

From the Department of Cell and Molecular Biology
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Structural and functional basis of mitochondrial tRNA processing

Sagar Sridhara



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Cover image: A schematic representation of the human mitochondrial tRNA processing
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Structural and functional basis of mitochondrial tRNA processing

THESIS FOR DOCTORAL DEGREE (Ph.D.)

by

Sagar Sridhara

Principal Supervisor:

Dr. B. Martin Hällberg
Karolinska Institutet
Department of Cell and Molecular Biology

Opponent:

Prof. Martin Ott
Stockholm University
Department of Biochemistry and Biophysics

Co-supervisors:

Dr. Linda Reinhard
Karolinska Institutet
Department of Cell and Molecular Biology

Examination Board:

Prof. Stefan Åström
Stockholm University, The Wenner-Gren Institute
Department of Molecular Biosciences

Prof. Nils-Göran Larsson
Karolinska Institutet
Department of Medical Biochemistry and
Biophysics

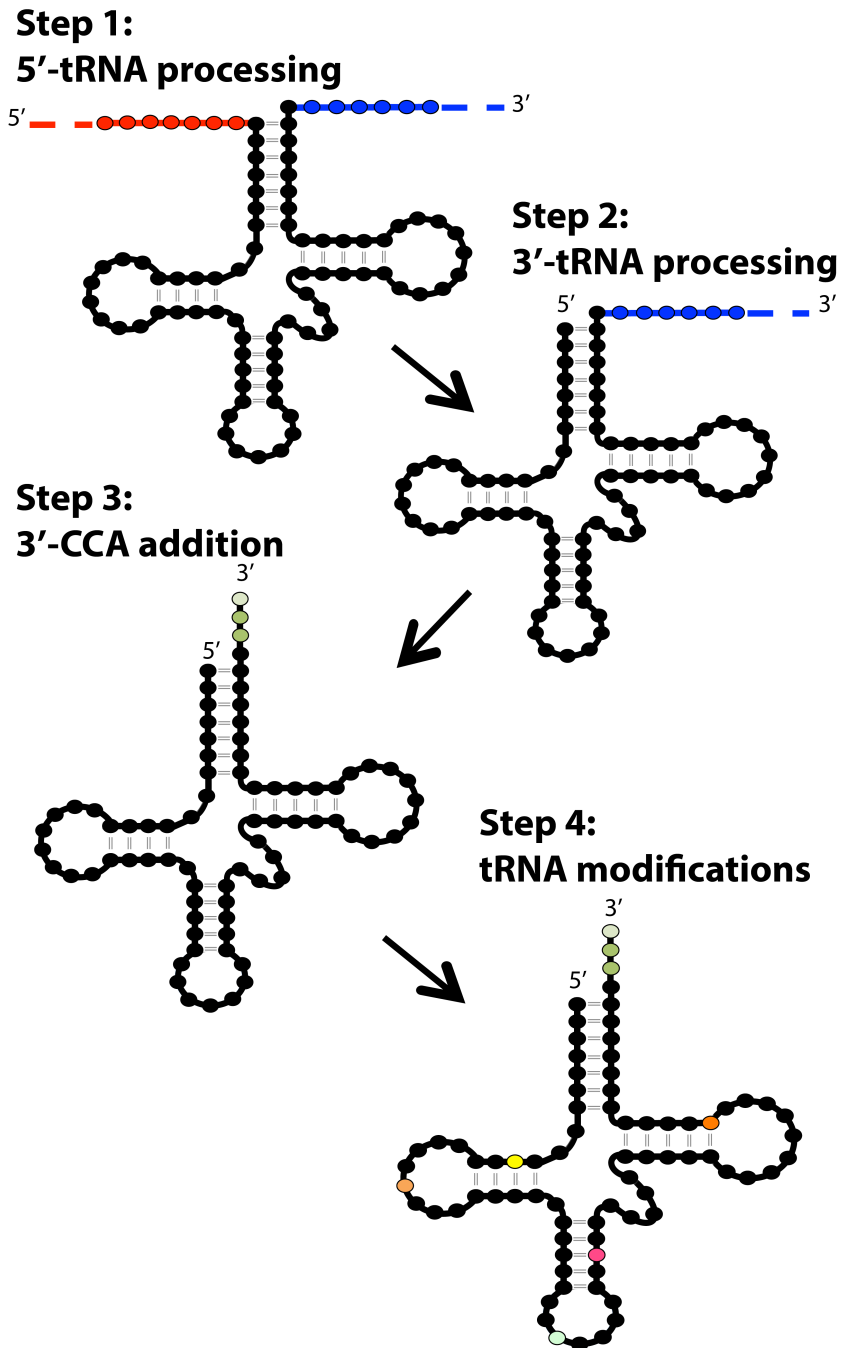
Dr. Lena Ström
Karolinska Institutet
Department of Cell and Molecular Biology

Prof. Maria Selmer
Uppsala University
Department of Cell and Molecular Biology

ಈ ಪ್ರಬಂಧ ಬರೆಯುವುದರ ಹಿಂದೆ ಹಲವಾರು ವ್ಯಕ್ತಿಗಳ ಪಾತ್ರವೂ ಇದೆ. ನನ್ನ ತಂದೆ, ತಾಯಿಯವರ ತ್ಯಾಗ, ನನ್ನ ಅಕ್ಕ, ಭಾವ ಹಾಗೂ ಕುಟುಂಬದವರ ಪ್ರೀತಿಗೆ ನಾನು ಚಿರಋಣಿ.

This thesis is my small gift to Science!

TRANSFER RNA PROCESSING



The major steps required for maturation of tRNAs involves the processing of 5'- and 3'- extensions, followed by 3'-CCA addition and post-transcriptional modifications.

ABSTRACT

The mammalian mitochondria are subcellular organelles, generating energy in the cell by the synthesis of adenosine triphosphate (ATP) via oxidative phosphorylation (OXPHOS). The maintenance of proper mitochondrial (mt) structure and function requires more than 1000 proteins generated from the nuclear DNA (nDNA) expression and 13 protein subunits generated from the mitochondrial DNA (mtDNA) expression. The transcription of the two strands of mtDNA generates two long polycistronic precursor ribonucleic acid (pre-RNA) molecules harboring the precursor messenger RNAs (pre-mRNAs), precursor ribosomal RNAs (pre-rRNAs) and precursor transfer RNAs (pre-tRNAs) at a stretch. The processing of pre-tRNAs punctuating the pre-rRNAs and most of the pre-mRNAs causes the release of each of the RNA species that undergo their own respective maturation pathways to attain the functional state. This thesis explores the molecular mechanism of pre-tRNA processing in human mitochondria, critical for several downstream processes such as mitoribosome biogenesis and the maturation of mt-mRNAs and mt-tRNAs.

The first step of pre-tRNA processing is performed by the human mitochondrial ribonuclease P (mt-RNase P) composed of three protein subunits: MRPP1, MRPP2 and MRPP3 that excises the tRNA at the 5'-end. In **Paper I**, the high-resolution crystal structure of the MRPP3 protein is described. We observed that the MRPP3 protein is unable to perform 5'-tRNA cleavage on its own because of its distorted active site. Unlike its structural homologue: protein-only RNase P 1 (PRORP1) found in the plant mitochondria and chloroplasts, MRPP3 requires a subcomplex of MRPP1 and MRPP2 (MRPP1/2) and pre-tRNA substrate to undergo conformational changes in the presence of metal ions to achieve an active state. In **Paper II**, previously unknown central role of MRPP1/2 complex as a maturation platform for mt-tRNAs is reported. We show that the MRPP1/2 complex is not just an essential component of human mt-RNase P, but also significantly enhances the 3'-processing by the ELAC2 protein in 17 out of 22 5'-processed mt-tRNAs. Moreover, the CCA addition to the 3'-processed tRNAs was shown to be possible while the tRNA remained bound to the MRPP1/2 complex. Thus, the MRPP1/2 sub-complex hosts the pre-tRNA substrate through three major steps of the processing activities instead of just one as thought previously.

The human mt-tRNA genes in the mtDNA are locations for several disease-causing mutations causing mt encephalomyopathies. In **Paper III**, the role of disease-causing tRNA(Lys) acceptor stem mutations is evaluated in terms of its effects on mt-RNase P activity. An overall impairment of RNase P processing was observed in tRNA acceptor stem mutants in relation to the wild type tRNA. The MRPP1/2 complex can bypass a single point mutation in the acceptor stem of substrate pre-tRNA and form a stable complex *in vitro*. However, the nuclease activity by MRPP3 is strongly affected. We speculate that either the MRPP3 protein is unable to form a complex with MRPP1/2-pre-tRNA or the re-organization of its active site upon binding to MRPP1/2-pre-tRNA is affected as a consequence of the acceptor stem mutation.

LIST OF SCIENTIFIC PAPERS

- I. Reinhard L*, **Sridhara S*** and Hällberg BM.
Structure of the nuclease subunit of human mitochondrial RNase P.
Nucleic Acids Res. 43(11), 5664-72 (2015).
(*Joint first authors)

- II. Reinhard L*, **Sridhara S*** and Hällberg BM.
The MRPP1/MRPP2 complex is a tRNA maturation platform in human mitochondria.
Nucleic Acids Res. Accepted (2017). doi: 10.1093/nar/gkx902 (Epub ahead of print).
(*Joint first authors)

- III. **Sridhara S**, Reinhard L and Hällberg BM.
Clinically relevant mutations in the acceptor stem of mitochondrial tRNA(Lys) strongly
affects RNase P activity.
Manuscript.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
CCA-E	Cytidine-Cytidine-Adenine adding enzyme
ELAC2	ElaC ribonuclease 2 (Gene)
HSD17B10	Hydroxysteroid 17-beta dehydrogenase 10 (Gene)
MAMIT	Mammalian mitochondrial tRNA (database)
MRPP1	Mitochondrial ribonuclease P protein 1
MRPP2	Mitochondrial ribonuclease P protein 2
MRPP3	Mitochondrial ribonuclease P protein 3
mtDNA	Mitochondrial deoxyribonucleic acid
mt-mRNA	Mitochondrial messenger RNA
mt-rRNA	Mitochondrial ribosomal RNA
mt-tRNA	Mitochondrial transfer ribonucleic acid
MTS	Mitochondrial targeting sequence
NADH	Nicotinamide adenine dinucleotide
PRORP1	Protein-only ribonuclease P 1
POLRMT	Mitochondrial ribonucleic acid polymerase
OXPHOS	Oxidative phosphorylation
SAM	S-adenosyl methionine
TRMT10C	Transfer RNA methyltransferase 10C (Gene)
TRNT1	Transfer RNA nucleotidyl transferase 1 (Gene)

Databases:

MAMIT (mamit-trna.u-strasbg.fr)

MITOMAP (<https://www.mitomap.org>)

mitotRNAdb (mttrna.bioinf.uni-leipzig.de/mtDataOutput/Welcome)

Online Mendelian Inheritance in Man /OMIM (<https://www.omim.org>)

MalaCards: The human disease database (<https://www.malacards.org>)

1 INTRODUCTION

The work presented in this thesis provides a detailed description of the human mt-tRNA processing and maturation at a molecular level. In the introductory chapter 1, the mammalian mt system and its genome organization is described along with a brief overview of human mt-tRNAs. In chapter 2, the molecular players involved in the processing of mt-tRNAs are described in detail. The mt-tRNA mutations and its implications in pathology of medically relevant human diseases are covered in chapter 3. The strategy employed to systematically form and analyze protein-protein and protein-tRNA complexes are provided in chapter 4. This is followed by a summary of papers and a discussion of the future perspectives in chapter 5 and 6 respectively.

1.1 Mitochondria

The mammalian mitochondrion is a vital organelle in the cell performing a wide variety of functions including the synthesis of ATP via the transfer of electrons through a complex mechanism called OXPHOS. It is made up of two compartments called the outer membrane and the inner membrane (Figure 1), the latter forming structures called cristae [1]. The folded crista harbors assemblies of four respiratory chain complexes (complex I – IV) and one ATP synthase (complex V) that function as a bio-energetic system for ATP production [2, 3].

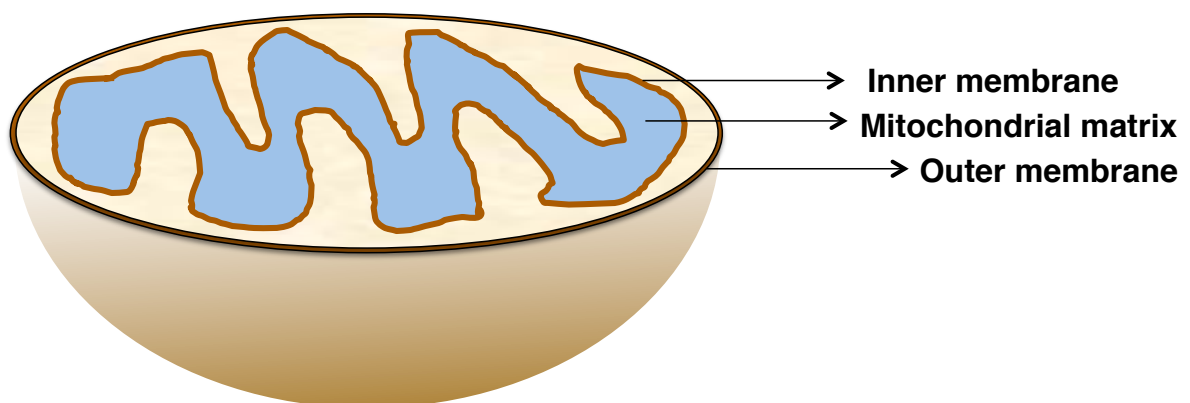


Figure 1: The cross-section of mitochondria shows inner membrane, outer membrane and mt matrix. The intermembrane space separates the two compartments.

