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DNA repair and mutagenesis in vertebrate mitochondria: evidence for asymmetric DNA strand inheritance

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A variety of endogenous and exogenous factors induce chemical and structural alterations in cellular DNA in addition to the errors occurring throughout DNA synthesis. These types of DNA damage are cytotoxic, miscoding or both and are believed to be at the origin of cancer and other age-related diseases. A human cell, aside from nuclear DNA, contains thousands of copies of mitochondrial DNA (mtDNA), a double-stranded, circular molecule of 16,569 bp. It has been proposed that mtDNA is a critical target of reactive oxygen species: by-products of oxidative phosphorylation that are generated in the organelle during aerobic respiration. Indeed, oxidative damage to mtDNA is more extensive and persistent as compared to that to nuclear DNA. Although transversions are the hallmark of mutations induced by reactive oxygen species, paradoxically, the majority of mtDNA mutations that occur during ageing and cancer are transitions. Furthermore, these mutations show a striking strand orientation bias: T→C/G→A transitions preferentially occur on the light strand, whereas C→T/A→G on the heavy strand of mtDNA. Here, we propose that the majority of mtDNA progenies, created after multiple rounds of DNA replication, are derived from the heavy strand only, owing to asymmetric replication of the DNA strand anchored to the inner membrane via the D-loop structure.

Key words: Mitochondrial DNA, oxidative DNA damage, uracil, abasic sites, DNA excision repair, DNA glycosylases.

Abbreviations: 5ohC, 5-hydroxycytosine: 5ohU, 5-hydroxyuracil; 8oxoA, 8-oxo-7,8-dihydroadenine; 8oxoG, 8-oxo-7,8-dihydroguanine; AP site, apurinic/apyrimidinic site; APEX1 or APE1, major human AP endonuclease 1; BER, base excision repair; CPD, cyclobutane pyrimidine dimer; DNA2, DNA helicase/nuclease 2; dRP, 5′-deoxyribose phosphate; dsDNA, double-stranded DNA; ETC, electron transport chain; Fapy, formamidopyrimidines; FEN1, DNA flap-structure endonuclease 1; H-strand, heavy strand of mtDNA; L-strand, light strand of mtDNA; LigIIIα, DNA ligase IIIα; LP-BER, long-patch BER; MGME1, mitochondrial genome maintenance exonuclease 1; MMR, mismatch repair; mtDNA, mitochondrial DNA; mtSSB, mitochondrial single-stranded DNA binding protein; MUTYH, human mismatch-specific adenine-DNA glycosylase; NER, nucleotide excision repair; NIR, nucleotide incision repair; NTHL1, human oxidative pyrimidine DNA glycosylase, homologue of *Escherichia coli* endonuclease III; OGG1, human 8oxoG-DNA glycosylase; OXPHOS, oxidative phosphorylation; PolG, mitochondrial DNA polymerase γ; POLRMT, mitochondrial RNA polymerase; RNase H1, ribonuclease H1; ROS, reactive oxygen species; SDM, strand displacement model; SP-BER, short-patch BER; ssDNA, single-stranded DNA; TFAM, mitochondrial transcription factor A; Tg, thymine glycol or 5,6-dihydroxy-5,6-dihydrothymine; TopoIIIα, Topoisomerase IIIα; UNG1, human mitochondrial isoform 1 of uracil-DNA glycosylase; εA, 1,N^6-ethenoadenine; εC, 3,N^4-ethenocytosine;

1. Introduction

Mitochondria are organelles that exist in the form of an extensive tubular network and can be found in most of eukaryotic cells. These organelles have a complex mesh-like structure across the cytosol and undergo continuous cycles of fission and fusion that break and connect the network, respectively. This process enables the regulation of cellular signalling, homeostasis and redistribution of mitochondrial genetic material. Mitochondria are cellular power plants, which oxidise or 'burn' carbohydrates, amino acids and fatty acids to synthesise adenosine triphosphate (ATP) in a process referred to as oxidative phosphorylation (OXPHOS) and the electron transport chain (ETC). Mitochondria are double-membrane organelles composed of a surrounding outer membrane and an inner membrane, which has five-fold larger surface area than the outer one. The inner membrane is extensively folded into compartments known as cristae. The space between the two membranes is termed the inter-membrane space, and the space inside the inner membrane is called the matrix. The five main OXPHOS complexes are concentrated in the cristae membranes. Complexes I, III and IV pump protons (hydrogen ions, H^+) from the matrix to the inter-membrane space to generate a proton concentration gradient across the inner membrane. Complex V (ATP synthase) then uses this gradient to produce ATP.

Mitochondria contain their own genomic DNA, referred to as mitochondrial (mt) DNA with unique replication, transcription and translational machinery. Human cell contains several thousand copies of mtDNA, which is organised as a small closed circular duplex DNA of 16,569 bp. The human mitochondrial genome encodes 13 structural proteins, which are essential subunits of OXPHOS complexes I, III, IV and V; two mitochondrial ribosomal RNAs and 22 mitochondrion-specific tRNAs (Mechanic et al. 2006). It is noteworthy that the mitochondrial genome employs a non-universal genetic code in which AUA is read as methionine, UGA is read as tryptophan, and AGA and AGG are read as STOP instead of arginines (Suzuki and Nagao 2011). Another intriguing peculiarity of the vertebrate mitochondrial genome is an unusual misbalance of nucleotide composition on the two strands of the mtDNA, thus leading to the separation into a heavy (H) and a light (L) strand upon ultracentrifugation in a caesium chloride gradient (Clayton 1996). It should be noted that the H-DNA strand is G+T-rich and composed of 31% of G, 13% of C, 25% of A and 31% of T, whereas the L-DNA strand is C+A-rich and composed of 31% of C, 13% of G, 31% of A, and 25% of T.

MtDNA is organised into nucleoids, a structural unit composed of DNA tightly packed with the mitochondrial transcription factor A (TFAM) protein (Bogenhagen 2012). The majority of nucleoids consist of only a single mtDNA molecule (Kukat et al. 2015; Kukat et al. 2011). Nucleoids are attached to the inner membrane on the side of the matrix and are partitioned into distinct mitochondrial compartments, cristae, to ensure that mtDNA is distributed evenly throughout the cell's mitochondria (Kopek et al. 2012). The mitochondrial nucleoid is a platform for transcription and replication of mtDNA that ensures accurate replication and effective partition of the genetic material. Replication of mtDNA is continuous and not dependent on the cell cycle; this arrangement is essential for maintaining a high copy number of mtDNA per cell (Sasaki et al. 2017). It is commonly accepted that mtDNA is bound to the mitochondrial inner membrane by a mechanism involving the control region sequence in mtDNA (Jackson et al. 1996). After replication termination, the separation of two newly synthesised molecules of mtDNAs is spatially linked to the division of the mitochondrial network, suggesting that mtDNA replication and mitochondrial fission are coupled processes (Garrido et al. 2003; Lewis et al. 2016). Fragmentation of the mitochondrial network via extensive fissions in the G2 phase of the cell cycle ensures homogenous partition of nucleoids across the mitochondria before cell division and makes certain that daughter cells receive equal numbers of mtDNAs during mitosis (Mishra and Chan 2014). Thus, the nucleoids in mitochondria of a cell are genetically uniform and can segregate as single units during mitochondrial fission (Ban-Ishihara et al. 2013; Garrido et al. 2003; Lewis et al. 2016).

More than 90% of the oxygen used for cell respiration is consumed by the ETC in mitochondria. Premature electron leakage from OXPHOS complexes I and III converts 1–2% of oxygen to reactive oxygen species (ROS) namely superoxide ($O_2^{-}\cdot$). The superoxide anions are converted to hydrogen peroxide (H_2O_2) by superoxide dismutases. Non-detoxified H_2O_2 can be converted to the hydroxyl radical (HO·) via the Fenton reaction and thereby damages all cellular components (Murphy 2009). Nucleoids are anchored to the inner membrane in close proximity to the

ETC, which is the main source of ROS within the cell. Although the mtDNA packaging into nucleotide provides certain protection, it is believed that ROS can preferentially target mtDNA over nuclear genetic material. In the 1970s, Harman proposed that the ROS-mediated damage is an important contributor to somatic mtDNA mutations that result in production of dysfunctional ETC components, which in turn generate an increased level of ROS, thus leading to a ‘vicious cycle’ responsible for aging (Harman 1972). This notion laid the foundation for the mitochondrial theory of aging which postulates that accumulation of ROS-induced mtDNA mutations leads to aging in humans and other animals (Miquel et al. 1980). Studies in the past have consistently shown more oxidative base damage in mtDNA than in nuclear DNA, in agreement with the hypothesis that endogenous DNA damage accumulates preferentially in mtDNA (Ames et al. 1995; Hudson et al. 1998). Moreover, environmental factors predominantly target mtDNA rather than nuclear DNA, suggesting that the mitochondrial genome is more susceptible to damage induced by exogenous agents (Yakes and Van Houten 1997). Although oxidative damage accumulates in cellular DNA with age (Bokov et al. 2004), the evidence that ROS-induced damage to mtDNA is a driving force behind aging is still lacking (Alexeyev 2009).

