

# Mitochondrial DNA maintenance: an appraisal

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**Abstract** Mitochondria play a crucial role in a variety of cellular processes ranging from energy metabolism, generation of reactive oxygen species (ROS), and Ca<sup>2+</sup> handling to stress responses, cell survival, and death. Malfunction of the organelle may contribute to the pathogenesis of neuromuscular disorders, cancer, premature aging, and cardiovascular diseases, including myocardial ischemia, cardiomyopathy, and heart failure. Mitochondria are unique as they contain their own genome organized into DNA–protein complexes, so-called mitochondrial nucleoids, along with multiprotein machineries, which promote mitochondrial DNA (mtDNA) replication, transcription, and repair. Although the organelle possesses almost all known nuclear DNA repair pathways, including base excision repair, mismatch repair, and recombinational repair, the proximity of mtDNA to the main sites of ROS production and the lack of protective histones may result in increased susceptibility to oxidative stress and other types of mtDNA damage. Defects in the components of these highly organized machineries, which mediate mtDNA maintenance (replication and repair), may result in accumulation of point mutations and/or deletions in mtDNA and decreased mtDNA copy number impairing mitochondrial function. This review will focus on the mechanisms of mtDNA maintenance with emphasis on the proteins implicated in these processes and their functional role in various disease conditions and aging.

**Keywords** CVD · mtDNA · Nucleoid · mtDNA replication · mtDNA repair

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## Introduction

Mitochondrial structural and functional alterations are implicated in the pathogenesis of a spectrum of conditions, such as neuromuscular [specifically, progressive external ophthalmoplegia (PEO)], cardiovascular [namely, myocardial ischemia, cardiomyopathies, and heart failure (HF)], and degenerative disorders, such as aging and cancer [1–5]. Mitochondria are unique organelles containing their own genome (mtDNA) along with multiprotein assemblies, which promote mtDNA replication, transcription, and repair [6–9]. mtDNA is compacted inside the mitochondria and packaged into DNA–protein complexes, termed mitochondrial nucleoids [10–12]. Mitochondrial nucleoid is a relatively stable genetic element, with an estimated average of mtDNA copy number in human cells between 1.4 and 7.5 per nucleoid [12].

Progress in mitochondria isolation and highly sensitive mass spectrometry (MS)-based proteomic techniques has resulted in the identification of multiple nucleoid-associated proteins (NAPs) [13]. Major mammalian NAPs include mitochondrial single-stranded (SS) DNA-binding protein (mtSSB), transcription factor A of mitochondria (TFAM, also known as mtTFA), both are the most abundant and tightly associated nucleoid proteins, mitochondrial DNA polymerase  $\gamma$  (POL $\gamma$ ) mitochondrial RNA polymerase (POLRMT), and mitochondrial DNA helicase Twinkle [11–13]. In addition to their role in mtDNA packaging, POL $\gamma$ , mtSSB, POLRMT, and Twinkle are major components of the mitochondrial replisome, which promotes mtDNA replication [9, 14].

Advances in genomics and proteomics technologies have shown that the human mitochondria contain over 1500 distinct proteins [15, 16]. Only 13 of these proteins, which are subunits of the electron transport chain (ETC),

are encoded by mtDNA, while the vast majority of mitochondrial proteins, including those implicated in mtDNA maintenance, are encoded by the nuclear genome (nDNA), synthesized on cytosolic ribosomes and imported through complex pathways [17, 18]. Thus, mitochondrial function depends on the qualitative and quantitative status of the mitochondrial genome and its intensive interaction with the nuclear genome to coordinate expression and assembly of numerous proteins encoded by both genomes [19, 20]. Notwithstanding, the role of mtDNA mutations as well as the alterations in the machineries implicated in mtDNA maintenance in the pathogenesis of degenerative and CVD remains largely unclear.

In this paper, we will first overview the mitochondrial replisome, with emphasis on the proteins implicated in both mtDNA replication and repair, such as POL $\gamma$ , mtSSB, TFAM, and mitochondrial DNA helicases, followed by a review of the mechanisms of mtDNA repair and their functional roles in health and disease.

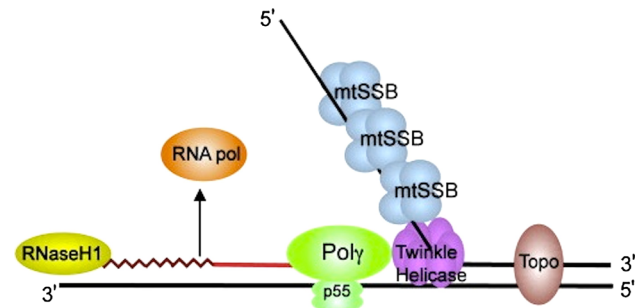
### Replication of mtDNA

Replication of mtDNA, vital for mitochondrial maintenance, is promoted by a multiprotein machinery, the mitochondrial replisome. The replicative helicase Twinkle acts ahead of the mtDNA replication fork translocating on one DNA strand in the 5'–3' direction and unwinding dsDNA. The formed ssDNA loop is coated by tetrameric mtSSB. Using the ssDNA released by Twinkle as a template, the POL $\gamma$  holoenzyme catalyzes the extension of the RNA primer synthesized by POLRMT; the primer is then degraded by RNase H1. Finally, topoisomerases relieve the torsional tension in mtDNA generated by its unwinding (Fig. 1) [21].

The basal mtDNA replication machinery, which has been reconstituted *in vitro* with purified recombinant proteins, consists of POL $\gamma$  holoenzyme, mtSSB, the replicative DNA helicase Twinkle, and POLRMT [9, 22, 23]. Among these core replicative proteins, POL $\gamma$ , Twinkle, and POLRMT share homology with the bacteriophage T7, the gp5 DNA polymerase, the gp4 primase-helicase, and the gp1 RNA polymerase, respectively, while mtSSB is a homolog of eubacterial SSBs [24].

#### DNA polymerase $\gamma$

The only DNA polymerase found in mammalian mitochondria is the POL $\gamma$ , the enzyme promoting mtDNA replication and repair [25, 26]. The POL $\gamma$  holoenzyme is a 245-kDa heterotrimer composed of a catalytic subunit (POL $\gamma$  A also referred to as POLG1) and two accessory subunits (POL $\gamma$  B also called POLG2) [27, 28]. 140-kDa POL $\gamma$  A is encoded by the *POLG1* gene and 55-kDa POL $\gamma$



**Fig. 1** Schematic diagram of a mitochondrial DNA replication fork showing the critical proteins required for DNA replication. The nascent DNA synthesized by DNA polymerase  $\gamma$  [POL  $\gamma$ /p55 (green)] is shown as a solid red line, while the RNA primer (jagged red line) created by the mitochondrial RNA polymerase (orange) is being degraded by RNase H1 (yellow). The mitochondrial DNA helicase (purple) unwinds the downstream DNA forming a single-stranded loop, which is coated with mtSSB (light blue). Topoisomerases (brown) work to relieve torsional tension in the DNA created by unwinding. Adapted from Kasiviswanathan et al. [21] with permission from Elsevier

B is encoded by the *POLG2* gene. POL $\gamma$  A has three polymerase motifs mediating DNA synthesis and a 3'  $\rightarrow$  5'-exonuclease domain promoting proofreading during replication. POL $\gamma$ B tightens DNA binding and increases the processivity of DNA synthesis. One POL $\gamma$ B subunit located close to POL $\gamma$ A enhances POL $\gamma$ A-DNA binding, while the more distantly located POL $\gamma$ B subunit accelerates polymerization rate [29–32].

#### mtSSB

mtSSB (the mammalian ortholog of yeast Rim1p) is tightly bound to mtDNA in a high-salt resistant fashion and copurifies with nucleoids isolated from human cells [33–36]. Human mtSSB shares sequence and structural homology with *Escherichia coli* SSB [37]. According to crystal structure analysis, human tetrameric mtSSB is wrapped by SS mtDNA [38]. mtSSB is an abundant NAP; the presence of thousands of mtSSB molecules/mtDNA has been reported, although it might be an overestimation [12, 39].

Deletion of yeast mtSSB or RNAi-mediated ablation of *Drosophila* mtSSB has led to a total loss or significant depletion of mtDNA, respectively, highlighting the critical role of mtSSB in mtDNA replication and maintenance [40–42]. Also, mtSSB may play a role in the stabilization of the D-loop structure and mtDNA replication intermediates.

#### TFAM

Human TFAM (the orthologue of yeast Abf2p) was originally purified and characterized as a mitochondrial transcription factor [43]. Subsequently, it has been cloned and

**Table 1** Human DNA helicases potentially involved in mtDNA maintenance

Helicase	Molecular size, kDa (aa)	Gene/chromosomal locus	Unwinding polarity	Other activities	Localization
Twinkle	~77 (684)	<i>C10orf2</i> (also known as <i>PEO1</i> or <i>TWINKLE</i> ) 10q24	5′–3′	ATP-independent strand annealing	Mitochondria
DNA2	~120 (1060)	<i>DNA2</i> 10q21.3-q22.1	5′–3′	ATP-independent strand annealing 5′–3′ endonuclease 3′–5′ exonuclease	Nuclei, mitochondria
PIF1	~70 (641)	<i>PIF1</i> 15q22.31	5′–3′	ATP-independent strand annealing	Nuclei, mitochondria
RECQL4	~130 (1208)	<i>RECQL4</i> 8q24.3	3′–5′	ATP-independent strand annealing	Nuclei, mitochondria

identified as a member of the high-mobility group (HMG)-box protein family containing two HMG-boxes flanking a linker region and a 29 residue carboxyl-terminal tail required for activation of transcription [44–46].

