUG but some with AUU or AUA, which normally encodes for isoleucine. Moreover, AGA and AGG are used as stop codons instead of coding for arginine, whereas UGA encodes tryptophan instead of a stop codon as in the cytosol[137,138]. The bicistronic transcripts *MT-ND4/ND4L* and *ATP6/ATP8* has partly overlapping ORFs and it is not known how they are translated[135,137]. Although less studied, the same features can be detected in *Dm* mitochondrial mRNAs[139].

2.5.3.2 *Mitochondrial tRNAs*

In the cytosol there are 50 different tRNAs, whereas mitochondria only contain the 22 encoded by the mitochondrial genome. The ability to recognise several codons, including the various start codons, is enabled by wobbling bases at the amino acid attachment site[140]. Mutations in tRNAs serve as major causes of mitochondrial disease and include, for instance, wobble modification defects of $tRNA^{Leu}$ and of $tRNA^{Trp}$[141–143]. The cloverleaf structure is conserved although several nucleotides have been exchanged[143–146]. In addition, the D and T loop can vary in size, possibly facilitating the interaction with aminoacyl-tRNA synthetases[143]. Maturation of mitochondrial tRNA molecules includes 3′ CCA addition by TRNT1 and various base modifications, such as methylation or pseudouridylation by PUS1[140,146–151].

2.5.3.3 *Mitochondrial rRNAs*

The mitochondrial 12S and 16S rRNAs undergo modifications but to a lesser extent than their cytosolic counterparts. Only 10 modifications are known so far[152,153]. Together with $tRNA^{Val}$, they are the only RNA components in the mitochondrial ribosome and the preserved modifications must therefore be essential for proper stability, function, and assembly into the mitochondrial ribosome. For instance, folding of 16S to generate the peptidyl transferase centre of the mitochondrial ribosome only occurs at the very end of proper ribosome assembly and is a conserved process also found in the cytosol and bacteria[154–156]. The modifications include methylation of nucleobases, 2′-O-ribose methylation and pseudouridylation[152,157–162]. Several of the enzymes responsible for the modifications have been characterised[152,157–160,163] but work remains to fully elucidate the function and how they are regulated.

2.5.3.4 *Mitochondrial non-coding RNAs*

Transcription of mtDNA generates non-coding RNA. Mitochondrial small non-coding RNAs (sRNAs) constitutes 3,1 % of the total number of cellular sRNAs and the majority originate from tRNA genes[39]. In addition to the 3′ UTRs found in a few mitochondrial mRNAs, long non-coding RNAs (lncRNAs), found in the mitochondrial transcriptome, include short inverted repeats that form 16S extensions[164] and *lncND5*, *lncND6* and *lncCytB* that are regulated by RNase P and ELAC2 as their coding equivalent. The lncRNAs can form intermolecular duplexes and, although primarily speculated, might pair with their complementary mRNAs to stabilise or prevent translation[165].

2.5.4 Mitochondrial transcript stability

The leucine rich pentatricopeptide repeat containing (LRPPRC) protein belongs to the family of RNA-binding pentatricopeptide repeat (PPR) proteins and are mostly found in the plant kingdom[166]. In metazoan, LRPPRC is situated in the mitochondrial matrix[167] where it binds

to and stabilises mRNAs after their release from the polycistronic transcripts. Disruption of LRPPRC expression results in a decrease of mRNA steady-state levels that ultimately leads to aberrant translation in both mice and *Dm* models[168–170]. By combining RNase footprinting and photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) Siira and colleagues demonstrated that LRPPRC functions as an RNA chaperone. By binding to mRNA LRPPRC stabilises the mRNA secondary structure, suggested to facilitate translation[171]. LRPPRC needs to be stabilised too and this is performed by stem-loop-interacting RNA binding protein (SLIRP). Although not as severe, disruption of SLIRP expression also affects mRNA stability and translation[172,173].

The FASTK protein family also contribute to mitochondrial mRNA stability. FASTK protects the 3′-UTR of ND6 and thus prevents its degradation[174] and disruption of FASTKD4 decreases steady-state levels of several mitochondrial RNAs[126,127]. The 16S rRNA is stabilised by FASTKD2 together with PTCD1 and RPUSD4[175–177]. Expression of FASTKD1 and FASTKD3 instead have a negative influence on mitochondrial transcript stability[126,178]. As mentioned earlier, several members of the FASTK protein family have been suggested to participate in non-canonical processing of the polycistronic transcript[126–129] and it is possible that this function affects mRNA steady-state levels.

The requirement of scaffold proteins is unique to mitochondrial RNA molecules and the purpose remains unknown. One theory is that they are needed to keep a pool of mature transcripts ready to be used quickly upon shifting demands of cellular energy[170], or they could protect against damaging components such as ROS.

2.5.5 Mitochondrial RNA polyadenylation

The only known maturation step of mitochondrial mRNAs is 3′ polyadenylation. However, its function is largely unknown[40,135,179]. The reaction is catalysed by polyadenylic acid RNA polymerase (MTPAP)[180] that generates a 30-70 nucleotides long poly(A) tail. Polyadenylation has been studied in different model systems and the variation in poly(A) tail length might differ depending on mRNA and cell type, although a functional relevance cannot be excluded[135,169,170,179,181]. Polyadenylation is necessary to complete the stop codon of several mitochondrial mRNAs[135]. However, this requires the addition of only two adenines. In human[182] and mice[170] *MT:ND6* is not polyadenylated but instead has a long non-coding 3′UTR[135], whereas *mt:nd6* in *Dm* is fully polyadenylated[169,183]. In humans, *MT:ND5* has a shorter tail with only a few nucleotides and *MT-CO1* has been found both polyadenylated and non-polyadenylated[39,135,184]. Mitochondrial rRNAs have shorter poly(A) tails and the 12S rRNA is only a few nucleotides long[135,182].

The crystal structure of MTPAP was established in 2011[185] and further explored by Lapkouski and Hällberg in a study published 2015[186]. The protein functions as a dimer, of unknown reason and not seen in other adenylases. MTPAP has a strong preference for adenosines, although it can use other nucleosides as substrates *in vitro*. The specific RNA-recognition motif (RRM) domain in MTPAP has also been suggested to act in protein-protein interactions[187]. No direct binding partners of MTPAP have so far been found but pull-down

experiments in human cell-cultures have suggested that MTPAP forms transient complexes with proteins of the mitochondrial RNA degradosome and that they together regulate poly(A) tail length[188,189]. Another protein that regulates MTPAP processivity is LRPPRC that has been found to promote polyadenylation in both mice[170] and *Dm*[169].

Cytosolic mRNA poly(A) tails are required for stability, nuclear export, and translation[190], whereas in bacteria polyadenylation promotes degradation[191]. The evidence of such involvement of polyadenylation in mitochondria is poor and is further studied in this thesis. Nearly all mRNA molecules in eukaryotic cells have a stable poly(A) tail. However, in bacteria only a small population of RNA is found to be polyadenylated. This suggested that polyadenylation in bacteria is a transient process and in accordance with this theory it was later found that the poly(A) tail destabilises full length RNA and decay intermediates. The process is conducted by the bacterial ortholog poly(A) polymerase (PAPI) and is followed by exoribonucleolytic cleavage by polynucleotide phosphorylase (PNPase) or RNase II[191,192]. A small population of polyadenylated and partly truncated transcripts have also been found in human mitochondria[182] and suggested to be processing intermediates[123]. In **paper III** we show that the metazoan mitochondrial RNA degradosome influence MTPAP activity but mitochondrial mRNAs with no poly(A) tail is still efficiently degraded. In addition, antisense transcripts that are rapidly targeted for degradation under physiological conditions only contain a few adenines attached to their 3′ end, suggesting that degradation occurs before polyadenylation[47]. These data suggest that polyadenylation is not a signal for degradation in mitochondrial mRNAs. On the contrary, polyadenylation has been suggested to be a signal for degradation of tRNAs with structural defects[193].

In the cytosol mRNA polyadenylation is a two-step process that starts with an endonucleolytic cleave at the 3′ end of the immature mRNA followed by polyadenylation that enables translation in the cytoplasm[190]. Defective polyadenylation can disturb translation in mitochondria[194,195] but a direct mechanism has never been proposed. We have previously shown in MTPAP knock-down (KD) and knock-out (KO) *Dm* models that loss of polyadenylation can both increase and decrease translation rate depending on transcript[196]. Moreover, a study of the mitochondrial mRNA poly(A) tail interactome identified several mitochondrial ribosomal subunits[197] and truncated transcripts have been suggested to cause ribosome stalling[198]. In paper **IV** I investigated the role of MTPAP and polyadenylation in translation and found alterations in the proteome of the translation apparatus and transcript specific consequences upon translation when the poly(A) tail is lost. This suggests that polyadenylation has adopted a role in translation and is further discussed in **paper IV**.

