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Off-target effects of drugs that disrupt human mitochondrial DNA maintenance

Matthew J. Young¹

¹To whom correspondence should be addressed.

Department of Biochemistry and Molecular Biology, Southern Illinois University School of Medicine, Carbondale, Illinois 62901

Telephone number: (618) 453-6437

E-mail: matthew.young@siu.edu

1 **Abstract**

2 Nucleoside reverse transcriptase inhibitors (NRTIs) were the first drugs used to treat human
3 immunodeficiency virus (HIV) the cause of acquired immunodeficiency syndrome. Development
4 of severe mitochondrial toxicity has been well documented in patients infected with HIV and
5 administered NRTIs. *In vitro* biochemical experiments have demonstrated that the replicative
6 mitochondrial DNA (mtDNA) polymerase gamma, Polg, is a sensitive target for inhibition by
7 metabolically active forms of NRTIs, nucleotide reverse transcriptase inhibitors (NtRTIs). Once
8 incorporated into newly synthesized daughter strands NtRTIs block further DNA polymerization
9 reactions. Human cell culture and animal studies have demonstrated that cell lines and mice
10 exposed to NRTIs display mtDNA depletion. Further complicating NRTI off-target effects on
11 mtDNA maintenance, two additional DNA polymerases, Pol beta and PrimPol, were recently
12 reported to localize to mitochondria as well as the nucleus. Similar to Polg, *in vitro* work has
13 demonstrated both Pol beta and PrimPol incorporate NtRTIs into nascent DNA. Cell culture and
14 biochemical experiments have also demonstrated that antiviral ribonucleoside drugs developed to
15 treat hepatitis C infection act as off-target substrates for POLRMT, the mitochondrial RNA
16 polymerase and primase. Accompanying the above-mentioned topics, this review examines: 1)
17 mtDNA maintenance in human health and disease, 2) reports of DNA polymerases theta and zeta
18 (Rev3) localizing to mitochondria, and 3) additional drugs with off-target effects on
19 mitochondrial function. Lastly, mtDNA damage may induce cell death; therefore, the possibility
20 of utilizing compounds that disrupt mtDNA maintenance to kill cancer cells is discussed.

21

22

1 **The origin of mitochondria and off-target effects of antibiotics**

2 Mitochondria are best known for their role in generating energy by oxidative
3 phosphorylation (OXPHOS), the process of coupling substrate oxidation to the production of the
4 energy-rich molecule adenosine triphosphate (ATP). In addition to generating the bulk of the
5 cell's energy supply mitochondria are important sites of calcium homeostasis, nucleotide and
6 amino acid metabolism and biosynthesis of heme, iron-sulfur clusters, and ubiquinone.

7 Mitochondria are eukaryotic organelles that share bacterial features such as a double-membrane
8 structure and a circular multi-copied genome or mitochondrial DNA (mtDNA). The
9 endosymbiotic theory hypothesizes mitochondria descended from an ancient alpha (α)-
10 proteobacteria that developed a symbiotic relationship with an ancient nucleated cell (1). Support
11 for the endosymbiotic hypothesis comes from striking similarities revealed between the
12 mitochondrial and the *Rickettsia prowazekii* genomes (2). Over time mitochondria lost most of
13 their proto-bacterial genome to the nucleus. One thousand one hundred and forty-five nuclear-
14 encoded mitochondrial gene products must be imported into mitochondria following translation
15 on cytoplasmic ribosomes and estimates place the total mitochondrial proteome at ~1500 gene
16 products (3, 4). Currently, there are ~170 known mitochondrial disease genes associated with
17 ~500 clinical phenotypes suggesting that most medical specialists could see patients with
18 mitochondrial disease (5, 6). The α -proteobacterial endosymbiont origin of mitochondria is
19 supported by observations that certain antibiotics have off-target effects on mitochondrial
20 ribosomes. Similar to bacterial translation, mitochondrial translation is initiated with an *N*-
21 formylmethionine and mitochondrial but not cytoplasmic translation is sensitive to bacterial
22 antibiotics such as chloramphenicol (CAP) and aminoglycosides (7-9). Additionally,
23 mitochondrial ribosomes are resistant to inhibitors of eukaryotic translation such as emetine and

1 cycloheximide (8).

2 **Mitochondrial disorders and the importance of mtDNA maintenance in human health**

3 The haploid human nuclear genome consists of ~3 billion base pairs (bp) of DNA and
4 contains ~20,000 protein-coding genes and ~23,000 non-coding genes. Examples of non-coding
5 genes include transfer RNA (tRNA), ribosomal RNA (rRNA), micro RNA (miRNA),
6 miscellaneous RNA (miscRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA),
7 small cytoplasmic RNA (scRNA), and long non-coding RNA (lncRNA). In comparison, the
8 mitochondrial genome harbors only 13 genes for polypeptides, 2 genes for rRNA, and 22 genes
9 for tRNA on ~16,600 bp and mutations associated with maternally-inherited mitochondrial
10 disorders have been identified in all 37 open reading frames. Similar to practically all
11 prokaryotic genes, human mtDNA genes lack introns. *The 13 polypeptide-encoded genes code*
12 *for subunits of the mitochondrial inner membrane (MIM) OXPHOS machinery.* While the size
13 and coding capacity of mtDNA is much less than the nuclear genome our maternally inherited
14 genome is critical to cellular viability as exemplified by the numerous disease mutations
15 associated with it and by observations that knocking out mtDNA maintenance genes results in
16 embryonic lethality in various mouse models (10). Currently, greater than 660 mtDNA mutations
17 are associated with disease phenotypes (www.mitomap.org). The most common
18 encephalopathies caused by mtDNA point mutations include Leigh Syndrome, Leber Hereditary
19 Optic Neuropathy, MERRF (myoclonic epilepsy with ragged red fibers), MIDD (maternally
20 inherited diabetes and deafness), MELAS (mitochondrial encephalomyopathy, lactic acidosis,
21 and stroke-like episodes), nonsyndromic hearing loss, and NARP (neuropathy, ataxia, retinitis
22 pigmentosa) (11). Maintenance of the mitochondrial genome is also required to avoid apoptosis
23 induced by mtDNA damage (12, 13).

1 Molecules of mtDNA associate with various DNA-binding proteins on the matrix-side of
2 the MIM and form protein-mtDNA structures known as nucleoids (14-17). Utilizing live-cell
3 fluorescence microscopy or immunocytochemistry, nucleoids can be visualized as foci or puncta.
4 Furthermore, a single cell can contain several thousand copies of mtDNA which are distributed
5 within hundreds of individual mitochondria or throughout an elaborate mitochondrial reticular
6 network (17-20). Localization of mtDNA at the MIM is likely important to coordinate mtDNA
7 replication and transcription with mitochondrial translation, cytoplasmic translation, and
8 mitochondrial protein import and assembly (18, 21). Nuclear-encoded mitochondrial
9 transcription machinery is imported into the organelle to transcribe mtDNA genes. Nuclear-
10 encoded mitochondrial ribosomal subunits assemble with mtDNA-encoded rRNAs following
11 protein import to form the translation machinery necessary to synthesize the 13 mtDNA-encoded
12 polypeptides. Therefore, the MIM OXPHOS energy-generating process is strictly dependent on
13 mtDNA maintenance and pharmacological blocks to mitochondrial genome replication would be
14 devastating to this energy-generating process.

15 **Mitochondrial reactive oxygen species (ROS) and base excision repair (BER)**

16 Aberrant electron leakage from the OXPHOS machinery to molecular oxygen (O_2) can
17 generate reactive oxygen species (ROS) which, if not detoxified, cause damage to intracellular
18 molecules such as DNA, RNA, lipids, and proteins (22). The close proximity of mtDNA-
19 containing nucleoids to the OXPHOS machinery generating ROS has been suggested to inflict
20 more damage on the mitochondrial genome than on the nuclear genome (12). ROS-induced DNA
21 damage includes a large quantity of mutagenic oxidized bases and the mutation rate of human
22 mtDNA has been estimated to be 20 to 100-fold higher relative to nuclear DNA. Nuclear-
23 encoded base excision repair (BER) machinery is imported into the mitochondrion to assist with

1 mending abnormal and oxidized base lesions. During mitochondrial short-patch BER, an
2 oxidized base may first be excised by a monofunctional DNA glycosylase such as UNG1 or
3 MUTYH, Figure 1 A. DNA glycosylase cleaves the damaged base *N*-glycosidic bond generating
4 an abasic or apurinic/apyrimidinic (AP) site then this site is cleaved by an AP endonuclease to
5 generate a 3'-OH and nonligatable 5'-deoxyribose phosphate (dRP) moiety. Next, the catalytic
6 subunit of the replicative mitochondrial 5'-3' DNA polymerase gamma (Poly) fills in the gap via
7 its DNA polymerase activity and removes the dRP group via its 5'-deoxyribose phosphate (dRP)
8 lyase activity leaving a 5'-phosphate. Lastly, DNA ligase III seals the nick and the damage is
9 repaired (23). Alternatively, a bifunctional DNA glycosylase harboring an intrinsic lyase activity
10 can cleave the *N*-glycosidic bond and incise the AP site; however, the ends generated by the
11 incision are nonligatable and must be processed by either AP endonuclease or polynucleotide
12 kinase 3'-phosphatase then Poly can fill the gap and ligase can seal the nick (24, 25). Figure 1 A
13 is a simplified cartoon of short-patch BER. Details regarding mitochondrial short-patch and
14 long-patch BER pathways have been thoroughly reviewed (25-27).

15 **Poly and the replisome**

16 Human Poly is the replicative mitochondrial DNA polymerase that harbors 3'-5'
17 exonucleolytic proofreading activity and participates in mtDNA repair (28). Poly is a
18 heterotrimer consisting of one 140-kDa catalytic subunit, p140 encoded by the nuclear *POLG*
19 gene, and a 110-kDa homodimeric processivity subunit, p55 encoded by the nuclear *POLG2*
20 gene. MtDNA disorders can be caused by genetic defects in nuclear genes, and a class of genes
21 specifically linked to instability of mtDNA has emerged over the last 16 years which includes
22 *POLG* and *POLG2*, Table 1 (28). Nuclear mitochondrial disease genes are associated with a

1 complex spectrum of early onset and late onset type phenotypes. One subclass of disorders,
2 mtDNA depletion syndromes, may arise due to defects in genes encoding mtDNA replication
3 machinery (ex. *POLG*, Alpers-Huttenlocher syndrome) or enzymes required for nucleotide
4 synthesis (ex. *TK2*). MtDNA depletion syndromes in of themselves are variable and clinical
5 manifestations may include myopathy, encephalomyopathy, neurogastrointestinal, or
6 hepatocerebral phenotypes (29). In addition to the 5'-3' DNA polymerase, 3'-5' exonuclease, and
7 5' dRP lyase activities mentioned above the p140 catalytic subunit harbors reverse transcriptase
8 (RT) activity (30-32). The RT activity or RNA-dependent DNA polymerase activity is similar to
9 viral enzymes such as human immunodeficiency virus RT (HIV-RT). Unfortunately, as will be
10 discussed below, biochemical experiments have demonstrated that Poly is sensitive to inhibition
11 by metabolically active forms of anti-HIV nucleoside reverse transcriptase inhibitors (NRTIs)
12 known as nucleotide reverse transcriptase inhibitors (NtRTIs). Treatment of HIV-infected
13 patients with NRTIs is accompanied by loss of mitochondrial function and NRTI toxicity mimics
14 mitochondrial genetic diseases and induces similar symptoms such as mtDNA depletion (31).
15 One explanation as to why Poly harbors RT activity may be to replicate past ribonucleotides
16 (ribonucleoside monophosphates) that are evenly distributed between the two strands of mtDNA
17 (32, 33). The homodimeric Poly p55 subunit imparts high processivity onto the holoenzyme by
18 increasing the binding affinity to DNA (17, 34). Processivity is a measurement of the extent of
19 Poly DNA synthesis during a primer-template binding event. Poly functions in conjunction with
20 several replisome components including: 1) topoisomerase, 2) mitochondrial single-stranded
21 DNA binding protein (mtSSB), 3) Twinkle mtDNA helicase, 4) RNaseH1, 5) mitochondrial
22 RNA polymerase (POLRMT), and 6) mitochondrial DNA ligase III, Figure 1 B. Additional
23 factors critical for mitochondrial genome maintenance include: the multifunctional mitochondrial

1 transcription factor A (TFAM) with significant roles in mtDNA replication and packaging, the
2 RecB-type mitochondrial genome maintenance 5'-3' exonuclease 1 (MGME1), the RNA and
3 DNA 5' flap endonuclease (FEN1), and the helicase/nuclease, DNA2 (35-37). MGME1, FEN1,
4 and DNA2 have all been implicated in mtDNA BER (26). Furthermore, DNA2 has been shown
5 to stimulate Poly activity and to co-localize with Twinkle in the mitochondrial nucleoid, which
6 suggests an important role in the replisome (38). Some of the genes encoding components of the
7 mtDNA replication machinery may have been acquired as part of a protomitochondrial genome,
8 in the form of integrated phage genes from a T-odd lineage, which were then transferred to the
9 eukaryotic nucleus (39). This hypothesis is based on the shared conservation of primary protein
10 amino acid sequences of T-odd bacteriophages with mitochondrial Poly, POLRMT, and Twinkle
11 helicase.

