Regulation of Human Mitochondrial DNA Replication and Transcription

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It always seems impossible until it's done – Nelson Mandela

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ABSTRACT

Mitochondria are organelles in eukaryotic cells, which through oxidative phosphorylation (OXPHOS) produce most of the ATP used to drive cellular processes. The organelle contains its own genetic material, mitochondrial DNA (mtDNA), which encodes 13 key components of the OXPHOS machinery. For its maintenance and expression, mtDNA is dependent on a large number of nuclear factors. Our understanding of these processes has progressed significantly during the last years, but much is still unknown.

The mitochondrial genome is completely coated by TFAM, which acts to compact mtDNA molecules into nucleoid structures. In this thesis we have examined how nucleoid formation contributes to regulation of mitochondrial replication and transcription. Our studies demonstrate that TFAM packaging regulates mtDNA availability, thereby directing levels of replication and transcription *in vitro*. These findings therefore reveal that TFAM has the potential to function as an epigenetic regulator of mtDNA transactions.

Second, we investigate the characteristics of a newly discovered mutation in TFAM that causes severe mtDNA depletion and early onset-liver failure in infants. Using a combined effort with biochemical, biophysical and cell biology techniques, we demonstrate that the mutant form of TFAM impairs transcription initiation from mitochondrial promoters. The mutant protein also impairs compaction of mtDNA.

Finally, we investigate a replication pre-termination event that leads to the formation of a displacement loop (D-loop) structure in mtDNA. We demonstrate that replication initiated at the origin of heavy-strand replication

and transcription coming from the opposite direction (initiated at the heavy strand promoter) are both terminated at an evolutionary conserved sequence, which we term coreTAS. We also provide data, which suggest that coreTAS plays an important role in the regulated switch between D-loop formation and full-length replication.

Keywords: mitochondria, mtDNA, TFAM, transcription, replication

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SAMMANFATTNING PÅ SVENSKA

Mitokondrier är organeller i eukaryota celler, som genom oxidativ fosforylering (OXFOS) producerar det mesta av det ATP som cellen använder för att driva olika processer. Organellen har sitt eget genetiska material, mitokondriellt DNA (mtDNA), som kodar för 13 viktiga komponenter i OXFOS-maskineriet. För dess underhåll och uttryck är mtDNA beroende av ett stort antal kärnfaktorer som styr processer såsom replikering och transkription. Vår förståelse av dessa processer har utvecklats avsevärt under de senaste åren, men mycket är fortfarande okänt.

TFAM binder till mtDNA på ett sekvensoberoende sätt. Tillsammans med mtDNA bildar TFAM ett kompakt nukleoproteinkomplex, en s.k. nukleoid. I denna avhandling har vi undersökt hur nukleoidbildning bidrar till reglering av mitokondriell replikation och transkription. Våra studier *in vitro* visar att TFAM genom att packa mtDNA kan reglera genomets tillgänglighet, vilket i sin tur styr replikations- och transkriptionsnivåer. Dessa resultat visar att TFAM har potential att fungera som en epigentisk regulator för mtDNA-transaktioner.

I den andra delen av denna avhandling undersöker vi hur en nyupptäckt mutation i TFAM kan orsaka en progressiv sjukdom med förlust av mtDNA och debut av leversvikt i tidig ålder. Med en rad biokemiska, biofysiska och cellbiologiska tekniker visar vi att den mutanta formen av TFAM är sämre på att stimulera transkriptionsinitiering från mitokondriella promotorer. Det mutanta proteinet påverkar också packningen av mtDNA. Som en konsekvens leder den sjukdomsframkallande mutationen i TFAM-genen till förlust av mtDNA och lägre nivåer av transkription.

I den avslutande, tredje delen av denna avhandling, undersöker vi mekanismerna för bildandet av en trippelsträngad DNA-struktur i mtDNA, en s.k. displacement loop (D-loop). Vi demonstrerar att både DNA-replikation och transkription termineras vid en evolutionärt konserverad, palindromisk sekvens i mtDNA, som vi kallar coreTAS. Vi visar vidare att processer vid coreTAS kontrollerar valet mellan fullängdsreplikation och D-loops-bildning.

LIST OF PAPERS

I. **In vitro-reconstituted nucleoids can block mitochondrial DNA replication and transcription**
Farge G*, **Mehmedovic M***, Baclayon M, van den Wildenberg SM, Roos WH, Gustafsson CM, Falkenberg M.
Cell Rep. 2014 July 10; 8(1):66-74

II. **Disease causing mutation (P178L) in mitochondrial transcription factor A results in impaired mitochondrial transcription initiation**
Mehmedović M, Martucci M, Spåhr H, Ishak L, Peter B, Mishra A, van den Wildenberg SM, Falkenberg M, Farge G.
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III. **Regulation of DNA replication at the end of the mitochondrial D-loop involves the helicase TWINKLE and a conserved sequence element**
Jemt E, Persson Ö, Shi Y, **Mehmedovic M**, Uhler JP, Dávila López M, Freyer C, Gustafsson CM, Samuelsson T, Falkenberg M.
Nucleic Acids Res. 2015 October 30; 43(19):9262-75

*Authors contributed equally

CONTENT

ABBREVIATIONS

aa	amino acids
Abf2p	autonomously replicating sequence-binding factor 2 protein
ADP	adenosine diphosphate
ATP	adenosine triphosphate
bp	base pairs
C	cytosine
CO_2	carbon dioxide
CoA	coenzyme A
CSB	conserved sequence block
CTD	C-terminal domain
cyt c	cytochrome c
D-loop	displacement loop
ddC	dideoxycytosine
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
dT	deoxythymidine
e^-	electron
EC	elongation complex
ETC	electron transfer chain
FAD	flavin adenine dinucleotide
G	guanine
G4	G-quadruplex
H-strand	heavy strand
H_2O	water; dihydrogen monoxide
HMG	high-mobility group

HSP	heavy strand promoter
IC	initiation complex
kDa	kilo Dalton
L-strand	light strand
LSP	light strand promoter
mRNA	messenger RNA
mt	mitochondrial
mtDNA	mitochondrial DNA
mtSSB	mitochondrial single stranded DNA binding protein
NAD	nicotinamide adenine dinucleotide
NAP	nucleoid associated protein
NCR	non-coding region
nm	nano meter
nt	nucleotide
NTD	N-terminal domain
NTE	N-terminal extension
O_2	oxygen; dioxide
O_H	origin of replication for heavy strand
O_L	origin of replication for light strand
OXPHOS	oxidative phosphorylation
Pi	inorganic phosphate
POLRMT	mitochondrial RNA polymerase
POLγ	mitochondrial DNA polymerase
R-loop	DNA-RNA hybrid loop
RITOLS	ribonucleotide incorporation throughout the lagging strand
RNA	ribonucleic acid
RNAP	RNA polymerase

rRNA	ribosomal RNA
SCM	strand coupled model
SDM	strand displacement model
SSB	single stranded DNA binding protein
ssDNA	single stranded DNA
TAS	termination associated sequences
TBB2M	mitochondrial transcription factor B2
TCA	tricarboxylic acid cycle (citric acid cycle)
TFAM	mitochondrial transcription factor A
tRNA	transfer RNA
TRX	thioredoxin
TSS	transcription start site
μm	micro meter

1. INTRODUCTION

1.1. The mitochondrion

The mitochondrion is an organelle existing in most eukaryotic cells. It is present in numbers ranging from hundreds to thousands of mitochondria per cell, depending of the energetic needs of the tissue. This cytoplasmic organelle is comprised of two membranes, an outer membrane that separates the mitochondrion from the cell cytoplasm and an inner membrane folded into itself to form invaginations named cristae where the cellular respiration takes place. The mitochondrial matrix harbors the mitochondrial genome and proteins involved in the maintenance and organization of mitochondrial DNA (mtDNA). Often referred to as the "powerhouse" of the cell, mitochondria are responsible for energy production in the form of ATP. Aside from this fundamental function in the cell, mitochondria are involved in numerous functions, among which they have a crucial role in cell signaling and apoptosis via regulation of cellular calcium and cytochrome c.

1.1.1. Origin of mitochondria

It is believed that the mitochondrion originated from an α-proteobacterium that was engulfed by an ancestral eukaryotic cell (Gray, Burger et al. 1999, Lang, Gray et al. 1999, Martin, Garg et al. 2015) about 1.5 - 2 billion years ago when the Earth's atmosphere first became oxygenated. In the now generally accepted theory of endosymbiosis, the mitochondrion provided the then anaerobic cell with energetic power in return for shelter and nourishment from the host (Alberts 2008). The groundbreaking discovery of the separate mitochondrial genome in the 1960's by Nass and Nass provided strong evidence for the endosymbiotic origins of mitochondria (Nass and Nass 1963, Nass and Nass 1963). The then bacterium contained its own genetic material, however through the evolutionary stretch of time, genes were lost or transferred to the nuclear genome (Gray, Burger et al. 1999) resulting in a smaller molecule containing only a remnant of its genetic information. The mitochondrion became an organelle supported by a nuclear encoded maintenance machinery (Gray, Burger et al. 1999, Andersson, Karlberg et al. 2003). However, three key components of the mitochondrial genome maintenance machinery – the mitochondrial DNA polymerase (POLγ), mitochondrial RNA polymerase (POLRMT) and the mitochondrial replicative

helicase (TWINKLE) do not derive from the α-proteobacterium, but share a high sequence homology and function to their counterparts from the T-odd family of bacteriophages. Hence, the mitochondrion that evolved through endosymbiosis had a contribution from three sources of genetic information – the eubacteria, phage, and the host cell (Shutt and Gray 2006).

1.1.2. Structure and dynamics of mitochondria

Separated from the cellular cytoplasm by the outer membrane, the inner membrane of the mitochondrion is highly folded into a string of invaginations denoted cristae (Palade 1952, Palade 1953, Sjostrand 1953, Berg 2019) (Figure1), where the respiratory chain is located. The process of ATP production is called oxygenated phosphorylation (OXPHOS) and is carried out by a series of protein complexes (Complex I-V) imbedded in the cristae (Palmer and Hall 1972). The number and size of mitochondria can vary in different cell types depending on the cellular energy demand. For example, in a cardiac muscle cell the number of cristae in the mitochondrion is three times larger than in a liver cell, where the amount of ATP is in higher demand for cardiac function (Alberts 2008). While the outer membrane of the mitochondrion is permeable to ions and proteins up to 5 kDa, larger proteins need to be actively transported through the membrane (De Pinto and Palmieri 1992, Mannella, Forte et al. 1992). The inner membrane is impermeable to hydrophilic molecules, whereas metabolites involved in energy conversion, and proteins necessary for mitochondrial maintenance and metabolism are imported via a variety of membrane transport proteins (Alberts 2008). The matrix, enclosed by the inner membrane, is host to an array of metabolic pathways (the β-oxidation of fatty acids, amino acid metabolism and the citric acid cycle) in addition to housing the mitochondrial genome, and the machinery required for its maintenance (Alberts 2008, Gustafsson, Falkenberg et al. 2016, Berg 2019).