Apart from generating the energy currency of the cell, the mitochondrion has a significant control over cellular homeostasis as a regulator of the level of amino acids, critical metabolites and several other cofactors. It serves as a source of nicotinamide adenine dinucleotide (NADH) for the cell and is also critical for metabolism of metals, oxidation of fatty acids, apoptosis and signal transduction [4-7]. It has a very high medical relevance as it is implicated in aging, diabetes, obesity, immunity, neurodegenerative disorders and a wide spectrum of human mt diseases [2, 8-13].

1.2 Organization of the mitochondrial genome

The mammalian mitochondrion harbors multiple copies of mtDNA organized as compact nucleoprotein structures called nucleoids in the mt matrix [14]. The mtDNA is a circular, compact, double-stranded molecule of around 16.5 kb that encodes 13 essential proteins, 22 tRNAs (Figure 2) and 2 rRNAs required for mitoribosome biogenesis and OXPHOS [15-17]. The remaining proteins needed for OXPHOS requires nuclear gene expression, the products of which are targeted to mitochondria. A total of around 1100 nuclear-encoded proteins are required for multiple purposes within mitochondria including OXPHOS, pre-RNA processing, mtDNA expression and maintenance [18, 19].

The mammalian mtDNA is composed of two strands, a heavy (H) strand and a light (L) strand, named historically due to their differing densities [20]. This difference is due to the variations in the nucleotide content between the two strands. The H strand is rich in its guanine content relative to the complementary L strand [21]. Each of the two strands harbors a main promoter called the heavy strand promoter (HSP) and the light strand promoter (LSP) at a non-coding region called displacement loop (D-loop). Thus, the D-loop serves as the transcription initiation sites. The H strand harbors 14 mt-tRNA genes (Table 1), 2 rRNA genes and all the mRNA elements (Table 2) except the gene coding for NADH dehydrogenase subunit 6 (ND6). The L strand harbors ND6 gene and the remaining 8 mt-tRNA genes (Table 1).

Name	Abbreviation	Location in mtDNA	Strand	D loop (bases)	Variable loop (bases)	TΨC loop (bases)
Mitochondrial tRNA Phenylalanine	mt-tRNA ^{Phe} (F/Phe)	577-647	Heavy	9	4	5
Mitochondrial tRNA Valine	mt-tRNA ^{Val} (V/Val)	1602-1670	Heavy	6	4	6
Mitochondrial tRNA Leucine(UUR)	mt-tRNA ^{Leu(UUR)} (L2/Leu2)	3230-3304	Heavy	10	5	7
Mitochondrial tRNA Isoleucine	mt-tRNA ^{Ile} (I/Ile)	4263-4331	Heavy	4	5	7
Mitochondrial tRNA Methionine	mt-tRNA ^{Met} (M/Met)	4402-4469	Heavy	5	4	6
Mitochondrial tRNA Tryptophane	mt-tRNA ^{Trp} (W/Trp)	5512-5579	Heavy	7	4	4
Mitochondrial tRNA Aspartate	mt-tRNA ^{Asp} (D/Asp)	7518-7585	Heavy	5	4	6
Mitochondrial tRNA Lysine	mt-tRNA ^{Lys} (K/Lys)	8295-8364	Heavy	3	5	9
Mitochondrial tRNA Glycine	mt-tRNA ^{Gly} (G/Gly)	9991-10058	Heavy	5	4	6
Mitochondrial tRNA Arginine	mt-tRNA ^{Arg} (R/Arg)	10405-10469	Heavy	5	4	3
Mitochondrial tRNA Histidine	mt-tRNA ^{His} (H/His)	12138-12206	Heavy	5	4	7
Mitochondrial tRNA Serine(AGY)	mt-tRNA ^{Ser(AGY)} (S1/Ser1)	12207-12265	Heavy	2	4	8
Mitochondrial tRNA Leucine(CUN)	mt-tRNA ^{Leu(CUN)} (L1/Leu1)	12266-12336	Heavy	7	4	7
Mitochondrial tRNA Threonine	mt-tRNA ^{Thr} (T/Thr)	15888-15953	Heavy	6	4	3
Mitochondrial tRNA Proline	mt-tRNA ^{Pro} (P/Pro)	15956-16023	Light	6	4	5
Mitochondrial tRNA Glutamate	mt-tRNA ^{Glu} (E/Glu)	14674-14742	Light	5	4	7
Mitochondrial tRNA Serine(UCN)	mt-tRNA ^{Ser(UCN)} (S2/Ser2)	7446-7514	Light	5	4	7
Mitochondrial tRNA Tyrosine	mt-tRNA ^{Tyr} (Y/Tyr)	5826-5891	Light	4	4	5
Mitochondrial tRNA Cysteine	mt-tRNA ^{Cys} (C/Cys)	5761-5826	Light	3	4	6
Mitochondrial tRNA Asparagine	mt-tRNA ^{Asn} (N/Asn)	5657-5729	Light	8	5	7
Mitochondrial tRNA Alanine	mt-tRNA ^{Ala} (A/Ala)	5587-5655	Light	5	4	7
Mitochondrial tRNA Glutamine	mt-tRNA ^{Gln} (Q/Gln)	4329-4400	Light	8	4	7

Table 1: A list of all 22 mt-tRNAs in human mitochondria arranged based on their location in the mt genome. The numbers of bases constituting the D loop, variable loop and TΨC loop are given for each mt-tRNA.

Abbreviation	Full name	Location in mtDNA	Strand	Respiratory chain component
Cyt b	Cytochrome b	14747-15887	Heavy	Complex III
ND6	NADH dehydrogenase subunit 6	14149-14673	Light	Complex I
ND5	NADH dehydrogenase subunit 5	12337-14148	Heavy	Complex I
ND4	NADH dehydrogenase subunit 4	10760-12137	Heavy	Complex I
ND4L	NADH dehydrogenase subunit 4L	10470-10766	Heavy	Complex I
ND3	NADH dehydrogenase subunit 3	10059-10404	Heavy	Complex I
COIII	Cytochrome C oxidase subunit III	9207-9990	Heavy	Complex IV
ATP6	ATP synthase 6	8227-9207	Heavy	Complex V
ATP8	ATP synthase 8	8366-8572	Heavy	Complex V
COII	Cytochrome C oxidase subunit II	7586-8294	Heavy	Complex IV
COI	Cytochrome C oxidase subunit I	5904-7444	Heavy	Complex IV
ND2	NADH dehydrogenase subunit 2	4470-5511	Heavy	Complex I
ND1	NADH dehydrogenase subunit 1	3307-4262	Heavy	Complex I

Table 2: A list of the 13 mt-mRNAs encoded in human mtDNA, their respective locations in mtDNA and the assemblies of their gene products into various respiratory chain complexes [15]. The data was compiled from Online Mendelian Inheritance in Man (OMIM) database [22]. The color code represents the clusters of genes that form different complexes of respiratory chain.

1.3 Crosstalk between mitochondria and nucleus

The mitochondrion and the nucleus are involved in cellular crosstalk as a means of regulation of mt function and possibly a complementary retrograde signaling mechanism to control nuclear gene expression [23]. The assembly of respiratory complexes involved in OXPHOS requires both mtDNA and nuclear gene expression, wherein, the products of the latter such as mitochondrial DNA-directed RNA polymerase (POLRMT), mitochondrial transcription factor A (TFAM) and mitochondrial transcription factor B2 (TFB2M) crucial for mtDNA transcription are imported into the mitochondria via protein import systems [21, 24, 25] (Figure 3).

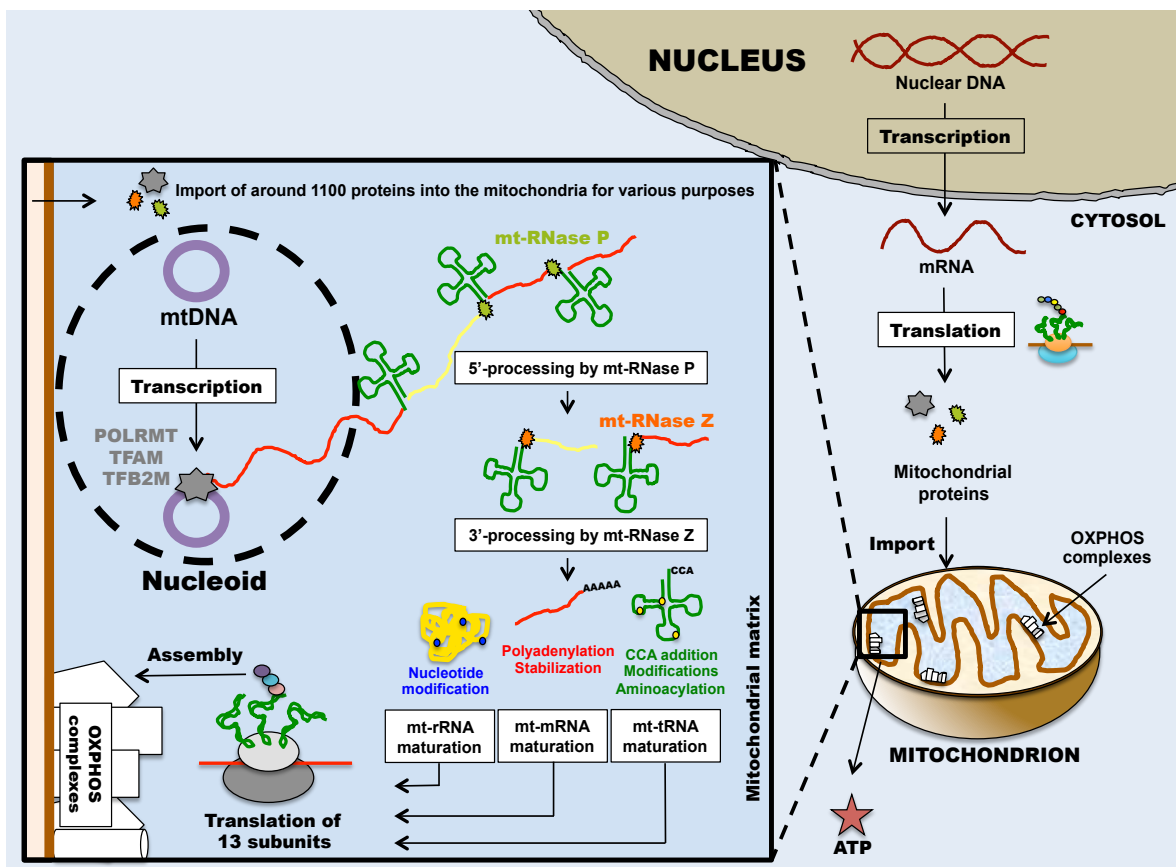


Figure 3: An illustration providing an overview of the path to generate ATP that entails both the mitochondrion and the nucleus. Several proteins produced in the cytosol enter the mitochondrion and perform mtDNA replication, transcription and processing of transcripts towards maturation of all RNA species, ultimately contributing to a proper mt function.

The nuclear encoded POLRMT is a single-subunit RNA polymerase that transcribes the H and L strands in the presence of TFAM and TFB2M to generate two long polycistronic transcripts that are substrates for extensive processing by mt-processing machinery [21, 26-29]. The precursor RNA processing occurs co-transcriptionally and is initiated in structures called as mitochondrial RNA granules (MRGs) [30-32] located near nucleoids. Except the mRNA clusters ATP6-COIII and ND5-Cytb, all elements are flanked by at least one tRNA gene. According to the tRNA punctuation model [17], the RNA processing is initiated on these tRNA sequences interspersed in between non-coding rRNA and most of the coding mRNA elements. The mt-RNase P and the mitochondrial ribonuclease Z (mt-RNase Z) are responsible for 5'- and 3'-processing activities that release individual mRNAs, rRNAs and tRNAs, each of which undergo their own maturation pathways [26]. The mature mt-rRNA and mt-tRNA^{Val} species participate in mitoribosome biogenesis and translate mt-mRNA elements to generate components of mt respiratory system [33, 34].