12 In agreement with the requirement for mtDNA replication re-initiation between
13 embryonic day (E)6 and 7.5 (40), p140 in animal cells was shown to be essential using *POLG*
14 knockout (KO) mice. The *POLG* KO results revealed embryonic lethality at E7.5–8.5 with
15 subsequent depletion of mtDNA (41). Comparatively, several studies have illustrated the
16 essential role of p55 in mtDNA replication: (i) two separate null mutations in the *Drosophila*
17 *melanogaster* *POLG2* gene lead to lethality in the early pupal stage of fly development (42), (ii)
18 homozygous *POLG2* KO mice are embryonic lethal at E8–8.5 (43) and (iii) in a porcine oocyte
19 knockdown model, oocyte maturation requires *POLG2* (44). Mouse RNaseH1^{-/-} embryos are null
20 at E8.5 and have decreased mtDNA content leading to apoptotic cell death (45). A mouse model
21 of Twinkle deficiency has been generated by transgenic expression of a Twinkle cDNA with an
22 autosomal dominant mutation found in patients (46, 47). These mice developed progressive
23 respiratory chain deficiency at 1 year of age in cerebellar Purkinje cells, hippocampal neurons,

1 and skeletal muscle. The affected cells accumulated multiple mtDNA deletions. These ‘Deletor’
2 mice recapitulate many of the symptoms associated with PEO and represent a useful research
3 model.

4 **Newly identified human DNA polymerases localizing to mitochondria - Pols β , θ ,** 5 **ζ and PrimPol**

6 Prior to 2013 human Pol γ was the
7 only polymerase out of the 17 known cellular DNA polymerases demonstrated to localize to
8 human cell mitochondria; however, mounting evidence suggests it is not the only one. Recently,
9 DNA polymerase beta (Pol β) was detected in mitochondrial extracts prepared from human
10 embryonic kidney cells (HEK-293T) and from various tissues obtained from mice (48). Analysis
11 of mouse tissue extracts revealed Pol β in brain and kidney mitochondria while none was
12 detectable in heart, liver, or muscle. As a key member of the nuclear BER machinery Pol β
13 provides the majority of the required 5'-dRP lyase activity in the nucleus; therefore, Pol β may
14 participate in mitochondrial BER. In a short-patch BER scenario, following the actions of a
15 monofunctional DNA glycosylase and an AP endonuclease Pol β could insert a nucleotide onto
16 the 3'-OH then remove the 5'-dRP group using its dRP lyase activity followed by the nick sealing
17 action of DNA ligase (Figure 1 A). As mentioned above mitochondrial ribosomes are sensitive to
18 CAP (CAP^S). MtDNA can develop resistance to CAP (CAP^R) through mutation of the mtDNA
19 16S rRNA gene changing the specificity of CAP for the mitochondrial ribosome and inhibiting
20 its binding (9, 48-50). In two HEK-293T Pol β KO cell lines very few CAP^R cells could be
21 isolated relative to the parental cell line when plated at high cell density. This finding suggests
22 that Pol β may mediate mtDNA mutational events. Utilizing *in vitro* biochemistry Pol β was also

1 demonstrated to interact with the mitochondrial Twinkle helicase and this interaction facilitated
2 Pol β strand displacement. Enhanced strand displacement suggests Pol β may participate in the
3 mitochondrial long-patch BER pathway (48). As many *Twinkle* gene-disease mutations result in
4 protein variants with partial helicase defects (51) it would be interesting to investigate strand
5 displacement using recombinant Twinkle disease variants and Pol β to provide insight into
6 possible mechanisms of *Twinkle*-related mitochondrial disease. Besides BER, other roles of Pol β
7 in mtDNA maintenance remain to be elucidated. Pol β is not likely a replicative mtDNA
8 polymerase as this enzyme lacks 3'-5' exonuclease proofreading activity, has low processivity,
9 incorporating few nucleotides each time it binds a primer-template, and has a high error rate
10 relative to the proofreading proficient Pol γ (24). However, *POLG*-related disease mutations that
11 abolish p140 activity and are associated with late age of onset may argue in favor of redundant
12 DNA polymerase function(s) in human cell mitochondria (48). Pol β ^{-/-} mouse embryos survive
13 the course of development but die immediately at the perinatal stage suggesting the cause of
14 death is a neonatal respiratory defect (52).

15 The DNA *primase* and translesion DNA *polymerase*, PrimPol, has been identified in
16 mitochondria isolated from HEK-293T cells (53). Translesion DNA polymerases are specialized
17 enzymes that pass through DNA damage. However, PrimPol is likely only required for mtDNA
18 repair and not for mtDNA replication, as *PRIMPOL*^{-/-} KO mice are viable. Like Pol β PrimPol is
19 localized to both the nucleus and the mitochondrion and lacks proofreading activity. Of note to
20 human genetic disease, mutation of *PRIMPOL* is associated with the ocular disorder high myopia
21 (54, 55). DNA polymerase theta (Pol θ) was recently identified in mitochondria isolated from
22 human cells (56). Pol θ is a proofreading-deficient and error-prone polymerase capable of

1 translesion DNA polymerization (57). In the nucleus, Pol θ is implicated in double-strand DNA
2 break repair, non-homologous end joining and maintenance of DNA replication timing. The
3 translesion DNA polymerase zeta (Pol ζ) is composed of two subunits the catalytic subunit Rev3
4 and the structural subunit Rev7. To date, no evidence for Rev7 localization to human cell
5 mitochondria has been described but the Rev3 subunit has been reported to localize to the
6 organelle and may play a role in protecting mtDNA from ultraviolet radiation-induced DNA
7 damage (58). Compared to Pol γ , Pol θ and ζ localize to both the nucleus and the mitochondrion,
8 have low fidelity, lack proofreading activity and have only moderate processivity (24, 57, 59);
9 therefore, their main roles are likely in assisting the core replisome in overcoming mtDNA
10 damage. Pol θ KO mice are viable whereas Pol ζ KO mice are embryonic lethal with a block in
11 embryo development not beyond 8 to 8.5 days *post coitus* (60, 61). Details regarding the
12 evidence supporting mitochondrial localization of the aforementioned human DNA polymerases
13 have been reviewed (62).

14 **Nucleoside reverse transcriptase inhibitors, NRTIs**

15 NRTIs were the first drugs used to treat HIV, the cause of acquired immunodeficiency
16 syndrome (AIDS). NRTIs remain effective today for treating HIV when combined with other
17 drugs. Highly active antiretroviral therapy (HAART) uses multiple drugs to act on different HIV
18 life-cycle stages. For patients with HIV infection, HAART regimens include treatment with
19 NRTIs in combination with non-nucleoside reverse transcriptase inhibitors (NNRTIs) or protease
20 inhibitors, PIs (63). NNRTIs and NRTIs primarily block HIV genome replication by inhibiting
21 the HIV-RT from transcribing the viral single-stranded RNA genome into DNA. FDA-approved
22 NRTIs used to treat HIV infection include: ddC (zalcitabine), 3TC (Epivir[®], lamivudine), AZT

1 (Retrovir[®], zidovudine), dDI (Videx-EC[®], didanosine), PMPA (Viread[®], tenofovir DF), d4T
2 (Zerit[®], stavudine), ABC (Ziagen[®], abacavir), and FTC (emtricitabine, Emtriva[®]), Table 2.
3 NRTIs may be administered to patients in fixed-dose combinations: Combivir[®] (Retrovir +
4 Epivir), Descovy[®] (tenofovir alafenamide + Emtriva), Epzicom[®] (Epivir + Ziagen), Trizivir[®]
5 (Retrovir + Epivir + Ziagen), and Truvada[®] (Viread + Emtriva),
6 <https://www.hiv.va.gov/patient/treat/NRTIs.asp>. The history of antiretroviral drugs and the
7 currently used antiretroviral therapies have been reviewed (64). A discussion of what is currently
8 known regarding NRTIs with off-target effects on mtDNA replication is discussed below.

9 Nucleoside analogs, including NRTIs, are taken up by cells then phosphorylated to active
10 nucleotide analogs by intracellular kinases (65). Nucleoside kinases such as DCK, CMPK1, and
11 nucleoside diphosphate kinases (NME) act on NRTIs like ddC and perform the first, second, and
12 third phosphorylation steps respectively generating the active NtRTI in the cytoplasm ex.
13 ddC_{ppp}, where ppp represent the triphosphate (66). NtRTIs can then be imported into
14 mitochondria and could compete with native nucleotides at DNA polymerase active sites to
15 inhibit mtDNA replication through chain termination and persistence in the mitochondrial
16 genome. Unlike natural deoxyribonucleotide triphosphate substrates, and with the exception of
17 FIAU, NtRTIs are chain terminators that lack the 3' hydroxyl group and therefore cannot be
18 extended by a polymerase once incorporated into DNA. Therefore, if these analogs are not
19 removed from DNA, replication will stall (Figure 1 B).

20 **Clinical evidence for NRTI disruption of mtDNA replication**

21 In clinical trials drugs that showed promise in AIDS therapy, such as fluoro-
22 dideoxyadenosine (FDDA), or in the treatment of chronic hepatitis B infection, such as FIAU,

1 toxicity was reported affecting peripheral nerves, liver, skeletal and cardiac muscle (67).
2 Toxicity to mitochondria was so severe that hepatic failure and death in some patients
3 necessitated discontinuation of their use (68). One long-term AZT use study of HIV-positive
4 patients concluded that AZT treatment caused toxic mitochondrial myopathy (69). In a follow-up
5 study investigating mtDNA content in muscle biopsies, mitochondrial genome depletion was
6 discovered in all HIV-positive patients who were treated with AZT and who displayed myopathy
7 and ragged-red fibers in comparison to controls (70). Another study investigated HIV-positive
8 patients who developed neuropathy 6 to 10 weeks after starting ddC and this investigation found
9 mitochondrial alterations and significantly reduced mtDNA copy number in nerve biopsy
10 specimens (71). These and other observations led to the Poly dysfunction hypothesis.

11 Hypothetically, poisoning of Poly would lead to decreased mtDNA, increased mitochondrial
12 stress due to compromised OXPHOS (as OXPHOS subunits are encoded by mtDNA), increased
13 cellular energy depletion (due to diminished ATP pools), and acquired mitochondrial disease
14 phenotypes (72). Key side effects of NRTIs are summarized in Table 2 and (72). Support for the
15 Poly dysfunction hypothesis comes from cell culture and biochemical work discussed below.