Early estimates of the mutation rate in mtDNA showed a 10-fold higher rate of evolution for mtDNA relative to the nuclear genome in somatic tissues of the different primates studied (Brown et al. 1982), indicating a highly increased mutation rate of mtDNA. More recent estimates revealed a 10- to 17-fold higher mutation rate of mtDNA in comparison with the nuclear genome (Tuppen et al. 2010). It has been suggested that the higher mutation rate of mtDNA is caused by ROS. Alternatively, other factors such as errors mediated by mitochondrial DNA replication machinery have been proposed as a primary source of mtDNA mutations (Zheng et al. 2006). MtDNA within a cell or individual can exist as a pure population of the wild type (homoplasmy) or a mixture of wild-type and mutant mtDNA, known as heteroplasmy. Because of continuous cycles of fusion and fission that enable mixing and homogenisation of the mitochondrial matrix, cells can maintain some level of heteroplasmy, in which functional and dysfunctional mtDNA variants coexist (Mishra and Chan 2014). For most of the known mtDNA mutations, no phenotypic alteration is ascribed unless mutant mtDNA molecules exceed 60% of total mtDNA (Gilkerson et al. 2008; Schon and Gilkerson 2010). Nevertheless, segregation of a heteroplasmic population of mtDNA in cells could result in an unequal partitioning of mtDNA variants either at cell division or by preferential replication of a specific mtDNA variant. Deep sequencing of mtDNA has uncovered the presence of low-abundance sequence variants in healthy individuals (Elliott et al. 2008; Payne et al. 2013); therefore, if the proportion of mtDNA containing a dysfunctional mutation segregates above the threshold level, then these heteroplasmic individuals might be at risk of a mitochondrial disease.

Recent advances in the understanding of the mechanisms of replication, repair and mutagenesis of the vertebrate mitochondrial genome revealed unexpected patterns that are incompatible with the hypothesis of oxidative-DNA-damage–driven mutagenesis. **In this review, we will discuss the basic mechanisms of mtDNA replication, repair, and mutagenesis and then propose a hypothetical model that addresses some unresolved issues**.

2. Replication of mtDNA

Mitochondria depend on the nuclear genome for mtDNA replication and maintenance because all the proteins required for replication, transcription and repair of the mitochondrial genome are encoded by nuclear DNA (Bogenhagen 2012). Nevertheless, the DNA replication machinery of mitochondria in mammalians is different from that used for nuclear DNA replication, and many mitochondrial replication factors are related to proteins identified in bacteriophages T3/T7 (Shutt and Gray 2006). DNA polymerase γ (PolG) is the high-fidelity replicative polymerase in mitochondria. Human PolG is a hetero-trimer consisting of one 140 kDa catalytic subunit (PolG1) and two p55 accessory subunits (PolG2) (Fan et al. 2006; Gray and Wong 1992; Yakubovskaya et al. 2006). The catalytic subunit has 5′→3′ DNA polymerase, 3′→5′ proofreading exonuclease and 5′-deoxyribose phosphate (dRP) lyase activities and contains the respective domains. The two accessory subunits are necessary for tight DNA binding to promote processive DNA synthesis (Young et al. 2011). TWINKLE is the mitochondrial DNA helicase, which is required for strand unwinding and separation

during mtDNA replication (Young and Copeland 2016). Mitochondrial topoisomerase I (mtTop1) catalyses transient cleavage and ligation of one strand of duplex DNA to relieve tension and DNA supercoiling during replication (Korhonen et al. 2003; Korhonen et al. 2008; Spelbrink et al. 2001). Transcription is tightly coupled with DNA replication in mitochondria because mitochondrial RNA polymerase (POLRMT) can act as a primase in mtDNA replication (Fuste et al. 2010; Wanrooij et al. 2008), in addition to PrimPol, a primase-polymerase member of the archaeo-eukaryotic primase superfamily (Rudd et al. 2014). Along with POLRMT, mitochondrial transcription factor A (TFAM) plays a central role in the production of truncated transcripts [from the L-strand promoter (LSP) in mtDNA] that are used to prime DNA synthesis during mtDNA replication (Shi et al. 2012). TFAM is a member of the high-mobility group (HMG) box domain family and performs an essential function in mtDNA packaging (Farge et al. 2014; Kukat et al. 2015). The single-stranded DNA (ssDNA)-binding protein (mtSSB) is an essential component of the mtDNA replisome. MtSSB binds to ssDNA and stimulates the activity of PolG (Farr et al. 1999; Korhonen et al. 2004). In addition to the above-mentioned proteins, mitochondrial genome maintenance exonuclease 1 (MGME1) (Kornblum et al. 2013), DNA ligase IIIα (Lakshmipathy and Campbell 1999; Puebla-Osorio et al. 2006), ribonuclease H1 (RNase H1) (Cerritelli et al. 2003; Holmes et al. 2015), DNA helicase/nuclease 2 (DNA2) (Copeland and Longley 2008), DNA flap-structure endonuclease 1 (FEN1) (Kalifa et al. 2009) and the mitochondrial isoform of topoisomerase IIIα (TopoIIIα) (Nicholls et al. 2018) are required for mtDNA maintenance and replication.

Three models of mtDNA replication have been proposed: the strand displacement model (SDM) (Robberson and Clayton 1972), the model of ribonucleotide incorporation throughout the lagging strand (RITOLS) (Yasukawa et al. 2006) and the strand-coupled model (Holt et al. 2000). In the widely accepted SDM model, mtDNA replication proceeds via asynchronous synthesis from sites O_H and O_L separated by a long distance (Gustafsson et al. 2016). In the beginning, replication is initiated at O_H by PolG, and a nascent H-strand is synthesised while the mtDNA is unwound by TWINKLE, and the displaced parental H-strand is coated with mtSSB, preventing POLRMT-initiated transcription. After the H-strand (leading strand) replication fork passes O_L, the latter adopts a stem-loop structure, which prevents mtSSB binding, and instead is recognised by POLRMT. The RNA polymerase initiates primer synthesis and then is replaced by PolG for synthesis of the L-strand (lagging strand) (Fuste et al. 2010). After that, the DNA synthesis continues on both the H- and L-strand until replication is terminated at O_H and O_L, respectively. RNase H1 is essential for replication termination through primer removal. The last two ribonucleotides of the RNA–DNA junction, left over after RNAse H1, are displaced by PolG into a flap-structure that is subsequently removed by DNA2, FEN1, and MGME1. The single-stranded break left after nucleases is sealed by DNA ligase IIIα. Of note, mtDNA replication termination results in the formation of a hemicatenane: two circular double-stranded (dsDNA) molecules interlocked at the O_H site via an ssDNA linkage. Recently, it has been demonstrated that the decatenation of mtDNA after replication termination is catalysed by TopoIIIα, which is essential for the segregation of nucleoids within the mitochondria (Nicholls et al. 2018). The mtDNA hemicatenane is unusual in the context of segregation of replicated circular dsDNA molecules because this process in general proceeds via catenanes, DNA rings mechanically interlocked via dsDNA linkage. At present, it is unclear how hemicatenane structures are generated during mtDNA replication, although the localisation of the ssDNA linkage in hemicatenanes to the O_H region suggests that they are formed at completion of mitochondrial-genome replication.

In addition to association with proteins, mtDNA contains specific regulatory DNA sequences and unusual structures, which participate in the regulation of DNA transcription and replication. For example, a substantial part of the mtDNA molecules bears a third strand of DNA, referred to as '7S DNA', which generates a displacement (D) loop covering much of the control region (CR) also known as the major non-coding region (NCR) (Nicholls and Minczuk 2014). The D-loop spans approximately 600 bp between O_H and TAS regions of mammalian mtDNA and is present in ~10% of the mtDNA pool in cultured human cells and in up to 90% of the mtDNA pool in *Xenopus* oocytes (Brown et al. 1978; Callen et al. 1983; Hallberg 1974). It is thought that the D-loop has many roles: it acts as a recruitment site for proteins involved in the organisation of mtDNA into nucleoid structures (He et al. 2007; Holt et al. 2007) and serves as a key component of the replication machinery (Antes et al. 2010; Clayton 1982). Recently, it was demonstrated that the D-loop has complex organisation: it contains an RNA strand on the strand opposite to the 7S DNA, thus forming an R-loop, which may play a part in

the organisation and segregation of mtDNA (Akman et al. 2016). Moreover, cellular turnover of 7S DNA is very rapid with a half-life of ~1 h in rodent cells (Gensler et al. 2001). Depletion of MGME1 causes large accumulation of 7S DNA, suggesting that this ssDNA nuclease participates in the D-loop turnover (Kornblum et al. 2013; Szczesny et al. 2013).

