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Review

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Starting the engine of the powerhouse: mitochondrial transcription and beyond

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Abstract: Mitochondria are central hubs for cellular metabolism, coordinating a variety of metabolic reactions crucial for human health. Mitochondria provide most of the cellular energy via their oxidative phosphorylation (OXPHOS) system, which requires the coordinated expression of genes encoded by both the nuclear (nDNA) and mitochondrial genomes (mtDNA). Transcription of mtDNA is not only essential for the biogenesis of the OXPHOS system, but also generates RNA primers necessary to initiate mtDNA replication. Like the prokaryotic system, mitochondria have no membrane-based compartmentalization to separate the different steps of mtDNA maintenance and expression and depend entirely on nDNA-encoded factors imported into the organelle. Our understanding of mitochondrial transcription in mammalian cells has largely progressed, but the mechanisms regulating mtDNA gene expression are still poorly understood despite their profound importance for human disease. Here, we review mechanisms of mitochondrial gene expression with a focus on the recent findings in the field of mammalian mtDNA transcription and disease phenotypes caused by defects in proteins involved in this process.

Keywords: inhibitor of mitochondrial transcription; mitochondria; mitochondrial disease; mitochondrial gene expression; mitochondrial transcription; PPR proteins.

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Introduction

Mitochondria are double-membrane enclosed organelles within eukaryotic cells and vital for mammalian life as they fulfill multiple functions including the conversion of energy from nutrients into the cellular energy currency, ATP, via the oxidative phosphorylation (OXPHOS) system. The biogenesis of the OXPHOS system is under dual genetic control as it requires the concerted expression of two cellular genomes, the nuclear (nDNA) and the mitochondrial genomes (mtDNA), to fulfill the bioenergetic demands of the cell.

Mutations in either mtDNA or nDNA genes encoding mitochondrial proteins cause a broad range of clinically heterogeneous mitochondrial diseases and among these, defects in mtDNA expression are major contributors to human disease (Suomalainen and Battersby 2018; Turnbull and Rustin 2016). Importantly, mutations may be present in all mtDNA molecules of a cell (homoplasmy) or only a subset (heteroplasmy), and the disease severity largely depends on the percentage of mutant mtDNA molecules (Sciaccio et al. 1994; Taylor and Turnbull 2005). Furthermore, mitochondrial dysfunction has been associated with common age-related diseases like Parkinson's disease, cardiovascular disease, obesity, type 2 diabetes, cancer, and the aging process itself (Larsson 2010; Nunnari and Suomalainen 2012). However, contrary to primary mitochondrial diseases, the causal contribution of mitochondrial dysfunction to many of these complex disorders remains unknown.

Mammalian mtDNA is a compact circular double-stranded genome of ~16.6 kb that encodes 11 messenger RNAs (mt-mRNAs), which are translated into 13 core subunits of the OXPHOS complexes, as well as 22 transfer RNAs (mt-tRNAs) and two ribosomal RNAs (mt-rRNAs) required to translate mtDNA-encoded genes within mitochondria (Figure 1A). There is only one large non-coding region (NCR) that contains essential regulatory elements including the heavy- and light-strand promoters for transcription (HSP and LSP, respectively) as well as the heavy (H)-strand origin of replication (O_H) (Figure 1B). Important

exact function is unknown (Cantatore and Attardi 1980; Chang et al. 1985; Chang and Clayton 1985; Falkenberg et al. 2007; Pham et al. 2006) (Figure 1B). Most human mtDNA replication events initiated at O_H are aborted at the TAS and lead to the formation of a triple-stranded structure called the displacement (D) loop (Bogehagen and Clayton 1978; Falkenberg 2018; Nicholls and Minczuk 2014) (Figure 1B). The aborted H-strand product is denoted 7S DNA and remains bound to the parental L strand, thereby displacing the H strand. Current models of mitochondrial replication, the details of primer formation and the mitochondrial replication machinery are discussed in detail elsewhere (Falkenberg and Gustafsson 2020).

Mammalian mt-mRNAs do not have introns, Shine Dalgarno sequences, conventional 5' and 3' UTRs, 5' 7-methylguanosine caps or base modifications. With the exception of *mt-Nd6*, all mt-mRNAs are polyadenylated in their 3' end and, for several of the transcripts, this process is essential as it completes the termination codon (Rackham et al. 2012; Ruzzenente et al. 2012). The relative abundance and half-lives of mature mt-RNAs varies considerably, with mt-rRNAs being the most abundant transcripts (Chujo et al. 2012; Mennuni et al. 2022; Mercer et al. 2011), underlining the importance of post-transcriptional processes in mt-RNA stability (Rorbach and Minczuk, 2012). Assembly of the mitochondrial ribosome (mitoribosome) initiates close to the mitochondrial genome as mt-rRNAs are formed by transcription (Bogehagen et al. 2014; Rackham et al. 2016). Mitoribosomes are anchored to the inner mitochondrial membrane (IMM) (Liu and Spemulli 2000) and it has been suggested that mtDNA-encoded proteins are inserted co-translationally into the IMM to assemble in the OXPHOS complexes (Richter-Dennerlein et al. 2015). Like the prokaryotic system, mitochondria have no membrane-based compartmentalization to separate the different steps of mtDNA maintenance and expression which include replication, transcription, RNA processing/stability, translation, and assembly. All of these processes require factors that are encoded in the nDNA, translated in the cytosol, and imported into the mitochondria by a complex ATP-dependent import machinery (Wiedemann and Pfanner 2017), usually via a mitochondrial targeting sequence (MTS) that is cleaved after import to produce a mature, functional protein. In yeast it is estimated that one quarter of the mitochondrial proteome (mitoproteome) is dedicated to maintain and express mtDNA (Gonczarowska-Jorge et al. 2017). The factors required for mtDNA expression are unique and differ completely from those acting in the nucleus as the mitochondrial gene expression system has adopted features from viral, eukaryotic nuclear, and bacterial gene expression machineries over the course of evolution. The core

machinery for mtDNA transcription and several of the factors required for post-transcriptional processes are known, and have been well studied *in vitro*, but the mechanisms regulating mtDNA gene expression are still poorly understood despite their profound importance for human disease. Here, we review our current understanding of mitochondrial gene expression with a focus on mammalian mtDNA transcription and disease phenotypes caused by defects in proteins involved in this process.

Three steps forward – current models of mitochondrial transcription

Mammalian mtDNA transcription is mediated by an exclusively mitochondrial DNA-dependent RNA polymerase (POLRMT) and involves three stages: initiation, elongation, and termination (Gustafsson et al. 2016; Hillen et al. 2018). To initiate transcription at the promoters, POLRMT requires two transcription initiation factors, the mitochondrial transcription factor A (TFAM) and B2 (TFB2M). Together, these three factors form the mitochondrial core transcription machinery as they are necessary and sufficient to initiate transcription from mtDNA fragments containing HSP and LSP *in vitro* (Figure 2A) (Falkenberg et al. 2002; Gustafsson et al. 2016; Litonin et al. 2010). Classical studies from the Attardi group actually identified two potential initiation sites of mitochondrial H-strand transcription, later denoted HSP1 and HSP2, by mapping *in vitro* 'capped' mitochondrial transcripts or nascent RNA molecules to mtDNA (Cantatore and Attardi 1980; Montoya et al. 1982). The HSP2-initiated transcript was mapped to start upstream of the 5' end of the *12S* mt-rRNA gene, resulting in the synthesis of a polycistronic molecule comprising almost the entire H strand. A much shorter transcript initiated from HSP1 was reported to start ~25 bp upstream of the mt-tRNA phenylalanine gene (*mt-Tf*) and to span the *mt-Tf*, *12S* mt-rRNA, *16S* mt-rRNA, and mt-tRNA valine (*mt-Tv*) genes (Montoya et al. 1982) (Figure 1A). The differential regulation of the two HSP promoters was suggested to explain mechanistically the increased abundance of steady-state levels of mt-rRNAs compared to other mt-mRNAs encoded on the same strand (Christianson and Clayton 1988; Kruse et al. 1989; Daga et al. 1993; Shang and Clayton 1994). However, as subsequent studies have shown conflicting results, there is an ongoing debate about the existence and physiological relevance of HSP2 (Shokolenko and Alexeyev 2017; Lodeiro et al. 2010; Zollo and Sondheimer 2017). For instance, reports of

HSP2-specific transcripts in reconstituted *in vitro* transcription systems are inconsistent (Gustafsson et al. 2016; Litonin et al. 2010), the precise location of HSP2 mapped *in vivo* and *in vitro* differ, and HSP2 has been reported to be repressed by TFAM *in vitro* (Lodeiro et al. 2010; Zollo and Sondheimer 2017). While these inconsistencies have been explained by differences in the requirements for each HSP and suggested as a potential regulation mechanism of mitochondrial transcription (Zollo and Sondheimer 2017), HSP2 inhibition by TFAM questions whether HSP2 can be active under physiological conditions since TFAM is substantially more abundant than TFB2M and POLRMT (Harmel et al. 2013). This argues in favor of a single HSP corresponding to the HSP1 sequence and generating the full-length polycistronic H-strand transcript.

Two models of initiation complex formation have been proposed for mitochondrial transcription that differ in the sequence of interaction between the core transcription machinery and the exact role the transcription factors fulfill. The first model, called the sequential model, is supported by *in vitro* cross-linking data, and proposes that TFAM binding to the promoter bends mtDNA allowing POLRMT recruitment to form the pre-initiation complex (pre-IC) (Figure 2A). Subsequently, TFB2M binds to the pre-IC and melts the promoters enabling transcription initiation (Gustafsson et al. 2016; Morozov et al. 2014). The second model is based on equilibrium binding experiments and differs from the sequential model in two ways: first, POLRMT and TFB2M can interact with the promoter independently of TFAM and therefore the initiation complex is not necessarily formed in a sequential manner, and second, TFAM is required for promoter melting (Ramachandran et al. 2017).

