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Structural and biochemical analysis of substrate recognition by mitochondrial transcription factor A (TFAM)

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Abstract of the Dissertation

Structural and biochemical analysis of substrate recognition of mitochondrial transcription factor A (TFAM)

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TFAM (mitochondrial transcription factor A) is a mitochondrial protein containing two tandem HMG (high-mobility group) boxes. It serves two essential functions in mitochondria: transcription activation and mtDNA (mitochondrial DNA) packaging. Interestingly, while its transcription activating function requires specific sequence recognition in order to specifically recognize the mitochondrial promoters, mtDNA packaging occurs mainly through non-sequence specific binding. However, the mechanism of nucleic acid recognition for both specific and nonspecific sequences has not yet been established despite the determination of TFAM crystal structures in complex with promoter sequences. The structures reveal that most of the interactions between TFAM and the promoters are non-sequence specific. However, I found two sequence specific interactions with two guanines separated by 10 variable nucleotides ($GN_{10}G$). These guanines hydrogen-bond to two TFAM residues in the HMG box 1 and 2. Intriguingly, these interactions are conserved in all TFAM structures with both promoter and nonspecific sequences, indicating that TFAM might recognize this $GN_{10}G$ consensus regardless of substrate specificity. Here, I present a crystal structure of TFAM in complex with a non-specific sequence (NS2) containing a $GN_{10}G$ consensus. The structure reveals consistent binding through the consensus, leading to the hypothesis that TFAM prefers to recognize the $GN_{10}G$ consensus on DNA substrates for its variable functions. *In vitro* transcription assays show that the $GN_{10}G$

consensus plays an important role in mitochondrial transcription, and EMSA (electrophoretic mobility shift assay) and other binding assays reveal that a $GN_{10}G$ consensus contributes to directing TFAM binding, indicating that it appears to have a role in mtDNA packaging. In addition, the TFAM-NS2 structure displays a unique interaction to a DNA nick composed of two DNA ends, which is consistent with previous studies about the preferential binding of TFAM to DNA damaged regions. End-joining assays suggest that TFAM is able to facilitate DNA ligation events, implicating its possible role in double-strand break repair in mitochondria. These studies will shed light not only on the mechanism of TFAM binding, but also on the involvement of TFAM in mitochondrial DNA repair.

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List of Abbreviations

TOM	Translocase of the outer membrane
TIM	Translocase of the inner membrane
OXPHOS	Oxidative phosphorylation
ATP	Adenine triphosphate
ETC	Electron transport chain
mtDNA	Mitochondrial DNA
dsDNA	Double strand DNA
ssDNA	Single strand DNA
Ni	Nickel
TBE	Tris/Borate/EDTA
K_D	Dissociation constant
DTT	Dithiothreitol
BSA	Bovine serum albumin
CytC	Cytochrome C
NADH	Nicotinamide adenine dinucleotide
MCU	Mitochondrial calcium uniporter
CaMC	Ca^{2+}-binding mitochondrial carrier
AGC1	Aspartate/glutamate carrier 1
ND1-6	NADH dehydrogenase 1-6
COI-III	Cytochrome c oxidase subunits I-III
Cytb	Cytochrome b
D-loop	Displacement loop
H-strand	Heavy strand
L-strand	Light strand
O_H	Origin of replication of H-strand
O_L	Origin of replication of L-strand
LSP	Light strand promoter
HSP	Heavy strand promoter
ROS	Reactive oxygen species
LHON	Leber heredity optic neuropathy
MELAS	Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like eposodes
LS	Leigh's disease
NER	Nucleotide excision repair
BER	Base excision repair
MMR	Mismatch repair
DSB	Double-strand break
HR	Homologous recombination
NHEJ	Non-homologous end joining
dL	Deoxyribonolactone
TWINKLE	Mitochondrial helicase
mtSSB	Mitochondrial single-strand binding protein
POLγ	DNA polymerase γ
NTP	Nucleotide triphosphate

TFAM	Mitochondrial transcription factor A
TFB1/2M	Mitochondrial transcription factor B 1/2
PORLMT	Mitochondrial RNA polymerase
NTE	N-terminal extension
NTD	N-terminal domain
CTD	C-terminal domain
RNAP	RNA polymerase
PPR	Pentatricopeptide repeat domain
CSBII	Conserved sequence block II
G	Guanine (nucleotide)
TEFM	Mitochondrial transcription elongation factor
MTERF	Mitochondrial transcription termination factor
HMG	High mobility group
NS	Nonspecific sequence
L (Leu)	Leucine
Abf2p	ARF-binding factor 2
ARF	ADP ribosylation factor
ADP	Adenine diphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
O.D.	Optical density
MBP	Maltose binding protein
TEV	Tobacco etch virus
S (Ser)	Serine
Y (Tyr)	Tyrosine
A (Ala)	Alanine
PEG	Polyethylene glycol
MME	Monomethyl Ether
SDS	Sodium dodecyl sulfate
EDTA	Ethylenediaminetetraacetic acid
C	Cytosine (nucleotide)
T	Thymine (nucleotide)
EMSA	Electrophoretic mobility shift assay
8-oxoG	8-oxoguanine
RMSD	Root-mean-square deviation
MMEJ	Microhomology-mediated end joining
R (Arg)	Arginine
Q (Gln)	Glutamine
E (Glu)	Glutamic acid
gg	*Gallus gallus*
sc	*Saccharomyces cerevisiae*
hs	*Homo sapiens*
mm	*Mus musculus*
xl	*Xenopus laevis*
dm	*Drosophila melanogaster*

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Chapter 1 Introduction

1.1 Mitochondria

1.1.1 Structure of mitochondria

The mitochondrion is an essential organelle involved in various cellular processes. Mitochondria are composed of unique structures compared to those of other organelles. They are composed of an outer membrane, an inner membrane, an intermembrane space, and the mitochondrial matrix. The outer membrane is the outermost enclosure of mitochondria. It is permeable to small molecules such as water, ions, sugars and small proteins. These molecules usually diffuse through a pore of a membrane transporter, porin, which is the most abundant protein in the outer membrane (Benz 1985, Hancock 1987, Weeber, Levy et al. 2002). However, large proteins targeting mitochondria are transported through a specific membrane transporter, TOM (translocase of the outer membrane) (Neupert 1997, Pfanner, Craig et al. 1997, Dekker, Ryan et al. 1998, Kunkele, Heins et al. 1998, Herrmann and Neupert 2000). In order for molecules to be transported into the matrix, they have to pass through another membrane, the inner membrane. However, in contrast to the permeable outer membrane, the inner membrane is impermeable to almost all molecules. Instead, a membrane transporter, TIM (translocase of the inner membrane), is responsible for the translocation of molecules into the matrix through the inner membrane (Neupert 1997, Pfanner, Craig et al. 1997, Herrmann and Neupert 2000). The inner membrane also harbors the essential machinery for oxidative phosphorylation (OXPHOS) to produce ATP: the electron transport chain (ETC) and the ATP synthase. The inner membrane adopts a highly folded structure. The folds, called cristae, increase the membrane surface, leading to more efficient ATP generation by providing more space where OXPHOS can take place. The intermembrane space is formed between the outer and the inner membranes. Due to

the permeability of the outer membrane to small molecules, the environment of the intermembrane space is similar to that of the cytosol. Finally, the mitochondrial matrix is the space enclosed by the inner membrane. The matrix contains various essential molecules such as mitochondrial DNA (mtDNA) and enzymes facilitating fundamental enzymatic processes (DNA replication, protein expression, citric acid cycle, and fatty acid oxidation) (Scalettar, Abney et al. 1991, Kuhlbrandt 2015).

1.1.2 Function of mitochondria

1.1.2.1 ATP production

Mitochondria play a key role in the generation of cellular energy, the ATP molecule. It is produced by OXPHOS through the action of the ETC and ATP synthase embedded in the inner membrane (Figure 1.1). The major role of the ETC in ATP production is the formation of a proton gradient by pumping out protons from the matrix to the intermembrane space. The ETC is composed of four major complexes (I ~ IV) with two electron carriers among the complexes (coenzyme Q (ubiquinone) and cytochrome C (CytC)). The ETC complexes are comprised of a number of polypeptides mostly encoded by the nuclear genome. The electrons are provided as NADH (Nicotinamide adenine dinucleotide), a product of the citric acid cycle in the mitochondrial matrix (Schultz and Chan 2001). The complex I (NADH:ubiquinone oxidoreductase) oxidizes NADH to NAD^+, and generates two electrons transferred to complex III by coenzyme Q and four protons translocated into the intermembrane space (Lenaz, Fato et al. 2006). Complex II (succinate:quinone oxidoreductase) is another electron entry point in the ETC. Instead of NADH, it oxidizes succinate also produced by the citric acid cycle, and then transfers the electrons through coenzyme Q. Unlike complex I, complex II does not pump out any protons (Cecchini 2003). The complex III (cytochrome bc_1 complex) transfers the electrons

to complex IV (cytochrome C oxidase) through another cofactor, CytC (Crofts 2004). The electrons moving through complex IV are finally transferred to oxygen, producing water molecules (Yoshikawa, Muramoto et al. 2006). Coupled with electron transfer, both complexes also translocate protons into the intermembrane space. Through the action of complex I, III and IV, a proton gradient across the inner membrane is established, and ATP synthase (sometimes called complex V) takes advantage of this proton gradient as the main force allowing it to produce ATP (Figure 1.1) (Junge and Nelson 2015).

1.1.2.2 Calcium signaling

In addition to ATP production, mitochondria also play an important role in various essential cellular functions, and calcium signaling is one of them. Calcium is an essential ion involved in various signaling pathways as a messenger. In mitochondria, the influx of calcium to the matrix activates three dehydrogenases in the citric acid cycle, pyruvate dehydrogenase, NAD+-dependent isocitrate dehydrogenase, and 2-oxohlutarate dehydrogenase (Denton and McCormack 1985, Hansford 1985, Denton and McCormack 1990, McCormack, Halestrap et al. 1990, Denton 1993, Traaseth, Elfering et al. 2004), resulting in more ATP production by facilitating NADH generation. The amount of calcium in the matrix is regulated mainly by the mitochondrial calcium uniporters (MCU) on the inner membrane (Kirichok, Krapivinsky et al. 2004, Baughman, Perocchi et al. 2011, De Stefani, Raffaello et al. 2011) and Na+/Ca2+ antiporter (Palty, Silverman et al. 2010). In addition, cytosolic calcium is also capable of activating Ca^{2+}-binding mitochondrial carriers (CaMCs) embedded in the inner membrane (Haynes, Picking et al. 1986, Nosek, Dransfield et al. 1990, Walker and Runswick 1993, Palmieri, Pardo et al. 2001, Satrustegui, Pardo et al. 2007). Among CaMC members, the

aspartate/glutamate carrier 1 (AGC1) is involved in glutamate-dependent respiration by transporting glutamate, a key mitochondrial substrate, into the matrix (del Arco and Satrustegui 1998, Del Arco, Agudo et al. 2000, Satrustegui, Pardo et al. 2007, Gellerich, Gizatullina et al. 2010).

1.1.2.3 Cell death

Mitochondria also play an important role in apoptosis. The essential step involved in cell death is the release of CytC to cytosol. As mentioned above, CytC is present in the intermembrane space and has a role in transferring electrons within the ETC. The release of CytC induces subsequent steps with Apaf-1 and caspase-9, and finally activates caspase-3, an essential protease for apoptosis (Li, Nijhawan et al. 1997, Rodriguez and Lazebnik 1999). CytC release is regulated by a protein family, Bcl-2 (Kluck, Bossy-Wetzel et al. 1997, Yang, Liu et al. 1997, Jurgensmeier, Xie et al. 1998, Luo, Budihardjo et al. 1998, Finucane, Bossy-Wetzel et al. 1999, Kuwana, Mackey et al. 2002). The Bcl-2 family induces a formation of supramolecular opening in the outer membrane, leading to increase in its permeability (Eskes, Antonsson et al. 1998, Eskes, Desagher et al. 2000, Wei, Lindsten et al. 2000, Nechushtan, Smith et al. 2001). Furthermore, mitochondrial fragmentation and cristae rearrangement can also induce the CytC release (Bossy-Wetzel, Barsoum et al. 2003, Youle and Karbowski 2005, McBride, Neuspiel et al. 2006). The mitochondrial fragmentation is a common event of cell death, which is caused by various mitochondrial fusion and fission factors (Frank, Gaume et al. 2001, Karbowski, Lee et al. 2002, Breckenridge, Stojanovic et al. 2003, Olichon, Baricault et al. 2003, Lee, Jeong et al. 2004, Sugioka, Shimizu et al. 2004). In cristae remodeling, some Bcl-2 proteins also play an important

role, which is independent from their other role in the formation of the outer membrane opening (Scorrano, Ashiya et al. 2002, Germain, Mathai et al. 2005).

1.2 Mitochondrial DNA

1.2.1 mtDNA structure

One of the key features of mitochondria is a double-stranded circular genome present in the matrix. Human mtDNA is circular-shaped genome of ~16.5 kbp. Two strands of mtDNA are separated by different cesium chloride gradients due to their biased [G+C] contents in each strand (Shadel and Clayton 1997), and thus the cytosine- and guanine-rich strands are called the light strand (L-strand) and the heavy strand (H-strand), respectively. mtDNA encodes a total of 37 genes: 13 proteins, 2 rRNAs and 22 tRNAs (Figure 1.2). Although mtDNA contains only 13 polypeptides, they are essential due to their involvement in ETC complexes: Seven are subunits of complex I (ND1~6 and ND4L), one is a subunit of complex III (Cytb), three are subunits of complex IV (CO I, II and III), and two are subunits of ATP synthase (A6 and A8) (Chomyn, Mariottini et al. 1985, Chomyn, Cleeter et al. 1986, Lenaz, Fato et al. 2006, Schon, DiMauro et al. 2012).

mtDNA harbors a triple-stranded region between 16024 and 576 of mtDNA (Arnberg, van Bruggen et al. 1971, Kasamatsu, Robberson et al. 1971, Anderson, Bankier et al. 1981, Walberg and Clayton 1981, Chang and Clayton 1985, Taanman 1999) (Figure 1.2). The region is established by a third strand (a short replicated H-strand) hydrogen-bonded to the L-strand. This strand induces the displacement of the non-templating H-strand, leading to the formation of a loop structure (called a displacement loop or D-loop). Even though the D-loop is located at a non-coding region (NCR), it harbors essential control sites, a origin of replication (H-strand

origin, O_H) and two promoter sites (Chang and Clayton 1985). Although the function of the D-loop has not yet been characterized, a study showed that the D-loop appears to be involved in mtDNA organization (He, Mao et al. 2007). In addition to O_H existing in the D-loop, mtDNA has another major origin of replication on the L-strand (O_L). Even though several models for mtDNA replication exist, both origins of replication play a key role in most replication models (Clayton 2003).

Three promoters are involved in transcription of the mtDNA (Figure 1.2). One of the promoters is located on the L-strand, the so called light strand promoter (LSP) (Montoya, Christianson et al. 1982). LSP is responsible for the transcription of 8 tRNAs and 1 polypeptide, and its transcription is terminated at a MTERF1 termination site located at the $tRNA^{Leu}$ gene (Attardi and Schatz 1988, Asin-Cayuela, Schwend et al. 2005, Shi, Posse et al. 2016). The other two promoters are placed on the H-strand, the heavy strand promoter 1 and 2 (HSP 1 and 2) (Montoya, Christianson et al. 1982, Montoya, Gaines et al. 1983), and transcription from both HSPs generates 2 rRNAs, 14tRNAs, and 12 polypeptides (Attardi and Schatz 1988). HSP1 is located 16 bp upstream of the $tRNA^{Phe}$ gene (Figure 1.2) (Martin, Cho et al. 2005), which is about 150 bp downstream of LSP. However, transcription reactions initiated at LSP and HSP1 are not overlapping since transcription occurs in opposite directions. In addition to HSP1, *in vitro* assays have shown the presence of another promoter on H-strand near the 5'-12S rRNA gene, HSP2 (Montoya, Christianson et al. 1982, Montoya, Gaines et al. 1983, Yoza and Bogenhagen 1984, Martin, Cho et al. 2005). Although the transcription initiated from HSP2 has been observed *in vitro*, its site has not yet been characterized. HSP1 transcription is responsible for two rRNAs (12S and 16S rRNA) and the $tRNA^{Val}$ and the $tRNA^{Phe}$, and frequently terminated at the MTERF1 binding site like LSP transcription (Attardi and Schatz 1988, Asin-Cayuela,

Schwend et al. 2005) (Figure 1.2). Transcription initiated at HSP2 yields a long polycistronic transcript covering almost all genes encoded on the H-strand. Although HSP2 transcription appears to be terminated after $tRNA^{Thr}$, its termination site has not been clearly determined. Sondheimer et al. have suggested that it is located at the promoter-distal site within the D-loop (Sondheimer, Fang et al. 2010).