TFAM is the main factor responsible for mtDNA packaging and fulfills the most stringent definition of a structural component of mitochondrial nucleoid [13]. Like other HMG proteins, TFAM binds, wraps, and bends dsDNA in a sequence-nonspecific manner [47]. TFAM binding to mtDNA induces DNA flexibility and promotes its compaction [48]. It has been demonstrated that TFAM binds mtDNA as a monomer [49, 50] occupying ~20–30 bp of mtDNA [48, 51]. Surface plasmon resonance analysis suggests that TFAM monomers can slide along mtDNA, but upon reaching a patch of TFAM they become immobile and aggregate forming higher order multimers [48]. Like mtSSB, TFAM is a highly abundant NAP, its amount appears to be sufficient to coat mtDNA in mouse and human cells [39, 51–53].

Transgenic mouse models confirm the essential role of TFAM in packaging and maintenance of mtDNA. *Tfam*<sup>-/-</sup> mice exhibit early embryonic lethality associated with a loss of mtDNA, while *Tfam*<sup>+/-</sup> mice have approximately twofold reduced mtDNA content [54, 55]. Consistently, human TFAM overexpression in mice has upregulated mtDNA copy number [53].

### DNA helicases

DNA helicases are ubiquitous and evolutionary conserved enzymes, which utilize the energy derived from ATP hydrolysis to catalyze unwinding of the duplex DNA [56–58]. Multiple DNA helicases are expressed in mammalian cells and participate in all DNA transactions, including replication, transcription, repair, and recombination [58–62]. Alterations in their expression and/or activity can have detrimental consequences to the cell contributing to the pathogenesis of various human diseases, including CVD. This section will focus on DNA helicases, which are

involved in mtDNA maintenance, such as Twinkle as well as the less characterized DNA2, PIF1, and RECQL4 (Table 1).

Typical replicative DNA helicases generate in the presence of Mg<sup>2+</sup> and/or NTP cofactors ring-shaped hexamers with a ~12–14 nm ring diameter [63]. Some replicative DNA helicases require the assistance of auxiliary factors, helicase loaders, for loading onto DNA, while other helicases, such as simian virus 40 large tumor (SV40 T) antigen and bacteriophage T7 gene 4 primase-helicase (T7 gp4), are able to load onto DNA without the help of a helicase loader [63–65]. Ring-shaped helicase hexamers have a central channel of ~2–4.5 nm capable to accommodate one DNA strand and displace the second DNA strand [63, 66, 67].

### Twinkle

The acronym Twinkle stands for T7 gp4-like protein with intramitochondrial nucleoid localization and is linked to its ability to form punctuate fluorescence in mitochondria resembling twinkling stars. The mtDNA helicase Twinkle belongs to the superfamily 4 (SF4) of bifunctional primase-helicases, which form ring-shaped hexamer structures to unwind DNA by the strand exclusion mechanism [68]. *Escherichia coli* replicative DNA helicase DnaB, main recombinase RecA, and the T7 gp4 essential for T7 DNA replication also belong to this superfamily. Twinkle, encoded by the *C10orf2* gene (also known as *PEO1* or *TWINKLE*) was originally identified as a locus responsible for PEO [69].

Twinkle shares 46 % amino acid sequence similarity and 15 % sequence identity with T7 gp4 [69, 70], and like T7 gp4, Twinkle has a modular molecular architecture: an N-terminal domain containing primase-like motifs connected by a short linker region to a C-terminal helicase domain characteristic to the SF4 helicases (Fig. 2a) [71].

The N-terminal domain contributes to efficient ssDNA binding and helicase activity [72]. The C-terminal region

of Twinkle containing five highly conserved helicase motifs displays the highest homology to T7 gp4 (56 % amino acid sequence similarity) [69]. While Arabidopsis phage T7 gp4/Twinkle homologue has both DNA primase and DNA helicase activities, human Twinkle lacks critical amino acid sequences found in T7 gp4 and does not have primase activity [33, 69, 73, 74].

Twinkle can form hexa- and heptamers; in contrast to T7 gp4, this oligomerization does not require the presence of NTP [71, 75]. However, in the presence of MgUTP, Twinkle assembles predominantly into hexamers [76]. The linker region of Twinkle is critical for its oligomerization, while the N-terminal domain contributes to hexamer stabilization [71, 72, 77]. Unlike the majority of replicative DNA helicases, Twinkle is able to load onto a closed circular ssDNA without the assistance of loader factors [78]. Although the precise mechanism of this process is unknown, it has been hypothesized that the ring-shaped Twinkle heptamer loses one of its subunits upon contact with DNA, the heptamer ring opens and encircles the DNA [63, 65, 76]. In addition, it is possible that a specific loader exists to enhance the loading efficiency of Twinkle during mtDNA replication initiation in vivo [76].

In vitro, Twinkle can unwind short (<20 bp) dsDNA fragments with 5′–3′ directionality in an NTP-dependent fashion [79–81]. It utilizes DNA fork-like substrates with 5′ and 3′ tails, but 5′-tail structures appear to be preferable substrates [68, 76, 79, 80, 82]. However, the functional significance of such substrate preference remains unclear. The presence of mtSSB or an ssDNA complementary to the unwound strand highly stimulates the Twinkle unwinding activity [76, 79]. Regardless of the loading mechanism, Twinkle once loaded on an mtDNA in the presence of mtSSB and POL $\gamma$  becomes a very processive helicase, unwinds dsDNA at the replication fork and supports a complete round of mtDNA replication without stalling and creating deletions in vitro [22, 78, 83].

In addition to helicase activity, Twinkle can catalyze an antagonistic reaction—annealing of two complementary ssDNA molecules [76]; it promotes this reaction in the presence or absence of cofactors, such as NTP and Mg<sup>2+</sup>. Although Twinkle binds both ssDNA and dsDNA with high affinities, the annealing activity can be inhibited only by ssDNA, implying the existence of distinct binding sites for ssDNA and dsDNA. Furthermore, using fluorescence anisotropy completion binding analysis, the presence of multiple ssDNA-binding sites on Twinkle has been suggested; one of these sites, exposed to an outer surface of ring-shaped Twinkle hexamers may be involved in ssDNA annealing [76].

Several other human DNA helicases also mediate complementary DNA strand annealing [62, 84–87]. Although the physiological significance of this ability

remains to be determined, it has been suggested that helicase-mediated DNA annealing may be implicated in the repair of damaged DNA replication forks or in the initiation of recombination-mediated replication [76, 85, 88]. Given the structural homology between Twinkle and *E. coli* recombinase RecA, a role for mitochondrial Twinkle in recombination events has been suggested. Consistent with this hypothesis, its overexpression in human cardiomyocytes (see below) has led to significant increase in mtDNA recombination intermediates [88].

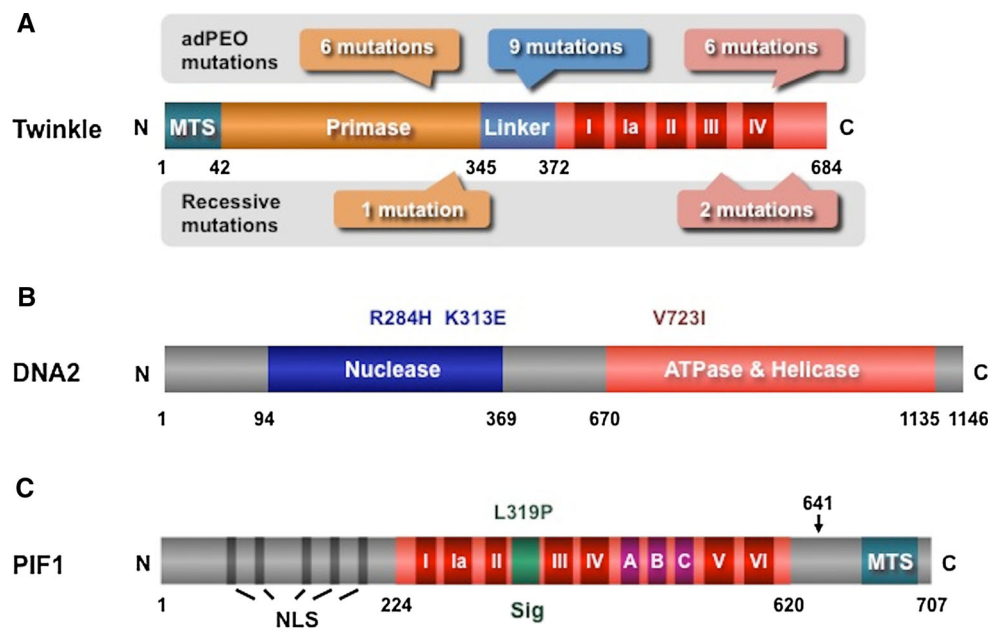
## DNA2

DNA2 is an evolutionary conserved DNA helicase/endonuclease, which was originally described in yeast *Saccharomyces cerevisiae* (yDna2) and subsequently in higher eukaryotes, including humans [89–94]. The yDna2 is a 172-kDa essential protein composed of the conserved nuclease, ATPase, and helicase domains [89, 95]. Importantly, the yDna2 endonuclease activity is indispensable, while its helicase activity is not, suggesting a more fundamental role for yDna2 endonuclease in vivo [96]. yDna2 endonuclease specifically cleaves ssDNA flaps, whereas its helicase activity is able to unwind dsDNA, forming 5′ flap DNA structures [95–97]. Interaction of the N-terminal region of yDna2 with ssDNA-binding replication protein A (RPA) stimulates yDna2-mediated cleavage of flap DNA substrates [97]. Genetic and biochemical studies suggest that yDna2 in cooperation with flap endonuclease 1 (Fen1) is involved in the processing of Okazaki fragments [98] during lagging strand replication [97, 99]. Furthermore, yeast *dna2* mutants are highly sensitive to DNA-damaging agents, such as X-ray irradiation and methyl methanesulfonate (MMS), suggesting that yDna2 is implicated in DNA double-strand break (DSB) and postreplicative repair [100].