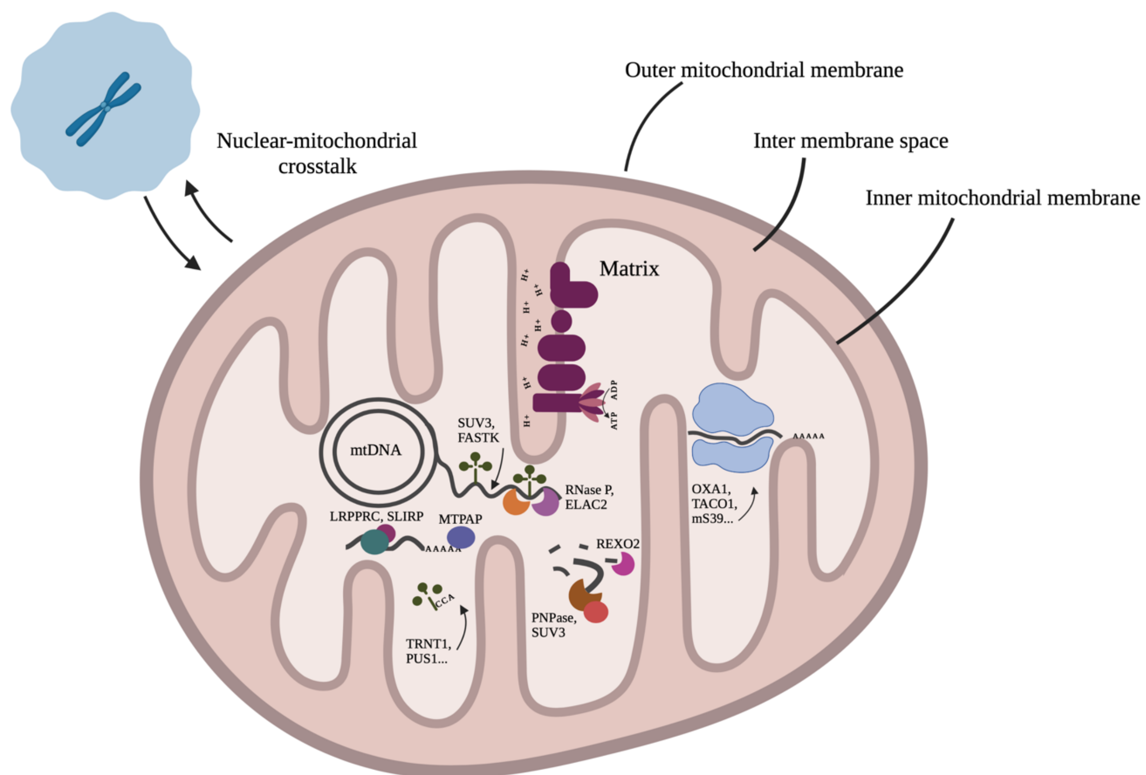

Figure 3. Mitochondrial RNA processing. The mitochondria are double-membrane organelles that are under dual genetic control. Mitochondrial gene expression takes place in the matrix and includes various steps that requires a machinery of enzymes imported from the nucleus. The figure illustrates some of these enzymes. *Transcription* generates long polycistronic transcripts that undergo processing to release the individual transcripts. The tRNAs are recognized by RNase P and ELAC2 that cut the tRNAs at their 5′ and 3′ ends, releasing the neighboring transcripts[120]. Transcripts not flanked by tRNAs are suggested to be recognized by proteins of the FASTK family[126–129]. SUV3 helicase facilitates release of the transcripts[119]. The released mRNAs are *stabilised* by the LRPPRC-SLIRP complex[168–170]. Further *maturation* includes mRNA polyadenylation by MTPAP[180] and tRNA modifications by for instance TRNT1[146] and PUS1[149]. Unwanted transcripts are *degraded* by the RNA degradosome consisting of PNPase and SUV3[47,188]. Short RNA products are degraded by REXO2[199–201]. It is not known how the mRNAs are recognized by the mitochondrial ribosome but TACO1[202,203] and mS39[204] might aid in the process. The mtDNA encodes core proteins of the RC complexes and ATP synthase that co-translationally are inserted into the IMM by the help of OXA1[205–207]. Created with BioRender.com

2.5.6 The mitochondrial RNA degradosome

Mitochondrial mRNAs originate from the same polycistronic transcripts, but display a high variability in steady-state levels upon physiological conditions, emphasising the importance to control their half-lives[39]. Redundant sense transcripts, antisense transcripts and other unwanted non-coding RNAs necessitates an active RNA degradosome that rapidly removes these products[208]. The metazoan mitochondrial RNA degradosome is suggested to be built by the ATP-dependent SUV3 helicase and PNPase that forms a heteropentameric complex that degrades RNA in a 3′ to 5′ direction[188,209]. SUV3 was initially identified in yeast as a suppressor of the *var1* (SUV3) phenotype. *Var1* encodes a mitochondrial ribosomal subunit and SUV3 was proposed to rescue impaired translation caused by manipulating Var1 expression[210]. SUV3 is mainly found in the mitochondrial matrix but has also been suggested to regulate the cell cycle and reside in the nucleoli[211]. In **paper I** I report an additional function for SUV3 in mitochondrial RNA processing. We disrupted the expression of SUV3

in *Dm* and detected an accumulation of unprocessed polycistronic transcripts, which suggests that SUV3 is important in the release of the individual transcripts. Disruption of SUV3 also hindered subsequent polyadenylation[119].

The other partner of the RNA degradosome, PNPase, was initially suggested to localise to the IMS[212,213]. This suggestion mainly arose from detecting a negative effect on import of the 5S rRNA upon disrupting PNPase expression[212]. In the ribosome of several kingdoms the 5S rRNA is found at the central protuberance region of the mitochondrial large subunit. However, it was later found that the 5S rRNA is replaced with mitochondrial encoded tRNA^{Val}[214,215] and consequently, there is no need of 5S import. In addition, the proposed function in RNA degradation occurs in the matrix. Therefore, there are some uncertainties considering the localisation of PNPase. PNPase has a phosphorolytic 3′ to 5′ exoribonuclease activity but has also been suggested to have an oligonucleotide polymerase activity[216]. Accordingly, *in vitro* studies have shown that PNPase can both degrade and extend 3′ poly(A) tails[189,217]. Consequently, it has been proposed that PNPsae regulates polyadenylation of MTPAP together with SUV3[188,189]. In **paper III** I report the role of PNPase *in vivo* using *Dm* as a model organism and we were able to confirm a role in RNA degradation together with SUV3. Moreover, we identified that PNPase influences MTPAP activity but the physiological relevance remains unclear[47].

There are several proteins that have been suggested to influence mitochondrial RNA degradation. Non-coding transcripts that are rich in guanines are prone to form four-stranded structures referred to as G-quadruplexes[218]. In mitochondria, these are removed by GRFS1, which simplifies degradation by the RNA degradosome[219]. LRPPRC instead stabilises and protects the transcripts from degradation[220]. The oligoribonuclease REXO2 functions as a complement to the RNA degradosome by degrading short RNA products in the mitochondria[199–201]. It is not known if deadenylation is required for degradation and so far no mitochondrial deadenylase has been determined although PDE12 has been suggested as a candidate[221,222] in addition to PNPase[47,189].

2.6 MITOCHONDRIAL PROTEIN SYNTHESIS

2.6.1 The mitochondrial ribosome

Recent advances in omics technologies and cryo EM have improved our understanding of mitochondrial translation. For instance, we are now able to capture detailed structures of different stages of mitochondrial ribosome assembly and translation. In 2015 the very first near-atomic visual description, with above 3.0 Ångström resolution, of the mitochondrial ribosome was obtained by Greber[223] and Amunts[224] with colleagues. As all ribosomes, the mitochondrial ribosome consists of a small (mtSSU) and large (mtLSU) subunit that assembles to form a monosome upon translation. However, the composition differs quite extensively from its bacterial ancestor. The amount of RNA components is almost half compared to other ribosomes and to compensate the loss of RNA about 30-70 additional mitochondrial ribosomal proteins (MRPs) have been recruited, depending on species. Some

of these proteins are unique for the mitochondrial ribosome and several of the conserved proteins have gained C-terminus and N-terminus extensions to replace lost functional domains[223–228]. Therefore, RNA-RNA and RNA-protein interactions have been replaced with protein-protein interactions. The two rRNA molecules encoded by the mtDNA are the only structural RNA components needed together with mitochondrial encoded $tRNA^{Val}$. As mentioned earlier, $tRNA^{Val}$ has replaced 5S at the central protuberance region of mtLSU[214,215]. The $tRNA^{Val}$ molecule can be replaced by $tRNA^{Phe}$ without disturbing the function of the mitochondrial ribosome[229]. The use of these two specific tRNA molecules can potentially be explained by their localisation in the mtDNA as they are neighbouring 12S and 16S and hence can be transcribed, matured and incorporated in the mitochondrial ribosome simultaneously[230]. The role of the tRNA molecule has not been elucidated but likely has a similar function as 5S that has been proposed to be important for mitochondrial ribosome stability[231].

The explanation of these adaptions of the mitochondrial ribosome is a subject of debate[230,232,233]. The initial genome reduction might have brought an advantage for energy production but later, with the advancement of the organism, forced the mitochondrial ribosome to be rebuilt. Nevertheless, the products produced by the mitochondrial ribosome also influenced the development of these adaptions. The mitochondrial ribosome must translate highly hydrophobic proteins and co-translationally these will be inserted in the IMM. The incorporation of mL45 facilitates binding of the mitochondrial ribosome to IMM[204,215,234]. In addition, hydrophobic residues coating the polypeptide exit tunnel regulate exit rate.

2.6.2 Mitochondrial translation

2.6.2.1 Regulation of mitochondrial translation

mtDNA only encodes a few components of the translation apparatus, meaning that numerous structural and regulatory proteins need to be imported from the cytosol. The signals and pathways regulating the coordination between cytosolic and mitochondrial translation in metazoan are largely unknown although feedback communication clearly exists as mitochondrial translation rate of individual transcripts is dependent on the import of nuclear encoded OXPHOS subunits[235,236]. The regulation of these signals has been suggested to be conducted by early OXPHOS assembly factors that recognise an imbalance in OXPHOS subunits. For instance, MITRAC15 regulates the assembly of CI and CIV and MITRAC12 controls CIV assembly[237]. Import of nuclear encoded subunits might also be regulated by translocases found in the mitochondrial membrane such as TIM21[237].

Our functional understanding of the mitochondrial ribosome has vastly improved with several structural intermediates of late-stage assembly and translocation intermediates being determined. As mentioned earlier, the assembly is believed to occur co-transcriptionally in the RNA granules[128,238,239] together with the stepwise post-transcriptional modifications of the rRNAs. The different rRNA modifications are also associated with step-wise recruitment

of MRPs[122]. This strict hierarchal assembly of the mitochondrial ribosome results in early, intermediate, and late assembly stages. The process requires assembly factors and might involve recruitment of already pre-assembled protein clusters. It is not known if these processes are strictly sequential or occur in several parallel pathways resulting in a mixed ribosomal population[240,241]. Additionally, numerous maturation factors, such as GTPases, ATP-dependent helicases and methyltransferases are important for ribosome maturation. Several of these factors are associated with the IMM, but further studies are required to elucidate their full roles[162]. Interestingly, there seems to be a surplus of mitochondrial ribosomal components, which are rapidly degraded if left un-used. Full assembly of the mitochondrial ribosome seems to be a slow process, predicted to take hours, in comparison to minutes in bacteria. Whether the excess in assembly intermediates is a reflection of this remains to be determined[240,242].