1.2.2 mtDNA maintenance

1.2.2.1 Mutations in mtDNA

mtDNA contains genes essential for the translation of 13 polypeptides which are subunits of the ETC and ATP synthase. Thus, mtDNA mutation precludes formation of the respiratory chain, resulting in serious defects in energy generation. One of the causes of DNA damage in mtDNA is the presence of reactive oxygen species (ROS), inducing various DNA lesions by oxidative attack (Krokan, Standal et al. 1997, Tuppen, Blakely et al. 2010). Mitochondria are a major source of ROS production in cells: ROS are produced by electron leakage from the ETC, especially from complex I and III (Chance, Sies et al. 1979, Fridovich 1995, Hansford, Hogue et al. 1997, Raha and Robinson 2000, Droge 2002, Crofts 2004, Gutterman 2005, Lenaz, Fato et al. 2006). In addition to oxidative damage, mtDNA is also damaged by various point mutations or large-scale deletions (Tuppen, Blakely et al. 2010). Alterations of even single nucleotides in mtDNA are able to cause various human mitochondrial diseases such as Leber's hereditary optic neuropathy (LHON), mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) and Leigh's disease (LS) (McFarland, Clark et al. 2002, Taylor, Giordano et al. 2003, Temperley, Seneca et al. 2003, McFarland, Schaefer et al. 2004, McFarland, Chinnery et al. 2007, Yang, Zhu et al. 2009). Large-scale deletions can be caused by mutations in genes involved in mtDNA replication and maintenance (Kaukonen, Juselius et al. 2000, Spelbrink, Li

et al. 2001, Hudson and Chinnery 2006), and flanking direct repeats in mtDNA (Schon, Rizzuto et al. 1989, Mita, Rizzuto et al. 1990, Samuels, Schon et al. 2004, Tuppen, Blakely et al. 2010). In addition, the deletions are accumulated and more severe with age (Bua, Johnson et al. 2006).

1.2.2.2 DNA repair in mitochondria

In order to maintain the integrity of mtDNA, mitochondria are to some extent capable of repairing damage to the mtDNA. However, this capacity is limited because mitochondria do not have all the DNA repair systems which are present in the nucleus. Nucleotide excision repair (NER) is responsible for repair of bulky UV-induced DNA damages (Rabik and Dolan 2007). Strikingly, NER does not exist in mitochondria (Clayton, Doda et al. 1974, Clayton, Doda et al. 1975, Kazak, Reyes et al. 2012). However, the observation of the repair of some NER-targeted damage, such as a cisplatin intrastrand crosslink, in mitochondria suggests that mitochondria appear to have alternative mechanisms to repair the damage (LeDoux, Wilson et al. 1992). Furthermore, mitochondria contain the cockayne syndrome proteins (CSA and CSB) which are involved in one of the NER pathways in nucleus, indirectly suggesting that mitochondria might have an NER-like repair system (Aamann, Sorensen et al. 2010, Kamenisch, Fousteri et al. 2010, Lagerwerf, Vrouwe et al. 2011, Cline 2012). It has also been observed that mitochondrial extracts are capable of repairing some mismatch damage, but the key proteins involved in the repair are different from those in the nuclear mismatch repair pathway (MMR) (Mason, Matheson et al. 2003, de Souza-Pinto, Mason et al. 2009). The repair of double-strand breaks (DSBs) in mitochondria by both homologous recombination (HR) and non-homologous end joining (NHEJ) has been studied. While HR has been shown in some organisms such as yeast, plant, and *Chlamydomonas reinhardtii* (Manchekar, Scissum-Gunn et al. 2006, Zhou, Liu et al. 2010, Davila, Arrieta-Montiel et al. 2011, Mileshina, Koulintchenko et al. 2011), HR in

mammalian mitochondria has not been frequently observed (Bacman, Williams et al. 2009, Alexeyev, Shokolenko et al. 2013). In addition, a recent study for NHEJ shows that mitochondria utilize alternative NHEJ (microhomology-mediated end joining) instead of classical NHEJ (Tadi, Sebastian et al. 2016). BER (base excision repair) is the best established repair mechanism in mitochondria. Several studies have shown that DNA damage can be repaired by the BER pathway (Pettepher, LeDoux et al. 1991, LeDoux, Wilson et al. 1992, Driggers, LeDoux et al. 1993, Bogenhagen 1999). BER is composed of two pathways: short-patch and long-patch BER. Although both pathways have been observed in mitochondria, the long-patch BER has to date only been characterized for a single type of DNA damage, 2-Deoxyribonolactone (dL) (Akbari, Visnes et al. 2008, Copeland and Longley 2008, Liu, Qian et al. 2008, Szczesny, Tann et al. 2008).

1.2.2.3 mtDNA replication

1.2.2.3.1 Components involved in mtDNA replication

Mitochondria contain many copies of mtDNA. Even though the number of mtDNA molecules is variable depending on the tissue, it is strictly regulated due to the relationship between mtDNA copy number and ATP generation (Moyes, Battersby et al. 1998, Dickinson, Yeung et al. 2013). The replication of mtDNA is an essential mechanism to maintain mtDNA. Various nuclear-encoded proteins are involved in mtDNA replication, such as the mitochondrial helicase (TWINKLE), the single-strand binding proteins (mtSSB), and DNA polymerase γ (POLγ). TWINKLE is a specific protein that unwinds double-strand mtDNA (dsDNA) (Spelbrink, Li et al. 2001). The unwinding activity of TWINKLE is dependent on NTP-hydrolysis, and always occurs in a 5’ to 3’ direction (Korhonen, Gaspari et al. 2003). The role of

mtSSB in replication is coating the displaced single-strand DNAs (ssDNAs) to prevent their renaturation, and in addition, it also has a stimulatory effect on the activity of TWINKLE (Van Tuyle and Pavco 1981, Van Tuyle and Pavco 1985, Yang, Curth et al. 1997, Korhonen, Gaspari et al. 2003, Kaguni 2004). POLγ is the sole DNA polymerase in mitochondria, which is composed of two subunits (POLγA and B). POLγA is a catalytic subunit of 140 kDa, containing polymerase, 3'-5' exonuclease and 5'-deoxyribose phosphate lyase activities (Gray and Wong 1992, Pinz and Bogenhagen 1998, Pinz and Bogenhagen 2000, Kaguni 2004). The accessory subunit, POLγB, has a role not only in binding to dsDNA but also in stimulating POLγA's catalytic activity and processivity (Lim, Longley et al. 1999, Kaguni 2004, Falkenberg, Larsson et al. 2007).

1.2.2.3.2 Replication models

Even though the mechanism of mtDNA replication has been studied for some decades, it has still been controversial. Several replication models have been suggested. The strand-displacement replication is the initial model suggested in the early 1970s (Robberson, Kasamatsu et al. 1972, Kasamatsu and Vinograd 1973). In this model, replication events take place from two major origins of replication (O_H and O_L). The initial replication starts at O_H located in the D-loop, and proceeds to around two thirds of the mtDNA to the location of O_L. The synthesis of the second strand is then initiated from O_L (Figure 1.3A) (Robberson, Kasamatsu et al. 1972, Kasamatsu and Vinograd 1973, Clayton 2003). The second model of mtDNA replication is a strand-coupled replication mechanism (Holt, Lorimer et al. 2000, Yang, Bowmaker et al. 2002). They observed the presence of lagging strands (replicated L-strand) coupled with the synthesis of the leading strand (replicated H-strand) (Figure 1.3B). The other replication model is proposed

from the strand-coupled model. Yang et al. showed that the some replicated fragments had resistance to RNase, which cleaves single-stranded RNA, but were digested by RNase H, which cleaves RNA in hybridized forms of RNA with DNA (RNA-DNA) (Yang, Bowmaker et al. 2002). It indicates that the synthesized lagging strands appear to be RNA. In addition, it was observed that long RNA strands are synthesized as lagging strands (Yasukawa, Reyes et al. 2006). Based on those observations, the other replication model, RITOLS, has been proposed (Figure 1.3C). In this model, the synthesis of leading strand is coupled with that of RNA as the lagging strands, and the synthesized RNA is then replaced by DNA (Yasukawa, Reyes et al. 2006, Holt and Reyes 2012). However, it has not yet been established what the RNA source is and how RNA is exchanged to DNA.

1.2.2.4 Mitochondrial transcription

1.2.2.4.1 Initiation

In order to express the genes encoded in mtDNA, mitochondrial transcription occurs at three promoters (LSP, HSP 1 and 2) and is initiated by three mitochondrial proteins (mitochondrial transcription factor A (TFAM), mitochondrial transcription factor B2 (TFB2M) and RNA polymerase (POLRMT)). TFAM is a HMG (high-mobility group)-containing protein responsible for the recognition of the promoters. Even though it has been argued that TFAM is not an essential component for mitochondrial transcription initiation, transcription activity is significantly stimulated in the presence of TFAM (Shutt, Lodeiro et al. 2010). TFAM bound to promoters then recruits other factors, TFB2M and POLRMT, and thus helps assemble the mitochondrial transcription initiation machinery (McCulloch and Shadel 2003, Yakubovskaya, Guja et al. 2014). A recent DNase I footprinting assays suggested the order in which these two

proteins are recruited: TFAM initially recruits POLRMT, and TFB2M is then recruited to this complex (Posse, Hoberg et al. 2014).

The mitochondrial transcription factor B (TFB) protein has two paralogues, TFB1M and TFB2M (Falkenberg, Gaspari et al. 2002, McCulloch, Seidel-Rogol et al. 2002). Sequence analysis reveals that both TFB1M and TFB2M are homologous to bacterial rRNA methyltransferases (Falkenberg, Gaspari et al. 2002), and i*n vivo* methylation assays further showed that both TFB proteins have rRNA methyltransferase activity (Seidel-Rogol, McCulloch et al. 2003, Cotney and Shadel 2006). However, while TFB1M plays an important role in methylation of 12S ribosomal RNA (Metodiev, Lesko et al. 2009), TFB2M has a fundamental role in mitochondrial transcription (Cotney and Shadel 2006, Metodiev, Lesko et al. 2009, Shutt, Lodeiro et al. 2010). The major role of TFB2M in transcription is melting the promoter sites and interacting with the priming substrates and DNA templating base to stabilize the open promoter complex and prevent re-annealing (Sologub, Litonin et al. 2009). TFB2M is able to bind to the promoter only when interacting with other transcription factors, TFAM or POLRMT (McCulloch and Shadel 2003, Sologub, Litonin et al. 2009).

POLRMT plays a key role in the synthesis of RNA transcripts. It shares homology with bacteriophage T7 RNA polymerase (T7 RNAP) (Masters, Stohl et al. 1987, Cheetham and Steitz 1999, Gaspari, Larsson et al. 2004, Mercer, Neph et al. 2011). However, there are several sequence and structural differences between both polymerases. Human POLRMT is broadly composed of three regions, an N-terminal extension (NTE), N-terminal domain (NTD), and C-terminal polymerase domain (CTD) (Arnold, Smidansky et al. 2012). However, the NTE does not exist in T7 RNAP (Cermakian, Ikeda et al. 1997). Human POLRMT contains a signal peptide targeting it to mitochondria, an unknown large flexible region and a pentatricopeptide

repeat domain (PPR). The large flexible domain appears to play an important role in promoter-specific transcription (Ringel, Sologub et al. 2011). In addition, its role is further suggested by a yeast homolog, Rpo41: it seems to be involved in RNA processing and translation after transcription, regulation of transcription initiation, and autoinhibitory role in transcription initiation (Rodeheffer, Boone et al. 2001, Rodeheffer and Shadel 2003, Paratkar, Deshpande et al. 2011). The PPR domain in the NTE appears to be involved in RNA-protein interaction (Small and Peeters 2000, Lightowlers and Chrzanowska-Lightowlers 2008). The NTD of human POLRMT is composed of 279 residues, but its role has not yet been clearly determined. Even though its sequence is not similar to the NTD of T7 RNAP, they share some structural features: an AT-rich loop and an intercalating β-hairpin (Ringel, Sologub et al. 2011, Arnold, Smidansky et al. 2012). The structural similarities in both structures led to the suggestion that the both NTDs might play a similar role. However, while the NTD of T7 RNAP has a role in promoter melting and recognition (Cheetham, Jeruzalmi et al. 1999, Brieba and Sousa 2001, Stano and Patel 2002), human POLRMT does not perform those functions by itself. Instead, two other factors, TFAM and TFB2M, are necessary for transcription initiation. The CTD is the core domain for RNA synthesis, and has highest similarity in both sequences and structures to T7 RNAP (Cermakian, Ikeda et al. 1997, Tiranti, Savoia et al. 1997), composed of finger, palm, and thumb subdomains like other typical polymerases.

Even though the structure of the mitochondrial transcription initiation complex has not yet been determined, each single crystal structure of TFAM, POLRMT, and TFB1M (a paralog of TFB2M) has been already solved (Ngo, Kaiser et al. 2011, Ringel, Sologub et al. 2011, Rubio-Cosials, Sidow et al. 2011, Guja, Venkataraman et al. 2013, Ngo, Lovely et al. 2014). Recently, Yakubovskaya et al. suggested a model of the transcription initiation complex by docking the

single crystal structures into EM maps of the complex with LSP (Yakubovskaya, Guja et al. 2014). Despite the lack of detailed information on interactions among those factors, this complex model shows how the three factors form the transcription initiation machinery.

1.2.2.4.2 Elongation

Even though the three initiation factors are capable of starting RNA synthesis, they are not sufficient to proceed to transcription of the promoter-distal genes. The transcription from LSP is frequently terminated at a guanine-rich sequence block, called the conserved sequence block II (CSBII) located downstream of LSP. When transcription proceeds to CBSII, a G-quadruplex structure is formed in synthesized RNA, stimulating transcription termination. These short RNAs are involved in mtDNA replication as primers (Chang and Clayton 1985, Xu and Clayton 1995, Xu and Clayton 1996, Pham, Farge et al. 2006, Wanrooij, Uhler et al. 2010, Wanrooij, Uhler et al. 2012). In addition to the early transcription termination from LSP, *in vitro* assays using HSP also showed that the promoter-proximal transcripts are more abundant than the promoter-distal ones (Minczuk, He et al. 2011). The mitochondrial elongation factor (TEFM) is an essential factor for mitochondrial transcription to proceed completely after transcription initiation occurs (Minczuk, He et al. 2011, Agaronyan, Morozov et al. 2015, Posse, Shahzad et al. 2015). TEFM interacts with the C-terminus of POLRMT (Minczuk, He et al. 2011), and enhances its processivity to overcome the early transcription termination happening from both LSP and HSP (Minczuk, He et al. 2011, Agaronyan, Morozov et al. 2015, Posse, Shahzad et al. 2015).

1.2.2.4.3 Termination

Transcription from three promoters is terminated at specific termination sites. The termination of HSP1 transcription takes place at the 3'-end of the $tRNA^{Leu}$ gene by MTERF1

(mitochondrial termination factor 1) (see Figure 1.2) (Kruse, Narasimhan et al. 1989, Fernandez-Silva, Martinez-Azorin et al. 1997). MTERF1 is the transcription termination factor recognizing and binding to the specific sequence at $tRNA^{Leu}$ gene site. The termination activity of MTERF1 is dependent on the orientation of its binding sequence with respect to the transcription direction (Asin-Cayuela, Schwend et al. 2005, Yakubovskaya, Mejia et al. 2010). In addition to HSP1 termination, LSP transcription is also terminated at the same site as HSP1 by MTERF1 (Asin-Cayuela, Schwend et al. 2005). Compared to the transcription termination of LSP and HSP1, the mechanism of HSP2 transcription termination has not yet been clearly established. One possible mechanism is through a rho-independent termination-like mechanism. Similar to the early termination of LSP transcription at CBSII, HSP2 transcription might be also terminated in the same way (Wanrooij, Uhler et al. 2010, Guja and Garcia-Diaz 2012). Sondheimer et al. suggested that a specific protein, the Leucine-rich pentatricopeptide-repeat containing protein (LRPPRC), binds to the distal termination site, and appears to be involved in HSP2 termination (Sondheimer, Fang et al. 2010).

