Molecular insights into primer removal during mtDNA replication

Ali Al-Behadili

علي محمد باقــر أحمـــد البــهادلــي

Department of Medical Biochemistry and Cell Biology
Institute of Biomedicine
Sahlgrenska Academy, University of Gothenburg

Gothenburg 2021

UNIVERSITY OF GOTHENBURG

Molecular insights into primer removal during mtDNA replication

ali.al-behadili@gu.se
ali.m.albehadili@gmail.com

ISBN 978-91-8009-224-1 (PRINT)
ISBN 978-91-8009-225-8 (PDF)
http://hdl.handle.net/2077/67330

Printed in Borås, Sweden 2021
Printed by Stema Specialtryck AB

“If you ventured in pursuit of glory, do not be satisfied with less than the stars.” Al-Mutanabi

To my beloved family

Molecular insights into primer removal during mtDNA replication

Ali Al-Behadili
Department of Medical Biochemistry and Cell Biology
Institute of Biomedicine
Sahlgrenska Academy, University of Gothenburg

ABSTRACT

Mitochondria are vital for cell survival, and the primary producers of ATP, the energy currency used for various metabolic processes. Mitochondria are unique from other cellular compartments because they have their own genomes of circular small double-stranded DNA (mtDNA) of approximately 16.6 kbp in size. The mtDNA is highly compact, containing no introns and little non-coding DNA. MtDNA has two non-coding regions: one large region known as the control region or the non-coding region that contains the promoters for transcription (LSP and HSP) and the origin of replication of the H strand (OriH), and a smaller region containing the origin of replication for the L-strand (OriL). MtDNA is replicated by a set of replication factors distinct from those needed for DNA replication in the nucleus. A fundamental step in mtDNA replication is the processing of the RNA primers needed for replication initiation.

In this thesis, we could demonstrate that Ribonuclease H1 (RNase H1) is essential for the process of replication initiation at OriH. We could also elucidate the role of RNase H1 during primer removal and ligation at the mitochondrial origin of light-strand DNA synthesis (OriL) and explain the pathogenic consequences of disease-causing mutations in RNase H1.These findings have taken the field of mitochondrial DNA transcription and replication forward and generated knowledge to build further research.

In the last project, we studied EXOG, a mitochondrial exonuclease.

We demonstrated that EXOG could interplay with RNase H1 and other

mitochondrial nucleases *in vitro* and identified a possible pathway for EXOG to function in.

Keywords: mtDNA, RNA primer, RNase H1
ISBN 978-91-8009-224-1 (PRINT)
ISBN 978-91-8009-225-8 (PDF)

SAMMANFATTNING PÅ SVENSKA

Mitokondrier uppfyller en kritisk funktion för cellöverlevnad. I mitokondrier används syre för att oxidera organiska födoämnen och därmed frigöra energi som kan omvandlas till adenosintrifosfat (ATP). Nedbrytning av ATP utgör i sin tur den omedelbara energikällan for cellens energikrävande processer.
Till skillnad från andra cellulära organeller, har mitokondrierna eget DNA (mtDNA), alltså arvsmassa. MtDNA är ett kort cirkulärt dubbelsträngat DNA, motsvarande 16.6 kbp i storlek. MtDNA kopieras med hjälp av en rad olika faktorer som skiljer sig från de som styr DNA kopiering i cellkärnan. Mutationer i någon av faktorer som är involverade i kopiering av mtDNA kan ge upphov till olika sjukdomar. Symtomen för dessa sjukdomar inkluderar muskelförtvining, demens och utvecklingsstörningar. Vår forsking har som mål att på detaljnivå försöka förstå hur mtDNA kopieras. Denna grundläggande kunskap kommer förhoppningsvis på längre sikt kunna hjälpa patienter med mitokondriellasjukdomar.

MtDNA kopiering (replikation) är en komplicerade process, som involverar olika steg. Ett grundläggande steg i mtDNA replikation är avlägsnandet av RNA-primerarna som är nödvändiga för att sätta igång kopieringen av mtDNA. I den här avhandlingen har vi studerat hur kopiering av mitokondriens DNA initieras samt hur RNA-primer avlägsnans. Vi har visat att ett enzym, Ribonuclease H1 (Rnase H1), behövs för både igångsättningen av mtDNA kopieringen samt RNA-primer borttagningen. Vi lyckades för första gången att återskapa denna process i provrör. I tillägg studerade vi de patogena konsekvenserna som uppstår till följd av sjukdomsframkallande mutationer i RNase H1.
Vi påvisade att RNase H1 avlägsnar det mesta av RNA-primern, förutom de tre återstående ribonukleotiderna. Dessa tre ribonukleotider måste avlägsnas med hjälp av ett annat enzym för att slutföra kopieringen.

I sista delarbete, studerade vi samspelet mellan ett mitokondriellt enzym kallad EXOG och RNase H1 i primerborttagningen. Vi visade att EXOG kan ta bort de återstående ribonukleiotiderna efter RNase H1 i provrör. Vi identifierade också en möjlig funktion för EXOG i mitokondrien.

Dessa fynd kan således frambringa mer klarhet inom forskningsområdet kring mitokondriell DNA replikering och har genererat relevant kunskap som kan nyttjas för fortsatta framtida forskning.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. RNase H1 directs origin-specific initiation of DNA replication in human mitochondria.

Posse, V., **A. Al-Behadili**, J. P. Uhler, A. R. Clausen, A. Reyes, M. Zeviani, M. Falkenberg ,C. M. Gustafsson.

PLOS Genetics, 2019; 15(1):e1007781.

II. A two-nuclease pathway involving RNase H1 is required for primer removal at human mitochondrial OriL.

Al-Behadili, A., J.P. Uhler, A.-K. Berglund, B. Peter, M. Doimo, A. Reyes, S. Wanrooij, M. Zeviani, M. Falkenberg.

Nucleic acids research, 2018;46(18):9471-83.

III. *In vitro* characterization of EXOG as a component of a mitochondrial oligonucleotide degradation pathway.

Al-Behadili, A., D. Erdinc, J.P. Uhler, I. Atanassov, T. J. Nicholls, M. Falkenberg.

Manuscript, 2021.

CONTENT

ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
bp	Base pair
CSB1-3	Conserved sequence blocks 1-3
D-loop	Displacement loop
dNTP	Deoxyribonucleosidetriphosphate
dsDNA	Double-stranded DNA
G	Guanine
G4	G-quadruplexes
HSP	Heavy strand promotor
H-strand	Heavy strand of mitochondrial DNA
kbp	Kilo base pair
kDa	Kilo Dalton
LSP	Light strand promotor
L-strand	Light strand of mitochondrial DNA
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
MTS	Mitochondrial targeting sequence
NCR	Control region of mitochondrial DNA
nt	Nucleotides
OriH	Heavy strand origin of replication
OriL	Light strand origin of replication
OXPHOS	Oxidative phosphorylation
Poly-dT	Polydeoxythymidine
RITOLS	The ribonucleotide incorporation model of the lagging strand
R-loop	DNA hybridized RNA loop
rRNA	Ribosomal RNA
SDM	Strand-displacement model of mtDNA replication
ssDNA	Single-stranded DNA
TAS	Termination-associated sequence
tRNA	Transfer RNA

1 INTRODUCTION

1.1 MITOCHONDRIA: ORIGIN AND FUNCTION

Mitochondria are subcellular organelles found in most eukaryotic cells. The discovery of mitochondria dates back to 1850, when Albert von Kollicker described the morphology of mitochondria as grains inside the cells (Scheffler 2008). In 1898, Carl Benda gave these organelles their name. Today, mitochondria are renowned as the cell's powerhouse, and they play a critical role in maintaining cellular metabolism and regulating cell survival and death. Mitochondria convert food molecules and oxygen into adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS). ATP functions as a cellular transporter for chemical energy, which is released when it breaks down into adenosine diphosphate (ADP) and inorganic phosphate (Pi) (Alberts 2015). In addition to OXPHOS, mitochondria play an essential role in the degradation of fatty acids to produce energy, amino acid metabolism and iron homeostasis (Sheftel and Lill 2009, Berg, Tymoczko et al. 2019).

Hypotheses proposing the origin of mitochondria have been around almost since their discovery. In 1890, Altman described mitochondria as bacteria-like colonies in the cytoplasm of host cells (Scheffler 2008). The idea that mitochondria were somehow related to bacteria was discussed without experimental grounds for several decades until, in 1963, Nass and colleagues observed fibres with DNA characteristics in chick embryo mitochondria (Nass and Nass 1963, Nass and Nass 1963). The discovery of separate mitochondrial DNA (mtDNA) shed new light on the origin of these organelles. Today, it is widely accepted that mitochondria originated from α-proteobacteria (Gray, Burger et al. 1999, Gray, Burger et al. 1999, Lang, Gray et al. 1999, Martin, Garg et al. 2015). During evolution, many of the genes in the α-proteobacteria were lost or transferred to the nucleus, leaving only the compact mtDNA molecule, which is essential for OXPHOS (Stewart and Larsson 2014).

The theory describing the origin of mitochondria is called the endosymbiotic theory.

1.2 THE STRUCTURE AND DYNAMICS OF MITOCHONDRIA

In the early 1950s, morphological studies of mitochondria using the electron microscope confirmed that mitochondria are membrane-bound organelles, with two membranes: an outer membrane and an inner membrane. The outer membrane surrounds the organelle, whereas the inner membrane is convoluted and folded into structures known as cristae (Figure 1) (Palade 1952, Palade 1953, Sjostrand 1953). Cristae are the site of OXPHOS (Palmer and Hall 1972). They are usually described as dynamic biochemical reactors, as they can modulate their shape under different physiological conditions to maintain cellular metabolism (Gilkerson, Selker et al. 2003, Cogliati, Enriquez et al. 2016). The outer membrane separates the mitochondria from the cytosol. It contains the voltage-dependent anion channel (VDAC) that allows the passage of metabolites (Colombini, Blachly-Dyson et al. 1996, Forte, Blachly-Dyson et al. 1996) and translocase proteins that can allow the passage of nuclear-encoded proteins, which are needed for mitochondrial functions (Pfanner and Wiedemann 2002).

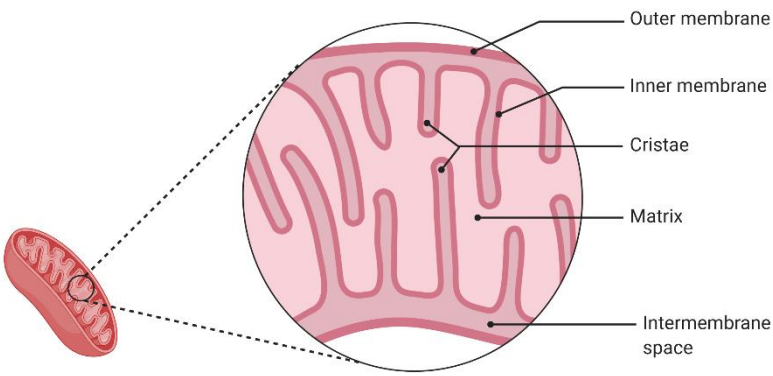

Figure 1. Schematic illustration of mitochondrial structure. The inner compartment of mitochondria, the matrix, is surrounded by the outer and inner membranes. The inner membrane is folded into cristae where OXPHOS takes place.

The internal space of the mitochondria has viscous material called the matrix. The matrix includes the enzymes required for different metabolic processes including the citric acid cycle, the β-oxidation of fatty acids and the amino acid metabolic pathway (Berg, Tymoczko et al. 2019). The mitochondrial genome and its maintenance machinery are also located in the matrix (Falkenberg 2018).

Although mitochondria have their own genome, which is replicated, transcribed and translated inside the mitochondria, they rely on nuclear-encoded proteins to function (Mercer, Neph et al. 2011, Barshad, Blumberg et al. 2018). About 1500 nuclear-encoded proteins are translated in the cytosol and imported into mitochondria via the import machinery located in the mitochondrial membranes (Dolezal, Likic et al. 2006). Intriguingly, all factors needed for replication, transcription and maintenance of mtDNA are of nuclear origin (Falkenberg and Gustafsson 2020).

Various cell types have different energy demands; therefore, the number of mitochondria per cell differs. For instance, high energy-demanding cells such as cardiomyocytes have around 5,000 mitochondria, while keratinocytes have just a few hundred (Alberts 2015). Mitochondria are very dynamic because of their ability to fuse and divide (fission). The fission and fusion of mitochondria allow for the redistribution of genomes, metabolites and proteins; hence, the mitochondria of a cell are denoted as a network rather than static units (Shaw and Nunnari 2002, Chen and Chan 2004, Mishra and Chan 2014).

1.3 MITOCHONDRIAL GENOME

1.3.1 CHARACTERISTICS OF THE MITOCHONDRIAL GENOME

The discovery of mtDNA in the 1960s provided overwhelming support for the prokaryotic origin of mitochondria and opened new horizons in the study of mitochondrial biogenesis (Nass and Nass 1963). MtDNA was first described as a circular, double-stranded molecule with a length of about 5µm (Hudson and Vinograd 1967, Radloff, Bauer et al. 1967). MtDNA molecules mainly exist as monomers but can also be catenated

circles (Clayton and Vinograd 1967, Hudson and Vinograd 1967, Clayton, Smith et al. 1968). Depending on the cell type, mammalian cells contain several hundred to hundreds of thousands of mtDNA molecules per cell (Bogenhagen and Clayton 1974, King and Attardi 1989, D'Erchia, Atlante et al. 2015). MtDNA is maternally inherited (Giles, Blanc et al. 1980). There are two possible mechanisms that can explain the inheritance pattern of mtDNA: (a) the mtDNA copy number is significantly downregulated during spermatogenesis, and (b) sperm mitochondria are actively degraded after fertilization (Kaneda, Hayashi et al. 1995, Larsson, Garman et al. 1996, Larsson, Oldfors et al. 1997, Sutovsky, Moreno et al. 1999).

In the early 1980s, human mtDNA was the first fully sequenced mitochondrial genome. It is 16569 base pairs (bp) in size, and it encodes two ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs) and 13 protein-coding genes (Figure 2) (Anderson, Bankier et al. 1981). The 13 proteins encoded by mtDNA are essential subunits of the OXPHOS system (Chomyn, Mariottini et al. 1983, Mariottini, Chomyn et al. 1983, Chomyn, Mariottini et al. 1985, Attardi, Chomyn et al. 1986, Chomyn, Cleeter et al. 1986, Mariottini, Chomyn et al. 1986). MtDNA is tremendously compact, lacking both introns and spacing between genes. The two strands of the circular human mtDNA can be separated by CsCl2 gradient density centrifugation due to the difference in their guanine and cytosine content (Corneo, Zardi et al. 1968, Berk and Clayton 1974). Consequently, the two strands are termed heavy (H) and light (L). The H-strand encodes 12 proteins, two rRNAs and 14 tRNAs, while the L-strand encodes one protein and eight tRNAs (Falkenberg and Gustafsson 2020).

As the H-strand is guanine-rich, it contains several sequence motifs that can form specific secondary structures called G-quadruplexes (G4s) (Wanrooij, Uhler et al. 2010, Wanrooij, Uhler et al. 2012, Bharti, Sommers et al. 2014).

