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# 2.13 Causes and Consequences of Perturbations of the Mitochondrial Genome

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## Abstract

Mitochondria are essential subcellular organelles, generating >90% of the cellular ATP needed to meet the functional needs of the organism and carrying out a multitude of other essential functions. Mitochondria harbor an essential, high copy number, small (16,569 base pair in humans), circular DNA genome required for well-coupled electron transport and oxidative phosphorylation. Damage to this genome or alterations in the number of genomes present in each cell can result from environmental exposures, heritable mutations, or endogenous factors. mtDNA damage, mutations, and depletion can compromise cellular respiration and give

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rise to mitochondrial toxicity and mitochondrial disease. In this chapter, we review the structure and composition of the mitochondrial genome, the processes that maintain mtDNA integrity, function, and stability, as well as exposure-related mtDNA toxicity.

### Key Points

- Mitochondria have their own, unique machinery for transcription and replication of the mitochondrial (versus nuclear) genome.
- There are many copies of the mitochondrial genome per cell.
- Mitochondrial DNA is very susceptible to damage from endogenous and exogenous sources.
- Many DNA repair pathways present in the nucleus are absent in the mitochondria.
- Cells employ a number of unique and different DNA damage removal and response pathways, including mitophagy and mitochondrial DNA export.
- Environmental factors can alter the number of mitochondrial genomes per cell.
- Mitochondrial DNA mutations cause many diseases, as can depletion of mitochondrial genomes.
- Toxic effects on the mitochondrial genome can be more problematic when they occur early in life, late in life, or for people with specific genetic backgrounds.

## 2.13.1 Introduction

With the exception of erythrocytes, mammalian cells typically contain hundreds to many thousands of copies of mitochondrial DNA (mtDNA). Damage to mtDNA can impede transcription and replication. Inhibition of transcription can alter oxidative phosphorylation (OXPHOS), and interference with replication can cause mtDNA depletion and, potentially, mutagenesis (Gustafson *et al.*, 2020; Nadalutti *et al.*, 2022). In animals, mtDNA mutagenesis occurs more rapidly with age, in age-related diseases, and over evolutionary time, than does nuclear DNA (nDNA) mutagenesis (Tuppen *et al.*, 2010; Marcelino and Thilly, 1999; Brown *et al.*, 1979; Allio *et al.*, 2017; Khrapko *et al.*, 1997; Sanchez-Contreras and Kennedy, 2022). mtDNA mutations cause disease in ~1 person in 5000, and many of these diseases are quite devastating (Schon *et al.*, 2012; Tuppen *et al.*, 2010). Therefore, it is critical to understand endogenous and exogenous processes and factors that affect the maintenance of mtDNA.

Mitochondrial DNA copy number (CN) varies several orders of magnitude between cell types, with oocytes, muscle and neuronal cells having by far the most (hundreds of thousands to possibly millions), and primordial germ cells the least abundant (as low as in the dozens). mtDNA CN also varies with cell cycle, among cells of the same type, and in disease (Aryaman *et al.*, 2018; Filograna *et al.*, 2021; Castellani *et al.*, 2020). Many other characteristics of mitochondria vary dramatically between cells (Monzel *et al.*, 2023). The combination of these features is highly influential in determining the tissue selective phenotypes of the various genetic and exposure-related forms of mitochondrial disease.

The mitochondrial genome is a closed circular genome that codes for 13 protein components of the electron transport chain, 22 transfer RNAs, and 2 ribosomal RNAs required for synthesis of the 13 polypeptides (**Fig. 1**A). Some variations in this structure have been detected (Kolesar *et al.*, 2013). The mtDNA is packaged in discrete DNA-protein structures termed "nucleoids," which are localized within the matrix of the mitochondrion. Mitochondrial nucleoids include numerous proteins involved in mtDNA maintenance, replication, and transcription (Lee and Han, 2017; Bogenhagen, 2012; Bogenhagen *et al.*, 2003; Spelbrink, 2010; Rebelo *et al.*, 2011), with each nucleoid containing 1–2 copies of mtDNA (Kukat *et al.*, 2011). The following sections describe the unique characteristics of this genome including structure, replication and repair, transcription and packaging, susceptibility to damage and damage response, and give an overview of endogenous and exogenous (environmental) factors that result in dysregulation of normal mtDNA maintenance and homeostasis processes and the related health effects.

## 2.13.2 Structure and Composition of the Mitochondrial Genome

The human mitochondrial genome was first sequenced in 1981 (Anderson *et al.*, 1981) and consists of 16,569 nucleotide base-pairs arranged as a closed circular loop containing a heavy (H) strand and a light (L) strand (**Fig. 1**A). The content and structure is generally quite similar in species used as models for human health studies, in particular vertebrates (Oliveira *et al.*, 2015), but there is significant variability in some species including yeast (Adams and Palmer, 2003; Nosek and Tomaska, 2003), which may be important to ecotoxicological researchers or others working with phylogenetically distant species. Within the small noncoding region of the genome is a GC-rich displacement loop (D-loop) containing the heavy-strand origin of replication sequence (**Fig. 1**A). mtDNA encodes 13 highly hydrophobic proteins, all of which are constituents of the 80+ protein subunit mitochondrial electron transport chain (ETC) that traverses the inner mitochondrial membrane (**Fig. 1**B). In addition to these 13 proteins, the mitochondrial genome encodes 22 tRNA and 2 rRNA required for the translation of these essential mitochondrial proteins. Recent research has revealed the presence of micropeptides and non-coding RNAs derived

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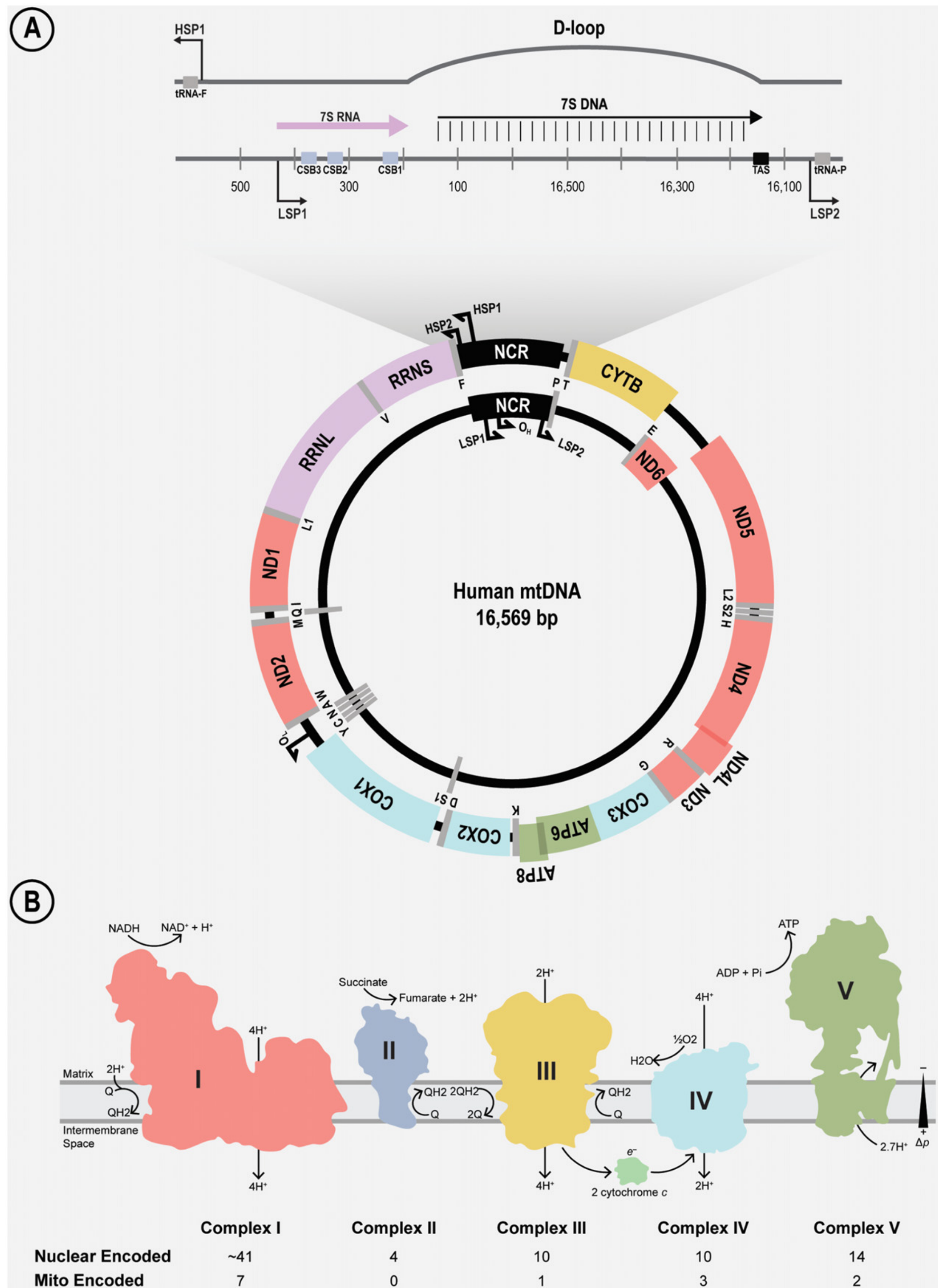


**Fig. 1** Mitochondrial DNA structure and contribution to the oxidative phosphorylation complexes. A. (Top) Detailed diagram of the non-coding region of the mitochondrial genome. Conserved Sequence Box (CSB) locations are annotated in blue and the Termination-Associated Sequence (TAS) is in black. CSBs are sequences that are highly conserved relative to the rest of the noncoding region. Their function is under investigation

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and appears to relate to regulating R loop formation and the transition from RNA to DNA synthesis. The TAS is a conserved sequence at which DNA replication often arrests, resulting in formation of the D loop. Black arrows denote the promoters LSP1, LSP2, and HSP1 positions above the genomic coordinates. 7S RNA is shown in pink and 7S DNA is in black. (Bottom) Schematic architecture of the human mitochondrial genome depicting the genes and the promoters for transcription (HSP1/2 and LSP1/2) and origins of replication ($O_H$ and $O_L$). B. Illustration of protein complexes of the oxidative phosphorylation complexes and proteins encoded by nuclear versus mitochondrial DNA. Indicated below each complex are the number of protein subunits encoded by the nuclear (nDNA) and mitochondrial (mtDNA) genomes. Protein coding genes in the mtDNA genomic map are color-coded to match the ETC subunits for which they encode proteins. Figure was adapted from Gustafson *et al.* (2020).

from mtDNA; their function and significance is just beginning to be explored (Gusic and Prokisch, 2020; Miller *et al.*, 2022). The fact that the mitochondrial genome is largely coding sequence is a potentially important consideration with regard to the potential toxicities associated with reactive genotoxic agents. In comparison to the nuclear genome, there are few sequences that are nonfunctional or under little selective pressure, suggesting reduced potential for "dilution" of DNA damage in the mitochondrial genome. **Fig. 1**B illustrates the respiratory complex-specific distribution of the 13 mtDNA encoded proteins of the mammalian mitochondrial ETC. The only respiratory complex that does not include protein subunits encoded by the mtDNA is complex II (succinate dehydrogenase), which is encoded entirely by the nuclear genome. All other protein subunits are encoded by the nuclear genome and imported electrophoretically into the mitochondrion facilitated by specific chaperone proteins, the flux being regulated by the mitochondrial membrane potential and pH gradient. Likewise, numerous additional proteins of the human mitochondrial proteome are also encoded by nDNA and translocated to their respective locales within the mitochondrial compartment. Efforts to identify all nuclear-encoded mitochondrial proteins are ongoing, and it is currently estimated that over 1100 nuclear encoded proteins are localized in mitochondria (Pagliarini *et al.*, 2008; Calvo *et al.*, 2016; Rath *et al.*, 2021; Cotter *et al.*, 2004).

The mitochondrial genome lacks the histone proteins that bind and compact the nuclear genome. Histones regulate nuclear replication, gene expression, and DNA repair processes, and also protect nuclear DNA from chemical attack via shielding. Instead, the mitochondrial genome is compacted and packaged into the nucleoid structures mentioned above and described in more detail in Section 2.13.5.1. The conformational status of nucleoids varies within a cell, with some more- and some less-compacted, and the degree of compaction has been associated with regulation of mtDNA replication and gene expression (Isaac *et al.*, 2024; Brüser *et al.*, 2021). The extent to which the mitochondrial nucleoid components may offer protection of mtDNA from genotoxic chemical exposure remains unclear, as does any potential role in regulating mtDNA repair or removal (see Section 2.13.5.2 for more detail).

## 2.13.3 mtDNA Replication

### 2.13.3.1 mtDNA Polymerase $\gamma$

Of the 17 mammalian DNA polymerases, DNA polymerase gamma (Pol$\gamma$) is the only replicative DNA polymerase known to function within mitochondria (Bebenek and Kunkel, 2004; Ropp and Copeland, 1996; Sweasy *et al.*, 2006; Wan *et al.*, 2013; Garcia-Gomez *et al.*, 2013). The polymerase $\gamma$ complex consists of one catalytic subunit (encoded by *POLG* at chromosomal locus 15q25) and a dimeric accessory subunit (encoded by *POLG2* at chromosomal locus 17q24.1). The 140 kDa catalytic subunit (p140) possesses DNA polymerase, 3′-5′ exonuclease and 5′ dRP lyase activities (Graziewicz *et al.*, 2006), while the 55 kDa accessory subunit (p55) is required for tight DNA binding and processive DNA synthesis (Lim *et al.*, 1999; Johnson *et al.*, 2000). The Pol$\gamma$ holoenzyme functions in conjunction with the mitochondrial DNA helicase, Twinkle, and the mitochondrial single-stranded binding protein (mtSSB) to form a minimal replication apparatus *in vitro* (Korhonen *et al.*, 2004).

*POLG*, the gene for the catalytic subunit of Pol$\gamma$, is one of several nuclear genes that is associated with mitochondrial DNA depletion or deletion disorders. To date, more than 300 disease mutations have been identified in the *POLG* gene and an up-to-date mutation database can be found at see "Relevant Websites" section which shows these mutations to be equally distributed over the length of the protein (Copeland, 2008; Copeland, 2012; Stumpf *et al.*, 2013; Young and Copeland, 2016; Rahman and Copeland, 2019). Disorders associated with *POLG* mutations include: 1) Myocerebrohepatopathy spectrum, 2) Alpers disease, 3) Ataxia neuropathy spectrum, 4) Myoclonus epilepsy myopathy sensory ataxia, 5) Autosomal recessive progressive external ophthalmoplegia, 6) Autosomal dominant progressive ophthalmoplegia, 7) Leigh syndrome, 8) Kearns-Sayre syndrome, and 9) Hepatocerebral mtDNA depletion syndrome (Wong *et al.*, 2008; Copeland, 2008; Copeland, 2012; Stumpf *et al.*, 2013; Young and Copeland, 2016; Rahman and Copeland, 2019).

### 2.13.3.2 The Mitochondrial DNA Helicase, Twinkle

Though a number of helicases are found within the mitochondria, the Twinkle helicase is the sole helicase involved in mtDNA replication. The Twinkle helicase, encoded by the *TWNK* gene, is a 5′ → 3′ helicase (Spelbrink *et al.*, 2001) with significant sequence homology to the C-terminal end of T7 gene product 4 (gp4) helicase-primase (Spelbrink *et al.*, 2001; Garrido *et al.*, 2003). The Twinkle helicase activity is specifically stimulated by human mtSSB (Korhonen *et al.*, 2003). Twinkle stimulates DNA

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synthesis by Pol$\gamma$ holoenzyme *in vitro* in the presence of mtSSB by supporting unwinding of dsDNA at the replication fork (Korhonen *et al.*, 2004). The primase domain of the Twinkle helicase is nonfunctional, and the primers required to initiate mtDNA synthesis are instead synthesized by the mitochondrial RNA polymerase (POLRMT). Similar to gp4 T7 DNA helicase (Toth *et al.*, 2003), the mitochondrial helicase can form a hexamer or heptamer complex, depending on salt and cofactors (Ziebarth *et al.*, 2007; Ziebarth *et al.*, 2010). However, the recent structure of the human Twinkle helicase revealed a more compact subunit structure as compared to the T7 DNA gp4 helicase (Riccio *et al.*, 2022).