1.4 Mitochondrial transfer RNAs

The tRNAs are a vital class of biomolecules participating in the ribosomal translation of mRNAs, delivering codon-specific amino acids aiding protein synthesis [35]. The secondary structure of tRNAs form a cloverleaf and has several subdomains: an acceptor stem, D arm, anticodon stemloop, TΨC arm and a variable loop (Figure 4). Noteworthy, a few alternate tRNA secondary structural folds exist [35, 36]. These tRNAs with varying sequence and structural features undergo tertiary folding to a 3-dimensional L-shaped structure (Figure 5) before aminoacylation [35].

The early discovery of structure of tRNAs from the mitochondria of parasitic nematode worm *Ascaris suum* was considered 'bizarre' as it completely lacked the TΨC arm and variable loop [37]. The sequence and structural features of human mt-tRNAs also widely varies, differing in the lengths of different subdomains (Table 1). The mitochondria of many eukaryotic organisms such as humans harbor a complete set of tRNAs within its genome [15].

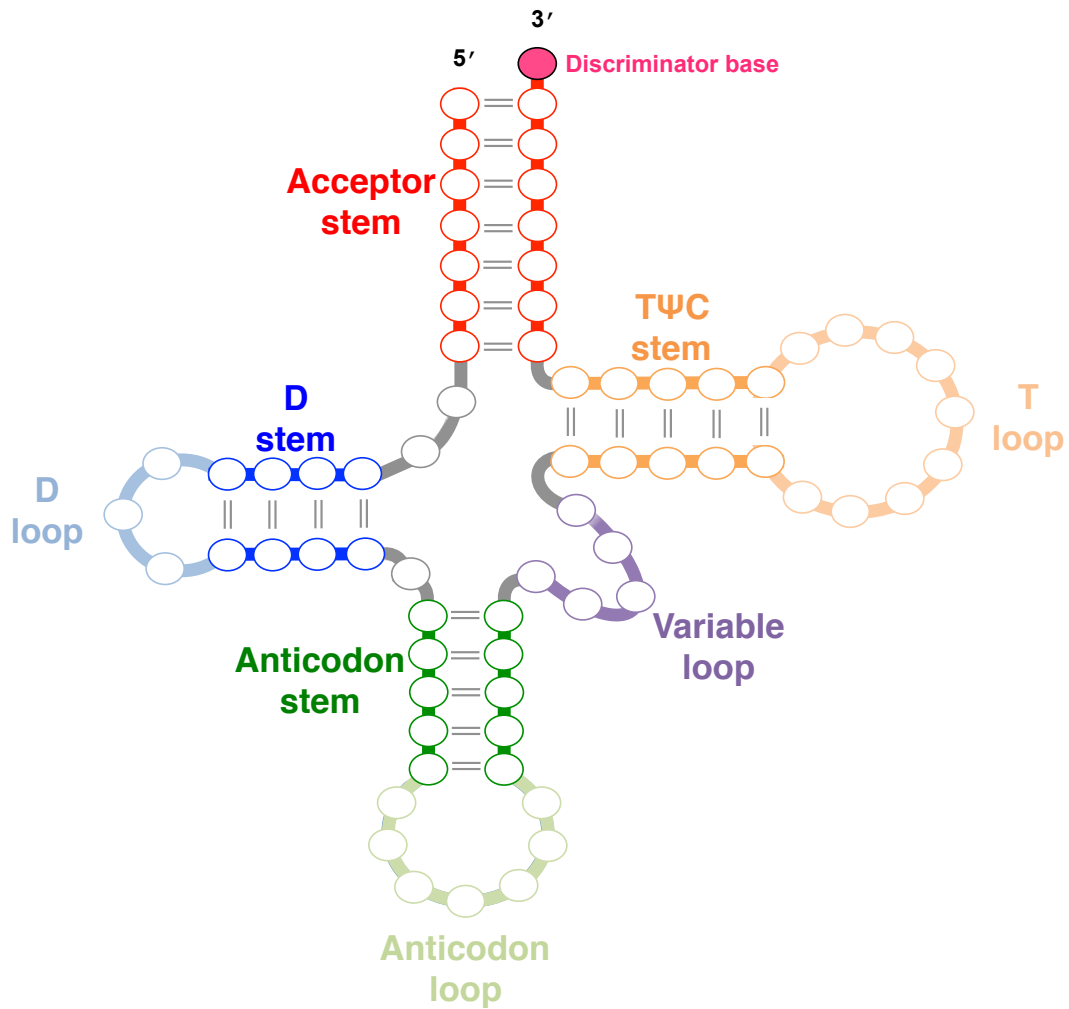


Figure 4: Color-coded cloverleaf representation of human mt-tRNAs harboring a 7-base pair acceptor stem, 4-base pair D stem, 5-base pair anticodon stem and 5-bp TΨC stem, all stabilized by WC base pairing. The mt-tRNA^{Ser(AGY)} lacks the D stem. In 22 mt-tRNAs, the D loop varies between 2 to 10 bases, variable loop varies between 4 and 5 bases and the T loop varies between 3 to 9 bases.

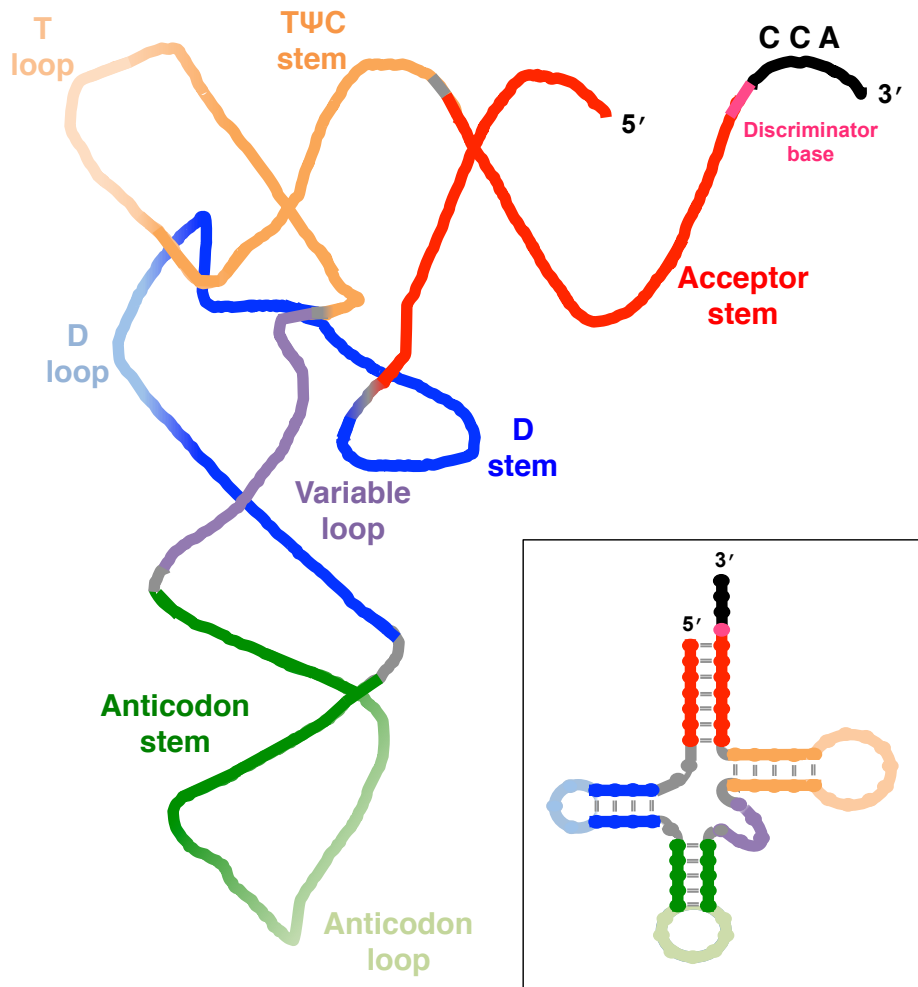


Figure 5: 3D color-coded representation of tertiary structure of mature tRNAs using X-ray structure of yeast tRNA^{Phe} [38] as reference. Inbox: Respective 2D color-coded cloverleaf-representation.

2 MITOCHONDRIAL TRANSFER RNA BIOGENESIS

The human mt-tRNA biogenesis is a systematic process involving several processing enzymes. The purpose of these mt-tRNAs is to aid the translation of 13 polypeptides encoded in mtDNA by delivering codon-specific amino acids to the mitoribosome. A thorough understanding of these mechanisms is crucial to address human mitochondrial diseases. In this chapter, the enzymes participating in these processes are discussed.

2.1 Human mitochondrial RNase P

2.1.1 Background

RNase P is an endonuclease performing the processing of 5'-pre-tRNAs across the three kingdoms of life [39-41] and was first discovered in bacteria [42]. Although the function of this enzyme is universally conserved, it displays stark structural diversity [43].

The bacterial RNase P is made up of a catalytic RNA component and a compact protein involved in the recognition of pre-tRNA 5'-leader sequences [44-46]. In higher organisms, the protein moiety constituting the RNA-based RNase P widely varies (Figure 6) with the archaeal RNase P composed of 4-5 proteins and eukaryotic nuclear RNase P composed of upto 10 proteins [40, 47-51]. While the bacterial RNase P RNA is active on its own *in vitro* [45], the RNA subunits of few archaeal and eukaryal RNase P display very low activity on its own [52, 53] highlighting the role of increased protein content in the latter cases. Thus, the evolution of RNase P supports the so-called 'RNA world hypothesis' that suggests a common primordial, prebiotic RNA to have evolved to function with proteins [43, 54, 55]. The existence of protein-only RNase P's (PRORPs) were long speculated [56] and supported by few reports [57-59], yet, their existence was fully consolidated much later with the discovery of PRORP in human mitochondria [60].

The three protein subunits: MRPP1, MRPP2 and MRPP3 constitute the human mt-RNase P [60, 61]. Unlike the RNase P's found in nature, the human mt-RNase P is not a ribonucleoprotein complex and is composed of only protein subunits (Figure 7) completely lacking any RNA subunit [60, 61]. The plant mt-RNase P also belongs to PRORP class of enzymes, wherein only a single protein subunit PRORP1 performs the 5'-pre-tRNA cleavage [62, 63].

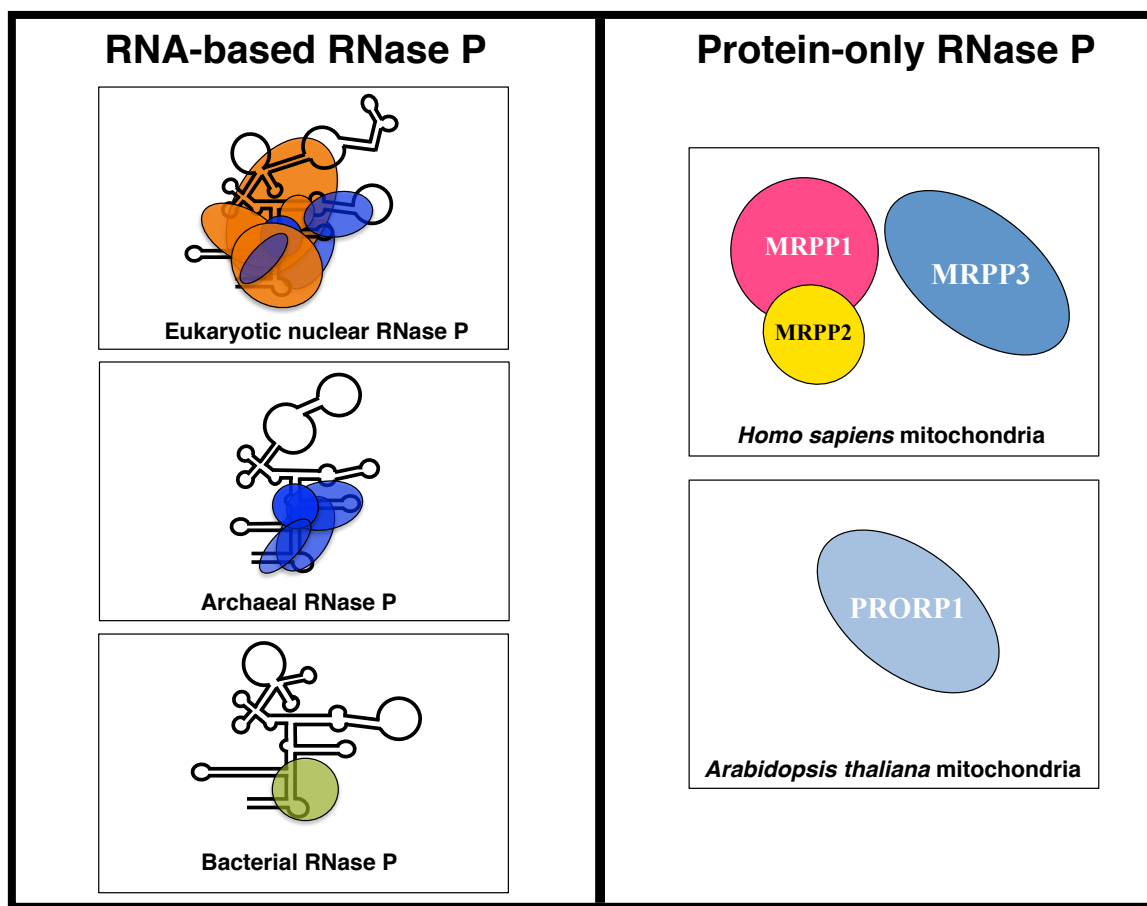


Figure 6: Structural diversity of RNase P across different kingdoms of life. The panel in the left provides three examples of RNA-based RNase P and the panel in the right provides two examples of PRORPs.