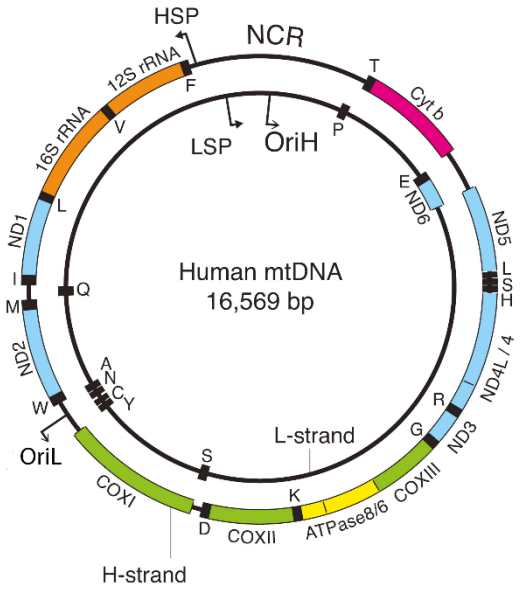

Figure 2. The mitochondrial genome has a size of 16569 base pairs in humans. The two strands are called heavy (H-strand) and light strand (L-strand). The long non-coding region (NCR) contains the origin of replication for the H-strand (OriH) and the transcription promoters for both strands. The genes encoded by mtDNA are: complex III cytochrome b (Cyt b)—pink; complex I NADH dehydrogenase (ND) genes—blue; complex IV cytochrome c oxidase (COX) genes—green; complex V ATP synthase (ATPase) genes—yellow; ribosomal RNA (rRNA)—orange; transfer RNA genes—black boxes.

MtDNA has two non-coding regions: one large region known as the control region or the non-coding region (NCR) (Anderson, Bankier et al. 1981), and a smaller region containing the origin of replication for the L-strand (OriL) (Tapper and Clayton 1981, Shadel and Clayton 1997, Uhler and Falkenberg 2015). The NCR (Figure 3) is one kilobase pair (kbp) in length and contains the origin of replication for the H-strand (OriH) as well as the H-strand promotor (HSP) and the L-strand promotor (LSP) for transcription of each mtDNA strand (Crews, Ojala et al. 1979, Montoya, Christianson et al. 1982, Chang and Clayton 1984). The NCR also contains three highly conserved sequence blocks (CSB 1, 2 and 3) and a termination-associated sequence (TAS) downstream of the three CSBs (Doda, Wright et al. 1981, Walberg and Clayton 1981). Early characterizations of mtDNA showed that the NCR

includes a short triple-stranded region; this region is denoted as the displacement loop (D-loop) (Kasamatsu, Robberson et al. 1971, Brown and Vinograd 1974). The D-loop results from the synthesis of 7S DNA. 7S DNA is a 600 nucleotide (nt) long DNA stretch that remains annealed to the complementary L-strand and is formed by replication initiation at OriH followed by early termination close to the TAS region (Brown, Shine et al. 1978, Gillum and Clayton 1978).

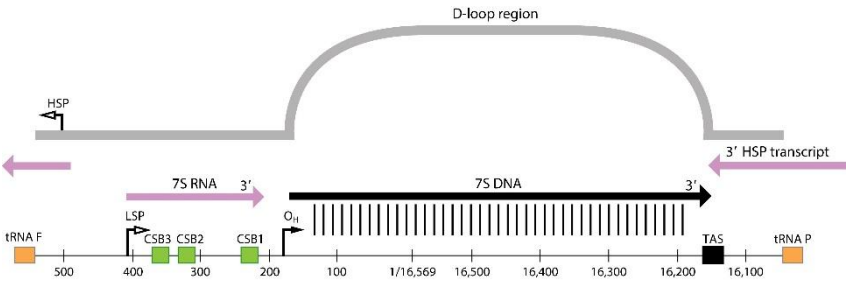

Figure 3. Schematic representation of the long non-coding region of mtDNA. It contains the transcription promoters HSP and LSP, the highly conserved sequence blocks (CSB1-3), the termination sequence (TAS), and the triple-stranded structure (D-loop).

1.3.2 MITOCHONDRIAL NUCLEOIDS

MtDNA is organized in DNA-protein complexes called mitochondrial nucleoids. The term was first used to describe the DNA containing small, rod-like structures in the mitochondrial matrix (Nass 1969). In the following decades, mtDNA-protein complexes were isolated from different organisms (Albring, Griffith et al. 1977, Barat, Rickwood et al. 1985, Miyakawa, Sando et al. 1987), and methods were developed to visualize nucleoids in cells directly (Garrido, Griparic et al. 2003, Ashley, Harris et al. 2005). Super-resolution microscopy techniques have indicated an average of one mtDNA molecule per nucleoid (Kukat, Wurm et al. 2011).

The nucleoid's major protein component is mitochondrial transcription A (TFAM), which is believed to be the mtDNA packaging factor (Farge, Laurens et al. 2012, Kukat, Davies et al. 2015) and regulates the mtDNA copy number (Ekstrand, Falkenberg et al. 2004). The TFAM-to-DNA

ratio affects both transcription and replication. High levels of TFAM (i.e., tight packaging) inhibit replication and transcription *in vitro* (Kaufman, Durisic et al. 2007, Farge, Mehmedovic et al. 2014). Nucleoids also contain proteins that are involved in mtDNA replication, transcription and components of the inner membrane, suggesting that mtDNA may be membrane-associated (Rajala, Gerhold et al. 2014). The organization of mtDNA into nucleoids can render the DNA more resistant to damage (Miyakawa 2017) and provide the proper microenvironment for mtDNA maintenance (Spelbrink 2010).

1.4 MITOCHONDRIAL TRANSCRIPTION

1.4.1 INTRODUCTION TO DNA TRANSCRIPTION

Transcription is a process in which genetic information stored in DNA is rewritten into single-stranded RNA. The RNA is then further processed into functional RNA molecules, such as tRNAs, rRNAs or non-coding RNAs. For protein-coding genes, the transcript (mRNA) is then translated into the corresponding amino acid sequence (Alberts 2015). The enzyme family that carries out the transcription is known as the RNA polymerases (RNAPs). RNAPs initiate transcription from the promotors. Once bound and the DNA strands are separated, the polymerases read the template strand one base at a time and catalyse the building of a nascent RNA strand in a 5'-3' direction.

In general, transcription consists of three steps: initiation at the promoter site, elongation and termination. The transcription machinery can differ in complexity; ranging from a single subunit polymerase system that can do all three transcription steps, as in viruses, to the multi-protein transcription systems found in eukaryotes (Berg, Tymoczko et al. 2019).

1.4.2 MITOCHONDRIAL TRANSCRIPTION MACHINERY

A unique mitochondrial RNAP was first reported in the yeast *Saccharomyces cerevisiae* and named Rpo41 (Greenleaf, Kelly et al. 1986). A decade later, mitochondrial RNAP was found in human cells and named POLRMT (Tiranti, Savoia et al. 1997). Both Rpo41 and

POLRMT show significant homology to RNAP in their T3 and T7 bacteriophages (Masters, Stohl et al. 1987, Tiranti, Savoia et al. 1997). In contrast to the RNAP in bacteriophages, POLRMT can bind to the promoter region in a sequence-specific manner; however, it is incapable of initiating transcription on its own. The additional factors needed for transcription initiation are TFAM and transcription factor B2 mitochondrial (TFB2M) (Falkenberg, Gaspari et al. 2002).

TFAM belongs to the high-mobility group (HMG-box) protein family. It can bind, unwind and bend mtDNA without sequence specificity. Therefore, it plays a vital role in mtDNA packaging into nucleoids (Kaufman, Durisic et al. 2007, Farge, Laurens et al. 2012, Farge, Mehmedovic et al. 2014). In transcription, TFAM binds to the promoter upstream of the transcription start site and recruits POLRMT (Morozov, Agaronyan et al. 2014, Posse, Hoberg et al. 2014).
TFB2M was initially identified based on its sequence homology with yeast mitochondrial transcription activator Mtf1 (Falkenberg, Gaspari et al. 2002). It has structural similarities to rRNA methyltransferases, a group of enzymes that methylate bases of the small subunit of rRNAs (Schubot, Chen et al. 2001). The recruitment of TFB2M to the initiation complex stimulates promoter melting and initiation of RNA synthesis (Posse and Gustafsson 2017).
TEFM is also essential for mitochondrial transcription (Minczuk, He et al. 2011), and it is the transcription elongation factor in mitochondria (Posse, Shahzad et al. 2015).

1.4.3 THE MODEL OF MTDNA TRANSCRIPTION

Both mtDNA strands are transcribed from their respective promoters (LSP and HSP) located in the NCR. Transcription yields large precursor polycistronic RNA molecules (Montoya, Christianson et al. 1982, Chang and Clayton 1984). These molecules are later processed into discrete transcripts via cleavage, polyadenylation and base modification (Sanchez, Mercer et al. 2011).

As discussed in the previous section, three core factors are needed for the initiation of mtDNA transcription. TFAM binds specifically to a sequence located 10–35 nt upstream of the transcription initiation site. These sites are located at both mitochondrial promoters, and TFAM binds them with high affinity (Fisher, Topper et al. 1987). Once bound to the promoter, TFAM induces a stable DNA U-turn and recruits POLRMT (Ngo, Kaiser et al. 2011). POLRMT has an N-terminal extension domain that mediates the interaction with TFAM and positions the active site near the point of transcription initiation (Morozov, Parshin et al. 2015). In complex with the DNA and TFAM, POLRMT undergoes a conformational change that enables the recruitment of TFB2M (Posse, Hoberg et al. 2014, Gustafsson, Falkenberg et al. 2016). TFB2M is needed for promoter melting and to form the first RNA phosphodiester bond (Lodeiro, Uchida et al. 2010, Posse and Gustafsson 2017).

After promoter escape by POLRMT, TFAM and TFB2M are released, and the elongation factor TEFM is recruited to form the elongation complex (Minczuk, He et al. 2011, Posse, Shahzad et al. 2015, Yu, Xue et al. 2018). Recent structural studies have shown that TEFM is necessary to stabilize the POLRMT domain responsible for separating the nascent RNA strand from the template (Hillen, Morozov et al. 2017). In this way, TEFM promotes POLRMT processivity and allows full-length transcripts (Posse, Shahzad et al. 2015, Hillen, Parshin et al. 2017). In the absence of TEFM, transcription from LSP is often terminated around CSB2. The terminated transcript is believed to play an essential role in mtDNA initiation at OriH, which is further discussed in the upcoming sections and Paper I.

1.5 MITOCHONDRIAL DNA REPLICATION: THE MODEL AND THE CORE MACHINERY

1.5.1 A SHORT INTRODUCTION TO DNA REPLICATION

DNA replication is the biological process of copying genetic information during cell division. DNA replication in all organisms is

described as a semiconservative process, which means that one parental strand is conserved in each of the two new daughter molecules (Alberts 2015). DNA replication consists of different steps. Hence, different factors are required to complete the process (Hamdan and Richardson 2009).

DNA polymerases are a group of enzymes responsible for copying DNA. They use the single-stranded parental sequence as a template and synthesise new DNA molecules in the 5'-3' direction. Therefore, additional factors called helicases must unwind the DNA duplex in front of the DNA polymerases in order to separate the parental strands. DNA polymerases cannot initiate DNA replication *de novo.* Instead, they require an RNA primer, a short double-stranded region consisting of RNA annealed to the DNA template (Berg, Tymoczko et al. 2019). RNA primers are often synthesised by specific RNA polymerases called primases. Other factors, such as single-stranded DNA binding protein (SSB), DNA polymerase processivity factors and topoisomerases, are essential for DNA replication in many organisms. The factors required for DNA replication together form the replisome. The front of the replisome is called the replication fork (Alberts 2015, Berg, Tymoczko et al. 2019).

Because of the nature of DNA and DNA polymerase directionality, the two newly synthesised strands must elongate in opposite directions: one in the 5'-3' direction of the advancing replication fork, called the leading strand, and the other in the 5'-3' direction away from the replication fork, called the lagging strand. The leading strand is synthesised continuously, whereas the lagging strand is synthesised in small fragments near the replication fork. These small fragments, called Okazaki fragments, are eventually ligated to become a single strand (Alberts 2015).

DNA replication studies in viruses and bacteria have served as a great model for understanding DNA replication in general. The T7 bacteriophage replication system resembles the mtDNA replication system, and understanding this system is essential to comprehend the

mechanism of DNA replication in the mitochondria. This is discussed further in the following section.

1.5.2 REPLICATION IN THE T7 BACTERIOPHAGE

The replisome of T7 bacteriophage is relatively simple. It can be reconstituted *in vitro* with only four factors: the T7 DNA polymerase (gp5), the polymerase's processivity factor thioredoxin (trx), the helicase/primase (gp4), and SSB (gp2.5) (Hamdan and Richardson 2009). The gp4 protein loads directly onto the DNA, the helicase domain unwinds the DNA duplex, and the primase domain synthesises the needed RNA primer to initiate replication (Figure 4) (Crampton, Ohi et al. 2006, Lee, Hite et al. 2006).

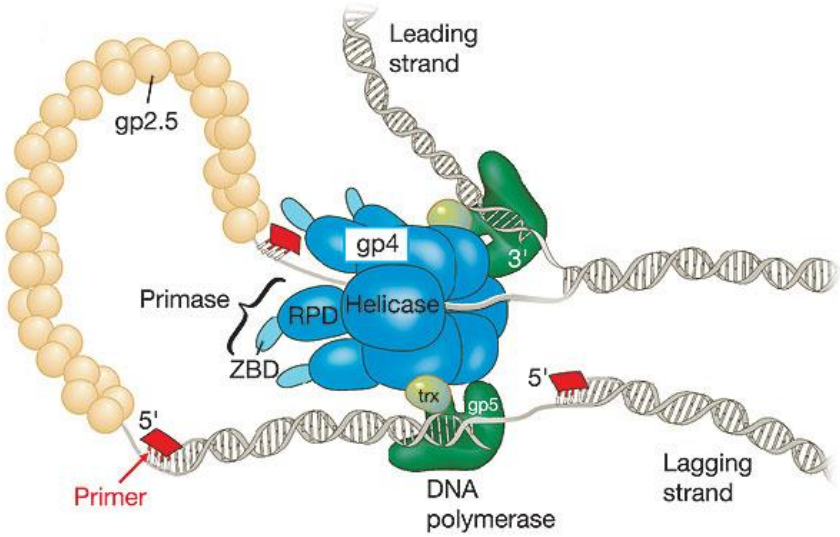

Figure 4. The T7 bacteriophage replication system. gp5 is the DNA polymerase in T7 bacteriophage. Gp4 is responsible for unwinding and primer formation. Trx interacts with gp4 to increase processivity. Gp2.5 is the T7 SSB (from (Lee, Hite et al. 2006) with permission).

Once loaded, gp4 makes contact with the polymerase gp5 and travels in front of the T7 replisome. The processivity factor trx physically interacts with gp5. Therefore, the processivity of the polymerase is increased from several nucleotides up to 1 kb.

The primase domain in gp4 also makes the primers for Okazaki fragments on the lagging strand. Gp 2.5 binds to the single-stranded DNA regions to keep the two strands of DNA separate, increase primase activity and coordinate leading and lagging strand synthesis (Kato, Ito et al. 2003, Crampton, Ohi et al. 2006, Lee, Hite et al. 2006, Hamdan, Loparo et al. 2009, Hamdan and Richardson 2009).

1.5.3 MTDNA REPLICATION MECHANISM

Compared with nuclear DNA replication, mtDNA replication is unique in terms of both the mechanism and the proteins involved. The mitochondrial replisome consists of factors that share homology with the T7 bacteriophage replisome, and they are discussed in detail in the following subchapter.

mtDNA is replicated asymmetrically, which means that both strands are synthesised continuously (Tapper and Clayton 1981). This replication model is called the strand-displacement model of mtDNA replication (SDM) and was first presented by Jarome Vinograd and colleagues in 1972 (Robberson and Clayton 1972). It is strikingly different from the nuclear DNA replication mechanism, but it is similar to the replication of ColE1 plasmid DNA (Masukata and Tomizawa 1990).