Diseases associated with mutations in the *Twinkle* gene include progressive external ophthalmoplegia, Perrault syndrome, infantile-onset spinocerebellar ataxia/IOSCA, and hepatocerebral mtDNA depletion syndrome (Spelbrink *et al.*, 2001; Tyynismaa *et al.*, 2004; Korhonen *et al.*, 2008; Holmlund *et al.*, 2009). A comprehensive study of recombinant disease variants overproduced and purified from *E. coli* revealed that 20 disease variants display some level of activity, from mild to moderate, in helicase activity, ATP hydrolysis, and stability and all the variants displayed efficient DNA binding (Longley *et al.*, 2010). The moderate defects demonstrated *in vitro* are consistent with the delayed onset of the autosomal dominant chronic progressive external ophthalmoplegia (CPEO) associated with mutation of *Twinkle*. The cryoEM structure revealed that a cluster of the disease mutations located at the subunit interface weaken subunit interactions (Riccio *et al.*, 2022).

#### 2.13.3.3 The Mitochondrial Single-Stranded DNA Binding Protein (mtSSB)

MtSSB was discovered in an analysis of protein-mtDNA complexes derived from rat liver mitochondria (Van Tuyle and Pavco, 1985). The asynchronous model of mtDNA replication (described below) predicts the existence of large regions of single-stranded DNA, and the abundant presence of 16 kDa mtSSB in these nucleoprotein fibrils strongly suggests that the mtSSB protein is an essential component of the mtDNA replication machinery. MtSSB coats displaced single-stranded DNA (ssDNA) and prevents the formation of secondary structures during mtDNA replication (Korhonen *et al.*, 2004; Gustafsson *et al.*, 2016). MtSSB is a 16 kDa protein that forms a homotetramer (Li and Williams, 1997; Longley *et al.*, 2009; Yang *et al.*, 1997). Single strand DNA regions are susceptible to hypermutation (Saini and Gordenin, 2020) and it is assumed that mtSSB protects ssDNA from this damage. However, single molecule imaging by atomic force microscopy reveals that mtSSB does not bind ssDNA in a cooperative manner, leaving the possibility that ssDNA regions are exposed to endogenous and exogenous damage (Kaur *et al.*, 2018). In addition to preventing secondary structures, mtSSB stimulates Pol$\gamma$ processivity and increases the helicase activity of the Twinkle helicase (Mignotte *et al.*, 1988; Genuario and Wong, 1993; Korhonen *et al.*, 2004; Kaguni, 2004; Kaur *et al.*, 2020). Mutations in mtSSB can cause mtDNA deletions and depletion and are associated with CPEO, optic atrophy, Kearns-Sayre syndrome, Pearson syndrome, and Leigh syndrome (Del Dotto *et al.*, 2020; Gustafson *et al.*, 2019; Gustafson *et al.*, 2021; Jurkute *et al.*, 2019; Piro-Megy *et al.*, 2020).

#### 2.13.3.4 Current Models of Mitochondrial DNA Replication

Two modes of DNA replication have been proposed for the mitochondrial genome, an asynchronous strand displacement model and a strand-coupled bidirectional replication model (Bogenhagen and Clayton, 2003a, 2003b; Holt and Jacobs, 2003). The currently favored method of replication is the asynchronous strand displacement model, in which the mtDNA is replicated in an asymmetric fashion wherein DNA synthesis is primed by transcription through the H strand origin within the D-loop (Shadel and Clayton, 1997). After two-thirds of the nascent H strand is replicated, the L strand origin is exposed, allowing initiation of nascent L strand synthesis (**Fig. 2**A). In the strand-coupled model, bidirectional replication is initiated from a zone near the origin of heavy strand replication ($O_H$) followed by progression of the two forks around the mtDNA circle (Bowmaker *et al.*, 2003). In both models, the DNA polymerization reaction is performed by Pol$\gamma$. While there is currently more evidence to support the strand displacement model of replication, there are certain elements in both models that are well supported by experimentation. Further studies are needed to determine which model predominates in nature. An important consideration for those using model organisms is that replication processes in yeast, *C. elegans*, and other simpler eukaryotes differ significantly from those observed in vertebrates (Ling and Yoshida, 2020; Lewis *et al.*, 2015).

Regardless of the mode of replication, both models require extension of an RNA primer and it is accepted that this primer is produced by the transcription machinery and extended by DNA polymerase $\gamma$. Full-genome replication is carried out by Pol$\gamma$, Twinkle helicase, mtSSB, topoisomerase, RNaseH1 and a number of transcription initiation and termination factors. RNA polymerase initiates transcription from the light strand promoter to generate near-genomic length transcripts (Clayton, 1984; Clayton, 1982). These transcripts from the LSP can be cleaved or prematurely terminated to generate primers for DNA replication (Lee and Clayton, 1996; Xu and Clayton, 1996). Further evidence has suggested that the RNA polymerase synthesizes a 25 nt RNA primer at the origin of light strand replication ($O_L$), after it becomes single stranded and adopts a stem-loop structure. This RNA primer is then extended by Pol$\gamma$ to complete the initiation of lagging strand DNA replication (Fuste *et al.*, 2010). Thus, RNA polymerase initiates RNA primers for DNA Pol$\gamma$ at $O_H$ and $O_L$.

Mutations in all major mtDNA replication proteins cause human disease by leading to mtDNA mutations or alterations in mtDNA CN (**Fig. 2**B and **Fig. 2**C), again highlighting the importance of mtDNA maintenance.

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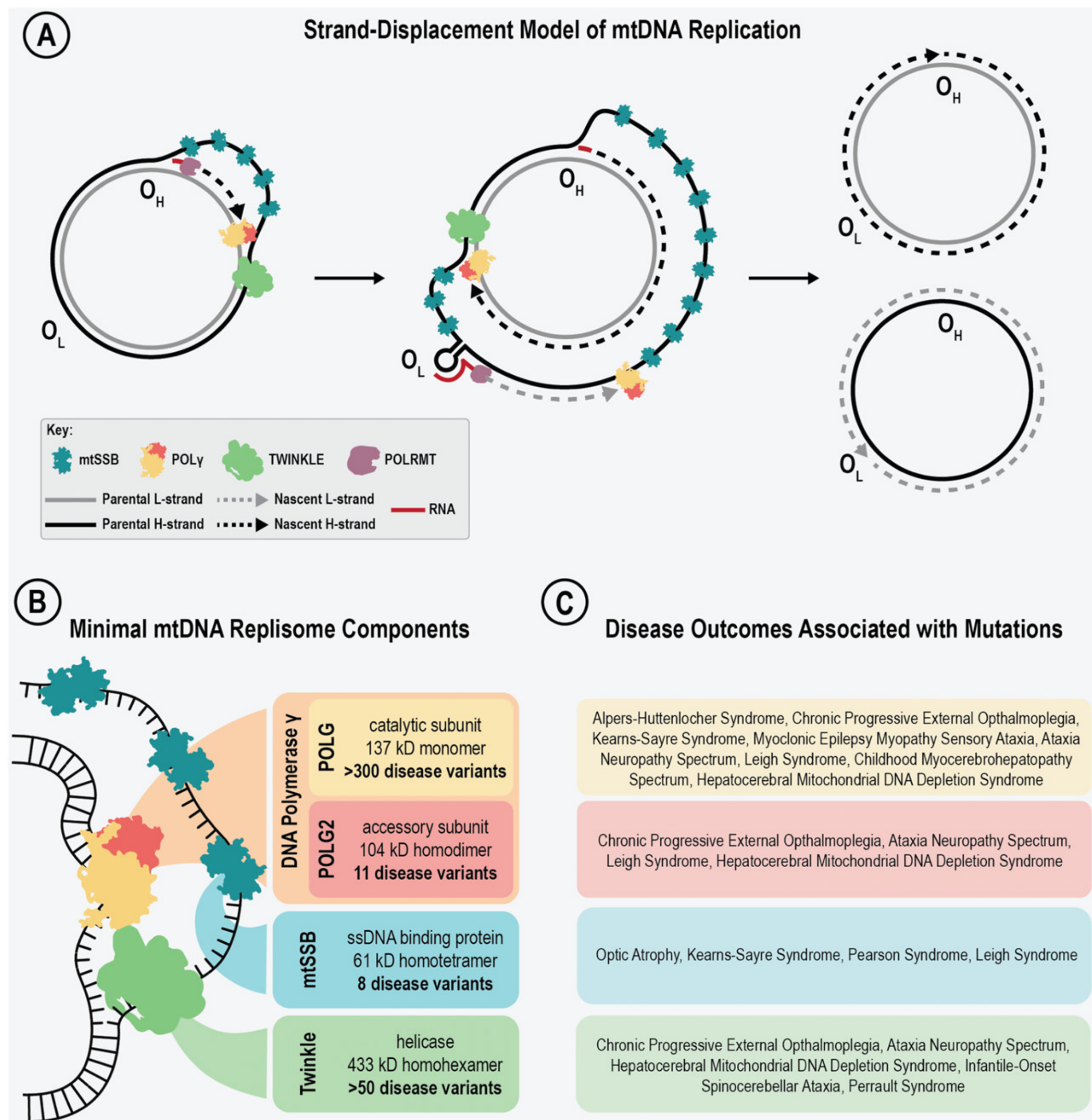


**Fig. 2** Mitochondrial DNA replication and related diseases. A. The strand displacement model of mtDNA replication. mtDNA replication is initiated at the heavy strand origin ($O_H$). The displaced strand is stabilized with mtSSB while the replisome, consisting of Polγ and Twinkle, proceeds unidirectionally to replicate one new strand. Once replication reaches the light strand origin ($O_L$), a stem–loop structure is formed and POLRMT initiates primer synthesis for the replication in the reverse direction. After completion of mtDNA strand synthesis, termination of mtDNA replication results in two circular daughter genomes. B. The minimal mtDNA replisome comprises the Polγ holoenzyme (consisting of POLG and POLG2), the mitochondrial single-stranding binding protein, and the Twinkle helicase. The Twinkle helicase unwinds mtDNA at the replication fork while mtSSB coats single-stranded DNA that is displaced following DNA unwinding. The Polγ holoenzyme is able to synthesize new strands of DNA. Each of these proteins is associated with numerous mutations that lead to disease. C. List of diseases that have been associated with mutations in the proteins that make up the minimal mtDNA replisome. These diseases have been shown to affect a wide range of tissue and cell types across the body. Figure was adapted from Gustafson *et al.* (2020).

### 2.13.3.5 Nucleotide Misincorporation During Replication

Mutations in mtDNA can arise through spontaneous errors of DNA replication or through unrepaired damage to mtDNA that introduces mis-coding lesions. Due to high nucleotide selectivity and exonucleolytic proofreading, the isolated catalytic subunit of Polγ exhibits exceptionally high fidelity of DNA replication, with nucleotide misinsertion events occurring only once per 500,000 nucleotides synthesized (Longley *et al.*, 2001). The intrinsic 3’ to 5’ exonuclease activity that contributes to replication fidelity can

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be completely eliminated by substitution of alanine for Asp198 and Glu200 in the conserved ExoI motif of human Pol$\gamma$ (Longley *et al.*, 1998b). Comparing the *in vitro* error rates for the exonuclease proficient and deficient forms of Pol$\gamma$ indicates that exonucleolytic proofreading contributes at least 20-fold to the fidelity of mtDNA synthesis (Longley *et al.*, 2001). Inclusion of the p55 accessory subunit decreases both frameshift and base substitution fidelity. Kinetic analyses indicate that p55 lowers fidelity of replication by promoting extension of mismatched DNA termini (Longley *et al.*, 2001). Nevertheless, the spectrum of base substitution errors made by highly purified Pol$\gamma$ copying DNA *in vitro* has been measured, and the resulting mutations represent over 85% of the mutations detected in native mtDNA that has been maintained *in vivo* (Zheng *et al.*, 2006). This result is remarkable, because mutations in native mtDNA represent the net sum of replication errors, nucleotide misincorporation in the context of unrepaired chemical damage to mtDNA, and purifying selection over many cell generations. Thus, spontaneous replication errors by Pol$\gamma$ account for the majority of base substitution mutations in mtDNA in human tissues (Ju *et al.*, 2014). By extension, spontaneous errors by Pol$\gamma$ are most likely responsible for the accumulation of point mutations and deletions in mtDNA during aging (Cortopassi *et al.*, 1992; Cortopassi and Arnheim, 1990; Michikawa *et al.*, 1999; Larsson and Clayton, 1995). Ultra-sensitive sequencing has determined that the frequency of point mutations increases approximately 5-fold over the course of 80 years of life (Kennedy *et al.*, 2013). These mutations are predominantly transition mutations, which is consistent with their proposed origin as common Pol$\gamma$-mediated misincorporation events. Interestingly, G to T transversion mutations that are commonly associated with oxidative damage (generated from reactive oxygen species as a by-product of the electron transport chain) do not significantly increase with age, suggesting that oxidative damage to mtDNA may not be a significant factor in aging (Kennedy *et al.*, 2013). Furthermore, Vermulst *et al.* (2007) determined in the mutator mouse (carrying an exonuclease- and proofreading-deficient Pol$\gamma$; this strain is described in more depth in Section 2.13.3.6) that point mutations do not drive the premature aging phenotype. Using a highly sensitive method called LostArc to detect mtDNA deletions, Lujan *et al.* (2020) found that although the frequency of specific, individual deletions does not correlate with age, ablation, which is defined as the total fraction deleted (summed across all individual deletions), increases substantially as a function of age in humans after 60 years of age.

There is an extensive literature describing how genotoxicants contribute to nuclear DNA mutagenesis. In contrast, the evidence for a contribution of chemical exposures to mtDNA mutagenesis is much more limited, with the nucleoside reverse transcriptase inhibitors (discussed in Section 2.13.7.3) being the clearest example. In general, it appears that mtDNA is resistant to point mutations and small deletions from DNA damaging agents, as initially reported in cell culture studies (Marcelino *et al.*, 1998; Mita *et al.*, 1988). In later *in vivo* studies, mice were treated with either benzo[*a*]pyrene or N-ethyl-N-nitrosourea. While both nuclear and mtDNA showed enhanced DNA damage, mostly in the form of covalent adducts, only in nuclear DNA did this damage translate to mutations (Valente *et al.*, 2016). This suggests that mtDNA repair, mitophagy, or other damage removal processes removed these adducts from the examined somatic cells before the damage could be fixed as a mutation. A subsequent study found a similar lack of chemical-induced mtDNA mutagenesis in the context of a 50-generation exposure (permitting examination of germ cells) to the nuclear DNA mutagens aflatoxin $B_1$ and cadmium, even in the context of deficiency in two mitophagy genes, in the nematode *Caenorhabditis elegans* (Leuthner *et al.*, 2022). There is also limited literature evidence for environmental stressors contributing to mtDNA mutagenesis in wildlife and human epidemiological studies (Leuthner and Meyer, 2021), again in contrast to the abundance of such studies in the context of nDNA mutagenesis. Additional work is needed to elucidate the potential contribution of exposures to mtDNA mutagenesis, and the mechanisms employed by cells to prevent mtDNA mutagenesis (Leuthner and Meyer, 2021).

MtDNA mutations have also been suggested to contribute to the development of cancer in some cases (Wallace, 2012). However, the generalized notion of a causal role for mtDNA mutations was challenged by analyses of colorectal tumor tissue which showed a decrease mtDNA mutagenesis as compared to adjacent normal tissue (Ericson *et al.*, 2012). The major reduction of mutations was due to a decrease in C:G to T:A transitions, which are associated with either oxidative damage or Pol$\gamma$ biosynthetic errors. Tumor cells are more reliant on glycolysis for energy production than normal cells, and this 'Warburg Effect' depresses mitochondrial respiration (Vander Heiden *et al.*, 2009). Reduced respiration lowers mitochondrial biogenesis and attendant DNA replication errors; it may also reduce reactive oxygen species (ROS) production and mtDNA damage. Taken together, decreased mitochondrial biogenesis and lowered oxidative damage likely explain the reduced mutagenesis.

Most DNA adducts pose a block to Pol$\gamma$ during DNA replication (Kasiviswanathan *et al.*, 2012; Kasiviswanathan *et al.*, 2013; Hanes *et al.*, 2006). However, *in vitro* assays of Pol$\gamma$ demonstrates some limited mutagenic bypass of UV-induced thymine dimers (Kasiviswanathan *et al.*, 2012), acrolein-derived DNA adducts (Kasiviswanathan *et al.*, 2013) and benzo[*a*]pyrene and benzo[*c*] phenanthrene diol epoxide adducts (Graziewicz *et al.*, 2004) (reviewed in Cline, 2012). These results suggest that long term exposure to these environmentally-induced adducts could participate in mtDNA mutagenesis. In translesion synthesis across 8-oxo-dG in the template, Pol$\gamma$ had low fidelity and incorporated a dATP opposite the lesion 10% of the time (Hanes *et al.*, 2006). With incorporation of 8-oxo-dGTP, the mitochondrial DNA polymerase was able to discriminate against this incorporation by 10,000-fold as compared to dGTP (Hanes *et al.*, 2006). However, once incorporated, the polymerase can extend this adduct 96% of the time (Hanes *et al.*, 2006).

