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Review

Different faces of mitochondrial DNA mutators[☆]

Karolina Szczepanowska, Aleksandra Trifunovic^{*}



Cologne Excellence Cluster on Cellular Stress Responses in Ageing-Associated Diseases (CECAD) and Institute for Mitochondrial Diseases and Ageing, Medical Faculty, University of Cologne, D-50931 Cologne, Germany

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ABSTRACT

A number of studies have shown that ageing is associated with increased amounts of mtDNA deletions and/or point mutations in a variety of species as diverse as *Caenorhabditis elegans*, *Drosophila melanogaster*, mice, rats, dogs, primates and humans. This detected vulnerability of mtDNA has led to the suggestion that the accumulation of somatic mtDNA mutations might arise from increased oxidative damage and could play an important role in the ageing process by producing cells with a decreased oxidative capacity. However, the vast majority of DNA polymorphisms and disease-causing base-substitution mutations and age-associated mutations that have been detected in human mtDNA are transition mutations. They are likely arising from the slight infidelity of the mitochondrial DNA polymerase. Indeed, transition mutations are also the predominant type of mutation found in mtDNA mutator mice, a model for premature ageing caused by increased mutation load due to the error prone mitochondrial DNA synthesis. These particular misincorporation events could also be exacerbated by dNTP pool imbalances. The role of different repair, replication and maintenance mechanisms that contribute to mtDNA integrity and mutagenesis will be discussed in details in this article. This article is part of a Special Issue entitled: Mitochondrial Dysfunction in Aging.

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1. Mitochondrial DNA

Part of mitochondrial uniqueness that separates them from any other structure within animal cells is their specific origin from ancestral endosymbiotic bacteria and mitochondrial genome is its most important legacy. Mitochondrial genomes from different organisms can vary greatly in a structure, size and coding capacity ranging from merely 6 kb in *Plasmodium falciparum* [1] to 11.3 Mb in some flowering plants [2]. However, most animal mitochondrial DNA (mtDNA), including human are compact, supercoiled and circular molecules of approximately 16 kb in size. They are characterized by high gene density and absence of introns [3]. With exception of approximately 1 kb noncoding regulatory fragment, so-called D-loop, it is entirely transcribed and only the full expression of mitochondrial genome will let the cell to maintain the proper respiratory capacity [3].

In contrast to nuclear DNA, mitochondrial genome is characterized by coexistence of numerous identical DNA molecules. The mtDNA copy number in most human cells ranges from 10^2 to 10^4 per cell and can reach 10^{6-7} in oocytes, often constituting up to 1% of total DNA cell mass [4]. Although mtDNA was initially considered to be naked, unprotected, and vulnerable to damage, research over the last decades has shown that mtDNA is protein-coated and packaged into nucleoids [5]. The protein that packages mtDNA in animal mitochondria is called

mitochondrial transcription factor A (TFAM) and it owes this to its ability to bind, wrap, bend, and unwind DNA without sequence specificity [6]. A large number of other mitochondrial proteins have been ascribed to nucleoids based on their interactions with mtDNA [7]. However, association of a protein that is essential for mtDNA maintenance does not necessarily mean that it has a role in structural organization of the nucleoid. Therefore, this view has been challenged by latest results using stimulated emission depletion (STED) microscopy and showing that mitochondrial nucleoids have a uniform mean size in a variety of mammalian species, and this size corresponds to a single mtDNA molecule wrapped by TFAM [8].

MtDNA is replicated independently of cell cycle and irrespectively to the replication of nuclear genes [9]. The proper balance between mtDNA replication, mitochondrial dynamics, mitophagy and mitochondrial biogenesis ensure the continuous turnover of mtDNA [9]. The importance of mitochondrial genetic information is stressed by the fact that mitochondria preserved very complex and unique machinery to maintain and express the content of mtDNA [9]. Many of the proteins involved in this processes will be discussed in more details later.

Although mitochondria persist their own genome, its coding capacity allows only small, essential, subset of proteins that is vital for mitochondrial function in energy production. In humans and most other animals, these are 13 protein subunits of the respiratory chain (ND1-ND6, ND4L, CytB, COX I-III, ATPase6 and ATPase8). Besides them, mitochondrial genome encodes for all RNA species (2 rRNA and 22 tRNA) required for mitochondrial genes expression. As a consequence, an overwhelming majority of ≈ 1100 mitochondrial proteins, including

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^{*} Corresponding author.

E-mail address: aleksandra.trifunovic@uk-koeln.de (A. Trifunovic).

those involved in maintenance and expression of mitochondrial genome, are encoded by nuclear genes, synthesized on cytoplasmic ribosomes and imported into mitochondria. Remarkably, almost a quarter of these proteins, as estimated in budding yeast, seem to be involved in maintenance and expression of mtDNA [10]. This raises a question: why would eukaryotic cells invest more than 250 proteins to be able to make just a handful of them inside mitochondria? This question is intimately linked to another one: why mtDNA has been so carefully maintained during evolution? Even after years of research we do not have a definite answer to these queries although some suggestions have been made to relate it to high hydrophobicity of mtDNA-encoded proteins or to the need for additional layer of regulation of the oxidative phosphorylation capacity.

2. Mitochondrial DNA mutations

Since the identification of the first human pathological mtDNA mutations in 1988 [11,12], it became increasingly clear that mtDNA mutations are at the core of many human diseases and their frequency is very high in general population, reaching > 1:200 in live births. Currently there are more than 600 different point mutations reportedly associated with different human diseases (source: Mitomap.org). Although many have not been definitively shown to cause disease, for some the evidence is more compelling. Unfortunately, a couple of unique features of mtDNA genetics and inheritance still makes it very difficult to predict the course of the disease, prenatal diagnosis and/or genetic counselling in everyday clinical practice. First of all, mtDNA does not follow the Mendelian rules of inheritance. In most animals, as in humans, mtDNA is maternally inherited. Therefore, a mother carrying an mtDNA mutation can transmit it to her children, but only her daughters can further transmit it to the next generation. As each cell contains ~10,000 copies of mtDNA a pathogenic mutation could be present in all or just few of copies of the molecule. Existence of two or more different populations of mtDNA in a single cell is called heteroplasmy in contrast to homoplasmy where all mtDNA molecules are identical. This leads us to yet another problem of the mtDNA complexity: the threshold effect. Threshold effect represents the minimal critical level of a pathogenic mutation in mtDNA that should be present in the cell or tissue to have a deleterious effect. A certain proportion of mutant mtDNA must be present before reduction of OXPHOS activity is observed, and the threshold is lower in tissues that are more dependent on oxidative metabolism. Different thresholds exist for different types of mtDNA mutations, ranging from 90% for some tRNA mutations [13,14] to 60% for large mtDNA deletions [15]. The last but not least problem of mtDNA genetics is mitotic segregation. Random distribution of mtDNA molecules during cell division can result in increased amount of mutant mtDNA molecules in one of the daughter cells. This can lead to a cell carrying low levels of mutated molecules giving rise to one of relatively high levels, which in turn will affect oxidative phosphorylation in that cell. Finally, given that there are hundreds of genes in nuclear genome bigger than the whole mtDNA, it is remarkable how this small piece of DNA can cause so many different metabolic diseases, be causatively linked to numerous age-associated disease and influence aging process itself.