Accordingly, mtDNA replication starts with the initiation of H-strand (leading strand) replication at OriH. Then, the replisome proceeds unidirectionally to displace the parental H-strand, and the nascent H-strand is synthesised. When the replication machinery has synthesised around 11 kb of the mitochondrial genome, it passes OriL, which becomes single stranded and forms a stem-loop structure. A short primer is synthesised at the stem-loop, which is used to initiate light strand (lagging strand) DNA synthesis (Fuste, Wanrooij et al. 2010). Once initiated, both strands are synthesized continuously in opposite directions until they have reached full circle. After completing mtDNA synthesis, replication is terminated at either OriH or OriL, depending on the initiation site (Figure 5) (Falkenberg and Gustafsson 2020). An essential feature of the SDM is that the displaced H-strand is coated with

the mitochondrial single-stranded binding protein (mtSSB) (Miralles Fuste, Shi et al. 2014).

Two alternate mtDNA replication models have been proposed, namely the ribonucleotide incorporation model of the lagging strand (RITOLS) and the strand-coupled mtDNA replication model (Holt, Lorimer et al. 2000, Yang, Bowmaker et al. 2002, Yasukawa, Yang et al. 2005, Yasukawa, Reyes et al. 2006, Reyes, Kazak et al. 2013). These two models are mainly based on the mtDNA replication intermediates observed using neutral 2D agarose gel electrophoresis.

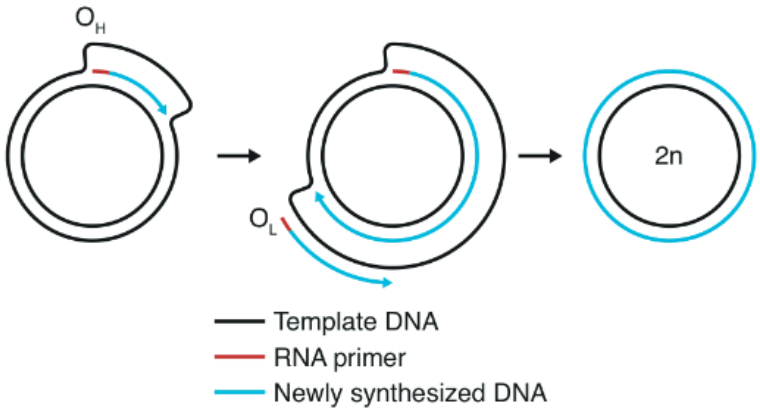

Figure 5. The strand displacement model of mtDNA replication. Replication is initiated at OriH (O_H). The replication machinery proceeds in one direction to displace the parental H-strand. When two-thirds of the genome is synthesised, OriL (O_L) is activated. The replication of the strands proceeds in two opposite directions until two new daughter molecules are formed (2n).

According to the strand-coupled mtDNA replication model, replication is initiated from a region called OriZ, and OriH instead functions as a pause site for the replisome (Bowmaker, Yang et al. 2003). However, this model is questioned because the intermediates on which the replication mechanism was initially proposed were not observed during *in organello* mtDNA labelling (Reyes, Kazak et al. 2013).

RITOLS is similar to the SDM except for what coats the displaced H-strand. This model assumes that ribonucleotides cover the parental H-strand. The ribonucleotides in the RITOLS are processed transcripts

(including tRNA and rRNA), which successfully penetrate the lagging strand template as the replication fork advances; they remain hybridised until they are replaced during the synthesis of the lagging strand, degraded or further processed (Yasukawa, Reyes et al. 2006, McKinney and Oliveira 2013, Reyes, Kazak et al. 2013, Ciesielski, Oliveira et al. 2016).

This model is disputed because the enzymes required to hybridise the RNA intermediates to the DNA and process them later have not been identified (Holt and Jacobs 2014). Furthermore, genome-wide mapping of mtSSB occupancy showed a high degree of protein binding downstream of OriH, which gradually decreased to OriL. From OriL, the protein binding increased again and then dropped gradually to OriH. This suggests that the *in vivo* binding profile of mtSSB is in good agreement with the SDM (Miralles Fuste, Shi et al. 2014).

Besides the *in vivo* evidence of mtSSB occupancy, the SDM is supported by electron microscopy and 5'-end mapping, as well as the biochemical reconstitution of mtDNA replication *in vitro* using recombinant proteins (Clayton 1982, Brown, Cecconi et al. 2005, Wanrooij, Fuste et al. 2008, Wanrooij and Falkenberg 2010, Falkenberg and Gustafsson 2020).

1.5.4 THE MTDNA REPLISOME

The nuclear-encoded polymerase POL γ synthesises the mtDNA. It was first identified in 1977 after isolation from HeLa cells (Bolden, Noy et al. 1977). Together with POL γ, the mtDNA helicase Twinkle and mtSSB are also needed to make the minimal mtDNA replisome (Figure 6) (Korhonen, Pham et al. 2004). Mitochondrial primase activity was first described as early as 1985 (Wong and Clayton 1985), and subsequent work revealed that POLRMT is the protein responsible for primer formation at both origins of replication (Tiranti, Savoia et al. 1997, Wanrooij, Fuste et al. 2008)

The mitochondria DNA polymerase POL γ

POL γ is a heterotrimer enzyme consisting of a 140 kDa catalytic subunit called POL γA and two 55 kDa accessory subunits termed POL γB (Gray and Wong 1992, Fan, Kim et al. 2006, Yakubovskaya, Chen et al.

2006). POL γA possesses DNA polymerase and 3'-5' exonuclease activities (Longley, Ropp et al. 1998). Exonuclease activity serves as a proofreading mechanism to ensure high fidelity, thus minimising point mutations in the mtDNA (Longley, Nguyen et al. 2001, Trifunovic, Wredenberg et al. 2004). Additionally, exonuclease activity is essential for the proper ligation of mtDNA replication intermediates (Macao, Uhler et al. 2015).

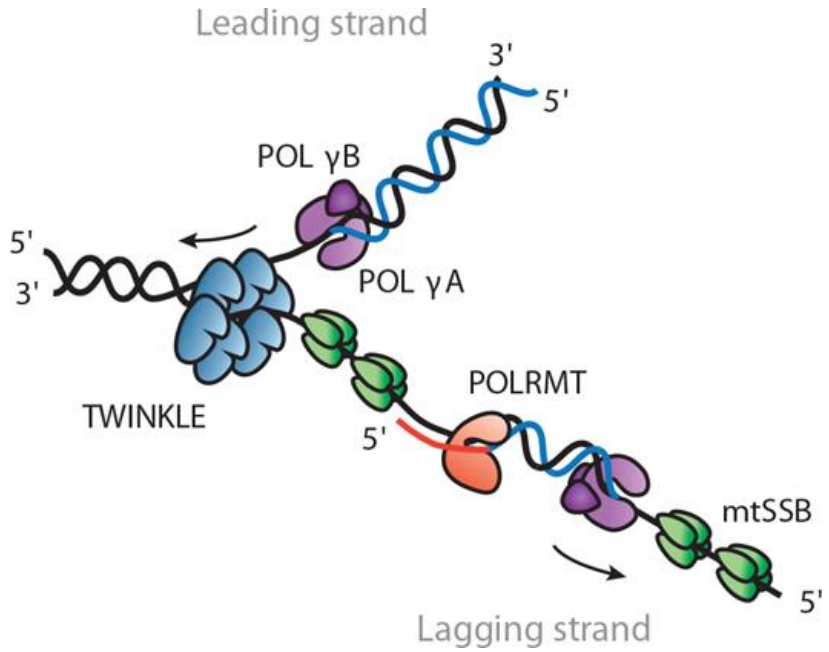

Figure 6. The core mtDNA replication proteins. TWINKLE helicase (blue) unwinds the double-stranded DNA in the 5'-3' direction. mtSSB (green) binds the single-stranded DNA. POL γ synthesises the DNA. POLRMT provides the needed RNA primer.

The accessory subunit POL γB increases the DNA binding affinity, stimulates the exonuclease and polymerase activities of POL γA and improves nucleotide incorporation (Johnson, Tsai et al. 2000, Carrodeguas, Pinz et al. 2002). Therefore, it is also known as the processivity factor. (Lim, Longley et al. 1999). The binding of POL γB to the mtDNA is required for the function of the replisome, as it ensures

the proper coordination of POL γA and TWINKLE at the replication fork (Farge, Pham et al. 2007).

The mitochondrial DNA helicase TWINKLE

TWINKLE (T7 gp4-like protein with intramitochondrial nucleoid localisation) is the replicative helicase in the mitochondria. The 70 kDa protein is nuclear-encoded and colocalises with the mtDNA in DNA–protein complexes (Spelbrink, Li et al. 2001). TWINKLE shares structural similarity with the T7 helicase gp4 and is required to unwind the double-stranded DNA template ahead of POL γ (Korhonen, Pham et al. 2004). It unwinds the DNA in the 5'-3' direction; this activity is stimulated by mtSSB. It can load on DNA, but it requires a fork-like structure with a single-stranded 5' -end and a short 3'- tail to start unwinding (Korhonen, Gaspari et al. 2003, Jemt, Farge et al. 2011).

The mitochondrial single-stranded DNA binding protein mtSSB

Human mtSSB is a small protein (15 kDa) which is homologous to *Escherichia coli* SSB (Tiranti, Rocchi et al. 1993). It forms a tetramer that binds ssDNA (Curth, Urbanke et al. 1994). MtSSB stimulates the activity of both POL γ and TWINKLE (Korhonen, Gaspari et al. 2003, Oliveira and Kaguni 2011). Hence, mtSSB stimulates mtDNA replication elongation and prevents re-annealing of the separated mtDNA strands. MtSSB binds to the H-strand in a gradient from OriH to OriL. Therefore, restricting the initiation of L-strand replication to OriL and preventing any unspecific primer formation are essential (Miralles Fuste, Shi et al. 2014). Recently, we have observed that mtSSB stimulates initiation at OriH (paper I).

Other proteins involved in mtDNA replication

Alongside the core proteins of the mtDNA replisome, additional proteins are required for proper mtDNA replication. These include the factors needed for primer removal (discussed in the following subchapter) and for ligation and separation of the newly synthesised molecules.

DNA ligase 3 (LIG3) is believed to be the enzyme that ligates the nascent mtDNA. The *LIG3* gene encodes a nuclear and mitochondrial variant of the enzyme (Lakshmipathy and Campbell 1999). Depletion of LIG3 leads to reduced mtDNA content and accumulation of nicked mtDNA (Lakshmipathy and Campbell 2001).

When the replication process is completed, the two daughter mtDNA molecules are still interconnected in a region close to OriH and must be separated. Topoisomerase TOP3α was recently found to be the main enzyme in this process (Nicholls, Nadalutti et al. 2018). TOP3α belongs to a class of topoisomerases called type 1A topoisomerase. This family separates the two interlinked DNA molecules by cleaving one of the two strands and let it pass the other strand through the nick (Pommier, Sun et al. 2016). In the absence of TOP3α, catenated mtDNA molecules accumulate, indicating the essential role of this protein in the separation of mtDNA molecules (Nicholls, Nadalutti et al. 2018).

1.6 PRIMER FORMATION AND REMOVAL DURING MTDNA REPLICATION

1.6.1 OVERVIEW OF PRIMER FORMATION

MtDNA replication starts with the synthesis of an RNA primer, which is subsequently elongated by POL γ. As discussed earlier, POLRMT is responsible for all mitochondrial transcription, including primer formation (Wanrooij, Fuste et al. 2008, Fuste, Wanrooij et al. 2010).

POLRMT transcription from LSP creates the near-full-length transcripts that are processed to liberate mRNAs and tRNAs and the pre-terminated transcript that can serve as the primer for replication initiation at OriH (Chang and Clayton 1985, Chang, Hauswirth et al. 1985). The premature transcription termination is caused by a G4 structure that forms at CSB2, located about 120 nt downstream LSP (Figure 7) (Pham, Farge et al. 2006, Wanrooij, Uhler et al. 2010). After termination, this transcript remains hybridised to the DNA template, creating an RNA–DNA hybrid termed the mitochondrial R-loop (Xu and Clayton 1996, Wanrooij, Uhler et al. 2012).

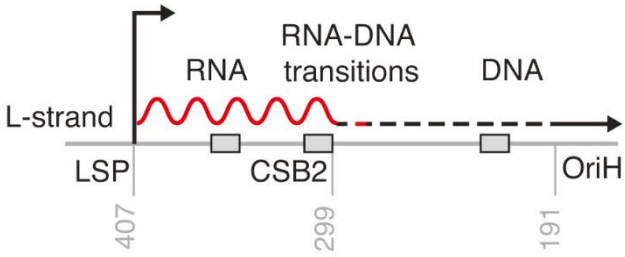

Figure 7. OriH priming. Transcription (red wavy line) from LSP is terminated at CSB2. This serves as an RNA primer after further processing. RNA to DNA transition sites are located close to the CSB region.

Recently, we have elucidated that POL γ cannot directly elongate these R-loops, until they are processed by a particular factor. This process is discussed in detail in paper I. TEFM modulates the level of premature transcription termination, as it facilitates the bypass of secondary structures by POLRMT (Posse, Shahzad et al. 2015). Hence, it can control the switch from transcription to replication (Agaronyan, Morozov et al. 2015).

After initiation at OriH, the replication machinery proceeds unidirectionally, and when it passes OriL, this origin becomes single stranded and forms a hairpin structure (Figure 8). Earlier studies identified a stretch of six bases of thymine (poly-dT) within the loop where RNA formation is initiated (Tapper and Clayton 1981, Wong and Clayton 1985, Wong and Clayton 1985, Kang, Miyako et al. 1997). Subsequent work has revealed that POLRMT specifically recognises the poly-dT stretch in the loop region (nucleotide positions 5747–5751) and generates short RNAs (~25 nt) that can be elongated by POL γ (Wanrooij, Fuste et al. 2008, Fuste, Wanrooij et al. 2010). In accordance with this, RNA–DNA transition sites were mapped just downstream of the OriL stem-loop *in vivo* (Fuste, Wanrooij et al. 2010).

Briefly, H-strand and L-strand DNA replication require a single priming event at the origins of replication, which are OriH and OriL, respectively.

In contrast to the short L-strand replication primer, the RNA primer generated at OriH is longer and requires processing before elongation by POL γ.

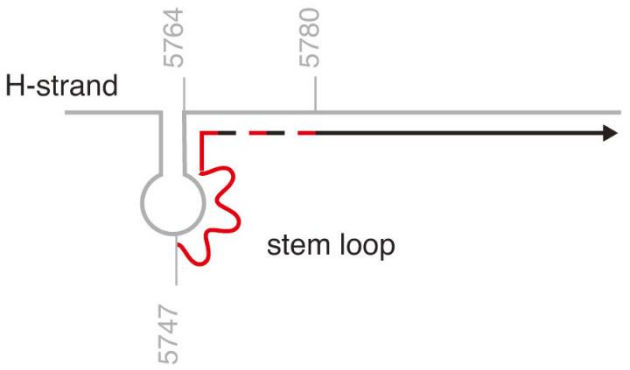

Figure 8. OriL priming involves forming a DNA stem-loop structure that directs a short RNA primer synthesis by POLRMT.