In addition to mutagenic bypass by the wild type Pol$\gamma$, evidence suggests that certain disease alleles of *POLG* can exacerbate mtDNA genome stability. Although no *POLG* disease mutation has shown the mutagenic level of point mutations displayed by the mutator mouse (Trifunovic *et al.*, 2004), the autosomal dominant Y955C *POLG* allele demonstrates enhanced point mutations (Ponamarev *et al.*, 2002). Studies of a spectrum of homologous *POLG* disease mutations in the yeast MIP1 gene demonstrated several alleles that increase the mutation load in mtDNA (Stumpf *et al.*, 2010).

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There are several examples of unique gene-environment interactions causing enhanced mutagenesis with *POLG* disease alleles (Chan, 2017). The common oxidized base 8-oxo-dGMP can exist in two conformations in DNA, *anti* and *syn*. The *syn* conformation is more mutagenic because it permits mis-pairing of the 8-oxo-dGMP with an A instead of a C. In the presence of 8-oxo-dGMP in the template, the *POLG* Y955C mutation allows more *anti* to *syn* conformational shift of the 8-oxo-dG base, leading to an increase in mutagenic dATP mis-incorporation opposite the lesion (Graziewicz *et al.*, 2007). Methyl methanesulfonate (MMS) damages nucleotide bases in double and single stranded DNA but causes hypermutation in single stranded DNA regions due to the inability of BER to repair these lesions in ssDNA (Yang *et al.*, 2010). MMS treatment in yeast with disease harboring MIP1 mutations demonstrated a unique mutation signature attributed to the uncoupling of the polymerase from the helicase causing extended ssDNA regions (Stumpf *et al.*, 2010; Stumpf and Copeland, 2014). Finally, while nucleoside analogs have been shown to inhibit the wild type Pol$\gamma$ (section 2.13.7.3), several disease variants of *POLG* have been shown to enhance the inhibition of DNA replication (Sohl *et al.*, 2013). In one case, stavudine, d4T, was implicated in a death of a patient with the homozygous R964C *POLG* variant. The R964C *POLG* polymerase is impaired in activity (14% of normal: (Yamanaka *et al.*, 2007)), and also shows a three-fold lower d4TTP discrimination during insertion as compared to the wild type Pol$\gamma$ (Bailey *et al.*, 2009).

### 2.13.3.6 Pol$\gamma$ Exonuclease Activity, Disease, and mtDNA Mutagenesis

To test the potential effects of eliminating mitochondrial proofreading function on disease, several groups have employed mouse models with disrupted Pol$\gamma$ exonuclease activity. In the first mouse model, a transgenic Pol$\gamma$ variant that eliminated Pol$\gamma$ exonuclease activity was targeted to the heart, where it caused severe cardiomyopathy accompanied by mtDNA mutations and deletions (Zhang *et al.*, 2000). Several years later, two independent groups created knock-in mice homozygous for mutations that disrupted exonuclease function (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005). These mice exhibited premature aging between six and nine months, characterized by graying hair, loss of hair and hearing, curvature of the spine, enlarged hearts, and decreased body weight and bone density (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005). These observations have not only shown that exonuclease deficiency in Pol$\gamma$ does not cause embryonic lethality, but they also have stimulated discussion about the role of mtDNA mutations in aging and mitochondrial disease.

The degree of increased mtDNA mutagenesis in Pol$\gamma$ exonuclease deficient mice was originally unclear. The increase in mtDNA mutagenesis reported in the mutant mice (three- to eight-fold increase) is similar to the accumulation of mutations detected in two- to three-year-old wild-type mice (three- to eleven-fold) (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005; Vermulst *et al.*, 2007; Trifunovic *et al.*, 2005). However, mutation frequencies in young, wild-type mice are at or below the limit of detection using methods based on PCR cloning and sequencing, which introduces mutations at a rate of $1.3 \times 10^{-4}$ mutations per base pair (Kujoth *et al.*, 2005). This limitation was alleviated by an alternative method of quantifying mutation frequencies called the "random capture method," where the frequency of mutations that cause resistance to restriction endonuclease digestion is enriched, allowing more accurate estimations of mutation frequency ($7.1 \times 10^{-7}$ mutations per base pair in wild-type mice and $1.6 \times 10^{-4}$ mutations per base pair in young heterozygotes) (Vermulst *et al.*, 2007). Mutation frequency in homozygous mutant mice was confirmed using next-generation sequencing technology (Williams *et al.*, 2010). The mutation frequency of heterozygotes, which were asymptomatic, was much higher than aged wild-type mice ($5.4 \times 10^{-6}$ mutations per base pair) (Vermulst *et al.*, 2007). Therefore, it was concluded that the increase in mutation frequency that occurs in older mice is not sufficient to cause phenotypes associated with aging. However, it is still possible that the extremely high mutation rate that occurs in homozygous Pol$\gamma$ exonuclease-deficient mice is sufficient to promote or accelerate the aging process. These mice have subsequently been described as "mutator" mice and used for many studies.

In addition to detecting point mutations, the random capture assay detected a 90-fold increase in mtDNA deletions in homozygous *POLG* exonuclease-deficient mice as compared to age-matched wild-type or heterozygote mice (Vermulst *et al.*, 2008). While mtDNA deletions in wild-type and heterozygotes mostly occur between direct repeats of six or more nucleotides, mtDNA deletions in homozygous mutants occur independently of direct repeats (Vermulst *et al.*, 2008). The mechanism for deletions between direct repeats is often suggested to be to strand-slippage, where a mispriming event occurs downstream of the correct target, a process that appears to be significantly dampened by the proofreading exonuclease function of a polymerase. Interestingly, the lack of increase of deletions in the heterozygote suggests that the wild-type copy of Pol$\gamma$ is able to protect against deletions that are caused by the exonuclease-deficient variant, suggesting an interplay between separate domains of both enzymes similar to the idea of *extrinsic* proofreading (Nick McElhinny *et al.*, 2006). The role of the exonuclease in the formation of mtDNA deletions between direct repeats was tested in yeast and shown to suppress the formation of deletions (Stumpf and Copeland, 2013).

Deletions in mtDNA are also a common occurrence in adult onset *POLG* disorders, as well as disorders involving *POLG2*, the Twinkle helicase, and others (Rahman and Copeland, 2019; Cohen *et al.*, 2018). Deletions in mtDNA due to *POLG* mutations have been detected and characterized using recent advances in next generation sequencing (Persson *et al.*, 2019). Deep sequencing of mitochondrial disease patients with *POLG*, Twinkle or *MGME1* mutations shows that deletions preferentially form on the major arc during strand displacement synthesis by a copy choice mechanism similar to strand slippage mechanism (Lujan *et al.*, 2020; Persson *et al.*, 2019). A more sensitive method, LostArc, revealed tens of thousands unique deletions in *POLG* patients with CPEO, totaling 35 million deletions (Lujan *et al.*, 2020). The pattern, location and sequence contexts at the break points implicate Pol$\gamma$ as the driver of deletion formation and also strongly support the asynchronous strand displacement model of mtDNA replication.

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### 2.13.4 DNA Repair Pathways in Mitochondria

Mitochondrial DNA incurs chemical damage from both endogenous and exogenous sources. A major category is oxidative mtDNA damage, which is mostly caused by reactive oxygen species generated through leakage of electrons from the electron transport chain. Mitochondria have certain DNA repair systems to counter such damage (**Fig. 3**). Several comprehensive reviews have been published that describe the types of damage detected in mtDNA and mitochondrial DNA repair pathways (Liu and Demple, 2010; Kazak *et al.*, 2012; Alexeyev *et al.*, 2013; Akbari *et al.*, 2022; Fu *et al.*, 2020).

#### 2.13.4.1 Base Excision Repair (BER)

Mitochondria possess a robust system for base excision repair. BER is initiated by a host of DNA glycosylases, which recognize numerous types of base damage. Mitochondrial base excision repair can proceed via two pathways, single-nucleotide-BER (SN-BER) or long-patch BER (LP-BER) (Copeland and Longley, 2008). In both repair pathways, an oxidized or damaged base is recognized and cleaved by a specific glycosylase, leaving an abasic site that is cleaved on the 5′ end by AP endonuclease to generate a nick with a 5′deoxyribose phosphate (dRP) flap. During single nucleotide BER, cleavage of the 5′dRP moiety can be performed by the mitochondrial DNA polymerase, Pol$\gamma$ (Longley *et al.*, 1998a). Alternatively, DNA polymerase $\beta$ has been shown to be localized to the mitochondria and is much more efficient in dRP lyase activity (Baptiste *et al.*, 2021; Sykora *et al.*, 2017; Prasad *et al.*, 2017). There is also evidence for other DNA polymerases being present and playing roles in DNA repair in mitochondria (Krasich and Copeland, 2017).

LP-BER activity in mitochondrial extracts has been described, and the mitochondrial proteins required for LP-BER have been identified (Akbari *et al.*, 2008; Liu *et al.*, 2008; Szczesny *et al.*, 2008). LP-BER requires an activity to remove the displaced 5′DNA strand, commonly known as a 5′-flap structure, and Liu *et al.* (2008) found that FEN-1 in their mitochondrial preparations that could carry out this activity *in vitro*. Furthermore, DNA2, originally identified as a yeast nuclear DNA helicase with endonuclease activity, has also been implicated in mitochondrial LP-BER, as well as having a possible role in mtDNA replication (Zheng *et al.*, 2008). In this capacity, DNA2 functions with FEN-1 to process 5′ protruding flaps due to strand displacement synthesis during LP-BER prior to ligation by ligase III. Alternately, the 5′ end may be processed by EXOG to produce a substrate for ligation (Tann *et al.*, 2011).

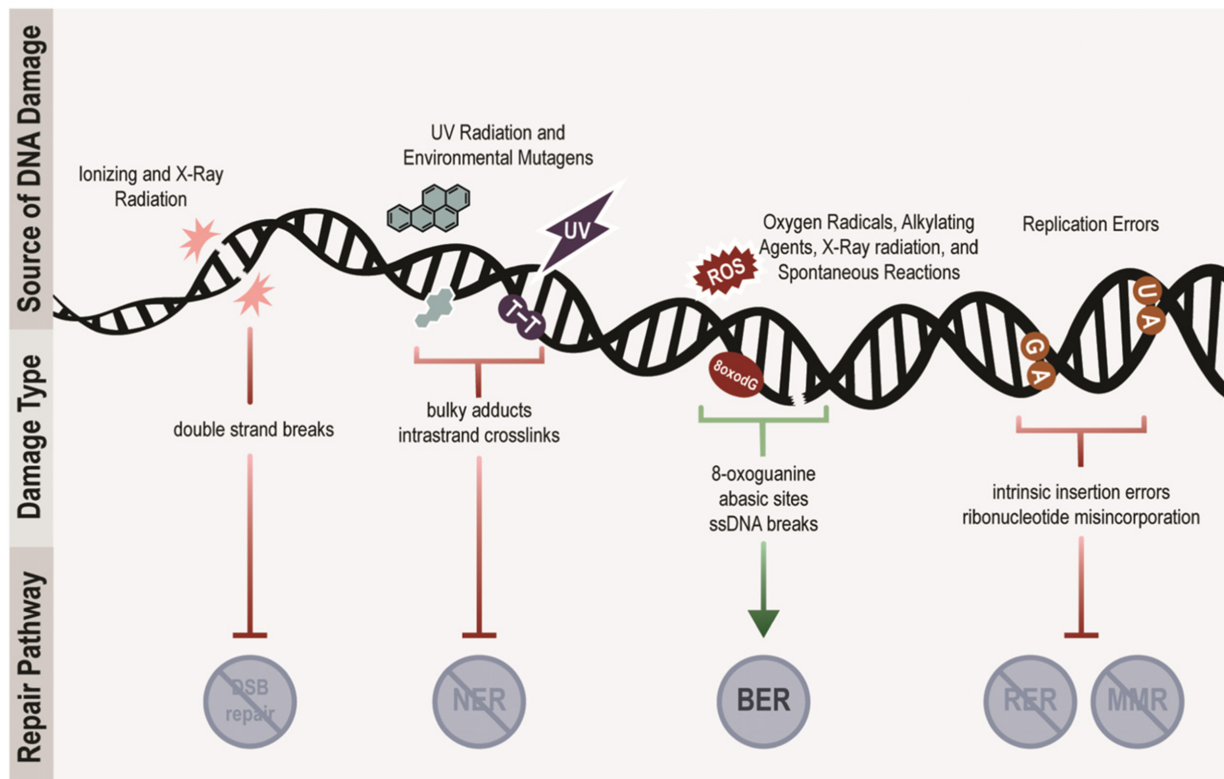


**Fig. 3** Mitochondrial DNA repair pathways present in vertebrate cells. Environmental and endogenous factors can cause mtDNA damage. Many forms of DNA damage are irreparable in the mitochondrial genome, as numerous DNA repair pathways are absent or very limited, such as Double Strand Break (DSB) repair, Nucleotide Excision Repair (NER), Mismatch Repair (MMR), and Ribonucleotide Excision Repair (RER). The only DNA repair pathway known to exist in mitochondria is Base Excision Repair (BER). Figure was adapted from Gustafson *et al.* (2020).

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#### 2.13.4.2 Nucleotide Excision Repair (NER)

It was demonstrated in 1975 that mitochondria cannot repair UV-induced pyrimidine dimers (Clayton *et al.*, 1975), suggesting that they lack a nucleotide excision repair (NER) system. NER is the repair system that removes UV-induced pyrimidine dimers from the nuclear genome. It also removes adducts formed by many other environmentally important mutagens including a subset of polycyclic aromatic hydrocarbons, mycotoxins such as aflatoxin $B_1$, some aromatic amines, and some of the DNA damage caused by certain drugs including cisplatin. Subsequent studies have validated the lack of NER in mitochondria and shown that UV exposure promotes C to T mutations in mtDNA (Pascucci *et al.*, 1997). Alkylation damage to mtDNA has also been investigated, and such lesions were found not to be efficiently repaired by NER in mitochondria (Croteau *et al.*, 1999).

#### 2.13.4.3 Mismatch Repair (MMR)

The presence of mismatch repair (MMR) pathways required for the removal of base mismatches and short insertions and deletions in nuclear DNA is well established. However, there is only limited evidence of MMR machinery in mitochondria. The presence of MMR in mitochondria has been reported in *S. cerevisiae* and *S. pombe* (Chi and Kolodner, 1994a, 1994b; Sia et al., 2000) but not in higher eukaryotes. *S. cerevisiae* encodes msh-1, a homologue of *E. coli* MutS, and mutations in msh-1 induce a higher mutation rate in yeast mtDNA (Reenan and Kolodner, 1992; Vanderstraeten *et al.*, 1998). However, no homologue of msh-1 has been found in animal cells and the concordance of *in vitro* Polγ generated mutation with mutations detected from *in vivo* sources suggest the absence of mammalian MMR in mitochondria (Zheng *et al.*, 2006). Thus, since Polγ is a high-fidelity DNA polymerase (Longley *et al.*, 2001) the higher mutation rate observed in mtDNA (Brown *et al.*, 1979) is likely due to the absence of MMR in mitochondria.