The mitochondrion is a dynamic organelle that undergoes continuous changes via fission and fusion, which allows for reorganization and redistribution of its content and is often discussed as ‘the mitochondrial network’ rather than distinct units (Shaw and Nunnari 2002, Chen and Chan 2004).

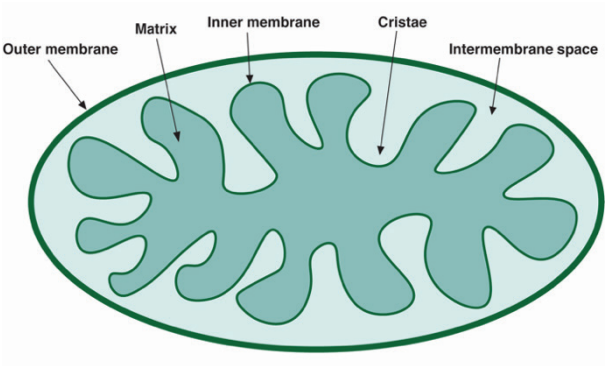

Figure 1. Elliptical in shape, the mitochondrion has two membranes. The outer membrane encloses the inner membrane which is invaginated to form structures called cristae. The matrix of the mitochondrion is surrounded by the inner membrane.

The mitochondrial proteome has been identified to have approximately 1100 proteins, many still of unknown function (Meisinger, Sickmann et al. 2008, Pagliarini, Calvo et al. 2008), and of which 99% are encoded by the nuclear genome, whereas only 13 proteins are encoded in the mtDNA. Interestingly, several hundred of the nuclear encoded proteins are needed for mtDNA gene expression (Sickmann, Reinders et al. 2003, Foster, de Hoog et al. 2006). All nuclear encoded mitochondrial proteins, including factors required for replication and transcription of mtDNA are translated in the cytoplasm and transported to the mitochondrion (Falkenberg and Gustafsson 2020).

1.2. Metabolism

1.2.1. The citric acid cycle and β-oxidation pathway

β-oxidation of fatty acids

Fatty acids are activated on the outer membrane of mitochondria by linking a fatty acid to coenzyme A (CoA) via a thioester bond, utilizing one ATP molecule in the process. The activated fatty acid is then translocated through both membranes into the matrix. Oxidation of the fatty acid occurs in four steps: oxidation by FAD to generate $FADH_2$, hydration, oxidation by NAD^+ to generate NADH and finally thiolysis by CoA to produce acetyl CoA, which then enters the citric acid cycle for further oxidation. As a result of these reactions the fatty acid is shortened by two carbon atoms, and the cycle is

repeated until the whole fatty acid chain is degraded. For example, complete oxidation of an activated fatty acid consisting of sixteen carbon atoms requires seven reaction cycles and will generate 106 molecules of ATP in total. Because this series of reactions occurs on the second (β) carbon atom it is denoted β-oxidation pathway (Berg 2019).

The citric acid cycle

Glycolysis is an ancient metabolic pathway utilized by a multitude of organisms, and is the first step in glucose breakdown. It is an anaerobic chemical sequence of reactions where one glucose molecule is metabolized to produce two molecules of pyruvate and two molecules of ATP (Berg 2019). Glycolysis takes place in the cytosol of the cell and pyruvate is subsequently transferred to the mitochondrial matrix for oxygenated metabolism (Berg 2019) via the citric acid cycle, also known as the tricarboxylic acid cycle (TCA) or Krebs cycle, as the final step in eukaryotic metabolism (Alberts 2008, Berg 2019). Metabolites from glycolysis and the β-oxidation pathway are converted to acetyl coenzyme A (acetyl CoA) in the matrix before they enter the TCA. Not only does the TCA provide electrons to the respiratory chain, or electron transport chain (ETC), for ATP synthesis, it is also an important source of precursors for building blocks in biosynthesis like nucleotide bases, amino acids and organic heme groups. In a series of oxidation-reduction reactions, the acetyl CoA is oxidized to two carbon dioxide (CO_2) molecules and one ATP molecule, generating high energy electrons that are carried via NADH and $FADH_2$ to the ETC to be utilized for ATP synthesis in a process called oxidative phosphorylation (discussed in the next paragraph) (Alberts 2008, Berg 2019).

1.2.2. Oxidative phosphorylation (OXPHOS)

First proposed by Peter Mitchell in 1961, the chemiosmotic hypothesis, where oxidation is coupled through an electron and proton transfer over the inner membrane, is a mechanism that explains a long-standing conundrum in cell biology (Mitchell 1961, Alberts 2008). OXPHOS takes place in the inner membrane of mitochondria and comprises a chain of reactions where electrons harvested in the TCA are carried through four protein complexes (Complex I-IV) to reduce oxygen to water while simultaneously generating a proton gradient and synthesizing ATP via ATP synthase (Complex V) (Mitchell 1966, Berg 2019). Figure 2 depicts a schematic of the OXPHOS pathway.

The electron transfer chain (ETC), or respiratory chain, consists of four large electron-carrier protein complexes (Complex I-IV) and an ATP synthase (Complex V). All enzymes with the exception of Complex II, have subunits encoded in both the nucleus and mtDNA. Complex I, III and IV are proton pumps, whereas ATP synthase consists of an ion channel and a catalytic ATP synthase domain (Berg 2019). Electrons (e^-) harvested in the TCA, via electron carriers NADH and $FADH_2$, are ferried through ETC to reduce O_2 to two molecules of H_2O. NADH releases electrons through Complex I (NADH ubiquinone oxidoreductase) which in turn carries the electron to ubiquinone, or coenzyme Q (Q). As NADH is oxidized to $NAD^+ + H^+$, Complex I pumps the protons through its ion channel to the intermembrane space. Q functions as an electron mediator between the complexes that passes electrons to Complex II and III. $FADH_2$ enters the ETC via Complex II (Succinate ubiquinone reductase) where it is oxidized, releasing electrons and protons. Unlike Complex I, III and IV, Complex II is not a proton pump, but is only responsible for transferring the electrons received from $FADH_2$ to Complex III via a second Q in the membrane. The H^+ released at Complex II is pumped out of the matrix via Complex III (Ubiquinol-cytochrome c oxi-reductase) and electrons flow from Complex I-II through to the hydrophilic cytochrome C (cyt c), thereby reducing it. Cyt c is located in the intermembrane space and anchored to Complex IV and the membrane (Saraste 1999, Berg 2019). Complex IV (cytochrome c oxidase) oxidizes cyt c, as the name implies, passing the electrons to the highly potent electron acceptor O_2 and reduces it to H_2O. As the last proton pump in the chain, it contributes to the electro-proton gradient (Saraste 1999, Berg 2019).

The difference in polarity and pH of the intermembrane space renders the proton-motive force to be exploited by the ATP synthase to release H^+ back into the matrix, harvesting the energy produced to phosphorylate $ADP + P_i$ to ATP, completing the OXPHOS pathway. Interestingly, ATP is not released from the ATP synthase catalytic site lest protons pour back through to the matrix. Thus, the role of the gradient is to release the ATP from ATP synthase, not to create it. The ATP synthase and the rest of the respiratory chain are biochemically separate systems, linked only by the proton-motive force created through electron transfer (Berg 2019).

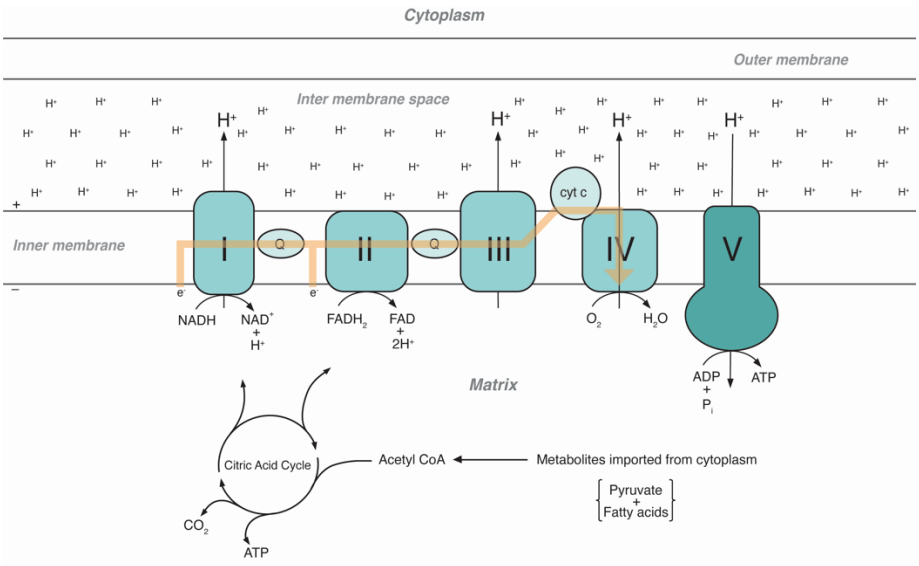

Figure 2. Metabolites derived from food are converted to acetyl CoA and enter the citric acid cycle. Oxidation of acetyl CoA provides electrons and proton ions used by the OXPHOS pathway to create an electro-motive force to fuel the production and release of ATP. The path of the electron transfer is marked in orange and ATP synthase colored in a darker shade.

1.3. The mitochondrial genome

In addition to the eukaryotic nuclear genome, mitochondria have retained genetic material through evolution following endosymbiosis. The mitochondrial genome was first discovered in the 1960's (Nass and Nass 1963, Nass and Nass 1963) and the complete sequence of the human mitochondrial DNA (mtDNA) was obtained circa 20 years later (Anderson, Bankier et al. 1981). Depending on tissue and cell type, cells can have a wide range of mtDNA molecules present, ranging from 1000 to 10 000 copies, and up to 100 000 in oocytes (Bogenhagen and Clayton 1974, Shmookler Reis and Goldstein 1983, Piko and Taylor 1987).