1.6.2 OVERVIEW OF MITOCHONDRIAL PRIMER REMOVAL

Once the replisome has synthesised the full-length mtDNA strands, the RNA primers at both origins must be substituted with DNA, as long stretches of RNA impair mtDNA stability (Wanrooij and Chabes 2019) and can obstruct the replication machinery (Lima, Rose et al. 2007). LIG3 discriminates against RNA (Cotner-Gohara, Kim et al. 2010), suggesting that incomplete RNA primer removal leads to ligation defects (Uhler and Falkenberg 2015). Lessons learned from the nucleus indicate that primer removal involves an interplay between the replicating polymerase and nucleases that can specifically cleave the RNA primer (Zheng and Shen 2011). In the mitochondria, POL γ idles at the 5'- end of the RNA primer, and intrinsic 3'-5' exonuclease activity disengages the strand displacement activity to facilitate both RNA primer removal and ligation (He, Shumate et al. 2013, Macao, Uhler et al. 2015).

A nuclease should meet several standards before it qualifies as a mitochondrial primer processing factor. It must have mitochondrial localisation, it should possess enzymatic activity to process primer

substrates, and the loss of function of such nuclease should cause mtDNA defects *in vivo* (Uhler and Falkenberg 2015). Different factors have been implicated in playing an essential role in the primer removal process in the mitochondria. Ribonuclease H1 (RNase H1) and mitochondrial genome maintenance exonuclease 1 (MGME1) are the main enzymes implicated in primer processing in the mitochondria. The nucleases implicated in primer removal will be discussed in the following section.

1.6.3 NUCLEASES IMPLICATED IN PRIMER PROCESSING

RNase H1

RNase H endonucleases cleave the RNA strand of an RNA–DNA hybrid. Therefore, they have been implicated in primer removal during nuclear lagging-strand DNA replication. There are two classes of RNase H proteins in mammals: RNase H1 and RNase H2. Only RNase H1 is present in the mitochondria (Cazenave, Frank et al. 1994, Cerritelli and Crouch 1998, Cerritelli and Crouch 2009).

RNase H1 consists of a mitochondrial targeting sequence (MTS), a conserved N-terminus called the hybrid binding domain that enhances the recognition and binding of the RNA–DNA hybrid, a spacer region and a conserved C-terminal catalytic domain responsible for RNA cleavage and substrate binding (Cerritelli and Crouch 1998, Nowotny, Cerritelli et al. 2008). *In vitro*, RNase H1 hydrolyses the RNA in RNA–DNA hybrid substrates without sequence specificity. It requires four consecutive ribonucleotides flanking the cleavage site, and it leaves several ribonucleotides attached to the 5'- end of the DNA strand after cleavage (Lima, Rose et al. 2007). RNase H1 can process long RNA hybrids, indicating a remarkable *in vitro* activity to process the primers both at OriH and OriL in the mitochondria (Gaidamakov, Gorshkova et al. 2005). Furthermore, *in vivo* studies indicate substantial evidence that RNase H1 is the main enzyme implicated in primer removal in the mitochondria. Patients harbouring RNase H1 mutations showed chronic progressive external ophthalmoplegia, which is common in mitochondrial disorders and is frequently associated with defects in

mtDNA replication (Reyes, Melchionda et al. 2015, Bugiardini, Poole et al. 2017, Sachdev, Fratter et al. 2018, Carreno-Gago, Blazquez-Bermejo et al. 2019, Kierdaszuk, Kaliszewska et al. 2020).
Evidence from knock-out mouse models also suggests that RNase H1 has an essential function in mtDNA replication. Loss of RNase H1 causes embryonic lethality (Cerritelli, Frolova et al. 2003) and primer retention at both mtDNA origins of replication (Holmes, Akman et al. 2015). Recently, we have reconstituted the primer removal process at OriL *in vitro*. We showed that RNase H1 is crucial for the process, as patients with RNase H1 mutations showed free unligated 5' - ends close to OriL (paper II).
RNase H1 is not only involved in primer removal, but recent work has revealed that it is also necessary to process the R-loops for generating the primer needed for the replication of the H-strand. This suggests that RNase H1 is essential for both primer formation and removal (Paper I).

Mitochondrial genome maintenance exonuclease 1–MGME1
MGME1 was first discovered in 2013. It is an exonuclease with a strong preference for single-stranded DNA, and it localises exclusively in the mitochondria (Kornblum, Nicholls et al. 2013, Szczesny, Hejnowicz et al. 2013). Loss-of-function mutations of MGME1 can cause mitochondrial disease, with patients exhibiting abnormal mtDNA rearrangements (Kornblum, Nicholls et al. 2013, Nicholls, Zsurka et al. 2014).
Analysis of MGME1 patient cells showed an 11 kb truncated linear mtDNA fragment with ends close to OriH and OriL (Nicholls, Zsurka et al. 2014). The OriL end of the linear deletion maps precisely within the RNA primer-DNA transition site, while the more heterogeneous OriH ends span largely within a 100 nt range between OriH and CSB2 (Nicholls, Zsurka et al. 2014). The linear fragment found in MGME1-deficient cells resembles that found in mice and flies which express a 3′-5′ exonuclease deficient version of POL γ (Trifunovic, Wredenberg et al. 2004, Bratic, Kauppila et al. 2015).

MGME1 has been shown to physically interact with POL γ (Nicholls, Zsurka et al. 2014). *In vitro*, MGME1 works in combination with POLγ to facilitate an efficient cleavage of 5'- DNA flaps formed by the strand displacement activity of POL γ (Uhler, Thorn et al. 2016). However, it cannot process short RNA flaps, but it can cut within the DNA of an RNA–DNA hybrid, a few nucleotides downstream of the RNA–DNA junction (Szczesny, Hejnowicz et al. 2013). Taken together, the mtDNA deletions, depletions, and rearrangements observed in the patients and the biochemical properties of MGME1, it is conceivable that MGME1 play a crucial role in primer flap processing at OriH.

Other factors involved in primer removal

Different studies have demonstrated that nucleases play a critical function during primer removal. Flap-structure specific endonuclease 1 (FEN1) is one of them. FEN1 is a 5'-3' specific endonuclease with suggested dual mitochondrial and nuclear localisation (Liu, Qian et al. 2008). It cuts both short RNA and DNA 5' flaps. Therefore, it is implicated in the processing of Okazaki fragments in the nucleus (Stodola and Burgers 2017).

Because of the cleavage pattern of RNase H1, FEN1-like activity is needed in the mitochondria. During OriL primer removal, FEN1 could remove the residual ribonucleotides left by RNase H1 and support proper ligation *in vitro* (paper II). However, the function of FEN1 inside the mitochondria is debated because the variant that is imported is truncated and has lost the nuclease activity (Kazak, Reyes et al. 2013).

The human nuclease/helicase DNA2 is another proposed factor for primer removal in the mitochondria. Mutations in DNA2 lead to mtDNA instability in patients with mitochondrial myopathies (Ronchi, Di Fonzo et al. 2013, Phowthongkum and Sun 2017). DNA2 can cleave substrates with 5' or 3' flaps with equal efficiency (Masuda-Sasa, Imamura et al. 2006). The role of DNA2 in mtDNA primer removal was initially based on its involvement in the nuclear primer removal process, evidence from knock-down studies in cells and the mtDNA defects seen in patients with DNA2 mutations (Masuda-Sasa, Imamura et al. 2006, Copeland

and Longley 2008, Masuda-Sasa, Polaczek et al. 2008, Zheng, Zhou et al. 2008). However, DNA2 did not support ligation when used together with POL γ and LIG3 in vitro (Zheng, Zhou et al. 2008), and it did not support ligation when used together with RNase H1 (paper II).

Recently, endonuclease/exonuclease G (EXOG) has been proposed to play a role in primer removal in the mitochondria (Wu, Lin et al. 2019). EXOG is a 5'-3' nuclease with both exonuclease and endonuclease activities (Cymerman, Chung et al. 2008). EXOG depletion leads to increased single-strand breaks in the mitochondrial genome (Tann, Boldogh et al. 2011). A recent analysis indicated that EXOG could remove the RNA at RNA–DNA junctions that resembled FEN1 activity on these substrates (Wu, Lin et al. 2019). We investigated the possibility of EXOG working with RNase H1 to remove the RNA primer *in vitro*, and the work is presented in paper III. However, *in vivo* evidence for the role of EXOG in primer removal has yet to be clarified.

1.7 MITOCHONDRIAL DISORDERS

Mitochondrial diseases are a heterogeneous group of disorders in which the oxidative phosphorylation system does not function properly due to mtDNA defects. Several tissues are typically affected, in particular tissues with a high-energy demand such as the central nervous system, skeletal muscle and heart. Clinical symptoms are wide-ranging and include progressive external ophthalmoplegia, fatigue, heart failure, diabetes, deafness, and kidney failure (Greaves, Reeve et al. 2012). Furthermore, the age of onset, disease progression, and mortality rates vary greatly across affected individuals.

Mitochondrial diseases can be caused by primary mutations in the mtDNA itself. However, the vast majority are caused by mutations in nuclear genes that encode mitochondrial proteins needed for mtDNA maintenance (Copeland 2012, Copeland and Longley 2014). These mutations in turn lead to secondary mutations in the mtDNA, and/or mtDNA depletion. In the past decades, disease-causing mutations have

been identified in genes for the mitochondrial DNA polymerase POL γ, the replicative helicase TWINKLE, the RNA polymerase POLRMT, and the nucleases MGME1 and RNaseH1 (Zeviani, Fernandez-Silva et al. 1997, Spelbrink, Li et al. 2001, Copeland and Longley 2003, Trifunovic, Wredenberg et al. 2004, Kornblum, Nicholls et al. 2013, Reyes, Melchionda et al. 2015). There is evidence that mtDNA mutations and depletion are also implicated in normal ageing, common neurodegenerative diseases and cancer (Krishnan, Greaves et al. 2007, Greaves and Turnbull 2009).

2 AIM

The overall aim of this thesis is to gain molecular insights into the process of primer removal during mtDNA replication. In the three papers of this thesis, we have addressed different questions regarding the mechanisms of this process. In papers I and II, we studied the role of RNase H1 in two crucial steps of primer processing: formation and removal. In paper III, we demonstrated the activity of EXOG on various substrates, including the intermediates produced during primer removal. The specific aims for each study are listed below.

Paper I: To investigate the role of RNase H1 in processing the R-loop in the CSB region and how it can direct origin-specific replication initiation

Paper II: To reconstitute RNA primer removal at OriL and define the importance of RNase H1 in this process

Paper III: To characterise the activity of EXOG on different substrates representing intermediates that evolve during mtDNA maintenance

3 RESULTS

3.1 PAPER I

RNase H1 directs the origin-specific initiation of DNA replication in human mitochondria

A unique feature of mtDNA replication is that the mitochondrial transcription machinery produces the primers required to initiate DNA replication at OriH. As previously discussed, most LSP transcription events are prematurely terminated after approximately 120 nucleotides, close to CSB2. CSB2 is guanine rich, and during its transcription, the nascent RNA folds into a G-quadruplex structure together with the non-template DNA, forming a stable triple-stranded RNA–DNA hybrid called an R-loop (Wanrooij, Uhler et al. 2012). Previous efforts to link transcription termination at CSB2 to the initiation of mtDNA synthesis *in vitro* have failed, indicating that POL γ cannot directly use the prematurely terminated transcripts as primers (Gustafsson, Falkenberg et al. 2016). Therefore, we wanted to address whether RNase H1 could cleave the R-loops and thereby support the initiation of mtDNA replication. We used our *in vitro* transcription system to reconstitute R-loop formation and monitored the ability of RNase H1 to process the R-loops.

We found that RNase H1 could cut the R-loops at multiple locations, creating new RNA 3'-ends. Unprocessed R-loops could not be used to prime DNA synthesis, but after RNase H1 cleavage, the addition of POL γ and dNTPs led to the initiation of mtDNA synthesis *in vitro*. When we included mtSSB, the efficiency of reactions was increased, demonstrating that mtSSB stimulated the initiation process.

We could also map the transition from the primer RNA to the DNA, and we found that the main points of replication initiation were located downstream of CSB3, with a second less abundant cluster of initiation downstream of CSB2.

Chromosome replication in *Escherichia coli* is initiated at a specific origin. However, in the absence of RNase H activity, this specificity is lost, and initiation occurs from multiple sites (Ogawa, Pickett et al. 1984). Similar to the situation in bacteria, we found that patient cells with reduced RNase H1 activity had lost the specificity of replication initiation, and we could map RNA–DNA transition sites outside the CSB region. Thus, our findings suggest that RNase H1 represses the initiation of mtDNA synthesis from locations outside the OriH region and restricts the initiation of replication to OriH.

3.2 PAPER II

A two-nuclease pathway involving RNase H1 is required for primer removal at human mitochondrial OriL

As described previously, mtDNA contains two origins of replication, OriH and OriL. Replication initiates at OriH, and the replication machinery proceeds in one direction to displace the parental H-strand while synthesising the nascent H-strand. Following the synthesis of approximately two-thirds of the genome, the replication machinery passes OriL, which becomes single stranded and adopts a stem-loop structure. POLRMT binds OriL and synthesises a short RNA primer used to initiate DNA synthesis of the nascent L-strand by POL γ in the opposite direction.

The RNA primer must be exchanged with DNA at the end of replication to ensure proper circularization. In this paper, we used a defined *in vitro* DNA replication system to reconstitute the initiation, elongation and termination of DNA replication at OriL. Our system contained purified recombinant proteins (POLRMT, POL γ, RNase H1 and LIG3) and OriL-encoding templates. We also analysed the consequences of pathological RNase H1 mutations associated with adult-onset mitochondrial encephalomyopathy.

In our experiments, we demonstrated that after initiation of replication at OriL, RNase H1 could process the RNA primer *in vitro*. However, the enzyme failed to remove the last one to three ribonucleotides at the

RNA–DNA junction, because the substrate specificity of RNase H1 requires at least four consecutive ribonucleotides flanking the cleavage site (Cerritelli and Crouch 1998). Because the remaining RNA residues would block ligation by LIG3, another factor must be required for the removal of the last one to three ribonucleotides. A prominent candidate for this function is FEN1, a flap endonuclease implicated in Okazaki fragment maturation in the nucleus (Zheng and Shen 2011). Recently, FEN1 was suggested to have mitochondrial localization (Kazak, Reyes et al. 2013). Indeed, the addition of FEN1 to our *in vitro* system efficiently removed the last ribonucleotides at the 5'- end of the nascent L-strand, thus allowing efficient ligation. The ligation was not observed when we used DNA2 and MGME1 in combination with RNase H1. However, the mitochondrial localization of FEN1 has been questioned, and we are therefore open to the possibility that a second, FEN1-like nuclease can assist in primer processing (Al-Behadili, Uhler et al. 2018). Thus, our findings suggest a two-step mechanism for primer removal at OriL, requiring both RNase H1 and FEN1-like activity.

We could also demonstrate that the pathological RNase H1 mutations impaired RNA primer removal both *in vivo* and *in vitro*. Inefficient removal of the RNA primer led to the formation of unligatable nascent L-strand 5'- ends. We mapped these ends using primer extension and showed they were close to OriL. *In vitro*, the combination of FEN1 with the mutant forms of RNase H1 could not yield ligated products, indicating that RNase H1 is the main enzyme involved in primer removal at OriL, and its activity cannot be compensated for.

3.3 PAPER III

In vitro characterization of EXOG as a component of a mitochondrial oligonucleotide degradation pathway

RNase H1 is incapable of fully processing the RNA primer. RNase H1 cleaves the RNA part in RNA-DNA hybrids but leaves one to three ribonucleotides at the 5'- end of the DNA. The removal of the residual ribonucleotides by a FEN-1-like nuclease is essential for ligation. A

dimeric nuclease called EXOG has been recently proposed to provide FEN-1-like activity in the mitochondria (Wu, Lin et al. 2019). EXOG has both 5'-3' exonuclease and endonuclease activities (Cymerman, Chung, et al. 2008), and interacts with POL γ and LIG3, indicating a possible role for EXOG in mtDNA maintenance (Szczesny, Brunyanszki, et al. 2014).