Although, historically most frequently detected somatic mtDNA mutations were large deletions that result in smaller, circular mtDNA molecules [16,17], it is increasingly recognized that the most common types of mutations observed in the mtDNA are single nucleotide substitutions, single base insertions or single base deletions [18] and that most of us carry appreciable numbers of low-level mtDNA variants in our cell [19]. The frequency of somatic mtDNA mutations can exceed the mutation frequency of the nuclear genome by several orders of magnitude [20]. Although initially this difference was attributed to increased mtDNA damage from elevated concentrations of endogenous reactive oxygen species produced as by-products of oxidative phosphorylation [21,22], many recent studies argue against any significant contribution of oxidative damage in mtDNA mutation accumulation [23–25]. In

recent years, advantages in the whole genome analysis through development of next-generation sequencing techniques, allowed also very detailed analysis of mtDNA mutational spectra in various tissues. These studies demonstrated that mtDNA mutations indeed increase with increasing age [23–25]. Surprisingly, G → T mutations, considered the hallmark of oxidative damage to DNA, do not follow this trend and overall represent a minor portion of all detected mutations [25]. Predominant mutations detected in these studies are transition mutations, consistent with misincorporation by DNA polymerase γ or deamination of cytidine and adenosine as the primary mutagenic events in mtDNA [23–25]. Overall these results argue against oxidative damage being a major cause of mtDNA mutagenesis and suggest that replication errors and/or spontaneous base hydrolysis are responsible for the bulk of accumulating point mutations in mtDNA. Therefore, this review focuses on recent advances in understanding the role of different proteins involved in maintenance, repair and replication of mtDNA and their possible role in creation of mtDNA mutations.

2.1. Lack of sophisticated packing and histone protection

In a contrast with highly regular packing of nuclear DNA, mtDNA exists in a histone-free form indicating that it is deprived of the effective protection against damage. MtDNA itself sets up into the uniform and compact structures suggesting the regular and firm packing [8]. Despite the lack of histones mtDNA is packed in nucleoids by TFAM that binds and wraps mtDNA molecules providing tight and efficient bundling [8]. Therefore, besides being an essential regulator of mtDNA transcription and replication in higher eukaryotes, TFAM is believed to ensure the histone-like protection and provide proper architecture to mtDNA [26]. Abundance of TFAM changes the mtDNA levels in a mutual, directly proportional and dose-dependent manner [6,26], hence, the loss of TFAM in mammalian mitochondria results in complete loss of mtDNA and embryonic lethality [27]. Tissue specific loss of TFAM leads to cardiomyopathy, muscle weakness, Parkinson-like neuronal dysfunction and diabetes (for review see [26]). In agreement, TFAM overexpression increases mtDNA levels, diminishes oxidative damage and ameliorates mitochondrial deficiency [28,29]. Recently, the role of TFAM in modulation of mitochondrial base excision repair has also been suggested [30]. TFAM downregulation associates with higher 8-oxoguanine (8oxoG) incision activity without changes in OGG1 protein levels and results in accumulation of mtDNA damage [30]. This is in agreement with a previous *in vitro* study stating that TFAM favours binding to DNA containing 8oxoG or cisplatin adducts [31].

Taken together these data suggest that low levels or complete loss of TFAM may potentially cause a variety of mtDNA mutations. MtDNA point mutations and small deletions may be generated due to the lack of structural protection against the physical and chemical mutagens. MtDNA deletions and rearrangements may result from affected mtDNA replication and repair on misfolded mitochondrial nucleoids. Finally the complete loss of mtDNA may take place due to the extensive degradation of uncoated mtDNA molecules and/or mtDNA missegregation into the daughter cells. Thus TFAM deficiency constitutes very potent although nonspecific mtDNA mutator model. On the other hand, overexpression of TFAM or posttranslation modulation of its activity might provide interesting antimutator properties.

2.2. Limited mtDNA repair

To prevent the accumulation of DNA damage, that may exert dramatic repercussion on a cellular function, five different types of DNA repair emerged in animal cells, exhibiting complementary, yet in some instances overlapping, substrate specificity: direct reversal, base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER) and DNA double-stranded break repair. Of the repair mechanisms that have been described in mitochondria, BER is the most documented.

2.2.1. Base excision repair

BER repairs a broad range of base modifications, fixes abasic sites and single-strand breaks in DNA. Thus BER is a major weapon correcting the hydrolytic, alkylated and oxidative lesions as well as ROS induced DNA breaks [32]. Mitochondrial BER machinery includes: DNA glycosylases that recognize and remove mutated base in a damage-specific manner (OGG1, UNG, NTH1, NEIL1/2, MUTYH, MTH1), AP endonuclease that process the abasic site (APE1), POLG that re-synthesizes lacking DNA patches and DNA ligase (LIG3) that seal the DNA fragments back [33]. There are evidences for both short-patch and long-patch BER repair pathways in mitochondria [33]. Interestingly, some studies show that the efficiency of mammalian mitochondrial BER pathway changes with age in a tissue-specific manner [34].

OGG1, 8-oxoguanine DNA glycosylase specifically recognized and removes 8oxoG adducts, the major mutagenic lesion generated by ROS [35]. 8oxoG adducts undergo pairing with adenine or cytosine during replication thus generating the T:A and G:C transversions [35]. However, OGG1 does not recognize adenines mispaired with 8oxoG bases. Therefore adenine has to be firstly removed by MUTYH to allow subsequent pairing [36]. These adducts were found in association with neurodegenerative diseases, some types of cancer and insulin insensitivity [37].

Although OGG1 acts mostly in nucleus, the mitochondria targeted isoform (*Ogg1-2a*) has been also identified (Nishioka et al, 1999; Panduri et al, 2009). *In vitro* studies showed that the presence of 8oxoG adducts in a DNA template prevents POLG processing through the lesion, suggesting that POLG cannot bypass these lesions without preceding action of BER machinery [38]. A number of studies analysing the effect of *OGG1* overexpression in isolated cells suggest that OGG1 protects against oxidant-induced mitochondrial dysfunction and intrinsic apoptosis, increases membrane potential, decreases mitochondria fragmentation and upregulates mtDNA levels [39–42]. For example, myotubes isolated from *Ogg1* overexpressing animals have increased ATP levels and mitochondrial mass, insulin sensitivity is enhanced while levels of mitochondrial ROS and palmitate-induced mtDNA damage were reduced [42]. However, these data are obtained from cells exposed to high oxygen concentrations present in cell culture (20% O₂), and it is difficult to understand their physiological relevance as most of cells in our bodies are confronted to 2–3% O₂ and therefore are never exposed to that level of oxidative stress. In agreement with this, some contradictory results were obtained in fruit fly cell lines overexpressing *OGG1*. Here, decreased accumulation of 8oxoG adducts in mtDNA was detected while cells became more susceptible to oxidative stress inflicted by paraquat and hydrogen peroxide treatment [43]. Those findings suggest that although OGG1 may indeed reduce 8oxoG adducts in mtDNA it rather affects overall mitochondrial function and decreases cellular survival.