#### 2.13.4.4 Ribonucleotides in mtDNA and the Lack of Ribonucleotide Excision Repair (RER)

It was first reported in 1973 that mitochondrial DNA contains approximately 10 ribonucleotides per genome (Grossman *et al.*, 1973). A more recent analysis revealed that the average mitochondrial genome contains as many as 30 ribonucleotides (Yang *et al.*, 2002). DNA polymerases discriminate against ribonucleotides with as much as a 10,000-fold preference for deoxyribonucleotides, despite the presence of ribonucleotides at much higher concentrations compared to their deoxyribonucleotide counterparts (Gao *et al.*, 1997; Nick McElhinny *et al.*, 2010). Efficient discrimination of ribonucleotides by these enzymes is controlled by specific amino acid residues, which sterically block entry of the incoming ribonucleoside 5′-monophosphate (rNMP) into the enzyme's active site. The structure of T7 DNA polymerase indicates that Glu480 interacts with the ribose ring of the incoming dNTP as well as through hydrogen bonding to Tyr530 (Doublie *et al.*, 1998). Glu895 of human DNA Polγ is analogous to *E. coli* pol I Glu710 and T7 DNA polymerase Glu480, and alteration of Glu to Ala results in a 100-fold loss of discrimination against ribonucleotides, but also at a cost of greatly reduced DNA polymerase activity (Kasiviswanathan and Copeland, 2011). The human Pol γ discriminates ribonucleotides efficiently but differentially depending on the identity of the base. While UTP is discriminated by 77,000-fold compared to dTTP, the discrimination drops to 1100-fold for GTP versus dGTP (Kasiviswanathan and Copeland, 2011). Since much of the discrimination is affinity (Km)-mediated as opposed to capacity (Vmax)-mediated, the actual discrimination *in vivo* depends on the concentrations of deoxyribonucleotides relative to ribonucleotides. Mitochondrial rNTP/dNTP ratios in rat tissue varied depending on the specific nucleotide and tissue. In general, the ATP/dATP ratio is ~1000, UTP/dTTP is 9–73, CTP/dCTP is 6–12, and GTP/dGTP is 2–26 in these rat tissues (Wheeler and Mathews, 2011). Thus, in consideration of the relatively high ATP concentration as well as kinetic discrimination against ATP, Polγ has the theoretical potential to incorporate at least 1 rATP for every 10 template T residues (Kasiviswanathan and Copeland, 2011). Analysis of human mtDNA reveals that ribonucleotides are distributed evenly between the heavy and light strand and throughout the genome and are not repaired (Berglund *et al.*, 2017). Eliminating rNMPs from mitochondrial DNA in SAMHD1$^{-/-}$ mice has no effect on its stability, indicating that rNMPs are well tolerated and not a threat to mtDNA stability (Wanrooij *et al.*, 2020).

RNase H enzymes can remove the RNA incorporated into DNA, and this 'repair' process is termed RER (Nick McElhinny *et al.*, 2010; Sparks *et al.*, 2012). Eukaryotic cells contain two RNase H enzymes: RNase H1 that functions processively to cleave long stretches of RNA/DNA hybrids, and RNase H2 that removes singly incorporated ribonucleoside 5′-monophosphate residues in DNA. Whereas both the H1 and H2 enzymes are found in the nucleus, only RNase H1 is found in mitochondria (Cerritelli *et al.*, 2003), and mechanisms to remove singly incorporated rNMP residues from mtDNA appear to be lacking (Berglund *et al.*, 2017). However, single ribonucleotides in the template DNA strand can be easily bypassed during replication by Polγ (Kasiviswanathan and Copeland, 2011).

#### 2.13.4.5 Double-Strand Break Repair

Double strand breaks (DSBs) in mtDNA can result from defective replication or by various endogenous or exogenous sources, such as reactive oxygen species, ionizing radiation, or various chemotherapeutic agents. Formaldehyde exposure was also shown to cause DSBs in mtDNA (Nadalutti *et al.*, 2020). These dsDNA breaks can lead to intra- and inter-molecular recombination products in mtDNA (Bacman *et al.*, 2009). While yeast mtDNA appears to undergo DSB repair (Bacman *et al.*, 2009), there is limited

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evidence to support double strand break repair in the mitochondria of vertebrates (Fu *et al.*, 2020; Fontana and Gahlon, 2020; Van Houten *et al.*, 2016). Due to the high copy number of mtDNA, the cell is not obligated to repair DSBs, but appears to have developed mechanisms to remove linearized mtDNA resulting from these breaks (discussed in Section 2.13.6.4).

## 2.13.5 Transcription, Epigenetics, and Packaging of the Mitochondrial Genome

### 2.13.5.1 mtDNA Transcription

The genes for the 13 polypeptides, 2 rRNAs and 22 tRNAs are distributed such that most of the genes (12 polypeptides, 2 rRNAs and 14 tRNA genes) are encoded on the heavy strand, while 8 tRNA genes and the ND6 gene are encoded on the light strand (Anderson *et al.*, 1981). For both strands, transcription is initiated from the control region and results in a polycistronic message that is processed post-transcriptionally. Transcription of the light strand initiates from the Light Strand Promoter (LSP1) and proceeds counterclockwise as depicted in **Fig. 1**A. A second light-strand promoter (LSP2) has also been recently identified and *in vitro* transcriptional activity of this promoter has been shown (Tan *et al.*, 2022); however, *in vivo* transcription initiation at this promoter still needs to be investigated. H-strand transcription can initiate from one of two promoters, HPS1 and HSP2, and proceeds in a clockwise direction; however, the importance of the HSP2 promoter *in vivo* remains controversial (Tan *et al.*, 2023).

For mtDNA transcription, Transcription Factor A Mitochondrial (TFAM) binds upstream of the promoter sites (Ngo *et al.*, 2014; Hillen *et al.*, 2017) and the mitochondrial RNA polymerase (POLRMT) binds the C-terminal domain of TFAM to form the preinitiation complex (Morozov *et al.*, 2014). Transcription Factor B2 Mitochondrial (TFB2M) is recruited to the preinitiation complex to cause melting of the strand and binding of the ssDNA to the active site of the POLRMT (Sologub *et al.*, 2009). After transcription is started, TFB2M dissociates from POLRMT which initiates the elongation step along with the mitochondrial transcription elongation factor (Minczuk *et al.*, 2011; Posse *et al.*, 2015; Jiang *et al.*, 2019). Transcription is terminated by the mitochondrial transcription termination factor; however, this has only been shown for LSP1 transcript termination (Kruse *et al.*, 1989; Terzioglu *et al.*, 2013). An important consideration for those using model organisms is that mtDNA transcription appears to differ significantly in at least some invertebrate species from that observed in mammals (Blumberg *et al.*, 2017).

In addition to producing protein-coding RNAs, tRNAs, and rRNAs, mtDNA transcription provides the RNA primers required by Pol$\gamma$ for mtDNA replication, as well as producing a relatively high abundance of R loops, which are strands of RNA hybridized with double-stranded DNA, resulting a triple helix. The normal function of R loops in mtDNA is not clear, but they are speculated to play roles in mtDNA segregation, organization, and replication (Holt, 2019). R loop homeostasis is important; deletion of RNAse H1, which removes RNA from mtDNA, results in persistence of RNA in mtDNA, which blocks subsequent mtDNA replication (Holmes *et al.*, 2015), and a mitochondrial disease-causing variant of RNase H1 results in mtDNA aggregation (Akman *et al.*, 2016).

### 2.13.5.2 mtDNA Packaging and Epigenetic Regulation

The presence of canonical epigenetic DNA methylation in mtDNA, such as the CpG methylation found in the nuclear genome, remains controversial. mtDNA methylation and the mitochondrial presence of DNA methyltransferases have been reported (Shock *et al.*, 2011; Saini *et al.*, 2017; Singh and Storey, 2022), but other studies have refuted the existence of CpG methylation in mitochondria (Mechta *et al.*, 2017; Matsuda *et al.*, 2018; Bicci *et al.*, 2021). Additionally, it has been proposed that even if the mitochondrial genome does contain methylated DNA, it is unlikely that it would be biologically meaningful, given the low abundance of DNA methylation observed on mtDNA (Goldsmith *et al.*, 2021). There also remains controversy regarding the existence of non-CpG methylation in the mitochondrial genome (Guitton *et al.*, 2022; Patil *et al.*, 2019).

Rather than canonical nuclear methods of DNA packaging around histones, mtDNA becomes compacted into a nucleoid structure. The mitochondrial nucleoid comprises many proteins, of which TFAM is the most abundant. TFAM is a high-mobility group (HMG) protein that contains two HMG boxes capable of binding DNA, and plays a role in nucleoid packaging that is distinct from its transcription factor activity. Following multimerization (polymerization?) with additional TFAM molecules, TFAM promotes looping of the DNA which initiates the compaction of mtDNA into the nucleoid structure (Ngo *et al.*, 2014). TFAM is sufficient to coat and compact the mitochondrial genome *in vitro* (Bogenhagen *et al.*, 2008; Kaufman *et al.*, 2007; Kukat *et al.*, 2015). Additionally, the concentration of TFAM can drive the accessibility of mitochondrial nucleoids, which in turn influences the replication and transcriptional activity (Isaac *et al.*, 2024). Mitochondrial nucleoids exist at varying degrees of compaction in the cell, and even within a single mitochondrion, multiple conformations of mtDNA nucleoids exist. Nucleoids that are larger and more loosely compacted are associated with active replication and transcription, confirmed by the incorporation of EdU and BrU labeling, while more closed-off and compact nucleoids are inactive in terms of replication and transcription (Brüser *et al.*, 2021). Spatial regulation of mitochondrial genomes within a cell has also been associated with differential regulation of mtDNA replication (Lewis *et al.*, 2016), though more work should be done to determine how nucleoid compaction is regulated spatially within mitochondria and across the cell.

The degree of compaction of the mitochondrial nucleoid is dynamic and varies throughout cellular differentiation (Isaac *et al.*, 2024). Nucleoid compaction is responsive to several exposures, including ethidium bromide, doxorubicin, and oxidative stress (Rebelo *et al.*, 2009; Bogenhagen, 2012; Ashley and Poulton, 2009; Valle *et al.*, 2005). Interestingly, TFAM has also been suggested to serve as an mtDNA damage-sensing protein as it has differential binding capabilities to various forms of DNA damage, which

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could serve to regulate nucleoid remodeling following DNA damage (Chew and Zhao, 2021). These findings suggest that mitochondrial nucleoid compaction may serve as a potential epigenetic mechanism in regulating genome accessibility, transcription, and replication, as well as damage accumulation, recognition, and response.

## 2.13.6 Additional Factors Contributing to Mitochondrial Genomic Stability and Copy Number

There are several important additional factors that may affect mtDNA damage levels, mtDNA turnover and CN, mtDNA mutations, and the biological manifestations of these alterations to mtDNA. Included among these are the high susceptibility of mtDNA to damage from many genotoxicants, and the persistence of some forms of mtDNA damage; the high, cell type-specific, and regulated mtDNA copy number in cells; the "threshold" effect whereby a certain percentage of mtDNAs must be damaged or mutated before phenotypes are observed; mtDNA degradation via mitophagy and replacement (biogenesis); the ability of cells to degrade mtDNAs internally; cytosolic export of mtDNA and mtRNA, and the ability of some cells to export or import mitochondrial genomes transcellularly.

### 2.13.6.1 Susceptibility of mtDNA to Damage and Persistence of Damage

Despite the high fidelity of mtDNA replication and the clear presence of some, but not all, mtDNA repair systems, point mutations and deletions in mtDNA do accumulate with age (Cortopassi *et al.*, 1992; Cortopassi and Arnheim, 1990; Michikawa *et al.*, 1999; Larsson and Clayton, 1995). In addition to Pol$\gamma$ error, some of this may be attributed to the accumulation of unrepaired oxidative damage that occurs as a byproduct of constitutive metabolic processes within the cell, whereas others may be reflective of the exposure history of the individual. Regardless, there are numerous examples demonstrating that mtDNA is often more susceptible to damage than nuclear DNA.

There are several factors that contribute to this enhanced sensitivity, including inefficient mtDNA repair capacities and the fact that mtDNA lacks introns that might be inconsequential substrates for nonspecific genotoxicities, "diluting" the impact of damage. Evolutionary evidence suggests that the frequency of point mutations in animal mtDNA exceeds that of nuclear DNA by a factor of ten, with some variability among phyla (Brown *et al.*, 1979; Allio *et al.*, 2017). Likewise, in multiple organisms such as yeast, rodents and humans, oxidative stress causes up to 10-fold more mtDNA damage than nuclear DNA damage (Yakes and Van Houten, 1997; Salazar and Van Houten, 1997; Santos *et al.*, 2002; Kang and Hamasaki, 2005). The same is true for damage caused by polycyclic aromatic hydrocarbon metabolites (Allen and Coombs, 1980) and cisplatin (Shu *et al.*, 2016). Once formed, the lack of nucleotide excision, mismatch repair, and canonical DSB repair in human mitochondria may render the individual more sensitive to important forms of xenobiotic-induced mitochondrial genotoxicity.

Previously, it has been speculated that the lack of a protective histone sheath in the mitochondrial genome would result in increased sensitivity of mtDNA to DNA damaging agents. However, this may not always be the case as we now know that mtDNA is coated, compacted, and possibly protected in nucleoids by TFAM. The extent to which the mitochondrial nucleoid proteins protect the mtDNA from various forms of DNA damage is not well studied, and likely depends on the specific type of DNA damaging event in question. It is possible that genotoxic events caused by enzymatic reactions may be blocked by the presence of TFAM and additional nucleoid proteins, but that small chemical compounds or damaging agents such as radiation would still be able to access and damage the mtDNA. The possibility that nucleoid proteins may protect the mtDNA from enzymatic events that may alter DNA is supported by the use of DNA methylation protection assays, such as Fiber-seq (Isaac *et al.*, 2024), which indicate that more compact nucleoids are less accessible to enzymatic processes that methylate DNA. The heterogenous nature of nucleoid compaction represents a potential driver of differential susceptibility between individual mitochondrial genomes to DNA damaging events. It is possible that the compactional status of individual genomes within a cell may confer differential damage accumulation and repair rates depending on how accessible each genome is. For example, open and actively replicating genomes may be more sensitive to damage than the population that is inactive and tightly compacted.

Differential persistence of mtDNA damage is also a factor in mtDNA vulnerability, as evidenced by the demonstration that repair of hydrogen peroxide-induced lesions in mtDNA occurs more slowly than repair of nuclear DNA damage (Yakes and Van Houten, 1997). In the case of acute doxorubicin-induced accumulation of 8-hydroxydeoxyguanosine (8-oxo-dG) adducts, repair of mtDNA damage in both heart and liver occurred at approximately the same rate as the loss of 8-oxo-dG adducts in nuclear DNA in both organs, the half-life for which was approximately 3 days (Palmeira *et al.*, 1997). However, repair was not complete after 7 days and residual 8-oxo-dG adducts accumulated with repeated weekly drug administration, with adducts to mtDNA being twice that of nuclear DNA in both heart and liver (Serrano *et al.*, 1999). Furda *et al.* (2012) reported that both hydrogen peroxide and MMS exposure resulted in subset of mtDNA damage that was persistent, despite the fact that most of the damage caused by these agents is expected to be efficiently repaired by BER. Overall, the conclusion is that *in vivo* mtDNA repair or replication is not sufficient and damage can accumulate under certain conditions. Of further concern is that at some point, a steady-state is reached wherein the cumulative concentration of residual adducts appears to be fixed and not restored even over the course of 25 drug half-lives, and well beyond the estimated rate of mitochondrial turnover, 2–4 days depending on tissue

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type and metabolic demand (Serrano *et al*., 1999; Kai *et al*., 2006; Lipsky and Pedersen, 1981). Although the underlying molecular mechanisms and conditions under which damage persistence occurs have yet to be defined, this persistence of unrepaired mitochondrial genomic damage warns of the risk of extended susceptibility to mitochondrial toxicity associated with chronic exposures to mitochondrial genotoxic agents.