After transcription is initiated, TFAM and TFB2M disengage from the initiation complex, resulting in transcription elongation. The transcription elongation factor (TEFM) binds POLRMT and interacts with mtDNA and the nascent mt-rRNA to form the processive elongation complex that transcribes the near-genome length polycistronic transcripts (Agaronyan et al. 2015; Jiang et al. 2019; Minczuk et al. 2011; Posse et al. 2015). Termination of mitochondrial transcription is not well understood but appears to be regulated in a strand-specific fashion, in contrast to mitochondrial transcription initiation. Termination of full-length LSP transcription is mediated by the only transcription termination factor identified to date: mitochondrial transcription termination factor 1 (MTERF1). MTERF1 binds the mt-tRNA leucine 1 (*mt-Tll*) sequence and was initially proposed to terminate HSP1-specific transcription downstream of the 16S mt-rRNA, (Fernandez-Silva et al. 1997; Gustafsson et al. 2016; Kruse et al. 1989)

explaining a key element of the two-HSP transcription model described above (Christianson and Clayton 1988; Camasamudram et al. 2003). However, loss of *Mterf1* in the mouse did not affect mt-rRNA and mt-mRNA levels or result in abnormal H-strand transcription. On the contrary, this knockout model evidenced that MTERF1 acts as a road-block terminating LSP transcription, preventing anti-sense mt-rRNA synthesis (Terzioglu et al. 2013). In line with this, MTERF1 was shown to block L-strand transcription *in vitro*, while H-strand transcription was only partially terminated (Asin-Cayuela et al. 2005; Terzioglu et al. 2013). Much less is known about termination of H-strand transcription. Tremendous efforts have been directed at identifying conserved sequence motifs within the NCR corresponding to a termination site and/or proteins serving as termination factors in various experimental systems such as human cell-lines, bovine mitochondria, mouse and rat (Camasamudram et al. 2003; Freyer et al. 2010; Jemt et al. 2015; Madsen et al. 1993; Sbisà et al. 1997; Selwood et al. 2001). Although different termination sites have been reported throughout the NCR, most H-strand termination events occur at the 3' end of the D-loop in the TAS region (Freyer et al. 2010; Jemt et al. 2015; Madsen et al. 1993; Sbisà et al. 1997; Selwood et al. 2001). Of note, termination of H-strand transcription might be dynamically regulated in response to mitochondrial stress as, when mtDNA synthesis or mitochondrial translation was inhibited, transcription passed the H-strand termination site resulting in the synthesis of an antisense D-loop transcript (Jemt et al. 2015; Selwood et al. 2001).

In addition to termination after full-length transcription from LSP and HSP, premature transcription termination of the L strand is needed to generate the polyadenylated 7S RNA transcript and the mtDNA replication primer for O_H (Figure 1B) (for detail, see Falkenberg and Gustafsson 2020; Gustafsson et al. 2016). The 3' end of the 7S RNA transcript maps to the NCR regulatory element CSB1 that has a palindromic sequence motif very similar to the TAS. However, the TAS alone was insufficient to terminate transcription *in vitro* suggesting that secondary nucleic acid structures are not mediating transcription termination at TAS or CSB1 (Jemt et al. 2015). Therefore, currently unknown protein(s) binding to TAS or recognizing the common palindromic motifs likely serve as the factor(s) terminating H-strand-polycistronic transcription and premature L-strand transcription for 7S RNA formation. Indeed, a number of studies observed protein activities binding to the identified sequence elements (Jemt et al. 2015; Polosa et al. 2005), unfortunately none of them have been purified and identified yet. During transcription of the guanine-rich CSB2 element, the nascent RNA folds into a G-quadruplex hybrid structure with the

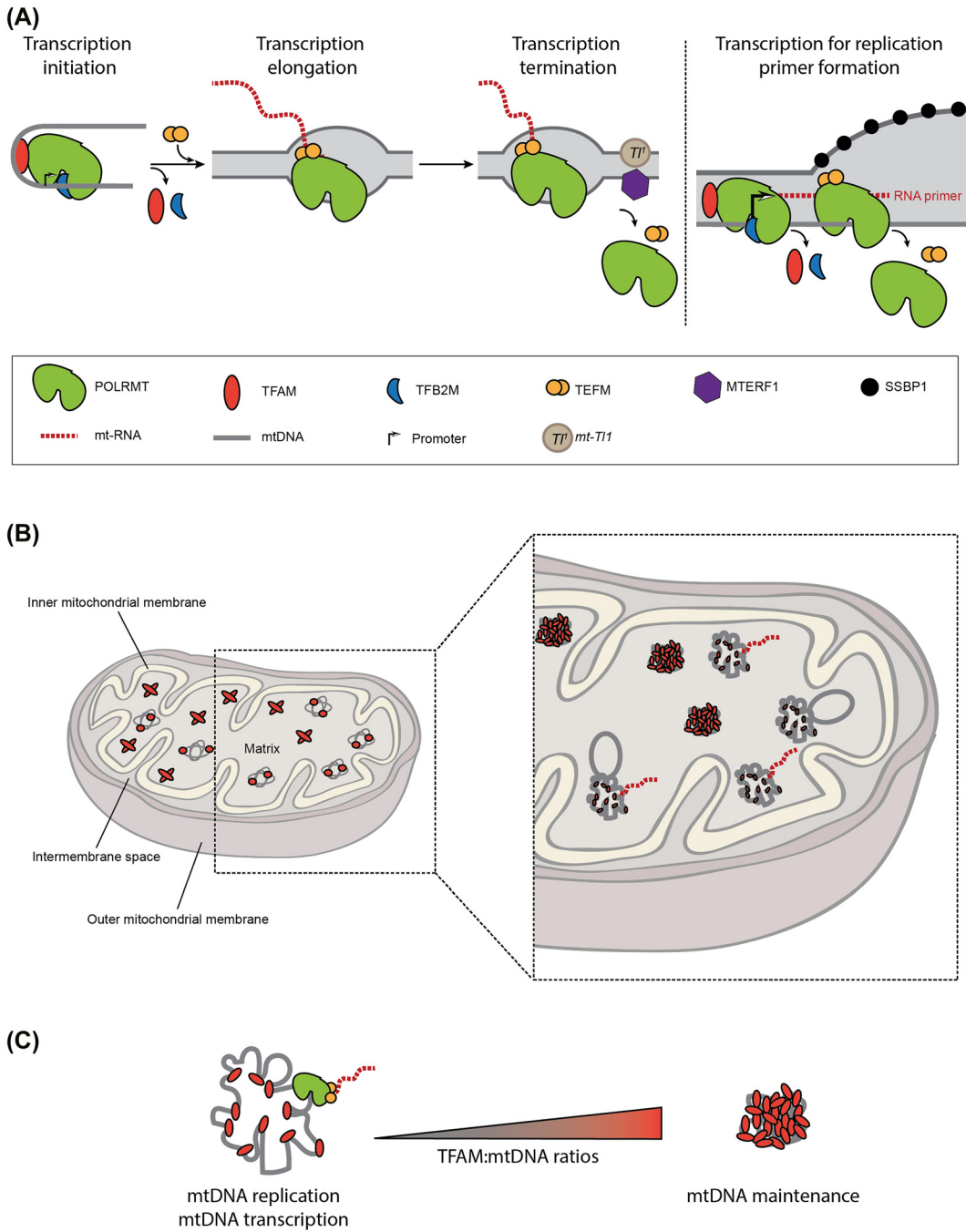


Figure 2: Model of mitochondrial transcription.

(A) The initiation complex formed by POLRMT, TFAM and TFB2M assembles in the promoter region. Then, TFAM and TFB2M disengage from the initiation complex and TEFM binds POLRMT to form the elongation complex. Transcription from LSP is terminated by MTERF1 that binds the *mt-T1* sequence. Transcription from HSP terminates in the TAS. (B) Mitochondrial DNA is organized into mitochondrial nucleoids containing one single copy of mtDNA condensed by TFAM. Each cell contains hundreds of mitochondrial nucleoids that can be subdivided based on their activity: larger active nucleoids engage in mtDNA transcription and/or replication, while more compact inactive nucleoids may represent a storage form protecting mtDNA from damage. Both populations reside side-by-side within the matrix. (C) Nucleoid compaction regulates mtDNA gene expression. Increased TFAM:mtDNA ratios lead to a higher degree of nucleoid compaction, abolishing mtDNA transcription and replication. Local variations of TFAM:mtDNA ratios might occur through an increase in TFAM expression, increased TFAM degradation by e.g. LONP1 and changes in mtDNA copy number.

non-template DNA, forming a stable R loop (Posse et al. 2019; Wanrooij et al. 2012; Wong and Clayton 1985; Xu and Clayton 1995). G-quadruplex formation at CSB2 coincides with the transcription of a poly-uracil sequence, leading to the dissociation of elongating POLRMT and premature transcription termination downstream of CSB2 (Pham et al. 2006). The R loop is then further processed by RNase H1 to yield 3' ends to serve as a primer (Posse et al. 2019). *In vitro* studies reported that human TEFM enables POLRMT to bypass the G-quadruplex structure in the CSB2 (Agaronyan et al. 2015; Posse et al. 2015), reducing premature transcription termination. Thus, TEFM was proposed as a key regulator of the switch between mtDNA replication and transcription (Agaronyan et al. 2015). However, loss of *Tefm* in the mouse heart leads to an accumulation of very short transcripts that terminate far before the proposed switch region at CSB2 and a drastic drop in mtDNA replication (Jiang et al. 2019), challenging the proposed model.

Thus, the hunt for the elusive mechanisms of mitochondrial termination continues and will be fundamental to understanding the regulation of mtDNA replication and gene expression.