Most studies *in vivo* suggest that OGG1 function does not affect overall mitochondrial function, nor significantly influences mtDNA mutation accumulation. OGG1 deficient mice have a 20-fold increase in G levels in the entire DNA pool, in comparison to wild-type mice [44]. Although it was initially suggested that the consequences of an OGG1 defect might be restricted to slowly proliferating tissues with high oxygen metabolism such as liver or heart, suggesting primary effect on mtDNA maintenance [44], subsequent studies showed that mitochondria from *Ogg1*^{-/-} mice were functionally normal [45]. The authors found no differences in maximal phosphorylation rates, no differences in maximal activities of complexes I and IV and no indication of increased oxidative stress in mitochondria from *Ogg1*^{-/-} mice, as measured by protein carbonyl content [45]. Moreover, a loss-of-function mutation in *Ogg1* did not significantly influence the somatic mtDNA mutation frequency in flies with extremely reduced activity of manganese superoxide dismutase 2 (SOD2), a primary enzyme that detoxifies superoxide anion within mitochondria [23]. Although increased mtDNA damage was detected in

mice deficient of OGG1, but not in mice deficient of OGG1 and MUTYH, during early heart reperfusion, six weeks after reperfusion *in vivo*, this difference disappeared [46]. The lack of functional impairment after ischemia-reperfusion injury in *Ogg1* and *Ogg1/Mutyh* knockout models could not be explained by compensatory upregulation of other potential mitochondrial BER enzymes suggesting that repair of mitochondrial DNA oxidative base lesions may not be important for maintenance of cardiac function during ischemia and reperfusion [46]. Together, these findings indicate that oxidative stress is not a major cause of somatic mtDNA mutations [23,45,46].

UNG1, uracil DNA glycosylase removes uracil bases misincorporated into DNA strands. Beside uracil, UNG is also able to remove 5-fluorouracil and oxidation products of cytosine adducts from DNA [47]. Uracil appears in DNA as a result of spontaneous deamination of cytosine or due to misincorporation of dUMP instead of dTMP during replication. Therefore UNG prevents GC to AT transitions [47]. Nuclear (*UNG2*) and mitochondrial (*UNG1*) isoforms of UNG are generated by alternative splicing and transcription from different positions in the *UNG* gene [48].

Inactivation of *UNG1* gene in yeast generates a 3-fold increased in mtDNA point mutation rate [49]. In contrast, specific inhibition of UNG1 activity in human cells does not result in mtDNA mutator phenotype [50]. Similarly, UNG1 deficient mice are characterized by normal mutation rates in mtDNA and do not differ from their wild-type littermates [51]. However, upon exposure of UNG deficient fibroblasts to nitric oxide donor, the uracil/cytosine ratio in DNA and a cell death rate increased [51]. It seems like, as for most other mtDNA repair enzymes, that only extremely strong, non-physiological conditions, using hazardous mutagens results in oxidative damage of mtDNA. This suggests that, in general, mtDNA is well protected from oxidative stress, possibly via TFAM packing, and that mtDNA mutations accumulate as a result of other processes. However, the mtDNA mutator properties of UNG1 should not be completely dismissed, as it was shown that the expression of forebrain neuron-specific mutant version of UNG1, that is able to remove thymine instead of uracil from mtDNA, causes accumulation of AP sites in mtDNA leading to apoptosis and neurodegeneration [52].

NTH1, Endonuclease III-like 1 initiates the BER repair of oxidized ring pyrimidine residues. NTH1 recognizes and binds C5-C6 rings saturated pyrimidines and releases the lesion generating the AP site [53]. NTH was shown to recognize thymine glycol, cytosine glycol and cytosine hydrate that are formed as a result of aerobic ionizing radiation, oxidative stress and UV irradiation and might be prone to deamination [53]. Other DNA adducts targeted to NTH1 may include ring-opened formamidopyrimidine lesions that are formed at high rates upon oxidative stress and result from hydroxyl radical attack on guanine or adenine [54].

Null *Nth1* animals are healthy, fertile and overall phenotypically indistinguishable from their wild type littermates [53,55]. Furthermore, mouse embryonic fibroblasts isolated from NTH1 deficient animals did not show any sensitivity to hydrogen peroxide or menadione [55]. Remarkably, tissues of NTH1 deficient animals exhibited activity raised against oxidized thymine suggesting that compensatory enzyme activity exists in those animals [53,55]. This suggests that mtDNA repair enzymes might be not as specific as they are thought to be and they can take over the function of missing ones.

NEIL1/NEIL2 (Nei endonuclease VIII-like 1 and 2) are two DNA glycosylases with preference toward oxidized pyrimidine, formamidopyrimidine and 5-hydroxyuracil. They have associated DNA glycosylase/lyase activity towards mismatched thymine and uracil favouring removal of T:C and U:C mismatches [56,57]. Contrary to other DNA glycosylases of BER machinery NEIL1/2 exhibit activity toward DNA bubbles formed by mismatched double-stranded DNA. Therefore they might be able to repair oxidative lesions on replicated and transcribed DNA [57]. Furthermore, NEIL1/2 are able to repair DNA lesions independently of subsequent APE processing [160].

NEIL1 can complement the activity of OGG1 and NTH1, particularly when those two are downregulated or dysfunctional [54]. Remarkably, both *Neil1* $-/-$ and $+/-$ animals show metabolic syndrome characterized by severe obesity, dyslipidemia and liver failure, even when external stress is absent [58]. NEIL1 deficient animals also showed elevated steady-state levels of mtDNA deletions even without presence of any stressors, although the technique used in these studies (qPCR) is not quantitative and is prone to artefacts [58].

Human NEIL2 also colocalize to mitochondria and it seems to interact with the DNA end-processing enzyme polynucleotide kinase 3'-phosphatase (PNKP) [59]. Chromatin immunoprecipitation analysis showed association of NEIL2 and PNKP with the mitochondrial genes MT-CO2 and MT-CO3 (cytochrome c oxidase subunit 2 and 3); importantly, both enzymes also associated with the mitochondrion-specific DNA polymerase γ [59]. Depletion of NEIL2 or PNKP in HEK293 cells increases the rate of single strand breaks, further supporting their role in maintenance of mtDNA integrity [59].

Therefore, NEIL1/2 when downregulated could act as mtDNA mutators, even without exposure to additional stress, unlike other BER associated DNA glycosylases [45,51,53]. It is likely that this is related to their specific repair function during replication/transcription of mtDNA, in agreement with notion that mtDNA is prone to damage only when unwinded/uncoated during replication or transcription.