### 2.13.6.2 mtDNA Copy Number, Biogenesis, Heteroplasmy, and Threshold Effects

Exposure-related mitochondrial genotoxicity is complicated by the very high mtDNA CN, and an apparent degree of redundancy conferred by this high copy number that permits a certain degree of tolerance of mtDNA damage, mtDNA mutations, and loss of mtDNA (reduced mtDNA CN). MtDNA CN ranges from 100–10,000 copies in most somatic cells depending on cell type (Shoubridge and Wai, 2007; Zhang *et al*., 2018; Castellani *et al*., 2020), potentially ranging into the millions in long neurons (Misgeld and Schwarz, 2017), with even more variation when non-somatic cells are included. The reasons for such a high cell to cell variation in mtDNA CN are not fully understood but presumably relate to cellular function. Cells with high bioenergetic demand (e.g., nerve cells, muscle cells, kidney cells) and large size typically have high mtDNA CN (~$10^3$–$10^4$: (D'erchia *et al*., 2015)). Oocytes have very high copy number (~$10^5$–$10^6$), but primordial germ cells have very low CN (dozens or hundreds; the importance of these extremes for developmental and reproductive toxicity is discussed in Section 2.13.7.7). Two cell types have been identified that have few or no mtDNAs in mammals: red blood cells and sperm (red blood cells in non-mammalian vertebrates do have mitochondria). MtDNA CN also appears to be higher in stem cell compartments of mature tissues, compared to differentiated regions, and in liver correlates with oxygen tension gradients (Chen *et al*., 2020). All of these differences, along with the fact that mtDNA CN can be regulated by exposure to stressors, support that regulation of mtDNA CN is important for cellular function, and that there has been evolutionary pressure to maintain cell type-specific differences as well as the ability to increase CN when needed. On the other hand, there is often several-fold cell to cell variability between individuals in mtDNA CN even in cells of the same type (Grunewald *et al*., 2016; Trifunov *et al*., 2018; Nakano *et al*., 2011; Stringer *et al*., 2013; Shokolenko and Alexeyev, 2022). Furthermore, mtDNA CN can be reduced ~50% prior to observing bioenergetic changes (Luz and Meyer, 2016; West *et al*., 2015), and conversely, roughly doubled mtDNA CN had only minimal effect on mitochondrial function (Defoor *et al*., 2023). Although these results suggest that cells possess considerable functional leeway in responding to changes in mtDNA CN in terms of maintaining energy production, it should be noted that Luz *et al*. observed mild sensitivity to secondary mito-toxicant exposure, and West *et al*. observed strong activation of the innate immune system.

MtDNA CN is a function of mtDNA replication and mtDNA removal (degradation, export, etc., described in Sections 2.13.6.4 and 2.13.6.5). MtDNA replication, one part of mitochondrial biogenesis, is described as "relaxed," meaning that although it is increased prior to cell division (Trinei *et al*., 2006; Chatre and Ricchetti, 2013), it can occur independently of cell division and nuclear DNA replication. Thus, for example, mtDNA replication continues to occur regularly for decades in post-mitotic cells such as neurons in people, and may occur at any time during the cell cycle in dividing cells. The pathways that regulate mitochondrial biogenesis in response to cellular stimuli are well-studied (Jornayvaz and Shulman, 2010; Ploumi *et al*., 2017; Gureev *et al*., 2019), although how cell-to-cell variation in mtDNA CN is determined is not well understood. Environmental stimuli including toxic exposures can activate mitochondrial removal and biogenesis pathways, as reviewed (Meyer *et al*., 2017; Martinez-Garcia and Marino, 2020; Suliman and Piantadosi, 2016).

Associated with this high degree of variability in mtDNA CN is a high degree of heteroplasmy, defined as presence of more than one mtDNA sequence per cell or tissue. Recent advances in sequencing sensitivity have demonstrated that the presence of low levels of heteroplasmy is the rule rather than the exception (Stewart and Chinnery, 2021), and heteroplasmy increases with age (Glynos *et al*., 2023; Lujan *et al*., 2020). Presumably due to the high degree of redundancy (high CN), mutations to a low to intermediate proportion of mtDNA are adequately compensated by the abundance of non-mutated copies such that the overall functional phenotype is not altered until such a point that mutation frequency exceeds a critical threshold within a cell. It is only when a sufficient proportion (typically well over half, with less tolerance when the mutation or deletion is highly deleterious or the cell type is highly dependent on mitochondrial function) of mtDNA copies are mutated that the effect is manifested transcriptionally to result in an altered proteome, and dysfunction of the cell (Rossignol *et al*., 2003; Dimauro and Schon, 2003; Filograna *et al*., 2021). This "threshold effect" presumably extends to include decreases in mtDNA CN and presence of mtDNA damage.

Based on the threshold effect and the potential to replace damaged or mutated mtDNAs with new copies, purposeful manipulation of mtDNA copy number or turnover has been proposed as a way to alleviate mitochondrial disease (Filograna *et al*., 2021; Russell *et al*., 2020). The most successful such efforts to date have been based on exercise, but considerable effort is being made to find dietary and pharmaceutical interventions. Some success has also been reported in alleviating or preventing chemical-induced mitochondrial (geno)toxicity by elevating mtDNA CN or turnover (Liu *et al*., 2015; Zeissler *et al*., 2016; Miglio *et al*., 2009; Jeng *et al*., 2009; Skildum *et al*., 2011; Rehman *et al*., 2014; Guo *et al*., 2014; Deus *et al*., 2015; Kang *et al*., 2016).

### 2.13.6.3 Mitochondrial Dynamics, Complementation, and Mitophagy

Mitochondria are highly dynamic, fusing, dividing, and moving around cells. Mitochondrial fission is the separation of a mito-chondrion into two distinct daughter mitochondria, while mitochondrial fusion is the joining of two mitochondria into one. Fusion and fission can occur quite rapidly (within seconds of a stimulus), and in many cell types is constantly ongoing

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(Westermann, 2010; Quintana-Cabrera and Scorrano, 2023). Cellular transport of mitochondria can be extensive (consider that in neurons, mitochondria must travel from the cell bodies where much biogenesis occurs to synapses, some of which can be more than a meter away, and that many neurons have several meters of total axonal length per cell (Misgeld and Schwarz, 2017)). Mitochondrial fusion, fission, and transport contribute to mitochondrial homeostasis and the threshold effect in a number of ways.

First, beyond simple redundancy, another phenomenon that likely contributes to the threshold effect is functional complementation. Functional complementation occurs upon mixing of $>1$ mitochondrion, such that functional deficits in one are complemented/compensated for by the presence of intact components (mtDNA, proteins, etc.) in another. This is possible because the mitochondrial network is highly dynamic, such that the contents of the organelles are mixed and exchanged by nearly continuous cycles of mitochondrial fission and fusion. Thus, functional complementation serves to minimize or delay mitochondrial dysfunction despite the ongoing accumulation of mutations and deletions in mtDNA. Specifically, mitochondrial fusion can exert a protective effect in the face of mtDNA mutagenesis (Chen *et al.*, 2010) and pre-existing large mtDNA mutations (Meshnik *et al.*, 2022), and, in disease models, manipulation of mitochondrial fission and fusion can partially rescue disease phenotypes (Chen and Chan, 2009; Haroon *et al.*, 2018). Ironically, however, the protective function of mitochondrial fusion in these cases means that cells tolerate (fail to eliminate) deleterious mtDNA mutations, permitting the persistence of a higher level of heteroplasmy. Mitochondrial fission also regulates persistence of mtDNA mutations (Lieber *et al.*, 2019), likely by facilitating mitophagy, discussed next.

Mitochondrial dynamics contribute to the threshold effect in another very important way. The cell also has the ability to purge damaged mtDNA and mitochondria through a specialized autophagy event termed mitophagy (Youle and Narendra, 2011; Nunnari and Suomalainen, 2012). Several mitophagy subpathways exist (Choubey *et al.*, 2021; Evans and Holzbaur, 2020; Hamacher-Brady and Brady, 2016). Ubiquitin-mediated mitophagy pathways include the PINK-1/Parkin mitophagy pathway, which is triggered following depolarization of the mitochondrial membrane potential (Narendra *et al.*, 2012), as well as the MAPL (also known as MUL1) mediated-ubiquitination pathway (Yun *et al.*, 2014; Braschi *et al.*, 2009). Mechanisms of receptor-mediated mitophagy include BNIP3-, NIX- (also known as BNIP3L)-, FUNDC1-, BCL2L1-, and FKBP8-mediated mitophagy (Bhujabal *et al.*, 2017; Cai and Jeong, 2020; Uoselis *et al.*, 2023; Shirane and Nakayama, 2003; Murakawa *et al.*, 2015; Liu *et al.*, 2012; Imazu *et al.*, 1999; Chen *et al.*, 1997). Lipid-mediated mitophagy also exists, wherein phospholipids such as cardiolipin can be externalized to the outer mitochondrial membrane and subsequently trigger mitophagy (Chu *et al.*, 2013). In addition to mitophagy pathways, damaged mitochondria and mitochondrial components can be removed from mitochondria via mitochondria-derived vesicles (MDVs), which are small ($<150$ nm in diameter) vesicles that bud off of mitochondria and contain mitochondrial components that are subsequently delivered to lysosomes for degradation (Popov, 2022). Of note, it is unclear whether any of these pathways are able to respond directly to mtDNA toxicity *per se*; to date, there is only evidence for response to the downstream or co-occurring cellular consequences of mtDNA toxicity, such as loss of mitochondrial membrane potential. Nonetheless, the evidence is quite clear that mitophagy can serve to remove damaged (Leuthner *et al.*, 2022; Bess *et al.*, 2012) and mutated (Lieber *et al.*, 2019; Valenci *et al.*, 2015; Meshnik *et al.*, 2022; Pickrell *et al.*, 2015; Haroon *et al.*, 2018) mtDNAs, and to protect against the cellular effects of mtDNA toxicity (Dan *et al.*, 2020). However, because it is unclear whether mtDNA damage or mutations can be detected directly and removed, it is also unclear whether mitophagy is able to respond to mtDNA damage and mutations when they are present at low levels, or, in the case of an mtDNA mutation, are neutral. In addition, it is important to note that too much mitophagy may be problematic, for example by causing mtDNA depletion (Chen *et al.*, 2023).

### 2.13.6.4 mtDNA Degradation

MtDNAs carrying some forms of damage can be directly degraded instead of being repaired or removed by mitophagy (Zhao, 2019). To date, this has been shown largely in the context of oxidative mtDNA damage. Shokolenko and colleagues showed that $H_2O_2$ caused mostly abasic sites and single-strand breaks, rather than oxidized bases, in mtDNA, and that this led to rapid ($<1$ h) degradation of mtDNAs (evidenced as much lower-molecular weight mtDNA) and loss of mtDNA (Shokolenko *et al.*, 2009). Degradation could also be elicited by the use of an $H_2O_2$-producing enzymatic system (Shokolenko *et al.*, 2009) or enzymes that created strand breaks and abasic sites (Shokolenko *et al.*, 2013). MMS, an alkylating agent, led to mtDNA degradation only if BER was inhibited (Shokolenko *et al.*, 2009). Finally, they showed that the rate of mtDNA degradation of oxidative damage varied with level of damage and cell type (Shokolenko *et al.*, 2016). The Van Houten group also showed that $H_2O_2$ but not MMS (without inhibition of BER) exposure resulted in mtDNA loss in cell culture (Furda *et al.*, 2012). Interestingly, some damage was persistent after both agents, despite degradation in the case of the $H_2O_2$ exposure and BER operation in both cases. There is evidence that EndoG may play a role in degradation of oxidatively-damaged mtDNA (Wiehe *et al.*, 2018).

Linearized mtDNA resulting from damage and double strand breaks is rapidly degraded by two enzymes, MGME and the exonuclease of Pol$\gamma$ (Nicholls *et al.*, 2014; Peeva *et al.*, 2018; Nissanka *et al.*, 2018). MGME1 is a 5′-3′ exonuclease that efficiently eliminates linear mtDNA; its deficiency in disease states causes elongated 7S DNA and long persistent linear fragments (Nicholls *et al.*, 2014). The Moraes group published a role for the Pol$\gamma$ 3′-5′ exonuclease function in degrading linear mtDNA and showed that this was important to prevent mtDNA deletion formation (Nissanka *et al.*, 2018). Trombly recently confirmed the importance of Pol$\gamma$ in degrading mtDNA linearized by $H_2O_2$-induced DSBs (Trombly *et al.*, 2023). Overall, much remains to be learned about mtDNA degradation, and other mechanisms that may also play a role. For

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example, the p53-inducible appearance of intra-mitochondrial lysosome-like organelles suggests that these organelles could degrade mitochondrial genomes (Miyamoto *et al.*, 2011). Abdu *et al.* (2016) showed that in *C. elegans*, large amounts of mitochondria are internally isolated in lobes by primordial germ cells, after which those lobes are endocytosed and digested by neighboring endodermal cells. This mechanism offers yet another way to degrade mtDNAs —one which relates to the topic of cellular export and import of mtDNAs, which is addressed in the next section.

#### 2.13.6.5 mtDNA Export and Import

Beyond degradation pathways, there also exist pathways for exporting mitochondria and mitochondrial components, such as mitochondrial extrusion into the extracellular environment (Jiao *et al.*, 2021; Melentijevic *et al.*, 2017) and mitochondrial transfer between cells (Islam *et al.*, 2012; Hayakawa *et al.*, 2016; Shen *et al.*, 2018; Davis *et al.*, 2014; Saha *et al.*, 2022). Note that mitochondrial exchange between cells can serve either as a way for one cell to provide good mitochondria to a cell with defective mitochondria, or for a healthy cell to help another cell degrade its dysfunctional mitochondria ("transmitophagy"). Export of mtDNA is a "Damage-Associated Molecular Pattern" (DAMP) that triggers immune responses (West and Shadel, 2017; West, 2017), discussed in Section 2.13.7.6, but may also contribute to removal of damaged mtDNA. There is evidence that oxidized mtDNAs are preferentially exported as mtDAMPs (Mathew *et al.*, 2012; Xian and Karin, 2023), but whether mtDNA with other forms of damage is preferentially exported has not been tested. Extracellular export of damaged mtDNAs occurs by a wide range of mechanisms (Sanz-Ros *et al.*, 2023; Miliotis *et al.*, 2019; Misgeld and Schwarz, 2017) and has been most studied in neurons (Fairley *et al.*, 2022; Davis *et al.*, 2014), but has not been widely assessed in other cell types.

## 2.13.7 Effects of MtDNA Damage, Mutations, and Altered Copy Number

#### 2.13.7.1 Mitochondrial Versus Nuclear DNA Toxicities

There are important fundamental differences between the mitochondrial and nuclear genomes that must be considered when addressing phenotypic effects of genomic damage. While the primary adverse outcome of greatest concern in nuclear genomic toxicity is the introduction of mutations or epigenetic changes leading to unregulated cell replication and cancer, mitochondrial genotoxicity is manifested in a completely different manner. As described above, both mitochondrial bioenergetics and mtDNA CN differ between cancer and non-cancerous tissues, but direct contributions of mtDNA mutagenesis to cancers appears to be rare.

In the case of mitochondrial genotoxicity, a primary adverse outcome is bioenergetic failure of the affected tissues without significant association with cancer. Other changes include alterations in mtROS production and other functions of mitochondria, as described in more detail in subsequent portions of this Section. Bioenergetic failure is most thoroughly characterized in the diagnosis of patients with primary heritable mitochondrial disease who express a systemic and progressive multi-organopathy involving primarily nervous and muscular tissues including peripheral and optic neuropathy, skeletal and cardiac failure, lactic acidosis, lipodystrophy, hepatic steatosis, and gastrointestinal disturbances (Wallace, 2005). The diagnosis correlates well with mitochondrial dysfunction and failure to provide sufficient energy to sustain normal cell or tissue function. Not surprisingly, the same metabolic phenotypes that are associated with heritable mitochondrial diseases also manifest in cases of exposure-related mitochondrial toxicities (Cannon and Greenamyre, 2011; Tanner *et al.*, 2011), making the differential diagnosis nearly impossible without a genetic analysis and thorough patient and family history. The symptoms and diseases resulting from mtDNA mutations are described in detail in several excellent reviews (Schon *et al.*, 2012; Tuppen *et al.*, 2010; Ng *et al.*, 2021; Gorman *et al.*, 2016). Mitochondrial diseases often have variable ages of onset and severity, suggesting that the effect of the primary mtDNA mutation is modulated by other genetic factors, environmental factors or both. The potential role of environmental factors is both a concern (i.e., these patients may be at particular risk of exposure to certain pollutants and drugs) and an opportunity (opening the window for therapeutic treatments (Barcelos *et al.*, 2020; Ng *et al.*, 2021; Russell *et al.*, 2020)).

A second distinction is that unlike the nuclear genome, in mammals, mtDNA mutations are heritable only from the mother; paternal mtDNA is not transmitted to the offspring, resulting in a non-Mendelian pattern of inheritance. This pattern has been extensively characterized for the many forms of mitochondrial disease associated with specific point mutations or deletions in the mitochondrial genome (Wallace, 2005). From a toxicological standpoint, it stands to reason that a similar pattern of inheritance would apply to exposure-related mtDNA mutations.