In this paper, we performed an *in vitro* characterization of EXOG using various substrates. Our data demonstrate that EXOG can efficiently degrade RNA, DNA and short DNA flaps in the 5'-3' direction generating dinucleotides. We also examined the activity of EXOG on RNA-DNA hybrid substrates. We found that EXOG can cleave the residual ribonucleotides left by RNase H1, indicating a possible role during primer removal.

To identify possible EXOG protein partners, we performed a proximity-dependent Biotin identification (BioID) assay. We identified an *in vivo* interaction between EXOG and the mitochondrial ssDNA nuclease MGME1 that we followed up *in vitro*. We found that MGME1 produces cleavage products of approximately four nucleotides that EXOG could process into dinucleotides. This suggests that EXOG and MGME1 may work together.

An additional nuclease in mitochondria, REXO2, degrades RNA and DNA dinucleotides into mononucleotides (Nicholls, Spahr et al. 2019). However, the enzyme/s responsible for creating the dinucleotide substrates for REXO2 is still unknown (Nicholls, Spahr et al. 2019). Interestingly, we found that REXO2 can efficiently process the degradation products of EXOG into mononucleotides. These results suggest that MGME1, EXOG, and REXO2 constitute a pathway for the complete decay of DNA and RNA into mononucleotides in mammalian mitochondria.

4 CONCLUDING REMARKS

The mitochondria are vital for the function of eukaryotic cells. Their unique genome is distinct from the nuclear genome in terms of size, inheritance pattern, replication mechanism, and maintenance factors. In the last decades, significant discoveries have been made in mitochondrial biology, providing a solid base for understanding how mtDNA is replicated. However, many questions have yet to be answered. This thesis's main aim was to shed light on an essential yet poorly understood steps during mtDNA replication, namely primer formation and removal.

We reconstituted the entire replication initiation process at OriH, from primer formation to DNA synthesis. Our work showed that RNase H1 fulfils an essential intermediary step of cleaving the RNA transcript into a mature primer that can be used by POL γ. Furthermore, we showed that RNase H1 is required to restrict priming to the OriH region of mtDNA.

Once replication initiates, it is crucial that the RNA primer is removed to enable ligation of the nascent strands. We reconstituted the primer removal process during mitochondrial L-strand DNA synthesis *in vitro*, providing, for the first time a model that describes this process (Figure 9). We propose a two-nuclease pathway whereby RNase H1 removes the majority of the ribonucleotides, while a nuclease with FEN1-like activity removes the residual ribonucleotides that are resistant to RNase H1.

In the search for a FEN1-like nuclease, we identified EXOG as a possible candidate. We showed that EXOG could process the residual ribonucleotides during the primer removal process when combined with RNase H1 *in vitro*. Future work will focus on EXOG's involvement *in vivo*. We also demonstrated that EXOG activity is not restricted to primer removal, but may also be a crucial factor in the mitochondrial RNA and DNA decay pathways.

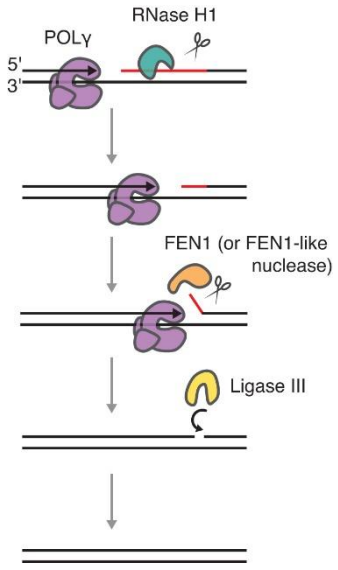

Figure 9. The model of primer removal at OriL. The RNA primer (red line) is removed by RNase H1, leaving behind residual ribonucleotides attached to the 5′ -end of the nascent L-strand. Replicating polymerase POL γ displaces the 5′-end during the completion of L-strand DNA synthesis, and a nuclease with FEN1-like activity removes the last ribonucleotides. Once a ligatable nick is produced, it is sealed by LIG3 (Ligase III).

Our findings provide answers to essential questions regarding mtDNA replication initiation and termination. However, as Ibn Al-Haytham, born c. 965 in Iraq and an early Arab scientist and pioneer in the theory of knowledge, once said; “Scientific findings should show us the path of ignorance, and inspire us to find new questions as long as we live. Once there is no question to answer, you are properly dead (Daneshfard, Dalfardi et al. 2016)". Fortunately, our findings also raise questions for further research. In particular, many unknowns surround the contrast between primer removal at OriH and that at OriL. The primer at OriH is much longer than the primer at OriL. Also, the 5'-ends of nascent H-strand DNA map approximately 100-150 nucleotides downstream of the RNA-DNA transitions at CSB2 and CSB3, which means that a long stretch of DNA is removed in addition to the RNA during primer processing at OriH. How this primer is processed, and why part of the DNA is also removed is not understood. MGME1 and RNase H1 have

been proposed to play a role in this process. However, MGME1 can only cut long DNA flaps and not dsDNA regions. Interestingly, EXOG can cut short flaps (1 nt flaps). Therefore, it would be of interest to study if EXOG can work together with MGME1 to process the DNA part of the primer at OriH.

ACKNOWLEDGMENT

First and foremost, I would like to express my deep gratitude to my supervisor **Maria Falkenberg**! You have been a great supervisor, mentor, and role model. Thank you for sharing your knowledge, guidance, and for your patience with me. You have truly inspired me during these years and shaped me to become a better researcher. Thank you deeply for believing in me and for all the invaluable support at moments I was about to give up. It has been an honour and a true pleasure to be under your supervision. I could not find a title that can describe a great supervisor like you, and I could not find enough gratitude words for your efforts to help me during the years.
I would also like to thank my co-supervisor **Chandrasekhar Kanduri**.
I also want to express my gratitude to **Claes Gustafsson.** Thank you so much for your knowledge sharing and for your support and encouragement during these years. Your door is literally open all the time, and it never gets closed. We talked several times about general issues, and every time I feel that I need to speak to you more and more.

My next thanks go of course to **Jennifer Uhler!** I was privileged to work with you. Your constant support, guidance and invaluable efforts during these years will never be forgotten. You took care of me from the very start and taught me a lot. This shaped and helped me massively during these years. I learned so much from you. Thank you **Jay**!
Thomas Nicholls, I was so lucky to start my PhD journey when you were in the lab, you shared your knowledge and I learned a lot from you. You kept your door open, and you were available for my questions via the e-mail. Thank you Tom! and thank you may not be enough.
Next, I would like to thank **Viktor Posse**, **Anna-Karin Berglund Bradley Peter** and **Anders R. Clausen**. Your invaluable contributions made my projects possible to conduct. It has been a true honour to work with you, and I learned a lot from you. I would further like to thank all my co- authors for their work and contribution to the projects presented in this thesis.

Bertil Macao, Thank you for all your help, ideas, suggestions and encouragement during the years. I disturbed a lot with technical issues, especially with the ÄKTA, and you were always available with a smile.
Örjan Persson I cannot imagine how difficult my journey in the lab would be without you. Your ideas helped me massively during the years. Your door was always open for my stupid questions or my complaining about failed experiments. Tack Örjan!
Emily Hoberg and Loiuse Jenninger, thank you for your help and friendly chats during the years. It was a great pleasure to know you.
Monica Olsson thank you for sharing your protein purification tricks.
Hector Diaz, Laleh Arabanian, Xie Xie, Yonghong Shi, Xuefeng Zhu and Zsolt Szilagyi thank you for excellent suggestions, massive help and great chats.
Benedict Tan, it has been very great working with you over the past years and thanks for all the help with broken sequencing gels!
Anup Mishra what a great person you are. I am really lucky to know you and have chats with you. Thank you for all support and encouragement and for having time for my random questions.
Maru Sánchez-Sandoval, Thanks for cheering me up even on my darkest days and sharing your nice stories, ideas and wisdom!
Layal Ishak شكراً جزيلاً لما تركتيه من اثر طيب في النفس، ولما قدمتيه من مساعدة قيمة
Anna Wramstedt Good luck with your thesis.
Carlos Pardo Hernández, my office mate. It was a great pleasure to share the moments during the last years with you at the same office. Thank you for the pleasant memories and teaching me some Spanish. I'll be available for the next FIFA world cup edition, to take the revenge. Good luck with your thesis.
Direnis Erdinc, Gunaydin. Thank you for your help with the BioID, and thank you for unforgettable memories in the lab. As the meaning of your name, your resistance to never give up is inspirational. Keep up the good work and good luck with your thesis.
Meenakshi Singh, Thank you for all the exciting stories, your support, and kind help. It had been a great pleasure to know you. You are a friend for life. Good luck with your thesis.

Majda Mehmodovic Congratulation Dr. Majda for great thesis defence and thank you for all your help and interesting stories during the years. It was beneficial that we had the process of writing nearly the same time, thank you for keeping your door open for any help.
I want to thank all the people working at the **Maria Falkenberg** and **Claes Gustafsson** labs, former and new colleagues. Thank you for making an excellent and friendly scientific environment. Thanks to all colleagues on our floor for contributing to a great workplace.

My previous supervisors, **Jonas Warringer**, **Per Sunnerhagen** and **Xiayong Wang**. Thank you for all support and all the knowledge you shared with me to prepare me for the PhD journey.

My dear university-friends: **Majd, Zaed, Kamal, Mustapha, Arman, Johan and Martin**. Thank you for all your encouragement, support and unforgettable memories.

My dear brothers and close friends: **Murtadha, Mohammed Almahdi and Ahmed**. I am lucky to have you in my life. Thank you for all your support and help. و ما أجمل الايام بصحبتكم، شكراً لكم وادامكم الباري لي ذخراً

My dear son **Alhussain**, you just asked me what I am doing, and I said I am writing a letter to you. You cannot read it now, but one day, hopefully, you will read it. I have been busy and away many times during the first four years of your life. I missed one birthday because of a deadline. So while I thanked everyone, I would like to apologize for missing such great moments with you. You are the pleasure of my life, the reason that keeps me motivated and only your smile can make me deeply happy. I love you! وكل الناس يا ابتي مياه، ووحدك زمزم يروي فؤادي

My dear love and soulmate, **Zainab**, you are the candle during darkest moments in life, and you are the cure for the heart. You are a special gift from the heavens. Your smile warms my heart, and your presence makes me whole. No greater light illuminates my path than the love you have

brought into my life. Thanks, my love. Sorry for being away and taken with the PhD journey, it is finally over, and we'll only have the great moments to remember. فكل مكان فيه شخصك جنة وكل زمان انت فيه ربيع

To a special one:
منذ نعومة اظفاري، اراك الملهم، ومنذ اولى لحظات الوعي، كنت انتظر اللحظة التي اهديك فيها كتاباً وضعت فيه الجهد. لا ادري ربما لأنني نشأت وانا اراك تكتب او انها لحظات حلم لأكون مثلك، لكنني كنت دائماً اتمنى ان اهديك كتاباً انا اكتبه.
هل تعرفني؟ لا ادري، لكنني لن انساك ما حييت.
حينما كنت طفلاً حاولت كثيراً ان اكتب واهديك، ربما لأسابق الزمن وانال كلمات المدح منك، سيدي اكتمل الكتاب الذي كنت احلم فيه، وها انا اكتب هذه الكلمات من القلب اليك. يوماً ما سيكون هذا الكتاب بين يديك، حينها ستلمع عيناي ويزهو القلب فرحاً.

To my parents, **Mohammed Baqer** and **Ateka** thank you for the never-ending love, continuous support and care! You have always been there for me. You mean everything to me.

والدي العزيز والدتي العزيزة بشكركما اختم هذا الجهد. وما الشكر بكافٍ، كيف لكلمات قواميس اللغات ان تسع الشكر لمجهودكما في صنعي وتربيتي؟
والدي العزيز، كيف لي ان اؤدي الشكر، وانا اتذكر ذلك الوجه المتعب القادم بكل لهفة ليهديني كتابي الاول؟ كيف لي ان اؤدي الشكر ولا زلت اتذكر جهدك بتلقيني فنون الخطابة، بتلك الليلة الممطرة، وفي خضم انشغالك بين طلب العلم وتوفير العيش الكريم لنا؟
كيف لي ان اؤدي الشكر، وانا اتذكر كل تلك اللحظات التي لم اكف فيها عن الطلب بأنانية، او التصرف بأنانية، ولم تكف عن تحقيق الطلبات؟
والدتي العزيزة، كيف لي ان اؤدي الشكر وكنت انت الراعية لي خطوة بخطوة، كشمعة تشتعل لتنير الدرب لنا ؟ كيف لي ان اؤدي الشكر وانا لا زلت اتذكر كم الليالي التي سرقت فيها راحتك لتعتكفي على تدريسي؟ كيف لي ان اؤدي الشكر، وانا اتذكر كل تلك الخيبات والالام التي سببتها لك في الرحلة نحو هذا الكتاب، رحلة الثلاثين عاماً؟
لن اؤدي الشكر بالتأكيد، حتى وان كتبت صفحات وصفحات، ولكنني اقدم قليلاً من الشكر مع الاعتذار لمقامكما عن كل تقصير، فاقبلا مني هذا القليل، فما هو الا صنيعتكما.
الهي مننك علي لا تعد .. ولا تحصى .. ولا ادري اي النعم اشكر .. وفقني على اداء نعمتك ..
الهي وفقني لاكون شاكرا لهما باليسير .. وادم ظل حنانهما ورأفتهما علي.

REFERENCES

Agaronyan, K., Y. I. Morozov, M. Anikin and D. Temiakov (2015). "Mitochondrial biology. Replication-transcription switch in human mitochondria." Science **347**(6221): 548-551.

Al-Behadili, A., J. P. Uhler, A.-K. Berglund, B. Peter, M. Doimo, A. Reyes, S. Wanrooij, M. Zeviani and M. Falkenberg (2018). "A two-nuclease pathway involving RNase H1 is required for primer removal at human mitochondrial OriL." Nucleic acids research **46**(18): 9471-9483.

Alberts, B. (2015). Molecular biology of the cell. New York, NY, Garland Science, Taylor and Francis Group.

Albring, M., J. Griffith and G. Attardi (1977). "Association of a protein structure of probable membrane derivation with HeLa cell mitochondrial DNA near its origin of replication." Proc Natl Acad Sci U S A **74**(4): 1348-1352.

Anderson, S., A. T. Bankier, B. G. Barrell, M. H. de Bruijn, A. R. Coulson, J. Drouin, I. C. Eperon, D. P. Nierlich, B. A. Roe, F. Sanger, P. H. Schreier, A. J. Smith, R. Staden and I. G. Young (1981). "Sequence and organization of the human mitochondrial genome." Nature **290**(5806): 457-465.

Ashley, N., D. Harris and J. Poulton (2005). "Detection of mitochondrial DNA depletion in living human cells using PicoGreen staining." Exp Cell Res **303**(2): 432-446.

Attardi, G., A. Chomyn, R. F. Doolittle, P. Mariottini and C. I. Ragan (1986). "Seven unidentified reading frames of human mitochondrial DNA encode subunits of the respiratory chain NADH dehydrogenase." Cold Spring Harb Symp Quant Biol **51 Pt 1**: 103-114.