2.6.2.2 Mitochondrial translation initiation

Translation initiation in mitochondria is comparable to its bacterial ancestor and includes recruitment of mRNA and association with initiation factors and $tRNA^{Met}$. In bacteria there are three initiation factors. IF2 recruits $tRNA^{Met}$ and stabilises the codon-anticodon reaction and IF3 primarily prevents pre-mature association between mtSSU and mtLSU. IF1 enhances the activities of IF2 and IF3[243,244]. Mitochondria have lost IF1 and its function seems to be transferred to mtIF2[245,246]. As in bacteria, $tRNA^{Met}$ needs to be formylated to be used for translation initiation[247–249] and loss of the responsible transformylase MTFMT causes mitochondrial disease[250]. Single-particle cryo-EM has revealed that mtIF3 is first recruited to the mtSSU MRP mS37, which induces a conformational change that allows interaction with mtIF2. Before association with $tRNA^{Met}$, mtIF3 has to leave[242] but whether the initiator tRNA and mRNA binds to the mtSSU prior to recruitment of the mtLSU or after, to form a working initiation complex, is not fully understood[162]. Another unsolved question of mitochondrial translation is how the leaderless mitochondrial mRNAs are recognised by the translation apparatus. Recent studies have found proteins that associated with both mitochondrial transcripts and the mitochondrial ribosome. TACO1 has been suggested to regulate translation by binding to *MT:CO1* prior to association with the mitochondrial ribosome and the GTP-hydrolysing MRP mS39 has recently shown to facilitate binding of mRNAs during translation initiation[204,234,251]. Purely speculative, these proteins might facilitate the recognition of transcripts by the mitochondrial ribosome.

2.6.2.3 Mitochondrial translation elongation and termination

The elongation process is comparable to that in bacteria and involves a number of elongation factors such as mtEFTu that delivers aminoacylated tRNAs to the matching mRNA codon at the decoding site[252,253]. When leaving the mitochondrial ribosome mtEFTU is recycled by the guanine exchange factor mtEFTs that enables mtEFTU to associate with GTP and bind a new aminoacylated tRNA[252–255]. Translation termination occurs when the ribosome reaches a stop codon that is recognised by ribosomal release factors.

Mitochondrial mRNAs carry both canonical and non-canonical stop codons, potentially necessitating several mechanisms for translation termination. mtRF1 and mtRF1a seem to recognise the canonical stop codons[256,257]. Currently two hypotheses can explain the recognition of the alternative stop codons. Either a mechanism of one nucleotide frameshift occurs that transforms the non-canonical stop codons to a conventional stop codon, recognised by mtRF1[258]. Alternatively, the alternative stop codon is also recognised by mtERF1, or by another elongation release factors such as ICT1[259–261]. Clearly, more detailed studies are required to elucidate the mechanism of mitochondrial translation termination. Many steps of mitochondrial gene expression occur simultaneously and this is also seen in the last step of translation as the emerging polypeptide is co-translationally inserted to the IMM by OXA1[205–207,262]. After release of the peptide the ribosomal subunits are dissociated and re-cycled by ribosomal recycling factors, such as mtRRF, mtEGF2[263] and GTPBP6[264], but currently not much is known about these processes.

2.6.3 Mitochondrial aminoacyl tRNA-synthetases

Aminoacyl synthetases (ARS) are responsible for charging tRNAs with their corresponding amino acid and are required for translating the genetic code to functional peptides. There are two groups of ARS based on their sequence motifs, which slightly changes the way they operate[265]. Aminoacylation involves an esterification reaction and is conducted in two steps. In brief, the first step constitutes the ARS binding to the cognate amino acid, in a reaction requiring ATP that is transformed to adenosine monophosphate (AMP) in the process. Secondly, the amino acid is transferred to its corresponding tRNA molecule and AMP is released. They are built simply by two domains, one catalytic and one binding the anticodon[266]. Mitochondria have their own set of ARS, referred to as ARS2. The nucleus encodes 19 specific mitochondrial ARS2 molecules, while two, GARS (glycine) and KARS (lysine), are shared between the cytosol and mitochondria. Finally, QARS2 (glutamine) is first aminoacylated with glutamic acid before undergoing transamidation to glutamine by hGatCAB[267]. In general, mitochondrial ARS2 have a reduced capacity to discriminate between amino acids, leading to an increase in wrongly aminoacylated tRNAs[268–270]. Cytosolic ARS have been shown to have a proof-reading ability to remove mismatched amino acids[266], but whether such function is retained in mitochondrial ARSs is unknown.

2.7 MITOCHONDRIAL DISEASES

2.7.1 Inborn errors of metabolism and the nature of mitochondrial diseases

IEM form a group of primarily monogenic disorders that most often disturb a single biochemical pathway, such as carbohydrate, amino acid, lipid or lysosomal metabolism[8]. The council of European union declared rare diseases, such as IEM, as a public health concern and therefore encourage newborn screening and continuous evaluation of included diseases and treatment[271]. In Sweden, newborn metabolic screening includes 24 curable diseases, such as phenylketonuria and congenital adrenal hyperplasia, and hopefully this list will continue

to expand. Mitochondrial diseases form a large subgroup within IEM and so far, does not have biomarkers or cure. The first mitochondrial disease was described in 1962 by Rolf Luft in a patient with a hypermetabolism[272]. About 30 years later, the first mitochondrial diseases caused by mutations in mtDNA were reported in Leber's hereditary optic neuropathy (LHON)[273] and mitochondrial myopathy[274]. Initially, mitochondrial diseases were described as primary defects in the final steps of cellular respiration and ATP production. However, over the years numerous additional mutations have been reported to affect OXPHOS, and factors involved in mitochondrial gene expression and dynamics have been shown to result in a wide range of clinical features[275]. In total, over 250 pathogenic variants of mtDNA and over 300 nuclear encoded genes have been described to cause monogenic mitochondrial diseases[276,277]. Since the metabolic pathways are linked, mitochondrial dysfunction is now also being described in other groups of IEM, as well as in more common diseases such as neurodegenerative disorders[278]. Advancements in understanding mitochondrial function can therefore be of value for a large group of patients.

The pathophysiology and molecular mechanisms causing mitochondrial diseases are complex but recent advancements in whole genome sequencing and other omics techniques have improved our knowledge dramatically[84,279,280]. However, the heterogenic nature of mitochondrial diseases is still difficult to understand. Mitochondrial dysfunction can affect a wide range of organs, at any age leading to that these patients can be found in almost all clinical specialties. Often the diseases are progressive and affect tissues with high energy demand, such as the nervous system, heart muscle, skeletal muscles, or liver. Children usually develop more severe symptoms than adults. Furthermore, mutations affecting the same biochemical pathway can have very different clinical presentations, which makes diagnosis even more complicated, and leaves numerous patients undiagnosed for several years[281]. Estimation of prevalence suggests that 9/100 000 adults will develop mitochondrial disease, while in children the number is suggested to be 5-15/100 000. Moreover, approximately 23 per 100 000 persons carry a pathogenic mutation that potentially can cause disease later in life or in future children[282,283].

The heterogenic clinical presentation makes it difficult to classify different groups of mitochondrial diseases. However, they can be grouped by their clinical presentation, although many have overlapping symptoms[279]. For instance, there are three groups of mitochondrial encephalomyopaties: Leigh syndrome, characterised by fast progressing encephalomyelopathy[284], mitochondrial epilepsy, and leukoencephalopathies. However, some patients with Leigh syndrome also present with epilepsy[279].

2.7.2 Diagnosis of mitochondrial diseases

The heterogenic nature of mitochondrial diseases can make it difficult to know when to start an investigation for mitochondrial dysfunction. However, there are some acute onset symptoms that proceed mitochondrial investigation such as stroke-like episodes, epileptic seizures difficult to treat, liver failure and unexplained lactic acidosis. Also, developmental delay or regression, muscle weakness, exercise intolerance and ptosis can be symptoms of mitochondrial disorders in children. Traditionally, diagnosing mitochondrial diseases have

been performed by a combination of metabolic screening, muscle biopsies, and neuroimaging. Although these methods still serve as valuable complements next generation sequencing, including whole genome and whole exome sequencing (WGS and WES), are the foremost used diagnostic tool today[285–287].

2.7.3 Pathologies of mitochondrial RNA processing

A growing number of factors involved in mitochondrial RNA processing have been linked to mitochondrial disease. Pathologic mutations can be found in enzymes of all stages of mitochondrial post-transcriptional RNA processing and some examples will be discussed here. Several mutations in ELAC2 have been reported and are associated with a severe form of infantile cardiomyopathy. ELAC2 cleaves the 3′ end of tRNAs in the polycistronic transcripts[120] and mutations cause an accumulation of RNA precursors, leading to impaired mitochondrial translation and CI deficiency[288,289]. Studies of the ELAC2 ortholog in *Dm* showed comparable symptoms, including cardiac hypertrophy and reduced contractility[290]. In addition, deficient ELAC2 expression has been associated with prostate cancer. The mechanism behind this association relies in the ability of ELAC2 to promote expression of the growth factor TGF-Beta that inhibits cell division[291].

Several pathogenic mutations also associate with enzymes responsible for RNA maturation, which emphasises the urgency of understanding these processes. In my thesis I present studies where the mechanism of several of these factors are investigated. In **paper III** the relationship between the RNA degradosome, LRPPRC and MTPAP is studied. Mutations in the *PNPT1* gene encoding PNPase results in severe neurological disorders such as encephalomyopathy[292], often with white-matter engagement[293], caused by OXPHOS deficiency. The mutations can be localised to different regions of *PNPT1* and disrupt the function of PNPase in various ways. PNPase is a trimeric, doughnut shaped protein, with a central channel that binds single-stranded RNA[294] and Golzarroshan and colleagues were able to show that two disease linked mutations cause loss of one of the subunits, significantly reducing the RNA binding capability[295]. Leigh syndrome is the most common mitochondrial disease in children[284] and mutations in LRPPRC can cause French Canadian type of Leigh syndrome[296–298]. Pathologic mutations in MTPAP cause spastic ataxia, optic atrophy[299,300], and encephalopathy[301] and the role of MTPAP in mitochondrial RNA processing is further discussed in **paper III** and **paper IV**.

Processing defects of rRNAs and tRNAs has also been implicated in mitochondrial disease. For instance, mutations that affect methylation of 12S can result in problems with the mitochondrial ribosome leading to OXPHOS defect in humans[302,303]. Additionally, tRNAs require several posttranscriptional modifications, such as uridine to pseudouridine by PUS1, which has been associated with myopathy, lactic acidosis, and sideroblastic anemia (MLASA)[148,304].