16 **Evidence for Poly-mediated NRTI toxicity from biochemical studies**

17 Poly-mediated NRTI mitochondrial toxicity requires that analogs be metabolized to
18 NtRTIs, imported into mitochondria then incorporated into mtDNA and persist there to block
19 further genome replication events. Support for NRTI toxicity caused by inhibition of Poly DNA
20 polymerase activity comes from extensive biochemical evidence. Pre-steady and steady-state
21 enzyme kinetic analyses have demonstrated that Poly is able to incorporate various anti-retroviral
22 NtRTIs (73-76). NtRTIs that have been tested *in vitro* for incorporation into nascent DNA by

1 Poly include: ddCppp, ddTppp, d4Tppp, ddAppp (the active form of ddI), (+) & (-)3TCppp,
2 PMPApp (PMPA triphosphate), AZTppp, CBVppp (the active form of ABC), and FIAUppp.
3 These biochemical studies agree that Poly incorporates NtRTIs during DNA replication;
4 however, the efficiency of analog incorporation is variable among the NtRTIs that have been
5 examined. Poly incorporates ddCppp, ddAppp (ddI), and d4Tppp analogs most efficiently while
6 3TCppp, PMPApp, AZTppp, and CBVppp (ABC) are modestly incorporated into DNA. Steady-
7 state and pre-steady-state kinetics have also demonstrated that FIAUppp is strongly incorporated
8 by Poly (73, 77). Mitochondrial toxicity, therefore, may be acquired due to a block in mtDNA
9 replication if chain-terminating NtRTIs cannot be removed. Indeed, biochemical evidence has
10 shown that Poly does not efficiently proofread NtRTIs incorporated into DNA. Pre-steady-state
11 measurements have demonstrated that a ddCp (ddC monophosphate) incorporated into the 3'-end
12 of a DNA oligonucleotide annealed to a DNA template essentially cannot be removed by Poly
13 proofreading activity even after 12-hour incubations with the DNA duplex (73). The remaining
14 NtRTIs analyzed for exonucleolytic removal had slow rates of excision and it has been estimated
15 that the half-life of the reaction to remove (+)3TCp or (-)3TCp is ~1 minute (78). The rate of
16 NtRTI excision could be detrimental *in vivo* by slowing the mtDNA replication machinery. If
17 Poly dissociates from mtDNA prior to cleaving an incorporated nucleotide analog then
18 replication would be terminated. When PMPA-terminated DNA substrate was tested for excision
19 in the presence of trap DNA, no Poly exonuclease activity was detected (73). This finding
20 suggests that NtRTI-containing duplex DNA is released from Poly prior to NtRTI excision and
21 perhaps a similar mechanism could happen *in vivo* with many copies of mtDNA. Similar
22 findings of slow rates of NtRTI excision were observed utilizing steady-state analyses.
23 Additionally, Poly exonuclease activity was inhibited at *in vivo* concentrations of the AZTppp

1 phosphorylated intermediate AZT monophosphate, AZTp (74). Perhaps *in vivo* intracellular
2 levels of AZTp allow for binding of the analog to the exonuclease active site and lower Pol γ 's
3 fidelity by blocking proofreading.

4 In 2015 crystal structures of Pol γ -DNA replication complexes separately bound to
5 ddCppp or to the natural substrate dCTP were solved (79). Within the DNA polymerase active
6 site the side chain of the p140 Y951 residue stacks with the incoming ddCppp nearly identically
7 to the natural dCTP substrate. The ribose sugar moieties of both nucleotides are located 3.5 Å
8 from the p140 Y951 hydroxyl group. In support of the p140 Y951 residue being the likely cause
9 of ddCppp toxicity, a biochemical study demonstrated that mutation of Y951 to phenylalanine
10 maintains DNA polymerase activity but renders p140 Y951F almost completely incapable of
11 incorporating ddCppp, CBVppp, 3TCppp, and d4Tppp (80). The p140 Y951F had a 2400-fold
12 increase in dCTP/ddCppp discrimination relative to wild-type p140. Therefore, the substitution
13 of the smaller phenylalanine side chain in the p140 Y951F variant must influence the structure
14 such that ddCppp is excluded from the DNA polymerase active site and not readily incorporated
15 into DNA.

16 Variability in mtDNA depletion has been observed in HIV-positive patients treated with
17 NRTIs and may result from a difference in treatment times or from genetic variations that have
18 increased susceptibility to NRTIs or both. A homozygous mutation encoding p140 R964C was
19 identified in a 34-year-old HIV-infected woman with a history of lactic acidosis induced by d4T
20 treatment (81). Recombinant p140 R964C displays 14% polymerase activity relative to WT
21 p140. Additionally, a patient-derived p140 R964C lymphoblastoid cell line (LCL) cultured with
22 d4T displays mtDNA depletion relative to a WT LCL suggesting p140 R964C is associated with

1 severe lactic acidosis induced by NRTI use. A pre-steady state analysis of Poly holoenzyme
2 harboring the p140 R964C variant determined that the substitution caused a 33% reduction in
3 dTTP incorporation efficiency and a 3-fold decrease in dTTP/d4Tppp discrimination relative to
4 WT suggesting p140 R964C has a higher propensity to incorporate d4Tppp (82). The p140 R964
5 residue is located in close proximity to the DNA polymerase active site. One explanation for the
6 mechanism of increased d4Tppp incorporation is that the p140 R964C substitution modulates
7 active site access increasing binding to d4Tppp. Also, a heterozygous mutation (C>T 2857/p140
8 R953C) was identified in an HIV-infected patient undergoing antiretroviral therapy who
9 displayed mitochondrial toxicity and mtDNA depletion (83). The recombinant R953C Poly
10 holoenzyme displayed an 8-fold weakened ability to bind to dCTP and a 4-fold decrease in its
11 ability to discriminate between dCTP and (-)-3TCppp relative to WT. Molecular modeling
12 revealed that a cysteine substitution at position 953 in p140 could abolish interactions between
13 p140 side chain residues in the active site thereby reducing the binding of an incoming
14 nucleotide. In another case-control study examining the relationship between p140 E1143D/G
15 substitutions, lipodystrophy, and d4T treatment it was concluded that HIV-infected patients
16 harboring an E1143D/G variant are 4-fold more likely to develop lipodystrophy and if treated
17 with d4T the risk of developing lipodystrophy increased (84).

18 **Evidence for NRTI disruption of mtDNA replication from cell culture and animal studies**

19 Support for intracellular NRTI mitochondria toxicity mediated by disruption of mtDNA
20 replication comes from observations that primary and immortalized cell lines undergo mtDNA
21 depletion upon exposure to various NRTIs. Table 3 lists examples of human cell lines exposed to
22 various nucleoside analogs in tissue culture. In some reports, mtDNA depletion was so severe

1 cell lines became rho zero completely lacking mtDNA. These findings are similar to what has
2 been reported with LA9 mouse cells exposed to ddC (85) and with treating human cell lines with
3 the mtDNA replication inhibitor ethidium bromide, EtBr (86). Low concentrations of EtBr either
4 partially or completely inhibit maintenance of the negatively supercoiled circular mitochondrial
5 genome but not nuclear DNA (nDNA). EtBr binds better to negatively supercoiled substrates
6 than to positively supercoiled ones and might enhance topoisomerase-mediated cleavage of
7 negatively supercoiled DNA; therefore, EtBr may act as a topoisomerase topological poison (87).
8 In agreement with Poly biochemical analyses, treatment of human cell lines with several
9 nucleoside analogs typically duplicate the finding that ddC causes the most severe inhibition of
10 mtDNA replication as indicated by mtDNA depletion. In an animal study investigating AZT
11 exposure by administering the drug in drinking water to rats, transmission electron microscopy
12 revealed widespread mitochondrial alterations in the heart following 35 days of treatment with 1
13 mg/ml AZT (88). In another four-month study investigating the treatment of BALB/C mice with
14 ddI, d4T, AZT, or 3TC, and with the exception of liver tissue from mice treated with 3TC,
15 mtDNA depletion was reported in liver, muscle, and cortical neurons. Also, cortical neurons
16 isolated from mice treated with ddI, d4T, and 3TC were reported to harbor an increased level of
17 mtDNA deletions (89).

18 **Other potential mechanisms of NRTI toxicity**

19 Other mechanisms of NRTI toxicity include increased frequency of mtDNA mutations
20 (perhaps from an altered Poly function), enhanced oxidative stress, and competition with
21 endogenous nucleotides for kinases required to phosphorylate and activate them thereby
22 lowering the *in vivo* concentrations of nucleotides available to replicate mtDNA (90). The recent

1 discovery of other cellular DNA polymerases localizing to human mitochondria also has
2 implications for NRTI toxicity as these enzymes may incorporate analogs. Purified Pol β is
3 considerably sensitive to NtRTIs including d4Tppp and ddCppp (75, 91) and compared to Pol γ is
4 less selective for and can incorporate AZTppp, PMPApp, L-FTCppp, and L-3TCppp, Table 2
5 and (76). In the nucleus Pols alpha (α), delta (δ), and epsilon (ϵ) harbor strong nucleotide
6 selection mechanisms and are less likely to incorporate NtRTIs (92). Incorporation of NtRTIs by
7 Pol β within the organelle would be complicated by 1) the sensitivity of analog incorporation by
8 Pol γ and 2) the lack of Pol β proofreading activity, which would likely contribute to NtRTI
9 persistence within mtDNA. Figure 1 A and B highlight key steps in BER and mtDNA replication
10 that could be negatively affected by NtRTIs. Finally, the mitochondrial localization of DNA
11 repair polymerases with flexible active sites could allow for accommodation of nucleotide
12 analogs and contribute to unwanted insertion of chain terminators. Pre-steady-state analyses of
13 PrimPol NtRTI incorporation kinetics revealed effective incorporation of CBVppp, followed by
14 ddCppp > ddAppp > AZTppp while d4Tppp, 3TCppp, PMPApp, and FTCppp were not readily
15 incorporated. From this study, it was determined that CBVppp is actually a better substrate for
16 PrimPol than for HIV-RT which may help to explain life-threatening sensitivity to this analog in
17 some patients (93).

18 **Evidence for POLRMT-mediated AVRN toxicity from biochemical and cell culture studies**

19 POLRMT directs polycistronic transcription from three promoters the heavy-strand
20 promoter 1 (HSP1), the HSP2, and the light-strand promoter, LSP (94), Figure 1 C. The two
21 mtDNA strands are named heavy (H) and light (L) based on the ability to separate them on
22 alkaline cesium chloride buoyant density gradients (95). RNA polymerase enzymes known as

1 primases synthesize RNA primers required for initiation of DNA replication. Evidence
2 supporting the role of human POLRMT as the mtDNA primase comes from the identification of
3 primers located adjacent to nascent H-strands isolated from human KB cell mitochondria (96),
4 from *in vitro* experiments demonstrating that POLRMT has primase activity (97), and from the
5 observation that replicating mtDNA obtained from mouse embryonic fibroblasts, and lacking
6 RNaseH1, retain unprocessed primers at origins of replication (98). The 5'-end of RNA primers
7 that have been mapped to the LSP therefore likely serve to initiate synthesis of nascent H-strand
8 mtDNA (96), Figure 1 B. Consequently, mtDNA replication is likely dependent on
9 mitochondrial transcription.

10 Sofosbuvir is an antiviral uridine analog inhibitor of hepatitis C virus (HCV) RNA-
11 dependent RNA polymerase (HCV non-structural protein 5B, NS5B) currently approved for use
12 to treat patients with HCV infections. A number of reports have described the potential use of
13 other antiviral ribonucleosides (AVRN) as anti-viral and anti-cancer agents; however, many of
14 these AVRN have had adverse toxic effects when administered to patients and did not pass
15 clinical trials or gain FDA approval (99, 100). For example, the AVRN analog BMS-986094
16 developed to treat HCV infection did not pass phase II development after nine patients became
17 hospitalized and one died (93). Utilizing a POLRMT *in vitro* biochemical system to measure
18 substrate utilization a panel of more than ten AVRN analogs were investigated that contained
19 moieties found in past and lead anti-HCV non-obligate chain terminators (100). Non-obligate
20 chain terminators are AVRN containing a 3'-OH yet prevent viral RNA elongation. Except for
21 one analog, all AVRN triphosphates (AVRNt) investigated were readily utilized by POLRMT
22 as off-target substrates and five analogs were strong non-obligate chain terminators of POLRMT
23 RNA elongation. Utilizing the human hepatoma cell line, Huh-7, the panel of AVRN were

1 metabolized to active triphosphates, presumably by intracellular kinases, and the levels of the
2 triphosphate forms varied from less than 0.15 μM to 3.5 mM. Cellular evidence for AVRNs
3 being used as substrates by POLRMT was demonstrated using Huh-7 cells pre-treated for 24
4 hours with EtBr to suppress mitochondrial transcription then cells were exposed to AVRNs for 1,
5 2, and 3 days. Mitochondrial transcription was impaired in cells exposed to 2'-C-
6 methyladenosine, 6-methylpurine-riboside, and 4'-azidocytidine. This study demonstrated that
7 toxic effects of AVRNs might result from inhibition of the mitochondrial transcription
8 machinery and mtDNA gene expression (100). Due to the close coupling of mitochondrial
9 transcription and mtDNA replication, prolonged exposure to AVRNs might also affect mtDNA
10 maintenance, Figure 1 B and C.