1.3 Mitochondrial transcription factor A (TFAM)

1.3.1 Introduction to TFAM

TFAM functions as a mitochondrial transcription initiation factor recognizing promoter sequences and recruiting other factors. However, TFAM also has another function as an mtDNA packaging factor. TFAM is a high-mobility group (HMG) containing protein composed of 246 amino acids (Parisi and Clayton 1991). The first 42 residues in the N-terminus constitute a signal peptide targeting TFAM to mitochondria, and it is cleaved after import into the mitochondrial matrix. TFAM is composed of two tandem HMG boxes connected by a ~30-residue long linker,

and a ~25-residue long C-terminal tail (Figure 1.4A). The HMG domain is a well-known DNA binding motif which has been already characterized in other DNA binding proteins (Stros, Launholt et al. 2007, Liu, Prasad et al. 2010). Since 2011, a total of four TFAM structures in complex with different DNA substrates have been determined to date: two with LSP promoter sequences, one with HSP1 sequences, and one with a nonspecific sequence (NS). Interestingly, despite various substrate sequences, all crystal structures reveal identical structural features: TFAM wraps up the DNA substrates and induces them to adopt a U-turn shaped structure (Figure 1.4B). DNA bending is induced by two intercalations of TFAM residues into two base stacking sites: Two leucine residues are responsible for the intercalations, L58 of HMG box 1 and L182 of HMG box2 (Ngo, Kaiser et al. 2011, Rubio-Cosials, Sidow et al. 2011, Ngo, Lovely et al. 2014). However, the DNA bending induced by the HMG boxes is not a unique structural property of TFAM. The structures of other HMG-containing proteins also present the DNA-bent structures caused by residue intercalation (Murphy, Sweet et al. 1999, Cerdan, Payet et al. 2001, Stott, Tang et al. 2006, Churchill, Klass et al. 2010, Sanchez-Giraldo, Acosta-Reyes et al. 2015). This similar structural property among HMG-containing proteins involved in various functions indicates that DNA bending by HMG boxes appears to play a role in the functions of those proteins.

1.3.2 Transcription activating function

TFAM plays a fundamental role in activating mitochondrial transcription. As mentioned above, major roles of TFAM in transcription are recognizing specific promoter sequences and recruiting two other transcription initiation factors. Even though there is some argument that TFAM is not essential for transcription initiation (Shutt, Lodeiro et al. 2010), it plays an essential

role in efficient transcription initiation (Gaspari, Falkenberg et al. 2004, Litonin, Sologub et al. 2010).

The specific binding of TFAM on promoters has been shown by DNase I footprint experiments (Fisher, Topper et al. 1987, Fisher and Clayton 1988). However, while TFAM binds to a specific site of ~23 bp at LSP, its binding at HSP1 is more diffuse, indicating that TFAM binds to HSP1 less specifically than LSP. The different binding specificity of TFAM between LSP and HSP1 also appears to be related to the distinct dependency of TFAM for transcription initiation at both promoters. *In vivo* studies show that LSP can be activated at lower TFAM concentrations than HSP1 (Bonawitz, Clayton et al. 2006, Litonin, Sologub et al. 2010), and *in vitro* transcription assays reveal that the absence of TFAM reduces transcription at LSP more than that at HSP1 (Shutt, Lodeiro et al. 2010). In addition, excessive amounts of TFAM are able to inhibit transcription from LSP significantly, but HSP1 transcription is much less affected (Shutt, Lodeiro et al. 2010). This indicates that LSP appears to be more sensitive to TFAM concentrations than HSP1.

The mechanism by which TFAM recognizes the promoters has not yet been characterized. It has been proposed that the C-terminal tail of TFAM plays a key role in promoter recognition (Dairaghi, Shadel et al. 1995). The elimination of the C-terminal tail reduces transcription activity and the specific binding affinity of TFAM. However, the crystal structures with LSP reveal that there is no sequence specific interaction between the C-terminal tail and LSP, conferring no role of the C-terminal tail in promoter recognition (Figure 1.5) (Ngo, Kaiser et al. 2011, Rubio-Cosials, Sidow et al. 2011). Instead, a study represented that the C-terminal tail is able to affect mtDNA structure such as the D-loop formation (Ohgaki, Kanki et al. 2007). Furthermore, the observation that the C-terminal tail physically interacts with TFB2M and

POLRMT indicates that its role appears to be the recruitment of these proteins for mitochondrial transcription initiation (McCulloch and Shadel 2003, Yakubovskaya, Guja et al. 2014). Besides the interaction with the C-terminal tail, most of the interactions between TFAM and the promoters are also non-sequence specific through phosphate ions and the sugar rings of DNA. Although there are a few hydrogen bonds, most of them are non-sequence specific through the minor groove.

1.3.3 mtDNA packaging function

In mitochondria, mtDNA is present in the matrix in a condensed form, called a nucleoid (Spelbrink 2010, Kukat and Larsson 2013), and TFAM plays a key role in mtDNA packaging (Alam, Kanki et al. 2003). TFAM is a very abundant protein in mitochondria: ~1000 molecules of TFAM are present per molecule of mtDNA, enough to fully coat the mtDNA (Ekstrand, Falkenberg et al. 2004, Kukat, Wurm et al. 2011). mtDNA coating by TFAM has been observed in various organisms such as human, mouse, frog, and chicken (Shen and Bogenhagen 2001, Takamatsu, Umeda et al. 2002, Alam, Kanki et al. 2003, Matsushima, Matsumura et al. 2003, Ekstrand, Falkenberg et al. 2004, Pellegrini, Asin-Cayuela et al. 2009, Kukat, Wurm et al. 2011). HMG boxes appear to play a role in non-sequence specific binding, followed by mtDNA packaging (Fisher, Lisowsky et al. 1992). In addition, Abf2p (ARF (ADP ribosylation factor)-binding factor2), a yeast homolog of TFAM, suggests that HMG boxes appear to be sufficient to drive this function. Abf2p also plays a fundamental role in yeast mtDNA packaging (Diffley and Stillman 1992, Brewer, Friddle et al. 2003). However, while it contains two tandem HMG domains like TFAM, the C-terminal tail does not exist (Diffley and Stillman 1991, Diffley and Stillman 1992), indicating that HMG boxes have an essential role in mtDNA packaging. Although the ability of TFAM to bind DNA nonspecifically has been established, the binding

mode is still being debated. While both *in vivo* and *in vitro* assays have shown that TFAM binds to DNA as a homodimer (Kaufman, Durisic et al. 2007, Gangelhoff, Mungalachetty et al. 2009), other *in vitro* studies and various crystal structures have revealed that TFAM acts as a monomer (Ngo, Kaiser et al. 2011, Rubio-Cosials, Sidow et al. 2011, Ngo, Lovely et al. 2014).

The condensation function of TFAM has an essential role in mtDNA maintenance. TFAM appears to be closely related to the mtDNA copy number (Larsson, Wang et al. 1998, Matsushima, Matsumura et al. 2003, Ekstrand, Falkenberg et al. 2004, Kanki, Ohgaki et al. 2004). The knockout of *TFAM* leads to the depletion of mtDNA in mouse, followed by defective energy generation, and even the heterozygous knockout of *TFAM* also reduces the mtDNA copy number although it is not lethal (Larsson, Wang et al. 1998). In addition, the downregulation and upregulation of *TFAM* expression are coupled with decreases and increases in the number of mtDNA, respectively (Kanki, Ohgaki et al. 2004).

1.4 Concluding remarks

Although TFAM has been investigated for a few decades, its binding mechanism to DNA substrates has not yet been characterized. Through analysis of the crystal structures determined to date, I have identified a specific sequence consensus that might facilitate the binding of TFAM. The consensus is composed of two guanine bases separated by 10 variable nucleotides ($GN_{10}G$). Here, I present a crystal structure with another nonspecific sequence and various biochemical assays to show that TFAM prefers to recognize this $GN_{10}G$ consensus, and that this interaction might play a role in TFAM's variable functions. In addition, the crystal structure also displays a unique binding manner recognizing between two DNA ends, implicating its involvement in double-strand break repair in mitochondria, and I also present another biochemical assay to test this hypothesis. These results provide not only a mechanistic

explanation for how TFAM might recognize its substrates to carry out its diverse functions on mtDNA, but also identify a novel potential functional role of TFAM in mitochondrial repair.

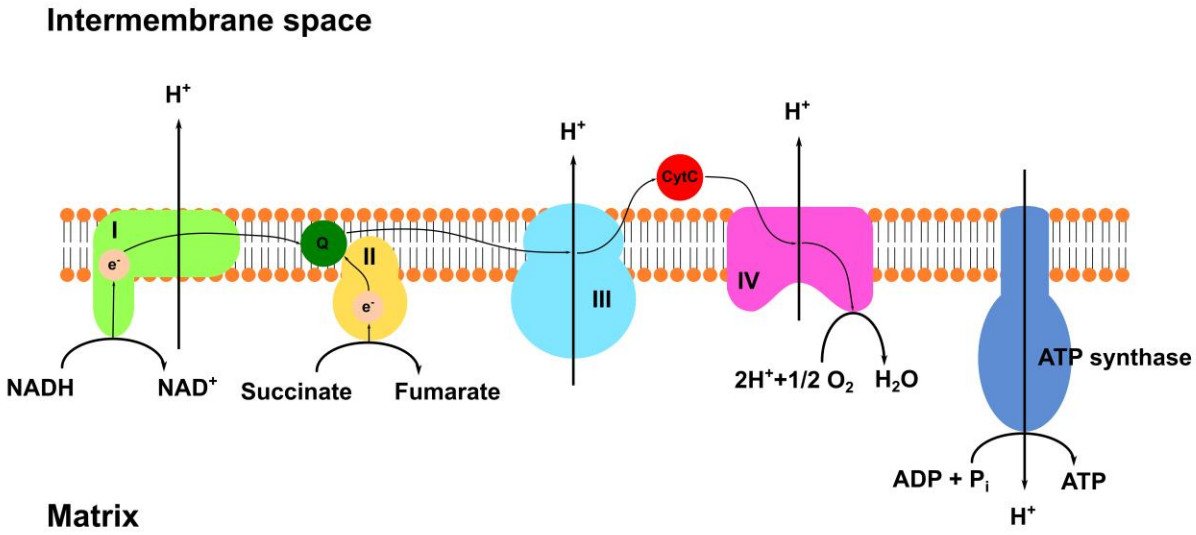

Figure 1-1. Oxidative phosphorylation
The electrons are provided as NADH and succinate from citric acid cycle, and transferred through the complexes imbedded in the inner membrane. Coupled with electron transfer, protons are also transported to intermembrane space, resulting in formation of proton gradient across the inner membrane. ATP synthase uses the gradient as main force to produce ATP in the matrix.

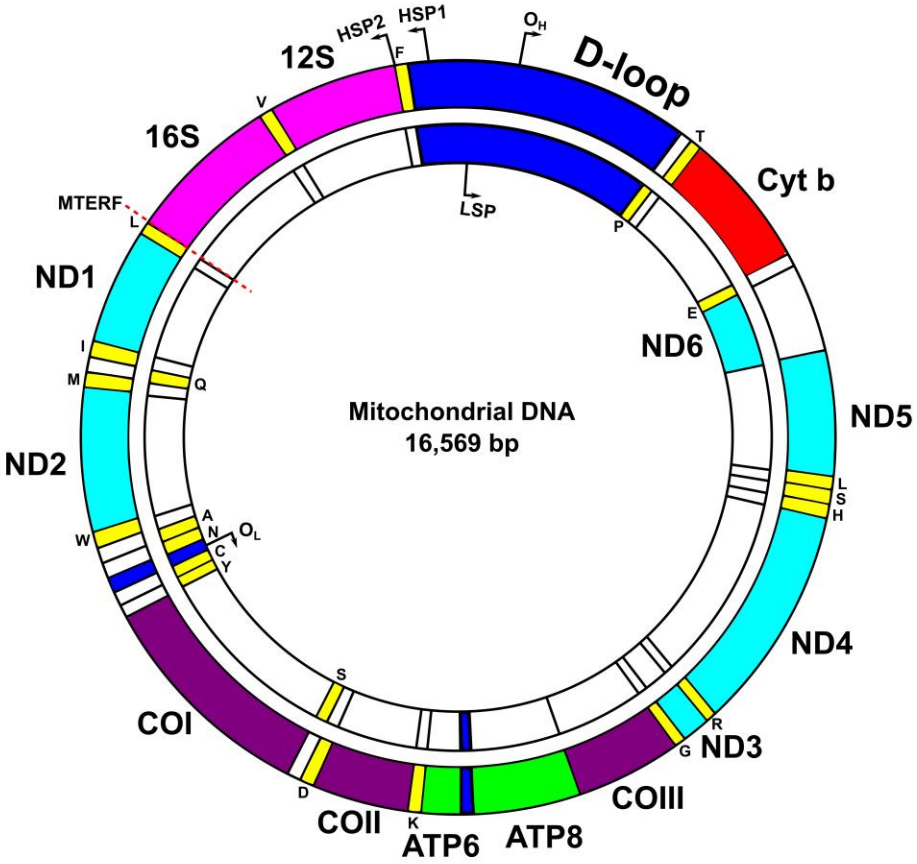

Figure 1-2. Mitochondrial DNA
The encoded genes in both H-strand (outer ring) and L-strand (inner ring) are colored: 13 proteins (cyan, purple, red and lime), 2 rRNA (pink), and 22 tRNA (yellow). The single letters labeled at tRNA sites represent one-letter symbols for amino acids. The blue colors represent non-coding regions. The replication origins and promoters are marked as bent arrows, and the red dot line shows the MTERF1 binding site. ND1-6, NADH dehydrogenase subunits 1-6; COI-III, cytochrome c oxidase subunits I-III; ATP 6 and 8, ATP synthase subunits 6 and 8; Cyt b, cytochrome b; D-loop, displacement-loop; O_H, origin of replication of H-strand; O_L, origin of replication of L-strand; HSP, heavy strand promoter; LSP, light strand promoter.

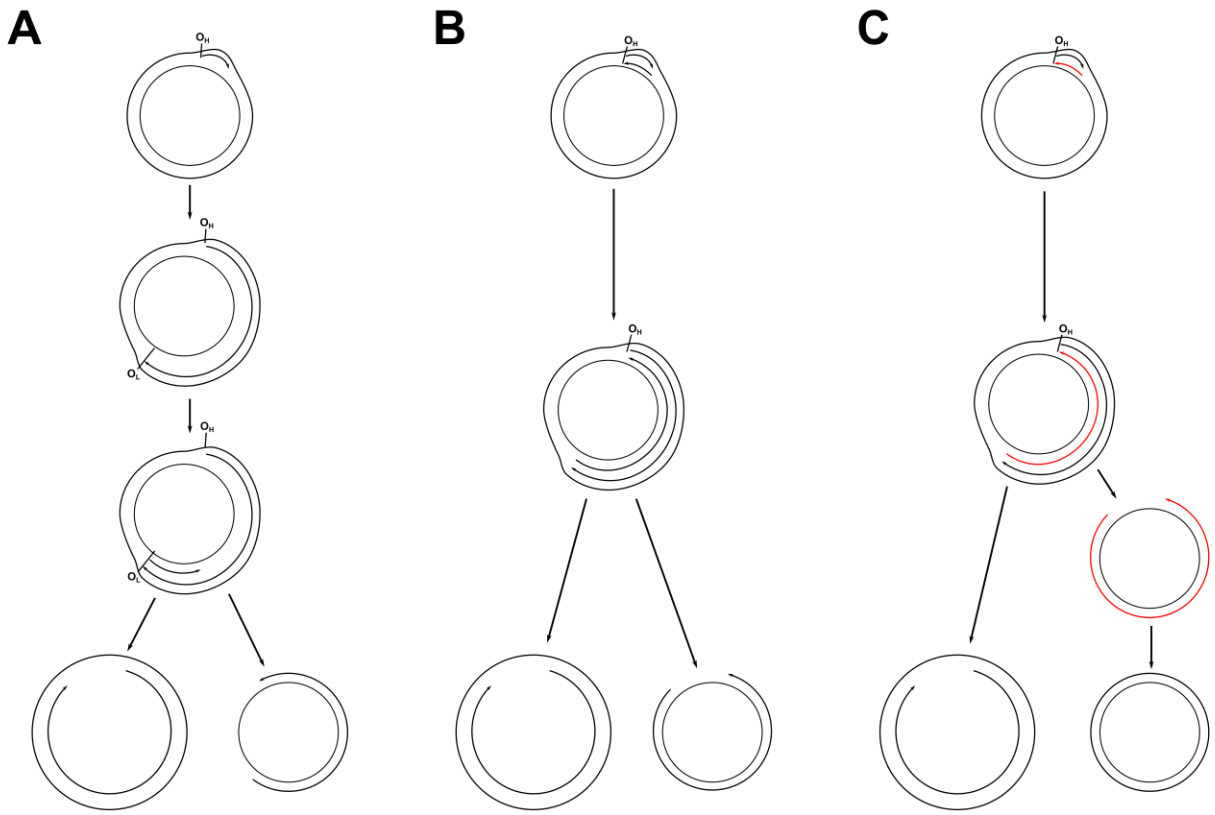

Figure 1-3. mtDNA replication models
(A) Strand-displacement model. The DNA synthesis at O_L begins when the synthesized strand from O_H reaches O_L site. (B) Strand-coupled model. Both strands are synthesized together at O_H. (C) RITOLS. The strand synthesis is the same as the strand-coupled model, but the lagging strand is initially synthesized as RNA strand. The red line represents RNA.