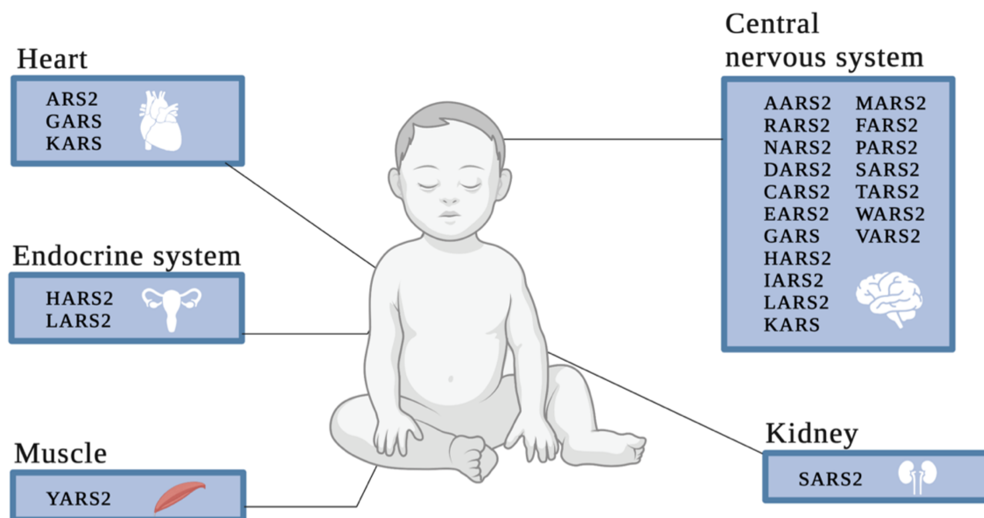

Figure 4. Pathologies of mitochondrial aminoacylation tRNA-synthetases. Pathogenic mutations in genes encoding ARS2 result in heterogenic clinical manifestations. The figure illustrates the different ARS2 and which organ system they affect. Although many pathogenic variants cause symptoms from the central nervous system the variation within these group is also diverse. For instance, deficiency of HARS2 can cause sensorineural hearing loss and ovarian dysgenesis while deficiency of DARS2 is associated with encephalopathy[305,306]. Created with BioRender.com

Pathologies of mitochondrial aminoacylation tRNA-synthetases

Pathogenic variants have been described in all ARS2 genes[279,305,306]. ARS2 diseases are distinctive in that they present with a surprising heterogenic clinical manifestation although they are all expected to affect mitochondrial translation similarly (**Figure 4**). The most common clinical presentation seen in these patients is encephalopathy, such as in the presence of DARS2[307,308] and FARS2 mutations[309]. But symptoms are diverse and mutations in for instance LARS2 cause infertility and hearing loss[310], while variants in AARS2 can lead to hypertrophic cardiomyopathy[311]. Additionally, mutations in the same gene can result in different symptoms in different patients. For instance mutations in WARS2 have been associated with encephalopathy and growth retardation[312], microcephaly, seizures and brain atrophy, hepatopathy, early onset Parkinsonism, as well as aggressive behaviour in children[313–317]. Mitochondrial diseases caused by mutations in ARS2 enzymes profoundly illustrates the importance of understanding tissue specificity and is further discussed in **paper II.**

2.7.4 Present and future treatment of mitochondrial diseases

Currently, symptomatic treatment is the main therapeutic approach that can be offered to patients with mitochondrial disease, but several pharmacological substances are tested in the laboratory and some have even advanced to clinical trials[318,319]. So far, mainly nutritional supplements are used in the clinic. The aim with these supplements is to interfere in mitochondrial pathways, such as to increase mitochondrial biogenesis or target mitophagy

via the mTOR pathway[279], but has failed to show important clinical results[320]. There are a few exceptions. Supplementation with CoQ in patients with primary CoQ deficiencies shows some symptomatic relief, but the results vary[321]. Also, the synthetic CoQ analogue 2,4-dihydroxybensoic acid has been shown to increase OXPHOS function in patient-derived fibroblasts[322], but has diverse results in patients, probably due to variation in remaining enzyme activity[323]. Treatment with deoxynucleoside monophosphate of patients with thymidine kinase 2 deficiency, which results in depletion of mtDNA, has also showed improvement of symptoms probably due to restoration of mtDNA levels[324].

In addition to pharmacological treatment options, the hopes for the future are to include genetic therapies that target and delete mutated mtDNA. CRISPR/Cas9 gene editing has shown to be very powerful, however, import to mitochondria of guide RNAs and the Cas9 enzyme complex is limited[325]. Instead there are other approaches under development that are showing promising results such as Zinc-finger nucleases and transcription activator-like effector nucleases (TALENs). These are mitochondrial-targeted restriction enzymes that are engineered to cut specific mtDNA sequences for destruction[326]. Although several promising therapeutic tools are on the horizon, most patients remain for now without cure. Understanding mitochondrial function upon physiological and pathological conditions, to be able to improve and develop treatment options and identify biomarkers, remains highly prioritised.

2.8 MITOCHONDRIA AND IMMUNITY

Mitochondria can both trigger and control immune responses and are therefore important regulators of immunity. The mechanisms and pathways are complex and will only be briefly summarised with some of the key features of mitochondrial immune functions. The mitochondrial network serves as a platform for diverse signals in innate immunity and one of the more well-studied mechanisms is the activation of the NLRP3 inflammasome. The NLRP3 inflammasome is a multi-protein complex that mediates caspase-1 activation and production of pro-inflammatory cytokines upon cellular damage and microbial infections[327]. The activation can occur via several pathways, including production of ROS in excess, calcium signalling, ATP and mitochondrial antiviral-signalling (MAVS)[328]. MAVS are found in the OMM and form prion-like aggregates when triggered[329], and besides activating the NLRP3 inflammasome, they are important signalling molecules for activation of the pattern recognition receptors (PPRs) RIG-I and MDA-5[330,331]. Also, intermediates of the TCA cycle can activate pro-inflammatory cytokines[332].

Intriguingly, release of mtDNA and RNA products have shown to elicit immune activation[1,328,333,334]. The role of mitochondrial RNA is less studied, but mtDNA functions as a damage-associated molecular pattern (DAMP) and triggers the expression of various PPRs, such as the toll-like receptor (TLR) family, and also activates the NLRP3 inflammasome[335]. In addition, the role of OXPHOS in controlling the function of immune cells, including those of adaptive immunity, is unclear and seems to depend on substrate availability[335].

2.9 THE EMERGENCE OF OMICS TECHNIQUES

Omics techniques have lately emerged as new tools to study biology. By applying computational data processing, the techniques enable studies of epigenomics, genomics, transcriptomics, proteomics, and metabolomics in any model system. In classic research studies are often performed to evaluate one particular protein or pathway of interest, while omics technologies enable studies of the whole landscape of molecules and how they are changing in different environments[336].

Biological processes rely on the transfer of information in a cascade of reactions, starting from gene expression and eventually reaching its destination, such as expression of a certain protein or metabolite. Omics techniques, especially if combined, will in this respect give a more complete and realistic image of the biological processes[337–339]. Importantly, outliers in a data set can indicate specific changes unique for that particular experimental set up, and often open up for additional research. For instance, in clinical studies, it can show changes upon advancement of disease that could act as useful biomarkers[340,341]. In addition, as omics technologies are further improved, they will provide an opportunity to develop precision medicine, where custom-made medicine can be specifically targeted to individual patients at the correct moment in time[339]. With a lack in suitable treatments, mitochondrial diseases are in great need for such advancements[84].

To perform omics studies, there has been an extensive development of different sequencing platforms, computer science techniques, as well as available databases, to mention a few[342]. With these cutting-edge technologies and high-throughput information a significant challenge relies in how to process and interpret the data, and ultimately translate it to the clinic[339]. This requires appropriate design of the experiments and validation of the results by traditional laboratory work.

3 RESEARCH AIMS

Previous studies of mechanisms and proteins involved in mitochondrial RNA processing have shown inconsistencies in the data, mainly because of the use of different model systems. The aim of the work presented in this thesis was to study factors suggested to be involved in RNA metabolism, and to understand their mechanism and role in mitochondrial gene expression. Several of these factors have also been suggested to be involved in mitochondrial diseases and clarifying their function might also elucidate their pathomechanism. All the studies are performed in the fruit fly to ensure comparable and physiologically relevant data.

In **paper I** the aim was to elucidate the role of *Dm*SUV3 helicase in mitochondrial RNA processing. SUV3 has been suggested to participate in RNA degradation together with PNPase and here we investigate this suggested function *in vivo* and evaluate any additional individual role of *Dm*SUV3.

Paper II describes the molecular diagnosis of two patients with a mutation in the mitochondrial tryptophanyl-tRNA synthetase, WARS2. In addition, we explore the benefits of using different model systems to study mitochondrial disease.

Paper III is a continuation of **paper I**, characterising the function of *Dm*PNPase, which, together with *Dm*SUV3, forms the mitochondrial RNA degradosome. In addition, an effort was made to map the role of *Dm*PNPase in relationship to *Dm*SUV3, BSF and *Dm*MTPAP to elucidate their function, in which order they work, if they collaborate, and how they influence mitochondrial RNA polyadenylation.

With **paper IV** we continued our work from **paper III** and investigated the role of *Dm*MTPAP and mitochondrial RNA polyadenylation upon translation.

4 MATERIALS AND METHODS

4.1 DROSOPHILA MELANOGASTER AS A MODEL ORGANISM

The fruit fly has been used as a model system to study various physiological processes ever since the lab of Thomas Hunt Morgan, over a century ago, showed how versatile and useful this little organism is[343,344]. It is easy to maintain, has a short generation time and only contains four chromosomes, which makes its genome rather easy to manipulate[345]. The four chromosomes of the *Dm* karyotype consist of the autosomal chromosomes 2, 3, 4 and the sex chromosomes X and Y. The X chromosome is also referred to as chromosome 1[346]. Chromosome 4 is profoundly small and lacks crossover events during meiosis and is therefore rarely used for genetic manipulation[347].

The various genetic tools that can be used include, for instance, insertion of *p-elements*[348], ends-in[349] and ends-out[350] targeted homologous recombination, or CRISPR/Cas9 gene editing[351,352]. The mitochondrial genome of humans and *Dm* is highly conserved, which makes the fly an excellent model to study and translate the results to human mitochondrial function. However, although the genes expressed are the same, gene order and expression pattern varies[44,95] which always have to be taken into account when translating results to the human system. The online bioinformatic database FlyBase, administered by Indiana University, Harvard University and University of Cambridge, is an immense tool for every drosophilist as it contains almost all needed information concerning genetic and molecular characteristics of any particular protein and fly.