hDNA2, the human orthologue of yDna2, is a ~120-kDa protein encoded by the *DNA2* gene [93, 94]. It has conserved nuclease/ATPase/helicase domains; however, in contrast to yDna2, it lacks the N-terminal RPA binding motif and nuclear localization sequences (NLS) (Fig. 2b) [93, 94, 101]. Similar to its yeast orthologue, hDNA2 displays in vitro ATPase, flap endonuclease, and 5′–3′ helicase activities [93, 94]. Like Twinkle, hDNA2 is also able to promote annealing of two complementary DNA strands in an ATP-independent manner. Interestingly, mutant hDNA2 variants, with deficient of either nuclease or ATPase/helicase activity, are able to catalyze single-strand annealing [102]. While the physiological role of hDNA2 strand annealing activity is unclear, it might be important for the function of hDNA2 in recombinational repair and/or telomere maintenance.

Consistent with the absence of NLS, it has been shown that hDNA2 is exclusively mitochondrial rather than a





**Fig. 2** The molecular structure of the human DNA helicases implicated in mitochondrial DNA replication and maintenance. **a** Twinkle helicase: the N-terminal region, containing mitochondrial targeting sequence (MTS) and primase-related domain, linker, and helicase motifs (I, Ia, II, III, and IV) are depicted. Dominant mutations responsible for autosomal dominant progressive external ophthalmoplegia (adPEO) and recessive mutations are also schematically shown. **b** DNA2 helicase: the conserved nuclease and ATPase/helicase domains are shown. Amino acid substitutions R284H and K313E within nuclease domain and V723I in ATPase/helicase

domain are also depicted. **c** PIF1 helicase contains the N-terminal nuclear localization sequences (NLS), helicase domain, containing the conserved SF1 motifs (I, Ia, II, III, IV, V and VI) and 3 motifs (a–c) shared with *E. coli* RecD helicase, a unique 21-amino acid signature sequence (Sig), and the C-terminal mitochondrial targeting sequence (MTS). Amino acid substitution L319P in a signature sequence and position of alternative splicing, which generates a 641-aa C-terminally truncated nuclear PIF1 $\beta$  are also shown. See text for details

nuclear protein [101]. In mitochondria, hDNA2 physically interacts with POL $\gamma$  and its helicase activity stimulates POL $\gamma$ -promoted displacement DNA synthesis, while hDNA2 endonuclease in cooperation with hFEN1 appears to be involved in processing RNA primers during mtDNA replication. In addition, hDNA2 and hFEN1 are synergistically implicated in intermediate processing in long-patch base excision repair (LP-BER) in mitochondria (see below). However, an exclusive mitochondrial localization of hDNA2 is still questionable; Duxin et al. [103] have presented immunohistochemical and biochemical evidence that hDNA2 is localized to both the mitochondria and the nucleus. Although the reason for the discordance in regard to the existence of nuclear hDNA2 remains unclear, it might be partially explained by the fact that different amounts of hDNA2 have been detected within the nucleus depending on the cell line and by the different antibodies employed in these studies [103]. Mitochondrial subfraction of hDNA2 is partially colocalized with the inner mitochondrial membrane (IMM) as well as with Twinkle. Importantly, the expression of disease-associated Twinkle mutants has led to the accumulation of hDNA2 in mitochondrial nucleoid, suggesting a crosstalk between these

two helicases [103]. Furthermore, hDNA2 silencing by RNAi resulted in reduction in mtDNA replication intermediates and in the efficiency of mtDNA repair. Finally, hDNA2 depletion has also been associated with accumulation of aneuploid cells and the appearance of internuclear bridges, suggesting that nuclear hDNA2, similar to its yeast orthologue, is implicated in genomic DNA and/or telomere maintenance [103].

In a follow-up study, Duxin et al. [104] have further explored the role of hDNA2 in genomic DNA replication and maintenance. They found that hDNA2 interacts with the replisome protein And-1 specifically in replicating, but not in resting human cells, contributing to Okazaki fragment processing. Importantly, both its helicase and nuclease activity are essential for this function and neither helicase nor nuclease hDNA2-deficient variants have been able to rescue hDNA2 depletion. Surprisingly, while depletion of the major replicative endonuclease FEN1 resulted, as expected, in reduced Okazaki fragment maturation, hDNA2 depletion has not led to a defective Okazaki fragment processing [104]. Moreover, in contrast to yeast, FEN1 overexpression has not been able to rescue genomic instability in hDNA2-depleted human cells. These findings

suggest that hDNA2 and FEN1 play distinct roles in DNA maintenance and replication [104].

Recently, it has been demonstrated that hDNA2 can be coimmunoprecipitated with well established nuclear replication factors including DNA polymerase  $\delta$ , the major polymerase implicated in the lagging strand nDNA synthesis, proliferating cell nuclear antigen (PCNA), RFC, and MCM [105]. Finally, recently discovered interactions between hDNA2 and several essential proteins involved in DSB repair pathway have provided mechanistical insights into its role in the maintenance of genomic stability (to be discussed later).

### PIF1

The Pif1 family represents an evolutionary conserved family of DNA helicases found in most eukaryotes from yeasts to humans [106–108]. In 1983, the first *PIF* gene was discovered in yeast *S. cerevisiae* in a genetic screen of genes essential for mtDNA maintenance [109]. Following this, it has been shown that the protein ScPif1, encoded by this gene, is a DNA helicase unwinding DNA substrates in the 5′–3′ direction [110]. Moreover, the *S. cerevisiae* has a second *Pif* gene, termed *RRM3* [111, 112]. Both proteins, ScPif1 and ScRrm3, are localized to mitochondria and nuclei. ScPif1 is implicated in mtDNA maintenance and its nuclear form is associated with a replisome and also inhibits telomerase, preventing *de novo* telomere formation during DSB repair. ScRrm3 plays a role in genome stability, while its mitochondrial function is currently unclear [106, 107].

The Pif1 family helicases share characteristic molecular structure: 7 conserved SF1 motifs (I, Ia, II, III, IV, V, and VI) and 3 motifs (A, B, and C) shared with *E. coli* RecD helicase. In addition, the Pif1 family contains a unique 21-amino acid signature sequence located between SFI motifs II and III with yet unknown function (Fig. 2c) [107, 113].

Mammals appear to have a single gene encoding PIF1 helicase. Human *PIF1* gene encodes a ~70 kDa protein hPIF1 sharing 24 % sequence identity with ScPif1 and 84 % sequence identity with its mouse homologue (mPif1) [114]. hPIF1 contains the N-terminal NLS and the C-terminal mitochondrial localization signal (Fig. 2c) [115]. Intriguingly, neither N- nor C-terminal regions of hPIF1 display any significant homology to other known Pif1 helicases.

hPIF1 is expressed in highly proliferating cells; like ScPif1, its expression is tightly regulated during cell cycle picking in late G<sub>2</sub>/M phase [114]. Two splice hPIF1 isoforms have been found: 707-aa full-length hPIF1 $\alpha$  located predominantly to mitochondria and 641-aa C-terminally truncated nuclear hPIF1 $\beta$  (Fig. 2c) [115].

The expression and purification of full-length hPIF1 have turned out to be challenging, mainly due to

aggregation and low solubility of the full-length protein. Hence, for initial biochemical characterization, its N-terminally truncated fragment, containing the helicase domain, has been employed [116]. This recombinant N-truncated hPIF1 unwinds both DNA/DNA and DNA/RNA substrates in the 5′–3′ direction [117, 118].

hPIF1 is colocalized with telomeres *in vivo* and its N-truncated form binds telomeric TTAGCG repeats with a 100-fold higher affinity compared to random DNA sequences and inhibits telomerase processivity *in vitro* [117]. In addition, FLAG-tagged hPIF1 has been coimmunoprecipitated with Myc-tagged human telomerase catalytic subunit (hTERT) suggesting a physical interaction between these two enzymes and a role for hPIF1 in telomere metabolism [114]. Recently, the full-length hPIF1 has been successfully purified and biochemically characterized [118–120]. Deletion analysis has shown that the PIF1 N-terminal (PINT) domain significantly enhances ssDNA binding and also promotes DNA strand annealing [118]. Similar to Twinkle and DNA2, hPIF1 DNA annealing activity is ATP independent, and it is rather inhibited by ATP. Interestingly, in contrast to ScPif1, both helicase and annealing activity of hPIF1 are inhibited by RPA [118]. The ATPase activity of the full-length hPIF1, which provides the energy to translocate along DNA and unwind the duplex DNA, has also been characterized. Binding to ssDNA greatly stimulates hPIF1 ATPase activity and this stimulation increases with an increasing length of ssDNA, due to an increase in hPIF1-ssDNA binding [120].