In respect to the nuclear genome, mammalian mtDNA is a small molecule (Figure 3). Human mtDNA is a closed circular double-stranded DNA (dsDNA) molecule with a size of 16 569 base pairs (bp) (Anderson, Bankier et al. 1981). The two strands of the circular mtDNA have different base compositions and can be separated on a cesium-chloride gradient. The strands have been named based on their guanine-cysteine composition, heavy-strand

(H-strand) on account of its G-rich content, and light- strand (L-strand) for its C-rich content respectively (Berk and Clayton 1974). The genetic organization of mtDNA is rather compact with both strands encoding genes, lacking introns and untranslated regions (UTRs). Also, some protein-encoding genes even overlap, often lacking stop codons that are instead added post transcriptionally during polyadenylation of mRNAs (Anderson, Bankier et al. 1981, Ojala, Montoya et al. 1981).

MtDNA encodes 37 genes in total, of which 13 code for protein subunits of the OXPHOS complexes, two for ribosomal RNA subunits (16S and 12S rRNA) for translation of the genetic code, and 22 for transfer RNAs (tRNAs) needed for protein biosynthesis (Gustafsson, Falkenberg et al. 2016). Most of the genes are encoded on the H-strand, whereas only one protein subunit and eight tRNAs are located on the L-strand. Although only 13 out of 90 proteins comprising the electron transport chain are encoded in mtDNA, they are nevertheless essential, with OXPHOS collapsing in the absence of mtDNA (Larsson, Wang et al. 1998). MtDNA also contains two non-coding regions (NCR).

1.3.1. The mitochondrial non-coding regions (NCRs)

A larger NCR, also called control region, is circa 1000 bp long containing two transcription promoters, the light-strand promoter (LSP) and the heavy-strand promoter (HSP), one for each strand respectively, and O_H (Montoya, Christianson et al. 1982). This control region, is known for its unique triple-stranded structure formed during replication initiation, as the nascent H-strand is annealed to the template strand while displacing the parental strand, creating the displacement loop (D-loop) spanning about 650 bp, from O_H to the termination associated sequence (TAS) (discussed later in this thesis and Paper III) (Arnberg, van Bruggen et al. 1971, ter Schegget, Flavell et al. 1971). The sequence of the NCR varies between species, but contains conserved sequence elements of vital importance (Sbisa, Tanzariello et al. 1997). These include the LSP and HSP promoters, three conserved sequence blocks (CSBI-III), and TAS (Bibb, Van Etten et al. 1981, Walberg and Clayton 1981, Sbisa, Tanzariello et al. 1997). Figure 3 (upper panel) illustrates a detailed organization of the O_H control region. A smaller NCR of about 30 nt, situated in a cluster of tRNAs circa two thirds downstream of O_H, is the origin of replication of the L-strand (O_L) (Tapper and Clayton 1981).

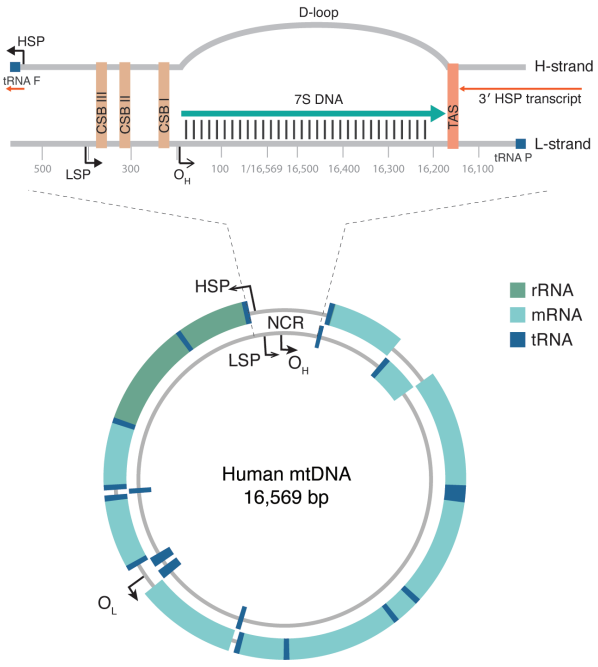

Figure 3. Schematic illustration of human mtDNA. H- strand and L-strand are represented by the outer and inner circle, respectively. Upper panel shows the non-coding region (NCR). The control region contains H-strand replication of origin (O_H), L- and H-strand promoters (LSP and HSP), conserved sequence boxes (CSBI-III), third DNA strand (7S DNA), and termination associated sequence (TAS). HSP transcript start and end at TAS is indicated (orange arrow). L-strand replication of origin (O_L) is indicated on the H-strand further down. (Figure adapted and courtesy of Prof. Falkenberg and Prof. Gustafsson (2020), www.tandfonline.com (with permission)).

1.4. Mitochondrial Transcription

1.4.1. Introduction to transcription

Genetic information stored in DNA needs to be expressed to have a functional role in cellular maintenance. The process of reading the genetic code is called transcription. Common to all organisms, the basic biochemistry of RNA synthesis is catalyzed by large enzymes, RNA polymerases (RNAP). Regardless of differences in size and makeup of these enzymes, the structural similarity of RNAPs from prokaryotes to eukaryotes remains, revealing a mutual evolutionary origin. The eukaryotic nuclear transcription mechanism involves many factors and three different RNAPs, while *Escherichia coli* (*E. coli*) has only one RNAP (Berg 2019). Because of their evolutionary

similarity to eukaryotic RNAP's and simplicity of the organisms, prokaryotic systems are used as models for research, and much of our knowledge about transcription processes stems from studying *E. coli* and bacteriophages. Unlike *E. coli* and the eukaryotic gene expression systems, T7 bacteriophage has only one factor involved, the T7 RNAP. This single subunit enzyme can recognize a promoter sequence, initiate, elongate and terminate transcription on its own. T7 RNAP has no homology, structural nor sequence, to multi-subunit RNA polymerases (Sousa, Chung et al. 1993). Its evolutionary homology to the mitochondrial RNA polymerase has made it the perfect model for mitochondrial transcription research.

1.4.2. Mitochondrial transcription

The mitochondrial promoter cassette is located in the control region. HSP and LSP are in close proximity, within ~150 nt of each other, oriented in opposite directions and of independent functionality (Montoya, Christianson et al. 1982, Bogenhagen, Applegate et al. 1984). Transcription initiated at LSP and HSP yields long polycistronic, near-genome length mRNA molecules of each strand. Termination of LSP transcription takes place at an MTERF1 binding site downstream of the 16S rRNA coding sequence (Asin-Cayuela, Schwend et al. 2005, Terzioglu, Ruzzenente et al. 2013, Shi, Posse et al. 2016). Transcription from HSP in the opposite direction is terminated at TAS (Figure 3 upper panel; Paper III of this thesis). In the mtDNA sequence, tRNAs often flank the non-tRNAs genes (Anderson, Bankier et al. 1981). To obtain individual rRNA, tRNA and mRNA molecules coding for specific genes, the tRNAs are excised from the transcripts, after which the non-tRNA molecules are further processed to produce mature RNA molecules (Ojala, Montoya et al. 1981). Transcription initiated at LSP has an additional role to the genome-length transcript production, namely synthesis of RNA primers for replication of the H-strand (mechanisms described in the next chapter of this thesis) (Falkenberg and Gustafsson 2020).

1.4.3. Mitochondrial transcription initiation factors

Mitochondrial RNA polymerase

Human POLRMT (140 kDa) is a single subunit DNA-dependent enzyme belonging to the polymerase A family of single subunit RNAPs. It is highly conserved and related to the bacteriophage T7 RNA polymerase (T7 RNAP) (Masters, Stohl et al. 1987, Tiranti, Savoia et al. 1997). The POLRMT enzyme is divided into three domains: N-terminal extension (NTE) containing

a tether-helix, N-terminal domain (NTD) and a C-terminal domain (CTD). The CTD of all single subunit RNAPs is highly conserved, folding into a "right hand" structure, with a catalytic palm domain and mobile fingers domain (Ringel, Sologub et al. 2011, Hillen, Morozov et al. 2017). Both CTD and NTD are conserved and structurally similar to the T7 RNAP. In contrast to T7 RNAP, POLRMT requires additional factors to initiate transcription at the promoter region (Falkenberg, Gaspari et al. 2002). The NTE is a feature unique to POLRMT (Gustafsson, Falkenberg et al. 2016). It seems to have an important role in polymerization specificity, where deletion of the NTE in mouse POLRMT showed increased catalytic activity and unspecific transcription initiation events in the absence of TFAM, even at non-promoter regions (Posse, Hoberg et al. 2014). The NTE has recently been visualized in a crystal structure of the transcription initiation complex, revealing a tether-helix involved in promoter recruitment, directly interacting with TFAM (Hillen, Morozov et al. 2017).

Mitochondrial transcription factor B2 (TFB2M)

In 2002, two additional mitochondrial transcription factors, transcription factor B1 and B2 (TFB1M and TFB2M respectively), were discovered based on a homology screen of the yeast transcription factor Mtf1 (Falkenberg, Gaspari et al. 2002). Both proteins are related to rRNA methyltransferases originating from the mitochondrial endosymbiont (Shutt and Gray 2006). TFB1M has retained its methyltransferase activity, and is involved in the stability of the 12 S rRNA small subunit involved in mitochondrial translation (Metodiev, Lesko et al. 2009). Only TFB2M has developed into a *bona fide* transcription factor, stimulating POLRMT-dependent transcription efficiency (Litonin, Sologub et al. 2010). TFB2M (45 kDa), a dimer, forms a heterotrimer with POLRMT on dsDNA and plays an indispensable part in the transcription initiation complex (IC). Upon binding to dsDNA, TFB2M melts the promoter DNA, creating a bubble (Posse and Gustafsson 2017) and exposing the start nucleotide for PORLMT to initiate transcription (Hillen, Morozov et al. 2017).

Mitochondrial transcription factor A (TFAM)

Mitochondrial transcription factor A (TFAM) was discovered as a factor stimulating POLRMT-dependent transcription from LSP and HSP (Fisher and Clayton 1985). TFAM is the third core component of the transcription IC (Shi, Dierckx et al. 2012) and essential for mtDNA maintenance (Larsson, Wang et al. 1998).