Throughout the work presented in this thesis flies were maintained on a standard yeast-sucrose-agar (10-5-1) nourishment and kept at 25 degrees, with 60% humidity, on a 12 hour:12 hour light:dark cycle. To minimise age gap within and between samples flies were laying eggs for maximum eight hours. Larvae were always collected at third instar larval stage (**Figure 5**) for analysis.

4.1.1 The GAL4-UAS system

The GAL4-upstream activating sequence (UAS) system was applied to target the expression of chosen genes in **paper I**, **paper II**, and **paper III**. The GAL4-UAS system enables tissue-specific silencing or overexpression of a gene of interest by utilising the yeast GAL4 transcription factor that is placed under the control of a native promoter in the tissue of choice. As examples, ubiquitous expression is often achieved by using the promoter of *daughterless* and specific expression in the nervous system often uses the *elav* promoter to ensure tissue-specific activity. Briefly, overexpression is achieved by the aid of a genomic enhancer, while silencing uses short hairpin RNA that is complementary to target mRNA and induces degradation and subsequent knock-down of gene of interest by RNA interference[353].

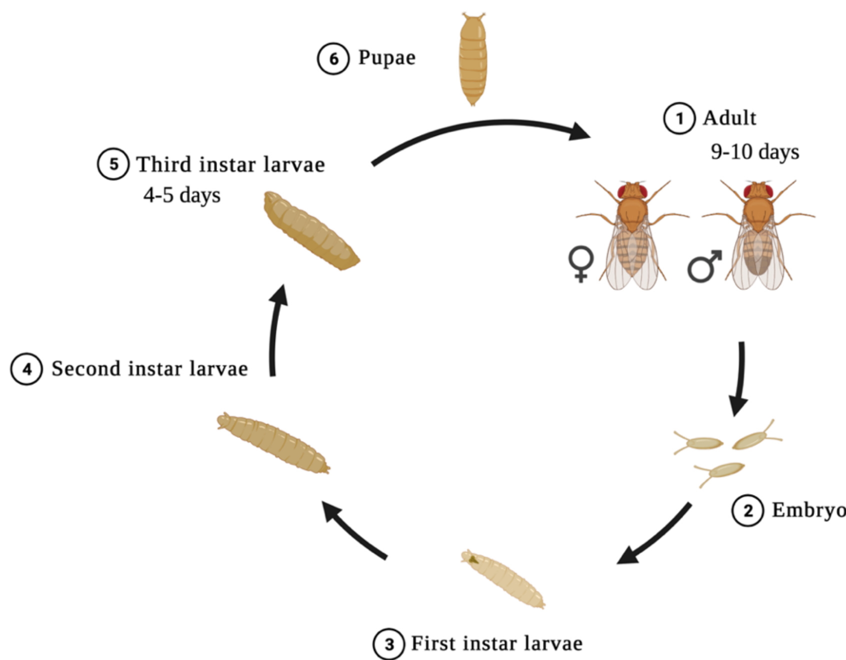

Figure 5. *Drosophila melanogaster* life cycle at 25ºC. The fruit fly has a short generation time and reaches adult stage 9-10 days after egg-laying when kept at 25ºC. The generation time increases slightly upon colder temperatures. The embryos develop into larvae that undergo three stages before pupating[343]. Created with BioRender.com

4.1.2 Generation of knock-out and knock-in models

The CG11337/*Dm*PNPase KO model studied in **paper III** was generated using CRISPR/Cas9. This method has revolutionised our possibility for genome editing in various organisms by simple measurements. The method has been adapted from a bacterial immune defence mechanism and only requires two components. Custom made small-guide RNAs (sgRNA) target the loci of interest, resulting in recruitment of the nuclease Cas9. Cas9 cuts the gene at the exact location of the sgRNAs and the genome is then repaired by the cells own system leaving DNA deletions in the target locus[351,352,354].

In **paper IV** CG11418/*Dm*MTPAP KO and knock-in (KI) flies were generated using ends-out homologous recombination[350,355]. In brief, the *Dm*MTPAP gene was replaced with an *attP* site to generate the KO. The endogenous locus was then targeted via the *attP* sites by genomic constructs, either containing CG11418/*Dm*MTPAP wild-type sequence, or a CG11418/*Dm*MTPAP mutant allele where two aspartate sites of the catalytic domain had been replaced with alanine. A silent XhoI was introduced into the wild-type allele to ensure differentiation between endogenous and "rescued" *Dm*MTPAP.

4.1.3 Generation of double-mutant models

To evaluate the role of and mode of collaboration between several proteins suggested to participate in mitochondrial RNA homeostasis, we generated a set of double-mutant *Dm* models in **paper III**. By silencing or overexpressing two factors of interest in the same animal we were able to study their interdependencies.

Stock keeping and genetic manipulation of *Drosophila* lines heavily relies on balancer chromosomes. These are inversions of large regions of fly chromosomes, preventing recombination during meiosis. These balancer chromosomes additionally carry easily traceable features, as well as recessive deleterious mutations. Together these features allow the researcher to follow and maintain specific genetic traits by visual inspection[346]. For instance, balancer chromosomes are used to maintain a desired lethal mutation in a fly population without the need to individually pick offspring in each generation. The allele of interest is paired to a balancer chromosome to prevent recombination, while flies homozygous for either the balancer chromosome or the lethal allele of interest are not viable. By breeding flies with different alleles of interest, balanced to different balancers, double mutant models could be generated and maintained. Common markers are *yellow* that causes yellow colour of the adult cuticle and is expressed on the X-chromosome and *Curly* that results in curly wings and is expressed on the second chromosome[346,356].

4.2 DEVELOPMENT OF NEUROEPITHELIAL STEM CELLS

To induce tissue specific cells, it is first required to generate induced pluripotent stem cells (iPSCs) that can differentiate to various cell types. Inductions is achieved by cultivating cells with reprogramming factors, such as transcription factors, growth factors and chemical compounds, that forces the cells to express a set of genes which changes the naiveness[357]. This ground-breaking discovery was awarded with the Nobel prize in 2012 to Shinya Yamanaka[358] and shared with Sir John Gurdon for his work on nuclear transfer[359,360]. Yamanaka and his research group used mouse fibroblast to show that reprograming to iPSCs was possible with just a small number of transcription factors, namely Oct3/4, Klf4, Sox2 and c-Myc[358]. Later they showed that c-Myc was redundant[361]. Differentiation into neuronal cells from human embryonic stem cells was accomplished in 2009 by Koch and colleagues[362]. Later Falk succeeded to cultivate neuronal precursor cells from adult patients differentiated cells[363,364], and hence there is now the possibility to avoid the usage of embryonic cells that often introduces ethical concerns.

In **paper II** patient fibroblasts were differentiated by my colleague to neuroepithelial stem cells (NESc) by culturing them in medium supplemented with various factors ensuring pluripotency and self-renewal potential. Upon removal of most of these factors, NESc undergo cell cycle arrest and neuronal differentiation into various neuronal subtypes, including neurones and to a lesser extent, glia cells.

4.3 MEASURE OXYGEN CONSUMPTION

Oxygen consumption measurement is a common strategy to evaluate OXPHOS function. In the studies presented in this thesis we employed the Oroboros oxygraphy (Oroboros Instruments GmbH) for this purpose, in both fruit flies and patient derived cells. The system is based on a Clark electrode, which utilizes a closed system to measure oxygen content upon addition of ADP or OXPHOS inhibitors [365]. In brief, an Oroboros measurement is taken place in a closed chamber and is initiated by permeabilization of samples with a detergent followed by the addition of substrates that either activate or inhibit each complex individually. The decline in oxygen over time is translated to oxygen consumption rate and indirectly represents the activity of each complex, hence it is a robust method to detect deficiencies of individual complexes.

4.4 STUDIES OF MITOCHONDRIAL RNA

4.4.1 Cloning and sequencing of mitochondrial mRNA poly(A) tails

To study disruption of polyadenylation two methods were employed. In **paper I** total RNA was isolated from *Dm* larvae and circularized by an RNA ligase prior to cDNA synthesis with gene-specific primers. The PCR product was then inserted to a plasmid followed by sequencing[183]. In **paper III** and **IV** a method called 3′ rapid amplification of cDNA ends (3′ - RACE) was employed. In this method the RNA poly(A) tail ends are ligated to a phosphorylated oligonucleotide linker prior to cDNA synthesis. The PCR reaction is then conducted using a gene-specific forward primer and a reverse primer complementary to the linker, followed by cloning and sequencing[184]. Analysis of poly(A) tail length was then facilitated by the detection of the linker.

4.4.2 Detection of mitochondrial double-stranded RNA

For detection of double-stranded (ds) RNA in **paper III** isolated RNA was treated with two different RNases, targeting either single-stranded (ss) RNA (RNase T1) or dsRNA (RNase III). A Northern blot analysis was then performed, using a mix of single-stranded mitochondrial RNA probes. Localisation of dsRNA within the cell was determined by immunohistochemistry, using the J2 antibody against dsRNA on the nervous system of dissected larval.

4.4.3 Ribosome footprinting

Ribosome footprinting, also referred to as ribosome profiling or Ribo-seq, is a method where RNA fragments protected by the ribosome are isolated and analysed. The technique was initially developed by Steitz and Kozak 60 years ago[366] and later refined by Ingola and Weissman to be able to apply the method to next generation sequencing[367,368]. The method is

carried out in **paper IV** to detect changes in translation upon loss of *Dm*MTPAP and subsequent mRNA polyadenylation. To summarise, mitochondria were isolated from *Dm* larvae and RNase treated to remove RNA outside of the mitochondrial ribosome. The sample was then run on a sucrose density gradient to separate the monosome from unassembled mitochondrial ribosome subunits. Fractions, corresponding to the monosome, were collected for further analysis, either by Western blot analysis, using antibodies against the mtSSU and mtLSU, or to prepare sequencing libraries to identify sequences protected by the ribosome. Sequences were then aligned to the mitochondrial genome, using bioinformatic tools[369,370].