11 **Other reports of drugs with off-target effects on mtDNA maintenance**

12 Four human cellular topoisomerases localize to mitochondria: TOP1mt, TOP2 α , TOP2 β ,
13 and a TOP3 α long isoform (101, 111). Tamoxifen a drug used to prevent breast cancer, tacrine a
14 drug used to treat Alzheimer's disease, and a fluoroquinolone broad-spectrum antibiotic, have all
15 been hypothesized to have off-target effects on mitochondrial topoisomerases (102-106). Mice
16 separately treated for 28 days with tamoxifen and tacrine displayed mtDNA depletion and both
17 of these drugs were demonstrated to inhibit *in vitro* topoisomerase-mediated plasmid DNA
18 relaxation (103, 104). The fluoroquinolone ciprofloxacin, an inhibitor of bacterial type II
19 topoisomerase DNA gyrase, was reported to induce double-strand mtDNA breaks when mouse
20 L1210 cells were exposed to various concentrations of the drug (106). The pyrrole alkaloid
21 lamellarin D and the chemotherapy drug doxorubicin have both been shown to poison
22 mitochondrial and nuclear topoisomerases (101).

23 Menadione (vitamin K3, VK3) has been demonstrated to inhibit the growth of human

1 cancer cell lines derived from various tissues and induces an increase in ROS leading to
2 apoptosis. In an *in vitro* biochemical assay VK3 selectively inhibited Poly DNA polymerase and
3 RT activities but did not inhibit the activity of other DNA polymerases tested including Pols
4 α , β , δ , ϵ , eta (η), iota (ι), kappa (κ), and lambda (λ). The authors proposed that suppression of
5 mtDNA replication and repair could trigger ROS production leading to apoptotic cell death
6 (107). Although the neurotoxicant 1-methyl-4-phenylpyridinium ion (MPP⁺) does not directly
7 inhibit the catalytic activity of Poly, MPP⁺ was reported to cause mtDNA depletion by
8 destabilizing the mtDNA displacement-loop, a mtDNA replication intermediate, thereby
9 inhibiting mitochondrial genome replication (108). Acetaminophen (APAP or paracetamol) is a
10 commonly used over the counter drug used for fever and pain relief. Mice treated with 300
11 mg/kg of acetaminophen had mtDNA depletion as quantitated using a slot blot hybridization
12 technique (109). The depletion is likely due to mtDNA strand breaks caused by the production of
13 ROS, reactive nitrogen species (RNS), and other reactive metabolites followed by rapid
14 degradation of damaged mtDNA by endogenous mitochondrial endonucleases (102).

15 Troglitazone is an anti-inflammatory and anti-diabetic drug that was withdrawn from the market
16 due to serious hepatotoxicity. Primary human hepatocytes exposed to troglitazone had increased
17 mtDNA depletion, decreased ATP production, and decreased cellular viability (110). ROS and
18 oxidative stress were hypothesized to be the source of mtDNA depletion causing mtDNA strand
19 breaks and cytotoxicity and treatment with *N*-acetyl cysteine (NAC), a known ROS scavenger,
20 reduced the troglitazone-induced cytotoxicity. Cisplatin is a platinum-based FDA-approved
21 chemotherapeutic known to damage nDNA by forming inter-strand crosslinks. Patients treated
22 with platinum-based compounds often display peripheral neuropathy, which may result from
23 damage to dorsal root ganglion neuronal mtDNA (112). *In vitro* work has demonstrated cisplatin

1 or oxaliplatin block Poly DNA synthesis. Furthermore, cisplatin has been demonstrated to inhibit
2 rat neuronal mtDNA replication and mitochondrial transcription (112-114).

3 **Targeting mtDNA maintenance to kill cancer cells**

4 Cancer cells display uninhibited DNA replication; therefore, DNA polymerases and DNA
5 repair proteins have been exploited as therapeutic targets to combat certain types of cancer (115,
6 116). NRTI-sensitive mitochondrial DNA polymerases afford a unique opportunity to target
7 cancer cell mitochondria as certain cancers have an increased reliance on OXPHOS and nDNA
8 polymerases are less sensitive to NRTI inhibition (66, 75). In a study comparing normal
9 hematopoietic cells to a panel of 542 primary acute myeloid leukemia (AML) samples, it was
10 recently discovered that 55% of the AML samples had increased mtDNA biosynthesis gene
11 expression. Upregulated genes included *POLG*, *POLG2*, *POLRMT*, *Twinkle*, *TFAM*, *SSBP1*,
12 *DGUOK*, *TK2*, nucleotide transporters (*SLC25A33*, *SLC25A36*, and *SLC29A3*) and nucleoside
13 kinases (*CMPK1* and *NME1-NME2*). When treated with ddC AML cells preferentially activated
14 the analog and blocked mtDNA replication and OXPHOS in comparison to hematopoietic cells.
15 Cytotoxicity was preferentially activated in NRTI-treated AML cells and an AML animal model
16 treated with low doses of ddC (35 and 75 mg/kg/day over 11 days) resulted in decreased
17 mtDNA, decreased mtDNA-encoded cytochrome oxidase subunit 2 (COX II), and induced tumor
18 regression without apparent toxicity (66).

19 Targeting mtDNA maintenance has also been exploited to treat cancer cell lines with a
20 mitochondrial-targeted cisplatin (117). Nucleotide excision repair (NER) machinery repairs
21 cisplatin-nDNA adducts; however, mitochondria lack NER machinery to deal with this type of
22 damage. Most cancer cells have an increased mitochondrial membrane potential relative to non-
23 cancer cells and triphenylphosphonium (TPP) cations are targeted to mitochondria due to their

1 size, lipophilic properties, and delocalized positive charge. An engineered TPP-tagged cisplatin,
2 Platin-M, caused increased cytotoxicity relative to cisplatin only treatment in several cancer cell
3 models: cisplatin-resistant A2780/CP70 ovarian cancer, prostate cancer PC3 (inherently resistant
4 to cisplatin therapy), and SH-SY5Y neuroblastoma cells. Furthermore, encapsulating Platin-M
5 into specialized nanoparticles enhanced cytotoxicity. SH-SY5Y cells treated with Platin-M and
6 Platin-M encapsulated in nanoparticles were annexin V-positive and propidium iodide-negative,
7 indicative of early apoptosis. Treatment with both Platin-M and Platin-M encapsulated in
8 nanoparticles weakened mitochondrial citrate synthase activity and diminished bioenergetic
9 parameters: spare respiratory capacity, coupling efficiency, and basal respiration. PC3 cells
10 treated separately with cisplatin, Platin-M, and Platin-M encapsulated inside of nanoparticles
11 were subjected to subcellular fractionation then platinum concentrations in various fractions
12 were quantified. Cells treated with Platin-M and Platin-M encapsulated in nanoparticles,
13 contained platinum-mtDNA adducts while cells treated with cisplatin contained mostly platinum-
14 nDNA adducts. These findings support that cisplatin is likely released from Platin-M within
15 mitochondria then binds to mtDNA and inhibits replication.

16 **Conclusions**

17 Evaluation of antibiotic and antiviral mitochondrial exposures using biochemistry and
18 human cell line and animal models is an important consideration for determining drug toxicity
19 because the complex mitochondrial network harbors multiple copies of OXPHOS complexes and
20 mtDNA that may cause a slow response to these agents. Chronic exposures to drugs may result
21 in long-term mtDNA and OXPHOS depletion. NRTIs may have tissue-specific toxicities such as
22 skeletal- and cardio-myopathies, peripheral neuropathy, and others (Table 2 and (67)). Side
23 effects may limit NRTI use in some individuals, cause organ failure and death in others or may

1 only result in minor discomfort (72). Gene variations (like those seen in *POLG* encoding p140
2 R964C, R953C, and E1143D/G) may exacerbate mitochondrial disease-like phenotypes in HIV-
3 infected patients treated with NRTIs. Also, valproic acid has been demonstrated to induce liver
4 failure in autosomal recessive *POLG* disease but may not be as toxic in autosomal dominant
5 disease. However, due to the potential for valproic acid to cause death by liver toxicity experts
6 recommend avoiding this drug (118). *POLG* is a highly polymorphic gene and the association
7 between disease-causing and non-disease causing substitutions are often unclear and dependent
8 on other complex factors. How other drugs or environmental factors interact with various genetic
9 variant backgrounds (so-called ecogenetic single nucleotide variants, ESNVs) and contribute to
10 mitochondrial disease manifestation is poorly understood (118, 119). Do ESNVs predispose
11 individuals to mitochondrial dysfunction via pharmacological or environmental toxicants while
12 individuals harboring other polymorphism remain resistant? ESNV-environment interaction is an
13 important area for future mitochondrial disease and mtDNA maintenance research. Evidence
14 suggests mitochondria are targets of environmental toxicants that disrupt mtDNA maintenance
15 and chemical exposures may cause increased and decreased mtDNA copy number. At low doses,
16 oxidative stress stimulates mtDNA replication but at high doses mtDNA depletion. Polycyclic
17 aromatic hydrocarbons cause more damage to mtDNA than to nDNA and a compilation of
18 studies comparing nDNA to mtDNA damage following chemical exposure has been reviewed
19 (120).

20 Evidence suggests five DNA polymerases localize to human cell mitochondria: Pol γ ,
21 Pol β , PrimPol, Pol θ , and Pol ζ . *In vitro* biochemistry measuring substrate binding and
22 incorporation lends strong support to Pol γ , Pol β , and PrimPol being off-targets for nucleotide
23 analogs (79, 91). Additionally, the Pol θ carboxyl-terminal polymerase domain has been

1 crystallized in a translesion DNA synthesis mode inserting ddAppp opposite a template abasic
2 site (121). Comparative investigations of mtDNA polymerase enzyme kinetics and determination
3 of crystal structures with and without lesions will assist in our understanding of the spectrum of
4 mtDNA polymerase toxicity. The overarching goal is that these structure-function studies will
5 assist with designing novel antiviral analogs with higher specificity to viral polymerases and less
6 mitochondrial off-target effects. In mice, mitochondrial Pol β was undetectable in heart, liver, and
7 muscle but was present in organelles obtained from brain and kidney (48). Potential questions for
8 future research include: 1) How are the other newly identified mtDNA polymerases distributed
9 among human organs and tissues and do they associate with other components of the mtDNA
10 repair or replication machinery? Knowledge of the distribution of mtDNA polymerases within
11 human tissues may assist with the prediction of tissue-specific toxicant effects. 2) Could
12 knowledge of mtDNA polymerases within different tissues be exploited to treat certain types of
13 cancers with NRTIs? 3) What analogs and toxicants are incorporated by the newly identified
14 mtDNA polymerases? And 4) Do ESNVs exist in any other genes of interest required for
15 mtDNA maintenance? Current next-generation sequencing technologies and continued research
16 utilizing *in vitro* biochemistry and model systems such as human cell lines and mice will be
17 essential to answer these questions and will be necessary for future investigations of
18 mitochondrial dysfunction and disease.