3. DNA damage and repair of mtDNA

3.1. The nature of DNA damage

DNA damage can be classified by its nature: spontaneous versus induced; by structure: complex versus singular, bulky versus nonbulky, base versus sugar damage, single versus clustered damage; and by biological consequences: innocuous versus toxic and/or mutagenic (Figure 1). It should be noted that DNA can undergo spontaneous decomposition because of its intrinsic chemical instability. Spontaneous hydrolysis of DNA in water under physiological conditions results in purine loss and cytosine deamination at significant rates leading to the appearance of abasic, i.e. apurinic/apyrimidinic (AP) sites and uracil residues, respectively (Lindahl and Andersson 1972; Lindahl and Nyberg 1974). Under physiological conditions, 1 per 100,000 purines is lost from DNA every 24 hours, resulting in abasic sites (Lindahl and Karlstrom 1973). Cytosine, adenine and guanine bases can undergo spontaneous loss of their exocyclic amino groups (deamination) giving rise to highly mutagenic uracil, hypoxanthine and xanthine residues, respectively, which lead to C*→T, A*→G and G*→A transitions, respectively (Hill-Perkins et al. 1986; Kamiya et al. 1992). Under typical cellular conditions, deamination of DNA-cytosine to uracil occurs in approximately one of every 10^7 cytidine residues in 24 hours, whereas guanine and adenine deamination occurs at 1/10 of this rate (Lindahl and Nyberg 1974). Moreover, deamination of C, A and G bases in ssDNA takes place 10-fold faster as compared to duplex DNA.

ROS generated during aerobic respiration in mitochondria is a major source of endogenous DNA damage. Studies on oxidative damage to mtDNA have revealed that oxidised bases occur more frequently and persist longer in mtDNA as compared to nuclear DNA damage (Richter et al. 1988; Yakes and Van Houten 1997). Approximately 80 types of base and sugar lesions induced by ROS have been identified (Bjelland and Seeberg 2003; Cadet et al. 2003) (Figure 1). ROS can damage nucleobases and sugar moieties in DNA either directly or indirectly. Hydroxyl radicals preferentially react with the C8 atom of purines in DNA to generate 8-oxo-7,8-dihydroguanine (8oxoG), 8-oxo-7,8-dihydroadenine (8oxoA) and formamidopyrimidines (Fapy) (Cadet et al. 2003; Dizdaroglu 2012) and with the C5=C6 double bond of pyrimidines to form glycols (Schuchmann et al. 1984; Téoule et al. 1977) (Figure 1). Abstraction of a hydrogen atom at the C1′ and C4′ positions of 2′-deoxyribose by ROS results in the formation of oxidised abasic sites: 2′-deoxyribonolactone and 2′-deoxypentos-4-ulose, respectively (Figure 1) (Dizdaroglu et al. 1977). The major endogenous oxidised bases 8oxoG, 5-hydroxyuracil (5ohU), and 5-hydroxycytosine (5ohC) are miscoding and, if not repaired, lead to mutation upon replication (Grollman and Moriya 1993; Kreutzer and Essigmann 1998; Kunkel and Bebenek 2000). C→T transitions and G→T transversions are the most common point mutations occurring in the tumour suppressor genes commonly mutated in human cancers (Pfeifer 2000). A C→T substitution could arise from mispairing of cytosine-derived lesions such as uracil, 5ohU and 5ohC with adenine (Kreutzer and Essigmann 1998). In contrast, the G→T transversion results from mismatched pairing of 8oxoG present in the template DNA strand with adenine in a newly synthesised strand (Grollman and Moriya 1993). It is noteworthy that the steady-state levels of 5ohU and 5ohC residues in the DNA of mammalian tissues and human cells are higher than those of 8oxoG (Wagner et al. 1992). Oxidation of adenine residues in DNA causes formation of 8oxoA and 2-hydroxyadenine (2-oxoA) (Kamiya et al. 1995). It should be noted that the formation of oxidatively induced adenine modifications including 8oxoA and FapyA is ~10-fold slower than that of related guanine degradation products after exposure of cellular DNA to either the hydroxyl radical or one-electron oxidants (Cadet et al. 2008; Cadet et al. 2010; Pang et al. 2014). Damage to the free nucleotide pool is also common and generates a similar spectrum of lesions (Cadet et al. 2003; von Sonntag 2006).

Indirectly, ROS can generate reactive aldehydes as products of membrane lipid peroxidation, which can react with DNA bases forming exocyclic etheno (ε) adducts 1,N^6-ethenoadenine (εA) and

3,N^4-ethenocytosine (εC) (Marnett and Burcham 1993) (Figure 1). Etheno adducts are ubiquitous and have been found in DNA isolated from tissues of untreated rodents and humans (Nair et al. 1995). Moreover, εA and εC levels are significantly increased by cancer risk factors related to oxidative stress and/or lipid peroxidation, such as dietary ω-6 fatty acid intake, chronic infections and inflammatory conditions (Bartsch and Nair 2000). The εA and εC residues in DNA are highly mutagenic; εC mostly produces C•G→A•T transversions and C•G→T•A transitions (Basu et al. 1993; Moriya et al. 1994), whereas εA residues are highly mutagenic in mammalian cells and yield T•A→A•T transversions (Levine et al. 2000; Pandya and Moriya 1996). Therefore, the processes that prevent mutagenic effects of ε-adducts when present in DNA should play an essential role in the maintenance of the stability of mitochondrial and nuclear genomes.

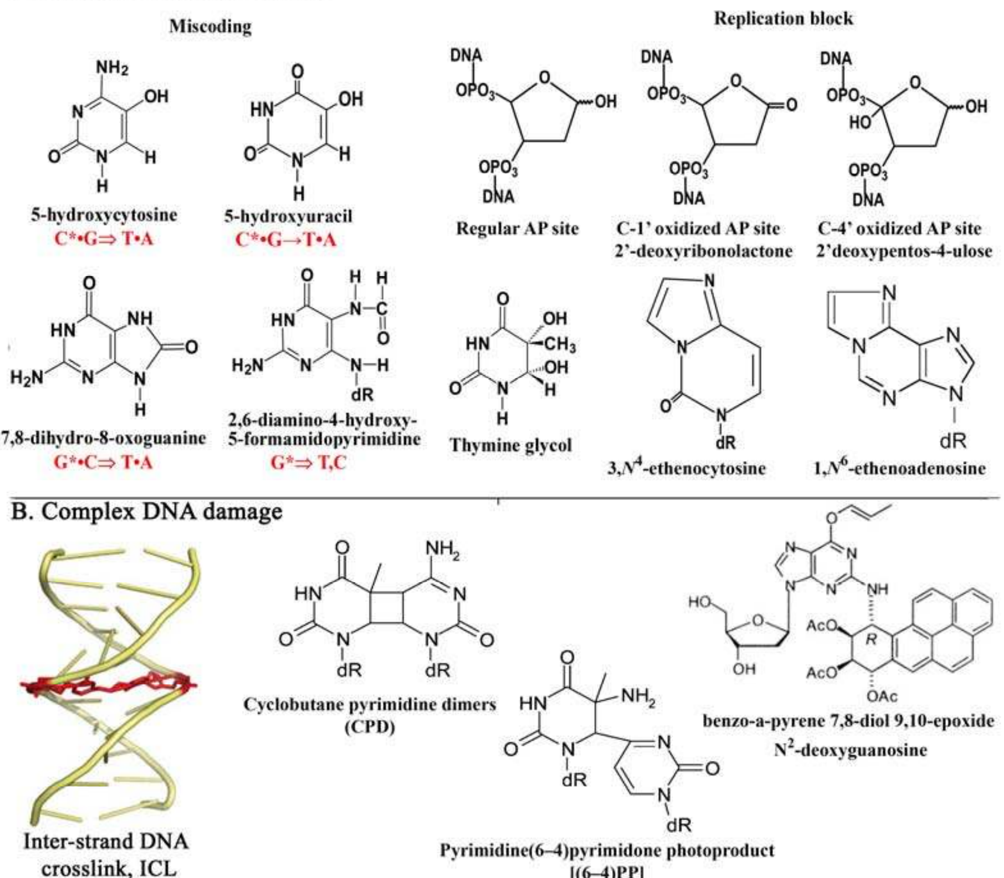

Figure 1. Schematic presentation of DNA damage induced by endogenous and exogenous factors. (**A**) Chemical structures of singular DNA base damage and sugar damage induced by ROS. (**B**) Chemical structures of complex DNA damage of bulky and clustered nature.