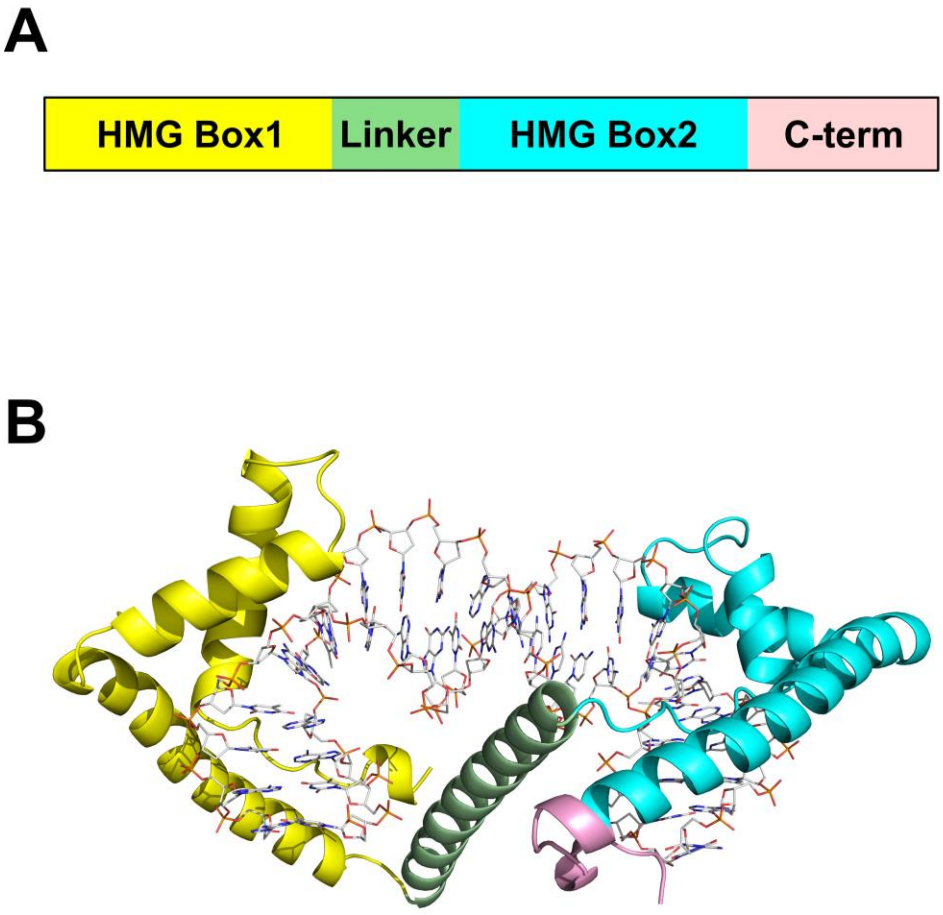

Figure 1-4. TFAM structure
(A) Domain architecture of TFAM. TFAM is composed of two tandem HMG boxes connected by a linker, and C-terminal tail. (B) TFAM-LSP structure. TFAM wraps up LSP substrate, and induces its U-turn shape by two intercalations of TFAM residues. PDB ID: 3TQ6

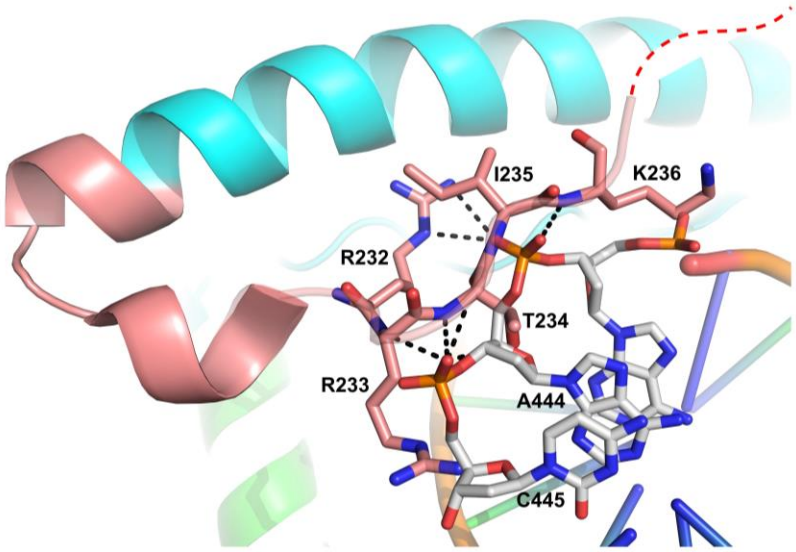

Figure 1-5. Interaction between C-terminal tail of TFAM and LSP
The C-terminal tail interacts with LSP through non-sequence specific contacts. The C-terminal tail is colored as pink, and the last nine residues which could not be built in the crystal structure are shown as the red dot line. PDB ID: 3TQ6.

Chapter 2 Materials and Methods

This chapter contains experimental procedures that will be incorporated in a manuscript currently in preparation: Choi WS and Garcia-Diaz M.

2.1 Protein expression and purification

TFAM (43~246) was cloned into a modified pET22 vector using EcoRI and XhoI. The plasmid was transformed into ArcticExpress (DE3). The protein was induced by 0.3 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) when the O.D. (Optical density) value was about 0.8, and the cells were then cultured at 16℃ for 15 hours. The protein was expressed as a fusion protein containing a histidine-tagged maltose binding protein (MBP) at its N-terminus. Cells were harvested and resuspended in a lysis buffer (20 mM Hepes pH 8.0, 1 M KCl, and 20 mM Imidazole). The cells were lysed by sonication and the supernatant was collected for the purification. TFAM was first purified by Nickel (Ni)-chromatography equilibrated with the lysis buffer. After the sample went through the column, the protein-bound resins were washed by a wash buffer (20 mM Hepes pH 8.0, 0.5 M KCL, and 20 mM Imidazole) to remove unbound-proteins and nonspecifically bound-proteins in column. The target protein was then eluted by an elution buffer (20 mM Hepes pH 8.0, 0.5 M KCL, and 0.5 M Imidazole). In order to remove the N-terminal tag (a histidine-tagged MBP) from TFAM, TEV (tobacco etch virus) protease was added to the eluted protein, and the mixture was incubated overnight at 4 ℃. TFAM was then purified by HiTrap Heparin HP (GE healthcare) equilibrated by a binding buffer (20 mM HEPES pH 8.0 and 1 mM DTT). Before the sample was loaded on the column, it was diluted 3~4 times with the binding buffer to reduce the salt concentration. The bound-TFAM was eluted at 61 mS/cm of conductivity by an elution buffer (20 mM HEPES pH 8.0, 1 M KCl, and 1 mM DTT).

TFAM was finally purified by a size-exclusion chromatography (Superdex 200 16/600 GL, GE healthcare) equilibrated with a buffer containing 20 mM Hepes pH 8.0, 150 mM KCl and 1 mM DTT. All TFAM variants (S61A, Y57A and S61A·Y57A) were generated by site-directed mutagenesis, and the mutations were confirmed by DNA sequencing. The purification procedure of these TFAM variants was the same as that of TFAM WT.

2.2 Crystallization of TFAM

The TFAM concentration for the crystallization screen was 10 mg ml^{-1}. Both LSP2 and nonspecific sequence (NS2) were separately mixed with TFAM as 1:1.5 (protein: DNA) molar ratio. The TFAM-DNA mixtures were incubated on ice for 30 min for stable complexes. Both complexes were crystallized on 24-well plates using a hanging-drop method. The crystallization conditions were 0.1M Hepes pH 7.5, 0.05 M $MgCl_2$, and 32.5% PEG MME 550 (LSP2) and 0.1 M Bis-Tris pH 6.5 and 24% PEG 2000 MME (NS2). The shapes of both crystals were the same, a rectangular plate. In order to freeze the crystals for x-ray diffraction, the crystals were prepared with cryo-buffers containing 0.1M Hepes pH 7.5, 0.05M MgCl2, and 36% PEG MME 550 (LSP2) and 0.1 M Bis-Tris pH 6.5, 30% PEG 2000 MME, and 20% Ethyl Glycol (NS2), respectively.

2.3 Data collection and structure determination

Data collection was carried out on beamline X29 (LSP2) and X25 (NS2) using the National Laboratory Synchrotron Light Source at Brookhaven National Laboratory. The crystals diffracted to 2.7 Å (LSP2) or 2.84 Å (NS2). The data were processed and scaled using HKL2000 (LSP2) (Otwinowski 1997), and XDS (Kabsch 2010) and SCALA (Evans 2006, Evans 2011) in the autoPROC pipeline (NS2) (Vonrhein, Flensburg et al. 2011) and respectively. Both structures

were phased by molecular replacement using Phaser (McCoy, Grosse-Kunstleve et al. 2007) with a TFAM-LSP structure (PDB ID: 3TQ6) as a template (Rubio-Cosials, Sidow et al. 2011). Further model building was carried out using COOT (Emsley, Lohkamp et al. 2010), and the structures were refined using Phenix (Adams, Afonine et al. 2010) and BUSTER (Bricogne G. 2011). The refined structures were finally validated using SFCHECK (Vaguine, Richelle et al. 1999), PROCHECK (Laskowski, Moss et al. 1993), and RAMPAGE (Lovell, Davis et al. 2003) implemented in the ccp4i program suite (Potterton, Briggs et al. 2003).

2.4 End-joining assay

A 237-bp substrate was obtained by PCR amplification using the following primers: 5' – AAACCGGGACATTCCCCTCTAG – 3' and 5' - GATGTCTGTGTGGAAAG GGCTG – 3'. The amplified product was then purified using the MiniElute® PCR purification kit (Qiagen). 50 nM of DNA substrate was mixed with a binding buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, and 10 mM DTT), and 0~300 nM TFAM in 20 µl. The mixture was incubated for 30 min on ice for stable binding of TFAM to DNA substrates, and 50 units of T4 DNA ligase was then added. The ligation reactions were performed at room temperature for 3 hours, and quenched by adding 80 µl of a stop solution (1% SDS, 20 mM EDTA, 300 mM sodium acetate), immediately followed by phenol-extraction. After vortexing, 85 µl of the aqueous solution (where the DNA product was included) was transferred to new 1.5 ml tubes. The product was further purified by ethanol-precipitation. The DNA pellet was resuspended in 10 µl of a loading buffer (1X Tris/Borate/EDTA (TBE) buffer and 5% glycerol), and loaded onto a 1.0 % agarose gel. The gel was run at 100 V for 45 min, and was then stained by SYBR® Gold (Invitrogen) and scanned at 473 nm using Typhoon 9000. The band intensities were quantified using ImageQuant (GE healthcare). The ligated ends were calculated by following equation:

$$\frac{1b + 2b + 3d}{a + b + c + d} \times 0.85\ (pmol)$$

The letters, *a*, *b*, *c*, and *d* represent the bands according to the number of ligation events (0, 1, 2, and 3 ligations, respectively). The 0.85 pmol was the amount of the whole DNA product loaded on gel, and in the calculation each band was weighted by the number of the ligation events.

2.5 *In vitro* transcription assay

LSP (171~470) and HSP1 (491~790) sites were cloned with NcoI and HindIII into pET-22 vectors. In order to produce run-off transcripts, LSP and HSP vectors were linearized with NcoI and HindIII, respectively. All transcription initiation factors were pre-mixed as 1:1:1 of the molar ratio (TFAM: TFB2M: POLRMT). The reaction volume was composed of a transcription buffer (20 mM Hepes pH 8.0, 40 mM KCl, 5 mM DTT, 1 mM EDTA and 10 mM $MgCl_2$), 20 ng (0.17 nM) of linearized LSP or HSP, 0.2 µM protein mixture, 0.3 µCi [P^{32}]-αUTP and 3 µl rNTP mixture (0.4 mM ATP, 0.15 mM CTP and GTP, 0.01 mM UTP) in 30 µl. The mixture was incubated at 32 ℃ for 30 min, and then quenched by 100 µl of a stop solution (20 mM EDTA, 1% SDS, 300 mM NaAc and 20 µg calf thymus DNA). The transcription products were ethanol-precipitated, and RNA pellets were resuspended in 20 µl of a RNA loading buffer. The samples were run on 10% polyacrylamide gels containing 8 M Urea and 1X TBE buffer at 200 V for 1.5 hours. The gel was dried for 2 hours and exposed to a phosphor screen (GE Healthcare) for 12 hours. The phosphor screen was scanned using Typhoon 9000 and the results were analyzed by ImageQuant (GE healthcare).

2.6 Electrophoretic Mobility Shift Assay (EMSA)

A 28-bp nonspecific oligonucleotide (DNA^{GG}) was selected from human mtDNA genome (6694-6721; within the cytochrome C oxidase subunit I gene), containing a $GN_{10}G$ consensus in the middle. DNA^{AA} has the identical sequence, but both guanines were replaced to adenines. Both substrates were labeled by Cy3 fluorescent tags at their 5'-ends, and the following complementary sequences were annealed: DNA^{GG}: 5'-/Cy3/AAAAAGAA**C**CATTTGGATA**C**ATAGGTAT-3'; 5' - ATACCTAT**G**TATCCAAATG**G**TTCTTTTT - 3' and DNA^{AA}: 5'-/Cy3/AAAAAGAA**T**CATTTGGATA**T**ATAGGTAT-3'; 5' - ATACCTAT**A**TATCCAAATG**A**TTCTTTTT - 3'. The underlined bold letters in DNA^{GG} and DNA^{AA} represent the $GN_{10}G$ consensus and the altered nucleotides, respectively. In order to reduce nonspecific interactions between TFAM and DNA, the NaCl concentration was optimized from 0 to 600 mM, and 500 mM NaCl was finally decided for further assays due to the first appearance of free DNA at this concentration. The assays for apparent K_D calculations were carried out with a binding buffer (10 mM Hepes pH 8.0, 2 mM DTT, 130 μg/ml BSA, 500 mM NaCl, 5% glycerol), 100 nM DNA substrate, and 0 ~ 600 nM TFAM. The reaction mixtures were incubated on ice for 30 min and at room temperature for 10 min for stable TFAM-DNA complexes. The samples were then mixed with 10μl of a loading buffer (5% glycerol and 1X TBE buffer). They were loaded onto 6% non-denaturing polyacrylamide gels, and run at 70 V for 1.5 hours. The results were recorded at 532 nm using Typhoon 9000 and analyzed by ImageQuant (GE healthcare). K_D values were calculated using Prism software (GraphPad Software Inc.).