More than two to tango – the core transcription machinery in mammalian mitochondria

Mitochondrial RNA polymerase

The catalytic component of mitochondrial transcription in *Saccharomyces cerevisiae* consists of a single nuclear-encoded polypeptide of ~145 kDa (Rpo41), first identified more than 35 years ago (Kelly and Lehman 1986), and the gene encoding the human homolog (MT-RPOL, mtRNAP or POLRMT) was discovered a decade later (Tiranti et al. 1997). POLRMT operates exclusively in mitochondria where it is the sole mammalian mitochondrial RNA polymerase, which is underscored by the embryonic lethality of homozygous disruption of *Polrmt* in mice (Kühl et al. 2016, 2014). POLRMT also generates the RNA primers required for mtDNA replication (Figure 1B) (Falkenberg 2018; Kühl et al. 2016), placing POLRMT at the heart of gene expression and genome maintenance in mitochondria. The catalytic part of POLRMT, consisting of an N-terminal domain (NTD) and a C-terminal domain (CTD), is homologous to T3/T7-bacteriophage RNA polymerase (T7-RNAP) (Masters et al. 1987), and is highly conserved across species (Ringel et al. 2011) suggesting that the switch from bacterial to phage RNA polymerase occurred early in the eukaryotic tree

(Shutt and Gray 2006). The mechanisms of substrate selection and binding as well as catalytic nucleotide incorporation seem to be conserved (Ringel et al. 2011; Schwinghammer et al. 2013); however, the mechanisms of promoter recognition, transcription initiation, transition to elongation, and transcription termination are very different from T7-RNAP because POLRMT depends on additional factors to perform these processes (Gustafsson et al. 2016; Hillen et al. 2018; Morozov et al. 2014; Schwinghammer et al. 2013; Shi et al. 2012; Sologub et al. 2009). Thus, POLRMT – similarly to the nuclear RNA polymerase II (Schier and Taatjes 2020) – depends on other factors to regulate mtDNA expression including the transcription factors TFAM, TFB2M, TEFM and MTERF1. The crystal structure of the elongation state of POLRMT bound to TEFM has been resolved (Hillen et al. 2017b) and, in this conformation, POLRMT differs considerably from the T7-RNAP elongating state (Hillen et al. 2018; Schwinghammer et al. 2013), underscoring core differences in the mechanism of POLRMT regulation. Other factors have been proposed to interact with POLRMT, mainly based on co-immunoprecipitation experiments: The mitochondrial topoisomerase I (TOP1MT) was identified as an exclusive mitochondrial protein acting as a negative regulator of transcription through interaction with POLRMT (Sobek et al. 2013). A proximity-biotinylation assay found POLRMT interacting with the mitochondrial genome maintenance exonuclease 1 (MGME1), proposed to play a role in the regulation of replication and transcription termination at the end of the NCR (Matic et al. 2018). The leucine-rich pentatricopeptide repeat-containing protein (LRPPRC) was suggested to directly interact with POLRMT and to stimulate mtDNA transcription (Liu et al. 2011), but a careful analysis of a mouse model with tagged LRPPRC could not confirm a direct role of LRPPRC in mitochondrial transcription and *de novo* transcription is not affected by knockout or overexpression of *Lrpprc* (Harmel et al. 2013; Ruzzenente et al. 2012). Studies in cell culture proposed that POLRMT interacts directly with mitochondrial dimethyladenosine transferase 1 (TFB1M) in the 28S mitochondrial ribosomes to augment its 12S rRNA methyltransferase activity, and that together they could provide a checkpoint for proper 28S and 55S mitochondrial ribosome assembly (Surovtseva and Shadel 2013). POLRMT might also bind a free pool of the mitochondrial ribosomal protein 12 (MRPL12), which was reported to act as an allosteric activator of transcription facilitating the transition from initiation to elongation (Nouws et al. 2016; Surovtseva and Shadel 2013; Wang et al. 2007). However, MRPL12 did not stimulate mitochondrial transcription in a reconstituted *in vitro* system (Litonin et al. 2010). Furthermore, this proposed interaction was not confirmed *in vivo* as POLRMT is found in

mtDNA-containing fractions in density gradient studies of mouse mitochondrial extracts, whereas MRPL12 is found in mtDNA-free fractions together with other ribosomal proteins (Kühl et al. 2016). During early mammalian development, the nuclear transcription factor TEAD4 was suggested to interact with POLRMT to regulate energy metabolism (Kumar et al. 2018), and interestingly, analysis of stress-induced changes of the human mitochondrial proteome reports the association of mitochondrial transcription rescue factor 1 (MTRES1) with POLRMT (Kotrys et al. 2019). MTRES1 was found to be a mtRNA-binding protein and its silencing caused a decrease in the LSP-proximal transcript 7S RNA despite normal POLRMT and TFAM levels. Moreover, MTRES1 was also shown to associate with the large subunit of the mitochondrial ribosome and to influence mitochondrial translation (Gopalakrishna et al. 2019). All these reported interactors highlight the central role of POLRMT beyond transcription including mtDNA replication and post-transcriptional mt-RNA regulation. However, the underlying mechanisms of these reported interactions are not fully understood and there might still be POLRMT-interacting proteins to be identified.

In addition to its bacteriophage-like core, POLRMT contains a unique N-terminal extension (NTE) of unknown *in vivo* function that varies across species and for which there is only limited high-resolution structural data (Ringel et al. 2011). The NTE of POLRMT may be involved in protein-protein contacts, potentially serving as a platform to recruit factors involved in co-transcriptional processes. In *S. cerevisiae*, the interaction of Rpo41 NTE with mitochondrial transcription factor 2 (Mtf2) might spatially and functionally link the core transcription complex to the RNA processing machinery and mitoribosomes (Rodeheffer et al. 2001). Mutations in Rpo41 that prevent the interaction with Mtf2 severely reduce translation without compromising mt-mRNA synthesis and accumulation, demonstrating the physiological importance of proper mt-mRNA channeling to smoothly couple transcription with translation. Importantly, there is no homolog of Mtf2 identified yet in humans. Recent *in vitro* data suggest that the NTE has a decisive role in promoter recognition and initiation of transcription (Hillen et al. 2018). Deletion of the majority of the NTE results in a polymerase that is catalytically active *in vitro* but unable to initiate promoter-directed transcription (Posse et al. 2014). It has been suggested that a deletion of a part of the NTE of *S. cerevisiae* Rpo41 results in decreased mtDNA stability (Kruszewski and Golik 2016). Interestingly, this mutation has no impact on mitochondrial transcription initiation *in vivo*, and the expression of the NTE *in trans* partially suppresses the mtDNA stability

defect. In mammals, the NTE also contains a structural element (tether helix) shown to interact with TFAM to recruit POLRMT to the promoters *in vitro* (Hillen et al. 2018).

Sequence analysis has identified that POLRMT contains a unique helical domain, the so-called PPR-domain, which is located in between the NTE and the NTD and consists of nine alpha-helices of which at least four comprise two pentatricopeptide repeat (PPR) motifs of unknown function (see Box 1 for further information on PPR proteins in mitochondrial gene expression) (Ringel et al. 2011). PPR proteins usually contain multiple tandem repeats of PPR motifs. In this sense POLRMT is atypical since it contains only a few PPR motifs gathered in a small region. However, despite the small number of PPR motifs in POLRMT compared to other PPR proteins, their conservation through evolution from yeasts to humans supports their functional importance (Kühl et al. 2011; Lipinski et al. 2011; Ringel et al. 2011). Crystal structure studies of human POLRMT show that its PPR-domain interacts tightly with the catalytic part of POLRMT and that the PPR-motifs are located adjacent to the exit point of newly synthesized RNA (Hillen et al. 2018; Ringel et al. 2011; Schwinghammer et al. 2013). Thus, one possibility is that this PPR domain could bind nascent mitochondrial transcripts to stabilize or channel them during synthesis. Of note, POLRMT's PPR motifs have also been proposed to mediate protein-protein interactions like tetratricopeptide repeat (TPR) domains. A patient was recently identified with a point mutation in the PPR domain of POLRMT (Table 1), underscoring the importance of understanding the function of this domain.

Mitochondrial transcription factor A

The first transcription factor identified in human mitochondria was the mitochondrial transcription factor A (TFAM or mtTFA) (Fisher and Clayton 1988) which is also the main regulator of mtDNA copy number (Ekstrand et al. 2004; Kanki et al. 2004; Larsson et al. 1998; Matsushima et al. 2003). In addition to its role in mtDNA transcription initiation (Falkenberg et al. 2002; Shi et al. 2012), TFAM serves as an essential regulator of mitochondrial function by packaging mtDNA into DNA-protein complexes called mitochondrial nucleoids (Figure 2B). The mitochondrial network within each cell contains hundreds of nucleoids and each nucleoid is mainly composed of a single mtDNA molecule and has a diameter of ~100 nm as determined by super-resolution microscopy (Brown et al. 2011; Kukat et al. 2011). TFAM is the major protein constituent of the mammalian nucleoid, present in about 1000 molecules

Table 1: Clinical and cellular phenotypes associated with novel pathogenic variants in the mitochondrial core transcription machinery.

ID	Mutant protein variant (allele 1 // allele 2)	Age at onset/clinical course	Clinical phenotype	Cellular phenotype	In vitro transcription	References
POLRMT P1	D870N//P566S/ S1193F recessive	1 y/alive 16 y	Proximal tubulopathy/renal Fanconi syndrome; mild developmental delay and hypotonia; short stature, chronic kidney disease	Mild CI/CIII respiratory chain defect in fibroblasts; CI deficiency in skeletal muscle; no mtDNA deletions or depletion; decreased transcript levels	---	Oláhova et al. (2021)
POLRMT P2	H250D//Δ742-7 recessive	Birth/alive 19 y	Intellectual disability; high frequency hearing loss; developmental delay, mild hypotonia, bilateral ptosis	Mild CI/CIII respiratory chain defect in fibroblasts and skeletal muscle; no mtDNA deletions or depletion; decreased transcript levels	---	Oláhova et al. (2021)
POLRMT P3	WT//Δ881-3 dominant	28 y/alive 57 y	Indolent PEO phenotype; bilateral ptosis	Mild CI/CIV defects in skeletal muscle; no mtDNA deletions or depletion; highly decreased transcript levels	---	Oláhova et al. (2021)
POLRMT P4	WT//S611F de novo dominant	10 m/alive 3 y	Global, mild developmental delay; hypotonia, language difficulties, short stature, medullary nephrocalcinosis; renal hypomagnesemia, elevated lactate	No data on RC; mtDNA and transcript analysis: n.d.	---	Oláhova et al. (2021)
POLRMT P5	F641L//F641L recessive	1 y/alive 10 y	Profound intellectual disability; microcephaly; severe global developmental delay, short stature, strabismus, no speech, generalized myopathy	No data on RC; mtDNA and transcript analysis: n.d.	---	Oláhova et al. (2021)
POLRMT P6	C925*//P810S recessive	Birth/alive 14 y	Bilateral club foot; dysmorphism; moderate intellectual disability; microcephaly; global developmental delay; short stature, no speech; focal epilepsy; hypotonia; unspecific organic aciduria, high lactate/pyruvate ratio	No mtDNA deletions or depletion; decreased transcript levels	---	Oláhova et al. (2021)
POLRMT P7	E149*//R1013C recessive	Early 40s/ alive 59 y	Predominant muscle weakness, muscle atrophy; normal RC and CoQ10 levels in skeletal muscle; elevated creatine kinase	mtDNA and transcript analysis: n.d.	---	Oláhova et al. (2021)
POLRMT P8	E149*//R1013C recessive	Early 40s/ alive 72 y	Proximal muscle weakness, right exotropia; mildly elevated creatine kinase	mtDNA and transcript analysis: n.d.	---	Oláhova et al. (2021)
TFAM P9	P178L//P178L recessive	Birth/died 2 m (liver failure)	Intrauterine growth restriction (IUGR); neonatal hypoglycaemia, hyperbilirubinemia, cholestasis and ascites; liver failure; normal echocardiogram, neurological exam and brain MRI	n.d.	n.d.	Stiles et al. (2016)