TFAM is a protein belonging to a family of high mobility group (HMG) proteins. HMG proteins can be categorized into three groups: HMG-A, HMG-N and HMG box (HMGB). HMGB is by far the largest group and plays many essential roles in DNA-dependent cellular processes and DNA maintenance (Malarkey and Churchill 2012). TFAM is part of the HMGB group. TFAM, is a protein of 246 aa (24 kDa), containing a leader peptide (42 aa) that is cleaved upon mitochondrial import. It contains two HMG boxes (HMG1 and HMG2), a positively charged (basic) 30 aa linker, and a flexible, basic 25 aa C-terminal tail extended from the HMG2 (Figure 4 A) (Parisi and Clayton 1991).

In 2011, the TFAM structure was solved by the Solá and Chan groups (Ngo, Kaiser et al. 2011, Rubio-Cosials, Sidow et al. 2011). This showed that upon binding to dsDNA, TFAM induces a sharp U-turn, bending the DNA 180° in total, with each HMG box responsible for about a 90° bend. Moreover, the two HMG boxes have been shown to bind in a cooperative manner, starting with HMG1 and further progressing with the linker and HMG2 (Rubio-Cosials, Battistini et al. 2018), thus inducing the U-bend in the TFAM/LSP complex. In solution, the linker is proposed to be an unstructured protein sequence, where it forms an α-helix first upon binding to DNA (Rubio-Cosials, Battistini et al. 2018). Mutations in the linker region demonstrate defective DNA bending and transcription activation (Ngo, Lovely et al. 2014), which could be important in nucleoid formation (Figure 4A).

The C-terminal tail of TFAM is essential for transcription activation (Dairaghi, Shadel et al. 1995), interacting directly with the backbone of dsDNA and POLRMT, recruiting it to the promoter region (Hillen, Morozov et al. 2017). Footprinting of TFAM shows specific TFAM binding sites upstream of the promoter (HSP and LSP), about -15 to -45 bp in relation to the transcription start site (TSS) (Fisher, Topper et al. 1987, Fisher, Parisi et al. 1989, Ghivizzani, Madsen et al. 1994, Dairaghi, Shadel et al. 1995, Posse, Hoberg et al. 2014, Posse and Gustafsson 2017). The U-bend is a favorable position in POLRMT recruitment, positioning the C-terminal tail in close proximity to the TSS and enabling transcription initiation, despite a distant binding position (Farge and Falkenberg 2019). Furthermore, TFAM has been proposed to bind to two additional sites (denoted site X and site Y) in the D-loop, located in between CSBI and CSBII (Cuppari, Fernandez-Millan et al. 2019), although the function of these TFAM binding sites have yet to be explained.

Besides its essential role in transcription initiation, TFAM is the major mitochondrial architectural protein, responsible for mtDNA compaction into nucleoids, regulating gene expression and mtDNA replication (discussed later in this thesis and Paper I) (Farge and Falkenberg 2019).

Additional factor involved in transcription

TEFM is a transcription elongation factor that interacts with POLRMT (Minczuk, He et al. 2011). TEFM removes pre-termination events occurring at CSBII (Posse, Shahzad et al. 2015, Hillen, Parshin et al. 2017) and stimulates transcription progression by POLRMT. Moreover, the TEFM structure has been solved in complex with POLRMT, forming the transcription elongation complex (EC) (Hillen, Parshin et al. 2017), suggesting that it replaces TFB2M after initiation.

1.4.4. Mitochondrial transcription initiation model

Studies based on structural, biochemical and biophysical analysis have provided a model for transcription initiation. Transcription initiation begins when TFAM binds to the designated TFAM binding site at the transcription promoter (either HSP or LSP), located about 10-15 bp upstream of the TSS (Gustafsson, Falkenberg et al. 2016). It then induces a U-turn bend in the DNA and recruits POLRMT to the promotor via direct contact involving the C-terminal tail of TFAM and tethers the enzyme via HMG2 and the tether helix of the NTE domain in POLRMT (Hillen, Morozov et al. 2017), anchoring POLRMT to the promoter over the TSS region. TFB2M binds to POLRMT inducing conformational changes within POLRMT, which then stabilizes the open DNA molecule. Via melting the DNA surrounding the TSS and trapping the non-template DNA in the open region, a bubble is created that POLRMT can utilize and begin nucleotide polymerization *de novo* (Hillen, Morozov et al. 2017, Posse and Gustafsson 2017) (Figure 4 B).

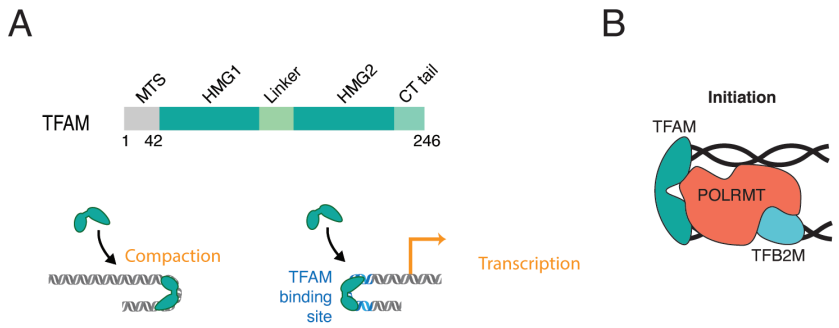

Figure 4. A) Schematic representation of TFAM sequence (upper), and mode of binding at non-specific DNA sequences and designated TFAM binding sites at the promoters (lower). B) Illustration of the mitochondrial transcription initiation complex. (Figure 4A courtesy of Prof. Falkenberg and Dr. Farge (2019, Int. J. Mol. Sci); Figure 4B courtesy of Dr. Viktor Posse (2017)).

1.5. Mitochondrial DNA Replication

1.5.1. Introduction to DNA replication

DNA replication is a highly coordinated mechanism involving a number of factors. All systems have the same core factors at the replication fork, however the number and nature of these factors along with lack of sequence homology can vary from system to system. The replisome consists of: a primosome (helicase and primase activity), a DNA polymerase, a polymerase accessory factor and a single stranded DNA binding protein (SSB) (Benkovic, Valentine et al. 2001, Berg 2019). Each strand of the double helix DNA structure needs to be separated, exposing a single stranded parental, also called template, strand where the DNA polymerase binds to synthesize a new matching daughter molecule in a 5' to 3' direction. DNA polymerases cannot initiate replication *de novo*, and therefore an RNA primer is needed and provided by a primase (Berg 2019). Helicases unwind the double stranded DNA using ATP hydrolysis to drive the strand separation in front of the DNA polymerase at the replication fork. SSB proteins bind to the exposed ssDNA to prevent secondary structure formation and protect it from nucleases. The leading strand is replicated continuously in the 5' to 3' direction, while the lagging strand, replicated in short pieces, called Okazaki fragments. Since DNA polymerases cannot move in the 3' to 5' direction, Okazaki fragments are synthesized in the opposite direction to the moving fork (Alberts 2008, Berg 2019).

To give insight into the eukaryotic replication mechanisms in the nucleus and mitochondria, organisms like *E. coli* and bacteriophages T4 and T7 have been extensively studied. A short description to the T7 replisome follows.

The T7 replisome and replication initiation

In vitro reconstruction of the T7 replisome only needs four proteins: the T7 DNA polymerase gene 5 protein (gp5), the *E. coli* processivity factor thioredoxin (TRX) (Tabor, Huber et al. 1987, Kulczyk and Richardson 2016, Kulczyk, Moeller et al. 2017), the hexameric primase/helicase T7 gene 4 protein (gp4) and an ssDNA binding protein T7 gene 2.5 protein (gp2.5) (Richardson 1983). The polymerase gp5 forms a complex with TRX and the interaction increases the processivity of the polymerase by 100-fold (Johnson and Richardson 2003). The polymerase has also a 3'-5' exonuclease activity involved in proofreading, increasing the fidelity of DNA synthesis (Tabor, Huber et al. 1987, Stano, Jeong et al. 2005). However, it lacks the ability to unwind dsDNA, hence a helicase is required. The ring-forming gp4 assembles on the lagging-strand as a hexamer and does not require a loading factor (Matson and Richardson 1983). During the unwinding of DNA in front of gp5, the helicase/primase gp4 uses the energy released from hydrolysis of dTTP to move in a 5' to 3' direction (Hamdan and Richardson 2009). In the T7 replisome, gp4 is in contact with two gp5/TRX complexes, one on each strand (Delagoutte and von Hippel 2003, Hamdan and Richardson 2009, Pandey and Patel 2014). Primase activity of gp4 synthesizes primers on the lagging-strand to facilitate gp5 creating Okazaki fragments. Finally, gp2.5 binds to the exposed ssDNA, preventing it from reannealing. Moreover, gp2.5 has been suggested to interact via its C-terminal tail with both the gp5/TRX complex and gp4 to coordinate both leading and lagging-strand synthesis. Deletion of its C-terminal tail showed a 4-fold drop in lagging-strand synthesis *in vitro*, while still retaining its ability to bind ssDNA (Lee, Chastain et al. 1998).

1.5.2. The core mitochondrial replication machinery

The human mitochondrial core replisome carries a high resemblance to the T7 phage replisome, which has therefore been implemented as a structure model for the mtDNA replication machinery. At least three factors from the human mitochondrial replisome are related to the T7 replisome: POLγ, replicative helicase TWINKLE and POLRMT (Lecrenier, Van Der Bruggen et al. 1997, Tiranti, Savoia et al. 1997, Spelbrink, Li et al. 2001). Figure 5 depicts a schematic of the replication fork.

DNA polymerase γ

Mitochondrial DNA polymerase γ is the only DNA polymerase involved in mtDNA replication, responsible for synthesis of both the H-stand and L-strand of the mitochondrial DNA molecule (Hance, Ekstrand et al. 2005, Falkenberg and Gustafsson 2020). The POLγ enzyme forms a heterotrimer consisting of one catalytic subunit (POLγA) and two accessory subunits (POLγB) (Gray and Wong 1992, Fan, Kim et al. 2006, Yakubovskaya, Chen et al. 2006).