4.5 ETHICAL CONSIDERATIONS

Work with patient material always needs to undergo ethical evaluation. Several aspects need to be addressed. Use of patient material in laboratory science is in Sweden under the control of law (2003:460). This law states that research can only be conducted if it is performed with respect to human integrity and rights, if the risks are low in comparison to benefits for the donor, if the research cannot be conducted satisfactorily with other material, and it must be performed by competent researchers. In **paper II** of this thesis fibroblasts derived from young children are studied and transformed to NESc. This brings several ethical dilemmas. When performing research on patients or patient material the principle of informed consent should be applied. This is not possible if the donor is a young child (or an adult who cannot consent for themselves), and here parents must be the child´s advocate. Research on mitochondrial diseases usually involves patient specific studies and laboratory findings can be surprising and unique for that patient. Potential results might not be useful for the patient but instead be incorporated with future research results. In addition, laboratory findings that bring negative news raise the questions of how, when and if this negative information should be passed on to patient and family. Moreover, introducing patient mutations in animals adds another dimension of ethical complexity as both the patient and animal must be considered. Nevertheless, the possibility to understand disease mechanisms and potentially develop future treatment usually motivates people to participate. To maintain a good ethical practice throughout the research patient confidentiality should be kept, the use and quality of the sample should be maximised to need as little as possible from the donor, information on the progress of the research should be offered, as well as the option to withdraw.

Working with fruit flies does not require an ethical permit. However, this does not mean that handling should not be performed with care and experiments should be planned to utilize the minimum number of animals used. The goal with the work of any animal model, including the fruit fly, is to generate an environment that resembles the natural conditions as far as possible and minimise suffering. By studying fly behaviour one quickly realizes that they are similar to other animals and, for instance, genes involved in pain are evolutionary conserved between flies and humans[371]. In my opinion this is enough to apply ethical considerations when working with the fly, no matter if it is regulated from higher instances or based on personal responsibility. I end this paragraph with a piece from William Blakes poem “The Fly” from year 1795. “*Am not I A fly like thee? Or art not thou a man like me?”*.

5 RESULTS AND DISCUSSION

5.1 PAPER I: SUV3 HELICASE IS REQUIRED FOR CORRECT PROCESSING OF MITOCHONDRIAL TRANSCRIPTS

In **paper I** the molecular role of ATP-dependent helicase SUV3 in mitochondrial RNA processing was investigated in a *Dm* model. Earlier studies in yeast and human cell lines suggested that, together with PNPase, SUV3 participates in the degradation of mitochondrial RNAs[188,372]. However, this had not been confirmed *in vivo*. Moreover, some previous data did not support a collaboration between SUV3 and PNPase. For instance, PNPase was suggested to localize to the IMS and be important for RNA import[212,213], while SUV3 were found in the matrix[211]. Moreover, SUV3 did not co-localize with PNPase in an RNAi screen for mitochondrial RNA binding proteins[211]. The helicase function can be useful in other processes, and we were therefore intrigued to investigate the consequences of disrupting expression of the SUV3 fly ortholog CG9791 (*Dm*SUV3) *in vivo*. By inducing RNAi, with use of the GAL4-UAS system, SUV3 was ubiquitously knocked-down in *Dm*. Our model was confirmed by quantitative (q) RT-PCR that showed a remarkable decrease of SUV3 transcripts in the *Dm* larvae. The KD of SUV3 resulted in larval lethality at the third instar developmental stage and we sought out to determine if this was caused by an OXPHOS deficiency. By performing blue native-PAGE (BN-PAGE) on mitochondrial preparations we were able to isolate OXPHOS complexes and perform in-gel activity assays, which revealed a strong defect of CI and CIV. Moreover, biochemical analysis of isolated complex activities and Western blot analysis confirmed the defects and detected deficiencies of CIII and CV but not of nuclear-encoded CII. This indicated a problem of mtDNA expression. By expressing a *Dm*SUV3-GFP construct in HeLa and Schneider 2R cells localization to the mitochondria was confirmed.

Mitochondrial RNA content in *Dm*SUV3 KD larvae was analysed by qRT-PCR and Northern blotting and showed a significant increase of mitochondrial mRNA but not rRNA steady-state levels. This supported a role of SUV3 in mitochondrial mRNA degradation. Interestingly, mtDNA levels were also increased and *in vitro* studies previously suggested that SUV3 can bind both RNA, as well as DNA, raising the possibility that SUV3 is involved in mtDNA homeostasis [373,374]. However, increased mtDNA levels are often a compensatory mechanism upon mitochondrial dysfunction. *De novo* transcription experiments on freshly isolated mitochondria suggested a mild defect upon loss of SUV3, and an increase in transcript stability, corroborating the increased steady state levels observed by Northern blot analysis. Additionally, unprocessed, primary transcripts accumulated in the absence of SUV3 but not in the absence of PNPase, suggesting that helicase activity might be required for efficient processing. Specifically, we found accumulation of tRNAs with non-coding flanking regions, tRNA-mRNA junctions, and antisense transcripts.

Circularisation and cloning experiments of mitochondrial transcripts revealed that the loss of *Dm*SUV3 also impaired their polyadenylation. This suggests that SUV3 is required for MTPAP activity as proposed previously[375].

To summarize, we used *Dm* to show the function of *Dm*SUV3 *in vivo* and propose that besides being a critical part of the mitochondrial degradosome, *Dm*SUV3 also is required for mitochondrial RNA processing. However, it remains to be elucidated if SUV3 works together with PNPase and how it influences MTPAP activity.

5.2 PAPER II: MUTATIONS OF THE MITOCHONDRIAL TRYPTOPHANYL-TRNA SYNTHETASE CAUSES GROWTH RETARDATION AND PROGRESSIVE LEUKOENCEPHALOPATHY

My postgraduate studies were performed in a research group with close ties to the Centre for Inherited Metabolic Diseases (CMMS) at Karolinska University Hospital. Here, WES and WGS is routinely performed to diagnose patients suspected to have a rare inherited metabolic disorder[285]. During the diagnostic process CMMS identified two siblings carrying the compound heterozygous variants, c.833T>G (p.Val278Gly) and c.938A>T (p.Lys313Met) in the gene encoding mitochondrial tryptophanyl-tRNA synthetase (*WARS2*). The siblings presented with severe neurological symptoms including growth retardation and progressive leukoencephalopathy. MRI scans of both siblings showed reduced supratentorial white matter volume but normal amount of myelinisation. Muscle biopsies could not detect an OXPHOS defect.

The p.VAL278Gly variant was first described in the current study whereas the p.Lys313Met variant had been recently reported[315–317]. Although no OXPHOS defect was observed in muscle or fibroblasts samples from either patient, acidic polyacrylamide gel electrophoresis, followed by Northern blot analysis, revealed a strong aminoacylation defect in fibroblasts from both siblings. To further understand the pathogenic mechanisms, we reprogrammed patient fibroblasts to iPSCs, followed by differentiation into NESc. WARS2 transcript levels were low in the NESc and we observed a pronounced defect in aminoacylation. Additionally, this defect was accompanied by a combined CI and CIV deficiency, supporting that neurones might be preferentially affected.

To study whether a partial defect in aminoacylation of $tRNA^{Trp}$ can have severe physiological consequences, we silenced the WARS2 ortholog in *Dm* by the GAL4-UAS system. The model was confirmed by measuring WARS2 mRNA levels by qRT-PCR and by aminoacylation gel electrophoresis. WARS2 deficiency caused pupal lethality and suppressed complex I and IV activity, comparable to results obtained in NESc. Pathogenicity of both variants was further confirmed in a yeast model by international collaborators, detecting impaired growth and respiratory rate deficiency.

Although the high variety of clinical manifestations in mitochondrial diseases remains to be unsolved, the heterogeneity of ARS2 patients may have several explanations. Firstly, the degree of enzyme activity will influence on the severity. Complete loss-of-function mutations

does probably not exist since this would not be compatible with life. However, different degrees of enzymatic activity, that allows prenatal development, could, together with tissue specific energy demands, influence the clinical manifestation[305]. In a group of children with mutations in *EARS2* some presented milder symptoms and with time partially recovered and regained developmental milestones[376]. GARS and KARS has no mitochondrial isoform[40] and is therefore used in both the cytoplasm and mitochondria. Mutations in *GARS* and *KARS* will therefore affect both mitochondrial and cytoplasmic translation which further complicates the pathological mechanisms. The non-canonical functions of ARS2 in other cellular pathways can also influence pathology but is mostly unexplored in these patients. For instance, some of them have been shown to participate as regulators at other levels of gene expression, such as RNA splicing but also in specific cellular events such as apoptosis[377].

In conclusion, **paper II** verifies a mitochondrial aminoacylation defect as the probable cause of mitochondrial disease in two patients with severe neurological symptoms and highlight the importance of using the right model system for studying mitochondrial diseases.

5.3 PAPER III: DEFECTS OF MITOCHONDRIAL RNA TURNOVER LEAD TO THE ACCUMULATION OF DOUBLE-STRANDED RNA IN VIVO

Mechanisms and factors involved in mitochondrial RNA degradation have remained elusive partly because the studies have been performed in different model systems. *In vitro* studies have suggested that PNPase forms a mitochondrial RNA degradosome together with SUV3[188]. **Paper III** is a continuation of **paper I** and in this study the role of PNPase as a putative partner of the mitochondrial RNA degradosome, together with SUV3, was investigated *in vivo*. In addition, the relationship of PNPase to other enzymes of mitochondrial RNA metabolism was studied to get a deeper understanding of their function, the order of their actions, and how they collaborate with each other. Previous studies focused on analysing each factor separately, limiting the ability to put their function in context with the whole RNA processing machinery. Here, we studied several factors involved in RNA metabolism by either deleting, silencing, or overexpressing two factors in the same *Dm* model. These flies were referred to as double mutants.

*Dm*PNPase was either silenced, using the GAL4-UAS system, or deleted (KO) by CRISPR/Cas9 gene editing. We localised *Dm*PNPase to the mitochondrial matrix using a *Dm*PNPase-GFP fusion protein in HeLa cells, as well as by subcellular fractionation and Western blot analysis. Disruption of *Dm*PNPase expression resulted in OXPHOS deficiency and larval lethality. qRT-PCR and Northern blot analysis showed increased steady-state levels of mitochondrial mRNAs, consistent with *Dm*PNPase being involved in mRNA degradation. Silencing of both *Dm*SUV3 and *Dm*PNPase resulted in a synergistic effect on mRNA levels, with a significant increase in steady-state levels compared to individual KD. In contrast, overexpression of both factors almost completely removed all mitochondrial mRNAs, supporting their role as the mitochondrial degradosome *in vivo*.