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3 **References:**

- 4
- 5 1. M. W. Gray, Lynn Margulis and the endosymbiont hypothesis: 50 years later. *Mol Biol*
- 6 *Cell* **28**, 1285-1287 (2017).
- 7 2. S. G. Andersson *et al.*, The genome sequence of *Rickettsia prowazekii* and the origin of
- 8 mitochondria. *Nature* **396**, 133-140 (1998).
- 9 3. S. E. Calvo, K. R. Clauser, V. K. Mootha, MitoCarta2.0: an updated inventory of
- 10 mammalian mitochondrial proteins. *Nucleic Acids Res*, (2015).
- 11 4. M. F. Lopez *et al.*, High-throughput profiling of the mitochondrial proteome using
- 12 affinity fractionation and automation. *Electrophoresis* **21**, 3427-3440 (2000).
- 13 5. C. Scharfe *et al.*, Mapping gene associations in human mitochondria using clinical
- 14 disease phenotypes. *PLoS Comput Biol* **5**, e1000374 (2009).
- 15 6. D. M. Turnbull, P. Rustin, Genetic and biochemical intricacy shapes mitochondrial
- 16 cytopathies. *Neurobiol Dis* **92**, 55-63 (2016).
- 17 7. D. C. Wallace, C. L. Bunn, J. M. Eisenstadt, Cytoplasmic transfer of chloramphenicol
- 18 resistance in human tissue culture cells. *J Cell Biol* **67**, 174-188 (1975).
- 19 8. N. A. Oliver, D. C. Wallace, Assignment of two mitochondrially synthesized
- 20 polypeptides to human mitochondrial DNA and their use in the study of intracellular
- 21 mitochondrial interaction. *Mol Cell Biol* **2**, 30-41 (1982).
- 22 9. D. C. Wallace, D. Chalkia, Mitochondrial DNA genetics and the heteroplasmy
- 23 conundrum in evolution and disease. *Cold Spring Harb Perspect Biol* **5**, a021220 (2013).
- 24 10. C. B. Park, N. G. Larsson, Mitochondrial DNA mutations in disease and aging. *J Cell*
- 25 *Biol* **193**, 809-818 (2011).
- 26 11. M. Pinto, C. T. Moraes, Mitochondrial genome changes and neurodegenerative diseases.
- 27 *Biochim Biophys Acta* **1842**, 1198-1207 (2014).
- 28 12. A. W. Tann *et al.*, Apoptosis induced by persistent single-strand breaks in mitochondrial
- 29 genome: critical role of EXOG (5'-EXO/endonuclease) in their repair. *J Biol Chem* **286**,
- 30 31975-31983 (2011).
- 31 13. J. H. Santos, L. Hunakova, Y. Chen, C. Bortner, B. Van Houten, Cell sorting experiments
- 32 link persistent mitochondrial DNA damage with loss of mitochondrial membrane
- 33 potential and apoptotic cell death. *J Biol Chem* **278**, 1728-1734. (2003).
- 34 14. D. F. Bogenhagen, D. Rousseau, S. Burke, The layered structure of human mitochondrial
- 35 DNA nucleoids. *J Biol Chem* **283**, 3665-3675 (2008).
- 36 15. T. A. Brown *et al.*, Superresolution fluorescence imaging of mitochondrial nucleoids
- 37 reveals their spatial range, limits, and membrane interaction. *Mol Cell Biol* **31**, 4994-5010
- 38 (2011).
- 39 16. F. Hensen, S. Cansiz, J. M. Gerhold, J. N. Spelbrink, To be or not to be a nucleoid
- 40 protein: a comparison of mass-spectrometry based approaches in the identification of
- 41 potential mtDNA-nucleoid associated proteins. *Biochimie* **100**, 219-226 (2014).

- 1 17. M. J. Young, M. M. Humble, K. L. DeBalsi, K. Y. Sun, W. C. Copeland, POLG2 disease
2 variants: analyses reveal a dominant negative heterodimer, altered mitochondrial
3 localization and impaired respiratory capacity. *Hum Mol Genet* **24**, 5184-5197 (2015).
- 4 18. J. N. Spelbrink, Functional organization of mammalian mitochondrial DNA in nucleoids:
5 history, recent developments, and future challenges. *IUBMB Life* **62**, 19-32 (2010).
- 6 19. F. J. Miller, F. L. Rosenfeldt, C. Zhang, A. W. Linnane, P. Nagley, Precise determination
7 of mitochondrial DNA copy number in human skeletal and cardiac muscle by a PCR-
8 based assay: lack of change of copy number with age. *Nucleic Acids Res* **31**, e61 (2003).
- 9 20. S. L. Archer, Mitochondrial dynamics--mitochondrial fission and fusion in human
10 diseases. *N Engl J Med* **369**, 2236-2251 (2013).
- 11 21. F. J. Iborra, H. Kimura, P. R. Cook, The functional organization of mitochondrial
12 genomes in human cells. *BMC Biol* **2**, 9 (2004).
- 13 22. D. C. Wallace, The mitochondrial genome in human adaptive radiation and disease: on
14 the road to therapeutics and performance enhancement. *Gene* **354**, 169-180 (2005).
- 15 23. M. J. Longley, R. Prasad, D. K. Srivastava, S. H. Wilson, W. C. Copeland, Identification
16 of 5'-deoxyribose phosphate lyase activity in human DNA polymerase gamma and its
17 role in mitochondrial base excision repair in vitro. *Proc Natl Acad Sci U S A* **95**, 12244-
18 12248 (1998).
- 19 24. K. Bebenek, T. A. Kunkel, Functions of DNA polymerases. *Adv Protein Chem* **69**, 137-
20 165 (2004).
- 21 25. M. Alexeyev, I. Shokolenko, G. Wilson, S. LeDoux, The maintenance of mitochondrial
22 DNA integrity--critical analysis and update. *Cold Spring Harb Perspect Biol* **5**, a012641
23 (2013).
- 24 26. W. C. Copeland, M. J. Longley, Mitochondrial genome maintenance in health and
25 disease. *DNA Repair (Amst)* **19**, 190-198 (2014).
- 26 27. B. Van Houten, S. E. Hunter, J. N. Meyer, Mitochondrial DNA damage induced
27 autophagy, cell death, and disease. *Front Biosci (Landmark Ed)* **21**, 42-54 (2016).
- 28 28. M. J. Young, W. C. Copeland, Human mitochondrial DNA replication machinery and
29 disease. *Curr Opin Genet Dev* **38**, 52-62 (2016).
- 30 29. A. R. Stiles *et al.*, Mutations in TFAM, encoding mitochondrial transcription factor A,
31 cause neonatal liver failure associated with mtDNA depletion. *Mol Genet Metab* **119**, 91-
32 99 (2016).
- 33 30. L. S. Kaguni, DNA polymerase gamma, the mitochondrial replicase. *Annu Rev Biochem*
34 **73**, 293-320 (2004).
- 35 31. M. A. Graziewicz, M. J. Longley, W. C. Copeland, DNA polymerase gamma in
36 mitochondrial DNA replication and repair. *Chem Rev* **106**, 383-405 (2006).
- 37 32. E. Murakami *et al.*, Characterization of novel reverse transcriptase and other RNA-
38 associated catalytic activities by human DNA polymerase gamma: importance in
39 mitochondrial DNA replication. *J Biol Chem* **278**, 36403-36409 (2003).
- 40 33. A. K. Berglund *et al.*, Nucleotide pools dictate the identity and frequency of
41 ribonucleotide incorporation in mitochondrial DNA. *PLoS Genet* **13**, e1006628 (2017).
- 42 34. S. E. Lim, M. J. Longley, W. C. Copeland, The mitochondrial p55 accessory subunit of
43 human DNA polymerase gamma enhances DNA binding, promotes processive DNA
44 synthesis, and confers N-ethylmaleimide resistance. *J Biol Chem* **274**, 38197-38203
45 (1999).

- 1 35. H. B. Ngo, G. A. Lovely, R. Phillips, D. C. Chan, Distinct structural features of TFAM
2 drive mitochondrial DNA packaging versus transcriptional activation. *Nat Commun* **5**,
3 3077 (2014).
- 4 36. C. Kornblum *et al.*, Loss-of-function mutations in MGME1 impair mtDNA replication
5 and cause multisystemic mitochondrial disease. *Nat Genet* **45**, 214-219 (2013).
- 6 37. L. Kalifa, G. Beutner, N. Phadnis, S. S. Sheu, E. A. Sia, Evidence for a role of FEN1 in
7 maintaining mitochondrial DNA integrity. *DNA Repair (Amst)* **8**, 1242-1249 (2009).
- 8 38. L. Zheng *et al.*, Human DNA2 is a mitochondrial nuclease/helicase for efficient
9 processing of DNA replication and repair intermediates. *Mol Cell* **32**, 325-336 (2008).
- 10 39. T. E. Shutt, M. W. Gray, Bacteriophage origins of mitochondrial replication and
11 transcription proteins. *Trends Genet* **22**, 90-95 (2006).
- 12 40. J. B. Stewart, N. G. Larsson, Keeping mtDNA in shape between generations. *PLoS Genet*
13 **10**, e1004670 (2014).
- 14 41. N. Hance, M. I. Ekstrand, A. Trifunovic, Mitochondrial DNA polymerase gamma is
15 essential for mammalian embryogenesis. *Hum Mol Genet* **14**, 1775-1783 (2005).
- 16 42. B. Iyengar, N. Luo, C. L. Farr, L. S. Kaguni, A. R. Campos, The accessory subunit of
17 DNA polymerase gamma is essential for mitochondrial DNA maintenance and
18 development in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* **99**, 4483-4488.
19 (2002).
- 20 43. M. M. Humble *et al.*, Polg2 is essential for mammalian embryogenesis and is required for
21 mtDNA maintenance. *Hum Mol Genet* **22**, 1017-1025 (2013).
- 22 44. S. K. Lee *et al.*, Polymerase subunit gamma 2 affects porcine oocyte maturation and
23 subsequent embryonic development. *Theriogenology*, (2014).
- 24 45. S. M. Cerritelli *et al.*, Failure to produce mitochondrial DNA results in embryonic
25 lethality in Rnaseh1 null mice. *Mol Cell* **11**, 807-815 (2003).
- 26 46. H. Tyynismaa *et al.*, Mutant mitochondrial helicase Twinkle causes multiple mtDNA
27 deletions and a late-onset mitochondrial disease in mice. *Proc Natl Acad Sci U S A* **102**,
28 17687-17692 (2005).
- 29 47. H. Tyynismaa, A. Suomalainen, Mouse models of mitochondrial DNA defects and their
30 relevance for human disease. *EMBO Rep* **10**, 137-143 (2009).
- 31 48. P. Sykora *et al.*, DNA polymerase beta participates in mitochondrial DNA repair. *Mol*
32 *Cell Biol*, (2017).
- 33 49. S. E. Kearsey, I. W. Craig, Altered ribosomal RNA genes in mitochondria from
34 mammalian cells with chloramphenicol resistance. *Nature* **290**, 607-608 (1981).
- 35 50. H. Blanc, C. W. Adams, D. C. Wallace, Different nucleotide changes in the large rRNA
36 gene of the mitochondrial DNA confer chloramphenicol resistance on two human cell
37 lines. *Nucleic Acids Res* **9**, 5785-5795 (1981).
- 38 51. M. J. Longley, M. M. Humble, F. S. Sharief, W. C. Copeland, Disease variants of the
39 human mitochondrial DNA helicase encoded by C10orf2 differentially alter protein
40 stability, nucleotide hydrolysis and helicase activity. *J Biol Chem* **285**, 29690-29702
41 (2010).
- 42 52. N. Sugo, Y. Aratani, Y. Nagashima, Y. Kubota, H. Koyama, Neonatal lethality with
43 abnormal neurogenesis in mice deficient in DNA polymerase beta. *EMBO J* **19**, 1397-
44 1404 (2000).
- 45 53. S. Garcia-Gomez *et al.*, PrimPol, an Archaic Primase/Polymerase Operating in Human
46 Cells. *Mol Cell* **52**, 541-553 (2013).