Table 1: (continued)

ID	Mutant protein variant (allele 1 // allele 2)	Age at onset/clinical course	Clinical phenotype	Cellular phenotype	<i>In vitro</i> transcription	References
TFAM	P10 P178L//P178L <i>recessive</i>	Birth/died 4 m (liver failure)	IUGR; at 2 month: hypoglycaemia, hypoalbuminaemia, increased liver enzymes, alkaline phosphatase and direct bilirubin, jaundice, ascites, coagulopathy; cirrhosis, normal cardiac and neurological exams	mtDNA depletion in liver and skeletal muscle; mitochondrial myopathy in skeletal muscle; mild respiratory chain defect, increased CS levels; TFAM depletion, decreased OCR	n.d.	Stiles et al. (2016)
TFAM	P11 R232C//R232C <i>recessive</i>	Young adulthood/alive 28 y	Amenorrhea; uterine hypoplasia, POI, abnormal sex hormone levels, delayed sexual maturity	n.d.	n.d.	Ullah et al. (2021)
TFAM	P12 R232C//R232C <i>recessive</i>	12 y/alive 29 y	Recurring epileptic seizures since young adulthood; severe intellectual disability with non-interactive and aggressive behaviour; delayed sexual maturity	n.d.	n.d.	Ullah et al. (2021)
TFAM	P13 R232C//R232C <i>recessive</i>	16 y/alive 22 y	Recurring epileptic seizures since young adulthood; amenorrhea; uterine hypoplasia, POI, abnormal sex hormone levels; delayed sexual maturity	Stable TFAM levels; moderate mtDNA copy number decrease, nucleoid clustering, decreased OCR	n.d.	Ullah et al. (2021)
TFAM	P14 R232C//R232C <i>recessive</i>	12 y/alive 20 y	Delayed puberty, primary amenorrhea; POI, abnormal sex hormones; intellectual disability, sensorineural hearing loss	n.d.	n.d.	Tucker et al. (2020); Ullah et al. (2021)
TFB2M	P15 H264Y//H264Y <i>recessive</i>	46 m/alive 8 y	Language delay; deficits in social interaction; severe ASD; delayed fine motor function; intellectual disability	Stable TFB2M levels; increased mitochondrial transcript levels; increased mitochondrial membrane potential, increased OCR	n.d.	Park et al. (2018)
TFB2M	P16 H264Y//H264Y <i>recessive</i>	Younger sibling of P15	Similar to P15	n.d.	n.d.	Park et al. (2018)

In vitro transcription rates of the POLRMT combinations detected in the patients are given as – (minor decrease), -- (intermediate decrease) and --- (severe decrease). ASD, Asperger spectrum disorder; CI, complex I; CIII, complex III; CoQ10, coenzyme Q10; CS, citrate synthase; MRI, magnetic resonance imaging; m, month; n.d., not determined; OCR, oxygen consumption rate; PEO, progressive external ophthalmoplegia; POI, primary ovarian insufficiency; RC, respiratory chain; y, years.

per mtDNA molecule or 1 TFAM protein molecule per 16–17 bp of mtDNA in mammalian cells (Alam et al. 2003; Kukat et al. 2011). However, a large number of other mitochondrial proteins are – at least transiently – present in the nucleoid, such as key components of the mitochondrial transcription, replication, and post-transcriptional processing machineries (Bogenhagen et al. 2014).

TFAM contains two high mobility group (HMG)-box domains, HMG-A and HMG-B, separated by a linker and followed by a short C-terminal tail essential for transcription activation (Dairaghi et al. 1995; Fisher and Clayton 1988; Kanki et al. 2004; Parisi and Clayton 1991). Each HMG box domain bends mtDNA 90° leading to a 180° U-turn when one molecule of TFAM binds to mtDNA (Ngo et al. 2011; Rubio-Cosials et al. 2011), which allows promoter-specific transcription activation by recruiting of POLRMT and TFB2M as described above (Hillen et al. 2017a). Specific binding of TFAM to the two mtDNA promoters occurs between bases –10 and –35 upstream of the transcription-start site (Fisher et al. 1987; Kanki et al. 2004). The *S. cerevisiae* counterpart of TFAM, the autonomously replicating sequence (ARS)-binding factor 2 (Abf2p), is also a key packaging factor of the mitochondrial genome (Friddle et al. 2004), but has no role in transcription initiation (Cho et al. 1998).

Importantly, TFAM also binds, unwinds, and bends DNA without sequence specificity which facilitates its packaging function. Binding of single TFAM molecules to random patches of mtDNA followed by a combination of cross-strand binding, loop formation, and cooperative binding of TFAM molecules at sites already occupied leads eventually to full mtDNA compaction (Farge et al. 2014; Kaufman et al. 2007; Kukat et al. 2015). This process can be achieved by the action of monomeric TFAM alone (Kukat et al. 2015). Importantly, fully compacted nucleoids are inaccessible to the replication and transcription machinery (Figure 2B) (Farge et al. 2014) and might represent a type of “storage unit” to potentially protect a subset of mtDNA molecules from mutations caused by mtDNA replication. In this respect, the TFAM:mtDNA ratio has emerged as an important regulator of mitochondrial gene expression, facilitating a switch of nucleoids from an “on” to an “off” state to regulate the number of mtDNA molecules engaged in gene expression (Brüser et al. 2021; Farge et al. 2014). Changes in TFAM and/or mtDNA levels thus affect nucleoid compaction and mitochondrial gene expression, an observation that has been made *in vitro*, in primary human cells, budding yeast and the mouse (Bonekamp et al. 2021; Brüser et al. 2021; Farge et al. 2014; Kucej et al. 2008; Zelenaya-Troitskaya et al. 1998). Thus, titration of TFAM:mtDNA levels appears to be a very fundamental mechanism of regulation of mitochondrial gene expression. Upon relaxation, individual nucleoids may then engage in mtDNA

replication or transcription, however, it was unclear if dedicated nucleoid subpopulations – potential storage, transcription or replication forms – exist. A recent study used multi-color STED super-resolution microscopy to determine the activity of individual nucleoids in primary human cells (Brüser et al. 2021). Indeed, they observed that a large fraction of nucleoids remain replication inactive over extended periods of time, pointing to a potential storage pool of mtDNA with a lower mutation load. Inactive nucleoids also showed a higher TFAM:mtDNA ratio. However, those nucleoids are not spatially separated from replication active nucleoids, in line with *in vitro* studies which observed different degrees of compacted nucleoids side by side (Kaufman et al. 2007). Although more nucleoids were observed to be engaged in transcription than replication, active nucleoids tend to be involved in both, contrasting the idea of nucleoids dedicated to a specific process (Brüser et al. 2021). Another study using immortalized mouse embryonic fibroblasts reported that the vast majority of nucleoids are transcribed in actively dividing cells (Silva Ramos et al. 2019), thus regulation of nucleoid compaction might be linked to the proliferative state of the cell.

The essential role of TFAM in regulation of mtDNA copy number and mitochondrial gene expression is highlighted by numerous *in vivo* models. Homozygous disruption of *Tfam* is embryonically lethal in mice, however, heterozygous TFAM knockouts can well tolerate the reduced TFAM levels and the concomitant ~50% decrease in mtDNA levels (Larsson et al. 1998). Moderate overexpression of *Tfam*, resulting in ~50% increase in mtDNA copy number, was even reported to be beneficial in mouse models presenting various types of pathology, e.g. myocardial infarction, amyotrophic lateral sclerosis, mitochondrial disease, transient forebrain ischemia, premature ageing and age-dependent memory loss (Filograna et al. 2019; Hayashi et al. 2008; Hokari et al. 2010; Ikeuchi et al. 2005; Jiang et al. 2019; Morimoto et al. 2012; Nishiyama et al. 2010). The beneficial effect was linked to the increased absolute levels of mtDNA, although the proportion of mutant mtDNA remains unchanged (Filograna et al. 2019; Jiang et al. 2019). However, it is critically important to modulate TFAM levels *in vivo* within a physiological range to avoid excessive nucleoid compaction, as supra-physiological *Tfam* overexpression may result in inhibition of mitochondrial gene expression (Bonekamp et al. 2021; Ylikallio et al. 2010). Further studies are necessary to gain insight into the regulation of TFAM levels, but +AAA Lon protease (LONP1) has emerged as an important regulator of TFAM levels *in vitro* and *in vivo* (Bonekamp et al. 2021; Lu et al. 2013; Matsushima et al. 2003).

Mitochondrial transcription factor B2

In the early 2000, initial attempts to reconstitute mammalian mitochondrial transcription *in vitro* with TFAM and POLRMT were unsuccessful suggesting that additional core transcription factors were missing. The mitochondrial dimethyladenosine transferase 1 (TFB1M or mtTFB1) and the mitochondrial transcription factor B2 (TFB2M or mtTFB2) were identified based on homology sequence database search using as reference the *Schizosaccharomyces pombe* mitochondrial transcription factor 1 (Mtf1) (Falkenberg et al. 2002). In *S. pombe* and *S. cerevisiae*, Mtf1 and Rpo41 are recruited to the different mitochondrial promoters to initiate mtDNA transcription (Fisher et al. 1989; Savkina et al. 2010). TFB1M and TFB2M have structural homology to bacterial rRNA methyltransferases but they have non-redundant functions in metazoa (Adán et al. 2008). *In vitro* studies suggested that both, TFB1M and TFB2M, interact directly with POLRMT to form a heterodimer (Falkenberg et al. 2002). However, mouse knockout studies showed that TFB1M is not essential for mitochondrial transcription, but methylates the 12S rRNA, a modification essential for the assembly of the mitoribosome (Metodieiev et al. 2009). Whether TFB2M retains methyl-transferase activity remains unknown, but it is now clear that TFB2M is a *bona fide* transcription initiation factor in mammalian mitochondria (Litonin et al. 2010). A conditional knockout of *Tfb2m* in mouse pancreatic beta-cells leads to loss of mt-RNAs (Nicholas et al. 2017) and, consistent with the importance of the mitochondrial transcription machinery for mtDNA replication, loss of TFB2M in human cybrid cells causes mtDNA depletion (Inatomi et al. 2022). *In vitro* studies have shown that TFB2M interacts with the N- and C-terminal domains (NTD and CTD) of POLRMT as well as with the promoter DNA and priming nucleotide (Morozov et al. 2015) and analysis of a conditional knockout mouse of *Polrmt* in heart showed that TFB2M is dependent on POLRMT for its stability (Kühl et al. 2016). TFB2M induces conformational changes in POLRMT which enables promoter opening and the correct localization of the non-template strand (Hillen et al. 2017a).