2.7 EcoRI cleavage assay

A 100-bp substrate was obtained by PCR amplification of a modified pET22 vector using following primers: 5’ – CTGAAGCCAGTTACCTTTGAAAAAAG – 3’ and 5’ – TAATCTGCTGCTTGCAAATAAAAAAAC – 3’. The substrate sequence was designed to have a $GN_{10}G$ consensus using site-directed mutagenesis. $DNA2^{GG}$ had a $GN_{10}G$ consensus and an EcoRI cleavage site between the two guanines. $DNA2^{AA}$ had the identical sequence where both guanines were replaced to adenines. $DNA2^{GG}$: 5’-CTGAAGCCAGTTACCTTTGAAAAAAGAGTTGGTAGCTCTTGATC**C**AG*GAATTC*AA**C**CACCGCTGGTAGCGGTGGTTTTTTTATTTGCAAGCAGCAGATTA-3’; $DNA2^{AA}$: 5’-CTGAAGCCAGTTACCTTTGAAAAAAGAGTTGGTAGCTCTTGATC**T**AG*GAATTC*AA**T**CACCGCTGGTAGCGGTGGTTTTTTTATTTGCAAGCAGCAGATTA-3’. The underlined and bold letters indicate templating cytosines ($DNA2^{GG}$) and altered nucleotides ($DNA2^{AA}$), respectively, and the underlined italicized letters are the EcoRI cleavage site. The binding reaction was carried out in a mixture of binding buffer (10 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 100 µg/ml BSA, 50 mM NaCl), 100 nM DNA substrate and 0~ 50 nM TFAM in 20 µl. The concentration of TFAM used in the reaction was less than DNA substrate because huge amounts of TFAM could coat the substrates through nonspecific interactions, followed by nonspecific interference of EcoRI function. The reaction mixture was incubated for 30 min on ice for stable TFAM-DNA complexes. 0.5 unit of EcoRI was then added and incubated at 37 ℃ for 15 min. The reaction was quenched by 80 µl of a stop solution (1% SDS, 20 mM EDTA, 300 mM sodium acetate), immediately followed by phenol-extraction. After vortexing, 85 µl of aqueous solution was transferred to clean 1.5 ml tubes. The extracted DNA was ethanol-precipitated overnight. The DNA pellet was resuspended in 10 µl of a loading buffer (5% glycerol and 1X

TBE buffer) and loaded on 4% agarose gel. The gel was run at 100 V for 50 min, and then stained by SYBR® Gold (Invitrogen). They were scanned at 473 nm using Typhoon 9000. The results were analyzed by ImageQuant (GE healthcare).

2.8 Compaction assay

A modified pET22 vector was used as a substrate. The binding reaction was composed of a binding buffer (10 mM Tris-HCl pH 7.6, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$), 20 nM plasmid, and 75 μM TFAM in 10 μl. The amount of TFAM enough to coat 1~2 plasmids was used in order to avoid higher condensation of a plasmid by unnecessary nonspecific binding. The mixture was incubated on ice for 30 min for stable binding of TFAM to the plasmids, and 20, 2, 0.2, 0.02 units of DNase I were then added and incubated at 37 ℃ for 10 min. The digestion was quenched by 90 μl of a stop solution (1% SDS, 20 mM EDTA, 300 mM sodium acetate), immediately followed by phenol-extraction. After vortexing, 85 μl of upper solution was transferred to clean 1.5 ml tubes. The samples were then ethanol-precipitated overnight, and the DNA pellet was resuspended with 10 μl of a loading buffer (5% glycerol and 1X TBE buffer). The DNA was loaded on 0.75% agarose gel, and run at 45 V for 2.5 hours. The gels were then stained by ethidium bromide. The results were scanned at 532 nm using Typhoon 9000.

Chapter 3

This chapter contains a description of results that will be incorporated in a manuscript currently in preparation: Choi WS and Garcia-Diaz M.

3.1 Binding of TFAM to DNA substrates

3.1.1 Interactions between TFAM and LSP

TFAM is capable of both specific and nonspecific sequence recognition in order to fulfill its transcriptional activator and mtDNA packaging functions. It generates a clear DNase I footprint in a well-defined region upstream of LSP (Fisher, Topper et al. 1987), thus indicating that it is capable of specifically recognizing the promoter. However, the molecular mechanism by which TFAM recognizes specific sequences has not yet been characterized. Even though several crystal structures of TFAM in complex with promoter sequences have been determined (Ngo, Kaiser et al. 2011, Rubio-Cosials, Sidow et al. 2011), they failed to shed light on this question, as no obvious sequence-specific interactions were observed between TFAM and the DNA bases. Instead, TFAM interacts with the promoter sequences mostly through non-sequence specific contacts with the phosphates and deoxyriboses of DNA, as well as sequence-nonspecific hydrogen bonds to the DNA minor groove. The structures thus indicate that TFAM might recognize the promoter sequence through indirect readout mechanisms (that recognize sequence-dependent DNA conformations/energetics) rather than through specific base contacts (Travers 1989, Rohs, West et al. 2009, Rohs, Jin et al. 2010). Indeed, several studies have shown that some DNA-binding proteins utilize indirect readout as their specific binding mechanism (Otwinowski, Schevitz et al. 1988, Hizver, Rozenberg et al. 2001, Rohs, Sklenar et al. 2005).

3.1.2 Two sequence specific interactions in crystal structures of the promoter sequences

Even though interactions between TFAM and LSP are mostly non-sequence specific, close inspection of both structures of TFAM in complex with its LSP binding site (PDB ID: 3TMM and 3TQ6) revealed two sequence specific interactions: two TFAM residues, Ser61 and Pro178 specifically hydrogen-bond to two different guanine bases. The Oγ of Ser61 in HMG box 1 interacts with N2 of one guanine base. Interestingly, another residue, Tyr57, also hydrogen-bonds to the same base in both crystal structures (Figure 3-1A). The N2 of a second guanine base is recognized by the main chain carbonyl of Pro178 (Figure 3-1B).

In order to confirm this observation, I solved an additional structure of TFAM bound to its LSP binding site (LSP2), although with a different sequence configuration. I was able to obtain data to 2.7 Å (Table 1). Despite subtle differences in the sequence, the structure revealed the same specific interactions as the other two TFAM-LSP structures. Furthermore, an additional TFAM structure with the HSP1 binding site (Ngo, Lovely et al. 2014) presents the same interactions. Interestingly, these interactions reveal a clear consensus for TFAM binding, at least in the context of the crystal structures (Figure 3-1C). TFAM binding appears to be organized around a $GN_{10}G$ consensus, where two guanine bases are separated by 10 nucleotides. Yet, two hydrogen bonds are clearly insufficient to drive specific binding of TFAM to the promoter sequences, further suggesting that specific TFAM binding relies on indirect readout.

3.1.3 Binding to a $GN_{10}G$ observed in a crystal structure of a nonspecific sequence

Interestingly, I could also observe the same pattern of interactions around the $GN_{10}G$ consensus in a crystal structure of TFAM bound to a nonspecific sequence (NS) (PDB ID: 4NUU, Figure 1D). This indicates that the presence of a $GN_{10}G$ consensus is not correlated with specific substrates. Except for the $GN_{10}G$ consensus, I could not find any sequence conservation among the different sequences found in the crystal structures (Figure 3-1C). Although some sequences appeared to be conserved among promoter sequences (blue boxes), the interactions with these bases were not conserved or did not exist, indicating that these sequences did not contribute to specific binding of TFAM.

In addition, the interaction through a $GN_{10}G$ consensus appears to be related to DNA bending. The sharp bend of DNA is one of the structural features observed in all crystal structures, and induced by the intercalations of two leucine residues of TFAM (Leu58 of HMG box 1 and Leu182 of HMG box 2) into base stacking. Interestingly, the two guanines in the $GN_{10}G$ consensus are placed right at the two kink sites, and are involved in both intercalations (Figure 3-2A and B). This indicates that recognition of these two bases might be important to support the sharp bend observed in the crystal structure.

3.1.4 TFAM-NS2 structure presents a unique binding conformation

In order to further test whether binding to a $GN_{10}G$ consensus could drive TFAM binding to DNA, I crystallized TFAM in complex with a 22-mer nonspecific sequence (NS2) containing a $GN_{10}G$ consensus where all other nucleotides were randomized with respect to the sequence used for our LSP2 structure. I obtained crystals that diffracted to 2.84 Å and I solved the crystal

structure by molecular replacement using the TFAM-LSP (PDB ID: 3TQ6) structure as a search model (Rubio-Cosials, Sidow et al. 2011). Most TFAM structures have two TFAM-DNA complexes in the asymmetric unit. These two complexes interact through their U-turn shaped DNA molecules, forming a circular shape (Figure 3-3). The exceptions to this is one of the TFAM-LSP structures (PDB ID: 3TMM) (Ngo, Kaiser et al. 2011), crystallized with a 28-mer substrate. However, even though in this structure a single TFAM-LSP complex was present in the asymmetric unit, the crystal packing revealed the same identical circular-shaped conformation. In addition, the TFAM-HSP1 structure (PDB ID: 4NOD) has a total of four TFAM-HSP1 complexes in the asymmetric unit, but they exist as two circular-shaped ones in the unit.

Importantly, while our NS2 structure appeared to reveal the same TFAM-DNA conformation, attempts to model the DNA as in previous complexes resulted in clear negative and positive Fo-Fc peaks on the DNA molecules (Figure 3-4). In accordance with the observation in Fo-Fc electron density map, I re-built the nucleotide sequences and refined the structure, resulting in 20.6% and 24% of R_{work} and R_{free} (Table 1). In the newly refined structure, there was no negative or positive density on the DNA molecules in the Fo-Fc map. In addition, I performed occupancy refinement between two structures, and the result indicated that the latter structure is the only conformation in the crystals.

Strikingly, the TFAM-NS2 structure showed a unique binding conformation. All TFAM-DNA structures determined to date have revealed that a molecule of TFAM interacts with a molecule of DNA (1:1 ratio of TFAM to DNA) (Ngo, Kaiser et al. 2011, Rubio-Cosials, Sidow et al. 2011, Ngo, Lovely et al. 2014). However, in the TFAM-NS2 structure, the binding site of TFAM was composed of two halves of adjacent DNA molecules (Figure 3-5A). Half of a DNA

molecule interacts with HMG box 1 of a TFAM molecule, and the other half of the DNA molecule binds to HMG box 2 of the other TFAM molecule in the asymmetric unit (Figure 3-5B). However, the TFAM-NS2 structure also showed the conserved binding pattern through the $GN_{10}G$ consensus, although in this case this consensus was formed between two adjacent DNA molecules (Figure 3-5B). As shown above, two U-turn shaped DNA molecules interact through base stacking in an asymmetric unit, and therefore the $GN_{10}G$ consensus was recognized as those in a single DNA molecule. The conserved interaction through the $GN_{10}G$ consensus in the TFAM-NS2 structure further supports the hypothesis that TFAM prefers to bind through a $GN_{10}G$ consensus for both specific and nonspecific substrates, and that this consensus might play an important role to enable TFAM's functions.

3.1.5 The $GN_{10}G$ consensus plays an important role in mitochondrial transcription

I then investigated the role of the $GN_{10}G$ consensus in mitochondrial transcription initiation. The TFAM binding site in LSP has been well characterized, as TFAM binding results in a very clear 23 nucleotide footprint (Fisher, Topper et al. 1987, Fisher and Clayton 1988). In addition to the $GN_{10}G$ sequence expected to direct TFAM binding from footprinting studies and crystal structures (Ngo, Kaiser et al. 2011, Rubio-Cosials, Sidow et al. 2011) (corresponding to C427•C438 on the L-strand), several occurrences of a $GN_{10}G$ consensus appear around this footprint. In order to test the importance of each individual guanine pair, I carried out *in vitro* transcription assays after modifying the sequence of the transcription substrate. Replacement of both guanines in the expected guanine consensus to adenines (corresponding to C427•438T on the L-strand) resulted in a ~50 % decrease in transcription activity (Figure 3-6B). Conversely,

altering three additional surrounding guanine pairs around the TFAM binding site (corresponding to C434•445T, C420•431T and C436•447T on the L-strand) had almost no influence in transcriptional initiation activity (Figure 3-6B). Replacement of C420•431 did result in a ~30 % reduction of activity. However, this reduction is likely related to the fact that C420 is located in the binding site for the POLRMT/TFB2M complex (Sologub, Litonin et al. 2009). Thus, our results confirm that the C427•C438 guanine pair is important for proper transcriptional activity of TFAM. At the same time, the fact that eliminating the guanine pair does not completely eliminate initiation activity is consistent with the hypothesis that TFAM recognizes its binding site in great part through indirect readout. I further investigated the importance of the interaction through $GN_{10}G$ consensus by mutating a residue of TFAM involved in the hydrogen bond. I replaced Ser61 to alanine (S61A) in order to disrupt the hydrogen bond. However, it was not possible to eliminate the other hydrogen bond of Pro178 because the atom involved in the interaction is its main chain carbonyl. S61A led to a decrease of ~30 % in transcription activity (Figure 3-6C). Although this reduction was more modest than that observed with DNA mutations, it is likely that this is due to the fact that a hydrogen bond can still be established by Pro178.

I next examined the importance of different $GN_{10}G$ patterns for initiation from HSP1. Interestingly, the transcription initiation mechanism at HSP1 appears to be different from that at LSP. Furthermore, upstream of HSP1, TFAM binding only results in a diffuse footprint (Fisher, Topper et al. 1987), suggesting that TFAM binding at HSP1 is less specific than at LSP. Consistently, the presence of varying TFAM concentrations affects LSP and HSP1 initiation differentially (Shutt, Lodeiro et al. 2010). Nevertheless, the structural analysis still suggests that binding to a $GN_{10}G$ consensus might play a role in HSP1 transcription initiation. I thus

systematically altered six different consensus sequences close to the HSP1 transcription start site (Figure 3-7A). Strikingly, the mutation of C530•541 resulted in an almost complete abrogation of stimulation of initiation activity by TFAM (Figure 3-7B). Compared to this reduction, alteration of the C525•536, C537•548, and C542•553 pairs did not significantly affect initiation activity. However, alteration of C524•535 and C541•552 reduced the activity up to ~30 % and ~50 %, respectively. Once more, DNase I footprinting studies indicate that C552 is located in the binding site for the TFB2M/POLRMT complex (Sologub, Litonin et al. 2009). Replacement of Ser61 to Ala also reduced ~25% transcription activity at HSP1, consistent with the result obtained with LSP (Figure 3-7C).

The results of our *in vitro* transcription assays allowed us to conclude 1) that TFAM binding to a $GN_{10}G$ consensus plays a central role in both LSP and HSP1 transcription initiation, 2) that each promoter has a single specific consensus that results in productive TFAM binding for transcriptional activation despite the presence of several guanine pairs present around the TFAM binding sites, and 3) that binding to the $GN_{10}G$ consensus appears to be more important for HSP1 transcription initiation than for LSP. This is consistent with the fact that TFAM binding to LSP appears to be highly specific and mostly due to indirect readout, and might reflect a larger dependence on binding the correct $GN_{10}G$ pair for initiation at HSP1.

3.1.6 $GN_{10}G$ consensus contributes to the binding of TFAM

3.1.6.1 Electrophoretic mobility shift assay

TFAM binding does not only involve specific promoter regions. In the context of its mtDNA packaging function, TFAM must interact with multiple sequences throughout the mitochondrial genome. In order to study whether binding to a $GN_{10}G$ consensus can influence binding in a

nonspecific context, I studied whether the presence of a $GN_{10}G$ consensus could affect TFAM DNA binding affinity. Thus, I calculated binding affinities (apparent K_D) using electrophoretic mobility shift assays (EMSA). This assay used a 28-bp nonspecific oligonucleotide corresponding to a region of the human mtDNA (6694-6721 within the cytochrome C oxidase subunit I gene) that contains a single $GN_{10}G$ consensus (DNA^{GG}) and compared it to an identical sequence where this consensus had been eliminated by replacing the two guanines with adenines (DNA^{AA}).

Although TFAM was able to efficiently bind both substrates, TFAM appeared to exhibit a slight preference for the DNA^{GG} substrate (Figure 3-8A). In order to directly compare the binding affinities between DNA^{GG} and DNA^{AA}, apparent K_D values were calculated. This revealed that the binding affinity to DNA^{AA} was significantly lower than that to DNA^{GG} (Figure 3-8B and Table 2). In order to confirm whether this result was directly related to the binding to a $GN_{10}G$ consensus, I decided to calculate the binding affinities between DNA^{GG} and the S61A TFAM variant. This did not result in a significant difference in binding affinity (Figure 3-8C and Table 2), perhaps because the remaining hydrogen bond of Pro178 can partially maintain the interaction with the $GN_{10}G$ consensus. In addition to Ser61, as shown above, Tyr57 contributes to bind the guanine base. I therefore asked whether eliminating this residue could alter the binding affinity of TFAM for this substrate. Thus, I calculated the binding affinity of a Tyr57 mutant (Y57A). Interestingly, this resulted in a ~2.3 fold lower binding affinity than TFAM WT (Figure 3-8C). Importantly, both residues seem to cooperate in binding the guanine base, as replacing both residues with an alanine results in a synergistic effect: the binding affinity of S61A•Y61A was ~ 2.8 fold lower than that of TFAM WT (Figure 3-8C). To further confirm this, I asked whether the double mutant would display a preference for binding substrates with a

GN10G consensus. I compared binding affinities between DNA^{GG} and DNA^{AA} with TFAM WT and S61A•Y57A. The results showed that TFAM S61A•Y57A did not exhibit a preference for the substrate containing a $GN_{10}G$ consensus (Figure 3-8B and D), further supporting the hypothesis that both Ser61 and Tyr57 are involved in binding of the guanine residue.