MUTYH, mammalian homolog of *E. coli* MutY is another DNA glycosylase that takes part in correction of 8oxoG oxidative lesions. *MUTYH* prevents G:C to T:A transversions by recognizing and excision of the 2-hydroxyadenine or adenine incorporated opposite to guanine or 8oxoG, respectively [60,61]. In agreement with strong mutator phenotype, *MUTYH* deficient animals are more prone to spontaneous tumors due to increased nuclear DNA mutation rate [62]. The role of *MUTYH* in mitochondria is less clear as it is hard to separate it from the strong nuclear phenotype. There are some indications that mitochondrial *MUTYH* isoform is needed for mtDNA stability, but more experiments are needed to confirm this [63].

MTH1, is an 8-oxoGTPase present both in nucleus and mitochondria that works to primarily prevent oxidized dGTP from being incorporated into DNA and it also shows affinity to 8-oxo-ATP and 2-OH-dATP [64]. As a result oxidized purines cannot generate A:T to C:G and G:C to T:A transversions. Although it was shown that *Mth1* $^{-/-}$ mice are slightly more prone to certain types of tumors, it is likely that the phenotypes of these mice are related to nuclear DNA mutations, rather than mtDNA changes, as it was shown that the mice accumulate higher levels of mutations in nuclear encoded genes [65].

APE1, apurinic/aprimidinic (AP) endonuclease catalyzes incision of phosphodiester backbone of the AP site created when DNA glycosylase removes the damaged base from DNA. The lack of *APE1* in rodents is lethal while heterozygous animals showed reduced repair of certain BER lesions [66]. Although *APE1* primary localizes to nucleus in basal conditions, it was suggested that it shuttles into mitochondria upon mtDNA damage or elevated oxidative stress to participate in restoring of mitochondrial function [67,68]. Again here, it is difficult to estimate the significance of *APE1* for mitochondrial physiology and mtDNA maintenance since it harbours a complex function in transcription and repair of nuclear DNA [69].

LIG3, DNA ligase III (*LIG3*) provides sealing of discontinuous DNA strands and thus confers the last step of DNA lesions repair. *LIG3* is another factor of BER repair shared between mitochondria and nucleus compartment. Nuclear isoform of *LIG3* plays a role in a DNA replication, in some cases of double strand break repair and in broadly defined single stranded break repair including BER [70]. *LIG3*, the only vertebral mitochondrial DNA ligase identified so far [71,72], is absent in yeast who instead possess *Cdc9* ligase essential for the overall DNA sealing events in replication, repair and recombination of mtDNA [73]. Although initially it was suggested that *LIG3* is bound to XRCC1, a key factor for the single-strand break DNA repair in the nucleus, recent studies showed that is dispensable for the nuclear DNA repair, but is central and

essential for mitochondrial DNA maintenance [71,72]. Inside mitochondria *LIG3* interacts with tyrosyl-DNA phosphodiesterase 1 (TDP1), NEIL1/2 glycosylases, and *POLG* [74]. Downregulation of *LIG3* in human fibroblastoma cell lines decreased mtDNA, levels, diminished respiration and lead to accumulation of SSB in remaining mtDNA, while complete lack of *LIG3* in murine cells leads to full depletion of mtDNA, underlying the essential role of *LIG3* in mitochondrial genome maintenance [75,76].

2.2.2. Mismatch repair

The primary role of the MMR pathway is the repair of base-base mismatches and insertion/deletion loops. The key protein complexes of the MMR system are the MutS and MutL families which are highly conserved from lower organisms to eukaryotes [77]. There is little evidence that nuclear form of MMR exists in mtDNA, and most enzymes essential for the MMR are not found in mitochondria [78]. However some findings support the existence of MMR activity in mammalian mitochondria [79] and the Y-box binding protein 1 (YB-1) may facilitate a unique mitochondrial mismatch repair process in human cells [78]. It was also proposed that *POLG* proofreading activity that allows high fidelity replication, might minimize the need for mismatch DNA repair in mammalian mitochondria [80]. However, that does not explain why budding yeast mitochondria have canonical mitochondrial MMR, despite having similar *POLG* proofreading activity. Historically, first mtDNA mutators were isolated in yeast and mapped to *Mip1* gene, encoding *POLG* homolog, and to *Msh1* gene, encoding a mitochondrial homolog of Mut S factor from *E. coli* that acts in mismatch repair [81]. *MSH1* possesses DNA binding activity and dATPase activity that allows recognition, binding and removal of DNA mismatches. However the affinity of *MSH1* toward different mismatches vary with an overall preference for G:T and A:T transversions which overlaps with types of transversions permitted by *MIP1* [82]. This indicates that *MSH1* might act directly in post-replication proofreading of errors overlooked by *POLG* in yeast.

2.2.3. Nucleotide excision repair

Nucleotide excision repair (NER) provides mechanisms for excision of bulky adducts and helix-distorting lesions from DNA strand and is efficient toward DNA cross-links. Bulky adducts can be generated by exposure to UV light or to some of carcinogenic compounds. Mitochondria lack the NER mechanism responsible for the removal of UV-induced pyrimidine dimers and consistent with this, mitochondria display very poor repair of cisplatin intrastrand crosslinks [83]. As bulky adducts are not removed from mtDNA and to prevent replication/transcription stalling, it is likely that mtDNA bearing bulky lesions is likely directly targeted for degradation [84]. The only evidence to date for an NER-like mechanism in mitochondria is the presence of the Cockayne syndrome proteins, CSA and CSB, which in nucleus are required for the transcription-coupled NER (TC-NER) [85]. However, the role of CS proteins in mitochondria differ from their nuclear functions, as they seem to be associated with BER and transcription of mitochondrial DNA [85]. Although some other roles, like the one in repair of double strand breaks, have been proposed for the mitochondrial CS proteins, this has to be confirmed by further experimental evidence [85].

2.2.4. Single strand break repair

SSBR is often considered a subpathway of BER due to end processing events to restore the 5' and 3' termini of the DNA, before ligation proceeds [86]. However, there are other instances whereby end processing at an SSB may occur but is not necessarily preceded by the other steps of BER i.e. removal of a damaged base and cleavage at an abasic (AP) site [86]. SSBs are often a result of abortive topoisomerase 1 (TOP1) activity involving collision with RNA polymerases during transcription or with DNA polymerases during replication [86]. This type of break has become the focus of much attention in recent years, because

defects in this type of repair are associated with the two genetic disease: ataxia with oculomotor apraxia type 1 (AOA1, caused by mutations in aprataxin – *APTX*) [87] and spinocerebellar ataxia with axonal neuropathy type 1 (SCAN1, caused by mutations in *TPD1* – tyrosyl-DNA-phosphodiesterase 1) [88]. *APTX* removes 5'-AMP groups that arise from aborted DNA ligation reactions [89], while *TPD1* is a key enzyme for the repair of trapped TOP1 cleavage complexes [90]. As presence and activity of these enzymes have recently been identified in the mitochondrion and mutations in both genes cause ataxia, a feature very often associated with mitochondrial disease, it was suggested that both AOA1 and SCAN1 are primarily mitochondrial disorders [32]. Indeed, both *TPD1* and *APTX* seems to be important for the mtDNA maintenance as their loss leads to mtDNA depletion and/or increased number of mtDNA mutation [91,92].