Mitochondrial transcription elongation factor

TEFM is the most recently described mammalian mitochondrial transcription factor. It was identified by homology analyses as a putative human mitochondrial Holliday Junction resolvase (Minczuk et al. 2011). Although this specific activity was not found in recombinant TEFM,

silencing of *TEFM* in human cells showed a unique pattern of mt-mRNA abundance whereby promoter-proximal transcripts were increased whereas promoter-distal transcripts were decreased suggesting a role in transcription elongation (Minczuk et al. 2011). Biochemical characterization of TEFM activity *in vitro* found that TEFM increases the processivity of POLRMT in longer transcripts by enhancing the affinity of POLRMT to an elongating template and facilitating the passage over structured or modified DNA sequences like G-quadruplexes or 8-oxo-dG lesions (Agaronyan et al. 2015; Posse et al. 2015; Sultana et al. 2017). The crystal structures of TEFM and POLRMT with TEFM in an elongating complex provided important mechanistic insights that could explain how TEFM promotes transcription elongation (Hillen et al. 2017b). First, TEFM has two domains, an N-terminal domain that contains two globular hairpin-helix-harpin domains of unknown function, and a C-terminal dimerization domain that forms the catalytic core. TEFM dimers interact with POLRMT and the nucleic acids in the transcription bubble. These interactions contribute to the formation of a ‘sliding clamp’ with the downstream DNA that enhances the processivity of the transcription elongation complex. Second, TEFM binds the single-stranded non-template DNA preventing the collapse of the transcription bubble. Third, TEFM induces conformational changes in POLRMT that contribute to the formation of the RNA channel and prevent the formation of RNA G-quadruplexes during transcription elongation.

Not much is known about mitochondrial transcription elongation factors in yeast. In *S. cerevisiae* a similar role as modulator of the activity of Rpo41 in an ATP-independent manner was proposed for the DEAD box protein Mss116 (Markov et al. 2014), in addition to its role as a mitochondrial splicing factor (Séraphin et al. 1989). However, a recent study found that Mss116 interacts with the mitoribosomal large subunit and suggests rather a post-transcriptional role of Mss116 (De Silva et al. 2017).

Mitochondrial transcription termination factor 1

MTERF1 is not part of the core transcription machinery in mammalian mitochondria, but it is the only known factor involved in transcription termination to date. MTERF1 binds mtDNA in the mt-tRNA *mt-TII* sequence that is located in the H strand downstream of mt-rRNAs (Shang and Clayton 1994; Terzioglu et al. 2013) and was proposed to act as a physical roadblock for the transcription machinery. MTERF1 is a modular protein with eight mterf

motifs, each consisting of two alpha-helices followed by a 3_{10} helix. The interaction of MTERF1 with mtDNA is primarily mediated by electrostatic binding between the negatively charged mtDNA backbone with the positively charged grooves on the MTERF1 surface. MTERF1 binding to the *mt-TII* sequence leads to partial melting of the DNA double strand with eversion of three nucleotides which is required for transcription termination, but not for DNA binding (Yakubovskaya et al. 2010).

More recent studies have shown that MTERF1 not only blocks transcription of antisense mt-rRNAs but also directs polar replication fork pausing. This means that MTERF1 also acts as a roadblock for the replication machinery in the H strand preventing collisions with the transcription machinery transcribing the mt-rRNAs (Shi et al. 2016). Collisions of replication and transcription machineries can result in replication stalling and genome instability (Merrikh et al. 2012).

In vertebrates and plants other MTERF proteins are conserved (Linder et al. 2005), but none of the factors MTERF2-4 play a role in mammalian mitochondrial transcription termination (Cámara et al. 2011; Pellegrini et al. 2009; Wredenberget al. 2013).

Friendly competition – transcription to initiate mtDNA replication

The precise interplay between the aforementioned mitochondrial transcription factors and POLRMT is the foundation of controlled mtDNA transcription for gene expression and mtDNA replication. However, additional factors are involved in safeguarding promoter-specific transcription for mtDNA replication. POLRMT can initiate unspecific transcription upon binding ssDNA *in vitro* which could result from transient strand separation due to mtDNA replication or supercoiling tension (Wanrooij et al. 2008). This is normally prevented by the mitochondrial single-stranded binding protein (SSBP1) (Fusté et al. 2010), an essential component of the core replication machinery that shields ssDNA (Figure 2A) (Falkenberg and Gustafsson 2020). Indeed, a drastic increase of unspecific transcription initiation of mtDNA transcription was observed in a conditional mouse knockout model lacking SSBP1 in the heart (Jiang et al. 2021), pointing to a functional interplay between core components of the transcription and replication machineries.

We previously showed that POLRMT levels play a role in balancing mtDNA replication and transcription

(Kühl et al. 2016). *In vitro* transcription assays using a single template containing both, HSP and LSP, and varying the levels of POLRMT showed that transcription from LSP is better maintained than transcription from HSP when POLRMT:DNA ratios are low. Importantly, TFAM and TFB2M concentrations were maintained constant in this experiment. These results were consistent with *in vivo* data from a *Polrmt* knockout mouse where at low POLRMT levels, LSP-initiated transcription is preferentially maintained over HSP-initiated transcription, potentially to ensure mtDNA replication (Kühl et al. 2016). However, the mechanisms for this observed promoter preference remain unknown. Differences in promoter strength between HSP and LSP have been reported, but remain controversial, as *in vitro* transcription experiments can be strongly influenced by reaction conditions and the promoter template used (Morozov and Temiakov 2016; Shi et al. 2012). A recent study showed that the pre-IC complex has a similar topology in both promoters based on crosslinking studies between TFAM and promoter sequences. According to this model, transcription machineries can assemble simultaneously at HSP and LSP (Morozov and Temiakov 2016). On the contrary, previous studies showed that LSP is a stronger promoter and its activity is strictly dependent on TFAM binding even under permissive conditions that allow promoter breathing like supercoiling or low salt concentrations (Lodeiro et al. 2010; Shi et al. 2012). This suggests that different structural requirements can apply to each promoter.

Communication is key – coordination of nDNA and mtDNA gene expression

A long-standing open question in the field is how expression of nDNA-encoded and mtDNA-encoded proteins is coordinated to generate functional OXPHOS complexes. A multi-omic comparison of mouse hearts from five different conditional knockouts of genes each essential for a different step of mtDNA gene expression (*i.e.* *Tfam*, *Twnk*, *Polrmt*, *Lrpprc*, *Mterf4*) showed that the abundance of nDNA-encoded proteins of complexes of dual genetic origin, *i.e.* the OXPHOS complexes and the mitoribosome, are regulated at post-transcriptional steps and the abundance of the complexes reflects the expression of mtDNA (Kühl et al. 2017). On the one hand, all five knockout mouse strains have impaired mtDNA gene expression and, therefore, the mtDNA-encoded proteins are strongly reduced. Consequently, most of the OXPHOS complexes

containing mtDNA-encoded proteins decline (Complex I, III, IV and F₀ subunit of complex V; Figure 1A). On the other hand, disrupting mtDNA gene expression at different levels results in different mt-rRNAs levels that are mirrored by in the abundance of the mitoribosomal proteins, which are all nDNA-encoded (Kühl et al. 2017). That is, knockouts with reduced steady-state levels of mt-rRNAs were depleted of mitoribosomal proteins, whereas knockouts with increased mt-rRNA levels had increased levels of mitoribosomal proteins. These findings support a model whereby the few proteins encoded by the mtDNA are limiting factors regulating protein complex abundance in the complexes of dual genetic origin. In this way, nDNA encoded proteins are produced in excess, imported into mitochondria, and assembled into the complexes or degraded. This mechanism of stoichiometric regulation has been proposed to explain the non-exponential degradation of proteins that are subunits of multiprotein complexes in mammalian cells (McShane et al. 2016). In the mammalian mitochondrial OXPHOS system, this model is supported by the fact that mtDNA-encoded proteins are required for early steps of assembly of complexes I, III, and IV (Fernandez-Vizarra and Zeviani 2018; Guerrero-Castillo et al. 2017; Stroud et al. 2015), whereas in complex V, that can form sub-assembled complexes, the mtDNA-encoded subunits are part of the later assembly stages (He et al. 2018). Similarly, mitoribosome assembly initiates co-transcriptionally using mt-rRNAs as scaffolds for assembly (Rackham et al. 2016) and SILAC pulse-chase experiments showed that the mitoribosomal proteins, are indeed produced in excess and degraded when they are not assembled (Bogenhagen et al. 2018).

Nuclear control of mitochondrial gene expression has been proposed by two mechanisms. First, transcription factors acting in the nucleus have been reported to dually localize to mitochondria or bind mtDNA (Barshad et al. 2018; Basu et al. 2020; Szczepanek et al. 2012). However, contrary to the *bona fide* mitochondrial transcription factors described above, precise sub-mitochondrial localization, identification of mtDNA binding sites or interaction with core mitochondrial transcription machinery factors, and functional or mechanistic evidence of their role in mtDNA transcription has not been demonstrated yet. Second, nuclear transcription factors acting on the nDNA can regulate mitochondrial gene expression by activating or repressing genes involved in mtDNA gene expression under different physiological or pathological conditions. The nuclear respiratory factor NRF1 has been reported to activate the expression of *TFAM* (Virbasius and Scarpulla 1994) and some mitochondrial ribosome subunits (Cam et al. 2004). Consistently, knockdown of *NRF1* in human

cells has been shown to decrease transcript levels of *TFAM* (Cam et al. 2004), and the embryonic lethality in a *Nrf1* knockout mouse correlates with a depletion of mtDNA (Huo and Scarpulla 2001). The GA-binding protein A (GABPA or NRF2) also binds upstream sequences encoding the mitochondrial transcription machinery factors *TFAM* (Virbasius and Scarpulla 1994), *POLRMT* and *MTERF1*, as well as upstream of sequences of genes encoding factors of the mtDNA replication machinery including the DNA polymerase gamma (*POLG*), *SSBP1*, and the DNA replicative helicase *TWINKLE* (Bruni et al. 2010). NRF1 and GABPA have been reported to be downstream factors of PGC1-alpha, an important master regulator of mitochondrial biogenesis (McMeekin et al. 2021; Mootha et al. 2003a; Scarpulla et al. 2012). *TFAM* and *POLRMT* have also been described as target genes of the MYC proto-oncogene protein and knockdown of *MYC* in human cancer results in decreased protein levels of both factors (Oran et al. 2016). Interestingly, MYC has also been reported to be implicated in mitochondrial biogenesis and endoplasmic reticulum-stress responses, respectively (Morrish and Hockenbery 2014; Pakos-Zebrucka et al. 2016), and we found that loss of mtDNA gene expression with severe OXPHOS deficiency leads to elevated levels of MYC, *POLRMT* and cellular stress response programs (Kühl et al. 2017).