2.1.2 MRPP1

MRPP1, also called tRNA methyltransferase 10 homolog C (TRMT10C) is a nuclear-encoded RNA-methyltransferase (MTase) targeted to mitochondria that uses S-adenosyl methionine (SAM) as a co-factor to perform N¹-methylation of purines at position 9 of human mt-tRNAs, prevalently (19/22 mt-tRNAs) occupied by either a G or A [64-66]. The methylation of mt-tRNAs allows them to achieve proper folding [67, 68], yet is not a prerequisite for 5'-tRNA cleavage by mt-RNase P [64]. The proper functioning of MRPP1 protein is heavily dependent upon the expression of a multifunctional protein called MRPP2, the knockdown of which was shown to strongly influence the steady-state levels of MRPP1 [69]. Noteworthy, the steady-state levels of MRPP2 remained unaffected by knockdown of MRPP1 [69]. Thus, MRPP2 can also be attributed the methyltransferase function (Figure 7).

MRPP1 belongs to the Trm10 family of MTases classified under class IV SpoU-TrmD (SPOUT) superfamily, the characteristic feature of which is the formation of tight homodimers and the presence of a trefoil-knot structure in its SAM-binding catalytic domain [70-74]. Several isoforms of Trm10 homologs are present in humans, wherein MRPP1 evolved separately [75] and is suggested to differ from the other Trm10 isoforms such as TrmT10A and TrmT10B with regard to mechanism of pre-tRNA substrate recognition. The MRPP1 and MRPP2 proteins form a strong complex [64, 65] and is a subcomplex of mt-RNase P [60]. Thus, the tRNA binding, substrate specificity and methylation function resides in MRPP1 [71], while the precise mechanism by which MRPP2 promotes MRPP1 remains to be elucidated.

2.1.3 MRPP2

The nuclear encoded MRPP2 protein is localized to mitochondria [76] and acts on a wide variety of substrates to perform multiple functions within the cell. They include isoleucine degradation, metabolism of sex hormones, β -oxidation of fatty acids, metabolism of neuroactive steroids among others [77-83]. Owing to its multifunctional role in many cellular pathways, it has been assigned multiple names such as short-chain dehydrogenase/reductase 5C1 (SDR5C1), 17 β -hydroxysteroid dehydrogenase type 10 (17 β -HSD10), 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) and amyloid β -peptide-binding alcohol dehydrogenase (ABAD) [76, 84, 85]. It is a NADH-dependent protein required for the stabilization of MRPP1 and has an oligomeric state of a tetramer [69, 86]. Its exact role as a component of human mt-RNase P is so far unknown, but has been speculated to contribute to the substrate tRNA-binding in the MRPP1/2 complex via its Rossmann fold [60, 87, 88].

2.1.4 MRPP3

The KIAA0391 gene product coding for a large uncharacterized protein [89] was discovered to be the third component of human mt-RNase P (named as MRPP3) that along with MRPP1 and MRPP2 is essential for 5'-tRNA processing [60]. It is a nuclear-encoded mt protein [65] and is one of the few pentatricopeptide repeat (PPR) proteins found in mammalian mitochondria [90].

The PPR proteins were first discovered in plants [91] and are characterized by several PPR repeats of around 35 amino acids each. They constitute a large family of proteins that mostly localize to organelles such as mitochondria and chloroplasts, exhibiting RNA binding properties and involvement in RNA metabolism [92-98]. Although MRPP3 is a PPR protein, it requires its binding partners MRPP1/2 complex to present the pre-tRNA substrate for 5'-processing [99].

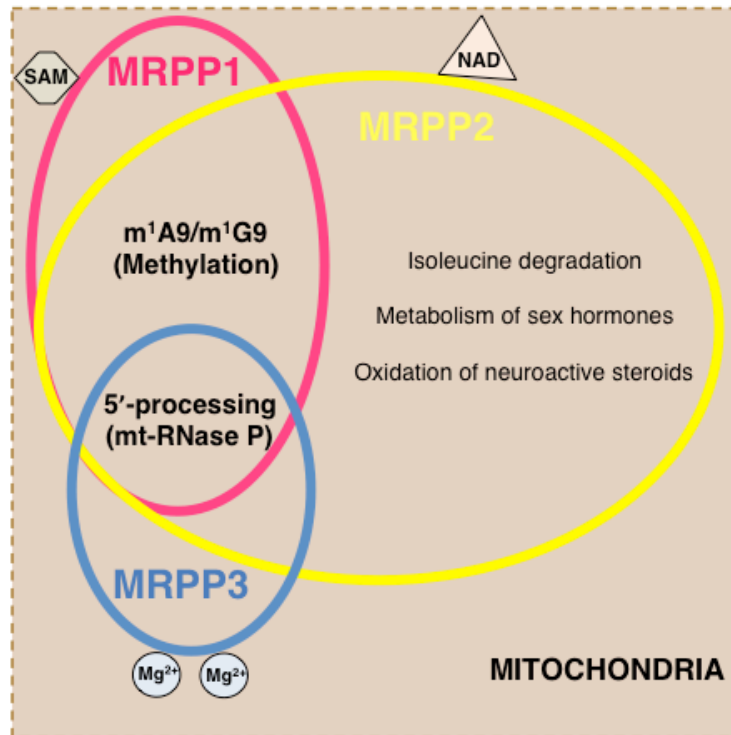


Figure 7: MRPP1, MRPP2 and MRPP3 constituting the human mt-RNase P localizes to mitochondria. The MRPP1/2 complex is responsible for methylation of human mt-tRNAs while the full MRPP1/2/3 complex performs 5'-processing of human mt-tRNAs. MRPP2 performs several other functions unrelated to tRNA processing.

During the purification of mt-RNase P, the interaction of MRPP3 with the MRPP1/2 sub-complex was observed to be very weak [60] suggesting that human mt-RNase P does not exist as MRPP1/2/3 complex, but rather as an intermediate transient complex. The C-terminal domain of MRPP3 also harbors conserved sequence blocks displaying four conserved amino acids initially speculated to perform metal-ion co-ordination to perform catalysis like a metallonuclease protein [100]. The crystal structure of *Arabidopsis thaliana* PRORP1 [62] served as a reference to speculate on the global architecture and mode of substrate catalysis of MRPP3. However, unlike MRPP3, PRORP1 can perform 5'-tRNA cleavage on its own using metal ions like Mg²⁺/Mn²⁺ [62, 63].

2.2 Human mitochondrial RNase Z

2.2.1 Background

The 3'-processing of tRNAs is performed by RNase Z that acts on 5'-processed substrates [99, 101, 102]. These enzymes either exist as RNase Z-S (short form) found in all kingdoms of life or as RNase Z-L (long form) found exclusively in eukaryotes [103]. The nuclear genome in humans encodes both forms of RNase Z called ELAC1 and ELAC2 [104]. The short form ELAC1 localizes predominantly to the cytosol and also to the nucleus while the long form ELAC2 localizes to both the nucleus and mitochondria [65, 104-109].

2.2.2 ELAC2

ELAC2 processes 3'-trailer sequences *in vitro* [110] and its silencing causes accumulation of unprocessed precursor transcripts [65, 101]. Thus, ELAC2 plays a major role in proper mitochondrial function [111]. Noteworthy, in some cases, ELAC2 is not involved in the 3'-processing such as in case of the mt-tRNA genes of tRNA^{Tyr} and tRNA^{Cys} that overlap by 1 nucleotide [15]. The generation of 3'-processed end of tRNA^{Tyr} is a result of addition of an adenosine by mitochondrial poly(A) polymerase (mtPAP) after the RNase P cleavage of downstream tRNA^{Cys} [112]. It was reported that a protein called PPR containing domain 1 (PTCD1) associates with ELAC2 [65]. The PTCD1 protein is characterized to negatively influence the levels of leucine tRNAs in mitochondria [113, 114]. So far, the precise interplay of ELAC2 with PTCD1 remains to be established. Either PTCD1 works in association with ELAC2 or as alternate 3'-processing machinery during the lack of ELAC2.

2.3 CCA addition

The maturation of mt-tRNAs is achieved by the addition of cytosine-cytosine-adenine (CCA) to the 3'-end of 5',3'-processed precursors and is performed by nucleotidyl transferases [115]. In human mitochondria, transfer RNA nucleotidyl transferase 1 (TRNT1), alternatively called as CCA-adding enzyme (CCA-E) is responsible for 3'-CCA addition, a pre-requisite for codon-specific amino acid attachment via aminoacylation [116]. TRNT1 performs just a single round of CCA addition due to its compact binding pocket. While doing so, TRNT1 interacts only with the sugar-phosphate backbone and not the nucleic acid template [117-120]. The tRNAs then undergo specific modifications at specific sites to attain proper folding, that are then recognized by the corresponding aminoacyl tRNA synthetases [109, 121].

2.4 tRNA modifications

The post-transcriptional modifications on various classes of RNA are carried out by a multitude of proteins. So far, around 130 different RNA modifications are reported, wherein tRNAs are the major targets of most common modifications such as methylation [122, 123]. The tRNA modifications are shown to be essential for stabilizing the tRNA tertiary structure, promote interaction with certain key enzymes, aminoacylation and accurate codon-anticodon decoding for an efficient translation [67, 68, 124-129]. Several tRNA-modifying enzymes are involved in mt-tRNA modifications, the lack of which leads to tRNA degradation [130] and cause several diseases [131]. The mammalian mt-tRNAs generally lacks a canonical D/T loop interaction, displaying an overall low melting temperature and hence is heavily reliant upon such modifications for stability and functional purposes [66, 128, 132, 133]. In mammalian mitochondria, more than 100 mt-tRNA post-transcriptional modifications are reported [66]. Several databases such as MODOMICS [134] and the RNA modification database [135] are dedicated towards careful documentation of reported modifications in bacteria, archaea and eukarya.

2.5 Aminoacylation

The aminoacylation of tRNAs is a process in which the mature tRNAs with a specific anticodon are charged with corresponding amino acids in two distinct steps, brought about by several aminoacyl-tRNA synthetases (aaRSs) [136]. The aminoacylation of 22 mt-tRNAs is carried out by 19 nuclear-encoded aaRSs targeted to mitochondria [26, 137]. Except the cases of mt lysine aaRS and mt glycine aaRS that share the same gene as their cytoplasmic counterparts, all 17 other aaRS machinery have distinct nuclear genes of their own [128]. Noteworthy, a single mt serine aaRS aminoacylates both the mt-tRNA^{Ser(AGY)} and mt-tRNA^{Ser(UCN)}, and a single mt leucine aaRS aminoacylates both the mt-tRNA^{Leu(CUN)} and mt-tRNA^{Leu(UUR)} [138]. As the mammalian mitochondria lacks glutamine aaRS, the aminoacylation of mt-tRNA^{Gln} deviates from the standard pathway wherein the synthesis of Gln-tRNA^{Gln} occurs by misacylation to Glu-tRNA^{Gln} followed by transamidation to Gln-tRNA^{Gln} [139].

The aaRS display a high degree of specificity towards their cognate tRNAs by recognizing key identity elements such as the anticodon triplet, the N1-N72 base pair in the acceptor stem and the discriminator base [136]. However, the mt-aaRSs differ from its cytoplasmic and bacterial counterparts in that it displays a more relaxed tRNA discrimination [140]. A wide range of human diseases are linked to mutations in mt-aaRSs [141, 142] including mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) and myoclonic epilepsy with ragged red fibers (MERRF) among many. The proper aminoacylation of mt-tRNAs is henceforth crucial for normal translation of 13 polypeptides required for OXPHOS.

3 HUMAN MITOCHONDRIAL DISEASES

The human mt diseases are caused either due to mutations in the nDNA, whose gene products play vital roles inside mitochondria or due to mutations in the mtDNA primarily affecting OXPHOS [12, 143]. A variety of such mt diseases displaying biochemical, genetic and clinical heterogeneity have been reported [12, 143] since the first clinical study was published in 1959 [144]. The databases such as MITOMAP [145] and MAMIT (Mammalian mitochondrial tRNA) database [146] serve as useful platforms with a compilation of all human pathologies originating due to mitochondria-associated mutations. Several strategies and therapies to address human mt diseases are currently under developmental stage [143, 147].