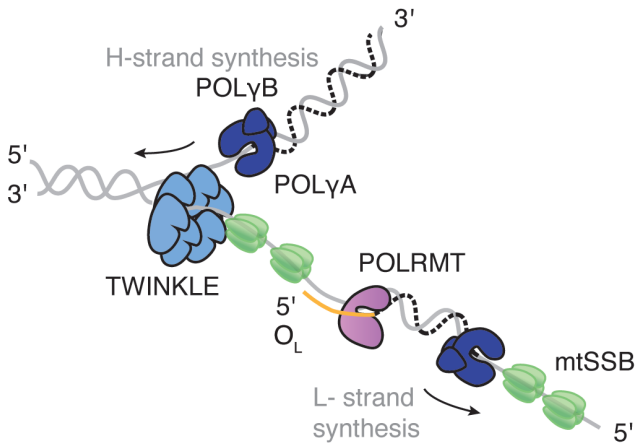

Figure 5. Illustration of the mtDNA replication fork. MtDNA helicase TWINKLE (light blue) loaded onto the parental H-strand moves in a 5'to 3' direction unwinding the dsDNA, making way for POLγ (dark blue) dependent DNA synthesis. MtSSB (green) binds to the displaced ssDNA (parental H-strand) and further stimulates POLγ replicative activity. L-strand DNA synthesis is also dependent on POLγ, using the displaced H-strand as template and POLRMT (purple) to synthesize RNA primer (orange) at O_L. (Figure courtesy of Prof. Falkenberg and Prof. Gustafsson (2020), www.tandfonline.com (with permission)).

The catalytic subunit POLγA (140 kDa) belongs to the family-A DNA polymerases, among which the T7 DNA polymerase (gp5) and bacterial DNA polymerase I are members (Gustafsson, Falkenberg et al. 2016). The enzyme also contains a 3' to 5' exonuclease proofreading domain, required for the high fidelity of DNA synthesis (Gray and Wong 1992, Longley, Nguyen et al. 2001). POLγB (55 kDa) binds to dsDNA in an unspecific manner, acts as a processivity factor for POLγA (Carrodeguas, Pinz et al. 2002), and it is essential for replisome function (Farge, Pham et al. 2007).

TWINKLE helicase

The mitochondrial replicative helicase TWINKLE is related to the T7 helicase/primase gp4 (Spelbrink, Li et al. 2001). TWINKLE (420 kDa) forms a homo-hexameric ring on ssDNA, and harbors three domains: an N-terminal domain (NTD), a C-terminal domain (CTD) and a flexible linker helix connecting the aforementioned two. It has a wide central channel that could accommodate both ssDNA and dsDNA (Fernández-Millán, Lázaro et al. 2015), indicating a conformational flexibility needed for assembly on DNA during its helicase activity. It has been observed that TWINKLE exists in two conformational states at physiological salt conditions, hexameric and heptameric ring-structures when unbound to DNA, and in presence of cofactors Mg^{2+} and NTPs (Ziebarth, Gonzalez-Soltero et al. 2010). TWINKLE is dependent on NTP hydrolysis for its motor function (Singleton, Sawaya et al. 2000, Peter and Falkenberg 2020). The helicase has a 5' to 3' polarity and the activity is stimulated by the mtSSB (Korhonen, Gaspari et al. 2003). Unlike the T7 gp4, TWINKLE has lost its primase function (Shutt and Gray 2006, Ziebarth, Farr et al. 2007, Holmlund, Farge et al. 2009). The primase role has instead been replaced by POLRMT in mammalian mitochondria (Wanrooij, Fuste et al. 2008).

Mitochondrial single stranded binding protein (mtSSB)

In contrast to the rest of the replication machinery, mtSSB is not related to T7 phage gp2.5, but is instead related to *E. coli* SBB (Lohman and Ferrari 1994). Forming a tetramer (60 kDa), mtSSB binds to ssDNA during strand displacement under replication progression, preventing formation of secondary structures and undesired priming (Mignotte, Barat et al. 1985, Tiranti, Rocchi et al. 1993, Miralles Fuste, Shi et al. 2014). MtSSB is known to stimulate processivity of POLγ and the helicase activity of TWINKLE (Korhonen, Gaspari et al. 2003, Falkenberg, Larsson et al. 2007).

POLRMT

POLRMT provides RNA primers necessary for mtDNA replication initiation at both O_H and O_L, although with two distinct mechanisms (discussed later in this thesis).

1.5.3. Strand displacement model of replication

The strand displacement model (SDM) of replication is generally accepted as the mitochondrial mode of replication, first presented in 1972 (Robberson,

Kasamatsu et al. 1972) based on electron microscopy observations of replicative intermediates. The model proposed that DNA synthesis of both H- and L- strands is a continuous procession without Okazaki fragment product formation during replication, on either strand (Tapper and Clayton 1981). With two dedicated origins of replication, one for each strand (O_H and O_L), replication of mtDNA is asymmetrical (Figure 6). However, the process of replication is synchronized in that the parental H-strand needs to be displaced for L-strand O_L to be exposed (Falkenberg and Gustafsson 2020). First initiated at O_H, replication progresses continuously, displacing the parental H-strand. During replication progression with POLγ and TWINKLE at the replication fork, the displaced parental H-strand is covered with mtSSB preventing unwanted priming and formation of secondary structures (Miralles Fuste, Shi et al. 2014). When the replisome has passed about two thirds of the mtDNA (~11 kbp) the O_L region becomes single stranded, forming a stem-loop structure, activating the O_L (Figure 6). The formation of the stem-loop has two purposes, it prevents mtSSB from binding and provides an initiation site for POLRMT (Fusté, Wanrooij et al. 2010, Miralles Fuste, Shi et al. 2014). POLRMT synthesizes a primer of about 25 nt, whereafter it is replaced by POLγ (Martens and Clayton 1979, Wanrooij, Fuste et al. 2008, Fusté, Wanrooij et al. 2010). Replication of the L-strand is continuous, oriented in the opposite direction of H-strand replication. The replication proceeds until the origin of replication is encountered again and replication termination occurs, producing two copies of mtDNA (Gustafsson, Falkenberg et al. 2016).

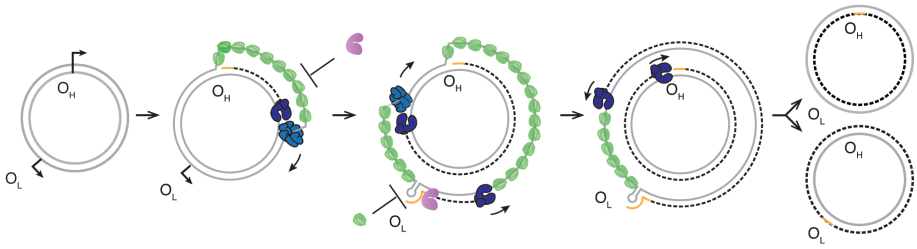

Figure 6. Strand displacement model of mtDNA replication. Upon replication initiation of the nascent H-strand at O_H the replication fork (POLγ – dark blue, TWINKLE – light blue) proceeds, continuously displacing the parental H-strand. MtSSB (green) binds to the displaced ssDNA preventing random priming by POLRMT (purple). As the replication machinery passes two-thirds of mtDNA, the exposed ssDNA on the displaced H-strand folds into a stem-loop structure at O_L and POLRMT can initiate primer synthesis for L-strand replication. The nascent L-strand is continuously synthesized by POLγ in the opposite direction (5' to 3'). Two new daughter molecules are formed when both replication events have reached full circle at the origin of initiation for each strand. (Figure courtesy of Prof. Falkenberg and Prof. Gustafsson (2020), www.tandfonline.com (with permission)).

Alternative models of replication

Two additional modes of replication have been proposed, both based on observations of intermediates on neutral two-dimensional agarose gel electrophoresis (2D-AGE). The strand-coupled model (SCM) suggests a synchronized replication of both H- and L-strands originating from one broad zone. Moreover, replication of the L-strand is postulated to initiate at multiples sites on the parental H-strand where DNA synthesis follows the more conventional mode of replication of lagging strand synthesis in Okazaki-like fragments that are later ligated to form one continuous strand (Holt, Lorimer et al. 2000). Evidence of any interaction between the leading and lagging POLγ enzymes during DNA replication is yet to be demonstrated. In contrast to SDM, the RITOLS model (ribonucleotide incorporation throughout the lagging strand) suggests that instead of mtSSB protecting the displaced parental H-strand, processed RNA is annealed (tRNA, poly adenylated mRNA, and rRNA), forming a temporary second strand (Yasukawa, Reyes et al. 2006, Reyes, Kazak et al. 2013). However, this mode of replication seems unlikely since RNA molecules are folded and modified during maturation and often bound to proteins under normal circumstances. Thus far, no molecular or biochemical explanation has been provided to how mature, processed RNA can be annealed to the displaced H-strand during replication (Falkenberg and Gustafsson 2020). Moreover, arguing against RITOLS is the existence and function of RNase H1 in mammalian mitochondria, an endonuclease actively degrading RNA molecules bound to

ssDNA (Al-Behadili, Uhler et al. 2018, Posse, Al-Behadili et al. 2019). Knockout of the RNase H1 gene in mice caused embryonic lethality due to mtDNA depletion, proving its essential function in mtDNA maintenance (Cerritelli, Frolova et al. 2003, Holmes, Akman et al. 2015).

1.5.4. Replication initiation at O_H and D-loop formation

RNA primer formation

As mentioned at the beginning of this chapter, DNA polymerases cannot synthesize DNA *de novo* and need RNA primers. Transcription initiation from LSP does not only result in genome length transcripts, but also provides primers for replication initiation at O_H (Gillum and Clayton 1979, Chang and Clayton 1985, Chang, Hauswirth et al. 1985). It is not yet understood how primer formation and transition between transcription and replication occurs, thus this subject is under heavy investigation (Falkenberg and Gustafsson 2020).

A triple-stranded structure with nascent RNA in a stable RNA-DNA hybrid formation, called an R-loop, was identified in early studies of the O_H replication initiation mechanisms (Xu and Clayton 1995) in the region containing CSBI-III, downstream of the LSP promoter (Figure 3, upper panel). Pre-terminated LSP transcripts have been mapped to CSBII (Pham, Farge et al. 2006). Studies of the CSB regions demonstrated that CSBII is involved in R-loop formation via G-quadruplex structures (G4) formed due to its G-rich sequence (Wanrooij, Uhler et al. 2012). These DNA G4-structures form hybrids with the nascent RNA transcript, promoting transcription termination (Pham, Farge et al. 2006, Wanrooij, Uhler et al. 2010, Wanrooij, Uhler et al. 2012). The R-loop formed cannot be used as a substrate for POLγ because it has no free 3'-end available (Posse, Al-Behadili et al. 2019), thus the R-loop needs to be processed by RNase H1 before it can be utilized by POLγ.