Furthermore, cells are exposed to environmental mutagens such as ionising radiation, UV light, alkylation and DNA-crosslinking agents. Ionising radiation creates DNA strand breaks, oxidised AP sites and bases and clustered lesions. UV radiation generates two most common DNA lesions: the cyclobutane pyrimidine dimer (CPD) and the pyrimidine (6–4) pyrimidone photoproduct [(6–4)photoproduct; 6–4PP]. Both photoproducts are cytotoxic (block DNA replication and transcription) and mutagenic, whereas CPDs are several-fold more frequent than 6–4PPs (Douki et al. 2000) (Figure 1). A hallmark of UV mutagenesis is the high frequency of C→T transitions at dipyrimidine sites in DNA, possibly owing to the extremely high deamination rate of cytosine residues within CPD sites in DNA (Peng and Shaw 1996). Mono-functional alkylating agents such as N-methyl-N′-nitro-N-nitrosoguanidine and methyl methanesulphonate react with DNA bases to generate 7-methylguanine, 3-methyladenine and O^6-methylguanine, which are the most abundant alkylation lesions (Pegg 1984; Singer 1976). The major adduct O^6-methylguanine mispairs with thymine during DNA replication, thus producing G•C→A•T transitions (Swann 1990). Bifunctional alkylating agents can generate a

covalent bond between nucleotides on opposite strands of a DNA duplex thereby forming an inter-strand DNA crosslink. Platinum compounds such as cis-diamminedichloroplatinum (II), also known as cisplatin, reacts with guanines and induces mainly DNA diadducts: 65% of d(GpG) intra-strand crosslinks, 25% of d(ApG) intra-strand crosslinks and 5–8% of inter-strand DNA crosslinks between the guanines in the sequence d(GpC). Inter-strand DNA crosslinks are highly lethal DNA lesions that block DNA replication, transcription and recombination by preventing strand separation (Figure 1).

3.2. DNA repair systems

For a more comprehensive review on the DNA repair pathways involved in the maintenance of mitochondrial genome, we suggest the reader several excellent reviews on this topic (Alexeyev et al. 2013; Kauppila and Stewart 2015; Kazak et al. 2012; Prakash and Doublie 2015; Stein and Sia 2017; Van Houten et al. 2016). DNA repair is essential for cell survival and tissue homeostasis. Cellular organisms must constantly contend with endogenous and exogenous DNA damage and for this reason, they have evolved multiple overlapping DNA repair systems to counteract the genotoxic effects of these insults. Modified base lesions and base mismatches are specifically recognised among the vast majority of regular matched bases by DNA glycosylases and AP endonucleases in the base excision repair (BER) and nucleotide incision repair (NIR) pathways, respectively (Couve-Privat et al. 2007; Krokan and Bjoras 2013) (Figure 2). In the BER pathway, a DNA glycosylase hydrolyses the *N*-glycosylic bond between the damaged base and sugar, leaving either an AP site or a single-stranded DNA break (Figure 2, steps 1 and 3). DNA glycosylases are classified as mono- and bi-functional based on their mechanism of action. Mono-functional DNA glycosylases cleave the *N*-glycosidic bond, release the modified base, and generate an AP site (Cunningham 1997). The resulting AP sites are then incised on the 5′ side by a major human AP endonuclease, APEX1 (also known as APE1, HAP-1, and REF-1), which generates a single strand break with 3′-OH and a blocking dRP group (Figure 2, step 4). Several studies have shown that the full-length form of the APE1 protein is present in the mitochondria of mammalian cells (Li et al. 2010; Tell et al. 2001; Vascotto et al. 2011). In fact, APE1 contains the mitochondrial targeting signal in the C-terminal part between residues 289 and 318 (Li et al. 2010). Bi-functional DNA glycosylases not only cleave the *N*-glycosidic bond but also have an AP lyase activity that eliminates the 3′ phosphate (β-elimination) or 3′ and 5′ phosphates (β,δ-elimination) of the resultant AP site in a concerted manner. β-Elimination produces a nick flanked by a 3′-terminal α,β-unsaturated aldehyde and a 5′-terminal phosphate, whereas β,δ-elimination yields a single-nucleoside gap flanked by two phosphates (Cunningham 1997; Dodson et al. 1994). The 3′-terminal phosphoaldehyde and 3′-phosphate are then removed by APE1 (Chattopadhyay et al. 2006) (Figure 2, step 6) and PNKP (Tahbaz et al. 2012), respectively, allowing a DNA polymerase to fill the gap before DNA ligase IIIα seals the resulting DNA nick (Demple and Harrison 1994). Two mono-functional DNA glycosylases have been identified in mitochondria: uracil-DNA glycosylase 1 (UNG1), which excises uracil residues derived from cytosine deamination (Anderson and Friedberg 1980; Nilsen et al. 1997) and mismatch-specific adenine-DNA glycosylase (MUTYH), a homolog of the *Escherichia coli* MutY DNA glycosylase, which excises adenine incorporated opposite to 8oxoG in duplex DNA (Ohtsubo et al. 2000; Takao et al. 1999). In addition, two bi-functional DNA glycosylases in mitochondria have been characterised: 8oxoG-DNA glycosylase 1 (OGG1), which excises 8oxoG residues opposite cytosine and FAPY residues in duplex DNA (de Souza-Pinto et al. 2001; Takao et al. 1998), and pyrimidine-specific DNA glycosylase NTHL1, a homolog of *E. coli* endonuclease III, which excises oxidative pyrimidine lesions (Karahalil et al. 2003). Regarding the biological role of DNA glycosylases, animal DNA glycosylase knockout models *Ung1*$^{-/-}$, *Ogg1*$^{-/-}$, *Mutyh*$^{-/-}$ and *Nthl1*$^{-/-}$, including double mutant *Ogg1*$^{-/-}$*Mutyh*$^{-/-}$ mice and *Nthl1*$^{-/-}$*Neil1*$^{-/-}$ mice manifest an increased spontaneous mutation rate in nuclear DNA and spontaneous carcinogenesis (Andersen et al. 2005; Chan et al. 2009; Nakabeppu et al. 2006; Xie et al. 2004). Paradoxically, the studies on the *Ogg1*$^{-/-}$ knockout (Itsara et al. 2014) and on the *Ogg1*$^{-/-}$*Mutyh*$^{-/-}$ double knockout (Halsne et al. 2012) failed to detect an increase in the spontaneous mutation rate in the mitochondrial genome. The intriguing question then arises: Why does the cell keep a specific repair system for mtDNA if it does not defend against induced or endogenous DNA damage? (Pawar et al. 2018) Nevertheless, it has been

demonstrated that expression of the OGG1 protein protects cells from ROS (Dobson et al. 2002; Lia et al. 2018; Wang et al. 2011a). In addition, *Ogg1*$^{-/-}$ mice and *Neil1*$^{-/-}$ mice are prone to obesity and insulin resistance, possibly due to the specific involvement of these proteins in the metabolic regulation via the maintenance of mtDNA (Sampath et al. 2011; Sampath et al. 2012).

