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## Mitochondrial DNA degradation: A quality control measure for mitochondrial genome maintenance and stress response

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### Abstract

Mitochondria play a central role in bioenergetics, and fulfill a plethora of functions in cell signaling, programmed cell death, and biosynthesis of key protein cofactors. Mitochondria harbor their own genomic DNA, which encodes protein subunits of the electron transport chain and a full set of transfer and ribosomal RNAs. Mitochondrial DNA (mtDNA) is essential for cellular and organismal functions, and defects in mitochondrial genome maintenance have been implicated in common human diseases and mitochondrial disorders. mtDNA repair and degradation are known pathways to cope with mtDNA damage; however, molecular factors involved in this process have remained unclear. Such knowledge is fundamental to the understanding of mitochondrial genomic maintenance and pathology, because mtDNA degradation may contribute to the etiology of mtDNA depletion syndromes and to the activation of the innate immune response by fragmented mtDNA. This article reviews the current literature regarding the importance of mitochondrial DNA degradation in mtDNA maintenance and stress response, and the recent progress in uncovering molecular factors involved in mtDNA degradation. These factors include key components of the mtDNA replication machinery, such as DNA polymerase  $\gamma$ , helicase Twinkle, and exonuclease MGME1, as well as a major DNA-packaging protein, mitochondrial transcription factor A (TFAM).

### Keywords

Mitochondrial DNA; DNA turnover; TFAM; DNA repair; DNA damage

### 1. Introduction

Mitochondrial DNA is a double-stranded circular molecule of 16,569 base pairs (bp) and is present in multiple copies in mitochondria [1–3]. mtDNA is essential for mitochondrial function and organismal health because it encodes 13 protein subunits of the electron transport chain system and a full set of tRNAs and rRNAs. Distinct from nuclear DNA (nDNA), the mtDNA genome exists as a heterogeneous population of molecules, known as heteroplasmy [4]. mtDNA copy number is specific to tissue type and to developmental stage and can number in thousands per cell. mtDNA is generally considered to be maternally inherited. In addition, the mtDNA genome contains no introns and very few noncoding

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intergenic nucleotides. It is replicated independently of the cell cycle and nDNA replication, although all the proteins involved in mtDNA replication are encoded by nDNA [3,5].

A distinctive property of the mitochondrial genome is that it has a much higher turnover rate than the nuclear genome [6,7]. Although this property has been known for decades, the basis of mtDNA degradation remains enigmatic. The identification of human diseases associated with mitochondrial DNA depletion has renewed interest in the topic [8,9], and inspired the development of mitochondrial-specific endonucleases targeting mutated mtDNA molecules [10–16]. Recently, mtDNA degradation has emerged as an important mechanism to counteract various insults to mtDNA. This review summarizes recent progress in understanding the molecular factors involved in mtDNA degradation in mammalian systems. For general discussion on mitochondrial genome maintenance and its relevance to human health, please see several excellent reviews [2,3,5,17].

## 2. Organization of mtDNA

### 2.1 Mitochondrial nucleoids and their DNA content

The mtDNA genome is organized in protein–DNA complexes known as nucleoids [18]. The term nucleoid was first used in 1959 in an electron microscopic study of rat oocytes [19], likely chosen in analogy to the organization of prokaryotic chromosomes. The understanding of the morphology and composition of mitochondrial nucleoids has advanced significantly over the past decade [18,20,21], driven primarily by the improved resolution of various imaging techniques. It is now known that nucleoid particles are located in the matrix between the cristae tubules, separated from the inner boundary membrane by cristae [22]. Historically, fluorescent DNA-binding probes, such as 4',6-diamidino-2-phenylindole (DAPI), were used to visualize nucleoids. The early studies demonstrated that nucleoids exist as compact spherical or ovoid structures in yeast [23] and human cells [24]. The visualization was also achieved by tagging the green fluorescent protein to nucleoid-specific proteins, such as mitochondrial transcription factor A (TFAM) and mitochondrial single-stranded DNA-binding protein (mtSSB) [21]. The reported sizes of nucleoids vary considerably, ranging from an average diameter of 0.1 to 0.9 $\mu$ m, due to the lateral resolution limit of approximately 250nm of wide-field or confocal microscopy [25] and different sample preparation methods. The more recently developed stimulated emission depletion (STED) microscopy is capable of achieving a 30–80nm resolution range [25], which is more suited to determine the size of nucleoids and to resolve nucleoid clusters. Two recent studies using STED microscopy [22,26] obtained a uniform mean size of approximately 100nm in a panel of cultured mammalian cells, similar to the dimensions of nucleoids reconstituted in vitro using mtDNA and TFAM.

Determining the number of DNA molecules has proven challenging. Satoh and Kuriowa were the first to report a calculated average of 1.4 mtDNA per nucleoid, with 4.6 mtDNA molecules and 3.2 nucleoids per mitochondrion in human ovarian carcinoma cells [24]. Subsequent studies reported a range from about 2.4 to 7.8 mtDNA molecules per nucleoid, depending on the cell type and the methods employed. Using the super resolution STED microscopy, Kukut et al. found that a surprisingly large fraction (26%–54%) of all nucleoids exist as nucleoid clusters in a panel of mammalian cells [26]. When using

bromodeoxyuridine to track newly synthesized DNA, the authors found 1.3 times more nucleoids using STED microscopy than with confocal microscopy, confirming the resolving power of STED microscopy. Notably, it was found that, on average, each mitochondrial nucleoid contains only a single mtDNA molecule in human and mouse fibroblasts [22,26], strikingly similar to the results by Satoh and Kuriowa almost 30 years ago [24]. It is conceivable that the DNA content could vary based on cell and tissue types, and the activity of DNA replication in nucleoids; additional research is required to clarify these questions.