D-loop formation

About 95% of the initiated replication events at O_H are terminated approximately 650 bp downstream of the mapped O_H position, producing a DNA fragment denoted 7S DNA (Figure 3 upper panel). The nascent 7S DNA remains annealed to the template strand displacing the parental H-strand and forming the so-called D-loop. The precise mechanism of D-loop formation (Clayton 1992) and its role in DNA maintenance is still not understood. The

question if 7S DNA can act as a primer for resumption of mtDNA synthesis *in vivo* still remains, but 7S DNA has been able to accomplish this *in vitro* (Eichler, Wang et al. 1977). At the end of the D-loop and mapping to the 3'-end of 7S DNA are short conserved termination associated sequences (TAS) believed to be involved in the termination of mtDNA replication (Doda, Wright et al. 1981, Walberg and Clayton 1981). It has been proposed that 7S DNA and mtDNA copy number are interlinked when observations of increased mtDNA copy number and reduced pre-termination events were observed after mtDNA depletion with the nucleotide analogue ddC (Brown and Clayton 2002). Analyses of the TAS region have suggested secondary structure formations, however whether these putative secondary structures can form *in vivo* is unknown (Brown, Gadaleta et al. 1986, Pereira, Soares et al. 2008). In Paper III in this thesis, we have identified two related 15 nt palindromic sequence motifs located at CBSI at the 5'-end of 7S DNA and at 3' of the 7S DNA located in the TAS region. We named this motif coreTAS. Interestingly, we could also map the HSP transcript 3'-end to coreTAS on the opposite strand and direction of 7S DNA termination, suggesting *trans*-regulation by these motifs. The function of these two motifs have to date not been elucidated, but could possibly be a binding site for yet to be determined regulatory proteins. A recently published study suggested that G4 structures in the control region, one at CSBII and three in the TAS region of the D-loop are involved in 7S DNA formation and transcription pre-termination. They propose that depending on the stability of G4 structures at CSBII, transcription can either favor full length transcription or RNA priming for DNA-replication, and at TAS favor either 7S DNA formation or full-length replication (Røyrvik and Johnston 2020). Clearly, more work needs to be done in order to understand the D-loop formation.

1.6. Nucleoid formation

1.6.1. An introduction into DNA compaction

Every living cell contains a genome that needs to be structured and compacted to fit into its cellular compartments. The genome is most often larger than the area of the cell it belongs to and in order to fit the genome inside the cell it needs to be compacted. To achieve this, cells use different approaches, with molecular crowding, DNA supercoiling and a variety of basic architectural proteins (Farge and Falkenberg 2019). Even though there is a clear lack of homology between architectural proteins of different organisms, the

mechanism by which they operate to condense the genomic material is highly conserved. It can be categorized into three groups: binding, bending and wrapping of DNA (van Noort, Snel et al. 2004, Dame, Noom et al. 2006). In bacteria these proteins are called bacterial chromatin proteins or nucleoid-associated proteins (NAPs) (Dillon and Dorman 2010). NAPs are highly conserved and at least one NAP is encoded in every bacterial species (Dorman 2014). Apart from their role in compacting DNA into nucleo-protein structures called nucleoids, NAPs also play an important role in gene silencing and overall gene regulation (Browning, Grainger et al. 2010). Some of the most studied and abundant bacterial NAPs are proteins with DNA-bending and DNA-bridging properties: histone-like nucleoid structuring protein (H-NS), factor for inversion stimulation (FIS), integration host factor (IHF) and the histone-like protein (HU) (Figure 7 A). While H-NS, FIS and IHF are found exclusively in *E. coli* and related enterobacteria, HU is among the most conserved NAPs in eubacteria (Dame, Luijsterburg et al. 2005, Skoko, Yan et al. 2005, Luijsterburg, White et al. 2008, Wang, Li et al. 2011, Krogh, Moller-Jensen et al. 2018). During cell growth, the structure of *E. coli* nucleoids is modified, where the nucleoids are more tightly compacted during the stationary phase compared to the exponential phase (Talukder and Ishihama 2015). In eukaryotes, the chromosomal DNA is wrapped around and compacted by histones (Figure 7 A) (Alberts 2008).

1.6.2. Mitochondrial DNA compaction

Abf2p and mtDNA compaction in yeast

The yeast model organism *Saccharomyces cerevisiae* has provided important insights into mammalian nucleoid formation (Miyakawa 2017). The mitochondrial DNA in yeast is a linear molecule as opposed to the circular mtDNA in mammals. About 15% of the total DNA content in yeast comprises of mtDNA, corresponding to circa ~50 copies of mtDNA per cell (Bendich 2010). The first protein to be associated with the nucleoid was *S. cerevisiae* autonomously replicating sequence-binding factor 2 protein (Abf2p) (Diffley and Stillman 1991). Abf2p is a small, architectural DNA binding protein that belongs to the HMG box protein family. It is highly abundant and coats the entire mtDNA, binding approximately ~25-30 bp per each molecule (Diffley and Stillman 1992). Upon deletion of the *abf2* gene rapid loss of mtDNA occurs (Newman, Zelenaya-Troitskaya et al. 1996), indicating the importance of this protein in mtDNA maintenance.

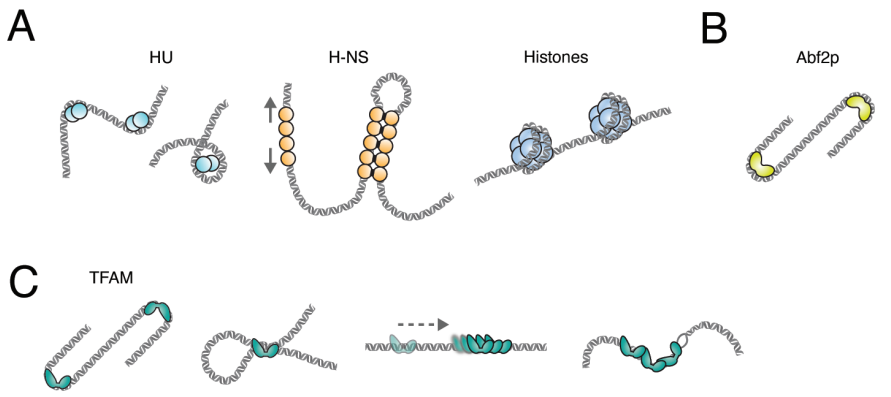

Figure 7. DNA binding and mode of compaction by architectural proteins. A) Bacterial protein HU and H-NS bind to DNA, forming bends and loops, while eukaryotic histones wrap DNA around the protein complex. B) Yeast Abf2p binds DNA and induces a 180° bend. C) Mode of compaction by TFAM, forming a U-turn bend in DNA, looping and local DNA melting to increase DNA flexibility. (Figure courtesy of Prof. Falkenberg and Dr. Farge (2019, Int. J. Mol. Sci.))

The mode of DNA compaction by Abf2p was elucidated with experiments using optical tweezer and visualization by atomic force microscopy suggesting that the compaction occurs when it induces strong bends in the backbone of DNA, whether it be linear or circular molecules. At high protein concentrations of Abf2p the DNA was compacted into a tight nucleo-protein structure (Brewer, Friddle et al. 2003, Friddle, Klare et al. 2004). More recently, the structure of Abf2p bound to dsDNA has been solved (Chakraborty, Lyonnais et al. 2017). It was found that just like its human orthologue TFAM, Abf2p induces a 90° bend with each HMG box, and an overall 180° U-turn bend (Figure 7 B).

1.6.3. The mammalian nucleoid

When mtDNA was first visualized, a lack of histones was noted and mtDNA looked “naked” (Nass, Nass et al. 1965). A mammalian mitochondrial DNA molecule has a contour length of approximately 5 µm, and therefore has to be compacted in order to fit into the space of the mitochondrion, which has a typical size of ~0.5 µm (Nass and Nass 1963, Nass and Nass 1963, Nass 1966). Depending on the tissue and cell type, a cell can contain between 1000 and 10 000 copies of mtDNA, organized in dynamic and inheritable units of DNA-protein complexes called nucleoids (Kuroiwa 1982, Legros, Malka et al. 2004). Nucleoids are often observed associated to the mitochondrial inner membrane (Kaufman, Newman et al. 2000, Hobbs, Srinivasan et al. 2001,

Legros, Malka et al. 2004, Chen and Butow 2005, Wang and Bogenhagen 2006) and were believed to contain multiple mtDNA copies per nucleoid (Iborra, Kimura et al. 2004, Kaufman, Durisic et al. 2007). However, this theory was disproved when the nucleoid was later visualized with higher resolution microscopy to reveal that only one molecule of mtDNA is present (Kukat, Wurm et al. 2011). The shape of the nucleoid shows an elliptical form with a uniform mean size of ~100 nm across tissues and mammalian species, suggesting a formidable evolutionary conservation of mtDNA maintenance and organization (Kukat, Wurm et al. 2011).

The first attempts to purify nucleoids identified two conserved mtDNA binding proteins, mtSSB and TFAM (Mignotte and Barat 1986, Alam, Kanki et al. 2003). Further key components involved in mitochondrial replication (POLγ, TWINKLE) and transcription (POLRMT, TFB2M and TEFM) were later identified to co-purify with the mitochondrial nucleoid (Kaufman, Newman et al. 2000, Spelbrink, Li et al. 2001, Garrido, Griparic et al. 2003, Wang and Bogenhagen 2006, Bogenhagen, Rousseau et al. 2008). Many other proteins have been associated with the nucleoid, among which are RNA-binding proteins, chaperones, proteases, and mitochondrial ribosomal proteins (Bogenhagen, Rousseau et al. 2008, Rorbach, Richter et al. 2008, He, Cooper et al. 2012, Hensen, Cansiz et al. 2014, Rajala, Hensen et al. 2015). However, observations have noted that with the exception of TFAM (Gustafsson, Falkenberg et al. 2016), nucleoid associated proteins are fleeting and different subsets of nucleoids can coexist within the human cells, underlining the dynamic disposition of these structures.

1.6.4. Mammalian mtDNA compaction

TFAM structure has been described earlier in this thesis. This paragraph concentrates on the role of TFAM in mtDNA compaction.