2.2.5. Mitochondrial recombination DNA repair (DSBR)

Irradiation, chemical agents and UV light can generate double strand breaks (DSB) in DNA molecules. DSB are the critical DNA lesions that might lead to genome rearrangements. Nuclear genome is well protected from DSB thanks to the homologous recombination (HR) and non-homologous end joining (NHEJ). It is still not completely clear if these pathways are present in mammalian mitochondria, as there are studies supporting both views [93–95]. Some claim that both DSBR pathways seems to be either entirely absent or heavily attenuated in mammalian mitochondria [93]. It is speculated that the organelle may encounter less of the adducts processed by these mechanisms, or that the high energy demand and complicated mechanics of these processes may make DNA repair not essential for the mitochondria, since it has access to multiple copies of its genomes [93]. Others propose that inefficient DSBR in mammalian mitochondria may significantly contribute to large scale deletions observed commonly in patients suffering from deficiency in mtDNA maintenance [94,95]. However, the enzymes involved in this process are still largely unknown, although a number of them, including *POLG* have been proposed to play a role in this process [95].

Taken together the evidence from research on different repair mechanism in mammalian mitochondria again suggests that majority of deleterious mutations arising from the deficient mtDNA repair are rather not connected with adducts arising from oxidative damage, but are linked to mtDNA replication.

2.3. Fidelity of mtDNA replication

Fidelity of DNA replication creates a major determinant of the genetic information stability. Proper balance between the efficiency and fidelity of DNA synthesis must be maintained by organism to avoid mutations that can lead to diseases, cancer and even death. On the other hand, low DNA synthesis fidelity provides diversity of genetic information, thus allowing flexibility of organisms in reaction to different environmental stimuli. Fidelity of DNA replication strongly depends not only on DNA polymerase, the key enzyme responsible for DNA synthesis fidelity, but also on other enzymes and factors involved in DNA maintenance, as well as on the type of damage in DNA and local DNA sequence environment.

In a light of recent findings it became increasingly clear that majority of mtDNA mutations arise as a consequence of errors during mtDNA replication with *POLG* as major mediator of mtDNA point mutations in humans [96]. It was suggested that most mtDNA point mutations are generated at the early stage of animal development as a result of insufficient or unfaithful replication [97]. Large mtDNA deletions that are often found in aged post mitotic tissues are initiated by single-stranded regions of mtDNA, possibly generated through *POLG* exonuclease activity [95]. These single strands could then anneal with microhomologous sequences on other single-stranded mtDNA molecules or within the noncoding region and subsequent repair, ligation

and degradation of the remaining exposed single strands would result in the formation of a circular mtDNA harboring a deletion [95].

Replication of the mitochondrial genome requires unique enzymatic machinery, composed of a set of factors encoded by nuclear DNA and recruited to mitochondria. *In vitro* studies showed that the basic replication fork of human mitochondria can be reconstituted using primed single-stranded DNA substrate and at least five mtDNA maintenance factors: catalytic and accessory subunit of DNA polymerase gamma (*POLG* and *POLG2*), mitochondrial RNA polymerase (*POLRMT*), replicative DNA helicase (*TWINKLE*) and the mitochondrial single-stranded DNA binding protein (*MTSSB*) [98]. Although this simple mitochondrial replisome is sufficient to duplicate a DNA substrate *in vitro*, proper maintenance and effective replication of the mitochondrial genome involves a much longer and still growing list of factors [99]. Remarkably, all those proteins were shown to significantly contribute to mutator mtDNA phenotypes in mammals.

2.3.1. Mitochondrial DNA polymerase – *POLG*

Out of 16 highly specialized eukaryotic DNA polymerases, *POLG* is the only one mediating all DNA synthesis events inside the mitochondria thus bearing substantial responsibility for maintaining mtDNA. Although *POLG* activity contributes just to 1–5% of the total cellular DNA polymerase activity, it plays a key role in the integrity and viability of eukaryotic cells [100]. Human *POLG* is a heterotrimer composed of a 140 kDa catalytic subunit (*POLG*) and a dimer of the p55 accessory subunit (*POLG2*) which increases the activity and processivity of the enzyme [101]. The *POLG* subunit contains two main domains: the 3'–5' exonuclease domain in the N-terminal part and the polymerase domain in the C-terminal part [101]. These two domains are connected by a linker region, which plays a role in DNA binding and processivity through its contacts with the proximal accessory subunit [101]. *POLG* possesses three catalytic activities: a DNA polymerase activity which synthesizes DNA, a 3'–5' exonuclease activity that proofreads nascent DNA molecule and eliminates mispaired nucleotides and a 5'-deoxyribose phosphate (dRP) lyase activity which catalyses the release of 5'-terminal dRP sugar moiety from incised apurinic/apyrimidinic sites during posttranslational BER [102,103]. The proofreading activity of *POLG* is essential for faithful DNA replication as it was estimated that reduces load of mismatches approximately 20-fold [103,104]. In addition, *POLG* exhibits a reverse transcriptase activity *in vitro* [105], which could be physiologically relevant as it was shown that the premature and accelerated aging of HIV-patients is likely caused by adverse effects of some HIV-antiviral drugs (nucleoside analog reverse-transcriptase inhibitors) that result in *POLG* – mediated loss of mtDNA integrity [106]. Importance of *POLG* for cell health and viability is further stressed by phenotypes presented by lower eukaryotic models lacking mitochondrial DNA polymerase. Depletion of *POLG* homolog, *MIP1*, in yeast is not lethal, but results in complete loss of mtDNA and lack of growth on nonfermentable carbon sources [107]. Disruption of *POLG* homolog in fruit flies results in lethality at pupa stage associated with loss of mtDNA, aberrant mitochondrial morphology and impaired cell proliferation in larval brain [108]. In contrast, *polg-1* deficient *C. elegans* mutants have normal development and regular morphology, but severe mtDNA depletion leads to sterility and shortened lifespan [109]. The overexpression of *POLG* is also not beneficial for cells and mitochondria. As shown in *Drosophila*, overexpression of *POLG* in nervous system shortens lifespan, while the constitutive overexpression reduces mtDNA copy number and results in lethality at pupal stage [110]. Similarly, the overexpression of *POLG* in yeast and roundworms is toxic (authors observations). In contrast, long-term overexpression of *POLG* in cultured human HEK293T cells did not have an impact on mitochondrial function, reflected by mtDNA content and oxygen consumption [104]. However, these cells were grown in high-glucose condition, making them less dependent on mitochondrial function.

The most common changes in mtDNA that contribute to inherited polymorphisms and disease-causing somatic mutations are G → A

transitions (see <http://www.mitomap.org/>). These mutations allow G:T mismatches to occur as a relatively frequent event and they overlap with the DNA base substitutions produced by POLG *in vitro* and recapitulate *in vivo* mtDNA mutations spectra produced by wild type or defective POLG [96,104,111]. Remarkably, the base substitution spectra generated by yeast MIP1 differs from those in human. This could be potentially explained by the lack of MMR activity in mammalian mitochondria that preferably and efficiently removes transitions in yeast mitochondria [82].