- 1 54. B. A. Keen, L. J. Bailey, S. K. Jozwiakowski, A. J. Doherty, Human PrimPol mutation
2 associated with high myopia has a DNA replication defect. *Nucleic Acids Res* **42**, 12102-
3 12111 (2014).
- 4 55. F. Zhao *et al.*, Exome sequencing reveals CCDC111 mutation associated with high
5 myopia. *Hum Genet* **132**, 913-921 (2013).
- 6 56. S. Wisnovsky, S. R. Jean, S. O. Kelley, Mitochondrial DNA repair and replication
7 proteins revealed by targeted chemical probes. *Nat Chem Biol* **12**, 567-573 (2016).
- 8 57. M. E. Arana, M. Seki, R. D. Wood, I. B. Rogozin, T. A. Kunkel, Low-fidelity DNA
9 synthesis by human DNA polymerase theta. *Nucleic Acids Res* **36**, 3847-3856 (2008).
- 10 58. B. Singh *et al.*, Human REV3 DNA Polymerase Zeta Localizes to Mitochondria and
11 Protects the Mitochondrial Genome. *PLoS One* **10**, e0140409 (2015).
- 12 59. Y. S. Lee, M. T. Gregory, W. Yang, Human Pol ζ purified with accessory subunits is
13 active in translesion DNA synthesis and complements Pol η in cisplatin bypass. *Proc*
14 *Natl Acad Sci U S A* **111**, 2954-2959 (2014).
- 15 60. N. Shima, R. J. Munroe, J. C. Schimenti, The mouse genomic instability mutation chaos1
16 is an allele of Polq that exhibits genetic interaction with Atm. *Mol Cell Biol* **24**, 10381-
17 10389 (2004).
- 18 61. G. Esposito *et al.*, Disruption of the Rev3l-encoded catalytic subunit of polymerase zeta
19 in mice results in early embryonic lethality. *Curr Biol* **10**, 1221-1224 (2000).
- 20 62. R. Krasich, W. C. Copeland, DNA polymerases in the mitochondria: A critical review of
21 the evidence. *Front Biosci (Landmark Ed)* **22**, 692-709 (2017).
- 22 63. D. Nolan, S. Mallal, Complications associated with NRTI therapy: update on clinical
23 features and possible pathogenic mechanisms. *Antivir Ther* **9**, 849-863 (2004).
- 24 64. A. K. Pau, J. M. George, Antiretroviral therapy: current drugs. *Infect Dis Clin North Am*
25 **28**, 371-402 (2014).
- 26 65. C. Zhu, M. Johansson, A. Karlsson, Incorporation of nucleoside analogs into nuclear or
27 mitochondrial DNA is determined by the intracellular phosphorylation site. *J Biol Chem*
28 **275**, 26727-26731 (2000).
- 29 66. S. U. Liyanage *et al.*, Leveraging increased cytoplasmic nucleoside kinase activity to
30 target mtDNA and oxidative phosphorylation in AML. *Blood* **129**, 2657-2666 (2017).
- 31 67. W. Lewis, W. C. Copeland, B. Day, Mitochondrial DNA depletion, oxidative stress and
32 mutation: mechanisms of nucleoside reverse transcriptase inhibitor toxicity. *Laboratory*
33 *Investigation* **81**, 777-790 (2001).
- 34 68. R. McKenzie *et al.*, Hepatic failure and lactic acidosis due to fialuridine (FIAU), an
35 investigational nucleoside analogue for chronic hepatitis B. *N Engl J Med* **333**, 1099-
36 1105 (1995).
- 37 69. M. C. Dalakas *et al.*, Mitochondrial myopathy caused by long-term zidovudine therapy.
38 *N Engl J Med* **322**, 1098-1105 (1990).
- 39 70. E. Arnaudo *et al.*, Depletion of muscle mitochondrial DNA in AIDS patients with
40 zidovudine- induced myopathy. *Lancet* **337**, 508-510 (1991).
- 41 71. M. C. Dalakas, C. Semino-Mora, M. Leon-Monzon, Mitochondrial alterations with
42 mitochondrial DNA depletion in the nerves of AIDS patients with peripheral neuropathy
43 induced by 2'3'-dideoxycytidine (ddC). *Lab Invest* **81**, 1537-1544 (2001).
- 44 72. C. A. Koczor, W. Lewis, Nucleoside reverse transcriptase inhibitor toxicity and
45 mitochondrial DNA. *Expert Opin Drug Metab Toxicol* **6**, 1493-1504 (2010).

- 1 73. A. A. Johnson *et al.*, Toxicity of antiviral nucleoside analogs and the human
2 mitochondrial DNA polymerase. *J Biol Chem* **276**, 40847-40857. (2001).
- 3 74. S. E. Lim, W. C. Copeland, Differential incorporation and removal of antiviral
4 deoxynucleotides by human DNA polymerase gamma. *J Biol Chem* **276**, 23616-23623.
5 (2001).
- 6 75. J. L. Martin, C. E. Brown, N. Matthews-Davis, J. E. Reardon, Effects of antiviral
7 nucleoside analogs on human DNA polymerases and mitochondrial DNA synthesis.
8 *Antimicrob Agents Chemother* **38**, 2743-2749 (1994).
- 9 76. J. A. Brown, L. R. Pack, J. D. Fowler, Z. Suo, Pre-steady-state kinetic analysis of the
10 incorporation of anti-HIV nucleotide analogs catalyzed by human X- and Y-family DNA
11 polymerases. *Antimicrob Agents Chemother* **55**, 276-283 (2011).
- 12 77. W. Lewis *et al.*, Fialuridine and its metabolites inhibit DNA polymerase gamma at sites
13 of multiple adjacent analog incorporation, decrease mtDNA abundance, and cause
14 mitochondrial structural defects in cultured hepatoblasts. *Proc. Natl. Acad. Sci. U.S.A.* **93**,
15 3592-3597 (1996).
- 16 78. J. Y. Feng, A. A. Johnson, K. A. Johnson, K. S. Anderson, Insights into the molecular
17 mechanism of mitochondrial toxicity by AIDS drugs. *J Biol Chem* **276**, 23832-23837.
18 (2001).
- 19 79. M. R. Szymanski *et al.*, Structural basis for processivity and antiviral drug toxicity in
20 human mitochondrial DNA replicase. *EMBO J* **34**, 1959-1970 (2015).
- 21 80. S. E. Lim, M. V. Ponamarev, M. J. Longley, W. C. Copeland, Structural Determinants in
22 Human DNA Polymerase gamma Account for Mitochondrial Toxicity from Nucleoside
23 Analogs. *J Mol Biol* **329**, 45-57 (2003).
- 24 81. H. Yamanaka *et al.*, Novel Mutation of Human DNA Polymerase gamma Associated
25 with Mitochondrial Toxicity Induced by Anti-HIV Treatment. *J Infect Dis* **195**, 1419-
26 1425 (2007).
- 27 82. C. M. Bailey, R. Kasiviswanathan, W. C. Copeland, K. S. Anderson, R964C mutation of
28 DNA polymerase gamma imparts increased stavudine toxicity by decreasing nucleoside
29 analog discrimination and impairing polymerase activity. *Antimicrob Agents Chemother*
30 **53**, 2610-2612 (2009).
- 31 83. M. Li *et al.*, The DNA Polymerase Gamma R953C Mutant Is Associated with
32 Antiretroviral Therapy-Induced Mitochondrial Toxicity. *Antimicrob Agents Chemother*
33 **60**, 5608-5611 (2016).
- 34 84. F. Chiappini *et al.*, Relationship between polymerase gamma (POLG) polymorphisms
35 and antiretroviral therapy-induced lipodystrophy in HIV-1 infected patients: a case-
36 control study. *Curr HIV Res* **7**, 244-253 (2009).
- 37 85. T. A. Brown, D. A. Clayton, Release of replication termination controls mitochondrial
38 DNA copy number after depletion with 2',3'-dideoxycytidine. *Nucleic Acids Res* **30**,
39 2004-2010 (2002).
- 40 86. M. P. King, G. Attardi, Isolation of human cell lines lacking mitochondrial DNA.
41 *Methods Enzymol* **264**, 304-313 (1996).
- 42 87. A. C. Gentry, S. Juul, C. Veigaard, B. R. Knudsen, N. Osheroff, The geometry of DNA
43 supercoils modulates the DNA cleavage activity of human topoisomerase I. *Nucleic Acids*
44 *Res* **39**, 1014-1022 (2011).
- 45 88. W. Lewis *et al.*, Mitochondrial ultrastructural and molecular changes induced by
46 zidovudine in rat hearts. *Lab Invest* **65**, 228-236 (1991).

- 1 89. Y. Zhang *et al.*, Long-term exposure of mice to nucleoside analogues disrupts
2 mitochondrial DNA maintenance in cortical neurons. *PLoS One* **9**, e85637 (2014).
- 3 90. E. E. McKee, A. T. Bentley, M. Hatch, J. Gingerich, D. Susan-Resiga, Phosphorylation
4 of thymidine and AZT in heart mitochondria: elucidation of a novel mechanism of AZT
5 cardiotoxicity. *Cardiovasc Toxicol* **4**, 155-167 (2004).
- 6 91. H. Pelletier, M. R. Sawaya, A. Kumar, S. H. Wilson, J. Kraut, Structures of ternary
7 complexes of rat DNA polymerase beta, a DNA template-primer, and ddCTP. *Science*
8 **264**, 1891-1903 (1994).
- 9 92. J. A. Brown, L. R. Pack, J. D. Fowler, Z. Suo, Presteady state kinetic investigation of the
10 incorporation of anti-hepatitis B nucleotide analogues catalyzed by noncanonical human
11 DNA polymerases. *Chem Res Toxicol* **25**, 225-233 (2012).
- 12 93. A. C. Mislak, K. S. Anderson, Insights into the Molecular Mechanism of Polymerization
13 and Nucleoside Reverse Transcriptase Inhibitor Incorporation by Human PrimPol.
14 *Antimicrob Agents Chemother* **60**, 561-569 (2015).
- 15 94. M. F. Lodeiro *et al.*, Transcription from the second heavy-strand promoter of human
16 mtDNA is repressed by transcription factor A in vitro. *Proc Natl Acad Sci U S A* **109**,
17 6513-6518 (2012).
- 18 95. H. Kasamatsu, J. Vinograd, Replication of circular DNA in eukaryotic cells. *Annu Rev*
19 *Biochem* **43**, 695-719 (1974).
- 20 96. D. D. Chang, D. A. Clayton, Priming of human mitochondrial DNA replication occurs at
21 the light-strand promoter. *Proc Natl Acad Sci U S A* **82**, 351-355 (1985).
- 22 97. S. Wanrooij *et al.*, Human mitochondrial RNA polymerase primes lagging-strand DNA
23 synthesis in vitro. *Proc Natl Acad Sci U S A* **105**, 11122-11127 (2008).
- 24 98. J. B. Holmes *et al.*, Primer retention owing to the absence of RNase H1 is catastrophic for
25 mitochondrial DNA replication. *Proc Natl Acad Sci U S A* **112**, 9334-9339 (2015).
- 26 99. J. J. Arnold, E. D. Smidansky, I. M. Moustafa, C. E. Cameron, Human mitochondrial
27 RNA polymerase: structure-function, mechanism and inhibition. *Biochim Biophys Acta*
28 **1819**, 948-960 (2012).
- 29 100. J. J. Arnold *et al.*, Sensitivity of mitochondrial transcription and resistance of RNA
30 polymerase II dependent nuclear transcription to antiviral ribonucleosides. *PLoS Pathog*
31 **8**, e1003030 (2012).
- 32 101. Y. Pommier, Y. Sun, S. N. Huang, J. L. Nitiss, Roles of eukaryotic topoisomerases in
33 transcription, replication and genomic stability. *Nat Rev Mol Cell Biol* **17**, 703-721
34 (2016).
- 35 102. K. Begriche, J. Massart, M. A. Robin, A. Borgne-Sanchez, B. Fromenty, Drug-induced
36 toxicity on mitochondria and lipid metabolism: mechanistic diversity and deleterious
37 consequences for the liver. *J Hepatol* **54**, 773-794 (2011).
- 38 103. I. Larosche *et al.*, Tamoxifen inhibits topoisomerases, depletes mitochondrial DNA, and
39 triggers steatosis in mouse liver. *J Pharmacol Exp Ther* **321**, 526-535 (2007).
- 40 104. A. Mansouri *et al.*, Tacrine inhibits topoisomerases and DNA synthesis to cause
41 mitochondrial DNA depletion and apoptosis in mouse liver. *Hepatology* **38**, 715-725
42 (2003).
- 43 105. S. Nadanaciva, K. Dillman, D. F. Gebhard, A. Shrikhande, Y. Will, High-content
44 screening for compounds that affect mtDNA-encoded protein levels in eukaryotic cells. *J*
45 *Biomol Screen* **15**, 937-948 (2010).