3.1.6.2 EcoRI cleavage assay

To further confirm the role of the $GN_{10}G$ consensus to drive TFAM binding, I carried out an additional assay. I used a 100-bp nonspecific sequence modified to contain a single $GN_{10}G$ consensus in the middle, and then an EcoRI cleavage site was placed between the two guanines. If TFAM binds to the $GN_{10}G$ consensus in the substrate, it will protect it from EcoRI cleavage (Figure 3-9A). I compared the protection level between $DNA2^{GG}$, containing a single $GN_{10}G$ consensus, and $DNA2^{AA}$, where the consensus was eliminated by replacing both guanines to adenines. The result showed a statistically significant difference in the protection of the two substrates (Figure 3-9B), further indicating that a $GN_{10}G$ consensus contributes to the binding of TFAM to a substrate.

3.1.7 TFAM binding to a circular DNA

I then investigated if a $GN_{10}G$ consensus would influence binding of TFAM on a circular substrate. In mitochondria, TFAM binds to and condenses a circular genome. In order to investigate whether binding to a $GN_{10}G$ consensus affects circular DNA condensation, I examined the ability of TFAM to protect a 6.5-kbp circular substrate from nuclease degradation (Figure 3-10A). Importantly, the substrate contains about 250 different $GN_{10}G$ consensus sites on each strand. In the absence of TFAM, DNase I treatment led to degradation of the plasmid

(Figure 3-10B, lane 2-5). At low DNase I concentration, both TFAM WT and S61A were capable of protecting the plasmid (Figure 3-10B, lane 8-9 and 12-13). However, at high DNase I concentrations (red boxes), TFAM WT protected the plasmid better than TFAM S61A (Figure 3-10B, lane 6-7 and 10-11). Thus TFAM WT appears to more efficiently condense a circular DNA than TFAM S61A, indirectly indicating that recognition of a $GN_{10}G$ consensus might be involved in TFAM's mtDNA packaging function.

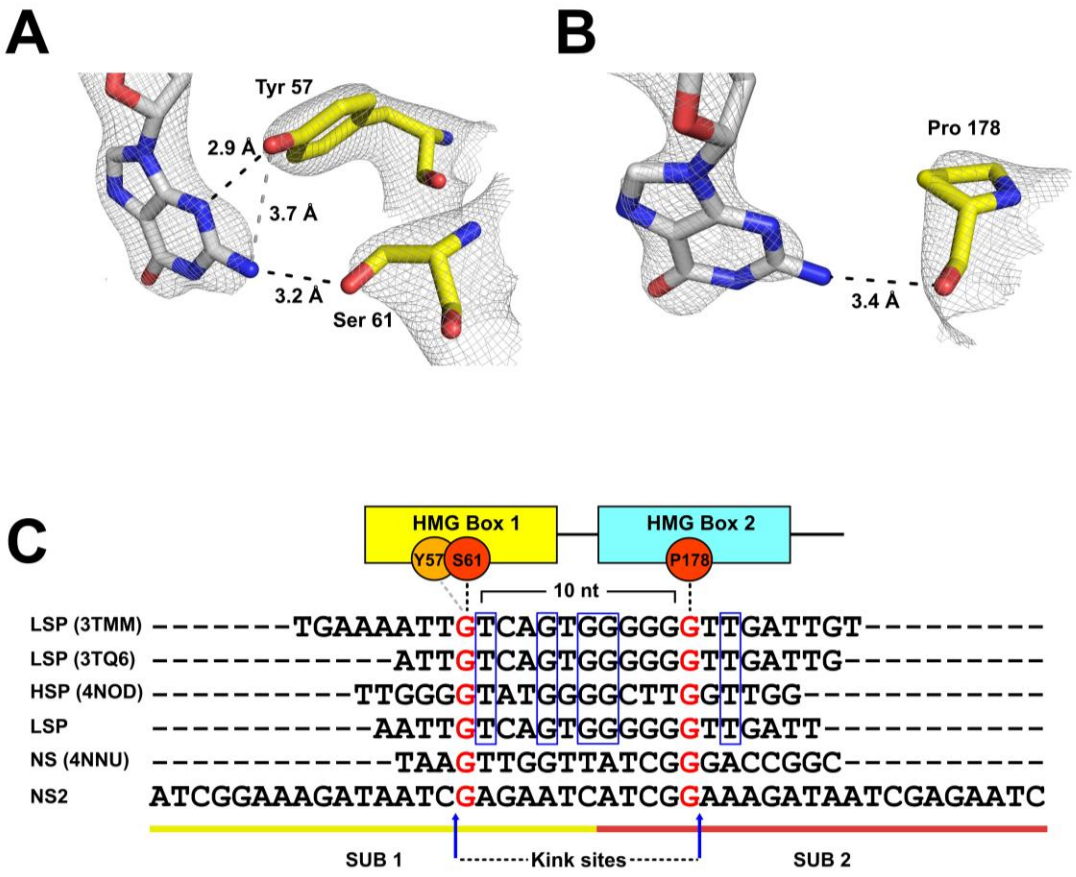

Figure 3-1. $GN_{10}G$ consensus
(A) Hydrogen bond between a guanine and Ser61 of HMG box1. In addition, Tyr57 also hydrogen-bonds to the same guanine despite non-sequence specific interaction. (B) Hydrogen bond of Pro178 with another guanine. This bond occurs through main chain carbonyl of Pro178. (C) Alignment of substrates used for the structure determination. The conserved guanine bases among all sequences are colored as red. The blue boxes represent the aligned nucleotides among promoter sequences. In the TFAM-NS2 structure, $GN_{10}G$ consensus recognized by a TFAM is composed of two adjacent DNA molecules (SUB1 and SUB2) in the asymmetric unit. The 2Fo-Fc maps are contoured at 1.5 σ.

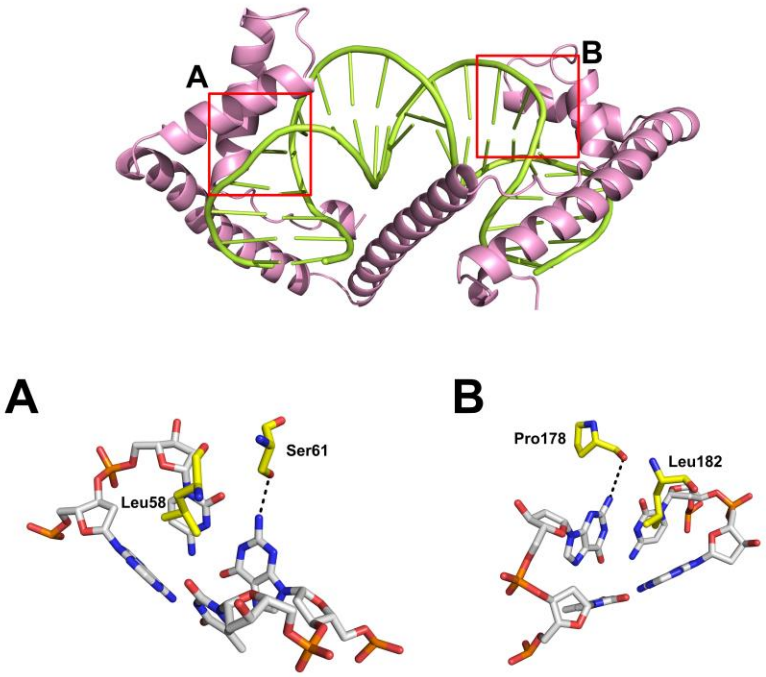

Figure 3-2. DNA intercalations occur at the $GN_{10}G$ consensus site
(A and B) Leu 58 of HMG box 1 and Leu 182 of HMG box 2 form two kink sites at the substrates by intercalating two base stacking regions. Those intercalations take place right next to two guanines of a $GN_{10}G$ consensus. The hydrogen bonds between guanines and TFAM residues (Ser61 and P178) are shown by dot lines.

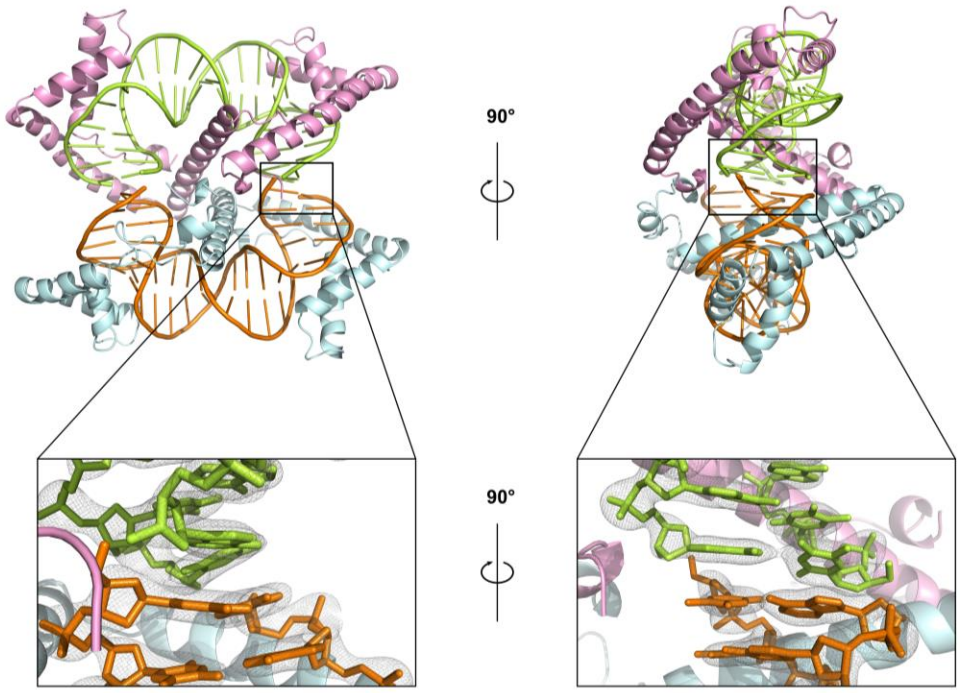

Figure 3-3. The asymmetric unit of TFAM-LSP structure
Two U-turn shaped DNA molecules in the two TFAM-LSP complexes interact with each other through base stacking interactions. The close-up view structures represent that the bases from two DNA ends form base stacking interactions. The 2Fo-Fc maps are contoured at 1.4 σ.

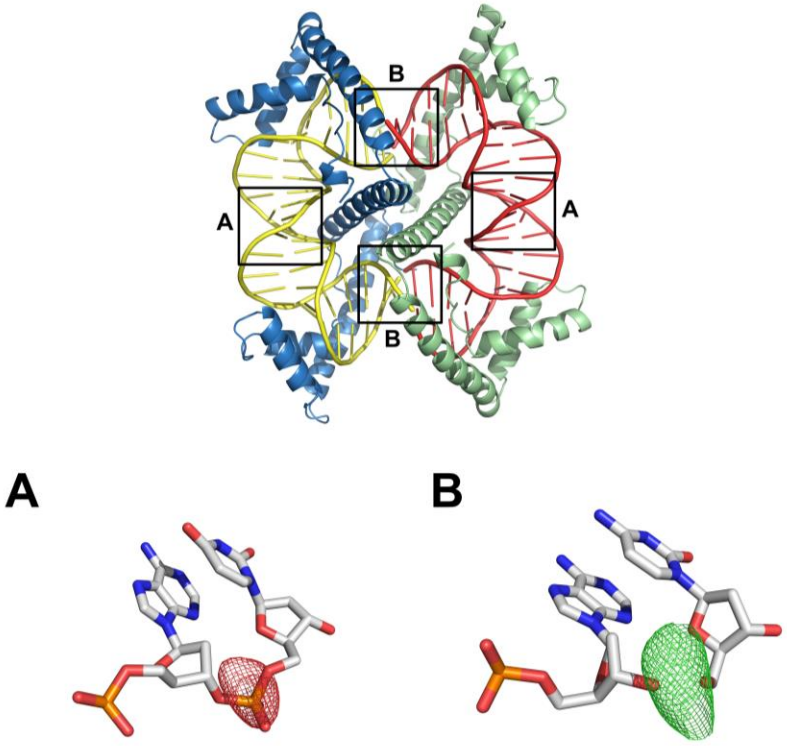

Figure 3-4. Fo-Fc peaks in the molecular-replaced TFAM-NS2 model
The TFAM-NS2 model by molecular replacement presents four interesting Fo-Fc peaks (in boxes) (A) Close-up view of a negative electron density. These peaks indicate that molecules are modelled in wrong place. In both DNA molecules, the negative densities are observed at the phosphates between 11^{th} and 12^{th} nucleotides. (B) Close-up view of a positive electron density. These peaks indicate lack of molecules which should be modelled, and are placed at the putative phosphate sites between two DNA ends.

Table 3-1. Data collection and refinement statistics

Crystal	TFAM-LSP2	TFAM-NS2
Space group	*P 2₁ 2₁ 2*	*P 2₁ 2₁ 2*
Cell dimensions		
a, *b*, *c* (Å)	113.497, 120.571, 55.215	115, 124.81, 55.18
α, β, γ (˚)	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Data collection[a]		
Resolution (Å)	50.00 – 2.70 (2.75 – 2.70)	39.813 – 2.838 (2.847 – 2.838)
Wavelength (Å)	1.075	1.10000
R_{merge}[b]	0.051 (0.787)	0.061 (0.636)
$I / \sigma I$	29.3 (2.2)	33.6 (4.0)
Completeness (%)	97.6 (92.9)	100 (100)
Multiplicity	7.6 (7.6)	12.8 (13.1)
Refinement[a]		
Resolution (Å)	32.80 – 2.70 (2.83 – 2.70)	39.81 – 2.84 (2.99 – 2.84)
No. reflections	20,969 (987)	19,494 (179)
R_{work} / R_{free}	0.2158 / 0.2454	0.2059 / 0.2395
No. atoms		
Protein	3192	3228
DNA	1797	1766
Water	84	73
Mean *B*-factors		
Protein	84.414	80.614
DNA	87.842	92.459
Water	60.266	64.200
R.M.S. deviations		
Bond lengths (Å)	0.0100	0.0101
Bond angles (˚)	0.96	1.05
Ramachandran		
Favored (%)	97.33	98.68
Outliers (%)	0.00	0.00
PDB ID		

[a] Values in parenthesis are for the highest resolution shell.
[b] The merging R-factor, R_{merge} describes the deviation of an individual intensity measurement from the mean value of all its symmetry-equivalent reflections.

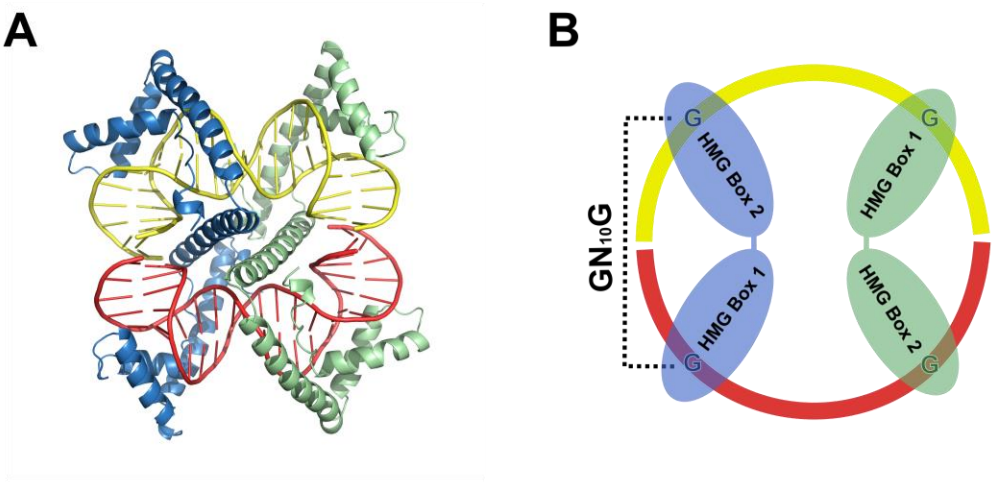

Figure 3-5. TFAM-NS2 structure
(A) Overall structure of TFAM-NS2. (B) Diagram of the binding conformation of TFAM in complex with NS2. The TFAM-NS2 structure represents that a molecule of TFAM binds to two halves of DNA molecules. Each HMG box in a TFAM is responsible for interactions with each adjacent DNA.