2.3.1.1. POLG mutators. Mutations that abolish MIP1 exonuclease activity in yeast result in the accumulation of base substitutions in mtDNA, hence they are named mtDNA mutators [112]. These MIP1 mutations are at least partially dominant, meaning that even in the presence of wild-type MIP1, mutations accumulate in mtDNA [112]. MIP1 carrying D171G, D230A or D347A mutation have even ~500 lower exonuclease activity and 100–200 fold increase in the number of point mutations, accompanied by overall high mtDNA integrity with almost no effect on MIP1 processivity [113]. MtDNA mutators in the polymerase domain mostly map in a close vicinity to the polymerase active site and their low fidelity may result from decreased selectivity toward dNTPs but also impaired DNA synthesis or DNA positioning on MIP1 active site [112,114]. They are characterized by modest increase of mtDNA point mutation frequency that is most likely compensated by proficient 3'–5' exonuclease activity, and different extents of decreased polymerization processivity, reflected by instability of mtDNA *in vivo* [114].

Similar to yeast, overexpression of exonuclease-deficient POLG (D198A, homologous to D171 in yeast) in human cells led to high accumulation of mtDNA point mutations [104]. Transient expression of POLG variants D890N or D1135A with mutations in polymerase domain inhibited endogenous mitochondrial DNA polymerase activity and caused mtDNA depletion [104]. Development of mtDNA mutator mice, the first *in vivo* mtDNA mutator model allowed further understanding of POLG contribution to mutational load in mammals [115]. The introduced mutation (D257A, homologous to D230A in yeast) mapped to the exonuclease domain and was designed to create a defect in the proofreading function of POLG, leading to the progressive, random accumulation of mtDNA mutations during the course of mitochondrial biogenesis [115]. As the proofreading in these mice was efficiently prevented, they develop an mtDNA mutator phenotype with a three to fivefold increase in the levels of random somatic point mutations [115]. Remarkably, these mutations accumulate at a higher rate during the time of development from oocytes to early embryonic life of mtDNA mutator mice, than during the rest of their life when mutations accumulate in rather linear fashion [115]. In addition to the standard circular chromosome, mtDNA mutator mice also harbour large linear mtDNA molecules caused by replication stalling [116]. These molecules are around 11–12 kb in length, and encompass the region between the origins of replication for the heavy (O_H) and the light strands (O_L). Around 25–30% of the mtDNA consists of these linear deleted molecules, and this ratio does not change as the animal age [115]. The POLG exonuclease activity may be involved in resolution of replication intermediates at O_L , which may explain why exonuclease deficiency will lead to replication stalling, thus leaving the mtDNA molecule susceptible to breakage at the stall site [116].

2.3.1.2. Can we increase POLG fidelity? Antimutators are the protein variants, the best established in DNA polymerases group, which reduce mutational load in DNA. Antimutator profile of DNA polymerases can be obtained by changes within its protein sequence that increase the proofreading efficiency, rise nucleotide selectivity or favour dissociation of polymerase from the primer termini prior to misincorporation [117]. Pioneering studies with bacteriophage T4 DNA polymerase (T4 Pol) established the paradigm that antimutator amino acid substitutions reduce replication errors by increasing proofreading efficiency at the expense of polymerase processivity [117]. Recently, an attempt has been

made to isolate and characterized antimutator alleles in *Mip1*, a yeast POLG homologue [118]. Discovered antimutator *Mip1* variants carried a change in the 3'–5' exonuclease domain and showed 2–15 fold decrease in the mtDNA point mutation frequency in an MMR deficient background [118]. Biochemical characterization of those variants revealed that stimulated DNA excision versus DNA synthesis could be beneficial for the proofreading ability of MIP1 [118]. Remarkably, at least one antimutator variant showed normal polymerase activity suggesting that baseline-proofreading properties of MIP1 are not optimal and can be still enhanced without decline in overall function of enzyme [118]. Therefore, it would be of great interest to determine whether the corresponding substitutions act as antimutators in human enzyme. Based on the finding that any modification of the balance between DNA synthesis and excision must be extremely subtle to favour exonucleolysis without disturbing processivity of DNA polymerization, the impact of these substitution in POLG function cannot be anticipated [118]. Moreover, although yeast and human mtDNA transactions share many features, substantial differences characterize the replisome, including the presence of an accessory subunit in human POLG and different DNA helicases. Finally, MMR does not exist in human mitochondria, and beside proofreading, the mechanisms by which human mtDNA point mutations are removed remain to be elucidated [118].

2.3.1.3. POLG mutations and disease. Mutations in POLG are an important cause of mitochondrial disorders and until today more than 200 different pathogenic POLG alleles have been identified (<http://tools.niehs.nih.gov/polg/>). Mutations in POLG can: (i) affect the polymerase or 3'–5' exonuclease activity; (ii) diminish the recognition of dNTPs; (iii) decrease the processivity of the enzyme; (iv) abolish the binding or partitioning of DNA template between two active sites; (v) or misfold the overall structure of POLG resulting in its loss [119]. As a consequence POLG mutations could lead to accumulation of mtDNA point mutation, increased frequency of mtDNA deletions/large-scale rearrangements, have a combined effect on accumulation of both point mutations and deletions, or lead to a depletion of mitochondrial genome [119]. The clinical identification of mitochondrial diseases associated with POLG mutations is difficult because POLG-related diseases evolve over time and have an overlapping range of symptoms with multiple organ system involvement and with different degrees of severity and timing of onset [120]. The most common disease caused by mutations in POLG gene is adult-onset progressive external ophthalmoplegia (PEO), that could be a result of dominant or recessive mutation associated with single or multiple mtDNA deletions and often varying degrees of tissue-specific mtDNA depletion [120]. A generalized myopathy is present in most patients PEO patients, and can also be associated with sensory ataxia, neuropathy, dysarthria, myopathy, Parkinsonism, premature ovarian failure and cataracts [120]. The Alpers syndrome that leads to hepatocerebral mtDNA depletion during infancy and death at an early age, is the most severe form of POLG disease [120]. Remarkably, studies in animal models show that disease-associated POLG mutations can significantly increase the vulnerability of mtDNA to lesions generated by exogenous mutagens [121,122].