Mice deficient in *mPif1* are viable, display no significant abnormalities, and normal life span [121]. Furthermore, treatment of *mPif1*<sup>-/-</sup> mouse embryonic fibroblasts (MEFs) with DNA-damaging agents, such as gamma-, UV-irradiation, or hydroxyurea, has not revealed any difference in their sensitivity compared to wild-type MEFs. Karyotyping of *mPif1*<sup>-/-</sup> MEFs or splenocytes has not detected any significant chromosomal rearrangements. Similar to hPIF1, mPif1 can be coimmunoprecipitated with mTERF and hTERF *in vitro*; however, *mPif1*<sup>-/-</sup> MEFs exhibited neither alterations in telomerase elongation activity nor in telomere lengths compared to wild-type MEFs [121]. These findings suggest that, in contrast to ScPif1, mPif1 has a dispensable redundant function, which might be compensated by the action of other mammalian helicases.

### Mitochondrial DNA repair

#### Sources and types of mitochondrial DNA damage

The maintenance of mtDNA integrity is critical for mitochondrial function and for cell and tissue viability. mtDNA damage can arise from both endogenous and exogenous

sources leading to mutations and alterations in mtDNA copy number. Major endogenous mtDNA damage is caused by ROS generated as byproducts during OXPHOS, which also target mitochondrial proteins and membrane lipids [122–127]. The close proximity of mtDNA to the main sites of ROS production and the lack of protective histones result in increased susceptibility of the mitochondrial genome to oxidative damage. Furthermore, in human cells, ROS-induced mtDNA damage is not only more extensive, but also persists longer following OS than nDNA damage [128].

Another source of point mutations and deletions in mtDNA is spontaneous errors of the mtDNA replication machinery [23, 129–131]. The intrinsic 3′–5′ exonucleolytic proofreading activity and high nucleotide selectivity of POL $\gamma$  are responsible for high fidelity of mtDNA replication [132, 133]. Also, aging-dependent accumulation of mtDNA mutations and deletion in human tissues appears to be caused by POL $\gamma$  spontaneous errors [134–136]. Furthermore, spontaneous replicative errors by POL $\gamma$  contribute to increased levels of mtDNA mutations as found in cancer cells [5]. Consistently, homozygous POLG exonuclease-deficient mice exhibit significantly higher frequencies of point mutations and deletions in mtDNA compared to aged-matched wild-type or heterozygous mice [137, 138].

Exogenous, environmentally induced, mtDNA damage can be caused by various genotoxic agents, such as industrial byproducts, ultraviolet and ionizing radiation, tobacco smoke, chemicals, environmental toxins, and therapeutic drugs [139–143]. A negative charge on the inner surface of the IMM, generated by  $\Delta\Psi_m$ , results in overwhelming accumulation (up to 1000-fold) of lipophilic cations in mitochondria [144]. Many toxic chemicals represent positively charged lipophilic substances and therefore tend to accumulate inside mitochondria affecting their function. Similar to ROS-mediated mtDNA damage, the lack of histones in mitochondria contributes to mtDNA vulnerability to exogenous genotoxic agents. These factors explain the significantly higher (10-fold or more) damage of mtDNA by alkylating agents, compared to that of nDNA [145].

A precise quantitative profiling of mtDNA damage represents an important but challenging task [143]. mtDNA constitutes a minor fraction of the total cell DNA, the relative amount of mtDNA to nDNA is different in different tissues. Furthermore, mtDNA lesions can undergo further alterations *in vivo* as well as during sample preparation. These difficulties have led in earlier studies to increasing artifacts, although the development of highly sensitive HPLC-MS, quantitative PCR, and repair enzyme-based techniques has allowed identification and quantification of various types of DNA lesions [146–153].

A wide spectrum of lesions, including base modifications, abasic [i.e., apurinic/apyrimidinic (AP)] sites, DNA strand breaks and DNA crosslinks, is generated upon oxidative DNA damage [146, 154]. Multiple ROS- and drug-induced defects have specifically been identified in mtDNA, which can lead to mtDNA mutagenesis in animal model and human disorders [143]. Among oxidative DNA damage, the 8-oxo-2′-deoxyguanosine (8-oxo-dG) has been one of the most studied DNA lesions. 8-oxo-dG is a mutagenic lesion: its mispairing with adenine leads to a G–C to T–A transversion upon a subsequent round of replication. Accumulation of 8-oxo-dG with age has been found in both nDNA and mtDNA with a greater extent in the latter [123, 155].

Oxidative-damaged DNA bases can lead to AP sites and to single-strand DNA breaks (SSBs), which are one of the most common types of mtDNA lesion [156–158]. ROS can damage not only bases, but also the sugar-phosphate backbone in mtDNA. OS induces SSBs in mtDNA with a higher frequency than in nDNA [158]. In addition to ROS-induced SSBs, they can also be formed in the process of repairing other DNA lesions [159]. Unrepaired SSBs can result in DNA replication fork stalling, increasing the risk of their conversion into DSBs, which are the most genotoxic DNA lesions [160, 161].

#### *Mitochondrial DNA repair pathways*

Despite the high vulnerability of mtDNA, it has been postulated that DNA repair is very inefficient or even absent in mitochondria. Damaged mtDNA molecules are degraded and replaced by newly synthesized DNA molecules using undamaged mtDNA copies as templates. Indeed, recently it has been shown that, in contrast to nDNA, degradation of unrepairable mtDNA, present in hundreds to thousands of copies per cell, represents a specific mitochondrial repair mechanism in response to OS [158, 162, 163]. Nevertheless, over the years, this view has changed and at present it is widely accepted that mammalian mitochondria possess almost all known nuclear DNA repair pathways, including base excision repair (BER), SSB repair, mismatch repair, and possibly homologous recombination (HR) and nonhomologous end joining (NHEJ) [23, 164–167]. Surprisingly, mitochondria lack a nucleotide excision repair system, able to repair bulky DNA lesions, including UV-induced photoproducts, cisplatin interstrand crosslinks, benzo[a]pyrene diol epoxide adducts, and some oxidative lesions [143, 168–173]. Albeit DNA repair mechanisms have primarily been studied in the cell nucleus, major pathways and proteins implicated in the maintenance of mtDNA have begun to be elucidated.

Several human mitochondrial proteins, which may contribute to various DNA repair mechanisms, have been

identified and their growing list is summarized in Table 2 [174–176]. None of the DNA repair proteins are encoded by mtDNA; on the contrary, they are encoded by nuclear genes and have to be imported to mitochondria. Furthermore, mitochondrial and nuclear DNA repair systems share many of these proteins and assessing their contributions to mtDNA maintenance is an important and challenging goal.

Here we will focus mainly on the mechanism of action and machinery of BER, the primary repair pathway in oxidative mtDNA damage. The potential role of other DNA repair mechanisms, such as mismatch repair and DNA break repair in mtDNA maintenance, has been recently reviewed in several excellent papers [23, 143, 165–167, 177].

### Base excision repair (BER)

BER is the predominant and best characterized mitochondrial DNA repair pathway, responsible for removal of non-helix distorting lesions and SSBs. BER repairs most common oxidative and alkylation damage in mtDNA, including 8-oxo-dG [178–182]. Importantly, the repair of 8-oxo-dG is more efficient in mitochondria than in the nucleus [183]. In the nucleus, BER comprises two subpathways, short-patch BER (SP-BER) and long-patch BER (LP-BER) [182]. Although only the SP-BER pathway, replacing a single damaged base, has initially been found in mitochondria, more recently LP-BER activity has been identified in mitochondrial extracts [181, 184–186]. Both mitochondrial SP-BER and LP-BER subpathways resemble the nuclear pathways and proceed through the following steps: lesion recognition, base excision, strand cleavage at the AP site, end processing, gap filling, and ligation (Fig. 3) [165, 167, 182]. These steps are promoted by the BER machinery assembled in a highly regulated fashion at the site of DNA damage. [182, 187, 188] A number of enzymatic activities promoting this repair pathway have been identified in human mitochondria (Table 2).

Base damage recognition followed by its removal is mediated by damage-specific DNA glycosylases, which hydrolyze the *N*-glycosylic bond between the damaged base and the DNA backbone. [189, 190] DNA glycosylases can be classified into two functional types: monofunctional enzymes, which remove damaged bases only from dsDNA cleaving the *N*-glycosylic bond and forming AP site due to their glycosylase activity; and bifunctional enzymes, which target damaged bases on both ssDNA and dsDNA cleaving the *N*-glycosylic bond and incising the formed AP site due to their glycosylase and intrinsic 3'AP-lyase activity [191]. Bifunctional DNA glycosylases play a predominant role in the repair of oxidative DNA damage.