Barat, M., D. Rickwood, C. Dufresne and J. C. Mounolou (1985). "Characterization of DNA-protein complexes from the mitochondria of Xenopus laevis oocytes." Exp Cell Res **157**(1): 207-217.
Barshad, G., A. Blumberg, T. Cohen and D. Mishmar (2018). "Human primitive brain displays negative mitochondrial-nuclear expression correlation of respiratory genes." Genome Res **28**(7): 952-967.

Berg, J. M., J. L. Tymoczko, G. J. Gatto and L. Stryer (2019). Biochemistry. New York, W.H. Freeman/Macmillan Learning.

Berk, A. J. and D. A. Clayton (1974). "Mechanism of mitochondrial DNA replication in mouse L-cells: asynchronous replication of strands, segregation of circular daughter molecules, aspects of topology and turnover of an initiation sequence." J Mol Biol **86**(4): 801-824.

Bharti, S. K., J. A. Sommers, J. Zhou, D. L. Kaplan, J. N. Spelbrink, J. L. Mergny and R. M. Brosh, Jr. (2014). "DNA sequences proximal to human mitochondrial DNA deletion breakpoints prevalent in human disease form G-quadruplexes, a class of DNA structures inefficiently unwound by the mitochondrial replicative Twinkle helicase." J Biol Chem **289**(43): 29975-29993.

Bogenhagen, D. and D. A. Clayton (1974). "The number of mitochondrial deoxyribonucleic acid genomes in mouse L and human HeLa cells. Quantitative isolation of mitochondrial deoxyribonucleic acid." J Biol Chem **249**(24): 7991-7995.

Bolden, A., G. P. Noy and A. Weissbach (1977). "DNA polymerase of mitochondria is a gamma-polymerase." J Biol Chem **252**(10): 3351-3356.

Bowmaker, M., M. Y. Yang, T. Yasukawa, A. Reyes, H. T. Jacobs, J. A. Huberman and I. J. Holt (2003). "Mammalian mitochondrial DNA replicates bidirectionally from an initiation zone." J Biol Chem **278**(51): 50961-50969.

Bratic, A., T. E. Kauppila, B. Macao, S. Gronke, T. Siibak, J. B. Stewart, F. Baggio, J. Dols, L. Partridge, M. Falkenberg, A. Wredenberg and N. G. Larsson (2015). "Complementation between polymerase- and exonuclease-deficient mitochondrial DNA polymerase mutants in genomically engineered flies." Nat Commun **6**: 8808.
Brown, T. A., C. Cecconi, A. N. Tkachuk, C. Bustamante and D. A. Clayton (2005). "Replication of mitochondrial DNA occurs by strand displacement with alternative light-strand origins, not via a strand-coupled mechanism." Genes Dev **19**(20): 2466-2476.

Brown, W. M., J. Shine and H. M. Goodman (1978). "Human mitochondrial DNA: analysis of 7S DNA from the origin of replication." Proc Natl Acad Sci U S A **75**(2): 735-739.

Brown, W. M. and J. Vinograd (1974). "Restriction endonuclease cleavage maps of animal mitochondrial DNAs." Proc Natl Acad Sci U S A **71**(11): 4617-4621.

Bugiardini, E., O. V. Poole, A. Manole, A. M. Pittman, A. Horga, I. Hargreaves, C. E. Woodward, M. G. Sweeney, J. L. Holton, J. W. Taanman, G. T. Plant, J. Poulton, M. Zeviani, D. Ghezzi, J. Taylor, C. Smith, C. Fratter, M. A. Kanikannan, A. Paramasivam, K. Thangaraj, A. Spinazzola, I. J. Holt, H. Houlden, M. G. Hanna and R. D. S. Pitceathly (2017). "Clinicopathologic and molecular spectrum of RNASEH1-related mitochondrial disease." Neurol Genet **3**(3): e149.

Carreno-Gago, L., C. Blazquez-Bermejo, J. Diaz-Manera, Y. Camara, E. Gallardo, R. Marti, J. Torres-Torronteras and E. Garcia-Arumi (2019). "Identification and Characterization of New RNASEH1 Mutations Associated With PEO Syndrome and Multiple Mitochondrial DNA Deletions." Front Genet **10**: 576.

Carrodeguas, J. A., K. G. Pinz and D. F. Bogenhagen (2002). "DNA binding properties of human pol gammaB." J Biol Chem **277**(51): 50008-50014.
Cazenave, C., P. Frank, J. J. Toulme and W. Busen (1994). "Characterization and subcellular localization of ribonuclease H activities from Xenopus laevis oocytes." J Biol Chem **269**(40): 25185-25192.

Cerritelli, S. M. and R. J. Crouch (1998). "Cloning, expression, and mapping of ribonucleases H of human and mouse related to bacterial RNase HI." Genomics **53**(3): 300-307.
Cerritelli, S. M. and R. J. Crouch (2009). "Ribonuclease H: the enzymes in eukaryotes." FEBS J **276**(6): 1494-1505.

Cerritelli, S. M., E. G. Frolova, C. Feng, A. Grinberg, P. E. Love and R. J. Crouch (2003). "Failure to produce mitochondrial DNA results in embryonic lethality in Rnaseh1 null mice." Mol Cell **11**(3): 807-815.

Chang, D. D. and D. A. Clayton (1984). "Precise identification of individual promoters for transcription of each strand of human mitochondrial DNA." Cell **36**(3): 635-643.

Chang, D. D. and D. A. Clayton (1985). "Priming of human mitochondrial DNA replication occurs at the light-strand promoter." Proc Natl Acad Sci U S A **82**(2): 351-355.

Chang, D. D., W. W. Hauswirth and D. A. Clayton (1985). "Replication priming and transcription initiate from precisely the same site in mouse mitochondrial DNA." EMBO J **4**(6): 1559-1567.

Chen, H. and D. C. Chan (2004). "Mitochondrial dynamics in mammals." Curr Top Dev Biol **59**: 119-144.

Chomyn, A., M. W. Cleeter, C. I. Ragan, M. Riley, R. F. Doolittle and G. Attardi (1986). "URF6, last unidentified reading frame of human mtDNA, codes for an NADH dehydrogenase subunit." Science **234**(4776): 614-618.

Chomyn, A., P. Mariottini, M. W. Cleeter, C. I. Ragan, A. Matsuno-Yagi, Y. Hatefi, R. F. Doolittle and G. Attardi (1985). "Six unidentified reading frames of human mitochondrial DNA encode components of the respiratory-chain NADH dehydrogenase." Nature **314**(6012): 592-597.

Chomyn, A., P. Mariottini, N. Gonzalez-Cadavid, G. Attardi, D. D. Strong, D. Trovato, M. Riley and R. F. Doolittle (1983). "Identification of the polypeptides encoded in the ATPase 6 gene and in the unassigned reading frames 1 and 3 of human mtDNA." Proc Natl Acad Sci U S A **80**(18): 5535-5539.

Ciesielski, G. L., M. T. Oliveira and L. S. Kaguni (2016). "Animal Mitochondrial DNA Replication." Enzymes **39**: 255-292.

Clayton, D. A. (1982). "Replication of animal mitochondrial DNA." Cell **28**(4): 693-705.

Clayton, D. A., C. A. Smith, J. M. Jordan, M. Teplitz and J. Vinograd (1968). "Occurrence of complex mitochondrial DNA in normal tissues." Nature **220**(5171): 976-979.

Clayton, D. A. and J. Vinograd (1967). "Circular dimer and catenate forms of mitochondrial DNA in human leukaemic leucocytes." Nature **216**(5116): 652-657.

Cogliati, S., J. A. Enriquez and L. Scorrano (2016). "Mitochondrial Cristae: Where Beauty Meets Functionality." Trends Biochem Sci **41**(3): 261-273.

Colombini, M., E. Blachly-Dyson and M. Forte (1996). "VDAC, a channel in the outer mitochondrial membrane." Ion Channels **4**: 169-202.

Copeland, W. C. (2012). "Defects in mitochondrial DNA replication and human disease." Crit Rev Biochem Mol Biol **47**(1): 64-74.

Copeland, W. C. and M. J. Longley (2003). "DNA polymerase gamma in mitochondrial DNA replication and repair." ScientificWorldJournal **3**: 34-44.

Copeland, W. C. and M. J. Longley (2008). "DNA2 resolves expanding flap in mitochondrial base excision repair." Mol Cell **32**(4): 457-458.

Copeland, W. C. and M. J. Longley (2014). "Mitochondrial genome maintenance in health and disease." DNA Repair (Amst) **19**: 190-198.
Corneo, G., L. Zardi and E. Polli (1968). "Human mitochondrial DNA." J Mol Biol **36**(3): 419-423.

Cotner-Gohara, E., I. K. Kim, M. Hammel, J. A. Tainer, A. E. Tomkinson and T. Ellenberger (2010). "Human DNA ligase III recognizes DNA ends by dynamic switching between two DNA-bound states." Biochemistry **49**(29): 6165-6176.

Crampton, D. J., M. Ohi, U. Qimron, T. Walz and C. C. Richardson (2006). "Oligomeric states of bacteriophage T7 gene 4 primase/helicase." J Mol Biol **360**(3): 667-677.

Crews, S., D. Ojala, J. Posakony, J. Nishiguchi and G. Attardi (1979). "Nucleotide sequence of a region of human mitochondrial DNA containing the precisely identified origin of replication." Nature **277**(5693): 192-198.

Curth, U., C. Urbanke, J. Greipel, H. Gerberding, V. Tiranti and M. Zeviani (1994). "Single-stranded-DNA-binding proteins from human mitochondria and Escherichia coli have analogous physicochemical properties." Eur J Biochem **221**(1): 435-443.

Cymerman, I. A., I. Chung, B. M. Beckmann, J. M. Bujnicki and G. Meiss (2008). "EXOG, a novel paralog of Endonuclease G in higher eukaryotes." Nucleic Acids Res **36**(4): 1369-1379.

D'Erchia, A. M., A. Atlante, G. Gadaleta, G. Pavesi, M. Chiara, C. De Virgilio, C. Manzari, F. Mastropasqua, G. M. Prazzoli, E. Picardi, C. Gissi, D. Horner, A. Reyes, E. Sbisa, A. Tullo and G. Pesole (2015). "Tissue-specific mtDNA abundance from exome data and its correlation with mitochondrial transcription, mass and respiratory activity." Mitochondrion **20**: 13-21.

Doda, J. N., C. T. Wright and D. A. Clayton (1981). "Elongation of displacement-loop strands in human and mouse mitochondrial DNA is arrested near specific template sequences." Proc Natl Acad Sci U S A **78**(10): 6116-6120.
Dolezal, P., V. Likic, J. Tachezy and T. Lithgow (2006). "Evolution of the molecular machines for protein import into mitochondria." Science **313**(5785): 314-318.

Ekstrand, M. I., M. Falkenberg, A. Rantanen, C. B. Park, M. Gaspari, K. Hultenby, P. Rustin, C. M. Gustafsson and N. G. Larsson (2004).

"Mitochondrial transcription factor A regulates mtDNA copy number in mammals." Hum Mol Genet **13**(9): 935-944.

Falkenberg, M. (2018). "Mitochondrial DNA replication in mammalian cells: overview of the pathway." Essays Biochem **62**(3): 287-296.

Falkenberg, M., M. Gaspari, A. Rantanen, A. Trifunovic, N. G. Larsson and C. M. Gustafsson (2002). "Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA." Nat Genet **31**(3): 289-294.

Falkenberg, M. and C. M. Gustafsson (2020). "Mammalian mitochondrial DNA replication and mechanisms of deletion formation." Crit Rev Biochem Mol Biol **55**(6): 509-524.

Fan, L., S. Kim, C. L. Farr, K. T. Schaefer, K. M. Randolph, J. A. Tainer and L. S. Kaguni (2006). "A novel processive mechanism for DNA synthesis revealed by structure, modeling and mutagenesis of the accessory subunit of human mitochondrial DNA polymerase." J Mol Biol **358**(5): 1229-1243.

Farge, G., N. Laurens, O. D. Broekmans, S. M. van den Wildenberg, L. C. Dekker, M. Gaspari, C. M. Gustafsson, E. J. Peterman, M. Falkenberg and G. J. Wuite (2012). "Protein sliding and DNA denaturation are essential for DNA organization by human mitochondrial transcription factor A." Nat Commun **3**: 1013.

Farge, G., M. Mehmedovic, M. Baclayon, S. M. van den Wildenberg, W. H. Roos, C. M. Gustafsson, G. J. Wuite and M. Falkenberg (2014). "In vitro-reconstituted nucleoids can block mitochondrial DNA replication and transcription." Cell Rep **8**(1): 66-74.

Farge, G., X. H. Pham, T. Holmlund, I. Khorostov and M. Falkenberg (2007). "The accessory subunit B of DNA polymerase gamma is required for mitochondrial replisome function." Nucleic Acids Res **35**(3): 902-911.

Fisher, R. P., J. N. Topper and D. A. Clayton (1987). "Promoter selection in human mitochondria involves binding of a transcription factor to orientation-independent upstream regulatory elements." Cell **50**(2): 247-258.
Forte, M., E. Blachly-Dyson and M. Colombini (1996). "Structure and function of the yeast outer mitochondrial membrane channel, VDAC." Soc Gen Physiol Ser **51**: 145-154.

Fuste, J. M., S. Wanrooij, E. Jemt, C. E. Granycome, T. J. Cluett, Y. Shi, N. Atanassova, I. J. Holt, C. M. Gustafsson and M. Falkenberg (2010).

"Mitochondrial RNA polymerase is needed for activation of the origin of light-strand DNA replication." Mol Cell **37**(1): 67-78.

Gaidamakov, S. A., Gorshkova, II, P. Schuck, P. J. Steinbach, H. Yamada, R. J. Crouch and S. M. Cerritelli (2005). "Eukaryotic RNases H1 act processively by interactions through the duplex RNA-binding domain." Nucleic Acids Res **33**(7): 2166-2175.

Garrido, N., L. Griparic, E. Jokitalo, J. Wartiovaara, A. M. van der Bliek and J. N. Spelbrink (2003). "Composition and dynamics of human mitochondrial nucleoids." Mol Biol Cell **14**(4): 1583-1596.

Giles, R. E., H. Blanc, H. M. Cann and D. C. Wallace (1980). "Maternal inheritance of human mitochondrial DNA." Proc Natl Acad Sci U S A **77**(11): 6715-6719.

Gilkerson, R. W., J. M. Selker and R. A. Capaldi (2003). "The cristal membrane of mitochondria is the principal site of oxidative phosphorylation." FEBS Lett **546**(2-3): 355-358.

Gillum, A. M. and D. A. Clayton (1978). "Displacement-loop replication initiation sequence in animal mitochondrial DNA exists as a family of discrete lengths." Proc Natl Acad Sci U S A **75**(2): 677-681.

Gray, H. and T. W. Wong (1992). "Purification and identification of subunit structure of the human mitochondrial DNA polymerase." J Biol Chem **267**(9): 5835-5841.

Gray, M. W., G. Burger, R. Cedergren, G. B. Golding, C. Lemieux, D. Sankoff, M. Turmel and B. F. Lang (1999). "A Genomics Approach to Mitochondrial Evolution." Biol Bull **196**(3): 400-403.

Gray, M. W., G. Burger and B. F. Lang (1999). "Mitochondrial evolution." Science **283**(5407): 1476-1481.

Greaves, L. C., A. K. Reeve, R. W. Taylor and D. M. Turnbull (2012). "Mitochondrial DNA and disease." J Pathol **226**(2): 274-286.

Greaves, L. C. and D. M. Turnbull (2009). "Mitochondrial DNA mutations and ageing." Biochim Biophys Acta **1790**(10): 1015-1020.