Both PNPase and SUV3 have shown to influence mRNA poly(A) tail length and form a complex with MTPAP in cell culture[189,375]. In **paper I** we reported that disruption of

*Dm*SUV3 shortened the poly(A) tail *in vivo*. In contrast, 3′-RACE, followed by cloning and sequencing, showed that loss of *Dm*PNPase KD and KO had the opposite effect on polyadenylation compared to *Dm*SUV3 KD, with poly(A) tails being significantly longer in *Dm*PNPase KD and KO samples. Surprisingly, overexpression of *Dm*PNPase and *Dm*SUV3 had very little effect on polyadenylation. To study if the poly(A) tail served as a signal for mRNA degradation, we generated a *Dm* model where we knocked out the fly ortholog of *Dm*MTPAP CG11418 and over expressed *Dm*PNPase. Despite a complete absence of a poly(A) tail we still observed significant degradation of mRNAs, supporting the notion that the poly(A) tail does not serve as a signal for degradation. However, we could not determine if degradation requires a deadenylase that first removes the poly(A) tail.

LRPPRC have shown to promote mRNA stability and polyadenylation[168–170]. To study the relationship between LRPPRC and the RNA degradosome we disrupted the expression of the fly ortholog BSF (here referred to as *Dm*LRPPRC) by RNAi and combined it with either *Dm*PNPase or *Dm*SUV3 KD flies. Indeed, both combinations stabilised mRNA steady-state levels, in comparison to *Dm*LRPPRC KD alone that decreased mRNA stability. In addition, the stimulated polyadenylation seen upon *Dm*PNPase KD was not detected when *Dm*LRPPRC was absent, which supported the idea that mRNA stabilisation by LRPPRC is required to stimulate *Dm*MTPAP activity[300].

Non-coding antisense RNAs are produced upon processing of the polycistronic transcript and are rapidly degraded[300]. In **paper I** we demonstrated that antisense RNA accumulated when silencing *Dm*SUV3. Northern blot analysis, using probes specific for antisense mRNAs, revealed a similar accumulation of antisense transcripts upon silencing of *Dm*PNPase. These findings indicated that the mitochondrial RNA degradosome is responsible for removing both sense and antisense mRNAs. To our surprise, also loss of *Dm*MTPAP resulted in the accumulation of antisense transcripts. Previous studies in mice suggested that mitochondrial transcription and polyadenylation occurs simultaneously[123]. Although purely speculative, the accumulation of antisense transcripts in all three models could indicate that RNA polyadenylation and turnover are also tightly linked events. Disruption of any participating enzyme would then have consequences on the others. This could result in the same molecular outcome, such as accumulation of antisense transcripts. Interestingly, antisense transcripts only presented with a few nucleotides long poly(A) tail. This suggests that they are not bound by *Dm*LRPPRC and stimulation of *Dm*MTPAP activity is lost. This is in agreement with previous results that also failed to detect antisense transcripts in LRPPRC-pulldown experiments, , followed by high-throughput sequencing analysis[171]. Taken together, our data suggests that antisense RNA is rapidly removed by the RNA degradosome, before being stabilised by LRPPRC and that in the absence of LRPPRC, MTPAP acts as an oligoadenylase. How these enzymes can discriminate between sense ant antisense transcripts remains unknown.

Transcription of both strands of a circular genome inevitably can lead to the formation of dsRNA and indeed, duplex mitochondrial RNA has previously been described[378,379] . By using ssRNA and dsRNA-specific nucleases we were able to demonstrate that disruption of *Dm*PNPase, *Dm*SUV3, or *Dm*MTPAP led to the accumulation of dsRNA, while loss of *Dm*LRPPRC did not show this, presumably due to the rapid degradation of all transcripts.

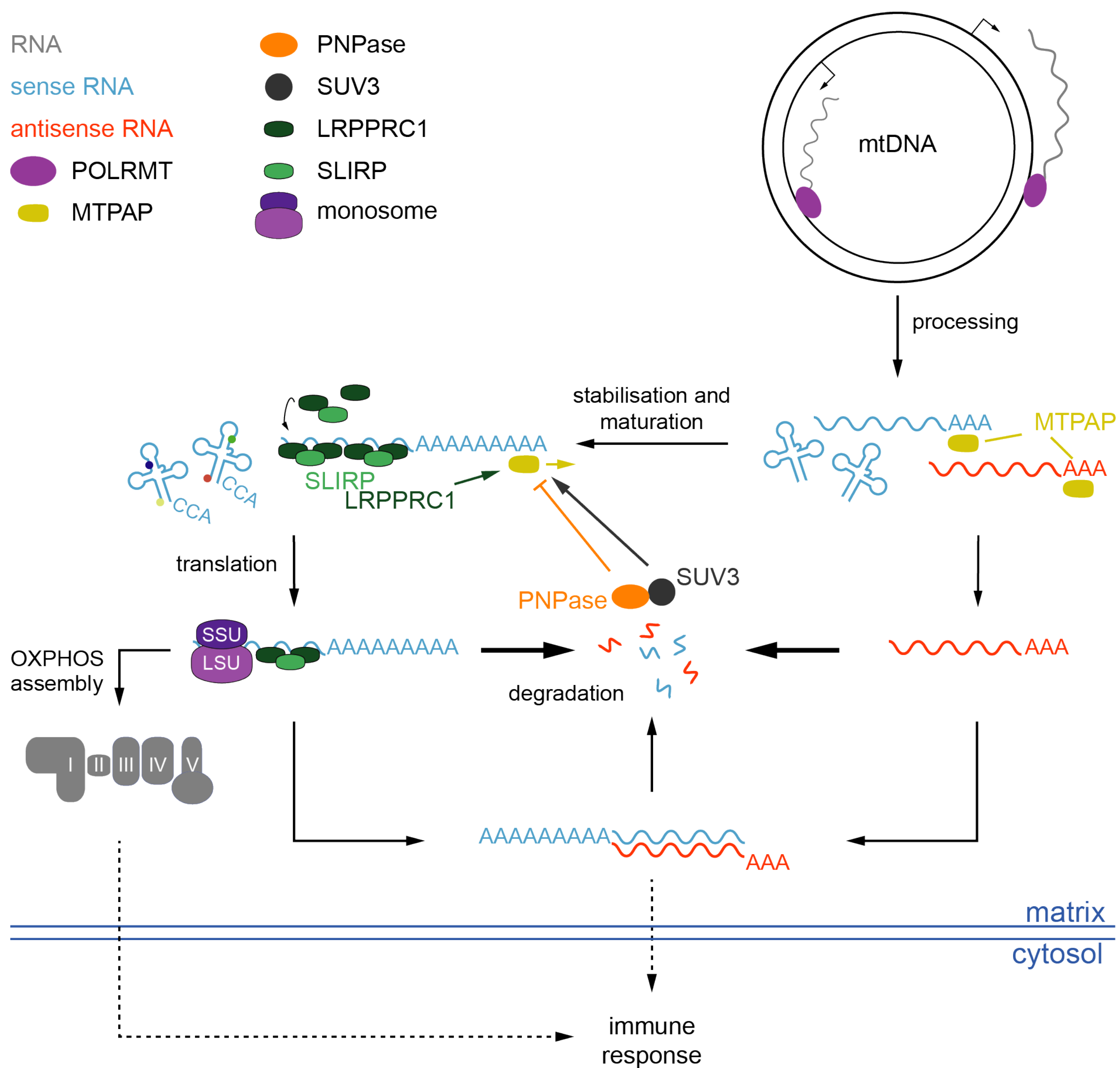

Figure 6. Summary of paper III. The figure illustrates the studied proteins and their involvement in mitochondrial RNA processing. Reprinted from Pajak A *et al.*[47] under the terms of the Creative Commons Attribution Licence 4.0 (CC BY).

The leakage of dsRNA from mitochondria has recently been reported to elicit an immune response in human cell culture, similar to the response of viral infections[333]. We performed immunofluorescence staining of our larvae and were likewise able to detect leakage of dsRNA into the cytosol. This was confirmed by antibody-based enrichment of dsRNA followed by RNA sequencing. In the previous cell culture study, leakage of dsRNA from mitochondria was proposed to be controlled by PNPase, since presence of dsRNA in the cytosol was only seen upon disrupting PNPase but not SUV3[333]. However, leakage of dsRNA was detected in all our *Dm* models that had an accumulation of antisense transcripts. Besides OXPHOS deficiency, other mitochondrial functions can be involved in the pathophysiology of mitochondrial diseases. This includes, for instance, maintenance of redox balance, calcium homeostasis[84] and one-carbon metabolism[380,381]. Defects in the immune system have also been implicated upon mitochondrial dysfunction in both human cell lines and animal models[382,383]. Moreover, infection sensitivity is detected in some patients with mitochondrial disease[384]. The significance of dsRNA leakage in patients with mitochondrial diseases

remains unclear. However, the results in **paper III** propose a potential disease mechanism and highlight the importance of studying a broad range of mitochondrial functions in order to understand pathology and develop treatment for mitochondrial dysfunction.

In summation, **paper III** demonstrates that PNPase and SUV3 form a mitochondrial RNA degradosome *in vivo*. Each factor has an opposing effect on mRNA polyadenylation, but the poly(A) tail is not a signal for degradation. LRPPRC protects mRNAs degradation and stimulates MTPAP activity. Finally, disruption of RNA processing can generate dsRNA that leaks to the cytosol and alters immunity. Illustration of the proteins and their function is presented in **Figure 6**.

5.4 PAPER IV: MITOCHONDRIAL TRANSLATION EFFICACY IS DEPENDENT ON RNA POLYADENYLATION

In **paper I** and **paper III** we demonstrated that poly(A) tail length can be modulated by several factors, and that in the absence of LRPPRC, mitochondrial transcripts only contain a short oligoadenylation. Although our group previously demonstrated that deletion of *Dm*MTPAP resulted in a complete loss of polyadenylation signal[196], these results could not exclude that MTPAP functions together with a putative oligoadenylase, responsible for the initial priming of polyadenylation. Support for such function stems from studies in human cells, where oligoadenylation was observed upon disruption of MTPAP expression[180,189,299], [196] leaving the existence of an oligoadenylase unanswered. In **paper IV** we addressed two questions concerning polyadenylation in mitochondria. Firstly, we wanted to study the existence of a putative oligoadenylase and further study the role of MTPAP and polyadenylation upon translation.