3.1 Significance, prevalence and occurrence

The heterogenous set of human mt diseases can occur at any stage of life and affects multiple organs including brain, heart and liver, all of which consume high amounts of energy [12, 148]. The onset of clinical symptoms is observed when the patient cells carry multiple copies of mutant mtDNA above a critical threshold in relation to the wild-type (referred to as heteroplasmy), when the disease phenotype becomes more apparent. Although the exact percentage of such mutant mtDNA causing disease phenotype varies, 70% or more is generally considered to be a critical threshold [149]. Noteworthy, even 25% mutant mtDNA or less was shown to cause disease [150, 151].

The population-based studies done in Sweden [152] and Australia [153] indicated that about 1 in 20,000 children are affected by such mt-diseases, prevalent also in adults [154]. A recent study found that about 1 in 5000 individuals were affected by mtDNA mutations and reported prevalence over time [155, 156]. Thus, these reports highlight an urgent need to develop strategies to tackle them.

3.2 Mutations in mtDNA and nuclear encoded genes

Early works indicated that specific mutations in mtDNA cause debilitating mt disorders [157, 158]. These mutations may have been acquired via natural maternal inheritance [159] or may have been sporadically introduced. The defective maintenance machinery of mtDNA may also lead to introduction of disease-causing mutations as a consequence of nDNA mutations [160]. An estimated 15% of all mt disorders are caused due to mtDNA defects [154]. Most of the disease-causing mtDNA mutations are localized within the 22 mt-tRNA genes [161] causing more than 130 human disorders ranging in severity from mild weakness to life-threatening cardiomyopathies [162, 163]. A compilation of all reported human pathologies as a result of mutations in the mt-tRNA genes is given in Table 3.

The mitochondrial disorders may also originate due to mutations in the nDNA [154] whose gene products are responsible for maintaining proper mt function by playing specific roles in processes such as mt transcription, processing of RNA species towards maturation and mt translation [26]. The clinical manifestations of such mutations range from biochemical defects to severe neurological disorders. The mutations in the key mt-tRNA processing enzymes such as MRPP1, MRPP2, MRPP3, ELAC2 and TRNT1 are implicated in several diseases and are compiled in Table 4.

mt-tRNA	Mutations in mtDNA	Pathology caused
Phe	T582C, G583A, A608G, G611A, T618C, G622A	EI, MELAS, MERRF, MM, TNS
Val	G1606A, G1642A, G1644T, T1659C	AMDF, EI, H, LS, MELAS
Leu(UUR)	G3242A, A3243G, A3243T, G3249A, T3250C, A3251G, A3252G, C3254G, G3255A, C3256T, T3258C, A3260G, T3264C, T3271C, delT3272, T3273C, C3275A, A3280G, G3283A, A3288G, T3291C, A3302G, C3303T	D, DM, DMDF, EI, G, IH, KS, LA, MELAS, MERRF, MERRF-like MELAS, MM, MMC, MTMD, PEM
Ile	A4267G, A4269G, T4274C, G4284A, T4285C, T4290C, T4291C, A4295G, G4298A, A4300G, G4309A, A4317G, C4320T	AMDF, CPEO, DM, ECM, EM, FICP, HCM, HHH, MICM, MM, PEO, SNHL
Met	T4409C, A4435G, G4450A	LHON, MM
Trp	G5521A, G5532A, InsT5537, G5540A, G5549A	CD, D, DEMCHO, LS, MILS, MM, NGI, PEM
Asp	A7256G	MM
Lys	A8296G, T8300C, A8308G, G8313A, T8316C, A8326G, G8328A, G8342A, A8344G, A8347G, T8355C, T8356C, G8361A, T8362G, G8363A	AR, C, DEAF, DMDF, EI, EM, HCM, LS, MELAS, MERRF, MICM, MNGIE, MS, PEO, SM
Gly	T9997C, A10006G, T10010C, A10044G	CIPO, EM, MHCM
Arg	A10438G	EM
His	G12147A, G12183A, G12192A	CM, MELAS, MERRF, PR, SNHL
Ser(AGY)	C12246A, G12207A, C12258A	CIPO, DMDF, EM, MM
Leu(CUN)	G12294A, T12297C, A12299C, G12301A, A12308G, T12311C, G12315A, A12320G, G12334A	AISA, CPEO, DCM, EI, MELAS, MERRF, MM
Thr	G15915A, A15923G, A15924G, delT15940, G15950A, A15951G	ADPD, LHON, LIMM, MM
Pro	T15965C, G15990A, C15995T	ADPD, C, MM, O
Glu	T14687C, T14693C, T14696C, A14709G, C14724T, C14739T	D, EI, EM, L, LA, MELAS, MM, PR, PRF
Ser(UCN)	InsG7472, A7480G, C7497T, A7510G, A7511G, A7512G	AMDF, DEAF, LA, MERRF-like, MM, PEM, RRF, SNHL
Tyr	T5843C, A5874G, G5877A, delT5885	CD, CPEO, EI, LW, MM
Cys	C5783T, A5814G, C5780T	DEAF, DCM, EM, MELAS, MM, PEO, SNHL
Asn	C5703T, A5692G, A5728G, A5693G, C5698T	CPEO, lethal MM, MM, MOF, PEO
Ala	C5591T, A5628G, C5650T	CEPO, MERRF, MM
Gln	C4332T, A4336G, InsT4370, T4381C	ADPD, CD, D, EM, LHON, MM

Table 3: A compilation of mtDNA mutations implicated in mt diseases compiled from MAMIT database [146]. The abbreviations are listed in the glossary in section 10.

Protein	#amino acids (including MTS)	Functions	Reported mutations	Pathological implications	References
MRPP1	403	m ¹ A9/m ¹ G9 methylation, 5'-tRNA cleavage (mt-RNase P)	R181L, T272A	Combined oxidative phosphorylation defect type 30	[69, 164]
MRPP2	261	m ¹ A9/m ¹ G9 methylation, 5'-tRNA cleavage (mt-RNase P), isoleucine degradation, metabolism of fatty acids, sex hormones and neuroactive steroids	V12L, V65A, D86G, L122V, R130C, Q165H, A154T, A157V, A158V, V176M, P210S, K212E, R226Q, N247A, E249Q	HSD10 disease (MHBD deficiency), colorectal cancer, alzheimer's disease, choreoathetosis, refractory epilepsy and learning disability, intractable epilepsy and global developmental delay	[69, 83, 85, 165-181]
MRPP3	583	5'-tRNA cleavage (mt-RNase P), possible modulation of rate of post-transcriptional modification	N437S, A485V	Perrault syndrome	[182, 183]
ELAC2	826	3'-tRNA cleavage (mt-RNase Z)	F154L, R211Q, S217L, L423F, G487R, T520I, A541T, E622V, R781H, G806R	Prostate cancer hereditary 2, combined oxidative phosphorylation deficiency, infantile hypertrophic cardiomyopathy and complex I deficiency, intellectual disability	[104, 111, 184, 185]
TRNT1	434	3'-CCA addition	T154I, M158V, L166S, R190I, I223T, I326T, K416E	Sideroblastic anemia with B-cell immunodeficiency, periodic fevers and developmental delays; retinitis pigmentosa with erythrocytic microcytosis	[186-190]

Table 4: A list of reported mutations in the key nuclear-encoded genes and the diseases they cause are compiled from MalaCards database [191]. MTS: mitochondrial target sequence.

4 METHODOLOGY

The research studies towards the structural and biochemical characterization of biomolecular complexes require significant quantities of pure proteins and tRNAs that were produced as shown below (Figure 8). The human mt-proteins were produced by the application of recombinant DNA technology [192-194], wherein they are expressed in *E. coli* bacterial cells and purified using advanced chromatographic techniques described below. The human mt-tRNAs were synthesized by *in vitro* transcription using T7 RNA polymerase and purified by a combination of glm-S based affinity purification setup [195] and chromatography.

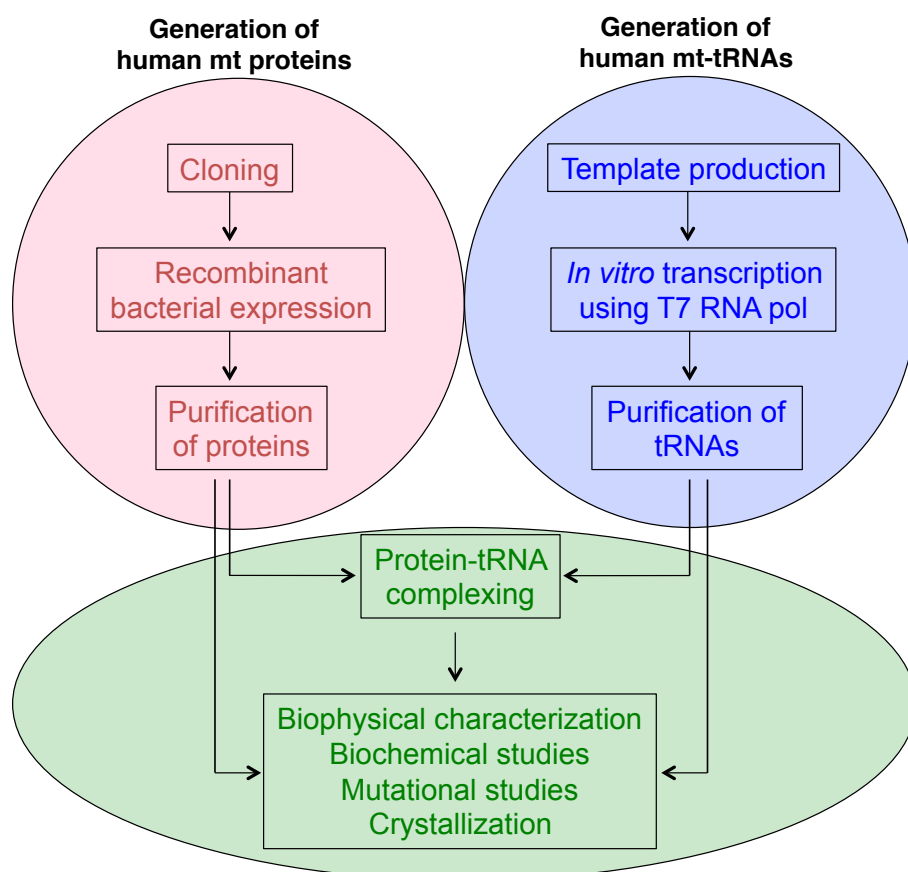


Figure 8: A flowchart showing the pipeline for generation of pure mt-proteins and mt-tRNAs. These biomolecular components will then be used for downstream studies.

4.1 Recombinant production of mt-proteins

The genes encoding human mitochondrial protein subunits are cloned into plasmids such as pNIC28-Bsa4 expression vector using standard molecular biology tools. The new recombinant plasmid is then transformed into *E. coli* bacterial cells of a particular strain such as *E. coli* BL21 (DE3), that consists of a re-engineered chromosomal setup, wherein the expression of encoded T7 RNA polymerase gene is controlled by a modified lac operon system. A lac repressor normally remains bound to this lac operon, blocking the expression of T7 RNA polymerase gene by *E. coli* RNA polymerase. The induction of cells by isopropyl β -D-1-thiogalactopyranoside (IPTG) causes the lac repressor to be released, resulting in the transcription of the T7 RNA polymerase gene by *E. coli* RNA polymerase (Figure 9). Thus, the newly synthesized T7 RNA polymerase binds to the T7 promoter region of plasmid DNA harboring the desired gene to produce the protein of interest [192].

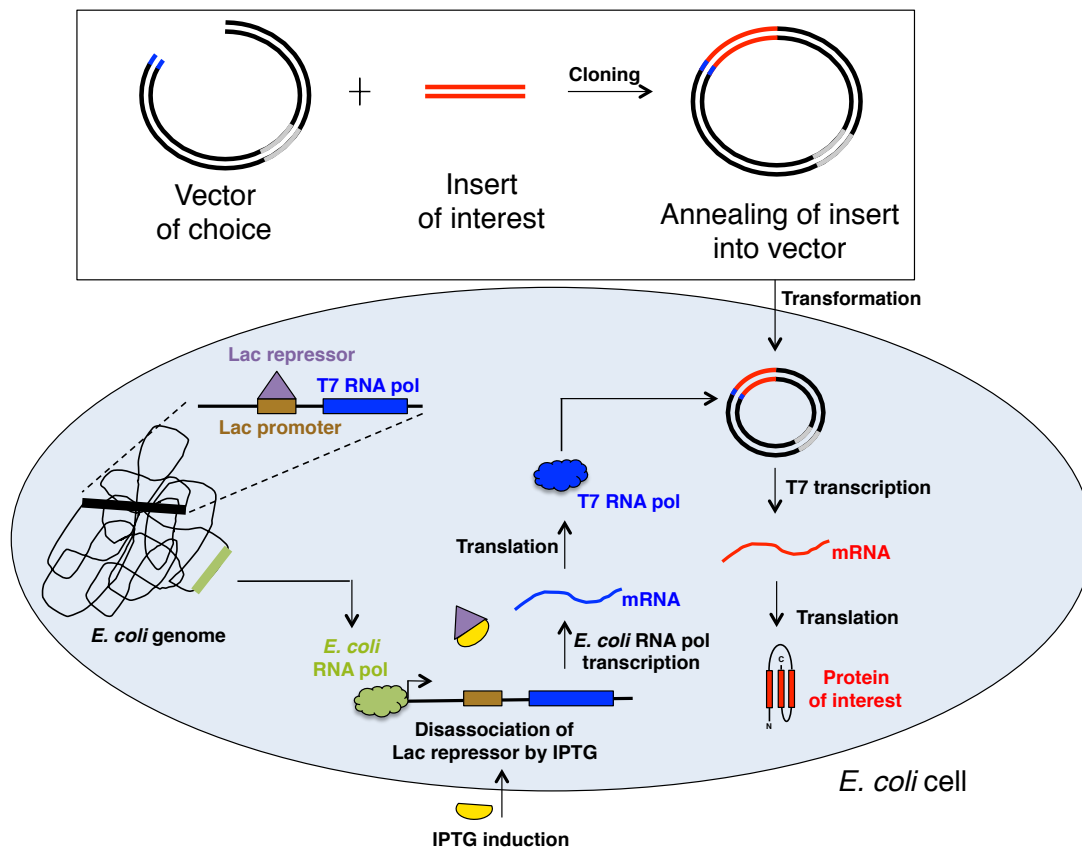


Figure 9: A scheme of recombinant expression of proteins in bacterial cells. The protein expression is induced when the bacterial cells are provided with rhamnose and IPTG.