## 2.2 Protein composition in mitochondrial nucleoids

The mtDNA genome is not naked but extensively coated with proteins. Early experiments showed that isolated mtDNA from HeLa [27] and rat cells [28] is associated with proteins and that certain regions of the *Drosophila melanogaster* genome are protected from trimethylpsoralen-induced DNA-interstrand crosslinking [29]. However, identifying nucleoid proteins is not as straightforward as it seems because of their dynamic composition and the low abundance of various proteins. The list of nucleoid proteins has been under constant expansion and revision, except for a list of core proteins that are essential for mtDNA maintenance and gene expression (for historical perspectives, please refer to previous reviews [18,20,30]). A layered structure of nucleoids was suggested by Bogenhagen and colleagues [31], which includes a set of core proteins for DNA replication and transcription, and peripheral proteins for translation, protein import, and metabolism.

Modern mass spectrometry-based methods have been the main driving force for identifying new nucleoid proteins. These methods generally involve two types of sample preparation methods. The first type involves formaldehyde cross-linking to covalently trap the interacting components followed by fractionation [31–33], and the second type employs known nucleoid proteins as bait to immunoprecipitate their interacting partners [33–35]. The former approach could produce false positives due to contamination from cytosolic and nuclear proteins, and the latter could generate false negatives owing to the failure of capturing transient or weak interactions. Consequently, there is a poor consensus among multiple data sets. Also, varying sensitivity of the instrumentation and different data processing methods may confound the analysis.

Recently, Han et al. used a proximity biotinylation method to identify a nucleoid proteome of 37 proteins [36], extending the specificity and coverage of previous mass spectrometry-based investigations [31,33] and those using immunoprecipitation-based approaches [34,35]. This method stands out, both in its sensitivity due to the ability to capture weak or transient interacting proteins in a cellular environment, and in its specificity due to avoidance of contaminants from cross-linking and fractionation steps. The selectivity of the data was further enhanced by filtering out by the nucleoid protein/matrix protein signal intensity ratios, although this data processing method could potentially miss some dual localized proteins [36]. Several new orphan nucleoid proteins were identified using this method, as summarized in Table 1. Interestingly, none of the published data sets identify any DNA repair factors in nucleoids, suggesting that these proteins exist in low abundance and are likely to interact transiently with DNA or other nucleoid proteins.

### 3. DNA turnover in mitochondrial DNA maintenance

#### 3.1 Mitochondrial DNA turnover

In 1969, Gross et al. reported that mtDNA in different rat tissues has a much shorter half-life (typically a matter of days) than nDNA (approximately 30 days) [6]. The mtDNA copy number varies considerably between cell and tissue types [87]. Even in a given tissue, mtDNA copy number varies over a 2–10-fold range [88]; variation in the range of 40%–150% in the average mtDNA content is considered clinically normal [89]. Current understanding of how mtDNA copy number is regulated remains incomplete [8]. Intriguingly, mitochondria can be partially or entirely depleted of their mtDNA in certain cells; mammalian erythrocytes contain no mitochondria and hence no mtDNA, and auxotrophic  $\rho^0$  cells lose their mtDNA content upon the induction of mtDNA depletion [90,91]. The major noncoding region of many mitochondrial genomes across species contains a triple-stranded D-loop, formed by stable association of a third, short DNA strand known as 7S DNA [92]. Curiously, 7S DNA is turned over even more rapidly relative to other parts of the mitochondrial genome, with a half-life of about an hour in rodent cells [93]. The reason for such a rapid turnover remains under investigation. The observations that organelle DNA abandonment exists across the species had led to the proposal that DNA degradation serves as a protective mechanism against mutagenesis and to reduce the energy cost of repair [93a].

#### 3.2 Mitochondrial DNA degradation under stress conditions

Similar to nDNA, mtDNA is susceptible to endogenous and exogenous chemicals [94–96]. It is well documented that a wide variety of environmental carcinogens and alkylating agents form covalent modifications preferentially with mtDNA relative to nDNA in mammalian cell culture and in experimental animals [94,95]. The ratio of the resulting mtDNA to nDNA lesion ratios ranges from several fold for alkylating agents [97–100] and aflatoxin B<sub>1</sub> [101], 50–100-fold for peroxidation-derived DNA adducts [102], to 50–200-fold for polycyclic aromatic hydrocarbons [103,104]. The higher ratios for the latter compounds have been attributed to their lipophilicity, and the persistence of the resulting lesions due to the lack of nucleotide excision repair in mitochondria. Moreover, several types of oxidative DNA lesions have been shown to exist in higher amounts in mtDNA relative to nDNA (see [94] and references therein). Contradictory results exist in the literature concerning the relative abundance in the two compartments of the lesion 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG, a commonly considered marker for oxidative DNA damage), likely because of the probability of artificial formation of 8-oxodG during sample preparation. Aside from measuring specific DNA lesions, multiple laboratories have reported a higher level of mtDNA lesions relative to nDNA in a wide variety of cells and tissues, using quantitative polymerase chain reaction (QPCR) methodology [105,106]. Cumulatively, these data emphasize the importance of mtDNA as a target for endogenous and exogenous DNA damaging agents.

The biological consequence of mtDNA damage remains less well understood than for its nuclear counterpart, due to the multicopy and heterogeneous characteristics of mtDNA. Several known pathways are known to cope with mtDNA damage. First, mtDNA lesions can

be repaired via several mitochondrial DNA repair pathways (see earlier reviews [2,5,107–112] and Chapter “Enzymology of mitochondrial DNA repair” by Alencar et al. of this volume), of which base excision repair (BER) is the most well understood. Second, during mtDNA replication, certain lesions can be bypassed by DNA polymerase  $\gamma$  (pol  $\gamma$ ) or other mitochondrial DNA polymerases now reported in mitochondria [113–115], or by a replication restart mechanism likely facilitated by PrimPol [116–119]. Lastly, the damaged mtDNA molecules can undergo a mitochondria-specific degradation pathway to maintain DNA integrity, which will be the focus of later sections. Notably, these pathways are not mutually exclusive, and can occur in parallel to cope with mtDNA damage.