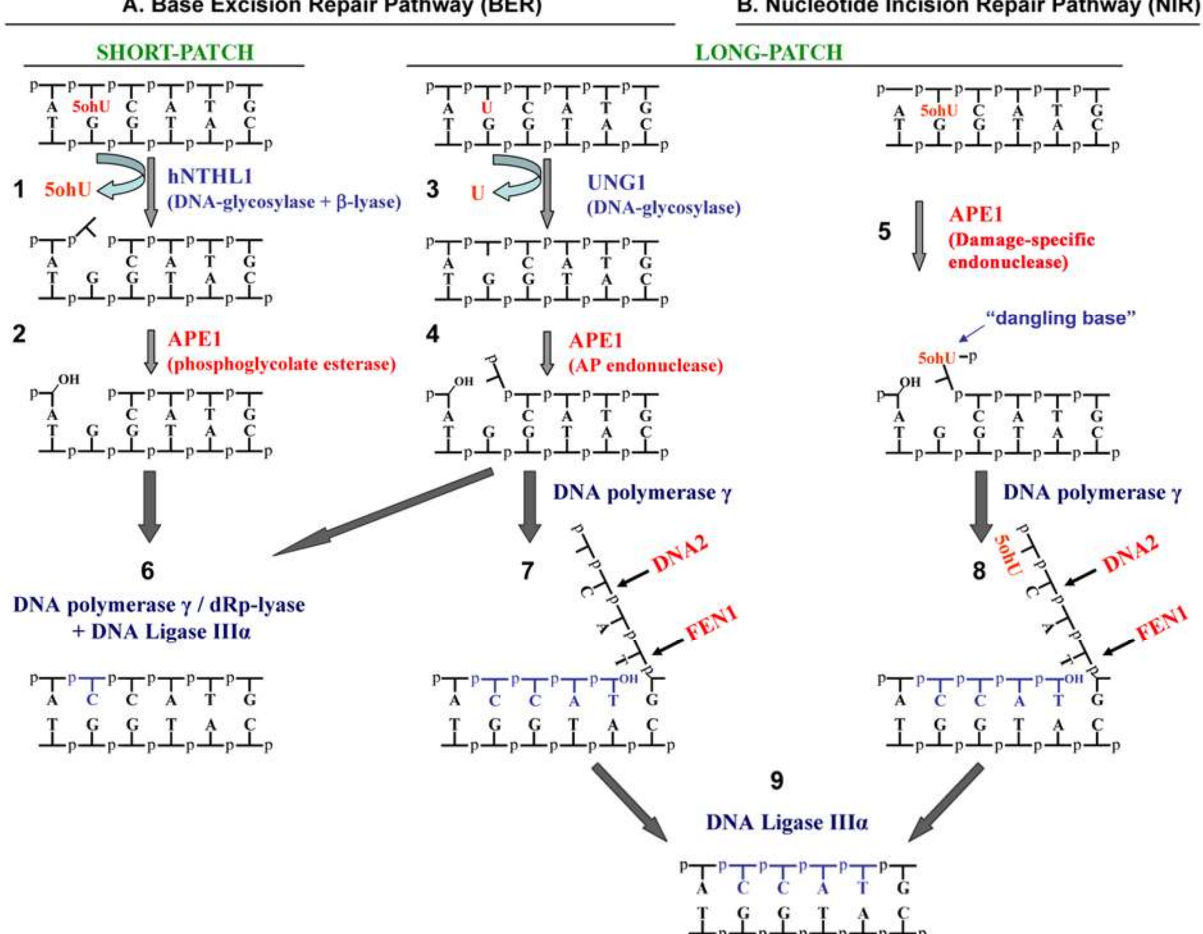

Figure 2. BER and NIR: two alternative DNA repair pathways for oxidative damage to mtDNA. (1–5) Upstream and (6–9) downstream steps of the BER and NIR pathways. In BER, (1) human bi-functional DNA glycosylase/AP lyase, hNTHL1, excises the 5-hydroxyuracil (5ohU) residue in DNA, thus forming a free 5ohU base and single-strand break in the form of one-nucleotide gap containing a 3′-α,β-unsaturated aldehyde and a 5′-phosphate; (2) the 3′ repair diesterase activity of human APE1 removes the 3′-blocking group to generate a 3′-OH terminus; (2) human mitochondrial mono-functional uracil-DNA glycosylase 1, UNG1, excises the uracil (U) residue in DNA, thus forming a free U base and abasic site (AP site); (4) APE1 cleaves duplex DNA on the 5′ side of the AP site and yields a single-strand break with a 3′ hydroxyl adjacent to a dRP. In NIR, (5) APE1 directly cleaves 5′ to the 5ohU base, thus generating a single-strand nick containing a 3′-OH and a 5′-phosphate with a dangling base. (6) In the SP-BER pathway, DNA polymerase γ inserts a single nucleotide and removes the dRP blocking residue, then DNA ligase IIIα seals the single-strand nick. (7 and 8) In the LP-BER and NIR pathways, DNA polymerase γ initiates strand displacement repair synthesis, coupled to DNA2- and FEN1-catalysed cleavage of unannealed 5′-flap structures containing a dRP residue in BER and a 5′-dangling base in the NIR pathway, respectively; (9) DNA ligase IIIα seals the single-strand nicks and restores genetic integrity of mtDNA.

In the downstream steps after sequential action of DNA glycosylases and AP endonucleases, two subcategories of the BER pathway are recognised: short- and long-patch BER (SP-BER and LP-BER, respectively; Figure 2). In SP-BER, only one nucleotide is removed upon damaged-base

excision, whereas in LP-BER, more than one nucleotide is replaced with a repair patch of 2–11 nucleotides. In the mitochondria, DNA polymerase γ (PolG) removes the blocking dRP residue by its AP lyase activity and then inserts a single nucleotide (Longley et al. 1998) (Figure 2, step 6). Nonetheless, the dRP lyase activity of PolG is weaker than that of nuclear DNA polymerase β (Polβ), which may play a back-up part in the mitochondrial SP-BER subpathway (Sykora et al. 2017). After filling of one nucleotide gap, the remaining nick is sealed by DNA ligase IIIα (LigIIIα) (Lakshmipathy and Campbell 2000). It should be noted that the oxidised AP sites and cleavage products of the NIR pathway cannot be removed by the dRP-lyase function of PolG and Polβ; therefore, these lesions are removed in LP-BER (Figure 2, steps 7 and 8). It is worth noting that among the types of oxidative DNA damage generated after oxidative stress, oxidised AP sites might represent the majority of sugar damage (Roginskaya et al. 2005), suggesting that SP-BER may be cytotoxic (Demple and DeMott 2002). During LP-BER, PolG initiates strand displacement synthesis and generates a 6- to 9-nucleotide ssDNA flap (Szczesny et al. 2008) containing the 5′-terminal blocking groups such as an oxidised dRP residue or nucleobase, which is removed by the concerted action of two nucleases, FEN1 and DNA2 (Duxin et al. 2009; Kalifa et al. 2009; Zheng et al. 2008) (Figure 2, steps 7 and 8). The resulting nick is sealed by LigIIIα (Figure 2, step 9). Of note, the LP-BER subpathway resembles Okazaki fragment maturation and involves replication-associated nucleases. Considering the pattern of occurrence of oxidised AP sites and the strong strand displacement activity of PolG, the LP-BER subpathway may be the predominant mode of repairing oxidative DNA base damage in mitochondria.

The DNA glycosylase–initiated BER pathway raises problems because it generates genotoxic abasic and 3′-blocking group intermediates. The findings that AP endonucleases can directly cleave DNA 5′ to various oxidatively damaged nucleotides, thereby generating 3′-OH and 5′-phosphate termini, together with genetic data on cellular resistance to oxidative stress are suggestive of the existence of an alternative to classic BER, referred to as the NIR pathway, which bypasses the abasic intermediates (Ischenko and Saparbaev 2002) (Figure 2, step 5). AP endonucleases have multiple repair activities and participate in both BER and NIR pathways. AP site cleavage (or AP endonuclease) and 3′-repair phosphodiesterase activities can be regarded as BER functions, whereas the nucleotide incision activity as the NIR function of the AP endonucleases (Gros et al. 2004). At low concentrations of Mg^{2+} (≤1 mM), APE1 switches its substrate specificity and recognises diverse types of DNA base lesions including α-anomeric nucleotides, oxidised pyrimidines such as 5,6-dihydrouracil, 5ohU and 5ohC (Daviet et al. 2007; Gros et al. 2004) (Figure 2, step 5), formamidopyrimidines (Christov et al. 2010), exocyclic DNA bases, thymine glycol, uracil (Prorok et al. 2013; Prorok et al. 2012) and bulky lesions such as benzene-derived DNA adducts (Guliaev et al. 2004). Of note, research into subcellular localisation of APE1 uncovered various patterns from mainly cytoplasmic to mixed cytoplasmic/nuclear and mainly nuclear localisation (Kakolyris et al. 1998). Moreover, in the cell types with high metabolic or proliferative rates, APE1 is predominantly localised in mitochondria and the endoplasmic reticulum (Tell et al. 2001; Tell et al. 2005; Tomkinson et al. 1988). Furthermore, when cells are exposed to oxidative-stress conditions, the level of APE1 in mitochondria significantly increases in a dose- and time-dependent manner (Frossi et al. 2002; Mitra et al. 2007), implying that APE1 participates in the maintenance of mtDNA under conditions of high energy metabolism and oxidative stress. In agreement with this notion, it has been demonstrated that down-regulation of APE1 in mouse embryonic fibroblasts causes reversible suppression of mitochondrial respiration and OXPHOS activity (Suganya et al. 2015). On the basis of these observations, we can speculate that in mitochondria, APE1 initiates the DNA glycosylase–independent NIR pathway and shifts the removal of oxidised bases and AP sites into long-patch repair synthesis similar to that in the LP-BER subpathway (Figure 2, steps 7–9).