In **paper IV** we introduced a catalytically inactive *Dm*MTPAP allele by homologous recombination into the *Dm*MTPAP KO model. This allowed us to investigate whether such an oligoadenylase works in a complex with *Dm*MTPAP, since any interaction sites were left intact. 3′-RACE followed by cloning and sequencing of mitochondrial mRNAs showed no sign of oligoadenylation, concluding that *Dm*MTPAP is the sole enzyme catalysing polyadenylation.

Loss of MTPAP resulted in a prominent decrease of OXPHOS subunits, specifically of CI and CIV, while CV was unaffected. Furthermore, proteome analysis showed increased levels of mitochondrial ribosomal subunits and associated factors, while the opposite was observed by Western blot analysis. This discrepancy requires further investigation but could be a consequence of technical normalisation of proteomic data and Western blot analysis. Nevertheless, loss of MTPAP activity influences mitochondrial translation and we therefore performed sucrose density gradients on isolated mitochondria to understand the consequences on monosome formation. Free mtSSU levels were increased in both *Dm*MTPAP KO and catalytic mutant larvae. Monosome assembly was still achievable but the increased amount of free mtSSU might indicate a mild assembly defect, as previously described upon disruption of mitochondrial gene expression[92].

The difference in the proteome from the total larval protein isolations between the *Dm*MTPAP KO and catalytic mutant was minor. However, when comparing the translation apparatus *Dm*MTPAP KO had a significant increase of GTP binding protein 10 (GTPBP10) and decreased levels of lactamase beta 2 (LACTB2). This proposes that the physical loss of *Dm*MTPAP has additional consequences on mitochondrial gene expression. GTPBP10 is a member of the Obg family of GTPases and is involved in the later maturation steps of mtLSU assembly. Disrupted expression of GTPBP10 impedes monosome assembly[385,386]. The increased level of GTPBP10 in *Dm*MTPAP KO larvae might indicate a need to inhibit monosome formation. It is unknown how the mitochondrial transcripts are recruited and recognized by the mitochondrial ribosome. A screen of the mitochondrial poly(A) tail interactome revealed associations with several proteins involved in translation, including MRPs and GTPBP10[197]. The unique MRP mS39 was recently shown to associate to LRPPRC and bind mRNAs during translation [204,215,234]. In yeast, several RNA maturation factors have been suggested to interact with the mitochondrial ribosome and form a supercomplex referred to as MIOREX[387,388]. It is possible that also in metazoan other RNA binding proteins, such as MTPAP, associate with the mitochondrial ribosome and facilitate recognition of mRNAs. Increase of GTPBP10 might signal the potential loss of an interaction between MTPAP and the mitochondrial ribosome (**Figure 7**).

LACTB2 is a ssRNA endoribonuclease found in mitochondria. The role in RNA turnover is not fully understood but overexpression in human cell culture resulted in decreased levels of mitochondrial transcripts while silencing had the opposite effect[389]. The relationship between LACTB2 and MTPAP remains to be elucidated.

Loss of polyadenylation can result in three different types of transcripts: (*A*) transcripts with no poly(A) tail but with a functional stop codon, (*B*) transcripts with no poly(A) tail and no functional stop codon, and (*C*) transcripts with no poly(A) tail, no stop codon and with additional truncations of the 3′end. To investigate how efficiently they are translated we performed mitochondrial ribosome profiling, identifying that *mt:nd2* and *mt:co2* were enriched on the mitochondrial ribosome in mitochondria lacking *Dm*MTPAP. Enrichment seemed to occur at the 3′ end suggesting that ribosome stalling happens upon late elongation or termination. Additionally, *mt:co1* and *mt:cytb* transcripts were severely reduced in the mitochondrial ribosome fractions suggesting a failure to load these transcripts. These results corroborate our previous findings that *de novo translation* of *mt:nd2* and *mt:co2* was increased while *mt:co1* and *mt:cytb* translation was decreased in *Dm*MTPAP KO samples[196]. However, we observed no difference in transcript occupation between the *Dm*MTPAP KO and catalytic mutant models. Finally, occupancy of the mitochondrial ribosome did not correlate with transcript steady-state levels[196] suggesting that translation initiation, rather than availability, contributes to the observed phenotype. The mechanisms of transcript loading and release are poorly understood necessitating further investigations. One possibility is that in the absence of polyadenylation the transcripts undergo different foldings[244,390–393]. Secondary structure of transcripts is determined by their sequence[394–396]. Mitochondrial G-rich transcripts have been shown to form G-quadruplexes upon transcription[219,397]. In the cytosol such formations affect translation[398]. Whether such structures are formed upon loss of polyadenylation and whether they affect mitochondrial translation remains to be determined.

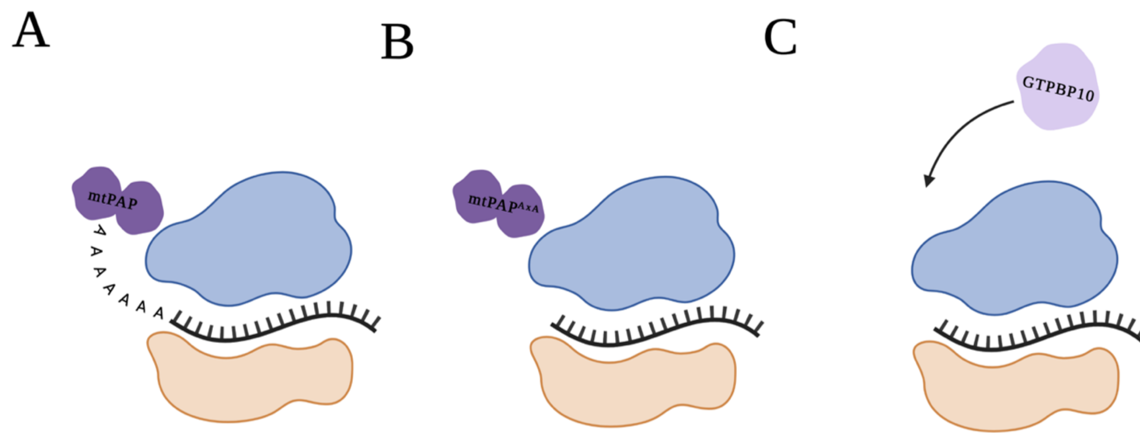

Figure 7. Hypothetical mitochondrial translation scenarios in *Dm*MTPAP models. A. Upon physiological conditions both MTPAP and a poly(A) tail can influence translation. **B.** In a catalytic mutant MTPAP model the poly(A) tail is lost but physical presence of MTPAP is retained and can influence translation. **C.** In a MTPAP KO model both MTPAP and the poly(A) tail is lost and cannot influence translation. MTPAP KO increases the level of GTPBP10 which might influence mitochondrial ribosome assembly. Created with BioRender.com

In **paper IV** we show that polyadenylation is catalysed by MTPAP alone and that loss of polyadenylation causes a translation defect of several transcripts. The physical absence of MTPAP have additional consequences on mitochondrial gene expression. Translation deficiency is caused by a chain of events starting with a lost factor, that either influence assembly of mtSSU, mtLSU or both, causing impaired monosome assembly and ultimately loss of protein synthesis. Where in this chain MTPAP and polyadenylation is important remains to be elucidated.

6 CONCLUSION AND FUTURE PERSPECTIVE

Previous studies of mechanisms and proteins involved in mitochondrial RNA processing have shown inconsistencies in the data, mainly because of the use of different model systems. The aim of the work presented in this thesis was to study factors suggested to be involved in RNA processing and understand the processes they perform and why these processes are significant for mitochondrial gene expression. Several of the studied proteins have been suggested to be involved in mitochondrial diseases. By analysing their individual functions, I attempt to untangle their pathomechanisms. All the studies are performed in the fruit fly to ensure comparable and physiologically relevant data.

In **paper I** and **III** degradation of unwanted mitochondrial RNA molecules was studied by characterizing the RNA degradosome suggested to consist of SUV3 helicase and PNPase. We confirmed that SUV3 and PNPase forms an RNA degradosome *in vivo* and discovered a previously unknown additional function of SUV3 in mitochondrial RNA processing. In addition, we found that both components of the RNA degradosome influence MTPAP activity and that LRPPRC promotes mRNA polyadenylation and hinders mRNA degradation. Disrupting the expression of SUV3, PNPase and MTPAP caused formation of dsRNA that leaked out in the cytosol and altered the expression of genes involved in the immune reactions against viral infections. Our studies thus suggest a tight interconnectivity between factors and processes involved in mitochondrial RNA processing. Further studies are required to determine if the proteins form complexes and if they share other downstream consequences upon disruption. In addition, identifying other factors involved in RNA metabolism, such as deadenylases, and mechanisms that signal degradation, would contribute with important puzzle pieces. The influence on expression of immune genes found in **paper III** implicates a potential important pathological mechanism in patients with mitochondrial diseases caused by disrupted mitochondrial gene expression. However, additional studies are required to unravel a potential causal link to the infection sensitivity seen in some patients with mitochondrial disease[384].

The role of mitochondrial RNA polyadenylation by MTPAP was further studied in **paper IV**. We confirmed that MTPAP is the only enzyme catalyzing RNA polyadenylation and showed that MTPAP activity is necessary for proper translation of several transcripts. Moreover, physical loss of MTPAP had additional consequences on the mitochondrial proteome, perhaps because of problems upon mitochondrial ribosome assembly. This result encourages further studies to determine the composition of the mitochondrial ribosome upon loss of MTPAP for instance by utilizing cryo-EM. Furthermore, understanding the mechanism of the poly(A) tail upon translation and determine why the need of polyadenylation differs between mitochondrial mRNA molecules require further studies.

Finally, in **paper II** we characterize two pathological variants in WARS2 by using several different tissues and model systems. We detected discrepancies in the results depending on the system used and therefore encourage careful choice of model system when studying

mitochondrial diseases. For instance, the use of “mini-organs” originating from patient fibroblasts can serve as reliable complement to animal models and can be an important tool to untangle the mystery of tissue heterogeneity among patients with mitochondrial diseases.

To summarize, the studies presented in this thesis contribute with novel data of how the availability of mature mitochondrial mRNAs are controlled and ultimately translated. In addition, it suggests pathological mechanisms of mitochondrial diseases.

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