An early report pointing to the existence of degradation of damaged mtDNA by Suter and Richter [120] showed that fragmented mtDNA contains a 15-fold higher level of 8-oxodG than the intact circular mtDNA in rat liver mitochondria, which suggested that “an efficient mtDNA repair or degrading system” exists in mitochondria [120]. Data from experimental animals also support the notion that mtDNA degradation is an important response to cellular stress. In mice, acute intragastric ethanol administration leads to mtDNA degradation in different tissues, including liver, brain, heart, and skeletal muscle [121,122]. Loss of mtDNA content was also observed after ischemia/reperfusion injury, followed by the restoration of mtDNA to a normal level 24h after the reperfusion [123].

A body of studies by Alexeyev and colleagues have shown unequivocally that mtDNA degradation is an active mechanism to counteract DNA damage [87,124–127]. Shokolenko et al. showed that hypoxanthine/xanthine oxidase-induced oxidative stress can lead to mtDNA degradation, and that accumulation of linear mtDNA molecules proceeds degradation [124]. When BER activity was inhibited by methoxyamine treatment, the activity of mtDNA degradation increased, suggesting that BER and mtDNA degradation cooperate to cope with DNA damage [124]. Subsequently, the same group verified that mtDNA degradation is a direct and immediate consequence of mtDNA damage after the induction of mitochondrial-specific DNA single-strand breaks and abasic sites [125]. The rate of degradation varies based on the type of mtDNA damage [125] and cell type [126]. In particular, Kozhukhar et al. demonstrated that BER and mtDNA degradation are major pathways to counteract mitochondrial abasic (AP) sites (*vide infra*), and that translesion bypass of AP sites occurs much less frequently [127].

Data from other laboratories also support the importance of mtDNA degradation. Kai et al. observed a rapid reduction in the amount of mtDNA after treating rat hepatocytes with 2', 3'-dideoxycytidine or ethidium bromide [128]. Furda et al. observed mtDNA loss and persistent mtDNA lesions in mouse embryonic fibroblasts upon H<sub>2</sub>O<sub>2</sub> treatment [129]. Although these authors did not observe a decrease in mtDNA level after treatment with methyl methanesulfonate in contrast to the results by Shokolenko et al. [124], the discrepancy can be explained by the different treatment times, the potential effect of compensatory DNA synthesis, and different methods in measuring the DNA copy number used in the two studies. Moretton et al. reported that rapid mtDNA loss occurs after the induction of double-strand breaks by expression of a mitochondria-targeted restriction enzyme [130]. Furthermore, that treatment of experimental animals with environmental carcinogens [131] or suppressing the expression of mitochondrial BER enzymes [132] did

not cause an increase in the mtDNA mutation load also suggests the existence of specific mechanisms to eliminate damaged mtDNA molecules. mtDNA degradation has emerged as an important mechanism for mtDNA maintenance and stress response. Degradation is believed to be nonspecific with regard to DNA lesion type, and activated in response to difficult-to-repair DNA lesions or excessive DNA damage [126,130].

#### 4. Molecular factors involved in mtDNA degradation

Critical to the understanding of the mechanism by which mitochondria destruct damaged DNA molecules, several recent studies have identified protein factors involved. These include key components in the mtDNA replication machinery, such as pol  $\gamma$ , Twinkle, and MGME1, and the DNA packaging protein TFAM, as summarized below.

##### 4.1 DNA polymerase $\gamma$

Encoded by the *POLG* gene, DNA polymerase  $\gamma$  has been considered historically as the sole DNA polymerase in human mitochondria [133,134]. It is now known that noncanonical DNA polymerases, such as PrimPol [116–119], pol  $\beta$  [113,114], and pol  $\theta$  [115], can also localize to mitochondria and potentially participate in special replication and repair events. Nonetheless, pol  $\gamma$  remains the only replicative DNA polymerase, as it is responsible for the bulk of the mtDNA synthesis in human mitochondria [133,134]. Pol  $\gamma$ , mtSSB, and helicase Twinkle constitute the minimal mitochondrial replisome [39]. The human pol  $\gamma$  holoenzyme consists of a catalytic subunit and a dimeric form of its accessory subunit [134]. The catalytic subunit is a 140kDa polypeptide (p140 or pol  $\gamma$ A) comprising an N-terminal exonuclease domain, a connecting linker region, and a C-terminal polymerase domain. The catalytic subunit has multiple enzymatic activities, including DNA polymerase activity, 3'  $\rightarrow$  5' exonuclease activity, and 5' dRP lyase activity. The accessory subunit, a 55kDa protein (p55 or pol  $\gamma$ B), is known to promote DNA binding and processive DNA synthesis [135].

Essential for high fidelity DNA synthesis, the exonuclease activity of pol  $\gamma$  proofreads the 3'-terminal nucleotide from both matched and mismatched primer termini, with a preference for the mismatched pairs. Moreover, the exonuclease activity cleaves single-stranded DNA, making it an excellent candidate for digesting linearized mtDNA fragments. Recently, results from several laboratories suggest the involvement of the exonuclease activity of pol  $\gamma$  in mtDNA degradation. Peeva et al. engineered human HEK-293 cells with mitochondrial-targeted restriction endonucleases [136]. Upon the induction of mtDNA double-strand breaks by restriction cleavage, progressive degradation occurs, to yield a mixture of DNA fragments ranging from a few hundred to several thousand base pairs downstream from the cleavage site. Ultra-deep sequencing revealed that mtDNA degradation occurs from both the 3'-end and 5'-end, suggesting the involvement of two types of exonucleolytic activities. Given the known specificities of mitochondrial nuclease MGME1 and pol  $\gamma$ , the authors verified the role of both enzymes in the process by generating MGME1-null cells and cells containing pol  $\gamma$  D274A (a known variant that is defective in its 3'  $\rightarrow$  5' exonuclease activity) using CRISPR–Cas9 technology. Results from Southern blotting and ultra-deep sequencing confirmed that the exonuclease activity of pol  $\gamma$  is required for removing linear

mtDNA species. The accessory subunit did not appear to be involved in the degradation process as judged from experiments using siRNA-mediated knockdown of the *POLG2* gene.