Bulky DNA adducts and DNA–DNA and DNA–protein crosslinks are substrates for nucleotide excision repair (NER). In the NER pathway, a multiprotein complex recognises and excises bulky DNA adducts in the form of short oligonucleotides that contain the lesion (Marteijn et al. 2014). NER is a major repair system that removes DNA damage induced by UV, by anticancer agents such as cisplatin and by many environmental carcinogens. In eukaryotic cells, NER involves dual incisions that bracket the lesion site and release a 24- to 32-nucleotide-long oligomer containing the damaged residues. At present, most of *in vitro* and *in vivo* evidence indicate that CPD and 6-4 PPthymidine dimers (Clayton et al. 1975), cisplatin intra-strand crosslinks, complex alkylation damage, and other

forms of damage (LeDoux et al. 1992; Pascucci et al. 1997) are not repaired in mtDNA, implying the absence of NER in mitochondria.

Base mispairs and short deletion-insertion loops are generated during DNA replication and homologous recombination. Mismatch repair (MMR) is an evolutionarily conserved system that recognises and repairs mismatches in a strand-specific manner. MMR machinery is coupled to DNA replication and can distinguish a newly synthesised strand from the parental strand. In human cells, two major heterodimers – Msh2/Msh6 (MutSα) and Msh2/Msh3 (MutSβ) – recognise DNA mismatches and trigger their removal by recruiting MutLα (MLH1/PMS2) and MutLβ (MLH1/PMS1) complexes (Jiricny 2006). To date, the main proteins involved in nuclear MMR have not been found in mitochondria. Nevertheless, repair factor YB-1, which has a binding activity towards mismatched DNA, has been identified in mitochondria, and its knockdown decreases the MMR activity in these organelles (de Souza-Pinto et al. 2009).

3.3. Alternative pathways of mtDNA maintenance

For the reason that thousands of copies of mtDNA are present in a cell, a significant loss of damaged DNA can be compensated by replication of remaining non-damaged molecules. Several studies have revealed that ROS induce degradation of mtDNA and mtRNA (Abramova et al. 2000; Furda et al. 2012; Rothfuss et al. 2009; Shokolenko et al. 2009). It is noteworthy that small numbers of 8oxoG residues were detected in circular mtDNA, whereas linear fragmented mtDNA contains many 8oxoG residue, and their number further increases after oxidative stress (Suter and Richter 1999). Therefore, the absence of significant mtDNA mutagenesis under oxidative stress conditions suggests that in mitochondria, most of damaged DNA molecules are degraded perhaps due to lesion-dependent transcription and replication blockage (Shokolenko et al. 2009). It is worth mentioning that it was demonstrated that mtDNA, but not nuclear DNA, is resistant to mutagenesis induced by powerful carcinogens, such as N-methyl-N′-nitro-N-nitrosoguanidine and ethylmethane sulphonate, most likely because of extensive degradation of alkylated mtDNA (Marcelino et al. 1998; Mita et al. 1988). According to these observations, it has been proposed that lesion-dependent DNA degradation can be considered a specific mitochondrial repair pathway (Alexeyev et al. 2013). Indeed, the ectopic expression of certain isoforms of OGG1 protects cells from increased oxidative stress (Dobson et al. 2002; Lia et al. 2018), maintains normal neuronal biogenesis (Wang et al. 2010) and promotes mitochondrial biogenesis during cell differentiation (Wang et al. 2011b). Thus, we may hypothesise that the DNA glycosylase–initiated BER pathway helps to destroy damaged mtDNA molecules rather than repair them. This mechanism may explain the absence of somatic enrichment for transversion mutations in mtDNA and the role of DNA glycosylases in metabolic regulation in mice (Sampath et al. 2011; Sampath et al. 2012).

Finally, it should be noted that the tight packaging of mtDNA into the nucleoid structure might offer efficient protection from exogenous DNA-damaging agents (Alan et al. 2016). The degree of compaction of mtDNA with TFAM – from fully compacted nucleoids to naked DNA – regulates transcription, replication and possibly DNA repair and degradation via regulated access to DNA (Farge et al. 2014).

4. Mutagenesis of mtDNA

In animal models and humans, point mutations and large deletions in mtDNA increase in frequency with age and have been implicated in the aetiology of age-related diseases (Greaves et al. 2014; Hahn and Zuryn 2019; Li et al. 2015; Sun et al. 2016). It is generally accepted that in the cell, mutations accumulate faster in mtDNA than in nuclear DNA. The high replicational turnover of mtDNA is likely the main contributor to the increased spontaneous mutation rate because of the inevitable introduction of DNA polymerase errors during the synthesis (Kennedy et al. 2013; Radzvilavicius et al. 2016). It must be emphasised that the types of mtDNA mutations are cell type and age specific. Large deletions are more likely to accumulate in non-dividing cells (muscle fibres and neurons) but not in actively proliferating cells, such as colon mucosal cells and cancer cells

(Khrapko and Turnbull 2014). Of note, pigmented neurons of the substantia nigra in the brain of old individuals contain a very high proportion of mtDNA deletions, but mtDNA from other types of neurons in the same subject lacks deletions (Kraytsberg et al. 2006). Recent breakthroughs in the DNA sequencing technology enabled detection of rare or sub-clonal mutations on a genome-wide level and characterisation of mutation spectra in mtDNA during aging and cancer (Ju et al. 2014; Kennedy et al. 2013; Williams et al. 2013). A study on the somatic mutations in mtDNA isolated from the pre-frontal cortex of a human brain from young and old individuals uncovered a 5-fold increase in the frequency of point mutations in an 80-year-old group (Kennedy et al. 2013). The majority of point mutations in the samples from young and old participants were transitions, whereas transversions – a mutational signature of oxidative DNA damage – were a minor mutation. Moreover, mutations accumulated asymmetrically in the H- and L-strands of mtDNA. In young human brain samples, C→T mutations were more likely to occur in the H-strand (Kennedy et al. 2013). In the brains of aged individuals, this pattern became more prevalent and was accompanied by $T_L \rightarrow C_L$ mutations in the L-strand, mirroring the nucleotide composition bias of the L-strand for A (~ 31%) over T (~ 25%). Quite unexpectedly, this strand bias was reversed in the mtDNA control region, which includes the H-strand origin of replication (O_H), promoters for transcription, and the D-loop. This asymmetrical pattern of mtDNA mutation in the human brain was confirmed in a subsequent DNA sequencing study (Williams et al. 2013). Furthermore, a previous study on mtDNA mutations in the human lung epithelium also indicates that G and T sites in the L-strand undergo transition mutations more frequently than C and A sites do (Zheng et al. 2006).

It was proposed that somatic mutations in mtDNA may contribute to tumour progression by fulfilling increased energy demand due to the uncontrolled cell proliferation associated with cancer (Wallace 2012). Recently, to address the participation of mitochondrial mutations in cancer, Ju and colleagues examined mtDNA sequences from 1675 cancer biopsies across 31 tumour types and compared the data to the corresponding normal tissue from the same patients (Ju et al. 2014). In total, 1907 somatic point mutations were identified, which manifested a replicative strand bias similar to the one described above, with the prevalence of $C_H \rightarrow T_H$ and $A_H \rightarrow G_H$ transitions in the H-strand and $T_L \rightarrow C_L$ and $G_L \rightarrow A_L$ substitutions in the L-strand. Moreover, this mitochondrial pattern of somatic mutations differs from that identified in the nuclear genome and most likely does not provide a selective advantage to cancer cells. ROS, cigarette smoke and UV light have little or no effect on mtDNA mutations in cancer, suggesting that an endogenous mutational mechanism linked to mtDNA replication is the major cause of mutagenesis in mitochondria (Ju et al. 2014). Elaborating on their model, Ju and colleagues proposed that during asynchronous replication of mtDNA, the displaced single-stranded H-strand is prone to cytosine and adenine deamination thus generating $C_H \rightarrow T_H / A_H \rightarrow G_H$ substitutions. In contrast, on the L-strand, template PolG predominantly generates T_L•G and G_L•T mismatches, which result in $T_L \rightarrow C_L / G_L \rightarrow A_L$ transitions (Ju et al. 2014). Remarkably, this strand bias is reversed in the D-loop region of mtDNA (Ju et al. 2014) and a similar reversion in the mutation pattern occurs in mtDNA of the aging brain as described above (Kennedy et al. 2013). This observation points to an important feature of mtDNA replication, because inversion of the control region in mtDNA of certain species of fish leads to a reversal of the strand bias in the mutation pattern (Fonseca et al. 2014; Fonseca et al. 2008). Moreover, the change in strand-specific compositional bias in the mitogenomes containing the inverted control region is in agreement with the asynchronous mode of mtDNA replication (SDM and RITOLS) but not with the strand-coupled model (SDM).

