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Review

# Mitochondrial DNA competition: starving out the mutant genome

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**High levels of pathogenic mitochondrial DNA (mtDNA) variants lead to severe genetic diseases, and the accumulation of such mutants may also contribute to common disorders. Thus, selecting against these mutants is a major goal in mitochondrial medicine. Although mutant mtDNA can drift randomly, mounting evidence indicates that active forces play a role in the selection for and against mtDNA variants. The underlying mechanisms are beginning to be clarified, and recent studies suggest that metabolic cues, including fuel availability, contribute to shaping mtDNA heteroplasmy. In the context of pathological mtDNAs, remodeling of nutrient metabolism supports mitochondria with deleterious mtDNAs and enables them to outcompete functional variants owing to a replicative advantage. The elevated nutrient requirement represents a mutant Achilles' heel because small molecules that restrict nutrient consumption or interfere with nutrient sensing can purge cells of deleterious mtDNAs and restore mitochondrial respiration. These advances herald the dawn of a new era of small-molecule therapies to counteract pathological mtDNAs.**

## Highlights

Selfish deleterious mtDNAs out-replicate wild-type molecules.

Glutamine addiction imparts sensitivity of mutant mtDNA to small molecules that restrict its utilization.

Targeting glutamine and glucose utilization as the 'Achilles' heel' of selfish selection sets the rationale for designing the next generation of therapeutics against mitochondrial diseases.

Recent data emphasize the role of key mitochondrial metabolites in mitonuclear signaling and epigenetic imprinting. They represent another important group of druggable targets for mitochondrial diseases.

## Manipulating mtDNA heteroplasmy

Since their discovery 35 years ago [1,2], mutant **mtDNA** (see Glossary) has emerged as a major cause of inherited metabolic diseases affecting approximately one in 8,000 individuals [3,4]. In most cases, the mutant molecules coexist with wild-type mtDNA (a state termed **heteroplasmy**), and high mutant loads can lead to severe disability and early death. Low levels of deleterious mtDNA variants, which nowadays are recognized to be pervasive in the human population [5,6], may also be problematic: by accumulating with age in tissues that are particularly affected in late-onset disorders, these mutants may contribute to common diseases [7–11]. Finally, it has become evident that dysfunctional mtDNA triggers multiple signaling pathways [12,13], some of which can alter cell homeostasis, and with time impact on disease pathogenesis and progression. Together, these findings have sparked growing interest in mutant mtDNA as a druggable target, stimulating the search for therapeutic strategies to combat deleterious mtDNAs.

By definition, cells of individuals with heteroplasmic disease-causing mutations contain a pool of unaffected mtDNA. Disabling the mutants, while permitting the wild type to propagate, would lead to a shift in the heteroplasmy ratio. Although there was a consensus that decreasing the mutant load would attenuate the biochemical defect in mitochondria and alleviate disease burden, many have questioned whether this could be achieved by pharmacological means. The mitochondrial genome is not only more difficult to modify than the nuclear DNA, but also **random genetic drift**, rather than active **selection**, was suggested to account for most of the fluctuations in the proportion of mutant and wild-type molecules [14–18]. This view has now changed, largely owing to growing evidence that multiple forces affect mtDNA heteroplasmy. On the one hand, mutated mtDNAs that have a **replicative advantage** will thrive, aided by reprogramming of cell metabolism that supports defective organelles; on the other hand, complex metabolic and

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genetic configurations can promote the selection of functional mitochondria with unadulterated mtDNA.

After introducing the fundamentals of pathological mtDNAs, we review the basis of the selection of variants that cripple mitochondrial energy production. We explain how such variants depend on rewiring of nutrient metabolism and how this could be exploited for therapeutic purposes by using small molecules that alter nutrient availability and sensing to counter mutant mtDNA. The review ends by indicating future research avenues that can further our understanding of the molecular basis of mtDNA selection and may lead to precision medicine for heteroplasmic mtDNA disorders.

## Mutant mtDNA and human disease

During evolution, mitochondria, which originated as free-living proteobacteria, have transformed into the modern double-membrane organelles that are present in the cytoplasm of almost all eukaryotic cells [19]. In the process, most of the original bacterial DNA has been transferred to the nucleus of the host cell such that the human mtDNA is, currently, a gene-dense and intronless circular molecule of 16.5 kilobases (kb) that has distinctive characteristics [20]. Unlike nuclear DNA, each cell contains thousands of copies of mtDNA that are transmitted exclusively from the mother [21], and each molecule continues to replicate throughout the life of the cell such that new variants can potentially emerge, at any time, via replication errors. All mtDNA gene products are crucial for mitochondrial energy production, and include 13 subunits of the **oxidative phosphorylation (OXPHOS)** system (Figure 1A) and RNA elements, two rRNAs, and 22 tRNAs, that are required for their translation [20]. Consequently, mutations in mtDNA can cause an energy crisis and lead to disease.