Greenleaf, A. L., J. L. Kelly and I. R. Lehman (1986). "Yeast RPO41 gene product is required for transcription and maintenance of the mitochondrial genome." Proc Natl Acad Sci U S A **83**(10): 3391-3394.

Gustafsson, C. M., M. Falkenberg and N. G. Larsson (2016). "Maintenance and Expression of Mammalian Mitochondrial DNA." Annu Rev Biochem **85**: 133-160.

Hamdan, S. M., J. J. Loparo, M. Takahashi, C. C. Richardson and A. M. van Oijen (2009). "Dynamics of DNA replication loops reveal temporal control of lagging-strand synthesis." Nature **457**(7227): 336-339.

Hamdan, S. M. and C. C. Richardson (2009). "Motors, switches, and contacts in the replisome." Annu Rev Biochem **78**: 205-243.

He, Q., C. K. Shumate, M. A. White, I. J. Molineux and Y. W. Yin (2013). "Exonuclease of human DNA polymerase gamma disengages its strand displacement function." Mitochondrion **13**(6): 592-601.

Hillen, H. S., Y. I. Morozov, A. Sarfallah, D. Temiakov and P. Cramer (2017). "Structural Basis of Mitochondrial Transcription Initiation." Cell **171**(5): 1072-1081 e1010.

Hillen, H. S., A. V. Parshin, K. Agaronyan, Y. I. Morozov, J. J. Graber, A. Chernev, K. Schwinghammer, H. Urlaub, M. Anikin, P. Cramer and D. Temiakov (2017). "Mechanism of Transcription Anti-termination in Human Mitochondria." Cell **171**(5): 1082-1093 e1013.

Holmes, J. B., G. Akman, S. R. Wood, K. Sakhuja, S. M. Cerritelli, C. Moss, M. R. Bowmaker, H. T. Jacobs, R. J. Crouch and I. J. Holt (2015). "Primer retention owing to the absence of RNase H1 is catastrophic for mitochondrial DNA replication." Proc Natl Acad Sci U S A **112**(30): 9334-9339.

Holt, I. J. and H. T. Jacobs (2014). "Unique features of DNA replication in mitochondria: a functional and evolutionary perspective." Bioessays **36**(11): 1024-1031.

Holt, I. J., H. E. Lorimer and H. T. Jacobs (2000). "Coupled leading- and lagging-strand synthesis of mammalian mitochondrial DNA." Cell **100**(5): 515-524.

Hudson, B. and J. Vinograd (1967). "Catenated circular DNA molecules in HeLa cell mitochondria." Nature **216**(5116): 647-652.

Jemt, E., G. Farge, S. Backstrom, T. Holmlund, C. M. Gustafsson and M. Falkenberg (2011). "The mitochondrial DNA helicase TWINKLE can assemble on a closed circular template and support initiation of DNA synthesis." Nucleic Acids Res **39**(21): 9238-9249.

Johnson, A. A., Y. Tsai, S. W. Graves and K. A. Johnson (2000). "Human mitochondrial DNA polymerase holoenzyme: reconstitution and characterization." Biochemistry **39**(7): 1702-1708.

Kaneda, H., J. Hayashi, S. Takahama, C. Taya, K. F. Lindahl and H. Yonekawa (1995). "Elimination of paternal mitochondrial DNA in intraspecific crosses during early mouse embryogenesis." Proc Natl Acad Sci U S A **92**(10): 4542-4546.

Kang, D., K. Miyako, Y. Kai, T. Irie and K. Takeshige (1997). "In vivo determination of replication origins of human mitochondrial DNA by ligation-mediated polymerase chain reaction." J Biol Chem **272**(24): 15275-15279.

Kasamatsu, H., D. L. Robberson and J. Vinograd (1971). "A novel closed-circular mitochondrial DNA with properties of a replicating intermediate." Proc Natl Acad Sci U S A **68**(9): 2252-2257.

Kato, M., T. Ito, G. Wagner, C. C. Richardson and T. Ellenberger (2003). "Modular architecture of the bacteriophage T7 primase couples RNA primer synthesis to DNA synthesis." Mol Cell **11**(5): 1349-1360.

Kaufman, B. A., N. Durisic, J. M. Mativetsky, S. Costantino, M. A. Hancock, P. Grutter and E. A. Shoubridge (2007). "The mitochondrial transcription factor TFAM coordinates the assembly of multiple DNA molecules into nucleoid-like structures." Mol Biol Cell **18**(9): 3225-3236.

Kazak, L., A. Reyes, J. He, S. R. Wood, G. Brea-Calvo, T. T. Holen and I. J. Holt (2013). "A cryptic targeting signal creates a mitochondrial FEN1 isoform with tailed R-Loop binding properties." PLoS One **8**(5): e62340.
Kierdaszuk, B., M. Kaliszewska, J. Rusecka, J. Kosinska, E. Bartnik, K. Tonska, A. M. Kaminska and A. Kostera-Pruszczyk (2020). "Progressive External Ophthalmoplegia in Polish Patients-From Clinical Evaluation to Genetic Confirmation." Genes (Basel) **12**(1).

King, M. P. and G. Attardi (1989). "Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation." Science **246**(4929): 500-503.

Korhonen, J. A., M. Gaspari and M. Falkenberg (2003). "TWINKLE Has 5' -> 3' DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein." J Biol Chem **278**(49): 48627-48632.

Korhonen, J. A., X. H. Pham, M. Pellegrini and M. Falkenberg (2004). "Reconstitution of a minimal mtDNA replisome in vitro." EMBO J **23**(12): 2423-2429.

Kornblum, C., T. J. Nicholls, T. B. Haack, S. Scholer, V. Peeva, K. Danhauser, K. Hallmann, G. Zsurka, J. Rorbach, A. Iuso, T. Wieland, M. Sciacco, D. Ronchi, G. P. Comi, M. Moggio, C. M. Quinzii, S. DiMauro, S. E. Calvo, V. K. Mootha, T. Klopstock, T. M. Strom, T. Meitinger, M. Minczuk, W. S. Kunz and H. Prokisch (2013). "Loss-of-function mutations in MGME1 impair mtDNA replication and cause multisystemic mitochondrial disease." Nat Genet **45**(2): 214-219.

Krishnan, K. J., L. C. Greaves, A. K. Reeve and D. Turnbull (2007). "The ageing mitochondrial genome." Nucleic Acids Res **35**(22): 7399-7405.

Kukat, C., K. M. Davies, C. A. Wurm, H. Spahr, N. A. Bonekamp, I. Kuhl, F. Joos, P. L. Polosa, C. B. Park, V. Posse, M. Falkenberg, S. Jakobs, W. Kuhlbrandt and N. G. Larsson (2015). "Cross-strand binding of TFAM to a single mtDNA molecule forms the mitochondrial nucleoid." Proc Natl Acad Sci U S A **112**(36): 11288-11293.

Kukat, C., C. A. Wurm, H. Spahr, M. Falkenberg, N. G. Larsson and S. Jakobs (2011). "Super-resolution microscopy reveals that mammalian mitochondrial nucleoids have a uniform size and frequently contain a single copy of mtDNA." Proc Natl Acad Sci U S A **108**(33): 13534-13539.

Lakshmipathy, U. and C. Campbell (1999). "The human DNA ligase III gene encodes nuclear and mitochondrial proteins." Mol Cell Biol **19**(5): 3869-3876.
Lakshmipathy, U. and C. Campbell (2001). "Antisense-mediated decrease in DNA ligase III expression results in reduced mitochondrial DNA integrity." Nucleic Acids Res **29**(3): 668-676.

Lang, B. F., M. W. Gray and G. Burger (1999). "Mitochondrial genome evolution and the origin of eukaryotes." Annu Rev Genet **33**: 351-397.

Larsson, N. G., J. D. Garman, A. Oldfors, G. S. Barsh and D. A. Clayton (1996). "A single mouse gene encodes the mitochondrial transcription factor A and a testis-specific nuclear HMG-box protein." Nat Genet **13**(3): 296-302.

Larsson, N. G., A. Oldfors, J. D. Garman, G. S. Barsh and D. A. Clayton (1997). "Down-regulation of mitochondrial transcription factor A during spermatogenesis in humans." Hum Mol Genet **6**(2): 185-191.

Lee, J. B., R. K. Hite, S. M. Hamdan, X. S. Xie, C. C. Richardson and A. M. van Oijen (2006). "DNA primase acts as a molecular brake in DNA replication." Nature **439**(7076): 621-624.

Lim, S. E., M. J. Longley and W. C. Copeland (1999). "The mitochondrial p55 accessory subunit of human DNA polymerase gamma enhances DNA binding, promotes processive DNA synthesis, and confers N-ethylmaleimide resistance." J Biol Chem **274**(53): 38197-38203.

Lima, W. F., J. B. Rose, J. G. Nichols, H. Wu, M. T. Migawa, T. K. Wyrzykiewicz, A. M. Siwkowski and S. T. Crooke (2007). "Human RNase H1 discriminates between subtle variations in the structure of the heteroduplex substrate." Mol Pharmacol **71**(1): 83-91.

Liu, P., L. Qian, J. S. Sung, N. C. de Souza-Pinto, L. Zheng, D. F. Bogenhagen, V. A. Bohr, D. M. Wilson, 3rd, B. Shen and B. Demple (2008). "Removal of oxidative DNA damage via FEN1-dependent long-patch base excision repair in human cell mitochondria." Mol Cell Biol **28**(16): 4975-4987.

Lodeiro, M. F., A. U. Uchida, J. J. Arnold, S. L. Reynolds, I. M. Moustafa and C. E. Cameron (2010). "Identification of multiple rate-limiting steps during the human mitochondrial transcription cycle in vitro." J Biol Chem **285**(21): 16387-16402.

Longley, M. J., D. Nguyen, T. A. Kunkel and W. C. Copeland (2001). "The fidelity of human DNA polymerase gamma with and without exonucleolytic proofreading and the p55 accessory subunit." J Biol Chem **276**(42): 38555-38562.

Longley, M. J., P. A. Ropp, S. E. Lim and W. C. Copeland (1998). "Characterization of the native and recombinant catalytic subunit of human DNA polymerase gamma: identification of residues critical for exonuclease activity and dideoxynucleotide sensitivity." Biochemistry **37**(29): 10529-10539.

Macao, B., J. P. Uhler, T. Siibak, X. Zhu, Y. Shi, W. Sheng, M. Olsson, J. B. Stewart, C. M. Gustafsson and M. Falkenberg (2015). "The exonuclease activity of DNA polymerase gamma is required for ligation during mitochondrial DNA replication." Nat Commun **6**: 7303.

Mariottini, P., A. Chomyn, G. Attardi, D. Trovato, D. D. Strong and R. F. Doolittle (1983). "Antibodies against synthetic peptides reveal that the unidentified reading frame A6L, overlapping the ATPase 6 gene, is expressed in human mitochondria." Cell **32**(4): 1269-1277.

Mariottini, P., A. Chomyn, M. Riley, B. Cottrell, R. F. Doolittle and G. Attardi (1986). "Identification of the polypeptides encoded in the unassigned reading frames 2, 4, 4L, and 5 of human mitochondrial DNA." Proc Natl Acad Sci U S A **83**(6): 1563-1567.

Martin, W. F., S. Garg and V. Zimorski (2015). "Endosymbiotic theories for eukaryote origin." Philos Trans R Soc Lond B Biol Sci **370**(1678): 20140330.
Masters, B. S., L. L. Stohl and D. A. Clayton (1987). "Yeast mitochondrial RNA polymerase is homologous to those encoded by bacteriophages T3 and T7." Cell **51**(1): 89-99.

Masuda-Sasa, T., O. Imamura and J. L. Campbell (2006). "Biochemical analysis of human Dna2." Nucleic Acids Res **34**(6): 1865-1875.

Masuda-Sasa, T., P. Polaczek, X. P. Peng, L. Chen and J. L. Campbell (2008). "Processing of G4 DNA by Dna2 helicase/nuclease and replication protein A (RPA) provides insights into the mechanism of Dna2/RPA substrate recognition." J Biol Chem **283**(36): 24359-24373.

Masukata, H. and J. Tomizawa (1990). "A mechanism of formation of a persistent hybrid between elongating RNA and template DNA." Cell **62**(2): 331-338.

McKinney, E. A. and M. T. Oliveira (2013). "Replicating animal mitochondrial DNA." Genet Mol Biol **36**(3): 308-315.

Mercer, T. R., S. Neph, M. E. Dinger, J. Crawford, M. A. Smith, A. M. Shearwood, E. Haugen, C. P. Bracken, O. Rackham, J. A. Stamatoyannopoulos, A. Filipovska and J. S. Mattick (2011). "The human mitochondrial transcriptome." Cell **146**(4): 645-658.

Minczuk, M., J. He, A. M. Duch, T. J. Ettema, A. Chlebowski, K. Dzionek, L. G. Nijtmans, M. A. Huynen and I. J. Holt (2011). "TEFM (c17orf42) is necessary for transcription of human mtDNA." Nucleic Acids Res **39**(10): 4284-4299.

Miralles Fuste, J., Y. Shi, S. Wanrooij, X. Zhu, E. Jemt, O. Persson, N. Sabouri, C. M. Gustafsson and M. Falkenberg (2014). "In vivo occupancy of mitochondrial single-stranded DNA binding protein supports the strand displacement mode of DNA replication." PLoS Genet **10**(12): e1004832.

Mishra, P. and D. C. Chan (2014). "Mitochondrial dynamics and inheritance during cell division, development and disease." Nat Rev Mol Cell Biol **15**(10): 634-646.

Miyakawa, I. (2017). "Organization and dynamics of yeast mitochondrial nucleoids." Proc Jpn Acad Ser B Phys Biol Sci **93**(5): 339-359.

Miyakawa, I., N. Sando, S. Kawano, S. Nakamura and T. Kuroiwa (1987). "Isolation of morphologically intact mitochondrial nucleoids from the yeast, Saccharomyces cerevisiae." J Cell Sci **88 (Pt 4)**: 431-439.

Montoya, J., T. Christianson, D. Levens, M. Rabinowitz and G. Attardi (1982). "Identification of initiation sites for heavy-strand and light-strand transcription in human mitochondrial DNA." Proc Natl Acad Sci U S A **79**(23): 7195-7199.
Morozov, Y. I., K. Agaronyan, A. C. Cheung, M. Anikin, P. Cramer and D. Temiakov (2014). "A novel intermediate in transcription initiation by human mitochondrial RNA polymerase." Nucleic Acids Res **42**(6): 3884-3893.

Morozov, Y. I., A. V. Parshin, K. Agaronyan, A. C. Cheung, M. Anikin, P. Cramer and D. Temiakov (2015). "A model for transcription initiation in human mitochondria." Nucleic Acids Res **43**(7): 3726-3735.

Nass, M. M. (1969). "Mitochondrial DNA: Advances, Problems, and Goals." Science **165**(3888): 25-35.
Nass, M. M. and S. Nass (1963). "Intramitochondrial Fibers with DNA Characteristics. I. Fixation and Electron Staining Reactions." J Cell Biol **19**: 593-611.

Nass, S. and M. M. Nass (1963). "Intramitochondrial Fibers with DNA Characteristics. Ii. Enzymatic and Other Hydrolytic Treatments." J Cell Biol **19**: 613-629.

Ngo, H. B., J. T. Kaiser and D. C. Chan (2011). "The mitochondrial transcription and packaging factor Tfam imposes a U-turn on mitochondrial DNA." Nat Struct Mol Biol **18**(11): 1290-1296.