As mentioned earlier, TFAM is the major component of the mitochondrial nucleoid. TFAM is ubiquitously present in mitochondria, about 1000 TFAM protein molecules per mtDNA molecule in mammalian cells. This gives a ratio of one TFAM molecule per 16-17 bp mtDNA, making it able to coat the entire genome (Kukat, Wurm et al. 2011). Besides sequence-specific binding of TFAM to the mitochondrial promoters, TFAM also demonstrates strong non-specific binding to dsDNA, an interesting characteristic for a transcription factor. The same dramatic U-bend is created at non-specific DNA as is seen upon promoter binding (Ngo, Lovely et al. 2014), suggesting

a mode of compaction similar to the bacterial HU protein (mentioned above). Furthermore, upon binding to DNA, TFAM creates small melting bubbles, making the DNA molecule more flexible and prone to compaction (Farge, Laurens et al. 2012, Traverso, Manoranjan et al. 2015). In recent years different techniques have been used to study mammalian mtDNA compaction to elucidate the mechanisms by which TFAM is packaging DNA. Although progress has been made, nucleoid formation is still not fully understood.

In 1992 a mechanism was proposed by which TFAM binds to DNA via bending and wrapping, and would package mtDNA in a similar manner to *E. coli* HU and yeast Abf2p (Fisher, Lisowsky et al. 1992). The solved structure of TFAM in complex with LSP and HSP, and subsequent structural studies, have shone light on the binding mechanisms (Ngo, Kaiser et al. 2011, Rubio-Cosials, Sidow et al. 2011, Ngo, Lovely et al. 2014, Rubio-Cosials, Battistini et al. 2018). Moreover, it has been demonstrated that TFAM binds readily in a positively cooperative manner by sliding on DNA until it encounters another TFAM molecule to bind next to, forming stable filaments of molecules on dsDNA (Figure 7 C), covering approximately 30 bp per TFAM molecule (Farge, Laurens et al. 2012). In addition, packaging of DNA via looping and cross-strand binding by TFAM (Kaufman, Durisic et al. 2007, Kukat, Davies et al. 2015) has been demonstrated. Dimerization of TFAM as an additional mechanism for compaction has been suggested (Ngo, Lovely et al. 2014, Kasashima and Endo 2015, Cuppari, Fernandez-Millan et al. 2019), conversely a dimer mutant (Ngo, Lovely et al. 2014) did not show an inability to compact DNA but rather a reduced capacity. Mentioned earlier in this thesis, the TFAM linker is important in coordination of TFAM DNA binding, it facilitates the seemingly important DNA bend which could be important in nucleoid formation as well. The C-terminal tail of TFAM has been suggested to play a part in stable DNA binding, where lack thereof decreased DNA binding by three-fold (Ohgaki, Kanki et al. 2007). The role of the C-terminal tail of TFAM in DNA packaging is an interesting question and further investigation is necessary to elucidate this.

1.6.5. TFAM in regulation of mtDNA maintenance

Throughout living organisms, DNA compaction is employed as a regulatory function of gene expression and DNA replication, thereby controlling cellular functions and energy requirements in response to environmental stimuli (Farge and Falkenberg 2019). In eubacteria that role is designated to H-NS (Brambilla and Sclavi 2015), in eukaryotes the nuclear genome is regulated by

histones (Finkelstein and Greene 2013). MtDNA regulation is believed to fall to TFAM via nucleoid formation and/or transcription initiation from LSP, which controls both DNA replication and gene expression (Gustafsson, Falkenberg et al. 2016). Studies have shown that TFAM is directly proportional to the copy number of mtDNA in mammalian mitochondria (Ekstrand, Falkenberg et al. 2004) where over-expression of TFAM increases mtDNA and knock-out of the *tfam* gene in mice leads to embryonic lethality due to mtDNA depletion (Larsson, Wang et al. 1998). In Paper I of this thesis we discuss what effect nucleoid formation *in vitro* has on gene expression and DNA replication, where small changes in TFAM levels show drastic effects on the genomes available for transcription and replication. Furthermore, different forms of the nucleoid have been detected in mammalian cells (Kukat, Davies et al. 2015), validating the *in vitro* observation in Paper I.

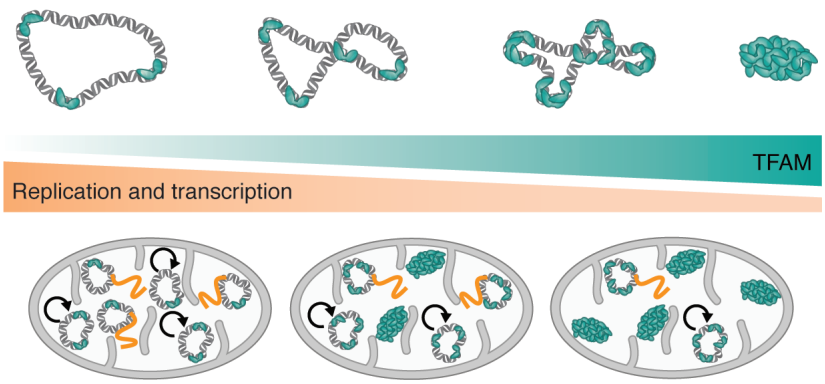

Figure 8. Model of regulation of mitochondrial maintenance. Small changes in TFAM regulate which mtDNA molecules are available for replication and transcription. (Figure courtesy of Prof. Falkenberg and Dr. Farge (2019, Int. J. Mol. Sci.))

This would suggest that TFAM has a role as an epigenetic regulator of mitochondrial DNA, controlling which molecules are accessible to DNA replication and/or transcription (Gustafsson, Falkenberg et al. 2016, Farge and Falkenberg 2019). Figure 8 demonstrates a model for the regulatory role of TFAM in mtDNA maintenance.

In addition, regulation of TFAM levels *per se* can affect nucleoid formation. TFAM is a substrate of the mitochondrial AAA+ protease LON, and is quickly degraded when not bound to DNA. Knockdown of LON in *Drosophila melanogaster* and human HeLa cells shows an increased level of

TFAM and mtDNA copy number (Matsushima, Goto et al. 2010, Lu, Lee et al. 2013) suggesting a controlled steady-state level of TFAM in mitochondria during normal conditions.

1.7. Mitochondrial disorders

Disorders related to mtDNA are a heterogenous group of diseases, which can affect one cell, tissue or be multisystemic. Mitochondrial diseases can arise in several ways: via defective nuclear encoded proteins involved in mitochondrial maintenance, mutations in the nuclear encoded OXPHOS proteins or via defects in the mitochondrial DNA itself (Larsson 2010, Nunnari and Suomalainen 2012). Mutations, deletions and/or depletion of the mtDNA mostly result from pathogenic defects in the replication and transcription machinery encoded in the nuclear genome. Inheritance of mtDNA diseases can occur in various ways. Diseases involving defective transcription and replication factors are inherited through the Mendelian pattern of inheritance, while defective mtDNA through maternal inheritance is more complicated, exhibiting a bottleneck pattern of inheritance (Chinnery, Thorburn et al. 2000). Mitochondrial related disorders are the most common inherited metabolic diseases, with a prevalence of 1 in 8000 being affected (Chinnery, DiMauro et al. 2004). All mitochondrial diseases are ultimately affecting the OXPHOS system and ATP production. Disorders related to OXPHOS insufficiency often affect skeletal muscles and/or the central nervous system and are therefore referred to as encephalomyopathies. Defects in mtDNA or mitochondrial maintenance factors have been linked to a variety of diseases, including autosomal dominant progressive external ophtalmoplegia (adPEO), MELAS (myopathy, encephalopathy, lactic acidosis and stroke-like episodes), MNGIE, and diseases like Parkinson's, Alzheimer's, diabetes, cancer, obesity, (Wallace 2010, Coskun, Wyrembak et al. 2012, Rahman and Copeland 2019) and the natural ageing process (Trifunovic, Wredenberg et al. 2004). Over 200 mutations linked to POLγ (POLγ and POLγB) and TWINLKE have been identified, involved aforementioned diseases as well as in adult-onset of progressive external ophtalmoplegia (PEO), mtDNA depletion syndromes (MDSs), Perrault syndrome and ataxia-related neuropathies (Spelbrink, Li et al. 2001, Fratter, Gorman et al. 2010, Rahman and Copeland 2019, Peter and Falkenberg 2020). Different maintenance factors can have mutations related to the same diseases, or the same mutations can have several different phenotypes. Only a few mutations in TFAM have been reported so far, two point-mutations and

one truncation, all related to mtDNA depletion (Guo, Zheng et al. 2011, Stiles, Simon et al. 2016, Tucker, Rius et al. 2020).

2. SPECIFIC AIMS

Many key components of the mitochondrial nucleoid and maintenance machinery have been identified. Even though major progress has been achieved in the mitochondrial field of research, the molecular functions of many factors involved in mtDNA regulation have yet to be investigated and fully understood. The aim of this thesis was to gain insight into the mechanisms behind the regulation of mitochondrial DNA levels, in major part by addressing the role of TFAM in mtDNA compaction, transcription, and replication. The specific aims are listed below.

Paper I: To study TFAM and its role in mtDNA regulation via compaction and nucleoid formation.

Paper II: To investigate the function and characteristics of the disease causing P178L TFAM mutant.

Paper III: To study the mechanism responsible for the pre-termination events occurring at the end of the mtDNA non-coding region (D-loop).

3. RESULTS AND DISCUSSION

3.1. Paper I

In vitro-reconstituted nucleoids can block mitochondrial DNA replication and transcription

Mitochondrial DNA is not a naked molecule, but is present in the matrix as a nucleo-protein complex called a nucleoid. Much about nucleoid formation, or mtDNA compaction, is still not understood. TFAM is the sole protein responsible for compaction of mtDNA into nucleoids. Studies have shown that TFAM levels *in vivo* are directly correlated to mtDNA copy number, where depletion of TFAM causes depletion of mtDNA, moreover overexpression of TFAM also can cause mtDNA depletion (Ektrand 2004; Pohjoismäki, J.L 2006). Thus, the theory that TFAM regulates mitochondrial genomic expression and copy number emerged. To elucidate this phenomenon, we employed biochemical analyses to study TFAM in its role in DNA compaction.

To determine if compaction of mtDNA can affect strand separation, a process required for both transcription and replication, we simulated DNA unwinding in a single molecule assay. We used optical tweezers to immobilize a dsDNA molecule and applied force to stretch the DNA, which caused strand separation. By adding TFAM, we saw that the protein prefers to bind cooperatively in patches and can indeed block the DNA unwinding. To verify the relevance of these observations for replication elongation, we analyzed mtDNA replication *in vitro*, using a reconstituted replication system containing POLγ, mtSSB and TWINKLE. As template we use dsDNA and TFAM was added to compact DNA. Replication progression decreased already at levels of TFAM corresponding to those observed *in vivo* (1 TFAM/20 bp) and was completely inhibited at 1 TFAM per 8 bp. Using an electrophoretic mobility shift assay (EMSA) we could visualize the nucleoid-like complex formed by TFAM. A stable nucleo-protein structure was initiated at 1 TFAM/20 bp and a full compaction was observed at 1 TFAM/12 bp, which coincided with the inhibition of the replication fork.