4.2 Purification of mt-proteins

The bacterial cells harboring the recombinant protein are lysed using homogenization or sonication and the cell lysate is subjected to centrifugation to separate the soluble fraction from undesired cell debris. The soluble fraction is then subjected to step-by-step purifications (Figure 10) starting with capture of desired macromolecule by methods such as immobilized metal-affinity chromatography (IMAC), followed by intermediate purification steps such as ion-exchange chromatography (IEC) and heparin affinity chromatography and a final polishing step by size-exclusion chromatography (SEC).

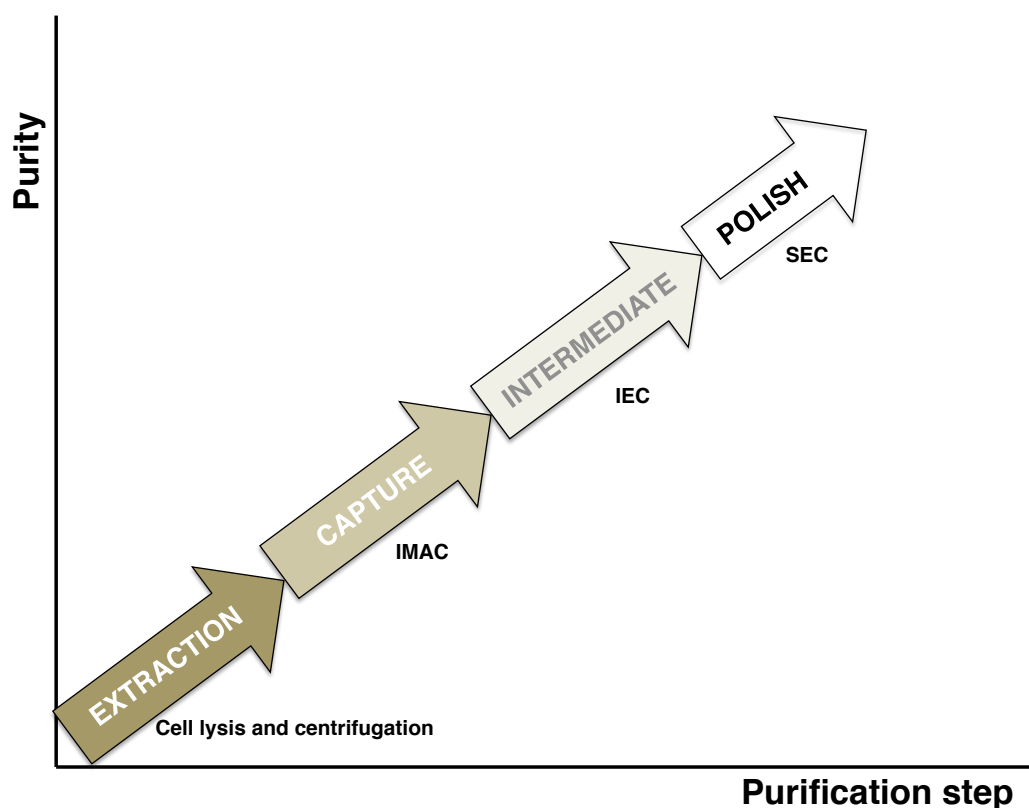


Figure 10: The pipeline for extraction and purification of proteins, recombinantly expressed in bacterial cells. The degree of purity is the highest in the final step of purification.

The recombinant proteins are generally expressed in fusion with affinity tags such as His-tag, consisting of six histidine (His)₆ residues [196]. The amino acids histidine and cysteine have a strong affinity to metal ions such as nickel or zinc at neutral pH [197]. In IMAC, hexahistidine tagged recombinant proteins are captured from a pool of bacterial proteins by immobilizing them on a metal-chelate resin and washing off the huge amounts of undesired bacterial proteins. The captured protein is released by the use of higher concentration of imidazole as an eluent. In Heparin chromatography, the resin made up of linear

polysaccharides employs a combined use of its negative charge and affinity properties to separate biological macromolecules [198]. IEC exploits the charge property of the target protein, wherein the protein is passed through a resin consisting of oppositely charged moieties. The efficient separation is achieved by differences in the degree of interaction of different molecules to the matrix of a particular charge [199]. As a final polishing step, SEC (also called gel filtration) offers a robust technique to obtain homogenous mixture of pure proteins, protein-protein or protein-nucleic acid complexes. The separation is based on the differences in migration pattern of molecules of varying sizes and shapes through medium of spherical particles [200].

4.3 *In vitro* synthesis of mt-tRNAs

The *in vitro* synthesis of human mt-tRNAs that we have used is based on the optimization of a protocol described for native affinity purification of RNA [195]. The design of a typical template for the transcription of tRNAs is as follows. It starts with a T7 promoter sequence immediately after a short T7 landing region, followed by the DNA sequence of choice, glm-S ribozyme gene sequence and MS2 coat protein sequence (Figure 11).

The first strategy employed here is to transcribe the template in the above-described set-up using T7 RNA polymerase and immobilize the transcription product onto a nickel-affinity resin using a hexahistidine-tagged form of the MBP-MS2 coat fusion protein (HMM) that binds to the MS2 coat protein-binding stem-loops at the 3'-end of transcription product (Figure 11). The addition of glucosamine-6-phosphate (GlcN6P) activates the glm-S ribozyme, releasing the desired tRNA in the flowthrough and washes [201, 202].

An alternate strategy of tRNA production by co-transcriptional glm-S cleavage was also used, wherein the GlcN6P is already provided during the *in vitro* transcription to facilitate cleavage already during the reaction. The transcription product containing a mixture of cleaved and uncleaved RNA species are then separated using ion-exchange chromatography (Figure 11). The use of chromatography can also be extended towards a traditional run-off transcription that will then normally give a heterogeneous 3'-end.

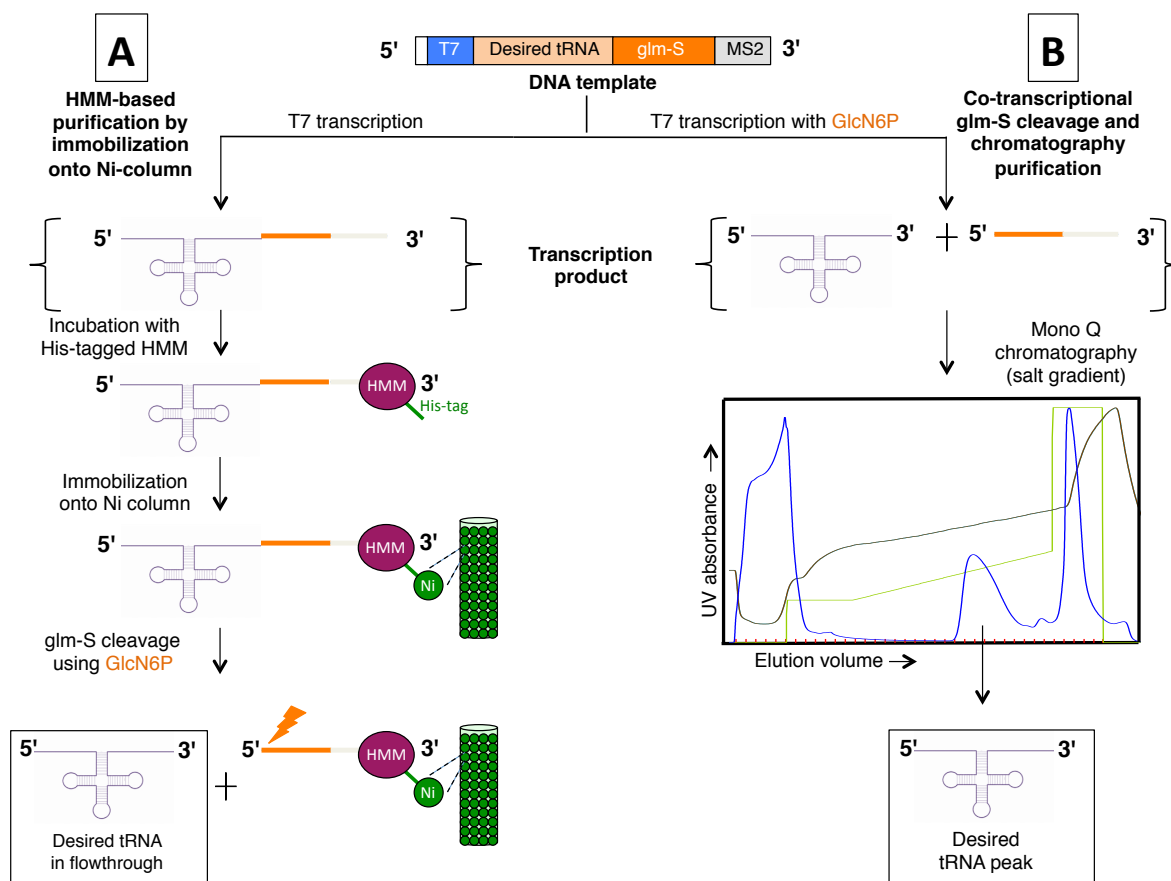


Figure 11: The pipeline for generation of pure mt-tRNAs. **A:** HMM-based tRNA production **B:** Co-transcriptional glm-S cleavage based tRNA production.

4.4 Crystallization of biological molecules and bio-molecular complexes

One of the strategies employed in the structure determination of proteins, nucleic acids and protein-nucleic acid complexes is by crystallizing them and collecting their X-ray diffraction data at high resolution. Such crystallization is achieved by subjecting the pure, homogenous macromolecular species to a supersaturated state using a suitable precipitating agent to allow nucleation and crystal growth [203, 204]. The successful crystallization depends on several factors such as purity and concentration of the macromolecule, temperature and composition of the precipitant solution. A wide range of commercial kits is available for initial crystallization screening.

The initial crystals obtained from the sparse matrix screenings are generally of poor-quality and hence the crystal conditions have to be optimized to obtain better quality crystals to collect the best X-ray diffraction data. The optimization of crystallization conditions involve systematic, incremental changes in various parameters such as pH, concentration of precipitant, concentration of macromolecule, presence of additives and temperature [205]. The quality of crystals can also be improved by strategies like seeding, wherein a crystalline

material is introduced into a crystallization drop as a 'seed' to promote crystal growth or by crystal dehydration or by several other post-crystallization methods such as soaking [206-209]. Thus, the successful structure determination by X-ray crystallography is based upon the generation of the best crystals of the macromolecular species, produced by the above-described methods.

5 SUMMARY OF PAPERS

5.1 PAPER I

Structure of the nuclease subunit of human mitochondrial RNase P

This paper describes the crystal structure of MRPP3 at high resolution and provides a comprehensive explanation as to why it needs MRPP1/2 and pre-tRNA substrate for 5'-processing.

The human mt-RNase P required an additional protein component from the mitochondrial extract along with MRPP1 and MRPP2 for 5'-processing of pre-tRNAs. This protein was identified to be the product of KIAA0391 gene expression and was named as MRPP3 [60]. The C-terminal domain of MRPP3 sequence showed conserved blocks and was suggested to co-ordinate metal ions to act as a protein metallonuclease [100]. In plant mitochondria and plastids, just one protein component PRORP1 was shown to perform 5'-processing of precursor tRNAs [63]. We designed the study to investigate the structural data of the human MRPP3 protein to understand as to why it is unable to function on its own.