The first reported mtDNA mutations associated with disease were so blatant and numerous there was little doubt they were pathogenic [1]. A population of molecules lacking several kb of DNA, alongside wild-type mtDNAs, was identified in the skeletal muscle of a third of adults with mitochondrial myopathy. These substantial deletions invariably result in the loss of key factors for OXPHOS, and, consequently, cause mitochondrial dysfunction. In the same year, the first pathological point mutation was also identified; in this case the variant, located in the gene encoding the ND4 subunit of respiratory chain complex I, typically affects all the mtDNAs (homoplasmy) and causes blindness [22]. Since these initial discoveries, the mitochondrial genome has become saturated with mutations, and pathological mtDNAs are now considered to cause a major class of mitochondrial disease. Notably, most of the mutations are located in tRNA genes, although they account for <10% of the mitochondrial genome (https://www.mitomap.org/MITOMAP).

mtDNA disorders display diverse clinical manifestations [3,4]. Heteroplasmic variants often cause multisystem diseases, although identical mutations can produce very different clinical outcomes, exemplified by the m.3243A>G variant in the $tRNA^{Leu(UUR)}$ gene. This mutation is associated with a deafness and diabetes syndrome, or, alternatively, neuromuscular disease [23,24]. Similarly, partial mtDNA deletions cause a wide spectrum of phenotypes, ranging from a severe infantile disorder that affects pancreas, liver, and hematopoietic system to serious, yet less life-threatening neuromuscular disease later in adulthood.

In addition to the specific mutation, differences in mutant load contribute to the clinical outcome [25,26]. The presence of thousands of mtDNAs in a typical cell creates a scenario more akin to population genetics, where the mutant load for a given variant ranges from 0.001% to 100%. Low mutant loads are generally harmless because nearly all deleterious variants are functionally recessive; in other words, they produce biochemical and clinical phenotypes only when their

## Glossary

**Autophagy:** from the Greek *auto*, 'self', and *phagein*, 'to eat', autophagy is a conserved process that degrades macromolecules and cellular components via the endosome lysosomal pathway.
**ATP:** the energy currency of the cell.
**Epigenetic modification:** non-heritable and reversible alterations to the DNA or ancillary factors that impact on gene expression. They do not change the DNA primary sequence, instead they regulate patterns of gene expression by altering DNA or factor accessibility and structure. Epigenetic tags include DNA methylation, histone modification (chromatin remodeling), and non-coding RNAs, which can be modified by nutrient availability or compounds that target nutrient metabolism.
**Heteroplasmy:** a state wherein two or more mtDNA variants coexist within one cell or mitochondrion. The presence of a single mtDNA genotype within a cell, tissue, or organism is termed homoplasmy.
**Mitochondrial DNA (mtDNA):** a genetic relic of the α-proteobacterial DNA after the endosymbiotic event that gave rise to the eukaryotic cell. In humans, it is a small intronless circular molecule akin to a plasmid (~16 kb), which encodes 13 structural components of the electron transport chain and ATP synthase, as well as tRNAs and two rRNAs that are required for the translation of the corresponding mRNAs.
**Mitochondrial membrane potential (MMP):** to produce ATP the mitochondria must generate and maintain a chemiosmotic gradient across the inner mitochondrial membrane. This is achieved by proton translocation across the inner mitochondrial membrane mediated by the respiratory chain; hence, respiratory chain defects limit the gradient, and *in extremis* the mitochondria become depolarized. Depolarization can also be achieved by chemical ionophores such as dinitrophenol and FCCP [carbonyl cyanide p-trifluoromethoxyphenylhydrazone].
**Oxidative phosphorylation (OXPHOS):** the combined process of generating an electrochemical proton gradient and its utilization to generate ATP by the mitochondrial enzyme ATP synthase (complex V). Oxidation of metabolites (from sugars, fats, and amino acids) yields electrons that are passed down the respiratory chain to oxygen as the terminal acceptor in a series of redox reactions which drive complex I, III, and IV proton pumps.