Human mitochondria contain two monofunctional DNA glycosylases: uracil-DNA glycosylase 1 (UNG1, also

known as UDG1) and MUTYH glycosylase (also known as MYH; homologue of the *E. coli* mutY glycosylase) [192, 193]. Mitochondria-specific isoform UNG1 is produced by alternative splicing [194, 195].

Four bifunctional DNA glycosylases have been detected in mammalian mitochondria: 8-oxo-dG DNA glycosylase 1 (OGG1; a homologue of *E. coli* mutM), NTHL1 (also known as NTH1; a homologue of *E. coli* endonuclease III), NEIL1, and NEIL2 (homologues of *E. coli* Fpg and Nei, respectively) [196–199]. Two alternatively spliced isoforms of OGG1 have differential intracellular localization:  $\alpha$ -OGG1 is localized to both mitochondria and nucleus, while  $\beta$ -OGG1 is localized to mitochondria [200]. Mitochondrial targeting sequence of NTHL1 targets this glycosylase to mitochondria [197, 201, 202]. NEIL1 and NEIL2 are localized to both mitochondria and nucleus [198, 203, 204]. In humans, OGG1 is mainly responsible for removal of oxidized purines, including 8-oxo-dG, while NTHL1 and NEILs recognize and excise oxidized pyrimidines [203–205].

AP site, formed by the action of monofunctional UNG1, is incised 5' to the lesion by AP endonuclease APE1 resulting in the formation of an SSB with a 5'-blocking deoxyribosephosphate (dRP) group and 3'-OH [206, 207]. APE1 is localized to both mitochondria and nucleus, and mitochondria-specific APE1 isoform is generated by proteolytic cleavage of the N-terminal 33-amino acid NLS [208–210].

The 5'-dRP group is cleaved by POL $\gamma$ , which possesses not only 5'-3' exonuclease but also dRP-lyase activity, resulting in a SS gap with 5'P and 3'-OH [211]. SSB produced by the action of bifunctional OGG1/NTHL1 or NEILs is processed by the phosphodiesterase activity of APE1 or by polynucleotide kinase 3'-phosphatase, respectively [182, 199, 212]. In both cases, a SS gap with 5'P and 3'-OH is generated.

The final steps of this repair pathway can be completed via SP-BER or LP-BER subpathway. In the SP-BER, resulting single-nucleotide gap is filled by POL $\gamma$ . During the LP-BER, POL $\gamma$  displaces 5'-strand forming so-called 5'-flap of 6–9 nucleotides in length [181, 185, 186]. Processing of displaced 5'-flap resembles Okazaki fragment maturation and is mediated by the concerted action of proteins implicated in DNA replication [165, 213]. The flap endonuclease FEN1 removes short displaced 5'-flaps, while DNA nuclease/helicase DNA2 can process long 5'-flaps [101, 181, 185, 186]. Consistent with their essential role in LP-BER, mitochondrial extracts immunodepleted for hDNA2 or hFEN1 have been less efficient in an in vitro LP-BER assay [101]. In addition, it has recently been shown that mitochondrial 5' exo/endonuclease G (EXOG) can remove 5' blocking nucleotides in LP-BER pathway independently of DNA2 and FEN1 [214]. EXOG has

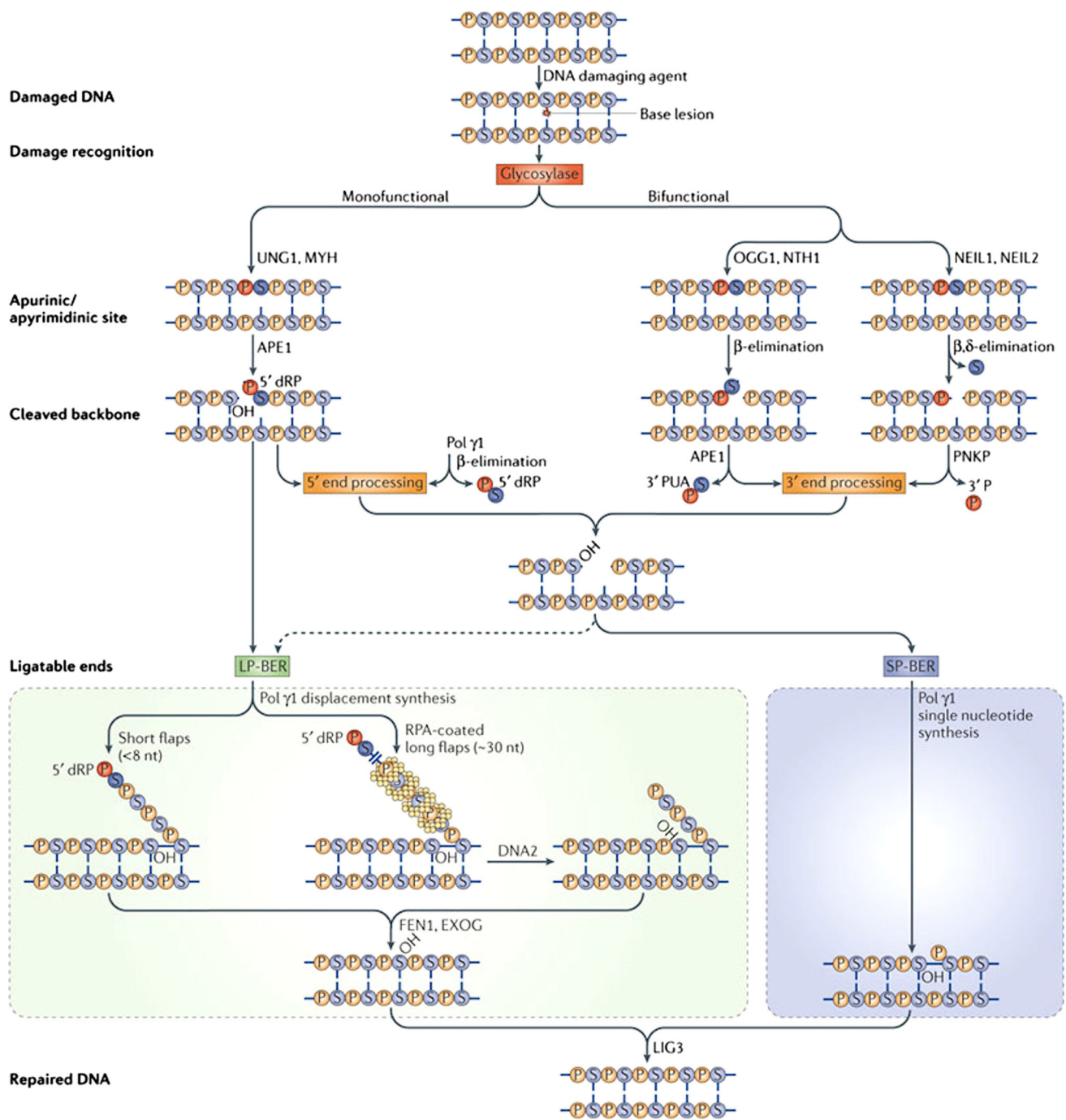


**Table 2** Human proteins potentially involved in mtDNA repair

Proteins	Gene/chromosomal locus	Functions
APE1 (also known as APE, Apn1)	<i>APEX1</i> 14q11.2	Apurinic/aprimidinic endonuclease; BER
UNG1 (also known as UDG1)	<i>UNG</i> 12q23-q24.1	Uracil-DNA glycosylase; monofunctional DNA glycosylase; BER
MUTYH (also known as MYH mutY homologue)	<i>MUTYH</i> 1p34.1	Monofunctional DNA glycosylase; BER
NEIL1 (also known as FPG1 or NEI1; Fpg/Nei homologue)	<i>NEIL1</i> 15q24.2	Bifunctional DNA glycosylase; BER
NEIL2 (also known as NEH2 or NEI2; Fpg/Nei homologue)	<i>NEIL2</i> 8p23.1	Bifunctional DNA glycosylase; BER
NTHL1 (also known as NTH1; endonuclease III homologue)	<i>NTHL1</i> 16p13.3	Bifunctional DNA glycosylase; BER
OGG1 (mutM homologue)	<i>OGG1</i> 3p26.2	8-Oxoguanine DNA glycosylase; bifunctional DNA glycosylase; BER
DNA2	<i>DNA2</i> 10q21.3-q22.1	DNA nuclease/helicase with strand annealing activity; LP-BER
FEN1 (also known as MF1 or RAD2)	<i>FEN1</i> 11q12	Flap structure-specific endonuclease; LP-BER
MLH1 (mutL homologue)	<i>MLH1</i> 3p21.3	Molecular matchmaker; MMR
MSH5 (mutS homologue)	<i>MSH5</i> 6p21.3	Mismatch recognizing protein; MMR
POL $\gamma$ A	<i>POLG1</i> 15q25	DNA polymerase $\gamma$ catalytic subunit
POL $\gamma$ B	<i>POLG2</i> 17q23-24	DNA polymerase $\gamma$ accessory subunit
mtSSB	<i>SSBP1</i> 7q34	Mitochondrial single-stranded DNA-binding protein
LIG3	<i>LIG3</i> 17q11.2-q12	DNA ligase III
TFAM (also known as mtTFA)	<i>TFAM</i> 10q21	Mitochondrial transcription factor A
RECQL4	<i>RECQL4</i> 8q24.3	DNA helicase with strand annealing activity
CSB (also known as ERCC6)	<i>ERCC6</i> 10q11.23	Cockayne syndrome group B protein with DNA binding and ATPase activity; BER (?)
EXOGL (also known as ENGL or ENDOGL1/2)	<i>EXOGL</i> 3p21.3	5'–3' exo/endonuclease; BER (?)
MGME1 (also known as DDK1)	<i>MGME1</i> 20p11.23	5'–3' exonuclease; BER (?)
TP53 (also known as p53 or TRP53)	<i>TP53</i> 17p13.1	Tumor suppressor protein; BER (?)