- 1 106. J. W. Lawrence, D. C. Claire, V. Weissig, T. C. Rowe, Delayed cytotoxicity and cleavage
2 of mitochondrial DNA in ciprofloxacin-treated mammalian cells. *Mol Pharmacol* **50**,
3 1178-1188 (1996).
- 4 107. R. Sasaki *et al.*, DNA polymerase gamma inhibition by vitamin K3 induces
5 mitochondria-mediated cytotoxicity in human cancer cells. *Cancer Sci* **99**, 1040-1048
6 (2008).
- 7 108. S. Umeda *et al.*, The D-loop structure of human mtDNA is destabilized directly by 1-
8 methyl-4-phenylpyridinium ion (MPP⁺), a parkinsonism-causing toxin. *Eur J Biochem*
9 **267**, 200-206 (2000).
- 10 109. C. Cover *et al.*, Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear
11 DNA damage in acetaminophen hepatotoxicity. *J Pharmacol Exp Ther* **315**, 879-887
12 (2005).
- 13 110. L. I. Rachek, L. V. Yuzefovych, S. P. Ledoux, N. L. Julie, G. L. Wilson, Troglitazone,
14 but not rosiglitazone, damages mitochondrial DNA and induces mitochondrial
15 dysfunction and cell death in human hepatocytes. *Toxicol Appl Pharmacol* **240**, 348-354
16 (2009).
- 17 111. H. Zhang *et al.*, Increased negative supercoiling of mtDNA in TOP1mt knockout mice
18 and presence of topoisomerases I α and I β in vertebrate mitochondria. *Nucleic*
19 *Acids Res* **42**, 7259-7267 (2014).
- 20 112. S. D. Cline, Mitochondrial DNA damage and its consequences for mitochondrial gene
21 expression. *Biochim Biophys Acta* **1819**, 979-991 (2012).
- 22 113. J. L. Podratz *et al.*, Cisplatin induced mitochondrial DNA damage in dorsal root ganglion
23 neurons. *Neurobiol Dis* **41**, 661-668 (2011).
- 24 114. A. Vaisman *et al.*, Effect of DNA polymerases and high mobility group protein 1 on the
25 carrier ligand specificity for translesion synthesis past platinum-DNA adducts.
26 *Biochemistry* **38**, 11026-11039 (1999).
- 27 115. R. R. Somasagara *et al.*, RAD6 promotes DNA repair and stem cell signaling in ovarian
28 cancer and is a promising therapeutic target to prevent and treat acquired
29 chemoresistance. *Oncogene*, (2017).
- 30 116. S. S. Lange, K. Takata, R. D. Wood, DNA polymerases and cancer. *Nat Rev Cancer* **11**,
31 96-110 (2011).
- 32 117. S. Marrache, R. K. Pathak, S. Dhar, Detouring of cisplatin to access mitochondrial
33 genome for overcoming resistance. *Proc Natl Acad Sci U S A* **111**, 10444-10449 (2014).
- 34 118. R. P. Saneto, R. K. Naviaux, Polymerase gamma disease through the ages. *Dev Disabil*
35 *Res Rev* **16**, 163-174 (2010).
- 36 119. Z. Zolkipli-Cunningham, M. J. Falk, Clinical effects of chemical exposures on
37 mitochondrial function. *Toxicology*, (2017).
- 38 120. J. N. Meyer *et al.*, Mitochondria as a target of environmental toxicants. *Toxicol Sci* **134**,
39 1-17 (2013).
- 40 121. K. E. Zahn, A. M. Averill, P. Aller, R. D. Wood, S. Doublé, Human DNA polymerase
41 θ grasps the primer terminus to mediate DNA repair. *Nat Struct Mol Biol* **22**, 304-311
42 (2015).
- 43 122. T. E. Shutt, M. F. Lodeiro, J. Cotney, C. E. Cameron, G. S. Shadel, Core human
44 mitochondrial transcription apparatus is a regulated two-component system in vitro. *Proc*
45 *Natl Acad Sci U S A* **107**, 12133-12138 (2010).

- 1 123. A. Reyes *et al.*, RNASEH1 Mutations Impair mtDNA Replication and Cause Adult-
2 Onset Mitochondrial Encephalomyopathy. *Am J Hum Genet* **97**, 186-193 (2015).
- 3 124. D. Ronchi *et al.*, Mutations in DNA2 link progressive myopathy to mitochondrial DNA
4 instability. *Am J Hum Genet* **92**, 293-300 (2013).
- 5 125. A. Besse *et al.*, The GABA transaminase, ABAT, is essential for mitochondrial
6 nucleoside metabolism. *Cell Metab* **21**, 417-427 (2015).
- 7 126. C. Rouzier *et al.*, The MFN2 gene is responsible for mitochondrial DNA instability and
8 optic atrophy 'plus' phenotype. *Brain* **135**, 23-34 (2012).
- 9 127. G. Birkus, M. J. Hitchcock, T. Cihlar, Assessment of mitochondrial toxicity in human
10 cells treated with tenofovir: comparison with other nucleoside reverse transcriptase
11 inhibitors. *Antimicrob Agents Chemother* **46**, 716-723. (2002).
- 12 128. B. Setzer, M. Schlesier, A. K. Thomas, U. A. Walker, Mitochondrial toxicity of
13 nucleoside analogues in primary human lymphocytes. *Antivir Ther* **10**, 327-334 (2005).
- 14 129. C. Rocher *et al.*, Influence of mitochondrial DNA level on cellular energy metabolism:
15 implications for mitochondrial diseases. *J Bioenerg Biomembr* **40**, 59-67 (2008).
- 16 130. N. Ashley, D. Harris, J. Poulton, Detection of mitochondrial DNA depletion in living
17 human cells using PicoGreen staining. *Exp Cell Res* **303**, 432-446 (2005).
- 18 131. E. Jemt *et al.*, Regulation of DNA replication at the end of the mitochondrial D-loop
19 involves the helicase TWINKLE and a conserved sequence element. *Nucleic Acids Res*
20 **43**, 9262-9275 (2015).
- 21 132. D. J. Medina, C. H. Tsai, G. D. Hsiung, Y. C. Cheng, Comparison of mitochondrial
22 morphology, mitochondrial DNA content, and cell viability in cultured cells treated with
23 three anti-human immunodeficiency virus dideoxynucleosides. *Antimicrob Agents*
24 *Chemother* **38**, 1824-1828 (1994).
- 25 133. M. Vazquez-Acevedo *et al.*, Characterization of a 5025 base pair mitochondrial DNA
26 deletion in Kearns-Sayre syndrome. *Biochim Biophys Acta* **1271**, 363-368 (1995).
- 27 134. M. Fernández-Moreno *et al.*, Generating Rho-0 Cells Using Mesenchymal Stem Cell
28 Lines. *PLoS One* **11**, e0164199 (2016).
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1 **Figure 1.** MtDNA maintenance and mitochondrial gene expression. **A.** Mitochondrial short-
2 patch base excision repair (BER) initiated with a monofunctional DNA glycosylase. The ROS
3 lightning bolt represents reactive oxygen species-induced mtDNA damage generating an
4 oxidized base lesion (star) that is subsequently removed and repaired by the BER machinery.
5 Two blue circles represent the double-stranded circular mitochondrial genome. A region of the
6 damaged mtDNA is shown below the circular genome to emphasize the BER pathway steps. AP
7 site, apurinic/aprimidinic site; AP endo, AP endonuclease; dRP, 5'-deoxyribose phosphate;
8 Pol γ / β the replicative mtDNA polymerase gamma or DNA polymerase beta; LigIII,
9 mitochondrial DNA ligase III. The NtRTIs lightning bolt represents nucleotide reverse
10 transcriptase inhibitors blocking Pol γ or β . **B.** Key components of the mtDNA replication and
11 repair machinery. The small purple line represents an RNA primer while the blue dashed line
12 represents newly synthesized mtDNA. TOPO, topoisomerase; Twinkle, Twinkle mtDNA
13 helicase; POLRMT, mitochondrial RNA polymerase and primase; RNaseH1, Ribonuclease H1;
14 mtSSB, mitochondrial single-stranded DNA binding protein. DNA polymerase beta, theta, zeta,
15 and the DNA *primase* and translesion DNA *polymerase* are represented by Pols β , θ , ζ , and
16 PrimPol respectively. These polymerases likely assist Pol γ with overcoming mtDNA damage.
17 The AVRNTs lightning bolt represents antiviral ribonucleotides blocking POLRMT activity. **C.**
18 Polycistronic mitochondrial transcription. Mitochondrial transcription (TS) occurs from three
19 promoters: 1) LSP, light-strand promoter, 2) HSP1, heavy-strand promoter 1, and 3) HSP2,
20 heavy-strand promoter 2. Three purple dashed lines represent transcripts synthesized from the
21 promoters. Although not visualized in the cartoon, mitochondrial TS initiation requires
22 mitochondrial TS factor A (TFAM) and either of mitochondrial TS factors B1 or B2 (TFB1M or

23 TFB2M). It is generally accepted that TFB2M is the primary factor for TS initiation (122).

24 Mitochondrial TS termination factor is represented by mTERF.

25 **Table 1. Nuclear genes identified in mitochondrial patients that affect mtDNA stability^a**
26

Gene	Disorder ^b	Chromosomal locus	Function
MtDNA replication and repair			
<i>POLG</i>	PEO / Alpers / ataxia	15q25	Poly catalytic subunit
<i>POLG2</i>	PEO	17q	Poly processivity subunit
<i>Twinkle</i>	PEO / ataxia	10q24	MtDNA helicase
<i>RNASEH1</i>	PEO / ataxia	2p25	Mitochondrial and nuclear RNaseH1 (123)
<i>DNA2</i>	PEO	10q21.3-22.1	Mitochondrial and nuclear helicase/nuclease (124)
<i>MGME1</i>	PEO, mtDNA depletion	20p11.23	RecB type exonuclease
<i>TFAM</i>	Neonatal liver failure mtDNA depletion	10q21.1	Mitochondrial transcription factor A (29)
Maintaining dNTP pools			
<i>ANT1</i>	PEO	4q35	Adenine nucleotide translocator
<i>TP</i>	MNGIE	22q13.33	Thymidine phosphorylase
<i>DGUOK</i>	MtDNA depletion	2p13	Deoxyguanosine kinase
<i>TK2</i>	MtDNA depletion	16q22-23.1	Mitochondrial thymidine kinase
<i>SUCLA2</i>	MtDNA depletion	13q14.2	ATP-dependent Succinate-CoA ligase
<i>SUCLG1</i>	MtDNA depletion	2p11.2	GTP-dependent Succinate CoA ligase
<i>RRM2B</i>	MtDNA depletion	8q23.1	p53-Ribonucleotide reductase, small subunit
<i>MPV17</i>	MtDNA depletion and deletion	2p23.3	Mitochondrial inner membrane protein
<i>ABAT</i>	MtDNA depletion	16p13.2	4-aminobutyrate aminotransferase (125)
Mitochondrial homeostasis and dynamics			
<i>OPA1</i>	Dominant optic atrophy	3q29	Dynamin-related GTPase
<i>MFN2</i>	Recessive optic atrophy	1p36.22	Mitofusin 2 (126)
<i>FBXL4</i>	MtDNA depletion, Encephalopathy	6q16.1-16.3	Mitochondrial LLR F-Box protein

27 ^aThe table is an updated version of Table 1 found in reference (28) and is reproduced with permission

28 ^bPEO, progressive external ophthalmoplegia; MNGIE, mitochondrial neurogastrointestinal
29 encephalomyopathy

Table 2. NRTIs with off-target effects on human DNA polymerases that localize to mitochondria