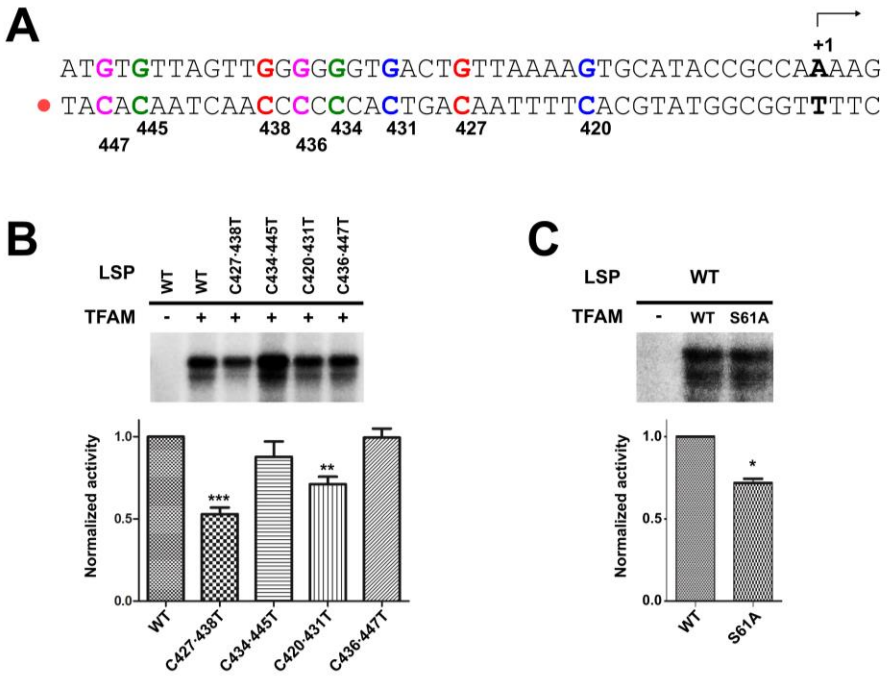

Figure 3-6. $GN_{10}G$ consensus and LSP transcription
(A) LSP sequence including the TFAM binding site. Each $GN_{10}G$ consensus has different color code: C420•431(blue), C427•438 (red), C434•445 (green), and C436•447 (pink). The nucleotide numbering at LSP is based on L-strand. The number +1 stands for the transcription start site. The arrow represents the direction of transcription. The red circle indicates the template strands for transcription. (B) Transcription assays with mutants of each $GN_{10}G$ consensus at LSP. The bar graph shows the relative transcription activities of LSP mutants compared to that of LSP WT. (C) LSP transcription with TFAM WT and S61A. *** P<0.001, ** P<0.01, * P<0.05.

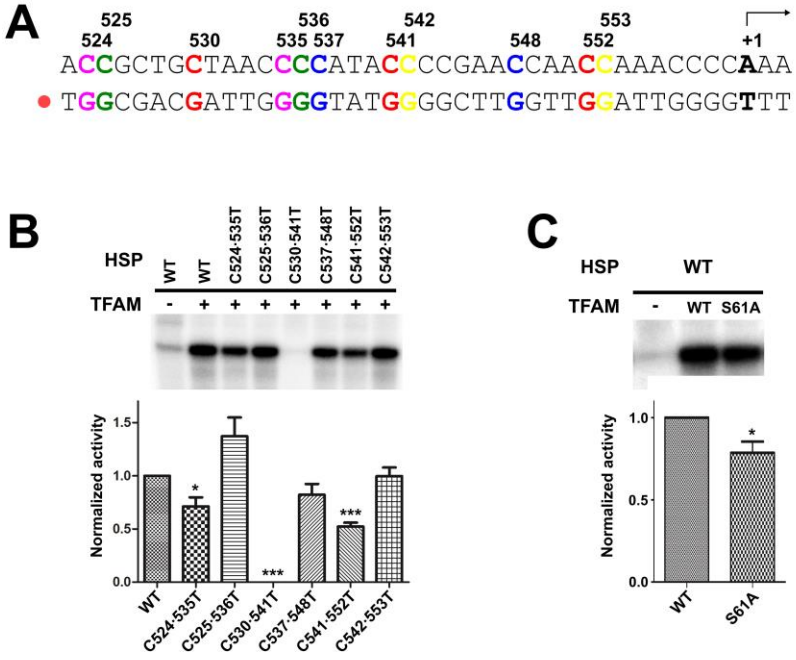

Figure 3-7. $GN_{10}G$ consensus and HSP1 transcription
(A) HSP1 sequence including putative TFAM binding site. Each $GN_{10}G$ consensus is shown in different color codes: C524•530 (pink), C525•536 (green), C530•541 (red), C537•548 (blue), C541•552 (red), and C542•553 (yellow). The color codes of C530•541 and C541•552 are the same due to C541 shared in both $GN_{10}G$ pairs. The nucleotide numbering at HSP1 is based on L-strand. The number +1 stands for the transcription start site. The arrow represents the direction of transcription. The red circle indicates the template strands for transcription. (B) Transcription assays with mutants of each $GN_{10}G$ consensus at HSP1. The bar graph shows the relative transcription activities of HSP1 mutants compared to that of HSP1 WT. (C) HSP1 transcription with TFAM WT and S61A. *** P<0.001, ** P<0.01, * P<0.05.

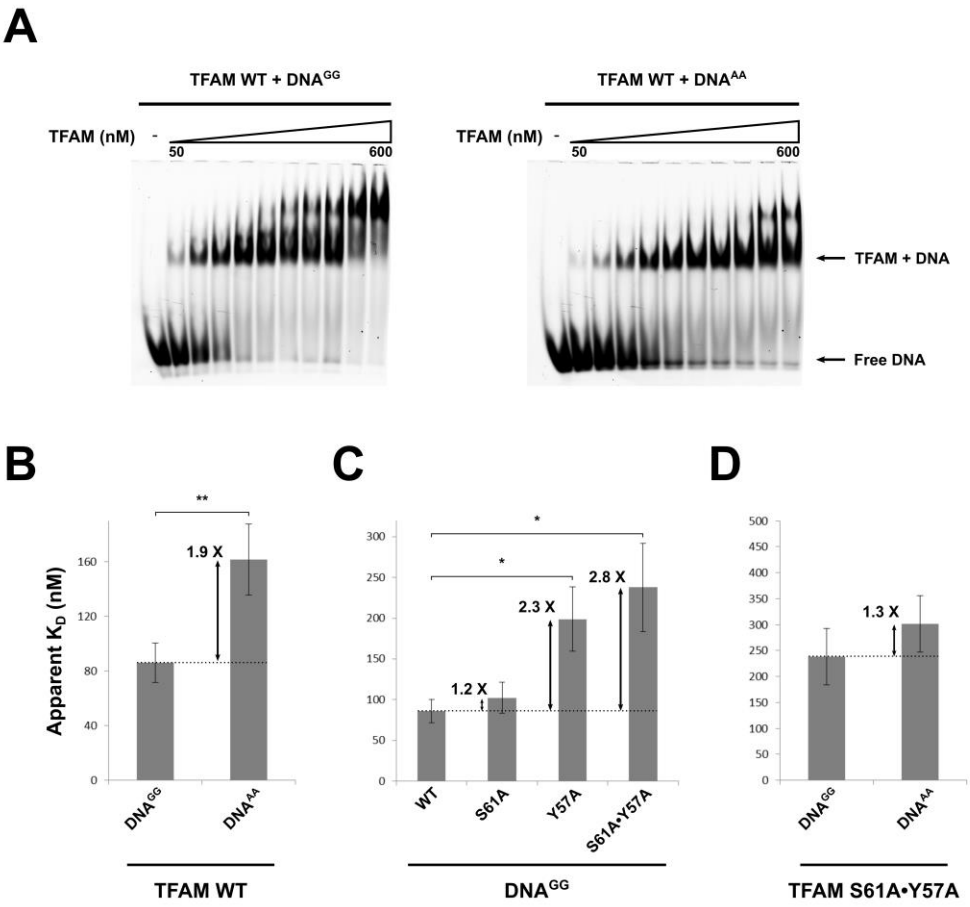

Figure 3-8. $GN_{10}G$ consensus contribute to directing TFAM binding
(A) EMSA with DNA^{GG} and DNA^{AA}. 0~600 nM of TFAM (0, 50, 150, 200, 250, 300, 350, 400, 500, and 600 nM) was used for the assays with100 nM of Cy3-labeled DNA substrates. (B) Apparent K_D values of DNA^{GG} and DNA^{AA} with TFAM WT. The binding affinity of DNA^{GG} is about two-fold higher than that of DNA^{AA}. (C) Apparent K_D values depending on mutations of TFAM residues. While the binding affinity of S61A is similar to that of WT, those of Y57A and S61A•Y57A are about 2.3 and 2.8-fold less than that of WT. (D) Apparent K_D values of DNA^{GG} and DNA^{AA} with TFAM S61A•Y57A. The double-mutant TFAM shows similar binding affinities between DNA^{GG} and DNA^{AA}.

Table 3-2. Apparent K_D between TFAM and DNA

TFAM	Substrate	K_D (nM)
WT	**DNA^{GG}**	86.08 ± 14.31
S61A		102.4 ± 19.11
Y57A		198.8 ± 39.51
S61A·Y57A		237.9 ± 54.33
WT	**DNA^{AA}**	161.67 ± 26.01
S61A·Y57A		301.1 ± 54.60

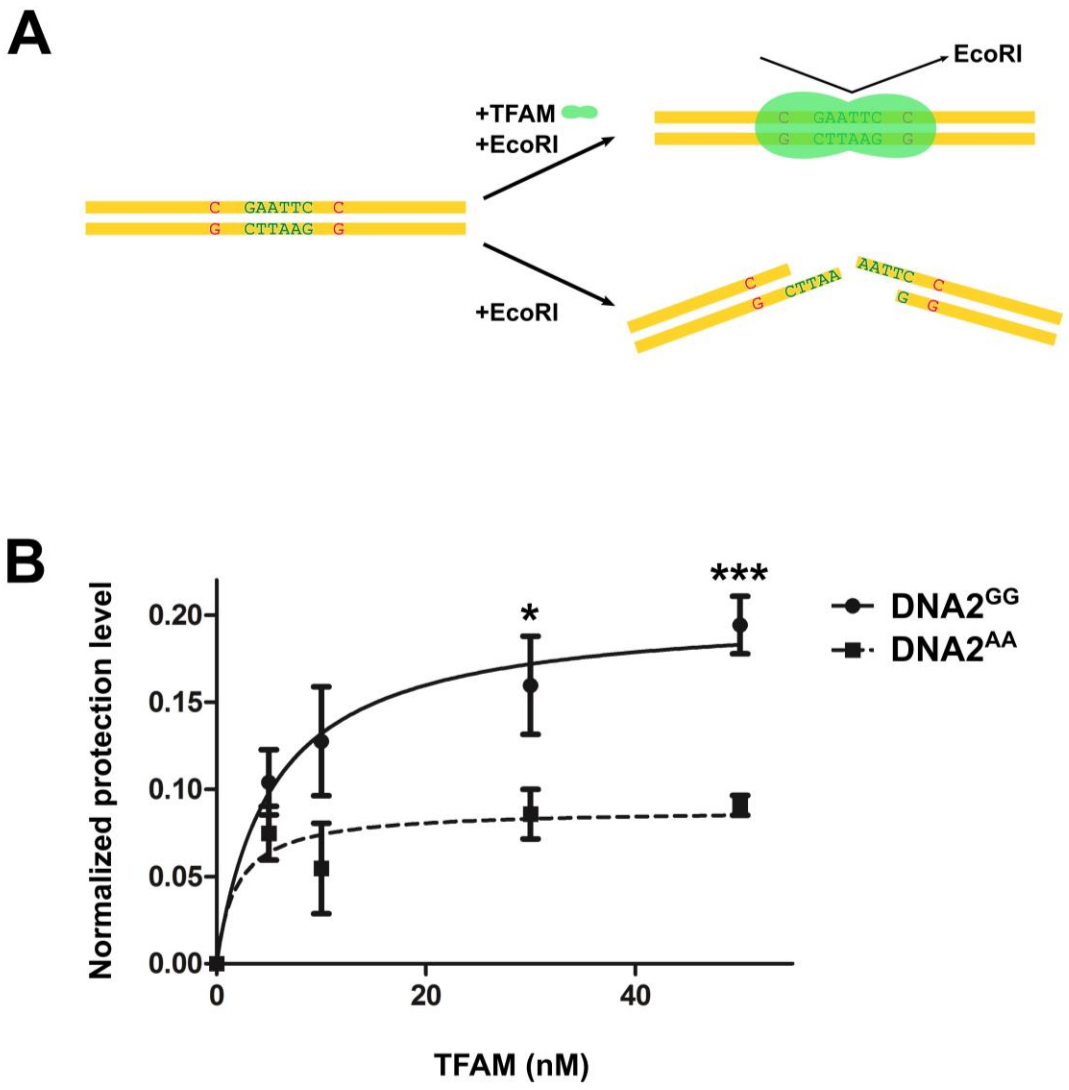

Figure 3-9. EcoRI cleavage assay
(A) Schematic experiment design. The nonspecific sequence contains a $GN_{10}G$ consensus in the middle, and an EcoRI site is placed between two guanines. If TFAM preferentially binds to the consensus, the substrate is protected from EcoRI cleavage. (B) Normalized substrate protection. $DNA2^{GG}$ is protected by TFAM better than $DNA2^{AA}$.

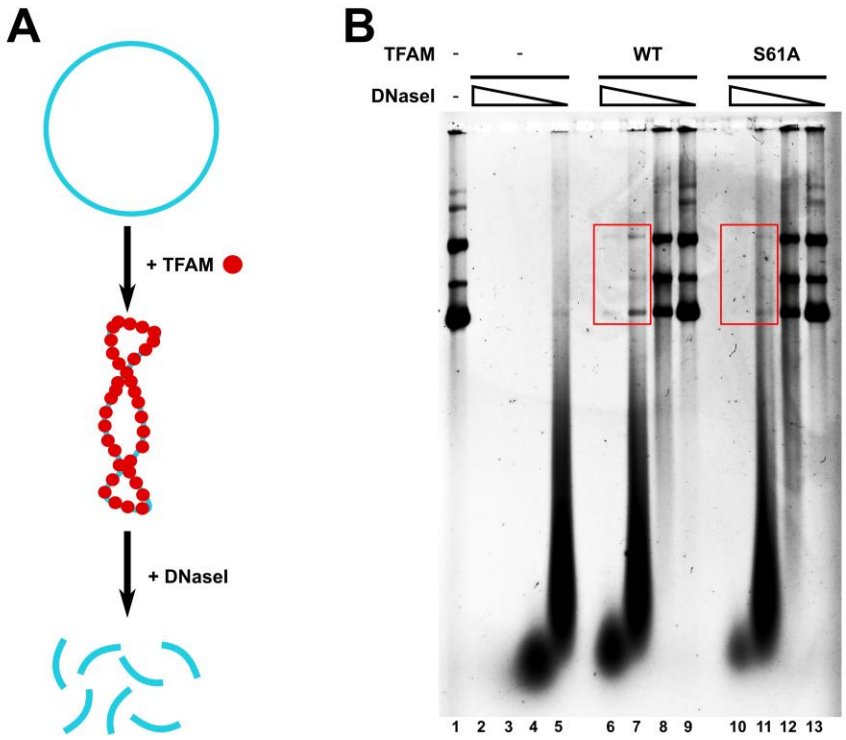

Figure 3-10. Compaction assay
(A) Schematic experiment design. A modified pET22 vector is used for this assay. The added TFAM then coats and condenses the plasmid, followed by its protection from DNase I degradation. (B) Plasmid protection by TFAM. In the absence of TFAM, all plasmids are degraded (lane 2~5). TFAM WT is able to protect the plasmid better than TFAM S61A (red boxes). The DNase I concentrations used in this assay are followed: 20, 2, 0.2, 0.02 units.