A third major distinction is that the mitochondrial genome is present at highly variable copy number in different cells (D'erchia *et al.*, 2015), as described above. MtDNA CN variation is relevant for toxicology because mtDNA CN is important to maintain mitochondrial function required for cell stress responsivity, and because mtDNA CN is responsive to stress and has therefore been proposed as a biomarker. It has also been suggested that the mtDNA bottleneck may also create windows of vulnerability to exposure to mitochondrial genotoxins due to the reduced CN and subsequent requirement for rapid replication that may result in misinsertions, deletions, and induction of mutations. Both mtDNA biogenesis and removal are regulated in response to a variety of pollutant and other stressors (Meyer *et al.*, 2017). As a result, measurement of mtDNA CN can be important both to interpret other measured parameters (e.g., mtDNA turnover may modulate age-related accumulation of mtDNA mutations: (Glynos *et al.*, 2023)) and to improve mechanistic understanding of stress response (e.g., some of the toxicity of the nucleoside reverse transcriptase inhibitors has been ascribed to blocking mtDNA replication: (Gardner *et al.*, 2014)). A large number of epidemiological

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studies have measured mtDNA CN in the context of exposures or disease, and changes have often been observed. However, two recent reviews pointed out that the changes observed have not generally been consistent (Reddam *et al.*, 2022; Smith *et al.*, 2023), a conclusion supported by a meta-analysis of studies that examined mtDNA CN as a biomarker or a number of different classes of pollutants (Aviles-Ramirez *et al.*, 2022). Similarly, even in human mitochondrial diseases and neurodegenerative/aging-related diseases associated with mitochondrial dysfunction, there is not a consistent pattern in mtDNA CN variation (Filograna *et al.*, 2021). These analyses, along with technical concerns (Picard, 2021) and theoretical (Meyer *et al.*, 2017) and empirical (Yuan *et al.*, 2016) support for single stressor exposures causing non-monotonic changes in mtDNA CN (increasing and then decreasing at higher exposure levels), lead us to suggest using mtDNA CN as a biomarker only with great caution.

#### 2.13.7.2 Toxicokinetic Considerations in Mitochondrial Genotoxicities

One important requirement of chemicals that directly affect the structure and/or fidelity of mtDNA is the delivery of the agent, or its reactive metabolite, to the mitochondrial compartment. The partitioning of chemical agents to the mitochondrial versus nuclear compartment is not a passive event, but rather is highly influenced by the bioenergetics of the functioning mitochondrion. In the process of transferring electrons along the ETC, protons are actively extruded from the matrix of the mitochondrion to the intramembrane space separating the inner and outer mitochondrial membranes. As a result, both pH (up to a full pH unit) and electrical (up to 100 mV) gradients are established across the inner mitochondrial membrane, which then influence the active flux of both weak acids and positively charged molecules. This strong electrochemical potential contributes to the import of both nuclear encoded proteins and metabolic substrates into the mitochondrial matrix under normal biological conditions. From a toxicological perspective, this electrochemical potential also effects the preferential partitioning of xenobiotics (drugs and environmental or industrial chemicals) between the mitochondrial and cytosolic compartments of the cell (Graziewicz *et al.*, 2004; Meyer *et al.*, 2013). ETC-facilitated electrophoresis across the inner mitochondrial membrane may contribute to the disproportionate accumulation of weak acids and heavy metals within the mitochondria of the cell (Bucio *et al.*, 1999; Castellino and Aloj, 1969; Gavin *et al.*, 1992). Ethidium bromide, paraquat, and 1-methyl-4-phenylpyridinium (MPP+) are three examples of xenobiotics that target mtDNA and preferentially accumulate in mitochondria. Based on these physical chemical phenomena, certain genotoxic exposures may manifest in a disproportionate effect on the mitochondrial, as compared to the nuclear, genome. The unique characteristics of some mitochondrial macromolecules may also contribute to sensitivity to damage. For example, the unique lipids present in the inner mitochondrial membrane may be particularly prone to oxidation-mediated production of aldehydes and other reactive products that cause mtDNA damage, as reviewed by Cline (2012) and Nadalutti *et al.* (2021).

Mitochondrial nucleoids are anchored to the inner aspect of the inner mitochondrial membrane in close proximity to the electron transport chain (although there is some evidence that nucleoids may be concentrated in submitochondrial compartments that are somewhat removed from the ETC: (Stephan *et al.*, 2019; Gerhold *et al.*, 2015)), which is the primary source for reactive oxygen species (ROS) generated within the cell. Much of this ROS generation owes to imperfect bioenergetics of electron transport and is exaggerated by inhibitors of the ETC such as rotenone, azide or cyanide (Wong *et al.*, 2017; Murphy, 2009; Korshunov *et al.*, 1997), and by physiological or "lifestyle" factors such as caloric intake and exercise that increase the state of reduction of the ETC (Fisher-Wellman and Neufer, 2012). Increased ROS in these scenarios results in part from the more-reduced state of components of the ETC upstream of the site of inhibition, which increases the likelihood that these electrons will "leak" onto oxygen. High-throughput screens suggest that perhaps 5–15% of pollutants are mitochondrial toxicants (Wills *et al.*, 2015; Datta *et al.*, 2016; Attene-Ramos *et al.*, 2015; Attene-Ramos *et al.*, 2013), and a high proportion of these are ETC inhibitors (Wills *et al.*, 2015), making this a potentially important mechanism of generation of oxidative mtDNA damage.

An alternate source of mitochondrial ROS generation is chemical agents that redox cycle at the ETC. Two key elements to redox cycling are an increase in abundance of unpaired electrons and the availability of an appropriate electron acceptor. An appropriate electron acceptor in this case is an agent with a redox half-potential that falls within the window of the electron transport chain (-350 mV to 0 mV). Redox cyclers include the dopaminergic neuron toxicants MPP+ and 6-hydroxydopamine, the herbicide paraquat, the plant toxin juglone, the combustion byproduct 2-nitrosofluorene, the *Pseudomonas aeruginosa* toxin pyocyanin, and doxorubicin (Klohn *et al.*, 1995; Drechsel and Patel, 2009; Lei *et al.*, 2014; Wallace, 2003; O'Malley *et al.*, 2003; Majiene *et al.*, 2019). Such "redox cyclers" generate excessive amounts of ROS such as superoxide anion, hydrogen peroxide, and hydroxyl free radical, all of which can interact with the mitochondrial genome to produce oxygenated adducts or deletions to the mtDNA. Interactions of ROS with mtDNA can result in large scale deletions that are common to most forms of oxidative damage (Cortopassi and Arnheim, 1990; Cortopassi *et al.*, 1992; Schon *et al.*, 1989). This biological production of ROS is thought to be the source for the association of mtDNA damage with the normal aging process.

A particularly interesting and well-studied example that illustrates the concepts of redox cycling and persistent effects of mtDNA damage is doxorubicin. Davies and Doroshow were the first to demonstrate mitochondrial site-specific redox cycling with doxorubicin (Adriamycin®) nearly 4 decades ago (Davies and Doroshow, 1986). Using freshly isolated beef heart submitochondrial particles and ETC complex specific inhibitors, these investigators demonstrated that both Adriamycin and the closely related chemotherapeutic Daunomycin stimulate oxygen consumption by submitochondrial particles when NADH is provided as reducing substrate, but not with succinate (**Fig. 4**). Inhibition of Complex I by rotenone and piericidin A accentuated the rate of oxygen consumption, indicating that the site of redox cycling was on the substrate side (upstream) of ubiquinone. Under conditions of reversed electron transport, quinone induced oxygen consumption was inhibited by rotenone,

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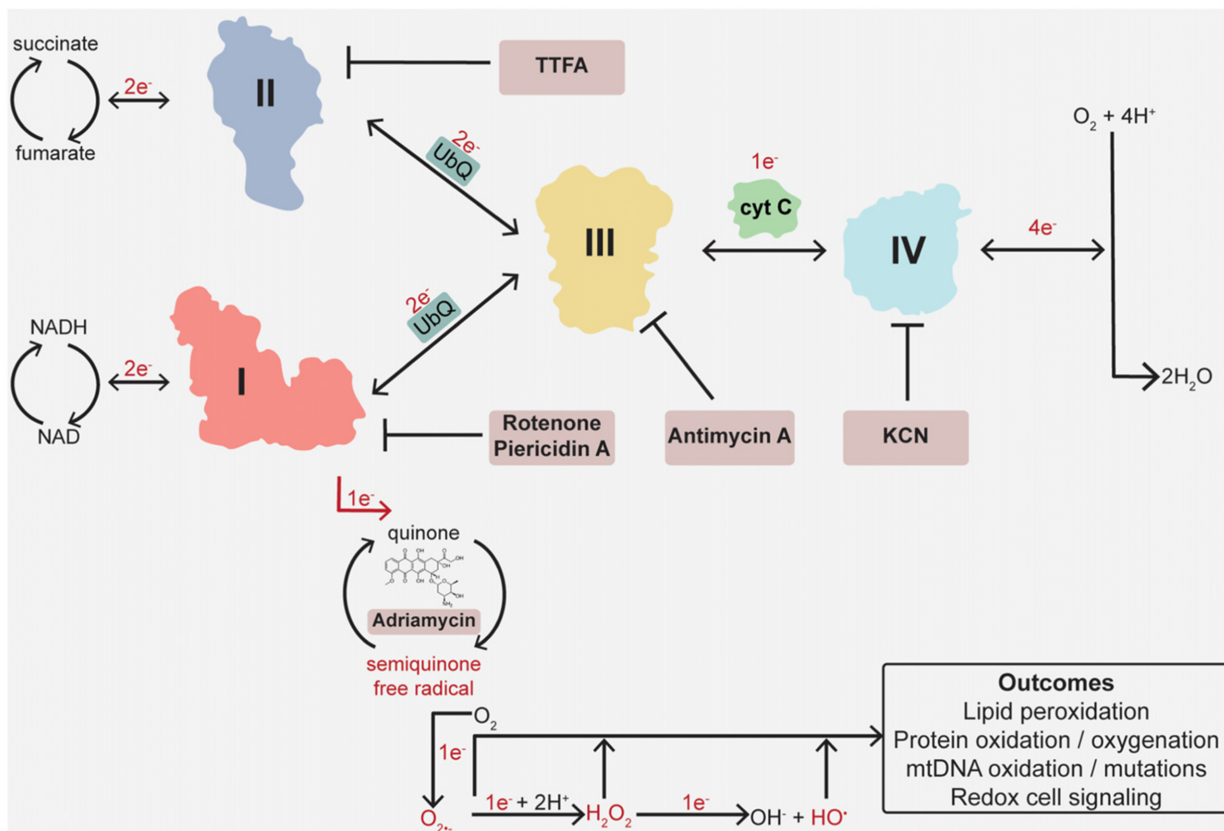


**Fig. 4** Mitochondrial electron transport and redox cycling. Illustration of electron flux on the mitochondrial electron transport chain (ETC) with sites of blockage by specific inhibitors of ETC complexes mentioned in the text, including thenoyltrifluoro acetone (TTFA), rotenone, piericidin A, and potassium cyanide (KCN). Blockage of electron transport leads to a more-reduced state of upstream ETC components. Adriamycin (doxorubicin) and related anthraquinones have been demonstrated to redox cycle on complex I, accepting unpaired electrons to form the highly unstable semiquinone free radical which spontaneously reduces molecular oxygen to the superoxide anion free radical ($O_2^{\cdot -}$). Subsequent univalent reductions lead to hydrogen peroxide and the highly reactive hydroxyl free radical ($^{\cdot}OH$). Any of these reactive oxygen species may lead to the oxidative damage of lipid, protein and/or DNA in proximity to the mitochondrial site of generation and may also participate in redox signaling resulting in transcriptional and other changes. Some redox cycling chemicals accept electrons from Complex III. Free radical species are indicated in red.

but not by TTFA, indicating complex I as the source for redox cycling. This complex I site-specific redox cycling of Adriamycin and related anthraquinones has been further substantiated through the measurement of the effect of ETC substrates and inhibitors on NADH consumption, semiquinone free radical generation, hydrogen peroxide production, and superoxide and hydroxyl anion free radical formation (Davies and Doroshow, 1986; Doroshow and Davies, 1986; Wallace, 2003; Berthiaume and Wallace, 2007a). Most interesting is that this redox-dependent mitochondrial genotoxicity is accompanied by significant changes in other genomic features. These include an increase in mtDNA CN and a shift in mitochondrial gene expression, nuclear epigenetic landscape, and mitochondrial proteomics towards non-oxidative bioenergetics (from fatty acid oxidation to glycolysis), all of which likely constitute a compensatory genomic remodeling of mitochondrial metabolic regulation (Berthiaume and Wallace, 2007b; Carvalho *et al.*, 2010; Ferreira *et al.*, 2017; Zhao *et al.*, 2014). These alterations, in turn, presumably contribute to the irreversible and cumulative nature of a critical toxic side effect of doxorubicin therapy, irreversible dilated cardiomyopathy (Wallace, 2003).

Exposure-related mitochondrial genotoxicity extends beyond agents that elevate oxidative mtDNA damage, and has been recognized for over a half century. Ferguson and von Borstel (1992) summarized the effects of a vast assortment of xenobiotics, including direct-acting genotoxic carcinogens, to induce cytoplasmic petite mutations in *Saccharomyces cerevisiae* that are incapable of growing on a non-fermentable substrate (glycerol). More than half of the chemicals tested were positive for inducing petite mutants in at least one strain of yeast. The authors suggest that this may actually be an underestimate due to less-than-optimal experimental conditions and the fact that the majority of petite-inducing chemicals are either direct mtDNA alkylating or strand-breaking agents or act by altering the structure or replication of the mitochondrial genome. Induction of petite mutants was found to be characteristic for chemical carcinogens (Egilsson *et al.*, 1979; Patel and Wilkie, 1982). Petite mutants, by definition, contain few or abnormal copies of mtDNA (Ephrussi and Hottinguer, 1950). Many of these petite mutants contain the same large deletions found in skeletal muscle of patients with CPEO and Kearns-Sayres syndrome. Both of these are heritable mitochondrial diseases indicating a fairly strong concordance between toxicological and genetic-based mtDNA damage with correspondingly similar metabolic phenotypes.

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Classification of mitochondrial genotoxicity has expanded beyond the induction of petite yeast mutants to include genetic damage in mammalian cells, either in culture or *ex vivo*, and include oxygenated purine and pyrimidine adducts, direct chemical adducts, and various point and long strand deletions. Occurrence and effects of mtDNA damage have been reviewed (Cline, 2012; Meyer *et al.*, 2013; Zhao and Sumberaz, 2020; Nadalutti *et al.*, 2022; Roubicek and Souza-Pinto, 2017). Perhaps because of lack of specific repair mechanisms as well as physical chemical partitioning within the cell as described in Section 2.13.6.1, mtDNA often, but not always, shows more chemical-induced genetic damage compared to nuclear DNA (Supplemental Table 1 in Meyer *et al.* (2013) and Table 1 in Zhao and Sumberaz (2020)). In some cases, specific sequence mutagenesis "hot spots," often repeat regions, have been identified in mtDNA (Schon *et al.*, 1989; Coller *et al.*, 1998; Khaidakov *et al.*, 2002; Khrapko *et al.*, 1997; Sharples *et al.*, 2000). Shu *et al.* (2016) found that cisplatin caused more mtDNA than nDNA damage, and that the light strand was more heavily targeted than the heavy strand at early timepoints. Scala *et al.* (2023) found an uneven distribution of 8-oxo-ddG across the entire mitochondrial genome, both at baseline and after antioxidant and ultraviolet radiation exposure, in cells in culture. However, damage and repair hotspots and coldspots have not been as carefully studied in the mitochondrial as in the nuclear genome.

We note that in many cases, as illustrated by the ETC inhibitors and redox cyclers, mtDNA genotoxicity is secondary to other forms of chemical-induced mitochondrial toxicity, a topic that is beyond the scope of this chapter but has been reviewed (Meyer *et al.*, 2013; Zolkipli-Cunningham and Falk, 2017; Meyer *et al.*, 2018; Jayasundara, 2017; Dreier *et al.*, 2019). Interestingly, it is also possible that oxidative stress could lead to mutagenesis not via causing oxidative DNA damage, but by damaging Pol$\gamma$, which decreases the polymerase's fidelity (Anderson *et al.*, 2020), or causing oxidation of the deoxyribose nucleoside triphosphate pool, which decreases POLG fidelity (Pursell *et al.*, 2008). Relatedly, we note that there are few if any chemicals that affect only mtDNA, without also affecting the nuclear genome to some extent, or also affecting other cellular macromolecules and processes, as highlighted in the examples given above of NRTIs and doxorubicin.

A third form of mtDNA toxicity is mediated by nucleoside analogs that interfere with Pol$\gamma$ mediated mtDNA replication and is discussed in the following section.