2.3.1.4. POLG mutation and aging. Ageing is associated with both mtDNA deletions and mtDNA point mutations. The highest levels of age-associated multiple mtDNA deletions are observed in post-mitotic tissues with high energy demands such as heart, skeletal muscle and brain [123,124]. The search for mtDNA point mutations, in tissue homogenates of ageing individuals gave rather disappointing results with very low levels of specific mutations (0.04–2.2%) [125]. When single cells were analysed, mtDNA point mutations were observed to accumulate to high levels in an age-dependent and tissue-specific manner [126,127]. Still there was an open question if mtDNA mutations could be a driving force of ageing? The creation of mtDNA-mutator mice has provided the first direct evidence that accelerating the mtDNA mutation rate can result in premature aging, consistent with the view that loss of

mitochondrial function is a major causal factor in aging [115]. The mtDNA mutator mice display a completely normal phenotype at birth and in early adolescence but subsequently acquire many features of premature aging. The increase in somatic mtDNA mutations in these mice is associated with reduced lifespan and premature onset of aging-related phenotypes such as weight loss, reduced subcutaneous fat, alopecia, kyphosis, osteoporosis, anaemia, reduced fertility and heart enlargement [115]. Premature ageing phenotypes in mtDNA mutator mice are not generated by a vicious cycle of massively increased oxidative stress accompanied by exponential accumulation of mtDNA mutations [128]. Instead, the observed phenotypes are a direct consequence of the accumulation of mtDNA point mutations in protein-coding genes, leading to a decreased assembly of the respiratory chain complexes, respiratory chain dysfunction and thus to premature ageing of mtDNA mutator mice [129]. The results indicating that mutations in the protein coding genes have the most deleterious impact on the somatic cells are in agreement with a study showing the existence of strong purifying selection against mutations within mtDNA protein-coding genes in the germline [130].

2.3.2. POLG2

To achieve the full processivity, mammalian POLG catalytic subunit requires interaction with the homodimer of 55 kDa POLG2 accessory subunit [101]. POLG2 binds POLG through the interaction with its linker region stimulating the holoenzyme affinity to DNA [98]. Remarkably, the 3′–5′ exonuclease proofreading activity of POLG is significantly reduced upon association with POLG2 to form the holoenzyme [131]. This reflects how enhanced processivity can compromise fidelity of DNA polymerases, highlighting again the importance of proper balance between efficiency and precision of DNA synthesis for the integrity of mtDNA. Therefore, POLG2 could be an interesting target for modulation of mtDNA proofreading, as some variants could potentially have mtDNA antimutator properties.

POLG2 genes have been identified in human, mouse, frog and fruit fly, but they are missing in the genomes of *Saccharomyces cerevisiae* and *Caenorhabditis elegans* [132]. *POLG2* disease mutation are rare and in most cases reported in patients suffering from PEO [133]. *POLG2* is absolutely required for viability and mtDNA maintenance and its loss leads to embryonic lethality around E8.0–8.5 [134], similar to deficiencies in other mitochondrial proteins necessary for mitochondrial maintenance, including POLG [135]. Knockdown of *POLG2* in cultured human cells increased nucleoid numbers, whereas its over-expression reduced the number and increased the size of mitochondrial nucleoids [136]. Both increased and decreased *POLG2* levels altered nucleoid structure and caused a marked decrease in 7S DNA molecules, which form short displacement-loops on mitochondrial DNA. Remarkably and in contrast to POLG, *POLG2* preferentially binds to plasmids with a short displacement-loop, supporting the view that the mitochondrial D-loop acts as a protein recruitment center, and suggesting that *POLG2* is a key factor in the organization of mitochondrial DNA in multigenomic nucleoprotein complexes [136].

2.3.3. Mitochondrial DNA helicase – TWINKLE

TWINKLE is a mitochondrial DNA helicase required for unwinding and subsequent separation of mtDNA strands [137]. TWINKLE has been originally identified in a screen for mutations in patients bearing mtDNA deletions [138]. TWINKLE mutations co-segregate with PEO, hepatocerebral syndrome associated with mtDNA deletions, spinocerebellar ataxia and Perrault syndrome. Muscle-specific conditional knockout mice showed that TWINKLE is the unique mtDNA replicative helicase indispensable to embryonic development and lack of TWINKLE results in a rapid loss of mtDNA [139]. Animals overexpressing TWINKLE with PEO patient mutations recapitulate phenotype presented by human patients with accumulation of mtDNA deletions and late-onset progressive respiratory deficiency [140]. Mice overexpressing wild type TWINKLE show 3-fold elevated

mtDNA levels and are protected against heart failure [140]. Similarly, overexpression of TWINKLE in cardiomyocytes of mice partially deficient in *SOD2* (*Sod2 +/-*) prevents stalling of replication, decreases mtDNA point mutation load and ameliorates the late onset cardiomyopathy [141]. This finding indicate that beside the role in fluency and precision of the mtDNA synthesis under normal conditions, TWINKLE could be important for the accuracy of mtDNA replication under various stresses.

2.3.4. Mitochondrial single-strand DNA binding protein – MTSSB

Although SSBP (single-stranded DNA-binding proteins) do not possess direct catalytic activity they are key players in the DNA metabolism as they prevent formation of secondary structures and premature re-annealing and protect DNA from nucleolytic damage. Accordingly, MTSSB plays an important role in mtDNA replication and repair. This small protein forms tetramers that coats single-stranded stretches in mtDNA and thus maintain two strands of double helix separated stimulating the activity of POLG and TWINKLE [137]. MTSSB directs the origin specific initiation of mtDNA replication both *in vitro* and *in vivo* [142]. Functional interactions between POLG and MTSSB in *Drosophila* embryos greatly enhance the overall activity of POLG by increasing primer recognition and binding, and stimulating the rate of initiation of DNA strands [143]. Correspondingly, knockdown of *Mtssb* in Schneider cells, leads to growth defects and depletion of mtDNA [143]. Recently an important role for MTSSB in BER has been proposed, as it was suggested that the massive amount of MTSSB in mitochondria effectively prevents processing of uracil and other types of damaged bases to avoid introduction of nicks in single-stranded mtDNA formed during replication [144]. Local enrichment of UNG1 at DNA-bound MTSSB may furthermore facilitate rapid access to- and processing of the damage once the dsDNA conformation is restored [144]. This could be of potential biological importance, since mitochondria have no or limited capacity for homologous recombination to process nicks at the replication fork.

2.3.5. Mitochondrial RNA polymerase – POLRMT

Beside its essential and basic function in mitochondrial transcription, POLRMT is also important and distinct role in mtDNA replication [145]. Indeed, deletion of a distinctive N-terminal extension of unknown function in the yeast POLRMT homologue (RPO41) does not affect the mitochondrial transcription, but results in the loss of mtDNA [146]. Although it remains to be tested if the amino-terminal extension in human POLRMT has also an exclusive role in the mtDNA replication, this result indicates that POLRMT might also be considered as a possible locus for mtDNA replication-related human diseases.