Chapter 4

This chapter contains a description of results that will be incorporated in a manuscript currently in preparation: Choi WS and Garcia-Diaz M.

4.1 TFAM binding to DNA ends

The TFAM-NS2 structure presents a different binding conformation from other TFAM-DNA structures determined to date: a molecule of TFAM binds to two adjacent DNA molecules in the asymmetric unit (See Figure 3-5). In addition to the binding of TFAM through the $GN_{10}G$ consensus established between two adjacent DNA molecules, I could find another interesting feature in the NS2 structure: it also reveals how TFAM could bridge two DNA ends (Figure 4-1). This mode of binding appears to be consistent with previous studies suggesting a binding preference of TFAM for damaged DNA sites: TFAM preferentially binds to damage-containing DNA such as 8-oxoG and cisplatin adducts (Huang, Zamble et al. 1994, Yoshida, Izumi et al. 2002, Canugovi, Maynard et al. 2010). Furthermore, proteins from the HMGB family (where TFAM is included) display high binding affinities to different damage-containing DNA sites (Stros, Launholt et al. 2007, Lange and Vasquez 2009). This is consistent with indirect readout being a feature of sequence recognition by this family of proteins.

The TFAM-NS2 structure further suggested that TFAM might have a binding preference for DNA ends. In the structure, the α-helix linker appears to bind to the minor groove of the resulting DNA nick after the two ends are brought together (Figure 4.1). However, there was no specific base contact between the linker and the DNA bases. The superposition of the α-helix linker between TFAM-NS2 and TFAM-LSP (PDB ID: 3TQ6) generated an RMSD of 0.408Å for 62 C-α atoms. In comparison with the RMSD of all C-α atoms between both structures (1.063

for 382 C-α atoms), it suggests that this minor groove interaction might be essential for DNA binding.

4.2 TFAM contributes to the ligation of DNA ends

Mitochondrial DSB repair has not yet been clearly characterized compared to nuclear DSB repair. Even though mitochondrial DSB repair has been observed *in vitro* assays (Coffey, Lakshmipathy et al. 1999, Lakshmipathy and Campbell 1999), the proteins involved in the repair have not yet been determined. Furthermore, recent *in vitro* assays show that mitochondria appear to preferentially repair sticky-ended breaks (microhomology-mediated end joining, MMEJ) rather than blunt-ended ones (nonhomologous end joining, NHEJ) (Tadi, Sebastian et al. 2016). In addition, little is known of the role of TFAM in mtDNA repair. Only its role in base excision repair (BER) has been characterized. In the presence of TFAM, the activities of enzymes involved in BER are reduced, indicating that TFAM appears to inhibit the BER pathway in mitochondria (Canugovi, Maynard et al. 2010). However, as mentioned above, the NS2 structure displays binding of TFAM to a DNA nick composed of two DNA ends, indicating a possible involvement of TFAM in DSB repair, especially NHEJ. In order to test this hypothesis, I performed an *in vitro* end-joining assay using T4 DNA ligase. The result shows that T4 DNA ligase alone could ligate the 237-bp DNA substrates. However, the addition of TFAM enhanced the ligations: increasing TFAM resulted in an increase in ligation events (Figure 4.2A), and the calculated ligation ends also showed that the increases in ligation were statistically significant (Figure 4.2B). This result indicates that TFAM could be involved in DSB repair in mitochondria.

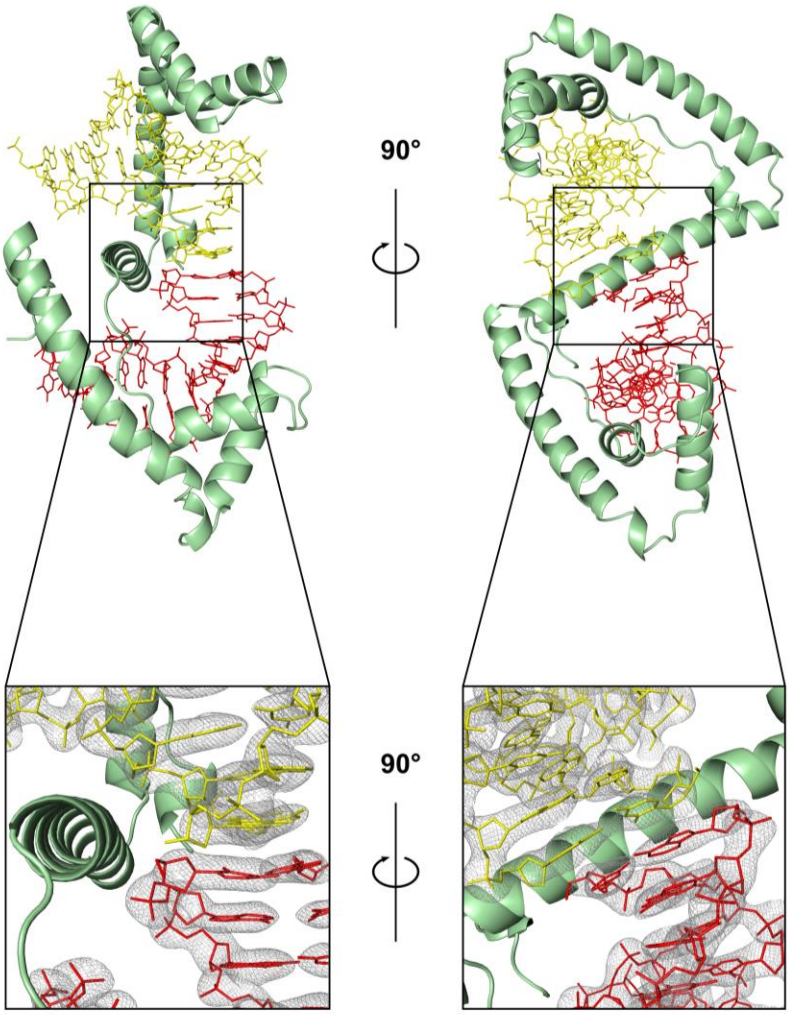

Figure 4-1. TFAM binds to a DNA nick
In the close-up view, TFAM appears to recognize the DNA nick through its α-helix, but there is no direct base contact between residues of α-helix and nucleotides

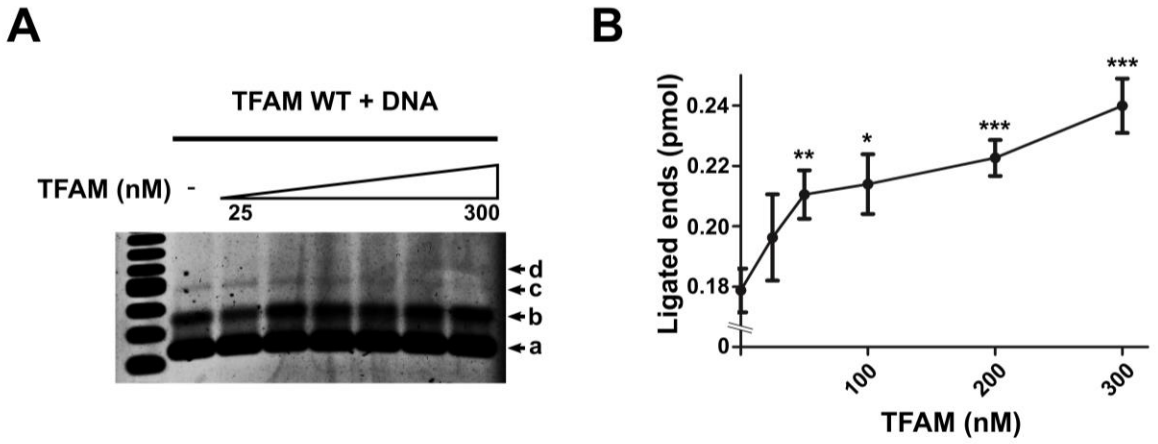

Figure 4-2. Contribution of TFAM to DNA end ligations
(A) End-joining assay. The DNA ligation events increase with TFAM concentration. The letters (a, b, c, and d) represent the number of the ligation events (0 to 3, respectively). (B) Quantification of the ligation. The graph represents the increase in DNA ligation with TFAM. Each ligated end is weighted by the number of ligation events (see chapter 2, End-joining assay). *** $P<0.001$, ** $P<0.01$, * $P<0.05$.

Chapter 5 Discussion

This chapter contains discussions that will be incorporated in a manuscript currently in preparation: Choi WS and Garcia-Diaz M.

TFAM is a bifunctional protein serving as a transcriptional activator and as an mtDNA packaging factor. However, the mechanisms by which TFAM differentially recognize substrates for its various functions are unknown. In this study, I provide some mechanistic insight into the DNA binding process of TFAM, and demonstrate that TFAM preferentially binds to a $GN_{10}G$ consensus on substrates.

The crystal structure of TFAM-NS2 showed two interesting features. Firstly, I observed consistent binding to a $GN_{10}G$ consensus in the NS2 structure which is also a feature of all crystal structures determined to date. It indicates not only that TFAM preferentially binds to a $GN_{10}G$ consensus, but also that binding to the consensus might be a common initial step for subsequent TFAM functions. In addition, the TFAM-NS2 structure showed a unique binding conformation where a molecule of TFAM binds between two ends of adjacent DNA molecules (see Figure 3-5 and 4-1). This binding conformation is likely that TFAM binds to a double-strand break (DSB) site. The role of TFAM in DSB repair system of mitochondria has not yet been characterized. However, the end-joining assays reveal that TFAM appears to contribute to the ligation of DNA ends, suggesting TFAM might be involved, in part, in DSB repair *in vitro*. In addition, previous studies showing the binding properties of TFAM and other HMGB proteins to damaged DNA sites (Huang, Zamble et al. 1994, Yoshida, Izumi et al. 2002, Lange and Vasquez 2009, Canugovi, Maynard et al. 2010) indirectly suggest its involvement in DNA repair in mitochondria.

In vitro transcription assays demonstrated the importance of a $GN_{10}G$ consensus for both LSP and HSP transcription initiation: the elimination of specific $GN_{10}G$ sites by converting guanines to adenines resulted in significant activity reductions at both LSP and HSP1. Nonetheless, the reduction was strikingly stronger for HSP1. This indicates that transcription initiation must obey a different mechanism at LSP and HSP1, further supported by previous studies showing different transcription levels(Montoya, Gaines et al. 1983, Falkenberg, Gaspari et al. 2002) and differential dependency on TFAM between two promoters (Shutt, Lodeiro et al. 2010). DNase I footprinting also revealed that the specificity of TFAM at both promoters appears to be different (Fisher, Topper et al. 1987): while TFAM bound to a specific site at LSP, its binding site at HSP1 was diffused. For LSP binding, it is likely that an indirect readout mechanism is involved in LSP recognition together with the binding to a $GN_{10}G$ consensus. *In vitro* transcription assays with LSP further support this hypothesis: despite the elimination of the specific $GN_{10}G$ consensus (C427•438), ~50% of transcription activity was still maintained. In contrast, the elimination of the specific $GN_{10}G$ consensus at HSP1 abolished the transcription activity. Together with the DNase I footprinting result, it indicates that the consensus might play a more important role in the recognition of HSP1 than that of LSP. Although the crystal structure bound to HSP1 was determined, the substrate sequence used for structure determination missed one of the two guanines. The crystal structure with a different HSP1 sequence including the specific consensus might provide a molecular explanation of different recognition mechanism at HSP1 from LSP.

Both EMSA and EcoRI cleavage assays showed the contribution of a $GN_{10}G$ consensus for the binding of TFAM to substrates. Although TFAM has the high basal binding affinity to DNA substrates through the nonspecific interactions, the presence of the consensus does have an

influence on the binding affinity of TFAM. In addition, the calculations of K_D values with TFAM variants show not only that Ser61 interacts with a guanine of a $GN_{10}G$ consensus, but also that Tyr57 contributes to the binding to the guanine. Intriguingly, replacement of Tyr57 has more influence on the binding affinity than that of Ser61, indicating that Tyr57 might have more roles in directing TFAM binding than Ser61. The alignment of TFAM sequences from various organisms also showed better conservation of Tyr57 than Ser61 (Figure 5-1, yellow shaded). Although some additional residues (Ser55, Arg59, Phe60, Glu63 and Gln64) are also conserved in most of TFAM sequences, most of them do not interact with DNA except for Ser55 (Figure 5-1, light green shaded). However, the interactions of Ser55 are not only conserved through all crystal structures, but also non-sequence specific. Ser61 appears to be well-conserved in the TFAM sequences serving a transcription activator function. It does not exist in chicken (gg), drosophila (dm), and budding yeast (sc) TFAM, and interestingly, those three TFAM molecules are not involved in mitochondrial transcription. Previous studies have shown that scTFAM and dmTFAM are not involved in mitochondrial transcription (Goto, Matsushima et al. 2001, Asin-Cayuela and Gustafsson 2007), and that ggTFAM also has a marginal effect on mitochondrial transcription (Matsushima, Matsumura et al. 2003). Compared to Ser61, Tyr57 is perfectly conserved throughout all TFAM sequences (Figure 5-1), implicating its common role in TFAM structure or functions. Ngo *et al.* suggested that Tyr57 is one of four hydrophobic residues stabilizing the L-shaped structure of a HMG box (Ngo, Kaiser et al. 2011). In addition, although the three TFAM molecules (sc, dm, and gg) are not involved in mitochondrial transcription, it has been well characterized that they play an important role in mtDNA packaging and maintenance (Diffley and Stillman 1992, Goto, Matsushima et al. 2001, Matsushima, Matsumura et al. 2003, Kucej, Kucejova et al. 2008). This indicates that the role of Tyr57 might be

associated with a structural aspect of TFAM binding, and also indirectly suggests that Tyr57 might play a role in mtDNA packaging function.

In summary, I show the role of a $GN_{10}G$ consensus in both mitochondrial transcription initiation and the direct binding of TFAM to DNA sequences through structural and biochemical results. Although our results could not clarify the mechanism whereby TFAM recognizes and differentiates its substrate for its dual functions, they propose that TFAM appears to prefer to bind the substrates through a $GN_{10}G$ consensus. It was proposed that TFAM slides along a substrate in order to search its target sites (Farge, Laurens et al. 2012). It is likely that a $GN_{10}G$ consensus might be one of the major targets of TFAM for its subsequent functions. In addition, the investigation about a relationship between the binding to a $GN_{10}G$ consensus and DNA bending would provide more critical explanation why TFAM binds its substrates through a $GN_{10}G$ consensus.

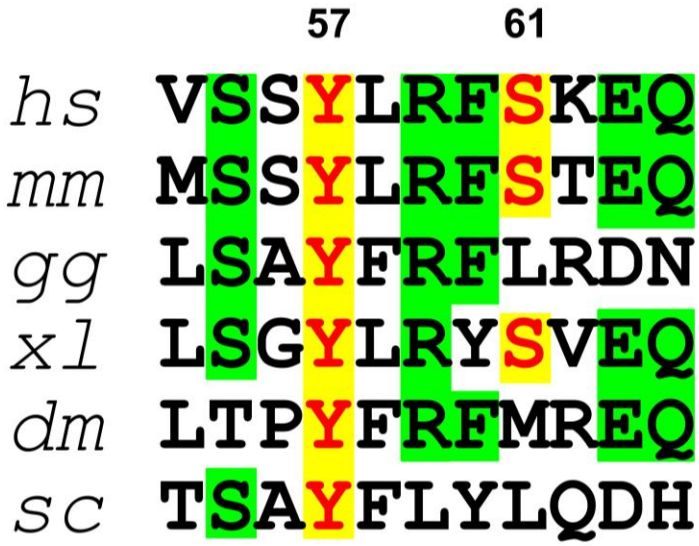

Figure 5-1. Sequence alignment around Ser61 and Tyr57
Ser61 and Tyr57 are shaded as yellow. While Ser61 is conserved in several organisms, Tyr57 is aligned perfectly in all TFAM. The residues partially aligned through TFAM are shaded as light green. However, most of them do not interact with DNA or interactions are not conserved. *hs, Homo sapiens; mm, Mus musculus; gg, Gallus gallus; xl, Xenopus laevis; dm, Drosophila melanogaster; sc, Saccharomyces cerevisiae.*

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