Nissanka et al. used the mtDNA mutator mice and the derived lung fibroblasts carrying an exonuclease-deficient pol  $\gamma$  to clarify the role of the exonuclease activity of pol  $\gamma$  in eliminating the fragmented mtDNA upon double-strand breaks [137]. In both cultured fibroblasts and the liver of the mutator mice, prolonged persistence of the mtDNA fragments was observed. Degradation did not depend on the DNA polymerase activity of pol  $\gamma$  or the origin of replication, suggesting that a separate population of pol  $\gamma$  molecules dock at the free double-stranded ends.

Currently, little is known regarding how the degradation mode of the replication machinery is regulated. Although the high-resolution crystal structures of apo pol  $\gamma$  and the pol  $\gamma$  ternary complexes have been solved [138] and revised [139–143], the existing structures do not shed light on the exonuclease mode of pol  $\gamma$ , or how the degradation mode is activated. It is conceivable that pol  $\gamma$  may adopt a different conformation in the degradative mode, and may cooperate with Twinkle and mtSSB to perform such functions [143]. Another potential factor regulating the pol  $\gamma$  degradative mode may be the level of deoxynucleoside triphosphates, reminiscent of the known activity of T4 DNA polymerase [144]. In addition, a switch between DNA synthesis and degradation by pol  $\gamma$  may depend on the homeostatic functions of autophagy, as has been observed in yeast [145]. All of these questions remain to be addressed.

## 4.2 MGME1

DNases play an essential role in mtDNA maintenance and repair (reviewed in [146]). Known mitochondrial DNases include APE1 [147], DNA2 [148,149], EXOG [150], ENDOG [151], FEN1 [152], MGME1 [76], and MRE11 [153,154]. EXOG, ENDOG, and MGME1 are localized exclusively to mitochondria, whereas the other enzymes are localized to both mitochondria and the nucleus. MGME1 (also known as Ddk1) belongs to the PD–(D/E)XK phosphodiesterase superfamily, which includes a broad spectrum of enzymes involved in DNA and RNA cleavage. MGME1 has a documented role in mtDNA degradation, evidenced from patients with loss-of-function *MGME1* mutations [75] and MGME1-depleted cells [76]. Patients carrying *MGME1* mutations develop multisystemic mitochondrial disorders characterized by external ophthalmoplegia, emaciation, and respiratory failure [75,155]. Muscle biopsies of the affected individuals show substantial mtDNA depletion and deletions. In fibroblast cultures from an affected patient, mtDNA repopulation is severely impaired after the nucleotide analog 2',3'-dideoxycytidine (ddC) treatment and subsequent withdrawal. In addition, the mtDNA depletion rate upon ddC treatment is significantly slower in the MGME-null cells than that of the control fibroblasts, arguing a role for MGME1 in mtDNA degradation. Furthermore, MGME1 is involved in maintaining 7S DNA, the single-stranded DNA species formed by premature replication termination at the end of the control region of mtDNA. Patients carrying *MGME1* mutations or MGME1-depleted cells exhibit an increase in 7S DNA levels [75,76]. The accumulated 7S DNA is elongated due to incomplete processing of 5'-ends by MGME1 [155]. Consistent

with these findings, *MGME1*-knockout mice show mtDNA depletion, deletion of the minor arc of mtDNA, and increased stability of 7S DNA [156].

*MGME1* is thought to be a component of the mitochondrial replisome, considering that *MGME1* interacts with all three core components (pol  $\gamma$ , mtSSB and Twinkle) of the minimal mitochondrial replisome [155–157]. In the context of degrading flap DNA during Okazaki fragment processing or long-patch BER, *MGME1* cooperates with the 3'  $\rightarrow$  5' exonuclease activity of pol  $\gamma$  to yield ligatable nicks in cells [158]. In cellular models containing restriction endonuclease-induced mtDNA double-strand breaks, *MGME1*, the 3'  $\rightarrow$  5' exonuclease activity of DNA polymerase  $\gamma$ , and Twinkle helicase together contribute to the removal of linear mtDNA [136]. The loss of *MGME1* activity does not affect the efficiency of mtDNA cleavage, but causes a substantial accumulation of the linear mtDNA fragments induced by engineered mitochondrial endonucleases [136]. In *MGME1*<sup>-/-</sup> mice, tissue-specific replication stalling has been observed in addition to the aforementioned effects on 7S DNA and mtDNA deletion [156].

Biochemically, recombinant *MGME1* cleaves single-stranded DNA (ssDNA) at the 5' - or 3' -terminus, and DNA flap substrates with a 5' - or 3' -flap [75,76]. *MGME1* can also degrade the DNA segment in RNA-DNA chimeric substrates at a position two to five nucleotides downstream from the RNA-DNA junction [75]. *MGME1* does not have endonucleolytic activity on a single-strand circular DNA [75,76]. In addition, *MGME1* processes DNA 5' - and 3' -splayed-arm substrates by digesting the ssDNA segment but pauses at the ssDNA-dsDNA junction [75]. *MGME1* is active in the presence of either Mg<sup>2+</sup> or Mn<sup>2+</sup>, with a different optimal concentration for each metal ion [76]. Together, these enzymatic activities suggest that *MGME1* is an excellent candidate for processing displaced DNA-containing Okazaki fragments on the lagging strand, and/or DNA flaps during long-patch BER [75].