Nicholls, T. J., C. A. Nadalutti, E. Motori, E. W. Sommerville, G. S. Gorman, S. Basu, E. Hoberg, D. M. Turnbull, P. F. Chinnery, N. G. Larsson, E. Larsson,

M. Falkenberg, R. W. Taylor, J. D. Griffith and C. M. Gustafsson (2018). "Topoisomerase 3alpha Is Required for Decatenation and Segregation of Human mtDNA." Mol Cell **69**(1): 9-23 e26.

Nicholls, T. J., H. Spahr, S. Jiang, S. J. Siira, C. Koolmeister, S. Sharma, J. H. K. Kauppila, M. Jiang, V. Kaever, O. Rackham, A. Chabes, M. Falkenberg, A. Filipovska, N. G. Larsson and C. M. Gustafsson (2019). "Dinucleotide Degradation by REXO2 Maintains Promoter Specificity in Mammalian Mitochondria." Mol Cell **76**(5): 784-796 e786.

Nicholls, T. J., G. Zsurka, V. Peeva, S. Scholer, R. J. Szczesny, D. Cysewski, A. Reyes, C. Kornblum, M. Sciacco, M. Moggio, A. Dziembowski, W. S. Kunz and M. Minczuk (2014). "Linear mtDNA fragments and unusual mtDNA rearrangements associated with pathological deficiency of MGME1 exonuclease." Hum Mol Genet **23**(23): 6147-6162.

Nowotny, M., S. M. Cerritelli, R. Ghirlando, S. A. Gaidamakov, R. J. Crouch and W. Yang (2008). "Specific recognition of RNA/DNA hybrid and enhancement of human RNase H1 activity by HBD." EMBO J **27**(7): 1172-1181.

Ogawa, T., G. G. Pickett, T. Kogoma and A. Kornberg (1984). "RNase H confers specificity in the dnaA-dependent initiation of replication at the unique origin of the Escherichia coli chromosome in vivo and in vitro." Proc Natl Acad Sci U S A **81**(4): 1040-1044.

Oliveira, M. T. and L. S. Kaguni (2011). "Reduced stimulation of recombinant DNA polymerase gamma and mitochondrial DNA (mtDNA) helicase by variants of mitochondrial single-stranded DNA-binding protein (mtSSB) correlates with defects in mtDNA replication in animal cells." J Biol Chem **286**(47): 40649-40658.

Palade, G. E. (1952). "The fine structure of mitochondria." Anat Rec **114**(3): 427-451.

Palade, G. E. (1953). "An electron microscope study of the mitochondrial structure." J Histochem Cytochem **1**(4): 188-211.

Palmer, J. M. and D. O. Hall (1972). "The mitochondrial membrane system." Prog Biophys Mol Biol **24**: 125-176.

Pfanner, N. and N. Wiedemann (2002). "Mitochondrial protein import: two membranes, three translocases." Curr Opin Cell Biol **14**(4): 400-411.

Pham, X. H., G. Farge, Y. Shi, M. Gaspari, C. M. Gustafsson and M. Falkenberg (2006). "Conserved sequence box II directs transcription termination and primer formation in mitochondria." J Biol Chem **281**(34): 24647-24652.

Phowthongkum, P. and A. Sun (2017). "Novel truncating variant in DNA2-related congenital onset myopathy and ptosis suggests genotype-phenotype correlation." Neuromuscul Disord **27**(7): 616-618.

Pommier, Y., Y. Sun, S. N. Huang and J. L. Nitiss (2016). "Roles of eukaryotic topoisomerases in transcription, replication and genomic stability." Nat Rev Mol Cell Biol **17**(11): 703-721.

Posse, V. and C. M. Gustafsson (2017). "Human Mitochondrial Transcription Factor B2 Is Required for Promoter Melting during Initiation of Transcription." J Biol Chem **292**(7): 2637-2645.

Posse, V., E. Hoberg, A. Dierckx, S. Shahzad, C. Koolmeister, N. G. Larsson, L. M. Wilhelmsson, B. M. Hallberg and C. M. Gustafsson (2014). "The amino terminal extension of mammalian mitochondrial RNA polymerase ensures promoter specific transcription initiation." Nucleic Acids Res **42**(6): 3638-3647.

Posse, V., S. Shahzad, M. Falkenberg, B. M. Hallberg and C. M. Gustafsson (2015). "TEFM is a potent stimulator of mitochondrial transcription elongation in vitro." Nucleic Acids Res **43**(5): 2615-2624.
Radloff, R., W. Bauer and J. Vinograd (1967). "A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells." Proc Natl Acad Sci U S A **57**(5): 1514-1521.

Rajala, N., J. M. Gerhold, P. Martinsson, A. Klymov and J. N. Spelbrink (2014). "Replication factors transiently associate with mtDNA at the mitochondrial inner membrane to facilitate replication." Nucleic Acids Res **42**(2): 952-967.

Reyes, A., L. Kazak, S. R. Wood, T. Yasukawa, H. T. Jacobs and I. J. Holt (2013). "Mitochondrial DNA replication proceeds via a 'bootlace' mechanism involving the incorporation of processed transcripts." Nucleic Acids Res **41**(11): 5837-5850.

Reyes, A., L. Melchionda, A. Nasca, F. Carrara, E. Lamantea, A. Zanolini, C. Lamperti, M. Fang, J. Zhang, D. Ronchi, S. Bonato, G. Fagiolari, M. Moggio,

D. Ghezzi and M. Zeviani (2015). "RNASEH1 Mutations Impair mtDNA Replication and Cause Adult-Onset Mitochondrial Encephalomyopathy." Am J Hum Genet **97**(1): 186-193.

Robberson, D. L. and D. A. Clayton (1972). "Replication of mitochondrial DNA in mouse L cells and their thymidine kinase - derivatives: displacement replication on a covalently-closed circular template." Proc Natl Acad Sci U S A **69**(12): 3810-3814.

Ronchi, D., A. Di Fonzo, W. Lin, A. Bordoni, C. Liu, E. Fassone, S. Pagliarani, M. Rizzuti, L. Zheng, M. Filosto, M. T. Ferro, M. Ranieri, F. Magri, L. Peverelli, H. Li, Y. C. Yuan, S. Corti, M. Sciacco, M. Moggio, N. Bresolin, B. Shen and G. P. Comi (2013). "Mutations in DNA2 link progressive myopathy to mitochondrial DNA instability." Am J Hum Genet **92**(2): 293-300.

Sachdev, A., C. Fratter and T. F. W. McMullan (2018). "Novel mutation in the RNASEH1 gene in a chronic progressive external ophthalmoplegia patient." Can J Ophthalmol **53**(5): e203-e205.

Sanchez, M. I., T. R. Mercer, S. M. Davies, A. M. Shearwood, K. K. Nygard, T. R. Richman, J. S. Mattick, O. Rackham and A. Filipovska (2011). "RNA processing in human mitochondria." Cell Cycle **10**(17): 2904-2916.

Scheffler, I. E. (2008). Mitochondria. Hoboken, N.J., Wiley-Liss.

Schubot, F. D., C. J. Chen, J. P. Rose, T. A. Dailey, H. A. Dailey and B. C. Wang (2001). "Crystal structure of the transcription factor sc-mtTFB offers insights into mitochondrial transcription." Protein Sci **10**(10): 1980-1988.

Shadel, G. S. and D. A. Clayton (1997). "Mitochondrial DNA maintenance in vertebrates." Annu Rev Biochem **66**: 409-435.

Shaw, J. M. and J. Nunnari (2002). "Mitochondrial dynamics and division in budding yeast." Trends Cell Biol **12**(4): 178-184.

Sheftel, A. D. and R. Lill (2009). "The power plant of the cell is also a smithy: the emerging role of mitochondria in cellular iron homeostasis." Ann Med **41**(2): 82-99.

Sjostrand, F. S. (1953). "Electron microscopy of mitochondria and cytoplasmic double membranes." Nature **171**(4340): 30-32.

Spelbrink, J. N. (2010). "Functional organization of mammalian mitochondrial DNA in nucleoids: history, recent developments, and future challenges." IUBMB Life **62**(1): 19-32.

Spelbrink, J. N., F. Y. Li, V. Tiranti, K. Nikali, Q. P. Yuan, M. Tariq, S. Wanrooij, N. Garrido, G. Comi, L. Morandi, L. Santoro, A. Toscano, G. M. Fabrizi, H. Somer, R. Croxen, D. Beeson, J. Poulton, A. Suomalainen, H. T. Jacobs, M. Zeviani and C. Larsson (2001). "Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria." Nat Genet **28**(3): 223-231.

Stewart, J. B. and N. G. Larsson (2014). "Keeping mtDNA in shape between generations." PLoS Genet **10**(10): e1004670.

Stodola, J. L. and P. M. Burgers (2017). "Mechanism of Lagging-Strand DNA Replication in Eukaryotes." Adv Exp Med Biol **1042**: 117-133.

Sutovsky, P., R. D. Moreno, J. Ramalho-Santos, T. Dominko, C. Simerly and G. Schatten (1999). "Ubiquitin tag for sperm mitochondria." Nature **402**(6760): 371-372.

Szczesny, R. J., M. S. Hejnowicz, K. Steczkiewicz, A. Muszewska, L. S. Borowski, K. Ginalski and A. Dziembowski (2013). "Identification of a novel human mitochondrial endo-/exonuclease Ddk1/c20orf72 necessary for maintenance of proper 7S DNA levels." Nucleic Acids Res **41**(5): 3144-3161.
Tann, A. W., I. Boldogh, G. Meiss, W. Qian, B. Van Houten, S. Mitra and B. Szczesny (2011). "Apoptosis induced by persistent single-strand breaks in mitochondrial genome: critical role of EXOG (5'-EXO/endonuclease) in their repair." J Biol Chem **286**(37): 31975-31983.

Tapper, D. P. and D. A. Clayton (1981). "Mechanism of replication of human mitochondrial DNA. Localization of the 5' ends of nascent daughter strands." J Biol Chem **256**(10): 5109-5115.

Tiranti, V., M. Rocchi, S. DiDonato and M. Zeviani (1993). "Cloning of human and rat cDNAs encoding the mitochondrial single-stranded DNA-binding protein (SSB)." Gene **126**(2): 219-225.

Tiranti, V., A. Savoia, F. Forti, M. F. D'Apolito, M. Centra, M. Rocchi and M. Zeviani (1997). "Identification of the gene encoding the human mitochondrial RNA polymerase (h-mtRPOL) by cyberscreening of the Expressed Sequence Tags database." Hum Mol Genet **6**(4): 615-625.

Trifunovic, A., A. Wredenberg, M. Falkenberg, J. N. Spelbrink, A. T. Rovio, C. E. Bruder, Y. M. Bohlooly, S. Gidlof, A. Oldfors, R. Wibom, J. Tornell, H. T. Jacobs and N. G. Larsson (2004). "Premature ageing in mice expressing defective mitochondrial DNA polymerase." Nature **429**(6990): 417-423.

Uhler, J. P. and M. Falkenberg (2015). "Primer removal during mammalian mitochondrial DNA replication." DNA Repair (Amst) **34**: 28-38.

Uhler, J. P., C. Thorn, T. J. Nicholls, S. Matic, D. Milenkovic, C. M. Gustafsson and M. Falkenberg (2016). "MGME1 processes flaps into ligatable nicks in concert with DNA polymerase gamma during mtDNA replication." Nucleic Acids Res **44**(12): 5861-5871.

Walberg, M. W. and D. A. Clayton (1981). "Sequence and properties of the human KB cell and mouse L cell D-loop regions of mitochondrial DNA." Nucleic Acids Res **9**(20): 5411-5421.

Wanrooij, P. H. and A. Chabes (2019). "Ribonucleotides in mitochondrial DNA." FEBS Lett **593**(13): 1554-1565.

Wanrooij, P. H., J. P. Uhler, Y. Shi, F. Westerlund, M. Falkenberg and C. M. Gustafsson (2012). "A hybrid G-quadruplex structure formed between RNA and DNA explains the extraordinary stability of the mitochondrial R-loop." Nucleic Acids Res **40**(20): 10334-10344.

Wanrooij, P. H., J. P. Uhler, T. Simonsson, M. Falkenberg and C. M. Gustafsson (2010). "G-quadruplex structures in RNA stimulate mitochondrial transcription termination and primer formation." Proc Natl Acad Sci U S A **107**(37): 16072-16077.

Wanrooij, S. and M. Falkenberg (2010). "The human mitochondrial replication fork in health and disease." Biochim Biophys Acta **1797**(8): 1378-1388.

Wanrooij, S., J. M. Fuste, G. Farge, Y. Shi, C. M. Gustafsson and M. Falkenberg (2008). "Human mitochondrial RNA polymerase primes lagging-strand DNA synthesis in vitro." Proc Natl Acad Sci U S A **105**(32): 11122-11127.

Wong, T. W. and D. A. Clayton (1985). "In vitro replication of human mitochondrial DNA: accurate initiation at the origin of light-strand synthesis." Cell **42**(3): 951-958.

Wong, T. W. and D. A. Clayton (1985). "Isolation and characterization of a DNA primase from human mitochondria." J Biol Chem **260**(21): 11530-11535.

Wu, C. C., J. L. J. Lin, H. F. Yang-Yen and H. S. Yuan (2019). "A unique exonuclease ExoG cleaves between RNA and DNA in mitochondrial DNA replication." Nucleic Acids Res **47**(10): 5405-5419.

Xu, B. and D. A. Clayton (1996). "RNA-DNA hybrid formation at the human mitochondrial heavy-strand origin ceases at replication start sites: an implication for RNA-DNA hybrids serving as primers." EMBO J **15**(12): 3135-3143.

Yakubovskaya, E., Z. Chen, J. A. Carrodeguas, C. Kisker and D. F. Bogenhagen (2006). "Functional human mitochondrial DNA polymerase gamma forms a heterotrimer." J Biol Chem **281**(1): 374-382.

Yang, M. Y., M. Bowmaker, A. Reyes, L. Vergani, P. Angeli, E. Gringeri, H. T. Jacobs and I. J. Holt (2002). "Biased incorporation of ribonucleotides on the mitochondrial L-strand accounts for apparent strand-asymmetric DNA replication." Cell **111**(4): 495-505.

Yasukawa, T., A. Reyes, T. J. Cluett, M. Y. Yang, M. Bowmaker, H. T. Jacobs and I. J. Holt (2006). "Replication of vertebrate mitochondrial DNA entails transient ribonucleotide incorporation throughout the lagging strand." EMBO J **25**(22): 5358-5371.

Yasukawa, T., M. Y. Yang, H. T. Jacobs and I. J. Holt (2005). "A bidirectional origin of replication maps to the major noncoding region of human mitochondrial DNA." Mol Cell **18**(6): 651-662.

Yu, H., C. Xue, M. Long, H. Jia, G. Xue, S. Du, Y. Coello and T. Ishibashi (2018). "TEFM Enhances Transcription Elongation by Modifying mtRNAP Pausing Dynamics." Biophys J **115**(12): 2295-2300.

Zeviani, M., P. Fernandez-Silva and V. Tiranti (1997). "Disorders of mitochondria and related metabolism." Curr Opin Neurol **10**(2): 160-167.
Zheng, L. and B. Shen (2011). "Okazaki fragment maturation: nucleases take centre stage." J Mol Cell Biol **3**(1): 23-30.

Zheng, L., M. Zhou, Z. Guo, H. Lu, L. Qian, H. Dai, J. Qiu, E. Yakubovskaya, D. F. Bogenhagen, B. Demple and B. Shen (2008). "Human DNA2 is a

mitochondrial nuclease/helicase for efficient processing of DNA replication and repair intermediates." Mol Cell **32**(3): 325-336.