To perform the study, we required MRPP3 (Uniprot: O15091; residues 45-583) along with the other two human mt-RNase P subunits: MRPP1 (Uniprot: Q7L0Y3; residues 40-403) and MRPP2 (Uniprot: Q99714; residues 1-261). A suitable pre-tRNA substrate was needed to evaluate the pre-tRNA 5'-processing activity. The *in vitro* transcribed pre-tRNA tyrosine substrate with suitable 5'- and 3'-extensions was produced by using a combination of glm-S based affinity purification setup [195] and chromatography purification. As MRPP1 and MRPP2 were reported to form a strong protein-protein complex [60, 64], we cloned them into a single pNIC-CTHF vector (Figure 12) such that untagged MRPP2 precedes C-terminally His-tagged, Flag-tagged MRPP1 separated by a ribosome re-initiation spacer.

The MRPP3 protein was cloned into pNIC28-Bsa4 vector (Figure 12), subjected to recombinant expression in *E.coli* KRX cells and purified using Ni-IMAC (Nickel-IMAC) and SEC. The full-length protein (45-583) lacking MTS was subjected to crystallization trials. However, no crystal hits were obtained. Hence, we created several N-terminal truncated variants of MRPP3 lacking one or more PPRs. Each of the truncated variants were cloned into pNIC28-Bsa4 vector and confirmed by DNA sequencing. These variants were expressed and purified like the WT protein and were subjected to crystallization trials. One variant (residues 207-583) generated crystals. This crystallization condition was optimized and X-ray diffraction data was collected to determine the structure of the protein.

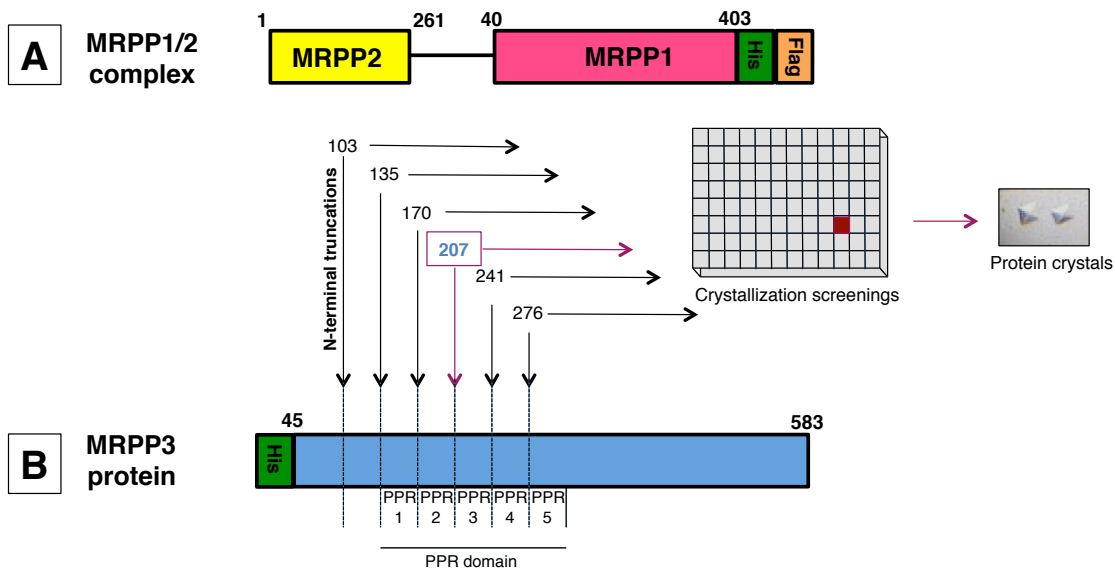


Figure 12: An illustration of constructs of human mt-RNase P subunits. **A:** MRPP1/2 subcomplex was produced using pNIC-CTHF-MRPP2-MRPP1 construct. **B:** An outlook of N-terminal re-engineered MRPP3 truncations created for crystallization. All variants were cloned into pNIC28-Bsa4 vector.

The overall structure of MRPP3 resembled the structure of *Arabidopsis thaliana* PRORP1 [62] containing three domains: N-terminal PPR domain, central domain and C-terminal metallonuclease domain. Yet, the structure of MRPP3 differed from PRORP1 both in terms of relative orientation of individual domains to each other and at molecular level. The active site of MRPP3 located in the metallonuclease domain lacked metal ions and showed severe distortion unlike PRORP1, whose active site coordinated two Mg^{2+}/Mn^{2+} ions via four conserved aspartate residues.

Initially, we suspected that perhaps the chelating crystallization condition may have caused the MRPP3 to loose Mg^{2+} ions causing the active site to be disorganized. Hence, we crystallized the MRPP3 protein in several non-chelating conditions including soaking the crystal in excess $MgCl_2$ and solved the structure. Still, the overall architecture of the active site remained the same confirming that our structure was not an artifact. We then created MRPP3 active site mutants to evaluate if its activity is indeed coordinated by four aspartates D409, D478, D479 and D499. By performing pre-tRNA activity assays we confirmed that the suspected four aspartates are indeed critical for catalysis. By following the architecture of the PRORP1 active site, we then extended our mutational analysis to evaluate if the active site of MRPP3 could be rebuilt so as to make it loose the requirement of the MRPP1/2 complex for pre-tRNA catalysis. However, all attempts to render MRPP3 active by itself were unsuccessful. We speculate that in the presence of the MRPP1/2 sub-complex and the pre-tRNA substrate, MRPP3 undergoes complex domain re-arrangements and re-organization of its active site to be able to perform the 5'-tRNA cleavage.

5.2 PAPER II

The MRPP1/MRPP2 complex is a tRNA-maturation platform in human mitochondria

This paper describes the previously unknown wider role of the human MRPP1/2 complex in the human mt-tRNA maturation pathway (Figure 13). The mt-tRNA processing and maturation is a systematic, ordered, step-by-step procedure starting with 5'-processing by mt-RNase P, 3'-processing by mt-RNase Z followed by 3'-CCA addition and the post-transcriptional modifications [26]. The MRPP1/2 complex along with MRPP3 constitutes the human mt-RNase P that performs 5'-tRNA processing [60, 61]. MRPP1 forms a strong protein complex with the multifunctional MRPP2 protein to perform methylation of mt-tRNAs at position 9 [64, 69]. Noteworthy, this methylation function of the MRPP1/2 complex is not a pre-requisite for RNase P activity [64]. The MRPP1/2 complex was thus by far only discussed in the context of mt-RNase P.

Our unsuccessful attempts in rebuilding the active site of MRPP3 by performing specific mutations as published in **PAPER I** highlighted the then unknown significance of MRPP1/2 sub-complex. During this work, we also observed that MRPP3 could perform multiple-turnover reactions unlike MRPP1/2 complex that performed single-turnover reactions. The MRPP1/2 complex stays with the pre-tRNA substrate even after MRPP3-led 5'-cleavage is complete. This led us to question as to how does the 5'-processed tRNA undergo 3'-processing by RNase Z while still remaining bound to MRPP1/2. The previous reports have shown that the ELAC2 protein functions as RNase Z in human mitochondria to perform the 3'-processing of 5'-processed mt-tRNAs [210]. There were no studies that evaluated the link between 5'- and 3'-processing events, giving an impression that these events were independent and uncoupled. Noteworthy, one study reported that the depletion of MRPP1 affected both 5'- and 3'-processing [101] without exploring this observation in detail.

To investigate these phenomena more closely and to bring clarity to the mt-tRNA processing pathway, we performed several *in vitro* activity assays and analytical size-exclusion chromatography experiments. We show that MRPP1/2 is not only participating in 5'-processing of mt-pre-tRNAs, but also hosts them in further steps. We show that ELAC2 led 3'-cleavage of mt-tRNAs is able to occur while they remain bound to MRPP1/2. Moreover, we have closely evaluated both the physiological and non-physiological conditions and report that the 3'-processing by ELAC2 is significantly enhanced in 17 out of 22 mt-tRNAs in the presence of the MRPP1/2 complex.

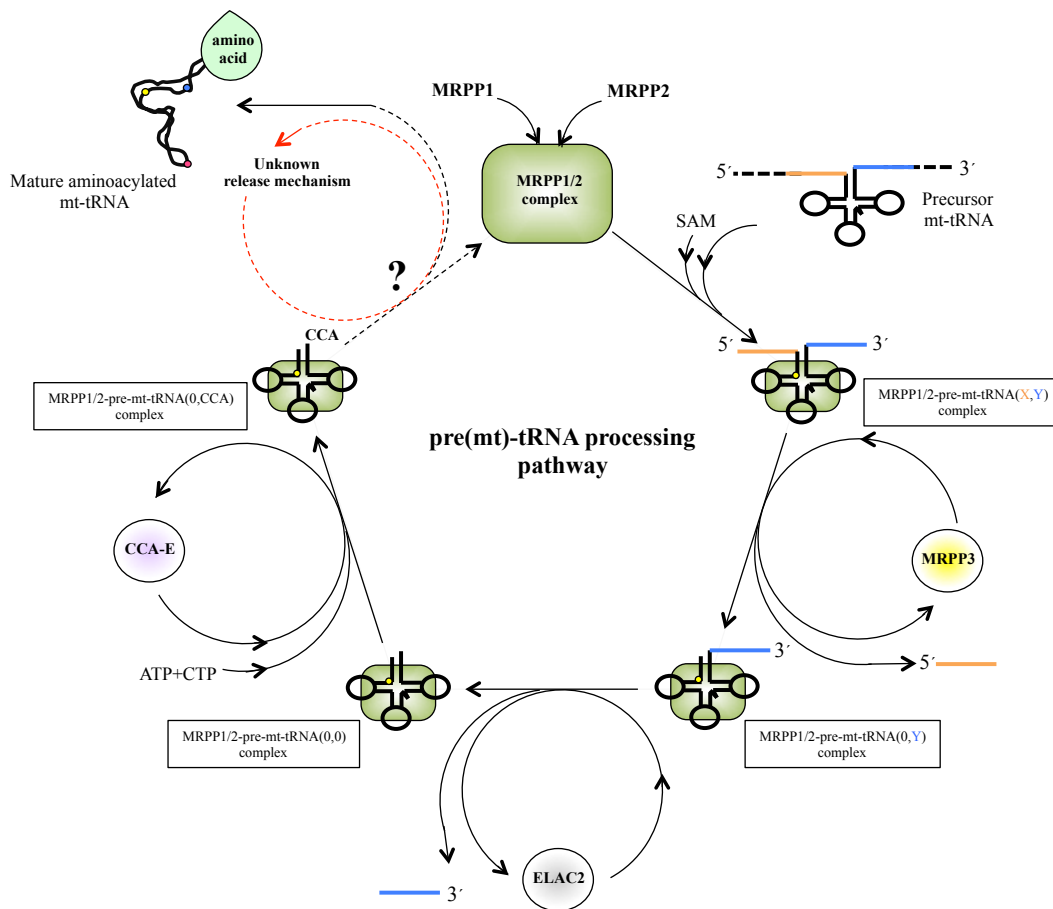


Figure 13: An illustration depicting MRPP1/2 complex as a platform for maturation of mt-tRNAs hosting them through 5'-processing with MRPP3, 3'-processing with ELAC2 and 3'-CCA addition with CCA-E. The exact mechanism by which MRPP1/2 releases tRNA is so far unknown and requires the evaluation of tRNA modifying enzymes, aminoacyltransferases and perhaps even the components of mitoribosome in this regard.

After the 3'-processing, MRPP1/2 continues to bind the 5'-, 3'-processed tRNA substrate and allows CCA-E to perform the 3'-CCA addition that is critical for aminoacylation of mature tRNAs [211]. Although CCA-E can perform the 3'-CCA addition on its own, it displays an error-prone behavior of addition of more nucleotides in non-physiological conditions. This behavior is completely abolished by the presence of the MRPP1/2 complex, wherein the proper functioning of CCA-E is extended across a broad range of physiological and non-physiological conditions. Thus, the relevance of the MRPP1/2 sub-complex in downstream tRNA maturation processes are validated using a combination of *in vitro* activity assays and complexing studies between various molecular players. At what stage does the sub-complex releases the mature tRNA remains an open-ended question that requires further evaluation in the context of processes such as mt-tRNA modifications and aminoacylation.

5.3 PAPER III

Clinically relevant mutations in the acceptor stem of mitochondrial tRNA(Lys) strongly affects RNase P activity

Mitochondrial diseases constitute a major class of commonly occurring human diseases requiring both clinical and biochemical characterization [128, 212]. The molecular causes of such pathologies are either due to mutations in the nuclear genes important for mt-function or due to mutations in the mtDNA, or both, giving rise to mutant proteins or mutant mt-tRNAs causing functional defects. Many mutations are localized within mt-tRNA genes in the mtDNA, with over 200 such mutations implicated in various mitochondrial diseases (Figure 14) [212].