Importantly, increasing *TFAM* and mtDNA levels does not result in an overall increase in mtDNA gene expression (Bonekamp et al. 2021), suggesting that important control points of mtDNA gene expression regulation also occur downstream of mtDNA copy number regulation. Consistently, expression quantitative loci analysis across 36 tissue/cell types identified several nuclei loci associated with expression levels of mt-RNAs, including different genes acting downstream of mitochondrial transcription such as *LRPPRC*, *MTPAP*, *TGBR4*, *PNPT1*, *FASTKD1*, *MRPP3*, and *MRPS35* (Ali et al. 2019). Regulation at the level of cytoplasmic or mitochondrial translation are also potential sites of control to coordinate the expression of both cellular genomes. For e.g. OXPHOS complexes assembly factors were reported to stall mitoribosomes translating *mt-CoI* when the nDNA-encoded subunits are absent, fine-tuning mitochondrial protein synthesis to the influx of nuclear-encoded OXPHOS proteins (Richter-Dennerlein et al. 2016). Interestingly, the mechanisms that coordinate mtDNA-encoded and nDNA-encoded mitochondrial gene expression seem to vary between species. In *S. cerevisiae* it was shown that expression of nDNA-encoded and mtDNA-encoded OXPHOS subunits are synchronized at the translational level and not at the transcriptional level (Couvillion et al. 2016). This synchronization was shown to be regulated unidirectionally by cytosolic ribosomal translation which is consistent with the

existence of translational activators for most mitochondrial messenger RNAs in yeast (Herrmann et al. 2013). In mammalian cells however, there is no rapid feedback between the two translation systems (Soto et al. 2021), consistent with the fact that mammalian mt-mRNAs have only very short 5' UTRs and to date only one translational activator for *mt-Co1* called TACO1 has been identified (Rackham et al. 2016; Weraarpachai et al. 2009).

The mitochondrial core transcription machinery – novel players in mitochondrial disease

Given the numerous metabolic processes carried out by mitochondria in different tissues, it is not surprising that mitochondrial dysfunction severely affects human health. Mitochondrial diseases are among the most common inherited metabolic disorders affecting 1 in 2000 individuals, but their extreme variability remains a clinical and therapeutic challenge (Russell et al. 2020; Suomalainen and Battersby 2018). Disease onset may occur from birth to late in adult life and different organs and cell types may be affected with varying degrees of severity. Furthermore, sporadic or inherited mutations in nDNA or mtDNA may cause mitochondrial disease, adding to their genetic complexity (Russell et al. 2020; Suomalainen and Battersby 2018). Consequently, mitochondrial diseases can be caused by any mode of inheritance. A common factor of mitochondrial disease is the observed defect in OXPHOS. However, the high degree of variability between afflicted tissues and disease severity in mitochondrial disorders points to the existence of genetic modifiers and tissue-specific mechanisms able to compensate for mitochondrial dysfunction (Suomalainen and Battersby, 2018). In the last 30 years, immense efforts have been directed at understanding the genetic basis of mitochondrial disease and potential cellular compensatory responses as well as identifying potential clinical treatments, which remain largely supplemental. An expert overview is given elsewhere in more detail (Russell et al. 2020; Suomalainen and Battersby, 2018).

Since the first discovery of disease-causing point mutations (Holt et al. 1988; Wallace et al. 1988), more than 300 different mutations in mt-tRNA, mt-rRNA and protein-coding genes were reported. A biochemical phenotype occurs once the heteroplasmy level reaches a certain threshold (Sciaccio et al. 1994; Taylor and Turnbull 2005). About 300 mutations in nuclear genes have also been linked to mitochondrial disorders that affect different

aspects of mitochondrial function such as OXPHOS function, regulation and biogenesis, mtDNA maintenance and expression, nucleotide transport/synthesis, protein quality control and membrane dynamics (Suomalainen and Battersby 2018). Variants in key components of the mitochondrial DNA replication machinery, POLG and TWINKLE, are frequent causes of mitochondrial disease (Copeland 2012), but only recently patients carrying pathogenic mutations in components of the mitochondrial transcription machinery were identified.

A large number of rare POLRMT variants were identified in a detailed study on eight individuals from seven unrelated families with a variable spectrum of neurological symptoms (Oláhová et al. 2021). Predominantly, patients presented with an early onset moderate to severe developmental delay (including fine/gross motor, language, and social/behavioral delay), mild to severe intellectual disability, low BMI, hypotonia and short stature, resulting in mild to severe disability. Other muscle symptoms were less frequent with no cardiac involvement. Massive parallel sequencing of all subjects identified recessive and dominant variants in the *POLRMT* gene leading to point mutations and deletions in different regions of the POLRMT protein, including c.1696C>T (p.Pro566Ser), c.3578C>T (p.Ser1193Phe), c.2608G>A (p.Asp870Asn), c.2225_2242_del (p.Pro742_Pro747del), c.748C>G (p.His250Asp), c.2641-1G>C. (p.Gly881-Lys883del), c.1832C>T variant (p.Ser611-Phe), c.1923C>G (p.Phe641Leu), c.2775C>A (p.Cys925*), c.2428C>T (p.Pro810Ser), c.445C>T (p.Gln140*) and c.3037C>T (p.Arg1013Cys) (Oláhová et al. 2021). An overview of the combination of mutations observed in the individual patients as well as the main clinical and molecular/biochemical findings is given in Table 1 and Figure 3A. All variants were predicted to negatively influence POLRMT protein function by either affecting the stability of the core structure, protein-protein or protein-DNA binding, or the conformational flexibility of the protein. Indeed, *in vitro* transcription data using mutant POLRMT variants indicated that RNA polymerase activity is severely affected, leading to a drastic reduction of *in vitro* transcription in patients P3, P6 and P7/8 (Table 1). *In vivo*, the effect is most probably modulated by specific, yet unidentified factors to be compatible with life (Oláhová et al. 2021). Indeed, mutant POLRMT variants led to decreased mt-RNA levels in patient fibroblasts, albeit not as extreme as observed *in vitro*. Analysis in fibroblasts of four of the patients (P1, P2, P3, P6) revealed a modest decrease of 31–66% of control mitochondrial transcript levels in patients P1, P2 and P6 and a severe loss of mt-RNA levels in patient P3 (5–34%). This resulted in a mild respiratory chain defect in P3, in line with previous findings that

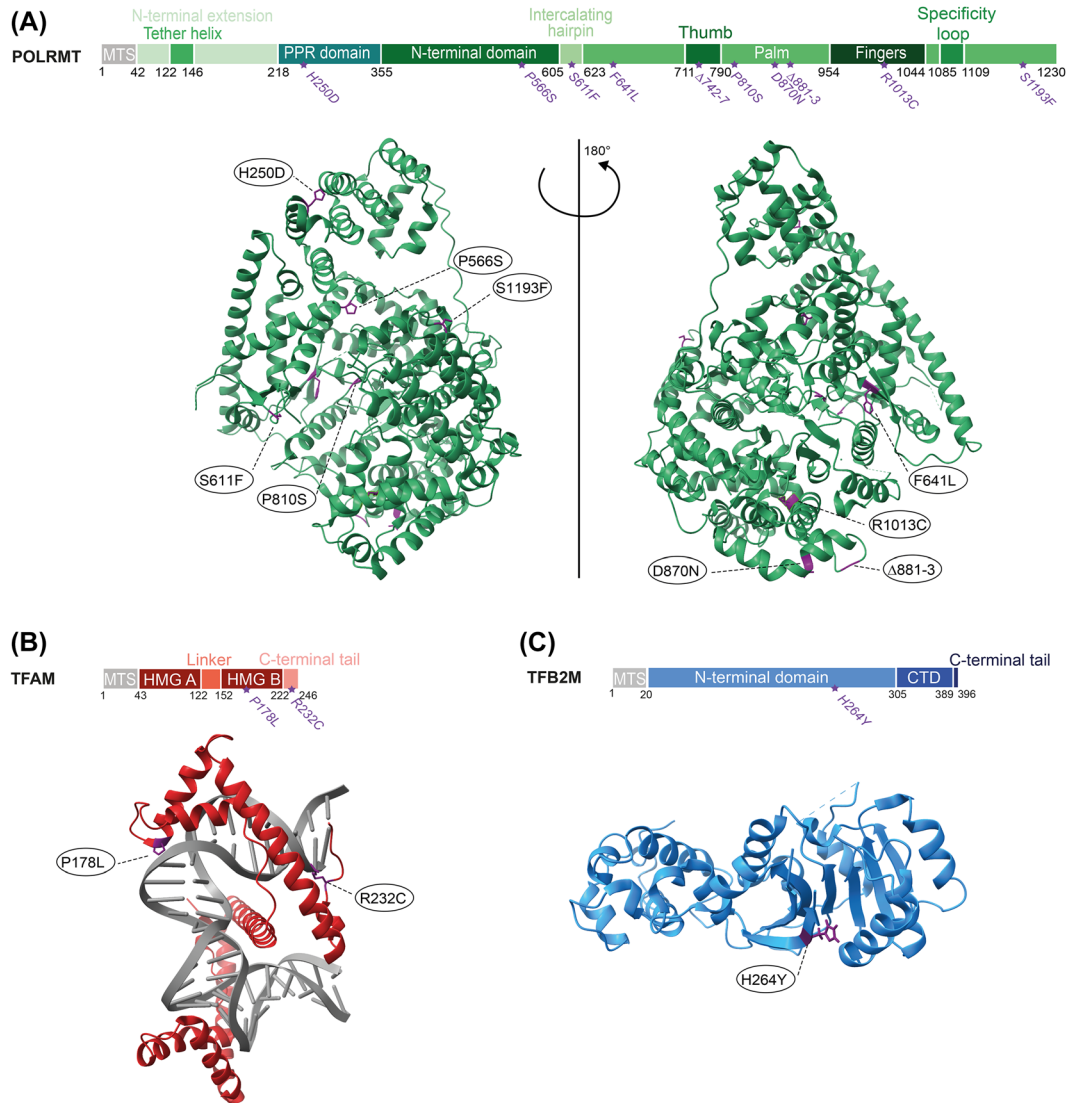


Figure 3: Pathogenic mutations in the core mitochondrial transcription machinery.