Drug	Target	Potential off-target^a	Mode of action	Side effects/toxicity/other notes	Experimental evidence for off-target effect	Reference
ddC , 2',3'-dideoxycytidine, zalcitabine, hivid	HIV-RT	Poly γ , PrimPol, Pol β	Deoxycytidine analog, chain-terminator	Peripheral neuropathy, sensorineural deafness, hypertrophic cardiomyopathy; according to the FDA ddC is no longer marketed	MtDNA depletion in various human cell lines; efficiently incorporated by Poly γ and PrimPol, <i>in vitro</i> (Poly 14-fold reduction in dCTP/ddCppp discrimination relative to PrimPol); Pol β incorporates and sensitive to ddCppp inhibition <i>in vitro</i>	(67, 73-75, 91, 93, 127-131)
ddI , 2',3'-dideoxyinosine, didanosine, Videx-EC [®]	HIV-RT	Poly γ , PrimPol, Pol θ ?	Deoxyadenosine analog, chain-terminator	Peripheral neuropathy, pancreatitis, hypertrophic cardiomyopathy, diabetes mellitus, hepatocellular failure, lactic acidosis; ddI is metabolized to ddAppp	Aberrant cristae and decreased mtDNA copy number in human cell lines; ddAppp (active form of ddI) incorporated efficiently by Poly γ and incorporated by PrimPol <i>in vitro</i> (Poly 233-fold reduction in dATP/ddAppp discrimination relative to PrimPol)	(67, 73, 93, 121, 127, 128, 132)
d4T , 2',3'-didehydro-2',3'-dideoxythymidine, stavudine, Zerit [®]	HIV-RT	Poly γ , Pol β	Thymidine analog, chain-terminator	Peripheral neuropathy, pancreatitis, hepatocellular failure, lactic acidosis, lipodystrophy; no longer recommended for administration	Aberrant cristae and decreased mtDNA copy number in human cell lines; incorporated efficiently by Poly γ <i>in vitro</i> ; Pol β incorporates and sensitive to d4Tppp inhibition <i>in vitro</i>	(67, 72-75, 127, 128, 133)
3TC , 2',3'-dideoxy-3'-thiacytidine, lamivudine, Epivir [®]	HIV-RT	Poly γ , Pol β	Zalcitabine/cytosine analog (see above), chain-terminator	Peripheral neuropathy, lactic acidosis, hepatomegaly with steatosis	Kinetic analysis with HeLa Poly γ , modest inhibition of Poly γ <i>in vitro</i> ; Pol β has a 9-fold reduction in dCTP/L-3TCppp discrimination in comparison to Poly γ <i>in vitro</i>	(67, 72-74, 76)

PMPA , (<i>R</i>)-9- (2-phosphonylmethoxypropyl)adenine, TFV, tenofovir	HIV-RT	Poly, Polβ	Deoxyadenosine analog, chain-terminator	Mitochondrial nephrotoxicity, kidney dysfunction; Viread®/TDF is a prodrug of PMPA	Modest inhibition of Poly <i>in vitro</i> ; Polβ has a 270-fold reduction in dATP/PMPApp discrimination in comparison to Poly <i>in vitro</i>	(72, 73, 76)
AZT , 3'-azido-2',3'-dideoxythymidine, zidovudine, ZDV, Retrovir®	HIV-RT	Poly, PrimPol, Polβ	Thymidine analog, chain-terminator, decreases levels of pyrimidines	Myopathy including ragged red fibers, decreased muscle mtDNA, bone marrow suppression, hypertrophic cardiomyopathy, sideroblastic anemia, pancytopenia, hepatocellular failure, lactic acidosis	Decreased mtDNA in cell culture, biochemical defects with Poly <i>in vitro</i> ; modestly incorporated by Poly; Polβ has an ~3850-fold reduction in dTTP/AZTppp discrimination relative to Poly and PrimPol has an ~60-fold reduction in dTTP/AZTppp discrimination relative to Poly <i>in vitro</i> .	(67, 73, 74, 76, 93, 134)
CBV , (-)-cis-2-amino-1,9-dihydro-9-(4-hydroxymethyl)-2-cyclopenten-1-yl)-6H-purin-6-one, carbovir active form of abacavir, ABC, see below	HIV-RT	Poly, PrimPol	Deoxyguanosine analog, chain-terminator	See below	Strongly incorporated by PrimPol <i>in vitro</i> and modest inhibition of Poly <i>in vitro</i>	(67, 73, 74, 93)
ABC , [(1 <i>S</i> ,4 <i>R</i>)-4-[2-amino-6-(cyclopropylamino)-9-puriny]-1-cyclopent-2-enyl]methanol, abacavir, Ziagen®	HIV-RT	Poly, PrimPol	Deoxyguanosine analog, chain-terminator	Increased myocardial infarction and congestive heart failure; Note: following intracellular phosphorylation ABC monophosphate is converted to CBV monophosphate by cytosolic enzymes then to CBVppp by cellular kinases	See CBV above	(72, 93)
FIAU , 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil, fialuridine, fluoriodoauridine	Hepatitis B, herpes virus DNA pols	Poly	Uridine analog, not a chain terminator as it contains a 3' OH, but	Severe lactic acidosis, liver failure, and steatosis, kidney failure, myopathy, peripheral neuropathy; discontinued use due to severe hepatotoxicity and death	Inhibition of Poly <i>in vitro</i> , cytotoxic to human Molt-4 cells, aberrant mitochondrial structures	(67, 68, 73, 75)

			impairs DNA elongation at adenosine tracts (77)			
FTC, 5-fluoro-1-[(2 <i>R</i> ,5 <i>S</i>)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine, RCV, emtricitabine, Emtriva [®] , coviracil, racivir	HIV-RT	Poly γ , Pol β	Deoxycytidine analog, chain termination	Lactic acidosis, hepatomegaly with steatosis	Pol β has a 100-fold reduction in dCTP/FTCppp discrimination in comparison to Poly <i>in vitro</i>	(72, 76)

^aThe Pol θ carboxyl-terminal polymerase domain has been crystalized inserting ddAppp opposite a template abasic site (121)

Table 3. NRTIs that disrupt mtDNA maintenance in human cell lines

Cell line	Source of cell line	Nucleoside Analog or agent studied ^a	Effect on mtDNA maintenance	Treatment time	Ref.
Molt-4	T lymphoblast	AZT, d4T, FLT, 935U83, FIAU, 524W91, 3TC, ddC, ddi	ddC and FLT, mtDNA depletion and cell death; d4T caused mtDNA depletion; FIAU did not alter ratio of mtDNA to nDNA but was cytotoxic; 524W91, AZT, 935U83 no detectable affect on mtDNA or cell growth	5 days (FIAU and ddC), 6 days (d4T), rest 7 days	(75)
HepG2	Hepatocellular carcinoma	PMPA, 3TC, ABC, ddC, ddi, d4T, and AZT	PMPA, 3TC, & ABC had no detectable effects on mtDNA levels; ddC > ddi > d4T > AZT depletion of mtDNA	9 days	(127)
Primary SkMC	Skeletal muscle cells	PMPA, 3TC, ABC, ddC, ddi, d4T, and AZT	PMPA, 3TC, ABC, AZT had no detectable effects on mtDNA levels; ddC > ddi > d4T depletion of mtDNA	9, 18, and 21 days	(127)
Primary RPTECs	Renal proximal tubule epithelial cells	PMPA, ddC, ddi, d4T, and AZT	PMPA & AZT had no detectable effects on mtDNA levels; ddC > ddi > d4T depletion of mtDNA	12 and 21 days	(127)
Lymphocytes	Primary peripheral blood lymphocytes	ddC, ddi, d4T, AZT	ddi > ddC > d4T deplete mtDNA; AZT did not affect mtDNA but increased lactic acid production and reduced cell counts	10 days	(128)
Lymphoblastoid cell line	Blood lymphocytes transformed with the Epstein Barr Virus	ddC	MtDNA depletion down to 20% of untreated cells	15 days	(129)
HCA2-htert	Fibroblast cell line immortalized by over-expression of human telomerase	ddC	Extreme mtDNA depletion	8 days	(130)
KP hMSC	Immortalized mesenchymal/stromal cell line	EtBr, AZT, d4T	MtDNA depletion EtBr > d4T > AZT	10 days	(134)
3a6 hMSC	Immortalized mesenchymal/stromal cell line	EtBr, AZT, d4T	MtDNA depletion d4T > EtBr; AZT no detectable mtDNA depletion	6 (d4T), 9 (AZT), or 10 (EtBr) days	(134)
HeLa	Cervical cancer cells	ddC	MtDNA depletion	3 days	(131)
CEM	Leukemia cell line	ddC, d4T, ddi	MtDNA depletion, potencies in reducing cell	4 days	(132)

			viability, mtDNA content and normal mitochondrial morphology were $ddC > d4T > ddi$		
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^aFLT, 3'-fluoro-3'-deoxythymidine; 935U83, 3'-fluoro-2',3'-dideoxy-5-chlorothymidine; 524W91, [(-) FTC], (-)-β-L-2',3'-dideoxy-5-fluoro-3'-thiacytidine; EtBr, ethidium bromide

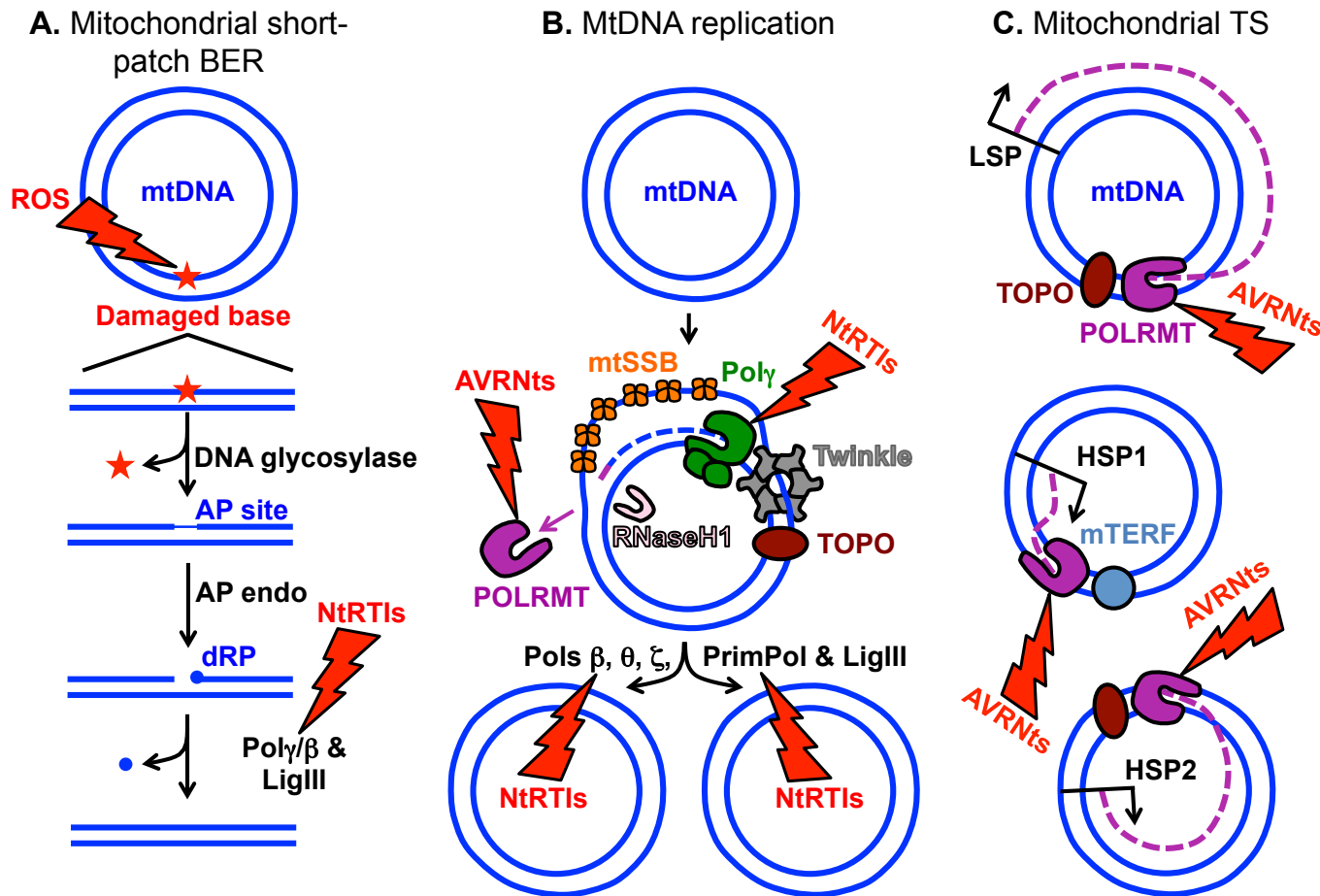


Figure 1.