In the PD-(D/E)XK phosphodiesterase superfamily, *MGME1* shares high sequence similarity with its orthologs from other species, and very low sequence similarity with other PD-(D/E)XK nucleases. Nonetheless, *MGME1* contains several motifs (Ia, I, II, III, and IV, depicted in Fig. 1A) that are characteristic of RecB-type and RecE-type nucleases. Recently solved X-ray crystal structures of *MGME1* have revealed the detailed architecture of the enzyme [159]. A Dali structural comparison search using the apo *MGME1* (PDB:5ZYW) resulted in a list of structurally similar proteins, including RecE (PDB: 3H4R) and RecB (PDB: 1W36) nucleases (Fig. 1B). The conserved motifs share a considerable structural similarity when comparing the catalytic domains of these proteins (Fig. 1C). The structure of *MGME1*-ssDNA complex closely resembles that of apo *MGME1*, except that the connecting loop (aa 255–264) between  $\beta$ 3 and  $\alpha$ 5 undergoes a large conformational change to allow DNA binding. The complex of *MGME1* with a 3' -flap DNA also superimposes well with the *MGME1*-ssDNA complex, suggesting a common mechanism to cleave the ssDNA segment of different substrates. *MGME1* uses a structural pin (F173) for duplex unwinding, as revealed in the structure of *MGME1* with 3' -flap DNA [159].

In evaluating the superimposition of structural components of several crystal structures, a two-metal ion catalytic mechanism has been proposed for *MGME1* [159]. The two-metal



ion mechanism, initially adopted from that of the exonuclease activity of *E. coli* DNA polymerase I, has been shown to be a common mechanism for many nucleases. In this model, metal ion A coordinates a water molecule to lower its pK<sub>a</sub>, and K253 acts as a general base to deprotonate the water molecule and to produce a strong nucleophile for the phosphoryl transfer reaction. Metal ion B also contributes to stabilizing the transition state by chelating the phosphate backbone and a conserved aspartate residue (D238). The solved MGME1 structures unveil the basis of the MGME1 3'-nuclease activity, although the mechanism of its 5'-nuclease activity remains to be clarified. At present, how MGME1 interacts with other components of the mitochondrial replisome is unknown.

### 4.3 TFAM

TFAM is a core mitochondrial transcription factor, responsible for recruiting mitochondrial RNA polymerase and transcription factor T2BM to activate transcription. Additionally, TFAM is an abundant protein that coats and packages mtDNA into nucleoids by imposing a U-turn on mtDNA [38,160] and cross-strand interactions [22]. TFAM knockout mice show embryonic lethality, indicating that TFAM is a critical protein for mitochondrial genome maintenance. Furthermore, TFAM has been proposed to play a role in regulating mtDNA repair [161], though the underlying mechanism remains to be clarified. Recently, my laboratory has identified a novel role for TFAM in facilitating the degradation of damaged mtDNA containing abasic (AP) sites [162], which are ubiquitous DNA lesions and an important intermediate during BER.

The importance of AP sites lies in their abundance and chemical reactivity. AP sites exist at a steady-state level of approximately 30,000 AP lesions per cell [163,164], and can number in the hundreds in the mitochondria of each cell [165,166]. AP sites are chemically reactive and labile, existing as an equilibrating mixture of a cyclic hemiacetal and a ring-opened aldehyde [167,168]. The latter functionality renders AP sites susceptible to reactions with amino groups in DNA, nuclear DNA repair enzymes, histones, small peptides, and endogenous polyamines (e.g., spermine), which can lead to DNA interstrand cross-links, DNA-protein cross-link intermediates, or single-strand breaks [167,169–177]. Several oxidized forms of AP sites can also yield stable DNA-protein cross-links [178,179].

AP sites and their derivatives pose tremendous threats to nuclear and mitochondrial genome integrity. AP sites are cytotoxic and mutagenic and can block nuclear DNA replication and transcription [180–182]. The DNA-protein cross-links formed between oxidized AP sites and histone proteins have the potential to alter histone epigenetic marks [183]. In mitochondria of human cells, an elevated level of AP sites leads to rapid loss of mtDNA, suggesting that the DNA degradation is unlikely due to autophagy, mitophagy, or apoptosis [126,130]. By inducing AP sites at either T or C residues in mouse embryonic fibroblasts, Kozhukhar et al. analyzed the relative contribution of DNA lesion bypass synthesis versus the combined contribution of BER and mtDNA degradation [127], and found that AP sites increase only moderately the overall mutation load, and that BER and mtDNA degradation are the major pathways to cope with abasic DNA [127]. Evidently, mitochondria are capable of abandoning the abasic DNA as a way to alleviate its cytotoxic effects.

What protein factors could be involved in abasic mtDNA degradation? Considering the abundance of TFAM and its role as a histone-like protein in packaging mtDNA, it is inevitable that TFAM encounters the ubiquitous AP sites. Recently, my laboratory has shown that TFAM accelerates the degradation of AP lesion-containing DNA using biochemical assays and mitochondrial extracts from human cells [162]. Based on the proximity of the  $\epsilon$ -NH<sub>2</sub> group of several lysine residues and the C1'-carbon of the nearby nucleotide residue in the TFAM-DNA crystal structures [38,160], we hypothesized that several lysine residues might react with AP sites nearby to form single-strand breaks (Fig. 2A). Site-specifically modified oligodeoxynucleotides with an AP lesion were designed and synthesized using light strand promoter (LSP) and nonspecific DNA sequences. On the basis of structural analysis, an AP lesion was positioned near K69 in the high-mobility group (HMG) 1 domain, or in K183, K186, and K189 in the HMG 2 domain (Fig. 2A). In reconstituted TFAM-DNA complexes, we found that the half-life of AP sites is reduced by 2- to 3-orders of magnitude, depending on the position of the AP sites and the sequence of the oligodeoxynucleotides. We were able to trap the reaction intermediates (TFAM-DNA cross-links) using NaBH<sub>3</sub>CN, a reagent that selectively reduces imine to amine, indicating that the reaction occurs via Schiff base intermediates (the proposed mechanism is shown in Fig. 2B). The reaction was observed with either recombinant TFAM or mitochondrial extracts, suggesting that TFAM interacts strongly with AP-DNA in the presence of other mitochondrial proteins. The TFAM-induced reduction of AP lesion half-life resembles that of histone proteins in reconstituted core particles, although the extent of decrease in AP lesion half-life appears to be more dramatic in TFAM-DNA complexes [172,184].