initially been discovered as a human paralogue of the yeast exo/endonuclease Nucp1 displaying ssDNA endonuclease and 5'–3' exonuclease activity [215]. Afterwards, it was demonstrated that its depletion affects the mitochondrial but not the nuclear genome, where FEN1 plays a main role,

and leads to mitochondrial dysfunction and apoptosis in various types of human cells. Furthermore, EXOG appears to interact physically with other repair proteins, such as POL $\gamma$ , APE1, and DNA ligase III [214]. Another recently discovered 5'–3' exonuclease MGME1 may also participate



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in this process; mutations in the *MGME1* locus have been identified in patients with multisystemic mitochondrial disease associated with mtDNA depletion and deletions [216, 217].

Finally, the processed 5' and 3' ends of mtDNA are sealed by mitochondrial DNA ligase III $\alpha$  completing SP-BER or LP-BER [218, 219]. Depletion of DNA ligase III $\alpha$  has resulted in reduced mtDNA integrity [220]. Intriguingly, mitochondria do not contain repair protein XRCC1, which

recruits DNA ligase I and/or DNA ligase III to complete BER in the nucleus; a mitochondrial factor for DNA ligase III $\alpha$  recruitment is currently unknown [143, 218, 221]. Importantly, significant reduction in levels of mitochondrial DNA ligase III in various cells derived from patients with Ataxia Telangiectasia has recently been found [222]. Moreover, it was hypothesized that this reduction compromises mtDNA repair and mitochondrial function and could contribute to neurodegeneration in these patients.

**Fig. 3** Base excision repair subpathways. Base lesions are recognized and removed by DNA glycosylases. Monofunctional DNA glycosylases remove damaged bases, and the DNA is subsequently cleaved by apurinic/apyrimidinic endonuclease 1 (APE1). Bifunctional DNA glycosylases go a step further and either cleave 3' to the damaged base ( $\beta$ -elimination) or remove the ribose altogether ( $\beta,\delta$ -elimination) and require the 3' end-processing activities of APE1 or polynucleotide kinase/phosphatase (PNKP), respectively. DNA gaps that have ligatable 5' ends only require single-nucleotide synthesis by DNA polymerase- $\gamma$ 1 (POL  $\gamma$ 1) for subsequent ligation by DNA ligase 3 (LIG3; short-patch base excision repair (SP-BER)), whereas 5' ends that cannot be ligated require POL  $\gamma$ 1-mediated displacement synthesis of two or more nucleotides to generate a single-stranded DNA (ssDNA) flap (long-patch base excision repair (LP-BER)). A small fraction of gapped DNA molecules containing 5' and 3' ends that could be repaired by SP-BER, nevertheless, still undergo LP-BER (represented by a dashed arrow). Short ssDNA flaps may be removed by flap endonuclease 1 (FEN1), whereas long ssDNA flaps may require DNA2 to first create a short stub, followed by full removal of the flap by FEN1. Alternatively, EXOG may be the major 5' flap-processing enzyme in mitochondria and may remove both short and long flaps. POL  $\gamma$ 1 and LIG3 are the only known polymerases and ligases involved in both replication and repair of mitochondrial DNA, and thus function in both SP-BER and LP-BER. 3' *PUA* 3'-phospho- $\alpha,\beta$ -unsaturated aldehyde, 5' *dRP* 5' deoxyribosephosphate, *MYH* MutY homologue, *NTH1* endonuclease III-like protein 1, *nt* nucleotide, *OGG1* 8-oxoguanine DNA glycosylase 1, *RPA* replication protein A, and *UNG1* uracil-DNA glycosylase 1. Adapted from Kazak et al. [165] with permission of nature publishing group

In addition to Twinkle, DNA2, and PIF1 helicases, human DNA helicase RECQL4, a member of the conserved RECQ helicase family, has recently been found to localize to mitochondria and play a role in the maintenance of mtDNA integrity [223, 224]. Five human RECQ members are implicated in DNA break repair and telomere maintenance; defects in three of them, bloom syndrome helicase (BLM), Werner syndrome helicase (WRN), and RECQL4, are associated with rare autosomal recessive disorders characterized by genome instability, cancer predisposition, and premature aging [60, 61, 225, 226]. Mutations in the *BLM* and *WRN* genes cause Bloom and Werner syndromes, respectively, while *RECQL4* mutations cause Rothmund-Thompson syndrome (RTS), RAPA-DLINO syndrome, and Baller-Gerold syndrome [227, 228].

Human RECQL4 displays DNA binding, DNA-stimulated ATPase, 3'-5' helicase, and DNA strand annealing activities [87, 229–231]. It has been demonstrated that RECQL4 is localized in the nucleus, nucleolus, and cytosol, and can relocate to DSBs induced by DNA-damaging agents or OS [232–237]. Consistent with its nuclear function, interactions between RECQL4 and nuclear replication and BER proteins have been shown [238–241]. However, it has been recently found that a significant fraction of RECQL4 localizes to the mitochondria and its N-terminal region appears to be responsible for this localization [223, 224]. RECQL4 is essential for mtDNA replication and, in the

absence of genotoxic stress, and it interacts with a fraction of p53 and relocates p53 to the sites of mtDNA replication optimizing the process. After DNA damage treatment, RECQL4-p53 interaction was disrupted and p53 accumulated in the nucleus [224]. Furthermore, expression of wild-type RECQL4 in fibroblasts from RTS patients has rescued hypersensitivity of these cells and has led to accumulation of p53 in the mitochondria, preventing its nuclear activation in RTS cells [224]. Croteau et al. [223], using both immunofluorescence and cellular fractionation, have confirmed that a fraction of RECQL4 localizes to mitochondria in human and mouse cells. Moreover, constructed RECQL4-deficient cells have displayed elevated mtDNA damage and reduced mitochondrial function. Although these studies suggest the involvement of RECQL4 helicase in mtDNA replication and in the maintenance of mtDNA integrity, the functional role of this DNA helicase in these processes remains yet to be determined.

Several additional proteins might be implicated in the BER pathway in mitochondria. They include poly(ADP-ribose) polymerase 1 (PARP1) and polynucleotide kinase 3'-phosphatase (PNKP) [199, 212, 242, 243], the tumor suppressor p53 [244–246], and the cockayne syndrome group B protein (CSB) [247, 248]. However, the mechanisms underlying their participation in mtDNA maintenance remain to be addressed by future research.

### Defects in mtDNA maintenance associated with human disorders

#### *POL* $\gamma$

Given that *POL* $\gamma$  is the only mitochondrial DNA polymerase, which functions in both mtDNA replication and repair, it is not surprising that hitherto most of the published reports have been focused on the analysis of disease-associated *POLG* mutations. Since the first *POL* $\gamma$  mutations were found to be associated with PEO [249], about 200 mutations in the *POLG1* and *POLG2* genes have been detected in association with various mitochondrial diseases (<http://tools.niehs.nih.gov/polg>) [129, 130, 250, 251]. Up to 150 disease-associated mutations in all domains of the catalytic subunit *POL* $\gamma$ A have been identified. [129] Albeit not all these mutations have been characterized biochemically, there is increasing evidence that they may contribute to disease pathogenesis. These rare hereditary mitochondrial diseases include neurodegenerative disorders, such as mtDNA depletion syndromes (e.g., Alpers-Huttenlocher or early childhood hepatocerebral syndromes) and mtDNA deletion disorders (e.g., ataxia neuropathy, autosomal recessive PEO [arPEO], and autosomal dominant PEO [adPEO]) (<http://www.genetests.org>) [250, 252, 253]. The number of patients affected with recessive pathogenic

*POLG* mutations appears to be higher than expected, with recent estimates approaching 2 % of the general population [254].

Alpers syndrome, a rare genetic autosomal recessive disorder, is associated with severe mtDNA depletion. The disease affects young children and is characterized by progressive cerebral degeneration resulting in dementia, deafness, cortical blindness, and liver failure, leading eventually to death. Analysis of skeletal muscle biopsy has shown significant decrease in mtDNA to  $\sim 1/3$  of normal levels, reduction of ETC activity, and loss of  $\text{POL}\gamma$  activity [255]. Currently, nearly 100 distinct mutations in the *POLG1* gene have been reported to be associated with Alpers and related early infantile hepatocerebral syndromes (<http://tools.niehs.nih.gov/polg>) [130, 250, 256–258]. All known *POLG1* mutations associated with Alpers syndrome are recessive, and many of them have also been identified in arPEO patients. Moreover, *POLG1* mutations, found in patients with adPEO, have also been associated with Parkinsonism and premature ovarian failure [259, 260]. The most common pathogenic *POLG1* mutation, A467T, results in decreased  $\text{POL}\gamma$  activity to 4 % of the wild-type enzyme, reduced stability, and in a compromised interaction between the catalytic and accessory subunits [261]. The second most common pathogenic *POLG1* mutation, W748S, associated with Alpers syndrome and ataxia, also leads to reduced DNA polymerase activity and processivity [262–264]. Intriguingly, several studied disease-associated mutations within the highly evolutionary conserved exonuclease domain have not resulted in compromised proofreading activity and increased mutagenesis [265–268].