To investigate if mitochondrial transcription could be affected in the same manner, we examined transcription *in vitro*, using a reconstituted system comprising TFAM, POLRMT, TFB2M, and a dsDNA template containing the mtDNA HSP/LSP promoter cassette. A drastic decrease in transcriptional activity was observed when the TFAM concentration was increased above the estimated *in vivo* level (1 TFAM/20 bp). Since TFAM is a transcription factor and part of the transcription initiation complex, it posed the question if the inhibition we observed was related to initiation or elongation. To elucidate this, we used a dsDNA template with a single stranded poly-dT stretch at the 3'-end, which POLRMT can utilize to initiate transcription independent of TFAM. Adding TFAM in increasing amounts, we again observed the same decrease and inhibition of transcription progression as previously. We concluded that compaction of DNA with TFAM inhibits transcription elongation.

To visualize DNA compaction at increasing TFAM concentrations, we used atomic force spectrometry. Here, we could discern that at concentrations similar to those observed *in vivo* (1 TFAM/20 bp), dsDNA was present at different states of compaction, ranging from fully compacted nucleo-protein complexes to almost naked DNA molecules. At TFAM levels of 1 TFAM/10 bp, which completely inhibits the transcription and replication machinery, nearly all DNA molecules were fully compacted into tight spherical nucleoid-like structures.

3.2. Paper II

Disease causing mutation (P178L) in mitochondrial transcription factor A results in impaired mitochondrial transcription initiation

A large number of mitochondrial disorders has been discovered since mitochondrial DNA was identified in 1960's. Disease causing mutations affecting mitochondrial replication factors are common, but for a long time, no mutations affecting TFAM were identified. Several cases of early onset liver failure in infants related to mtDNA depletion were reported in the 1980's and 1990's, but the genes and mechanisms involved remained unknown. Recently, a homozygous single point mutation in the nuclear encoded *tfam* gene was identified in two siblings that were diagnosed with early onset liver failure which progressed to death caused by mtDNA depletion (Stiles et al

2016). The mutation causes a single amino acid substitution from proline (P) to leucine (L) at position 178 in the primary protein sequence.

To understand the severe repercussions of this mutation for TFAM function, we cloned and expressed both the wild-type ($TFAM^{WT}$) and the mutant protein version ($TFAM^{P178L}$) in order to compare their activities. We monitored their ability to bind to dsDNA, compact DNA, and to activate transcription. We also analyzed effects on protein stability and performed structural modeling. $TFAM^{P178L}$ binds DNA with similar efficacy as $TFAM^{WT}$. Using acoustic force spectrometry and EMSA we found that the ability to form tight and stable nucleo-protein complexes was slightly impaired in $TFAM^{P178L}$. Earlier observations demonstrating that TFAM can inhibit replication progression through compaction (Farge and Mehmedovic 2014, Paper I) prompted us to investigate if $TFAM^{P178L}$ could do the same. Analysis of replication in a reconstituted *in vitro* system containing POL*γ,* mtSSB and TWINKLE, demonstrated that $TFAM^{P178L}$ was unable to efficiently block progression of the replication fork as compared to $TFAM^{WT}$. This observation agreed with the notion that P178L impaired TFAM's ability to compact DNA.

We obtained patient primary skin fibroblast cells and sought to confirm the reported mtDNA depletion. Using Southern blot to detect mtDNA levels, including the replication pre-termination product 7S DNA, we could indeed confirm mtDNA depletion in patient cells. While mtDNA levels were depleted, 7S DNA remained constant. Finally, we used *in vitro* transcription assays with POLRMT, TFB2M and TFAM on a dsDNA template, containing the cloned mitochondrial HSP/LSP promoter cassette, to monitor the effects the P178L mutation might have on transcription initiation. We observed a dramatic decrease in transcription activity with $TFAM^{P178L}$. Transcript run off from both HSP and LSP were reduced up to ~2.5-fold.

To understand why transcription is impaired by the P178L mutation, we modeled the structure of $TFAM^{P178L}$ based on the already solved crystal structures available. The proline at position 178 forms a turn between α6 and α7. A change from leucine at this position may therefore cause structural changes, including increased structural flexibility in the surrounding region, which may also affect the positioning of the C-terminal tail. These structural changes may disrupt important interactions between POLRMT and TFAM, causing reduction in transcription initiation.

3.3. Paper III

Regulation of DNA replication at the end of the mitochondrial D-loop involves the helicase TWINKLE and a conserved sequence element

Initiation of mitochondrial DNA replication at OriH is dependent on RNA primers, and thus on the transcription machinery responsible for their synthesis. The replication machinery progresses unidirectionally, but most initiated events are prematurely terminated approximately 650 bp downstream of the origin, and as a consequence a triple-stranded structure, referred to as the D-loop, is created (Arnberg, van Bruggen et al. 1971, Kasamatsu, Robberson et al. 1971, Robberson, Kasamatsu et al. 1972). The nascent DNA fragment is denoted 7S DNA and it remains annealed to the parental L-strand. The function of 7S DNA is still unclear, and whether the free 3'-end can be utilized for re-initiation of mtDNA replication *in vivo* is still unknown.

Using comparative bioinformatical analysis of the D-loop we looked for sequence elements that could potentially be involved in transcription and replication regulation in the D-loop region. We identified an evolutionary conserved 15 bp palindromic sequence element located in the extended TAS region (ETAS1) and denoted it coreTAS. We also identified a closely related palindromic motif on the opposite side of the D-loop, at CSBI. It should be noted the two palindromes are positioned on opposite strands of mtDNA (L- and H-strand respectively). Next, using 3' RACE (rapid amplification of cDNA ends) and Northern blotting, we mapped the exact 3'-end positions of 7S DNA and the opposing HSP transcript to coreTAS. Using the same methods, we mapped 7S RNA termination (not to be confused with 7S DNA) to the 15 nt palindrome at CSBI. Based on the similarity of the two motifs and the fact that both elements correspond to transcription termination sites, we speculate that the same mechanism could be responsible for the two transcription termination events. To investigate if coreTAS forms a secondary structure and can terminate replication and termination on its own *in vitro,* we employed reconstituted transcription and replication assays. We found that the coreTAS sequence itself cannot terminate replication nor transcription.

Temporary depletion of mtDNA in cells treated with ddC showed a subsequent increase in full-length DNA replication and reduced 7S DNA formation, i.e. pre-termination events. This observation agrees with data previously published (Brown and Clayton 2002). Correspondingly, we

observed a decrease in transcription termination of HSP transcripts at coreTAS. This led to the conclusion that these two events are functionally linked and regulated via coreTAS. Interestingly, we noticed that in cells recovering from mtDNA depletion, 7S RNA formation by termination at CSBI is lost, leading to the theory that the mechanism regulating 7S DNA and HSP transcription termination could also affect transcription from LSP. Finally, to investigate whether 7S DNA could be used as a primer to re-initiate replication from its 3'-end, chromatin immunoprecipitation (ChIP) followed by next-generation sequencing (ChIP-Seq) or quantitative PCR (qPCR) of POLγ and TWINKLE was employed. High peaks of both POLγ and TWINKLE were observed at O_H, consistent with frequent replication initiation events (Bogenhagen and Clayton 1978, Doda, Wright et al. 1981). However, a second peak of POLγ was observed occupying the end of 7S DNA, where TWINKLE was relatively low. We came to the conclusion that the termination event at the 3'-end of the D-loop is related to TWINKLE unloading. Also, during recovery of mtDNA after mild depletion, our results suggest that TWINKLE can reassemble at coreTAS and POLγ can use 7S DNA as a primer to continue full-length DNA synthesis. We speculate that the unloading of TWINKLE could involve an anti-helicase activity.

4. CONCLUDING REMARKS

Since the discovery of mtDNA more than 50 years ago, significant progress has been made towards understanding the mechanisms of mtDNA replication and expression. Nevertheless, many questions still remain open. The work in this thesis might give some answers regarding the regulation of mtDNA maintenance and D-loop formation.

We have demonstrated that TFAM, through compaction, can regulate mtDNA availability for genomic transactions. Slight variations in TFAM to mtDNA ratios can have extreme effects on transcription and mtDNA replication, and it is thus important to keep TFAM levels within a narrow range. This effect can be seen as a type of epigenetic regulation of mtDNA, where some genomes are silenced while others are available for further genetic interactions. However, why some mtDNA molecules are silenced whilst others are active is still unclear. Does TFAM package mtDNA into nucleoids at random, or is it a controlled action? If so, what other factors could be involved?

The reason for D-loop formation is still unknown. In this thesis we found that the new palindromic sequence located at the 3'-end of 7S DNA is involved in pre-termination of replication and in termination of HSP transcription. TWINKLE binds to the H-strand during replication progression and an anti-helicase activity at coreTAS, preventing further progression along the displaced parental strand, could be a possibility. More research is needed to understand the formation of the D-loop and its purpose in mtDNA replication regulation, as well the mechanism involved in terminating transcription at coreTAS and CSBI. Interestingly, one of the three G4 structures recently mapped in the TAS region (Røyrvik and Johnston 2020) believed to be involved regulation of 7S DNA formation coincides with the location of coreTAS identified in this study. Whether these two motifs have an interconnected role remains to be seen.

Lastly, we have shed some light on the mechanism behind the severe early onset liver failure caused by a mutation in the *tfam* gene. The mutation causes severe mtDNA depletion, but the underlying mechanism was unknown. We concluded that the most probable cause of the severe phenotype of the patients with the P178L mutation in TFAM was impaired transcription initiation. This

impairment might be due to a structural change in HMG2 of TFAM, but until the structure of this mutant is solved, this remains a speculation.

In addition, we also used a truncated TFAM mutant lacking the C-terminal tail ($TFAM^{\Delta 25C}$). Knowing its involvement in transcription initiation is crucial, we believed it to be a good control for impaired function. In contrast to previous reports, lack of the C-terminal tail did not affect DNA binding but compromised TFAM's ability to form stable nucleoid-structures, perhaps suggesting a role in cooperativity in addition to transcription initiation. More investigation into the importance of the C-terminal tail in nucleoid formation could elucidate this hypothesis.

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