Remarkably, substituting putative lysine residues with alanine did not completely abolish TFAM-DNA cross-link formation, suggesting the potential contribution of other lysine or arginine residues in TFAM to promote strand scission. This observation is consistent with the dynamic nature of TFAM-DNA complexes, which could lead to transient interactions between additional lysine or arginine residues and AP sites, perhaps through a butterfly-like TFAM-DNA complex breathing [185]. In addition, considering the flexibility of the inter-domain linker [185], other Lys or Arg residues in the region could potentially contribute to the observed reaction with TFAM variants. The potential for multiple residues to contribute to the AP-DNA strand cleavage, and the fact that TFAM coats nearly-uniformly the entire mtDNA molecule, suggest a ubiquitous role for TFAM-promoted AP-DNA degradation. The role of TFAM in accelerating the strand cleavage of AP-DNA also suggests that TFAM could be involved in the rapid DNA depletion resulting from mitochondrial AP sites [127]. Additional research is required to establish the role of TFAM in DNA degradation *in cellulo* and *in vivo*.

APE1 is known to localize to mitochondria and particularly relevant to AP lesion repair. We found that TFAM competes actively with APE1-mediated DNA cleavage using mitochondrial extracts from HeLa and HEK-293 cells [162], suggesting that TFAM interacts strongly with AP lesions under physiological concentration ratios of TFAM to APE1, and TFAM could play a regulatory role in mtDNA repair, as suggested previously [161]. Because mtDNA degradation and repair may occur in parallel to alleviate abasic DNA damage [127], it is plausible that TFAM could act redundantly with APE1 to trigger mtDNA turnover or to regulate the partitioning between mtDNA degradation and repair. The

cooperation between degradation and repair is likely to be regulated by additional factors, such as the total amount of TFAM protein (regulated by Lon protease [186,187]), the posttranslational modification of TFAM [187a,b], and the interactions between TFAM and its interacting partners.

#### 4.4 Interactions of replisomal components

It is well established that interactions among replisome proteins are critically important for their functions [143]. Relevant to mtDNA degradation, it has been shown that mtSSB stimulates the DNA polymerase and exonuclease activities of pol  $\gamma$  [188,189], albeit moderate in the human system relative to the high level of stimulation observed in the *Drosophila* system [190,191]. Similarly, mtSSB stimulates the DNA unwinding activity of the human mtDNA helicase, Twinkle [188,189]. The importance of Twinkle helicase is supported by the accumulation of linear mtDNA fragments in human cells after siRNA knockdown [136]. It is reasonable to presume that functional interactions between mtSSB and both Twinkle and pol  $\gamma$ , are vital for their roles in mtDNA degradation.

MGME1 interacts with all three replisome components: the catalytic subunit of pol  $\gamma$ , mtSSB and Twinkle [155,156]. Although MGME1 employs a structural pin for duplex DNA unwinding [159], structural comparison of MGME1 with RecBCD complex and the direct interactions between MGME1 and Twinkle have led to the hypothesis that Twinkle may mimic the helicase domains of RecBCD to assist the nuclease activities of MGME1 and pol  $\gamma$  [159]. The structural and biochemical basis of these interactions remain to be determined.

## 5. Conclusions and future perspectives

Prevailing evidence supports clearly the importance of mtDNA degradation as a unique mechanism for mtDNA maintenance and stress response. Studies of mtDNA turnover and the selective destruction of damaged mtDNA molecules have inspired the development of mitochondrial-specific endonucleases to target mutated mitochondrial molecules for therapeutic purposes [10–16]. Despite recent advances, a complete understanding of mtDNA degradation remains to be determined. Myriad questions remain to be addressed, including the additional factors involved, detailed molecular pathways, and regulatory mechanisms. The dynamics of mtDNA turnover require complex regulation on multiple levels, including, but not limited to, mtDNA replication and degradation, fission and fusion, and mitophagy. Additional complexity from tissue-specific factors also needs to be considered carefully. An understanding of these fundamental questions will provide additional insights into the development therapeutics for mitochondrial diseases.