PEO caused by mtDNA mutations and deletions as well as mtDNA depletion is characterized by bilateral ptosis, progressive external eye and skeletal muscle weakness, and exercise intolerance. Patients with PEO have skeletal muscles with red ragged fibers associated with reduced ETC activity [250, 251, 254]. The vast majority of PEO-caused autosomal dominant *POLG1* mutations are located in the polymerase domain leading to a severe reduction in polymerase activity and processivity. Two substitutions, R943H and Y955C, resulted in decrease polymerase activity to 1 % of that of the wild-type enzyme [269]. In addition, the Y955C substitution results in 10–100-fold increase in nucleotide misinsertions, in the absence of exonuclease proofreading [270].

### Twinkle

Defects in one of the main NAPs and active player at the mtDNA replication fork, mitochondrial DNA helicase Twinkle, can also cause mtDNA depletion and deletion contributing to human diseases. Since the discovery that

Twinkle and its defects are responsible for adPEO, over 30 different mutations in the *TWINKLE* gene have been identified resulting in deletions or depletion of mtDNA and alterations of mitochondrial function [14, 69]. These *TWINKLE* mutations are associated with neuromuscular disorders [271]. The majority of patients with PEO harbor heterozygous *TWINKLE* mutations with autosomal dominant inheritance when one mutant allele is sufficient to develop the disease in adulthood [272]. Rare homozygous patients manifest more severe phenotypes and develop the disease earlier than their heterozygous relatives [69, 273–275]. All dominant *TWINKLE* mutations identified in adPEO patients cause exercise intolerance and muscle weakness, being the ocular muscles the most severely affected [14, 69, 276]. AdPEO patients accumulate multiple deletions in mtDNA and have reduced mtDNA copy number in post-mitotic tissues. AdPEO-linked *TWINKLE* mutations are mainly mapped to sequences encoding the linker region and the C-terminal helicase domain (Fig. 2a). Several recessive *TWINKLE* mutations causing mtDNA depletion associated with severe epileptic encephalopathy or infantile onset spinocerebellar ataxia (IOSCA) have also been identified [274, 275, 277].

Reduced replication and depletion of mtDNA in human HF have also been found, which appear to be due to defective mitochondrial biogenesis. These defects that may be independent of downregulation of PGC-1 expression, often are present in the failing heart, suggesting that other mechanisms may account for the dysfunctional mitochondria associated with HF [278]. Furthermore, mtDNA depletion has been reported in patients with hypertrophic cardiomyopathy (HCM) [279]; however, it is not known if this is secondary to *TWINKLE* mutations. Research is undergoing in our laboratory to assess whether *TWINKLE* mutations play a primary role in HCM pathogenesis.

Biochemical analysis of PEO-linked Twinkle mutants has provided new insights into the disease underlying mechanisms and into the functional roles of Twinkle domains. All adPEO-associated mutations in the N-terminal region of Twinkle result in impaired helicase function mainly related to a dramatic decrease in ssDNA binding and ATPase activities [77, 280]. These mutants, with exception of R334Q, are unable to support  $\text{POL}\gamma$ -mediated replication in vitro, confirming the critical role of Twinkle helicase activity in mtDNA replication. Most of the adPEO-linked Twinkle linker domain mutants display impaired hexamerization in vitro [70, 77, 281]. Importantly, even the linker region mutants with mild Twinkle oligomerization defects (e.g., S369P and L381P) are characterized by decreased ATP binding and helicase activities [70, 77]. AdPEO-linked mutations in the C-terminal helicase domain of Twinkle have led to significantly reduced helicase activity due to disrupted Twinkle



hexamerization [70, 77, 81, 281]. Most of these mutations are mapped to the residues in the helicase domain, which together with the residues in the linker region are essential for Twinkle hexamerization. Systematic biochemical analysis of 20 recombinant PEO-related Twinkle mutants has shown that they have some decrease in ATPase and DNA-binding activities and thermal stability [282]. Surprisingly, all tested mutants retained DNA helicase activity under optimized in vitro conditions, even though mild-to-moderate defects in helicase activity have been observed. Importantly, these moderate alterations detected in vitro are consistent with delayed presentation of adPEO associated with *TWINKLE* mutations [282].

dPEO may occur as part of a syndrome involving severe mitochondrial cytopathy, such as Kearns-Sayre syndrome (KSS). KSS patients may manifest chronic PEO, bilateral pigmentary retinopathy, and cardiac conduction abnormalities including complete atrial-ventricular block. Significant amount of deleted mtDNA is frequently found in skeletal muscle of KSS patients, as well as concomitant severe reduction in specific mitochondrial respiratory enzyme activity suggesting that mtDNA deletions play a role in the pathogenesis of this syndrome [283]. Also, heteroplasmic G3249A mutation in transfer RNA(Leu) gene has been reported with a clinical phenotype resembling KSS [284]. Furthermore, mtDNA deletions and a *TWINKLE* gene mutation (G1423C) have been found in skeletal muscle of 2 Iranian patients (out of 16 screened) with chronic PEO [285], mutations in *ANT1* in one patient, in *TWINKLE* in two patients, and in *POLG1* in seven patients DNA from a group of 15 Italian and 12 British patients with PEO [286].

Cellular and transgenic mouse models have confirmed the critical role that Twinkle plays in mtDNA replication and maintenance, and recapitulated the main disease phenotypes associated with *TWINKLE* mutations. Overexpression of adPEO-associated *TWINKLE* mutations in cultured human or Schneider cells has caused mtDNA replication stalling, as evidenced by altered replication intermediate pattern. These alterations have led to depletion of mtDNA resembling adPEO cell phenotypes [77, 80, 281]. Furthermore, a similar reduction in mtDNA copy number has been induced by RNAi-mediated silencing of Twinkle expression in cultured human cells [287]. Consistent with a role of Twinkle as one of the major NAPs, overexpression of disease-associated Twinkle mutants in human cells has led to structural alterations in nucleoid [77].

Transgenic mouse models, which express PEO-causing *TWINKLE* mutations, have been generated. The so-called ‘deletor’ mice harboring a 13 amino acids duplication in the Twinkle linker region, which corresponds to an adPEO mutation, have accumulated multiple deletions in mtDNA and mitochondrial dysfunction [288]. Furthermore,

elevated mtDNA replication stalling has been found in the ‘deletor’ mice [77]. More recently, conditional *TWINKLE*-deficient mice have been generated [83]. The authors have shown that Twinkle is essential for mouse embryonic development and its loss causes embryonic lethality at ~E8.5 due to severe mtDNA depletion.

#### Other helicases

In contrast to mitochondrial POL $\gamma$  and Twinkle, two other DNA helicases DNA2 and PIF1, localized in the nucleus and in mitochondria, appear to be involved in both nuclear and mitochondrial genome maintenance; however, their roles in these processes are less well characterized. Using exome sequencing in patients with adult-onset mitochondrial disease featuring mtDNA deletions, heterozygous mutations in the *DNA2* gene have recently been identified [289]. Phenotypically, the disease has been characterized by ptosis and progressive myopathy. Mutations, which may lead to changes in R284H and K313E, both located within the nuclease domain, and V723I located within ATPase/helicase domain, have been found (Fig. 2b). Using three-dimensional structure of the yeast Upf1 helicase-RNA complex and human Upf1-ADP complex as templates, homology model for the hDNA2-ssDNA-ADP complex has been generated [289]. According to this model, R284 and K313 contact directly with ssDNA, therefore R284H and K313E mutations may interfere with ssDNA binding and impair DNA2 nuclease and helicase activity. The V723 is located in the interface of the ATPase and helicase domains, and the V723I mutation may affect hDNA2 ATP-dependent helicase activity [289]. Biochemical analysis of purified recombinant wild-type and mutant hDNA2 enzymes revealed that mutant proteins possess altered characteristics. All three disease-related hDNA2 mutants display significantly reduced nuclease activity; R284H and K313E have also shown severely impaired helicase and ATPase activities, while V723I has increased helicase activity and unchanged ATPase activity [289].

The physiological role that PIF1 nuclease/helicase plays and its contribution to the pathogenesis of human diseases is uncertain. Recently, the first mutations in the *hPIF1* gene associated with human disease have been reported. A genome-wide search for genes suppressing Alu-mediated recombination, which contributes to allelic loss of *BRCA1* in breast cancer, showed a potential role for hPIF in breast cancer predisposition [290]. Among several hPIF1 variants, the heterozygous hPIF1 variant L319P has been identified in three high-risk breast cancer families. Importantly, L319, located within a putative Pif1 family signature motif, is conserved in all known members of the PIF1 family helicases [107]. Consistently, functional analysis of the hPIF1 variant L319P in yeast demonstrated its failure to

complement Pif1 function in the nucleus or mitochondria [290]. Thus, these data suggest a role for hPIF1 helicase in genome and/or mtDNA maintenance; however, the precise mechanisms underlying this function remain to be determined.