### 2.13.7.3 Pol$\gamma$ – Mediated Replication Errors and Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

As described in Section 2.13.3, mutations in proteins responsible for mtDNA replication are commonly associated with mitochondrial DNA depletion or deletion disorders, many of which are implicated in the various inherited mitochondrial diseases. However, there are also a number of clear examples of chemical-mediated Pol$\gamma$-targeted mutations to mtDNA, the classic examples being fialuridine (1-[2-deoxy-2-fluoro-b-D-arabinofuranosyl]-5-iodouracil (FIAU) and other antiretroviral NRTIs). These nucleoside analogs, intended to inhibit viral reverse transcriptase (upon phosphorylation), are also recognized as substrates for mtDNA Pol$\gamma$ and mistakenly incorporated into the elongating strand of mtDNA during the replication cycle. Because many of these analogs are chain terminators, replication is halted and accumulation of truncated strands of mtDNA occurs.

Lewis and coworkers were first to provide a link between what was clinically observed as a "mitochondrial myopathy" in HIV patients receiving azidothymidine and the inhibition of Pol$\gamma$- mediated mtDNA replication (Lewis *et al.*, 2003; Lewis *et al.*, 1992; Lewis *et al.*, 1994). According to this "DNA Pol$\gamma$-hypothesis" (Lewis and Dalakas, 1995), by inhibiting Pol$\gamma$ replication and repair activities, NRTIs cause mtDNA depletion and mutagenesis. Associated with this reduction in mtDNA copy number is a decrease in the gene transcripts and an inability to synthesize the mtDNA encoded proteins that are essential to construction of fully functional electron transport chains. As a result, there is a loss of bioenergetic capacity and fidelity (coupling efficiency) in affected tissues leading to a decrease in ATP synthesis and an increase in ROS generation. The attending oxidative stress accounts for the increase in oxidative nucleotide adducts and mtDNA strand deletions associated with NRTI administration (De La Asuncion *et al.*, 1999). Not surprisingly, the clinical symptoms associated with FIAU and NRTI-induced mitochondrial toxicity closely resemble those of the primary genetic mitochondrial diseases, including the autosomal recessive mtDNA depletion syndrome (MDS).

Besides the "DNA Pol$\gamma$ hypothesis", other potential targets for nucleoside analog-induced mtDNA toxicity include the cytoplasmic and/or mitochondrial nucleoside kinases and the nucleoside or nucleotide transporter proteins. The pro-drugs are usually the unphosphorylated nucleosides, which are not substrates for Pol$\gamma$. To be recognized by and to inhibit Pol$\gamma$ activity, these nucleosides must first be phosphorylated at the 2′ position to the corresponding di-or tri-phosphorylated nucleotide. The kinases responsible for NRTI phosphorylation reside in both the cytoplasmic and mitochondrial compartments of the cell. In the case of thymidine kinases (TK) there are two discrete isoforms. TK1 is cytoplasmic and most abundant in undifferentiated, mitotically active tissues. TK2, on the other hand, localizes to the mitochondrial matrix and is most abundant in striated tissues such as skeletal and cardiac muscle. The nucleoside analogs are competitive substrates for both isoforms of TK and it has been postulated that inhibition of endogenous nucleoside phosphorylation, and thus disturbance of the mitochondrial nucleoside salvage pathway, may account for a significant portion of the observed NRTI-induced mitochondrial toxicity (McKee *et al.*, 2004; Kamath *et al.*, 2015). Mitochondrial TK2 seems to be the most critical isoform associated with robust mtDNA depletion and mitochondrial myopathy (Saada *et al.*, 2001) and a systemic phenotype that closely resembles both MDS and NRTI-induced mitochondrial genotoxicity. Correlative analyses suggest that tissue-specific susceptibility to NRTI-induced mitochondrial toxicity is determined in large part by the abundance of TK2 relative to TK1 (Arner and Eriksson, 1995), which is consistent with the mitochondrial myopathy clinical diagnosis.

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While the NRTI example makes clear that chemical exposure can cause mtDNA mutations, the evidence for pollutant-mediated mtDNA mutagenesis is very limited, as discussed in Section 2.13.3.2. However, there is also some evidence that other drugs—and thus potentially non-pharmaceutical chemicals—may affect other aspects of mtDNA maintenance (Young, 2017).

### 2.13.7.4 Effects of mtDNA Damage and Copy Number Alteration on Transcription and Bioenergetics

While there has been a fair amount of research conducted on the impact of mtDNA damage on the replicative capacity of Pol$\gamma$, less is known about the influence of mtDNA damage on mtDNA transcription and the response of transcription machinery such as POLMRT when encountering lesions. In general, mtDNA damage can reduce or alter mtRNA production. Reduced mtRNA production can lead to inhibition of replication (via reduced production of the mtRNA primer) or reduced production of mtDNA-encoded ETC components, which might in turn lead to reduced mitochondrial membrane potential and ATP:ADP ratios. Under extreme circumstances, there could even be increases in ROS and activation of mitochondrial unfolded protein responses due to imbalance of production of the nuclear- and mitochondrial-encoded ETC proteins. These deficiencies could elicit more downstream changes in other mitochondrial functions, including heme and iron sulfur cluster synthesis, pyrimidine and steroid synthesis, iron and copper homeostasis, thermogenesis and fever, one-carbon metabolism, innate immunity, provision of epigenetic cofactors, and ultimately cell viability (Gustafson *et al.*, 2020; Van Houten *et al.*, 2016; Nadalutti *et al.*, 2022; Zhao and Sumberaz, 2020). Finally, over time, cells may sense either mtDNA damage or the downstream effects of that damage, resulting in changes that may be compensatory or deleterious. Below, we briefly review evidence for these outcomes.

It is well documented that human nuclear RNA polymerases, bacterial RNA polymerases, and T7 RNA polymerases, which share structural similarities to POLRMT, can stall at lesions present on DNA and aid in facilitating repair pathways such as transcription-coupled nucleotide excision repair. POLMRT is capable of bypassing 8-oxo-dG lesions and non-bulky lesions *in vitro*, but it cannot bypass bulky lesions such as damage induced by UV exposure or abasic sites (Nakanishi *et al.*, 2012). Additionally, POLRMT arrests at aldehyde adducts *in vitro* and demonstrated higher sensitivity to arrest on transcripts generated from the LSP vs the HSP (Cline *et al.*, 2010). POLRMT pausing at DNA damage is likely associated with alterations in the production of RNA transcripts, which may protect against production of aberrant mtRNAs. However, it is not known if POLRMT pausing also serves to signal the presence of damaged DNA to repair machinery or other damage-response processes, in a manner analogous to transcription-coupled NER in the nuclear genome. Cline (2012) discusses the possibility of this and other possible mtDNA damage responses, and Nadalutti *et al.* (2022) discuss the evidence for a mtDNA damage-sensing mechanism. Scala *et al.* (2023) recently showed that in cells in culture, the HSP1 transcript was specifically upregulated after UV radiation exposure, highlighting the possibility that one cellular response to mtDNA damage may be differential regulation of transcription from different promoters.

Other mitochondrial proteins may play important roles in the recognition and transcriptional response to mtDNA damage. TFAM has differential binding capabilities to various forms of DNA damage (Chew and Zhao, 2021), which has led to the proposal that TFAM may even serve as a DNA damage sensor for the mitochondrial genome. TFAM has been shown to have affinity for bulky lesions such as cisplatin (Yoshida *et al.*, 2003), as well as bulged DNA (Wong *et al.*, 2009), oxidative lesions (Yoshida *et al.*, 2002), and alkylated DNA lesions (He *et al.*, 2021). TFAM's ability to bind to these lesions across the mitochondrial genome may mediate alterations in nucleoid compaction and accessibility to proteins involved in replication, transcription, and DNA repair (Cline, 2012). Any potential influence of mtDNA damage on TFAM's other function as a transcription factor have not been reported. TFAM can promote transcriptional mutagenic bypass in the context of O4 AlkylT lesions (He *et al.*, 2021; Nakanishi *et al.*, 2012). Overall, it is possible that mtDNA lesions could interfere with transcriptional regulation and initiation as well as transcript extension and termination, but more work is needed to understand the transcriptional response to mtDNA damage.

Reports of the downstream consequences of causing mtDNA damage and depletion by highly mtDNA-specific mechanisms serve as important proofs of principle of the potential consequence of mtDNA damage in the absence of nDNA damage or other macromolecular damage. Complete depletion of mtDNA can be accomplished with some cells, leading to $\rho^{\circ}$ ("rho zero") cells (King and Attardi, 1996); this eliminates the possibility of oxidative phosphorylation because critical components of Complexes I, III, IV, and V are not made. Introduction of high levels of mtDNA DSBs by transgenic expression of mtDNA-degrading enzymes led to mtDNA depletion, loss of oxidative phosphorylation, membrane potential, and loss of cell viability (Shokolenko *et al.*, 2013; Fu *et al.*, 2023; Srivastava and Moraes, 2005; Kukat *et al.*, 2008). Studies on the outcome of deletion of mtDNA-specific DNA repair capacities (without compromising nDNA repair) have demonstrated that loss of mtDNA repair capacity can cause mitochondrial dysfunction and cell death in a variety of circumstances (Tann *et al.*, 2011; Simsek *et al.*, 2011; Szczesny *et al.*, 2013 and reviewed in Van Houten *et al.*, 2016). Qian *et al.* (2019) used a highly specific mitochondrial ROS-generating system to cause specific mtDNA damage, and saw sustained ROS generation, loss of oxidative phosphorylation, mitochondrial fragmentation, and telomere damage. Additional examples were discussed by Nadalutti *et al.* (2022).

Most chemicals and other environmental stressors, while more environmentally realistic, are also less specific and cause lower levels of mtDNA depletion and damage, such that the threshold effect discussed earlier (Section 2.13.6.2) is a key consideration to interpreting this literature. However, many such studies using a wide range of stressors exist and in aggregate clearly demonstrate that such damage can also alter mtRNA levels, bioenergetics, other mitochondrial functions, and cell viability. A subset of examples of such studies is provided in the following paragraph.

Ultraviolet C radiation causes equivalent amounts of mtDNA and nDNA damage, but the photodimers formed are repaired in the nuclear but not mitochondrial genome. As expected based on the *in vitro* studies described above indicating that photodimers

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impede POLRMT, decreases in mtRNA counts following mtDNA damage caused by ultraviolet C radiation, as well as decreases in oxygen consumption and ATP levels have been documented in *C. elegans* (Bess *et al.*, 2012; Leung *et al.*, 2013), with similar results in cell culture (Bess *et al.*, 2013b). Cisplatin reduced mtDNA replication and transcription, and also caused mitochondrial vacuolization (Podratz *et al.*, 2011). Nadalutti *et al.* (2022) showed that $H_2O_2$-induced mtDNA damage reduced mtRNA levels, although some of this may have been the result of direct mtRNA oxidation. Furda *et al.* (2012) showed that $H_2O_2$ caused persistent mtDNA damage, mtDNA loss, decreased Complex I levels, large decreases in OCR, and increased glycolysis in cell culture, although MMS caused persistent mtDNA lesions but no mtDNA loss and mild or no mitochondrial dysfunction. Formaldehyde caused mtDNA DSBs and mitochondrial dysfunction in a primary cell culture model (Nadalutti *et al.*, 2020). NRTI exposure decreased oxygen consumption in a cell culture model, and this was partially rescued by overexpression of PGC-1$\alpha$, a stimulator of mitochondrial biogenesis (Liu *et al.*, 2015). Bleomycin caused both nuclear and mitochondrial DNA damage, as well as loss of mitochondrial membrane potential and apoptosis; however, the apoptotic response was blocked in $\rho^{\circ}$ cells (Brar *et al.*, 2012). Troglitazone damaged mtDNA and induced mitochondrial dysfunction and cell death in a hepatocyte model, and this result was particularly compelling in terms of demonstrating the importance of the mtDNA damage in comparison to other types of toxicity because the damage could be rescued with a mitochondrial-targeted DNA repair enzyme (Rachek *et al.*, 2009).

The literature on mitochondrial diseases caused by mtDNA depletion or mutations suggests that some tissues are also likely to be particularly vulnerable to pollutant-mediated mtDNA damage or depletion. There is some evidence for this, as mtDNA damage has been linked with neuronal toxicity, including dopaminergic neurodegeneration (Gonzalez-Hunt *et al.*, 2014; Qi *et al.*, 2023; Sanders *et al.*, 2014). Cisplatin, which as described above inhibits mtDNA replication and transcription (Podratz *et al.*, 2011) and also drives mitochondrial ROS production (Marullo *et al.*, 2013); these mitochondrial impacts may contribute to cisplatin-associated peripheral neuropathies (Podratz *et al.*, 2011). Doxorubicin has potent effects on heart muscle cells (Berthiaume and Wallace, 2007a), as do NRTIs (Poirier *et al.*, 2015). Thus, although this topic has not been thoroughly explored, it appears that highly energy-dependent cells are likely to often be the targets of chemical-mediated mtDNA toxicity.

There are a variety of processes that have evolved to protect mitochondria and mitochondrial DNA and help cells respond to mitochondrial damage. Some of these have been described here (e.g., mtDNA replication and repair fusion and fission, mitophagy and degradation), but there are others that are outside of the scope of this chapter. For example, the mitochondrial unfolded protein response (Shpilka and Haynes, 2018) upregulates a variety of stress response pathways, and promotes mtDNA repair over transcription (Dai *et al.*, 2023). Interestingly, the mtUPR also promotes tolerance of mtDNA deletions, presumably by permitting cell or organismal survival in spite of the mtDNA deletions (Lin *et al.*, 2016; Yang *et al.*, 2022). Signaling by reactive oxygen species, often generated in mitochondria, can activate a wide range of protective responses including antioxidant enzyme expression, mitochondrial uncoupling, altered metabolism, and xenobiotic detoxification (Sies and Jones, 2020; Hayes and Dinkova-Kostova, 2014), all of which could indirectly protect mtDNA. POLG, TFAM, and mtRNAs were upregulated after exposure to ultraviolet radiation (Leung *et al.*, 2013), suggesting that cells can detect and respond to mtDNA damage or its consequences, although the mechanism for these responses is unknown. Overall, it remains unclear whether these mitochondrial-protective responses are *bona fide* mtDNA damage response process in the sense of responding directly to the mtDNA damage itself, vs responding to downstream events such as decreased mitochondrial membrane potential. Regardless, these responses can lead to altered cellular physiology that is longer-lived than the initial insult, a topic addressed in the next section.

Many of the genes encoding proteins involved in processes that protect mitochondria and mtDNA are human disease genes, suggesting the possibility for gene-environment interactions in which some individuals would be differentially sensitive to mtDNA toxicity. Some such examples were noted in Sections 2.13.3.5 and 2.13.6.3, where we described potential sensitivity of individuals with mutations in mtDNA replication genes to exposure-mediated mtDNA mutagenesis, and some effects of mitophagy deficiency, respectively. Additional examples have been reported, although this is another area deserving of more study. Mitophagy (Parkin) deficiency led to loss of dopaminergic neurons in mtDNA mutator mice (Pickrell *et al.*, 2015) and in mice with mtDNA DSBs introduced via a mitochondrial-targeted restriction enzyme (Pinto *et al.*, 2018), although these genetic deficiencies do not cause dopaminergic neurodegeneration on their own. Mice with mitophagy deficiencies (PINK-1 and parkin) also had mtDAMP-mediated inflammatory phenotypes in the mtDNA mutator mouse background (Sliter *et al.*, 2018). Deficiencies in mitochondrial dynamics genes generally exacerbated the effects of UVC-induced mtDNA damage on development and ATP levels (Bess *et al.*, 2013a; Bess *et al.*, 2012) and neurodegeneration (Hartman *et al.*, 2019) in *C. elegans*, but were in some cases protective. Genetic disruption of TFAM expression in dopaminergic neurons (the "MitoPark" mouse) causes loss of these neurons (Ekstrand *et al.*, 2007) which is exacerbated by exposure to manganese (Langley *et al.*, 2018).