Novel, disease-causing mutations identified in (A) POLRMT (green), (B) TFAM (red) and (C) TFB2M (blue). Schematic representation of the domain structures of human POLRMT, TFAM and TFB2M modified from (Hillen et al. 2018). Disease-causing mutations are depicted in purple and are given in one letter code. Structural representations were performed in ChimeraX (Pettersen et al. 2021) using the PDB files 3SPA (human POLRMT), 3TMM (human TFAM) and 6ERO (human TFB2M). The mutation $\Delta 742-7$ is not marked in the structure. MTS, mitochondrial targeting sequence.

mammalian mitochondria have a great excess of transcripts *in vivo* (Lagouge et al. 2015). No profound mtDNA deletions or depletion were observed, although POLRMT is the sole mitochondrial primase (Kühl et al. 2016). Lower levels of RNA primer synthesis were apparently enough to sustain mtDNA levels, which is consistent with previous observations that LSP transcription is better maintained at low POLRMT levels to safeguard replication primer synthesis (Kühl et al. 2016; Oláhová et al. 2021).

Novel disease-causing mutations were also described for the two mitochondrial transcription factors, TFAM and TFB2M. A homozygous c.533C>T TFAM variant was identified in two siblings from a consanguineous family (Stiles et al. 2016). The patients presented with fatal neonatal-onset liver failure and died at the age of 2 and 4 months, respectively. Brain MRI was normal and renal and cardiac failure was diagnosed as secondary to the liver disease. The c.533C>T TFAM variant results in a p.Pro178Leu amino acid

change which is located in the HMG-B domain of the TFAM protein and expected to cause steric clashes with mtDNA (Figure 3B and Table 1). TFAM protein levels were severely decreased, leading to a drastic mtDNA depletion to 11% in the liver and 21% in the skeletal muscle of patients (Stiles et al. 2016), in line TFAM's function as a regulator of mtDNA copy number (Freyer et al. 2010; Larsson et al. 1998). Interestingly, some observations made in the patient mirror phenotypes from a variety of conditional *Tfam* knock-out mouse models. For instance, increased citrate synthase activity in patient skeletal muscle pointed to an increase in mitochondrial mass to partly compensate for reduced OXPHOS function, similar to observations made upon disruption of *Tfam* in the skeletal muscle of mice (Wredenberg et al. 2002). The absence of severe neurological symptoms in the patients may be explained by the fact that loss of mtDNA expression can be tolerated for a very long time in post-mitotic tissues (Sørensen et al. 2001; Viader et al. 2011; Wang et al. 1999), while rapidly dividing tissues, such as the liver, are severely affected (Larsson et al. 1998). Another TFAM variant, the homozygous c.694C>T (p.Arg232Cys) mutation (Figure 3B), was identified by whole exome sequencing as a potential disease causing gene in Perrault syndrome, a rare heterogeneous condition characterized by sensorineural hearing loss and premature ovarian insufficiency (Tucker et al. 2020). Three additional individuals from a consanguineous family carrying the same mutation were recently identified (Ullah et al. 2021). These patients presented with seizures and reproductive phenotypes with no signs of growth disorders (Table 1). The two females showed uterine hypoplasia, with small or undetectable ovaries and abnormal sex hormone levels. Unlike the c.533C>T TFAM variant, TFAM protein levels were unaltered in patient fibroblasts, but a moderate decrease in mtDNA copy number, nucleoid clustering and decreased oxygen consumption was detected (Ullah et al. 2021). Apparently, the resulting p.Arg232Cys TFAM protein is more stable, although it results in a point mutation in the C-terminal tails of TFAM, a region necessary for transcription activation (Dairaghi et al. 1995). Disruption of the *tfam* locus in zebrafish recapitulated the mtDNA depletion and ovarian dysgenesis phenotype which could not be rescued upon expression of the mutant TFAM variant, confirming the pathogenicity of the c.694C>T (p.Arg232Cys) TFAM variant and the important role of TFAM in gonad development (Ullah et al. 2021).

A rare homozygous c.790C>T (p.His264Tyr) variant of TFB2M was identified in a Korean patient with autism spectrum disorder (Park et al. 2018) (Figure 3C and Table 1). The mutation might affect the rigidity of the TFB2M hinge region, but there was no significant change in the protein

levels of TFB2M in patient fibroblasts and no changes in mtDNA copy number or mitochondrial mass were observed. However, there was a significant increase in mitochondrial transcript levels up to 4-fold of control concomitant with an increase in ROS levels, mitochondrial membrane potential and basal respiration. Overexpression of a wild-type *Tfb2m* construct led to a similar phenotype, albeit at lower levels. Further studies are required to understand the molecular and clinical phenotype of the c.790C>T TFB2M variant (Park et al. 2018).

Mitochondrial transcription – a promising drug target

The link between mitochondria and cancer cell growth has long been dominated by the Warburg effect. In the 1920s, Otto Warburg observed that cancer cells predominantly rely on glycolysis for energy conversion, even in the presence of oxygen, a process referred to as aerobic glycolysis or Warburg effect (Warburg 1924). Warburg proposed that defects in OXPHOS lead to tumorigenesis (Warburg 1956). However, recent observations argue that aerobic glycolysis result from oncogene activation (Zong et al. 2016). Indeed, tumors and tumor stem cells were shown to be highly dependent on OXPHOS (Birsoy et al. 2014; Kuntz et al. 2017; Shi et al. 2019; Viale et al. 2014; Zong et al. 2016) and many biosynthetic reactions occurring in fast-growing tumor cells require a fully functional mitochondrial metabolism to sustain biomass generation and aggressive cancer behavior (Vander Heiden and DeBerardinis 2017). Thus, mitochondria emerged as promising targets for cancer therapy. A variety of different strategies directly targeting the mitochondrial OXPHOS system, mitochondrial translation and redox capacity in cancer cells was put forward with promising results (Weinberg and Chandel 2015). However, the clinical use of mitochondrial poisons is limited by their toxicity in healthy tissues, especially in cell types with high mitochondrial ATP demand such as cardiomyocytes and neurons. Another problem are off-target effects: repurposing of tigecycline, an FDA-approved antibiotic, has shown that it inhibits mitochondrial translation and causes cytotoxicity against acute myeloid leukemia (AML) stem cells (Škrčić et al. 2011); however, it also caused acute side effects in a phase 1 dose-escalation study of AML patients due to its limited target specificity (Reed et al. 2016).

Recent studies have evidenced that mtDNA expression is important for tumor growth (Bralha et al. 2015; Stewart et al. 2015; Tan et al. 2015). Loss of TFAM reduced

tumorigenesis in a *Kras*-driven mouse model of lung cancer (Weinberg et al. 2010) and silencing *POLRMT* reduced cell growth in a cellular model of AML (Bralha et al. 2015). *POLRMT* was also identified as an important oncogene in lung and breast cancer and shown to be overexpressed in human lung cancer tissues and cell lines (Sotgia et al. 2012; Zhou et al. 2021). Interestingly, modulation of *POLRMT* levels directly influenced non-small lung cancer cell growth, as genetic inhibition of *POLRMT* potently inhibited cancer cell proliferation, migration and invasion, while *POLRMT* overexpression rendered cells more proliferative (Zhou et al. 2021). Similarly, elevated TEFM levels promote growth of hepatocellular carcinoma cell lines and primary tumors and are associated with poor patient prognosis (Fei et al. 2020; Wan et al. 2021). Since the mitochondrial transcription machinery in general, and *POLRMT* in particular, have emerged as key targets for cancer therapy, highly specific first-in-class inhibitors of *POLRMT* (“inhibitors of mitochondrial transcription”, IMTs) were developed (Bonekamp et al. 2020). These allosteric inhibitors bind specifically to the palm domain of *POLRMT* near the active center cleft, which leads to a rearrangement of the palm loop (Figure 4). This in turn leads to clashes of the loop with the template DNA strand and the nascent RNA, thus interfering with the binding of the DNA-RNA hybrid and RNA translocation.

IMTs dose-dependently impair mtDNA transcription *in vitro* and *in vivo*, causing OXPHOS dysfunction, severe depletion of cellular metabolites and ultimately apoptotic cell death upon sustained application. Importantly, IMT application in cell-derived xenograft mouse models significantly reduced tumor cell growth without any signs of acute or chronic toxicity in the time of treatment (Bonekamp et al. 2020). OXPHOS protein levels and mtDNA copy number were not affected in differentiated tissues like liver and heart. The high tolerability and favorable therapeutic properties of IMTs probably result from a combination of high on-target specificity and tissue-specific vulnerability to inhibition of mtDNA expression, as disruption of mtDNA expression is well tolerated for a long time in differentiated tissues such as brain, heart or skeletal muscle (Li et al. 2000; Sørensen et al. 2001; Viader et al. 2011). However, certain cell types within different tissues might be more resistant to IMTs than others. Interestingly, several mechanisms conferring resistance to IMTs in cancer cell lines were recently identified. Using a CRISPR-Cas9 whole genome screen, loss of genes belonging to the Van-Hippel-Lindau and mTORC1 pathways or chemical inhibition of these pathways mediated resistances to acute IMT treatment. Acquired IMT resistance using a dose-escalation approach occurred via compensatory upregulation of mtDNA levels

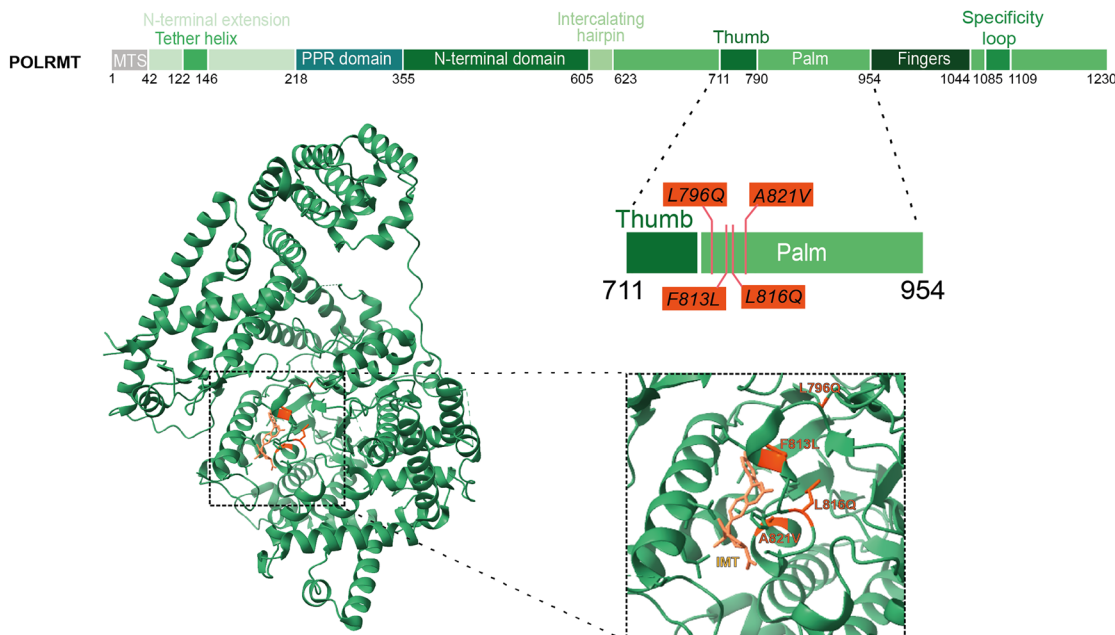


Figure 4: Inhibitors of mitochondrial transcription (IMTs).