#### *Mitochondrial genome maintenance in the injured heart*

At present, the precise mechanisms underlying abnormal mtDNA maintenance and mitochondrial biogenesis associated with CVD are poorly understood. In part because the number of studies on the contribution of abnormal mitochondrial proteins, implicated in mtDNA maintenance, to the pathogenesis of CVD has been rather limited.

Compared to other human cells, cardiomyocytes have been found to have one of the highest mtDNA copy number, ranging from 4000 to 34,000 mtDNA per cell, whereas skeletal muscle cells contain 1000–4000 mtDNA molecules per cell [88, 291, 292]. Earlier studies have demonstrated that a significant decrease in mtDNA content and mitochondrial genes expression result in impairment of mitochondrial function leading to cardiomyopathy and ultimately to HF [293, 294]. Consistently, treatment with chemotherapeutic inhibitors of DNA replication (e.g., the nucleoside-transcriptase inhibitors or doxorubicin) has led to mtDNA depletion associated with cardiomyocyte death and severe cardiac dysfunction [295–297]. Importantly, the Y955C *POLG* mutation characterized by 10–100-fold increase in nucleotide misinsertions, has been recapitulated in mouse model, and the observed phenotype has been linked to abnormal mtDNA replication and repair. Transgenic mice overexpressing the Y955C *POLγ* mutant in the heart have developed cardiomyopathy associated with mtDNA depletion and elevated levels of 8-oxo-dG in mtDNA [298].

Significant mtDNA depletion, reduced expression of mtDNA-encoded proteins, and elevated oxidative mtDNA damage have been detected in the human failing heart [299]. On the other hand, defects in mtDNA maintenance and mitochondrial biogenesis have been found to be independent of the master regulator of mitochondrial biogenesis PGC-1 $\alpha/\beta$ , whose expression has been unchanged in the failing heart, suggesting a novel underlying mechanism [300]. In earlier studies, approximately 30 % down-regulation of PGC-1 $\alpha$  expression in the human failing heart has been detected [301, 302]. This as well as the mechanism responsible for mtDNA depletion in the failing heart remains controversial and need to be addressed in future studies.

Interestingly, human heart mtDNA is organized in a highly complex branched network, containing abundant three- and four-way DNA junctions; these molecular structures resemble recombination intermediates [88, 303].

While human and mouse brain contain such complex mtDNA networks, they have not been found in mouse, pig, or rabbit hearts [88].

In human newborns hearts, as in human skeletal muscle and mouse heart, mtDNA displays a simple topological organization lacking complex three- and four-way DNA junctions. The characteristic adult cardiac mtDNA networks develop within the first years of life [304], and these changes in mtDNA organization correlate with an increase in mtDNA copy number; this mtDNA remodeling has been suggested to occur to meet the high energy demands of the adult human heart [304].

Genetic animal models have confirmed the role of mitochondrial proteins, involved in mtDNA transactions, in cardiac-specific topological organization of mtDNA. Transgenic mice overexpressing wild-type Twinkle helicase, have displayed a significant (up to a 3-fold) elevation in mtDNA copy number in the heart and muscle [287]. Cardiac-specific *TWINKLE* knockout in mice has caused drastic mtDNA depletion leading to severe cardiac mitochondrial dysfunction. In mouse hearts with deficient Twinkle, the levels of D-loop synthesis intermediates are hardly detectable confirming that Twinkle plays a critical role in mtDNA replication [83]. Moreover, mice overexpressing Twinkle or another major NAP, such as TFAM, have displayed complex branched mtDNA forms in the heart, skeletal muscle, and brain [88]. As previously mentioned, strand annealing activity of Twinkle in vitro suggests a potential direct involvement in mtDNA recombination pathway [76]. mtDNA recombination in the human myocardium may have a protective role against chronic ROS damage during a human lifetime [88, 305]. Importantly, mice overexpressing human TFAM seem to maintain high mtDNA content after MI and exhibits improved mitochondrial function in association with cardioprotection in ischemia-induced HF [306]. Similarly, in a mouse pressure overload model of HF, Twinkle overexpression has not only significantly increased mtDNA copy number, but also attenuated left ventricular fibrosis (a typical maladaptive alteration in hypertensive heart disease), ameliorating cardiac function and preventing HF progression [307]. Overexpression of Twinkle has not affected ETC activity, suggesting a novel mechanism of Twinkle overexpression-mediated cardioprotection after sustained pressure overload. Furthermore, Twinkle overexpression in *Sod*<sup>+/-</sup> mice has not only recapitulated in the mouse heart the complex mtDNA networks, characteristic of human hearts, but also prevented oxidative damage and reduced mtDNA mutation load [308]. In addition, overexpression of Twinkle in *Sod*<sup>+/-</sup> mice has ameliorated the cardiomyopathy, caused by *Sod* heterozygosity, by inhibiting cardiomyocyte death. Interestingly, the elevated apoptosis levels observed in *POLG* mutator mice have

suggested a functional crosstalk between cell death and mtDNA integrity [309, 310].

The effect of a dominant negative Twinkle mutation (duplication of 352–364 amino acid residues, dup352–364), detected in adPEO patients with a late-onset myopathy, has been studied in a transgenic mouse model [304]. This mutation altered Twinkle helicase activity leading to stalled mtDNA replication in vitro and in vivo [77]. It has been suggested that stalled mtDNA replication forks due to mutant Twinkle, could lead to DSBs, and their repair through recombination could generate multimeric mtDNA molecules [311]. Importantly, some patients harboring the Twinkle dup352–364 mutation have exhibited left ventricular hypertrophy, sinus bradycardia, and ischemic changes [271]. On the other hand, transgenic mice expressing Twinkle dup352–364 have displayed no obvious cardiac abnormality [288].

In contrast to wild-type Twinkle, Twinkle dup352–364 overexpression has failed to change mtDNA copy number and to induce the appearance of complex branched mtDNA molecules, resembling recombination intermediates [304]. Consistently, in cardiac tissues from adPEO patients carrying the Twinkle dup352–364 mutation complex, four-way mtDNA junctions have not been detected [304]. Interestingly, adPEO patient hearts expressing the POL $\gamma$  G848S mutant, a substitution in the POL $\gamma$  catalytic domain, which severely affects the polymerase activity, have contained X-forms of mtDNA, while dimeric and high molecular weight forms have significantly been reduced [304]. These findings suggest that Twinkle controls mtDNA copy number and the maintenance of complex mtDNA organization in the adult human heart through distinct mechanisms, which remain to be yet elucidated.

## Conclusions

Mitochondria are crucial for a variety of cellular processes ranging from energy metabolism and Ca<sup>2+</sup> handling to various signaling cascades, cell survival, and death. The dysfunctional organelle contributes to the pathogenesis of various human disorders, including CVD.

Defects in several components of the highly organized machineries that mediate mtDNA maintenance may result in accumulation of point mutations or deletion in mtDNA and decrease in mtDNA copy number impairing mitochondrial function and leading eventually to disease. These machineries have the capacity to repair certain types of mtDNA lesions through BER (including SP-BER and more recently discovered LP-BER subpathways), mismatch repair, and recombinational repair pathways. Multiple mutations in two major NAPs, POL $\gamma$  and Twinkle, which are exclusively located in the mitochondria, and involved

in both mtDNA replication and repair, have been associated with neuromuscular and neurodegenerative diseases. Among them, PEO is one of the better characterized, and PEO patients harboring certain Twinkle or POL $\gamma$  mutations may develop severe cardiomyopathy. Importantly, Twinkle dup352–364 and Y955C POL $\gamma$  mutations found in patients with adPEO have been recapitulated in mouse transgenic models and biochemical defects in these proteins have been linked to the disease phenotype.

The functional role that other DNA helicases, such as DNA2, PIF1, and RECQL4, play in the maintenance of mtDNA integrity is poorly understood; it is also unclear how their helicase activities are coordinated with Twinkle helicase function. The involvement of DNA2, PIF1, and RECQL4 in nuclear genome replication and/or repair further complicates the assessment of their mitochondrial function and their potential role in the pathogenesis of CVD. Targeting these proteins to the nuclear or mitochondrial compartment represents a potential regulatory mechanism, which remains to be assessed. Hitherto, only a few mutations in *DNA2* and *PIF1* have been found to be associated with myopathy and cancer predisposition, respectively. However, mtDNA mutations and mtDNA deletions have been found to be a driving force behind the observed premature aging phenotype of the mitochondrial mutator mice. Moreover, recent findings that Twinkle overexpression plays a cardioprotective role against maladaptive cardiac remodeling and HF progression in mouse models suggest that Twinkle may be an important target for the treatment of HF in humans.

Finally, despite significant progress in our understanding of the molecular mechanisms of mtDNA maintenance in health and disease, further research is warranted to determine potential target organ and tissue specificity and to translate this knowledge into clinically relevant therapeutic strategies.

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