2.4. Quality and quantity of dNTP pools

MtDNA maintenance depends not only on essential processing factors but also on the availability and quality of basic units required for DNA synthesis, dNTPs. Taking into account that mitochondria are separated from the rest of the cell, they have to also sustain and guard their own pool of nucleotides in order to synthesize nucleic acid and provide intermediates for metabolic reactions. Asymmetry in dNTP pools mainly causes mitochondrial DNA depletion syndromes (MDS), although in some cases increased levels of mtDNA mutations have also been described [147,148]. *TK2* (thymidine kinase 2), *SUCLA2* [adenosine diphosphate (ADP)-forming succinyl CoA ligase beta subunit], *SUKLG1* [guanosine diphosphate (GDP)-forming succinyl CoA ligase alpha subunit], *RRM2B* (ribonucleotide reductase M2 B subunit), *DGUOK* (deoxyguanosine kinase), and *TYMP* (thymidine phosphorylase) encode proteins that maintain the mitochondrial dNTP pool; and mutations in any of these genes result in depleting the mitochondria from DNA building blocks and, subsequently, can contribute to elevated mtDNA mutability or decrease the mtDNA synthesis rates, ultimately

resulting in mtDNA loss [148]. The dNTP pool asymmetry of mammalian mitochondria seems to be tissue specific, with dGTP present at a much higher concentration than dATP in mitochondria of postmitotic tissues, including heart and skeletal muscle, possibly increasing the frequency of G:T mismatches found in these organs [149]. Remarkably, these pool imbalances do not differ between young and old animals [149]. It is not known if dNTP pool imbalances play any role in the generation of the other specific types of mtDNA mutations that occur with age, such as transversion or deletion mutations [149].

Mitochondrial dNTP pools are maintained either through active transport of cytosolic dNTPs or through the purine and pyrimidine salvage pathways by action of two mitochondrial deoxyribonucleoside kinases, thymidine kinase 2 (*TK2*) and deoxyguanosine kinase (*DGUOK*). *TK2* and *DGUOK* mediate the first, and rate-limiting, steps in the phosphorylation of pyrimidine and purine nucleosides, respectively, in the mitochondrial matrix [150]. Mutations in both *TK2* and *DGUOK* genes lead to decreased synthesis of DNA building blocks inside mitochondria leading to mtDNA depletion syndrome in human infants. Although majority of *TK2* related MDS have purely myopathic form [151] an encephalomyopathic presentation with hypotonia, weakness and epilepsy was also reported [152]. Currently, two forms of *DGUOK* deficiencies have been described, the hepatocerebral MDS, which presents as a multisystem disorder and an isolated hepatic disease later in infancy or childhood [148].

SUCLA2 and *SUCLG1* encode subunits of succinyl CoA ligase (*SUCL*) that catalyzes the reversible conversion of succinyl-CoA and ADP or GDP to succinate and adenosine triphosphate or guanosine triphosphate. *SUCL* also forms a complex with the mitochondrial nucleoside diphosphate kinase, and the lack of this complex formation in *SUCL* deficiency has been suggested to disturb the kinase function, resulting in decreased mtDNA synthesis leading to depletion [153]. Mutations in both genes lead to a mitochondrial disorders manifesting as early onset encephalomyopathy (*SUCLA2*) or severe acidosis with lactic aciduria leading to death within 2–4 days postnatal (*SUCLG1*) [153].

RRM2b encodes the p53-inducible small subunit (p53R2) of ribonucleotide reductase, a cytosolic enzyme that catalyzes the terminal step of *de novo* synthesis of deoxyribonucleoside. As mutations in *RRM2b* are associated with severe depletion of mtDNA in both humans and the gene is expressed in post-mitotic cells it has a key function in the maintenance of dNTP pools needed for mtDNA replication, although the precise mechanism leading to this, primarily mitochondrial function are not fully understood [154].

Thymidine phosphorylase (*TYMP*) is part of the cytosolic pyrimidine salvage pathway required for the reversible reaction catalysing thymidine and phosphate to thymine and deoxyribose-1-phosphate. Remarkably, mutations in this gene cause MNGIE, mitochondrial neurogastrointestinal encephalopathy caused by the accumulation of excess thymidine in the blood, that is uptaken by mitochondria, where it stimulates the synthesis of unbalanced and elevated dTTP levels by mitochondrial thymidine kinase 2 (*TK2*). The imbalance caused by increased dTTP levels results in mtDNA depletion, often accompanied by multiple deletions [155]. Interestingly, skin fibroblasts from MNGIE patients do not show any signs of depletion or mtDNA deletions, yet presented an OXPHOS deficiency, that is a result of numerous mtDNA point mutation [147]. The majority of mtDNA mutations found in tissues from MNGIE patients are T-to-C transitions preceded by a short run of “As” [147]. These results support a mutagenic mechanism involving competition between dGTP and dATP for incorporation opposite to T [147].

The adenine nucleotide translocator (*ANT*) is a very abundant mitochondrial protein that primarily catalyses ADP/ATP exchange across the inner membrane. Mutation in *ANT1* (or *SLC25A4*), the heart and skeletal muscle specific isoform, have been associated with diseases, including autosomal dominant PEO, mitochondrial myopathy and cardiomyopathy, Sengers syndrome and Facioscapulohumeral muscular dystrophy (*FSDH*) (for review [156]). Multiple models have

been proposed to explain the *ANT1*-induced pathogenesis. Studies from yeast have suggested that in addition to altered nucleotide transport properties, the mutant proteins cause a global stress on the inner membrane. The mutant proteins likely interfere with general mitochondrial biogenesis in a dominant-negative manner, which secondarily destabilizes mtDNA [156].

2.5. A quality control for mtDNA molecules?

When the surplus of mtDNA damage exceeds the repair and buffering capacities of mitochondria, leading to respiratory chain deficiency and mitochondrial dysfunction, mutated mtDNA should be deleted from pool. So far there is no evidence that mutated mtDNA could be selectively degraded by any of the mitochondrial nucleases. Therefore damaged mtDNA has to be removed by some large-scale mechanism recognizing deleterious product of mtDNA genes expression, that is – defective respiratory chain. Combined action of mitochondrial fission and mitophagy could provide an elegant, self-purifying mechanism for removal of defective mtDNA molecules from the cellular pool. Nonetheless, mtDNA deletions often constitute a substantial proportion of total mtDNA array in patients with mitochondrial disorders and mtDNA mutations accumulate in both, disease and ageing. Therefore, it seems difficult to justify a significant role of mitophagy in mitochondrial quality control when persistent retention of mutated mtDNA, hosted by defective mitochondria is very common. Tissue specificity, stage of development and distinction between mtDNA deletions and point mutations can be substantial in a discourse of mtDNA damage fate in various cell types, especially in post-mitotic versus dividing somatic cells. Rigid retaining of each mtDNA mutation type could result from different selective advantages. Finally, the turnover of individual mtDNA molecules can vary from one cell type to another and could decline in a course of aging. Recently, possible mechanisms for expansion and fixing mtDNA mutations in a light of current findings have been reviewed in details [157–159] and will not be discussed in more details here.

3. Conclusions

In recent years, many novel factors contributing to formation of mtDNA mutations have been discovered. In many cases, the precise function and molecular mechanisms leading to mtDNA mutation accumulation are still not completely understood. What became clear is that unfaithful mitochondrial DNA synthesis is the main contributor to the mtDNA mutagenesis, while oxidative damage seems to play only minor role in this process. Further understanding of molecular mechanism of action will likely lead to discovery of potential therapeutic targets with hope of finding the new ways of treating mitochondrial disorders.

Transparency document

The [Transparency document](#) associated with this article can be found, in online version.

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