Schematic and structural depiction of novel first-in-class IMTs and their binding site in human *POLRMT*. Mutations conferring IMT resistance are shown in orange, IMT is shown in coral. Structural representations were performed in ChimeraX (Pettersen et al. 2021) using the PDB file 7A8P.

(Mennuni et al. 2022). The identified resistance mechanisms will be relevant for the further development of IMTs as future anti-cancer drugs.

Conclusion and outlook

Research from recent years has further expanded our understanding of the key players in the field of mammalian mitochondrial gene expression and their interplay. In the nucleus, protein-coding genes are typically present in two copies per cell and, therefore, activation of specific gene programs is controlled by different combinations of transcription factors binding to the promoter regions. In contrast to the nucleus, mtDNA is present in multiple copies per cell and all the genes are controlled by only two promoters. This means that mtDNA transcription can be regulated by engaging more mtDNA templates in

transcription, increasing transcription from HSP and LSP, or a combination of both. Therefore, mtDNA copy number and mitochondrial transcription have emerged as important regulatory points for mitochondrial gene expression, and ultimately, OXPHOS tuning in physiologic and pathologic conditions.

Pathogenic mutations causing a broad spectrum of mitochondrial diseases have been recently identified in multiple factors of the core transcription machinery and abnormal levels of key players of mitochondrial transcription have been increasingly described in cancer. These exciting findings will further our mechanistic understanding of the function and regulation of the mitochondrial core transcription machinery factors and their specific protein domains. Furthermore, the extreme discrepancy observed between *in vitro* activity of the proteins and the observed clinical phenotype in some of the patients points to the existence of yet unidentified cellular mechanisms able to

Box 1. Pentatricopeptide repeat (PPR) proteins are essential players in mammalian mitochondrial gene expression

PPR proteins are the largest known RNA-binding protein family, and are found in all eukaryotes where they localize to mitochondria or chloroplasts, being particularly abundant in higher plants (Small and Peeters 2000). They contain a set of related degenerate motifs of 35 amino acids that appear to be involved in RNA-protein interactions (Schmitz-Linneweber and Small 2008). PPR motifs are related to the tetratricopeptide (TPR) motifs, which serve as protein-interaction modules and multiprotein complex mediators (Schmitz-Linneweber and Small 2008). Although most PPR proteins appear to consist almost entirely of tandem arrays of motifs, some contain other functional domains such as an endonuclease or RNA polymerase. Each PPR motif forms a hairpin of two antiparallel α -helices connected by a short turn of two amino acids. Each α -helix is composed of four helical turns followed by a five-amino-acid loop (Yin et al. 2013). PPR proteins are RNA-binding adaptor proteins that mediate interactions between RNA substrates and the enzymes that act on them, involved in *e.g.* RNA processing, turnover, editing or translation. Interestingly, the majority of studied PPR proteins have been found to recruit additional proteins with RNA modifying or degrading functions or to block the access of these proteins to their target RNAs (Delannoy et al. 2007).

Plant PPR proteins bind RNA in a sequence specific manner, determined by amino acids located at specific positions of each motif. A combinatorial amino acid code via modular RNA recognition by plant PPR proteins has been described (Barkan et al. 2012) and further insights have been gained from a crystal structure (Yin et al. 2013). The binding to RNA is determined by amino acids located in specific positions of each motif. However, this code is not suited for all plant PPR proteins and is moreover not at all applicable in non-plant PPR proteins (Herbert et al. 2013; Spähr et al. 2016).

The family of non-plant PPR proteins is much smaller. Yeast genomes encode for at least ten in *S. pombe* to fifteen in *S. cerevisiae* PPR proteins, most of them are regulators of mitochondrial gene expression and appear to be involved in stability and/or translation of specific mt-mRNAs (Herbert et al. 2013; Kühl et al. 2011; Lipinski et al. 2011). To date there are seven PPR proteins known in mammalian mitochondria, all involved in different steps of mtDNA gene expression (Figure 5), however, the detailed molecular mechanisms by which these proteins regulate organellar gene expression remain to be discovered. Curiously, the way non-plant PPR proteins bind their target RNA is unknown and structural data is lacking. To date, four of the mammalian PPR proteins have been associated with patient mutations, those detected in POLRMT are described in the main text (Oláhová et al. 2021).

The leucine-rich pentatricopeptide repeat containing (LRPPRC or LRP130) protein was discovered when a recessive mutation causing an A354V amino acid substitution led to the French-Canadian variant of Leigh syndrome, a neurodegenerative disorder characterized by complex IV deficiency (Mootha et al. 2003b). Amongst all mammalian PPR proteins LRPPRC has the highest number of PPR motifs, namely 33 (Spähr et al. 2016), and is an essential factor coordinating mt-mRNA stability and polyadenylation (Ruzzenente et al. 2012; Sasarman et al. 2010). Deletion of *Lrpprc* in mouse causes a global decrease of all mt-mRNAs except *mt-Nd6* whereas mt-tRNAs are mostly unchanged and mt-rRNAs are increased in abundance (Ruzzenente et al. 2012).

A consanguineous family with three affected individuals homozygous for a missense variant p.(Ala485Val) in the metalloprotease domain of PRORP was reported with Perrault syndrome (Hochberg et al. 2021). The disease variant of PRORP produced ~35–45% less 5'-processed tRNA than the wild-type enzyme resulting in accumulation of unprocessed polycistronic mitochondrial transcripts and loss of OXPHOS components.

Lately, two potentially loss-of-function variants [c.415-2A>G, and c.1747_1748insCT (p.Phe583Serfs*3)] in *PTCD3* have been associated with Leigh syndrome along with combined OXPHOS deficiencies caused by a decrease of the small subunits of the mitoribosome (Borna et al. 2019). Complementation experiments rescued mt-12S rRNA transcript levels, as well as OXPHOS complex I and IV levels and activities.

	Step of mitochondrial gene expression	PPR protein	# Motifs/protein length (aa)	Primary target	Function	Mouse model/human disease gene	Refs.
Transcription	Mitochondrial DNA	POLRMT/ MT-RPOL	2/ 1230	mtDNA	Synthesis of polycistronic mt-RNA from HSP and LSP, as well as RNA primer for mitochondrial replication	yes/ yes	Kühl et al. 2014, 2016; Ringel et al. 2011; Schwinghammer et al. 2013
	Polycistronic RNA						
Processing	Precursor RNA	PRORP/ MRPP3	3/ 583	all mt-pre-RNAs	Ribonuclease component of mitochondrial ribonuclease P complex which cleaves tRNA molecules in their 5'-ends; PPR domain contacts the T arm of the pre-tRNA substrate in sequence non-specific manner	yes/ yes	Bhatta et al. 2021; Holzmann et al. 2008; Lopez-Sanchez et al. 2011; Rackham et al. 2016; Reinhard et al. 2015
	Mature RNA	PTCD2	5/ 388	mt-Nd5/CytB precursor	mt-mRNA processing	yes/ NR	Xu et al. 2008
Stabilization	Mature RNA	LRPPRC/ LRP130	33/ 1394	mt-mRNA (except mt-Nd6)	mRNA stability and polyadenylation; coordination of mitochondrial translation; functions in complex with SLIRP	yes/ yes	Mootha et al. 2003a; Ruzzenante et al. 2012; Sarsarmann et al. 2010
		PTCD1	8/ 700	mt-16S rRNA	RNA stability, pseudouridylation, and biogenesis of the ribosomal LSU	yes/ NR	Perks et al. 2018
Translation	Full-length protein	MRPS27	6/ 414	mt-12S rRNA, mt-7e and Ribosome	RNA-binding component of the ribosomal SSU	no/ NR	Davies et al. 2012
		PTCD3/ MRPS39	10/ 689	mt-12S rRNA	Associates with the 12S rRNA and the ribosomal SSU; essential for translation	no/ yes	Davies et al. 2009

Figure 5: Overview of various roles of the human PPR proteins during mitochondrial gene expression.

The seven known PPR proteins contain 2 to 33 PPR motifs and mainly act during different steps of mtDNA expression: transcription, mtRNA processing, stability or translation (see Box1); NR: not reported. The crystal structures of POLRMT and PRORP have been resolved (Bhatta et al. 2021; Ringel et al. 2011). However, both are atypical PPR proteins since they only contain few PPR motifs additional to their enzymatic domains, thus it is difficult to draw conclusions to the mode of action of PPR proteins that are completely covered with PPR motifs.

compensate or modulate defects in mitochondrial transcription *in vivo*. Mitochondrial DNA copy number and transcription are currently being targeted to modulate mitochondrial and common disease phenotypes. The deep mechanistic understanding of the mitochondrial transcription core machinery achieved in the last 30 years has allowed the development of highly specific novel drugs that are currently being prepared for clinical testing for cancer. Given the essential role of mitochondrial metabolism in a variety of metabolic states, there might be potential applications beyond cancer treatment.

There are still many exciting open questions to be elucidated in the field of mammalian mtDNA gene expression: Can mtDNA gene expression be modulated as a mechanism to fine tune cellular bioenergetic requirements? Are there tissue-specific mechanisms regulating the number of active nucleoids *in vivo*? How are mtDNA replication and transcription balanced in the cell? The answers to some of these questions in the years to come

will not only further our understanding of mitochondrial biology but also broaden the therapeutic application for mitochondrial and common disease treatments.

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