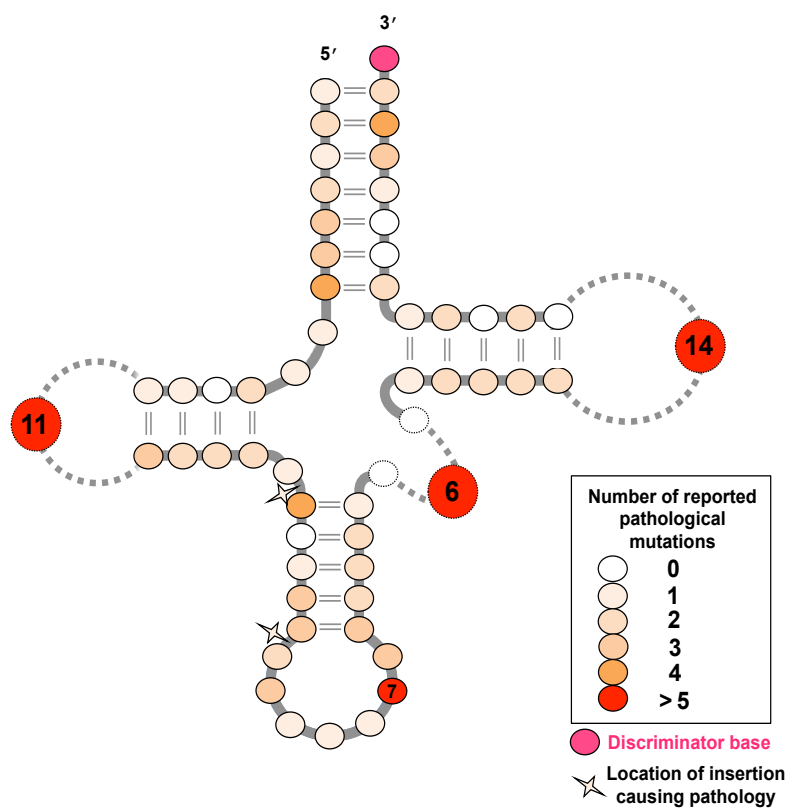


Figure 14: An illustration depicting the prevalence of reported pathological mutations at various locations of mt-tRNAs (data compiled from MAMIT database).

The 5'-tRNA cleavage by human mt-RNase P is the first step towards the release and maturation of individual RNA species from the polycistronic transcript. At least 28 mutations in the tRNA acceptor stem have been linked to various mitochondrial diseases such as MELAS and MERRF. We aimed to explore the consequences of mutations in the acceptor stem of the mt-tRNA^{Lys} in terms of RNase P processivity. The choice of tRNA lysine for this

study was motivated by the high number of reported pathologies caused by mutations in its acceptor stem.

We observed an overall reduced human mt-RNase P activity in mt-tRNA^{Lys} harboring pathological mutations in its acceptor stem. This observation is crucial not just from disease perspective, but also highlights the role of an intact acceptor stem as an identity element for stable mt-RNase P binding and catalysis. Our quest to identify the molecular cause of reduced RNase P processivity indicated that the nuclease activity of MRPP3 is affected, while the MRPP1/2 sub-complex can stably form a complex with the mt-tRNA^{Lys} irrespective of the presence or absence of the mutation. It remains to be established if the reduced 5'-processivity is because MRPP3 is unable to form a complex with MRPP1/2-pre-tRNA or because its active site reorganization is affected.

6 FUTURE PERSPECTIVES

This thesis summarizes the findings on the processing of human mt-tRNAs, crucial in health and diseases adding significant knowledge to the field of PRORP's.

In **PAPER I**, we have solved the crystal structure of MRPP3 showing a peculiarly distorted active site. We suggest that the activity of human mt-RNase P requires complex reorganization of active site of MRPP3 brought about by MRPP1/2 bound to pre-tRNA substrate. The understanding of the precise mechanism by which such a reorganization would occur requires the structural elucidation of human mt-RNase P – tRNA complex.

In **PAPER II**, we have unraveled the wider role of MRPP1/2 complex in tRNA maturation pathway. The obvious next step would be to investigate the mechanism of release of mature tRNA from MRPP1/2 sub-complex. In this regard, the role of tRNA modifying enzymes and aminoacyltransferases must be closely inspected. Perhaps like with MRPP3, ELAC2 and CCA-E, MRPP1/2 might allow tRNA modifying enzymes and aminoacyltransferases to perform their function while being bound to the tRNA substrate.

The tRNA^{Val} gene is present in between the genes encoding 12S and 16S rRNAs in the human mtDNA. The human mitoribosome structure revealed that tRNA^{Val} is an integral component of mitochondrial large subunit (mtLSU) [34]. The maturation of pre-tRNA^{Val} follows standard RNase P and RNase Z processing [213]. In this regard, it is noteworthy that in **PAPER II**, we observed strong enhancement in 3'-processing of tRNA^{Val} by MRPP1/2-ELAC2 in relation to ELAC2 alone. It remains to be established as to how the MRPP1/2 mediated processing of mt-tRNA^{Val} is co-ordinated with the mitoribosome biogenesis. Such a study would help us decipher the links between mtDNA mutations in tRNA^{Val} gene and implicated pathologies [214-218] perhaps resulting due to improper mitoribosome assembly.

The methylation of mt-tRNAs brought about by MRPP1/2 complex was shown to not be a pre-requisite for RNase P activity [64]. Given the recent knowledge of MRPP1/2 as a tRNA-maturation platform [99], one could evaluate if the presence or absence of methylation of mt-tRNAs would have an effect on downstream processing mechanisms.

In **PAPER II**, we observed that the tRNA^{Leu(UUR)} and tRNA^{Leu(CUN)} showed a moderate to no significant difference in MRPP1/2-led enhancement of ELAC2 processing. The ELAC2 protein was shown to associate with PTCD1 [65] that negatively regulates leucine tRNAs (both tRNA^{Leu(UUR)} and tRNA^{Leu(CUN)}) [113]. In this regard, the 3'-processing mechanism of leucine tRNAs in mitochondria must be closely inspected to evaluate if PTCD1 may act in association with ELAC2 or as an alternate to ELAC2 as speculated [65]. This evaluation

would thus require a careful and intense investigation of interplay between MRPP1/2 subcomplex, ELAC2, PTC1 and mt-leucine tRNAs.

The mtDNA mutations cause several life-threatening mitochondrial diseases and have severe impact on human health due to the lack of effective treatments. Several procedures are being developed to alleviate the effects of mutant mtDNA expression by employing strategies such as the expression of mt genes in the nucleus and the transfer of healthy mitochondria between cells [219]. Moreover, certain mutations in the nuclear encoded mt-RNase P proteins can cause processing defects leading to accumulation of precursor transcripts. The careful biochemical and structural characterization of biomolecules participating in cellular and organellar metabolic pathways are crucial for the development of such novel strategies.

7 POPULAR SCIENCE SUMMARY

Our body is made up of billions of cells and each cell has a nucleus (like a city council) and many small factories called ‘mitochondria’ where energy is produced. Each of these factories employs 22 tRNAs and several proteins (biological classes of molecules) to ultimately produce energy. It is a complex phenomena and involves multiple biological reactions. One such set of reactions involves the preparation of tRNAs by certain proteins whose journey starts from the nucleus.

These proteins prepare/process the tRNAs inside the mitochondria in 3 major steps (Figure 15) involving proteins like MRPP1, MRPP2, MRPP3, ELAC2 and CCA-E. In the first step, the proteins MRPP1 and MRPP2 (MRPP1/2) hold the tRNA and MRPP3 performs the first cut. Then, another protein called ELAC2 performs the second cut while MRPP1/2 continues to hold onto tRNA. In the third step, a special extension is added by another protein called CCA-E, this while MRPP1/2 is still present with the tRNA. After these three steps, other proteins act on the tRNA to make it ready to participate in other pathways towards energy production. We still do not know at what stage the tRNA leaves the MRPP1/2.

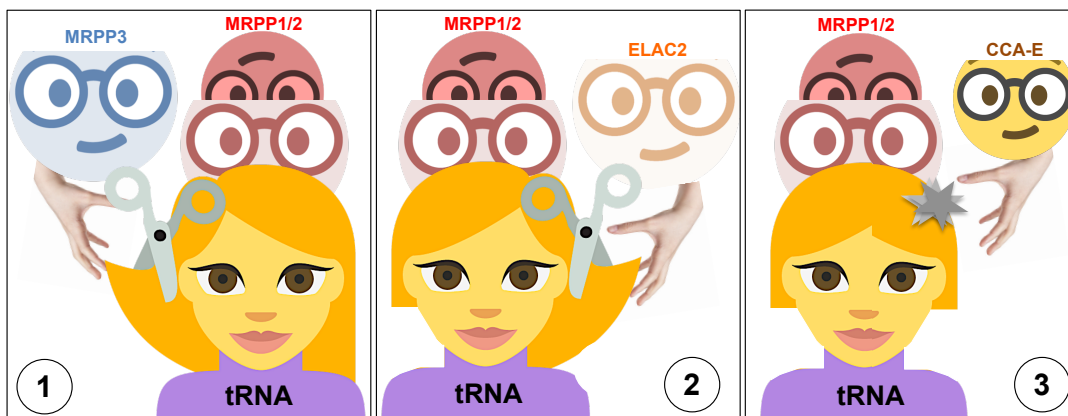


Figure 15: The major steps of tRNA processing explained using simple analogy. Step 1 refers to 5'-processing by MRPP1/2/3, step 2 refers to 3'-processing by MRPP1/2/ELAC2 and step 3 refers to 3'-CCA addition by MRPP1/2/CCA-E.

Sometimes, when there are problems in tRNAs or proteins, they cannot perform these activities and this can cause human diseases. We have explored how the problems in tRNAs can affect the first step. Thus, this thesis has contributed to our knowledge of the grooming processes of tRNAs in human mitochondria.

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Home Institution



Affiliations



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10 GLOSSARY

The following abbreviations were compiled from the MAMIT database [146].

ADPD (Alzheimer's **D**isease and **P**arkinson **D**isease)

AISA (**A**cquired **I**diopathic **S**ideroblastic **A**nemia)

AMDF (**A**taxia, **M**ental deterioration, **D**eafness)

AR (**A**xenfeld-**R**ieger anomaly)

CD (**C**ox **D**eficiency)

CIPO (**C**hronic **I**ntestinal **P**seudo**O**bstruction with myopathy)

CPEO (**C**hronic **P**rogressive **E**xternal **O**phthalmoplegia)

D (**D**iabete)

DCM (**D**ilated **C**ardio**M**yopathy)

DEAF (Maternally inherited **D**EAFness/Aminoglycoside-induced **D**EAFness)

DEMCHO (**D**EMentia, **C**HOrea)

DM (**D**iabetes **M**ellitus)

D MDF (**D**iabetes **M**ellitus, **D**eafness)

ECM (**E**ncephalo**C**ardio**M**yopathy)

EI (**E**xercise **I**ntolerance)

EM (**E**ncephalo**M**yopathy)

FICP (**F**atal **I**nfantile **C**ardiomyopathy **P**lus a MELAS-associated cardiomyopathy)

H (**H**emiplegia)

HCM (**H**ypertrophic **C**ardio**M**yopathy)

HHH (**H**ypertension, **H**ypercholesterolemia, **H**ypomagnesemia)

IH (**I**neffective **H**ematopoiesis)

KS (**K**earns-Sayre syndrome)

L (**L**eukoencephalopathy)

LA (**L**actic Acidose)

LHON (**L**eber **H**ereditary **O**ptic **N**euro**P**athy)

LIMM (**L**ethal **I**nfantile **M**itochondrial **M**yopathy)

LS (**L**eigh Syndrome)

LW (**L**imb **W**eakness)

MELAS (**M**itochondrial **E**ncephalomyopathy, **L**actic **A**cidosis and **S**troke-like episodes)

MERRF (**M**yo**C**lonic **E**pilepsy with **R**agged **R**ed **F**ibers)

MHCM (**M**aternally inherited **H**ypertrophic **C**ardio**M**yopathy)

MICM (**M**aternally **I**nherited **C**ardio**M**yopathy)

MILS (**M**aternally **I**nherited **L**eigh Syndrome)

MM (**M**itochondrial **M**yopathy)

MMC (**M**aternal **M**yopathy and **C**ardiomyopathy)

MNGIE (**M**itochondrial **N**euro**G**astro**I**ntestinal **E**ncephalomyopathy)

MOF (**M**ulti**O**rgan **F**ailure)

MS (**M**yo**C**lonic **S**eizures)

MTMD (**M**ultiple **T**umor in **M**itochondrial **D**iabetes)

NGI (**N**euro**G**astro**I**ntestinal syndrome)

O (**O**phthalmoplegia)

PEM (**P**rogressive **E**ncephalo**M**yopathy)

PEO (**P**rogressive **E**xternal **O**phthalmoplegia)

PR (**P**igmentary **R**etinopathy)

PRF (**P**rogressive **R**espiratory **F**ailure)

RRF (**R**agged **R**ed **F**ibers)

SM (**S**keletal **M**yopathy)

SNHL (**S**ensori**N**eural **H**earing **L**oss)

TNS (**T**ubulointerstitial **N**ephritis and **S**troke)