5. A hypothesis about the origin of mtDNA mutations

Here we propose a model of asymmetric DNA strand inheritance, which provides a simple explanation of the observed highly biased pattern of mutations in vertebrate mtDNA (Figure 3). Our model is consistent with the strand-asynchronous models of mtDNA replication: SDM and RITOLS (Falkenberg 2018; Holt and Reyes 2012; Pohjoismaki et al. 2018) but contains several new important features, which apparently have not been discussed before. In this model, we suggest that the single-stranded H-strand, anchored to the inner membrane through specific interactions with the control region or probably the D-loop structure, replicates in an uninterrupted manner to make new DNA progeny (containing the parental H-strand and a nascent L-strand; Figure 3, step 6). In contrast, the L-

strand will undergo only one cycle of replication, which will build a new mtDNA molecule (containing the parental L-strand and a nascent H-strand) not attached to the inner membrane (Figure 3, step 5).

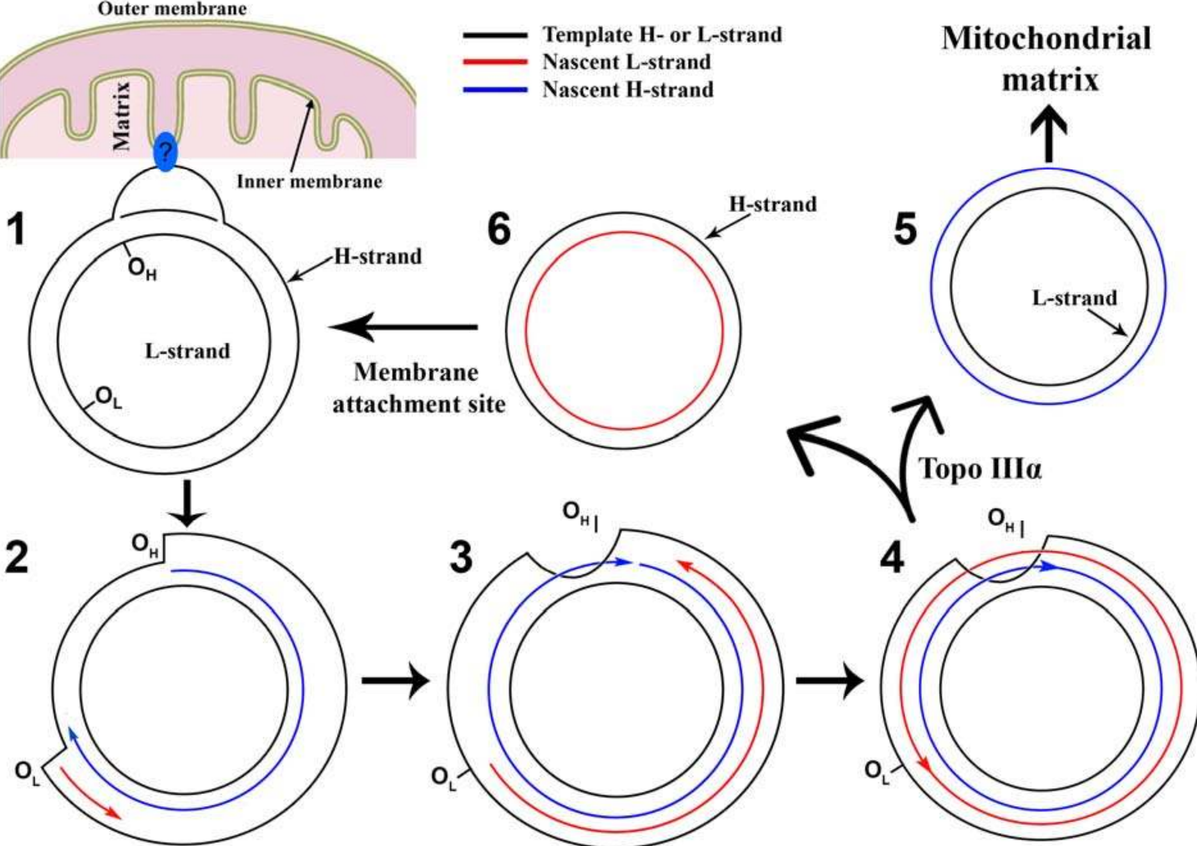

Figure 3. The model of mtDNA replication leading to asymmetrical DNA strand inheritance. (**1**) MtDNA is anchored to the inner membrane of a mitochondrion via interaction of the D-loop region with a specific site in the membrane. (**2**) Asynchronous replication of a leading H-strand (blue arrow) at the O_H site proceeds unidirectionally to displace the parental H-strand. When the O_L site is exposed, lagging L-strand (red arrow) replication starts in the opposite direction. (**3**) Replication of the leading H-strand proceeds further and terminates at O_H before the lagging L-strand replication reaches this site. Premature termination of H-strand synthesis produces a hemicatenane: two circular DNA molecules mechanically interlocked via a single-stranded linkage (Laurie et al. 1998). (**4**) Replication of a lagging L-strand terminates at the O_L site to complete the formation of a double-stranded hemicatenane composed of two interlocked mtDNA molecules. The hemicatenane is unlinked by mitochondrial topoisomerase IIIα to produce two separate mtDNA molecules: one containing the parental H-strand, and the other the parental L-strand. (**5**) The mtDNA molecule containing the old parental L-strand diffuses freely throughout the mitochondrial matrix and may get anchored to the inner membrane through the expression of OXPHOS complex proteins (Lynch and Wang 1993). (**6**) The mtDNA molecule containing the old parental H-strand stays attached to a specific site in the inner membrane and continues to replicate.

Thus, the L-strand–derived progeny will not proceed to the next DNA replication cycle and will probably diffuse in the mitochondrial matrix as unattached nucleoids. In our model of highly asymmetric DNA strand replication, the H-strand produces the majority of mtDNA offspring, and the L-strand yields only a minor proportion of the mtDNA progeny pool. Nevertheless, we hypothesise that the pool of free and membrane-non-bound mtDNA progeny generated by L-strand replication may compete for a specific membrane attachment site required for mtDNA replication. In the SDM and RITOLS models of mtDNA replication, the synthesis of a nascent L-strand on the H-strand template from O_L is delayed as compared with the synthesis of a nascent H-strand on the L-strand template from O_H (Figure 3, steps 2 and 3). Here, we suggest that replication of the H-strand is terminated with

DNA nick sealing before the completion of L-strand replication, and this event should generate hemicatenanes (two intertwined circular DNAs associated through a single-strand linkage; Figure 3, step 4), rather than catenanes (two intertwined circular DNAs associated through a double-strand linkage). In agreement with our hypothetical model, recently, it was demonstrated that mtDNA replication termination involves a hemicatenane formed at the O_H site and that TopoIIIα is essential for resolving this structure (Nicholls et al. 2018). It should be noted that in the past, Laurie and colleagues proposed a similar mechanism of the formation of hemicatenanes through alternative replication termination of circular DNA molecules (Laurie et al. 1998). In their model, the authors proposed that when two replication forks converge on a circular DNA, one of the advancing DNA polymerases is displaced from the template by the accumulated stress, so that only one fork advances. This situation induces branch migration in which both the leading and lagging ends of DNA strands of the abandoned fork are progressively displaced. When the displaced DNA strand ends anneal back to complementary single-stranded regions of the advancing fork, the replication of both strands can be finished, and this event results in hemicatenation (Laurie et al. 1998). Furthermore, formation of plasmid DNA hemicatenanes in *Xenopus* egg extracts can be triggered by the DNA polymerase inhibitor aphidicolin, which most likely promotes the asynchronous mode of DNA replication (Lucas and Hyrien 2000).