### 2.13.7.5 Long-Term Effects of mtDNA Damage on Mitochondrial Function

MtDNA damage can be quite persistent, especially when it is of a type that can't be repaired, as described above. However, ultimately, after days or weeks depending on the turnover kinetics of mtDNA in that particular cell type, mtDNA damage is expected to be diluted away via specific/selective or stochastic removal. Nonetheless, there is evidence that mtDNA damage or mitochondrial dysfunction (caused either indirectly by the mtDNA damage, or along with the mtDNA damage in the case of stressors that have multiple targets) can lead to highly persistent, even life-long or multigenerational effects. Well-studied examples discussed elsewhere in this chapter in which evidence derive both from animal models and people include the chemotherapeutic doxorubicin (Berthiaume and Wallace, 2007a; Berthiaume and Wallace, 2007b) and the NRTIs (Poirier *et al.*, 2015) (although the

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persistent effects of the NRTIs may be based in part on mtDNA mutagenesis, expected to be persistent). In both animal and human studies, Gulf War Illness is associated with mitochondrial dysfunction that occurs long (decades, in the case of the veterans) after exposure to a number of chemical agents and other stressors (Meyer *et al.*, 2023; Raju and Terry, 2021). There are also examples based so far only on laboratory models. Developmental (*in utero*) exposure to the Complex I inhibitor rotenone led to long-lasting alteration in mitochondrial function and gene expression in mice (Lozoya *et al.*, 2020). *In utero* (but not post-weaning) exposure to arsenic, which has many enzymatic targets including mitochondrial enzymes, caused later-life susceptibility to metabolic disruptions and fatty liver disease in the context of a high-fat diet (Ditzel *et al.*, 2016), also in mice. Early-life exposure of *C. elegans* to UVC radiation in a form designed to maximize mtDNA damage while permitting nDNA repair resulted in life-long decreases in steady-state ATP levels, increased susceptibility to later-life challenge with rotenone, and an apparent resurgence of mtDNA damage later in life (Hershberger *et al.*, 2021). Developmental delay in response to the UVC-induced DNA damage was mediated by redox signaling and ATFS-1 (Hershberger *et al.*, 2021). Developmental exposure to benzo[*a*]pyrene (which causes mtDNA damage and other mitochondrial toxicities, in addition to non-mitochondrial effects) resulted in mitochondrial impairment in offspring, despite this being a relatively rapidly-metabolized compound (Kozal *et al.*, 2023).

An interesting pattern is that many, but not all (e.g., Gulf War and doxorubicin studies), of these persistent effects occurred in the context of exposures that occurred early in development. Thus, while some persistent effects of mitochondrial or mtDNA toxicity may fall into the category of Developmental Origins of Health and Disease (Barouki *et al.*, 2012), not all do. In most cases, the mechanisms by which these persistent effects are mediated have not been worked out. However, there is good reason to expect that mitochondrial dysfunction (Weinhouse, 2017) and redox disruptions (Weinhouse, 2021) could result in changes to nuclear epigenetic patterns, which could be persistent. Indeed, the study on developmental rotenone exposure mentioned earlier identified a pattern of altered epigenetic marks that correlated with altered transcriptomics and phenotypic outcomes (Lozoya *et al.*, 2020). It should also be noted that these long-term changes may be deleterious, beneficial, or both, as reviewed (Meyer *et al.*, 2018).

### 2.13.7.6 mtDNA as a DAMP, Signaling Molecule, and Biomarker

As mentioned in Section 2.13.6.5, mtDNA can also be a "DAMP," serving as a signal to the innate immune system when released from mitochondria (West and Shadel, 2017), and evidence is emerging that this can be driven by exposure to mitochondrial toxicants (West, 2017). MtDNA can also be released extracellularly by neutrophils as a form of anti-pathogenic Neutrophil Extracellular Trap that involves only mtDNA, and not nDNA, and does not result in death of the neutrophil (Yousefi *et al.*, 2009). Other macromolecules unique to mitochondria, including n-formylated peptides and cardiolipin, may be released upon mitochondria dysfunction triggered by mtDNA damage and mutation or other forms of mitochondrial dysfunction. These activate the innate immune system, because the endosymbiotic origin of mitochondria results in their structure being misidentified by metazoan immune systems as bacterial or viral. More recently, double-stranded mtRNA has been added to the list of mitochondria-released signaling molecules (Tigano *et al.*, 2021). If such release occurs following chemical exposure but in the absence of a pathogen target, it may be deleterious to the cell and neighboring cells, because the inflammatory response can itself cause damage (Marchi *et al.*, 2023), a major contributor to chronic disease (Furman *et al.*, 2019). Interestingly, mtDNA damage-induced stress signaling also leads to enhanced nDNA repair, suggesting that mtDNA could serve as a generalized genotoxic stress sentinel (Wu *et al.*, 2019). Finally, the extracellular release of mtDNA under conditions of stress has led some to propose that circulating or excreted (urinary) mtDNA might serve as a biomarker (Miliotis *et al.*, 2019). However, there are many sources of circulating mtDNA, suggesting that it will rarely be a highly specific biomarker (Aucamp *et al.*, 2018), and many of the same caveats to using mtDNA CN as a biomarker in other contexts, discussed in Section 2.13.7.1, apply.

### 2.13.7.7 Developmental Sensitivity

It is a common theme in toxicology that developmental stages are often particularly sensitive to pollutant exposures (Cohen Hubal *et al.*, 2000; Heindel *et al.*, 2015; Ferguson *et al.*, 2017). This is true for both toxicokinetic reasons such as increased exposure or decreased metabolism/excretion, and toxicodynamic reasons such as the potential for developmental processes to be altered resulting in permanently changed cellular programming (epigenetic changes) or permanently altered tissue formation (teratogenesis). This has been less studied in the context of mtDNA toxicities, but examples do exist, such as the NRTI example given earlier in which doses that were not toxic to pregnant mothers caused toxicity in their children. Indeed, in addition to the reasons for developmental sensitivity mentioned above that are common to many developmental toxicities, there are reasons unique to mtDNA, resulting from the developmental biology of mitochondria.

There are many features of mitochondria that are different in early development and in stem cells, including energetics and morphology (Bukowiecki *et al.*, 2014; Van Blerkom, 2011; Harvey, 2019). For this chapter, we focus on the mitochondrial genome. At fertilization, vertebrate oocytes typically have hundreds of thousands or millions of mtDNAs (Zhang *et al.*, 2018). Sperm normally contribute no mtDNAs, either because they have none (Lee *et al.*, 2023) or because those they have are eliminated shortly after fertilization (Sato and Sato, 2013). Subsequent to fertilization, mtDNA replication in early development appears to absent or very limited except a small amount of replication in the one- and two-cell stages (Shoubridge and Wai, 2007), with total mtDNA CN constant in the developing embryo. MtDNAs are simply distributed into proliferating cells, with compounding reductions in mtDNA CN per cell in the daughter cells. Of particular importance for the next generation, the copy number in

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primordial germ cells drops to as low as fewer than 100, or perhaps in the low thousands, depending on the species (Zhang *et al.*, 2018; Shoubridge and Wai, 2007). This is one of several mechanisms that may contribute to the mtDNA "bottleneck" observed between generations, which can result in large changes in heteroplasmy (Zhang *et al.*, 2018). It also provides an opportunity either for particular vulnerability to mtDNA genotoxicants (because there are so few mtDNAs, the protection normally conferred by high redundancy may be reduced) or for identification of and selection against mtDNAs that are damaged or mutated, or cells carrying a high proportion of them. There is evidence for selective removal of highly deleterious mtDNA mutations (Fan *et al.*, 2008 and reviewed by Fu *et al.* (2020)), but the persistence of mtDNA mutation-based mitochondrial disease demonstrates that this process is not perfect. One study showed that exposure to the NRTI azidothymidine, the ATP synthase poison oligomycin, or the amino acid homocysteine led to changes in mtDNA CN immediately postfertilization in mice, demonstrating the ability of environmental factors including mtDNA genotoxicants to alter mtDNA at this early developmental stage (Mcconnell and Petrie, 2004).

Based on these considerations, a number of authors have suggested that developmental exposures to mtDNA toxicity (direct, or via more general mitochondrial toxicity) could be both particularly deleterious and lead to long-term effects, and provided a number of examples from the reviewed literature (Gyllenhammer *et al.*, 2020; Brunst *et al.*, 2015; Meyer *et al.*, 2013). Most recently, the Cardenas group reviewed the epidemiological evidence for impacts of early-life exposures on mtDNA CN (Smith *et al.*, 2023), finding that some exposures appeared to cause fairly consistent changes in different studies, while others were less consistent. Some stressors caused increases, and others decreases, in mtDNA CN. We suggest that this is an important area for future laboratory and epidemiological research.

### 2.13.7.8 Potential for Sensitivity in Older Individuals

Of particular concern, but considered less often than early life sensitivity, is the maintenance of mitochondrial bioenergetics and genomic homeostasis in the aging individual. This was first referenced by Raymond Pearl in his 1928 declaration of the "Mitochondrial Theory of Aging" (Pearl, 1928). According to this theory, life span is determined by the "rate of living": One is born with a fixed and finite mitochondrial complement and once this allocation is exhausted the organism can no longer survive. We have since learned, however, that mitochondrial competence is not fixed but instead is highly dynamic. A good example is that aerobic exercise increases mitochondrial competence and that associated with this is an increase in vitality, a decrease in several disease states such as diabetes and cancer, and a prolonged lifespan (Lopez-Otin *et al.*, 2016). An alternate and sometimes conflated theory is the "Free Radical Theory of Aging" proffered by Harman in 1956 (Harman, 1956). According to Harman "aging and the degenerative diseases associated with it are attributed basically to the deleterious side effects of free radicals on cell constituents and on the connected tissues". Miquel *et al.* (1980) expanded on this to postulate that mitochondria are the primary intracellular source of these free radicals and that mtDNA is the critical target for age-associated cell damage. Much of this latter doctrine is based on the well-established increase in mtDNA mutations that occur throughout one's lifespan, which are considered to be primary determinants of age-associated degenerative changes (Bratic and Larsson, 2013; Larsson, 2010). Although enhanced rates of ROS production may account for this accumulation of mtDNA mutations, the age-associated loss of mtDNA excision/repair capacity is also a major contributing factor (Leandro *et al.*, 2015; Gredilla *et al.*, 2010). There is also growing literature evidence for a decrease in both mtDNA CN and mtRNA levels in age (Short *et al.*, 2005; Welle *et al.*, 2003).

However, mitochondrial aging is more complex than simply mtDNA genotoxicity (Hebert *et al.*, 2010; Sun *et al.*, 2016). Phenotypic characteristics of aged mitochondria include structural, functional as well as genomic features. Electron microscopy reveals that mitochondrial size or volume is as much as two-fold greater in cells from aged individuals (Bueno *et al.*, 2015). This is accompanied by slight decrease in mitochondrial number per cell, which the authors suggest may reflect altered mitochondrial fusion, fission, and mitophagy. Tyrrell *et al.* (2020) report a significantly increased rate of PINK1/Parkin related ubiquitination of mitochondrial proteins, decreased mitochondrial membrane potential, and impaired ability to induce mitophagy after rotenone challenge in the cerebral vasculature from aged mice. Overall, the pattern is one of increased macromolecular damage, decreased mitochondrial turnover, and decreased mitochondrial abundance with age. A very recent publication demonstrates that induction of mitophagy by treatment with urothilin A reduced age-associated increases in cytosolic mtDNA, inflammation, and neurological decline (Jimenez-Loygorri *et al.*, 2024).

Associated with this decrease in mitochondrial abundance and macromolecular integrity is a decrease in both baseline and maximum mitochondrial respiration by cells from aged individuals (Bueno *et al.*, 2015). This loss of mitochondrial bioenergetic capacity with aging is well established in a variety of tissues and has been implicated as a major causative factor for a variety of degenerative changes associated with aging (Short *et al.*, 2005; Amorim *et al.*, 2022; Srivastava, 2017). This may also contribute to the increased incidence of prescription-related and environmental exposure related mitochondrial toxicities observed in older, geriatric populations (Will *et al.*, 2019). There is little doubt that changes in the molecular biology of mitochondrial genomics with age is a major factor contributing to the differential sensitivity of geriatric populations to mitochondrial toxicity.

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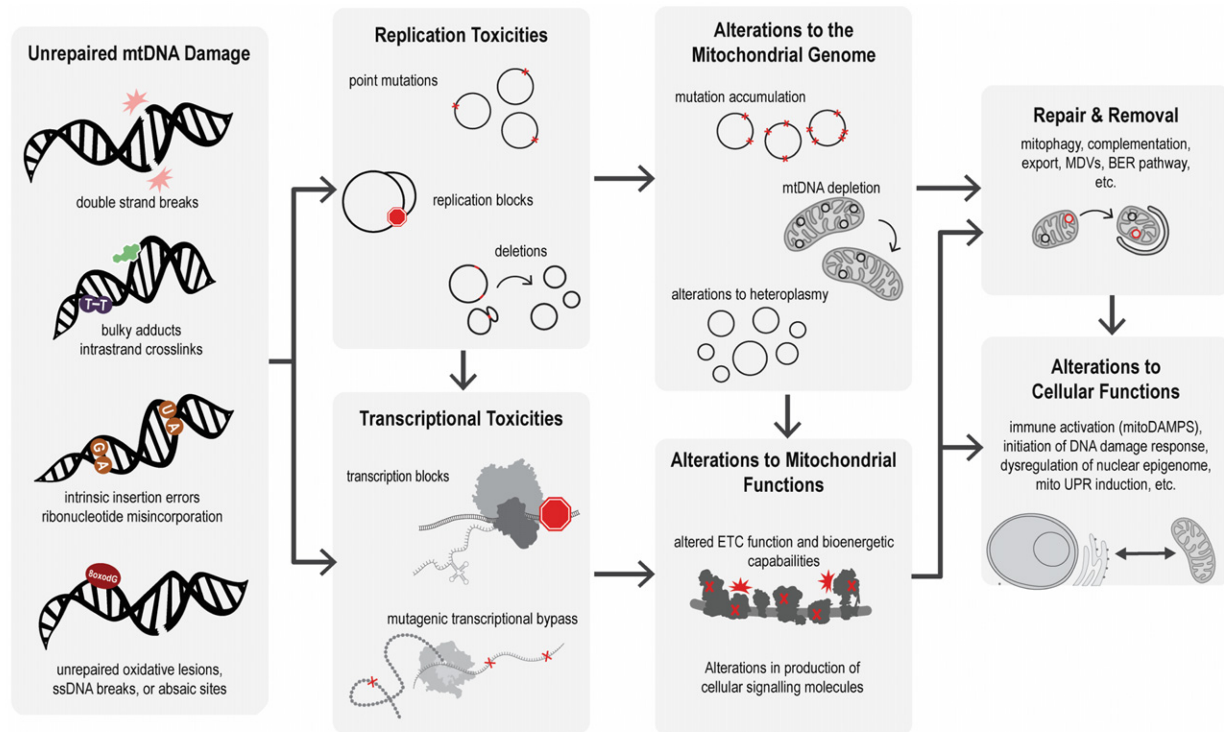


**Fig. 5** Outcomes of mtDNA toxicities. DNA damage that is not repaired can impact mtDNA replication and mtDNA transcription, as can large changes in mtDNA copy number. Disruptions to mtDNA replication can ultimately lead to alterations to the mitochondrial genome. Disruptions to mtDNA transcription can result in alterations to mitochondrial function, such as bioenergetic capabilities and the formation of key cell signaling molecules. Some forms of mtDNA damage can result in repair and removal of damaged genomes. Ultimately, unrepaired mtDNA damage can cause disruptions to broad cellular function and alter cell signaling pathways.

## 2.13.8 Summary

The mitochondrial genome is both structurally and functionally distinct from the nuclear genome, accounting for vital differences in the susceptibility and adverse phenotype associated with chemically-induced gene mutations. Although the same rules apply for chemical reactivity of genotoxic agents, numerous discrete features render mtDNA particularly susceptible. This includes the disproportionate accumulation of weak acids and cationic compounds into the mitochondrial matrix and the lack of intronic sequences in mtDNA and some DNA repair capacities. The high redundancy of mtDNA copies in each cell, which is a dynamic feature that differs among tissues, tempers this susceptibility, and affords a certain degree of threshold, non-linear dose-response relationships to chemical exposure. Unlike the nuclear genome, exceeding this threshold results in bioenergetic failure of affected tissues and a clinical phenotype not unlike those associated with heritable, primary mitochondrial diseases, where the critical adverse event is metabolic dysregulation and the accompanying neurologic, endocrine and myopathic deficits. Fig. 5 illustrates some of the effects of mitochondrial DNA toxicities discussed in this chapter. Despite recent advances in characterizing and understanding the molecular regulation of the mitochondrial genome, we are only on the cusp of fully appreciating the entire scope and significance exposure-related mitochondrial genomic toxicity as a determinant of individual and public health.

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Human DNA Polymerase Gamma Mutation Database