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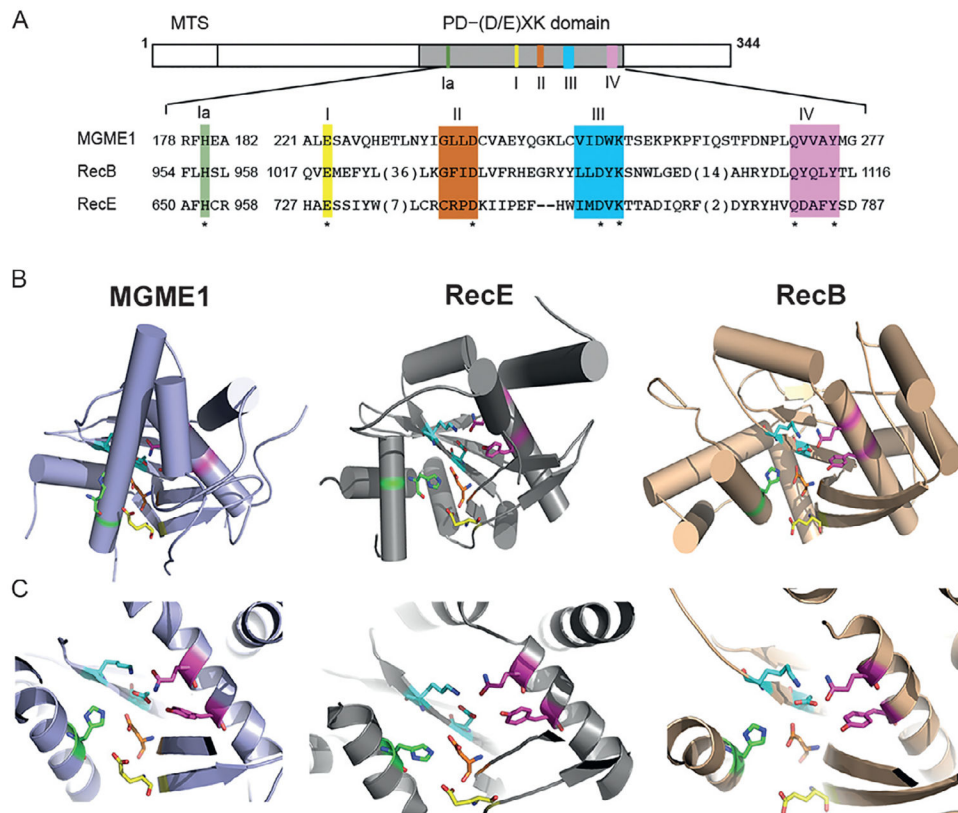
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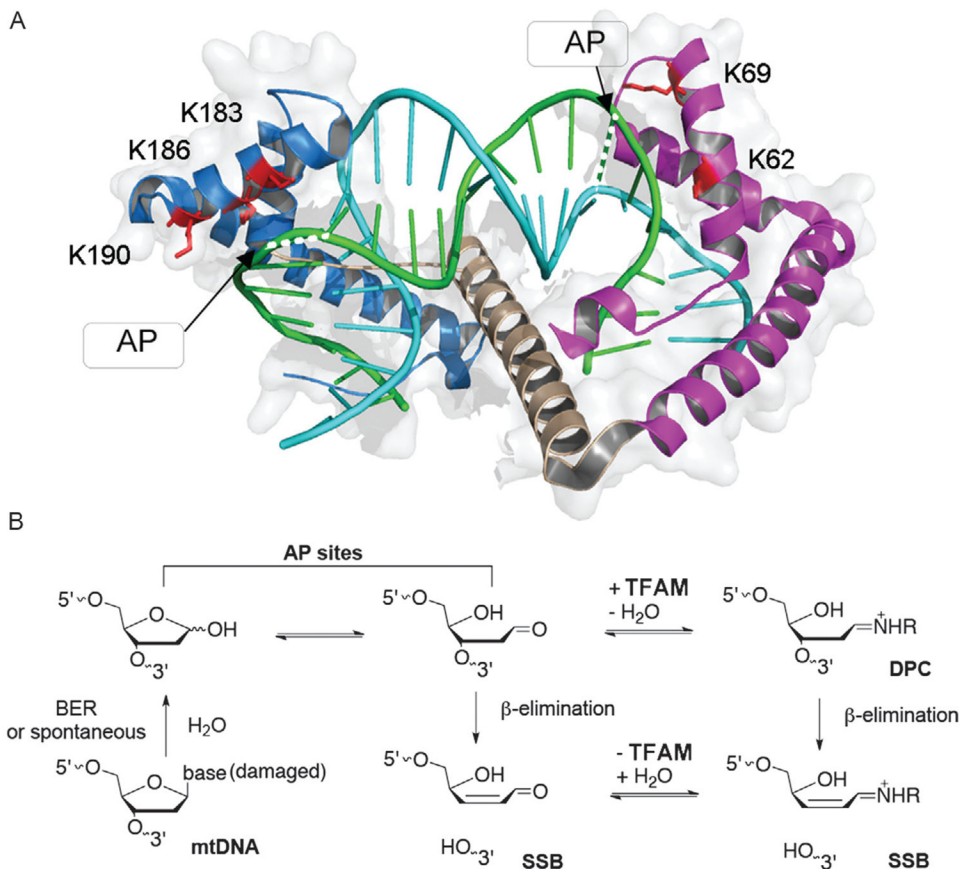
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**Fig. 1.** Domain illustration of MGME1 and structural comparison with RecE and RecB nucleases. (A) MGME1 contains characteristic of RecB-type and RecE-type nuclease motifs (Ia, I, II, III, and IV). (B) Overall view of MGME1 (PDB:5ZYW), RecE (PDB: 3H4R), and RecB (1W36) nucleases with their active sites highlighted in (C). Conserved residues at their active sites are highlighted.



**Fig. 2.** Structural basis and proposed mechanism of TFAM-promoted abasic DNA strand scission. (A) Crystal structure of TFAM in complex with DNA containing the mitochondrial light-strand promoter (LSP) sequence (PDB: 3TQ6). The two high-mobility group (HMG) box domains of TFAM are in purple (HMG1) and blue (HMG2), and the inter-domain linker is in wheat. Potentially reactive (with AP sites) lysine residues within the two HMGs are highlighted in red. The heavy strand is in cyan, and the light strand is in green with the position of AP lesions shown in the dashed lines. (B) The proposed mechanism of TFAM-mediated DNA strand cleavage at AP sites via Schiff base intermediates (DNA-TFAM cross-links, DPC) to form single-strand breaks (SSB).

Table 1

Summary of mitochondrial nucleoid proteins.