Our model also provides a simple explanation for the unusual strand bias in the somatic mutations observed in the mitochondrial genome of the aging brain and in cancer (Ju et al. 2014; Kennedy et al. 2013). According to our model, the prevalence of C→T/A→G transitions in the H-strand, and these types of substitutions ($C_L \rightarrow T_L/A_L \rightarrow G_L$) in the L-strand are rare or absent because the majority of mtDNA progeny in vertebrates are derived from the H-strand and because the propagation of mutations occurring in the L-strand is very limited. We will refer to this phenomenon as asymmetric DNA strand inheritance. We suggest that spontaneous cytosine deamination occurs on both mtDNA strands at similar rates, and after insertion of A opposite U by DNA polymerase, these conditions should induce C→T transitions in both the H- and L-strand. Nevertheless, the mutation that occurred in the H-strand, not that in the L-strand, will spread in the progeny owing to asymmetric DNA strand inheritance. This is why we observe the prevalence of C→T transitions in the H-strand and their mirror counterparts G→A transitions in the L-strand. Furthermore, it has been suggested that A→G transitions in mtDNA are due to the increased rate of deamination of adenines in the H-strand when it is exposed in the single-stranded form (Ju et al. 2014). On the other hand, spontaneous deamination of both A and G occurs at a 50-fold slower rate than that of C (Lindahl 1979), whereas *in vivo*, $A_H \rightarrow G_H$ transitions occur at the rates comparable to that of $C_H \rightarrow T_H$ (Ju et al. 2014; Kennedy et al. 2013). Besides, deamination of G forms a xanthine residue, which can mispair with T; however, $G_H \rightarrow A_H$ transitions occur at a much lower frequency than $A_H \rightarrow G_H$ transitions do. It should be stressed that in addition to cytosine deamination, the most frequent spontaneous lesion in DNA is an abasic site (AP) that results from depurination. The rate of spontaneous formation of AP sites is at least 10-fold greater than that of deamination of cytosines (Lindahl 1993). It is tempting to speculate that the majority of $A_H \rightarrow G_H$ transitions in mtDNA are caused by spontaneous formation of AP sites. It is noteworthy that yeast and human REV1, a translesion synthesis DNA polymerase, preferentially inserts C opposite AP sites (Choi et al. 2010; Haracska et al. 2002) and that in budding yeast, dCMP is inserted ('C-rule') opposite the AP-site in the single-stranded gap of a duplex plasmid (Gibbs and Lawrence 1995). According to the C-rule, the loss of adenines in mtDNA should give rise to A→G transitions, whereas the loss of guanines should be non-mutagenic (Zhang et al. 2006). Nonetheless, a recent study suggests that enzymatically created AP sites in mtDNA of mouse cells are weakly mutagenic, and that repair and DNA degradation take place more often than translesion synthesis of AP sites (Kozhukhar et al. 2016). More studies are necessary to identify the possible origin of $A_H \rightarrow G_H$ transitions in mtDNA.

The completion of a mitochondrial replication cycle takes approximately 1 hour (Berk and Clayton 1974), indicating that the speed of DNA synthesis is much slower than that of eukaryotic nuclear and bacterial genomes (Clayton 1982). The time-consuming and asynchronous mode of mtDNA replication may ensure high-fidelity copying of DNA, whereas the synchronous strand-coupled mechanism (SDM) is faster and more error-prone but enables quick restoration of the copy number after DNA damage (Torregrosa-Munumer et al. 2015; Yasukawa et al. 2005). In the RITOLS model, replication of a nascent H-strand starts early and generates partially replicated mtDNA in

which a displaced parental H-strand is covered by RNA. It should be noted that the H-strand is the template from which most mitochondrial proteins (12 out of 13) are transcribed, whereas only one protein-coding gene, *ND6*, is transcribed from the L-strand. One could say that the H-strand is preferentially anchored to the inner membrane via expression of the membrane-bound mitochondrial OXPHOS proteins (Lynch and Wang 1993) (Figure 3, step 1). In addition, high transcriptional activity may inhibit mitochondrial replication because mtDNA deletion mutants acquire a selective advantage in aging tissues (Kowald and Kirkwood 2014). Furthermore, we can speculate that active transcription of the H-strand serves to screen this portion of mtDNA for damage. If DNA transcription on the H-strand is blocked by a lesion, then this event may inhibit replication initiation from the O_H site via the interference with the D-loop structure. This mechanism of transcriptional DNA scanning may stop the replication machinery from copying damaged DNA templates and thus prevent mutation fixation.

In summary, our model of asymmetric DNA strand replication of vertebrate mtDNA explains the formation of hemicatenanes and the unusual mutation pattern. Besides, the proposed model may provide some insights into a putative evolutionary origin of cellular organelles. SsDNA bacteriophages such as M13 and φX174 share similarities with vertebrate mtDNA in the asymmetric mode of DNA replication and strand nucleotide compositional bias, suggesting that asymmetric strand replication may be a cause of the bias in strand nucleotide composition. Furthermore, the key proteins of mitochondrial transcription and replication machineries are derived from bacteriophages such as the T3/T7 lineage of coliphages (Filee and Forterre 2005; Filee et al. 2002), some cyanophages (Chan et al. 2011) and others phages rather than from an α-Proteobacterium as thought before (Shutt and Gray 2006). On the basis of these observations, we believe that mitochondria and some bacteriophages are remnants of an extinct ancient free-living unicellular organism that had a different type of genetic code. Mutational pressure and natural selection during a billion years of evolution led to the emergence of a single universal genetic code in almost all free-living organisms and to the extinction of all the species of unicellular organisms having alternative codes.

6. Conclusions

In this chapter, we reviewed the roles of DNA damage, DNA repair, and DNA replication in the maintenance of the mitochondrial genome in vertebrates. To summarise our review, we would like to highlight several key points:

- Replication of mtDNA proceeds through asymmetrical replication of the H-strand, thereby resulting in predominant biased inheritance of one strand in mtDNA.
- Asymmetrical DNA strand replication generates asymmetry in the somatic mtDNA mutational signature, which in turn may be an evolutionary cause of the strand nucleotide compositional bias of the animal mitochondrial genome.
- The formation of hemicatenanes during mtDNA replication points to premature termination of nascent–H-strand replication.
- Studies on DNA damage, repair and mutagenesis in mitochondria suggest that the spontaneous decay of mtDNA – rather than DNA polymerase errors – is a major source of endogenous mutations;
- The non-universal genetic code of animal mitochondria and the similarity of mtDNA transcription and replication machineries with those of bacteriophages are suggestive of an extinct ancient cellular organism with an alternative DNA coding system as a putative ancestor of mitochondria.

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Figure legends

Figure 1. Schematic presentation of DNA damage induced by endogenous and exogenous factors. (**A**) Chemical structures of singular DNA base damage and sugar damage induced by ROS. (**B**) Chemical structures of complex DNA damage of bulky and clustered nature.

Figure 2. BER and NIR: two alternative DNA repair pathways for oxidative damage to mtDNA. (1–5) Upstream and (6–9) downstream steps of the BER and NIR pathways. In BER, (1) human bi-functional DNA glycosylase/AP lyase, hNTHL1, excises the 5-hydroxyuracil (5ohU) residue in DNA, thus forming a free 5ohU base and single-strand break in the form of one-nucleotide gap containing a 3′-α,β-unsaturated aldehyde and a 5′-phosphate; (2) the 3′ repair diesterase activity of human APE1 removes the 3′-blocking group to generate a 3′-OH terminus; (2) human mitochondrial mono-functional uracil-DNA glycosylase 1, UNG1, excises the uracil (U) residue in DNA, thus forming a free U base and abasic site (AP site); (4) APE1 cleaves duplex DNA on the 5′ side of the AP site and yields a single-strand break with a 3′ hydroxyl adjacent to a dRP. In NIR, (5) APE1 directly cleaves 5′ to the 5ohU base, thus generating a single-strand nick containing a 3′-OH and a 5′-phosphate with a dangling base. (6) In the SP-BER pathway, DNA polymerase γ inserts a single nucleotide and removes the dRP blocking residue, then DNA ligase IIIα seals the single-strand nick. (7 and 8) In the LP-BER and NIR pathways, DNA polymerase γ initiates strand displacement repair synthesis, coupled to DNA2- and FEN1-catalysed cleavage of unannealed 5′-flap structures containing a dRP residue in BER and a 5′-dangling base in the NIR pathway, respectively; (9) DNA ligase IIIα seals the single-strand nicks and restores genetic integrity of mtDNA.

Figure 3. The model of mtDNA replication leading to asymmetrical DNA strand inheritance. (**1**) MtDNA is anchored to the inner membrane of a mitochondrion via interaction of the D-loop region with a specific site in the membrane. (**2**) Asynchronous replication of a leading H-strand (blue arrow) at the O_H site proceeds unidirectionally to displace the parental H-strand. When the O_L site is exposed, lagging L-strand (red arrow) replication starts in the opposite direction. (**3**) Replication of the leading H-strand proceeds further and terminates at O_H before the lagging L-strand replication reaches this site. Premature termination of H-strand synthesis produces a hemicatenane: two circular DNA molecules mechanically interlocked via a single-stranded linkage (Laurie et al. 1998). (**4**) Replication of a lagging L-strand terminates at the O_L site to complete the formation of a double-stranded hemicatenane composed of two interlocked mtDNA molecules. The hemicatenane is unlinked by mitochondrial topoisomerase IIIα to produce two separate mtDNA molecules: one containing the parental H-strand, and the other the parental L-strand. (**5**) The mtDNA molecule containing the old parental L-strand diffuses freely throughout the mitochondrial matrix and may get anchored to the inner membrane through the expression of OXPHOS complex proteins (Lynch and Wang 1993). (**6**) The mtDNA molecule containing the old parental H-strand stays attached to a specific site in the inner membrane and continues to replicate.