Protein name	Gene name	Biological function in mitochondria	Han et al. [36]	Bogenhagen et al. [31]	He et al. [35]	Rajala et al. [33]	Wang and Bogenhagen [34]
Transcription factor A, mitochondrial	<i>TFAM</i>	Transcription factor required for transcription [37,38]					
Single-stranded DNA-binding protein, mitochondrial	<i>SSBP1</i>	Single-stranded DNA binding protein required for mtDNA replication [39]					
Lon protease homolog, mitochondrial	<i>LONP1</i>	mtDNA binding and RNA binding, involved in degradation of other nucleoid proteins such as POLG, TFAM and PEOI [40–42]	×				×
DNA-directed RNA polymerase, mitochondrial	<i>POLRMT</i>	RNA polymerase required for transcription [37]					
Serine hydroxymethyltransferase, mitochondrial	<i>SHMT2</i>	Involved in thymidylate biosynthesis required to prevent uracil accumulation in mtDNA; also part of the mitochondrial folate pathway [43]					
Isoform 2 of putative ATP-dependent RNA helicase DHX30	<i>DHX30</i>	Known RNA binding protein that affects mtRNA abundance [44]					
GTPase Era, mitochondrial	<i>ERALI</i>	Binds to 12S-mtRNA [45,46]				×	×
G-rich sequence factor 1	<i>GRSFI</i>	Associated with nascent transcript binding and processing [47,48]	×			×	×
Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial	<i>DBT</i>	Mutation causes mtDNA damage accumulation and affects mtDNA copy number [49]					×
Twinkle protein, mitochondrial	<i>PEOI</i>	Helicase required for mtDNA replication [39,50]			×		
DNA polymerase subunit gamma-1	<i>POLG</i>	DNA polymerase required for mtDNA replication [51]			×		
RNA pseudouridylylate synthase domain-containing protein 3	<i>RPU/SD3</i>	RNA modification enzyme [52]		×	×	×	×
Mitochondrial ribonuclease P protein 1	<i>TRMT10C</i>	Associated with nascent transcript binding and processing [53]		×	×	×	×
2-Oxoglutarate dehydrogenase-like, mitochondrial	<i>OGDHL</i>	Oxoglutarate dehydrogenase degrading glucose and glutamate [54]		×	×	×	×
Aspartate aminotransferase, mitochondrial	<i>GOT2</i>	Known RNA binding protein [55]		×	×	×	×
Mitochondrial 10-formyltetrahydrofolate dehydrogenase	<i>ALDH1L2</i>	Mitochondrial form of 10-formyltetrahydrofolate dehydrogenase [56–58]		×	×	×	×
Monofunctional C1-tetrahydrofolate synthase, mitochondrial	<i>MTHFD1L</i>	Up regulated with mtDNA replication defects [59]		×	×	×	×



Protein name	Gene name	Biological function in mitochondria	Han et al. [36]	Bogenhagen et al. [31]	He et al. [35]	Rajala et al. [33]	Wang and Bogenhagen [34]
Dimethyladenosine transferase 2, mitochondrial	<i>TFB2M</i>	Transcription factor required for transcription [37]			×		
Peroxisome oxidase 5, mitochondrial	<i>PKDX5</i>	DNA binding, negative regulator of transcription [60]		×	×	×	×
NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial	<i>NDUF56</i>	A subunit of the NADH:ubiquinone oxidoreductase for the transfer of electrons from NADH to the respiratory chain [61]		×	×	×	×
Isoaleryl-CoA dehydrogenase, mitochondrial	<i>IVD</i>	Mitochondrial matrix enzyme for leucine catabolism [62–64]		×	×	×	×
Isocitrate dehydrogenase [NADP], mitochondrial	<i>IDH2</i>	Yeast homolog IDH2 interacts with TFAM and binds to mtRNA [65,66]		×	×	×	×
Adenylate kinase 4, mitochondrial	<i>AK4</i>	Nucleotides modification enzyme [67]		×	×	×	×
Mycophenolic acid acyl-glucuronide esterase, mitochondrial	<i>ABHD10</i>	Hydrolase removing glucuronide from mycophenolic acid acyl-glucuronide [68–70]		×	×	×	×
Methionine-R-sulfoxide reductase B2, mitochondrial	<i>MSRB2</i>	Predicted DNA binding, transcription factor activity [71]		×	×	×	×
DNA polymerase subunit gamma-2, mitochondrial	<i>POLG2</i>	DNA polymerase required for mtDNA replication [39,72]		×	×		
GripE protein homolog 2, mitochondrial	<i>GRPEL2</i>	Essential component of the PAM complex, a complex required for the translocation of transit peptide-containing proteins from the inner membrane into the mitochondrial matrix in an ATP-dependent manner [73,74]		×	×	×	×
Mitochondrial genome maintenance exonuclease 1	<i>MGME1</i>	Mitochondrial 7S DNA exonuclease [75,76]		×	×	×	×
Kynurenine—oxoglutarate transaminase 3	<i>CCBL2</i>	Known RNA binding protein that affects mtRNA abundance [55,77]		×	×	×	×
FAST kinase domain-containing protein 1	<i>FASTKD1</i>	Predicted RNA binding protein [52]		×	×	×	×
Carnitine O-acetyltransferase	<i>CRAT</i>	Transferase removing acyl groups from carnitine to coenzyme A (CoA) and regulates the acyl-CoA/CoA ratio [78,79]		×	×	×	×
UPP0562 protein C7orf55 (Protein FMC1 homolog)	<i>C7ORF55 (FMC1)</i>	Involved in the assembly/stability of the mitochondrial membrane ATP synthase [80]		×	×	×	×
Probable glutamate—tRNA ligase, mitochondrial	<i>EARS2</i>	tRNA binding for aminoacylation [81]		×	×	×	×
Decaprenyl-diphosphate synthase subunit 2	<i>PDSS2</i>	Similar to oxoglutarate dehydrogenase (OGDH) of the OGDH complex, which degrades glucose and glutamate [82–84]		×	×	×	×

Protein name	Gene name	Biological function in mitochondria	Han et al. [36]	Bogenhagen et al. [31]	He et al. [35]	Rajala et al. [33]	Wang and Bogenhagen [34]
Probable proline-tRNA ligase, mitochondrial	<i>PARS2</i>	tRNA binding for aminoacylation [36]		×	×	×	×
Polymerase delta-interacting protein 2	<i>POLDIP2</i>	Interacts with TFAM and SSBP1, DNA binding activity, cross-links to SSBP1, LRPPRC, LONP1, CLPX [85,86]		×	×	×	×

For simplicity, the list is constructed based on the list of proteins in Ref. [36]. For comparisons with non-mass spectrometry-based studies, please see Refs. [30,33].