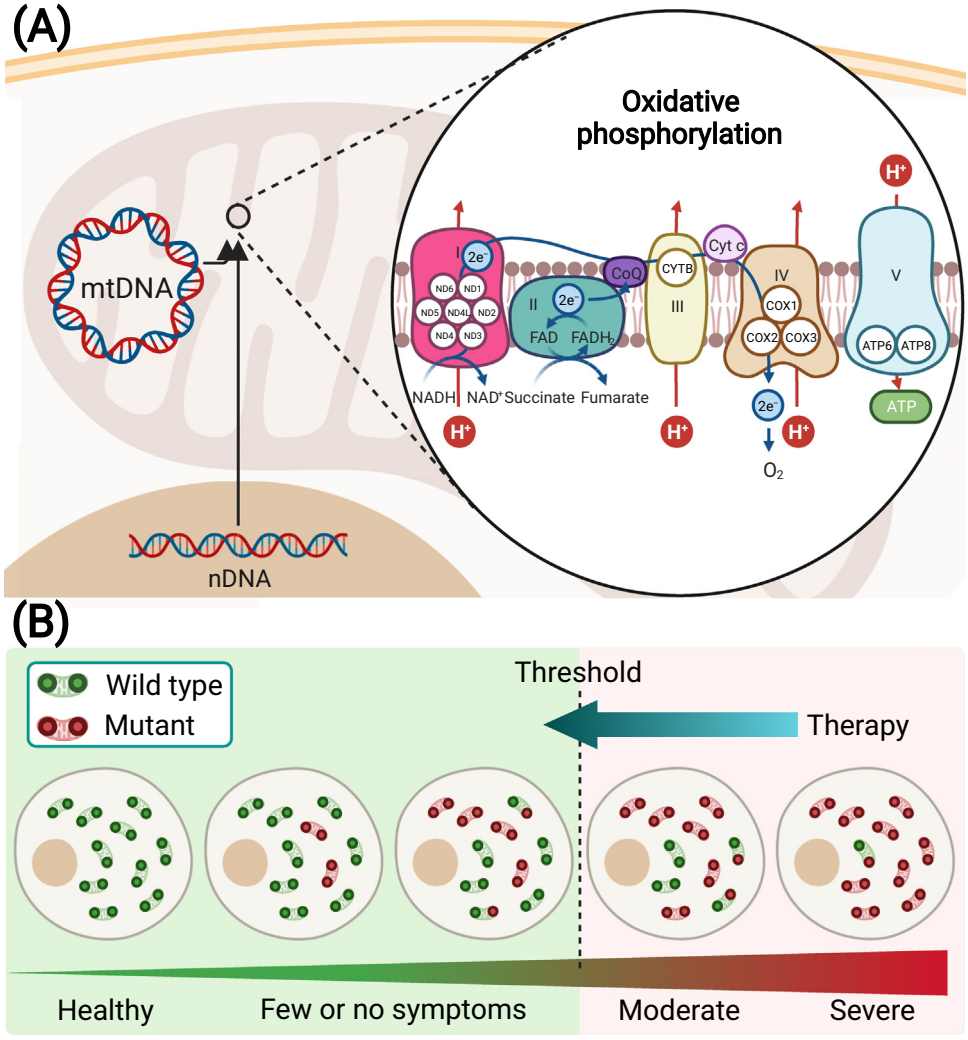


Figure 1. Impairment of oxidative phosphorylation (OXPHOS) in mitochondrial DNA (mtDNA) disorders depends on the level of mutant mtDNA. (A) mtDNA encodes essential subunits of the respiratory chain (complexes I, III, and IV) and ATP synthase (complex V), which together comprise the OXPHOS system. Therefore, OXPHOS is inoperable without mtDNA. (B) Cells typically contain thousands of copies of mtDNA, and the vast majority of deleterious mtDNA variants (red-filled circles) do not cause an appreciable OXPHOS deficiency unless they outnumber the wild-type (functional) mtDNAs (green-filled circles). There are often strict thresholds where a marginal change in the proportion of mutant molecules (mutant load) has a dramatic effect on biochemical function, and thus on cell and organ physiology. Consequently, reducing the mutant load below such a threshold can restore OXPHOS, and is expected to improve all reversible pathologies. Abbreviations: CoQ, coenzyme Q10; $e^-$, electron; $H^+$, proton; nDNA, nuclear DNA.

numbers exceed a critical threshold. This threshold usually falls within the range of 50–90% of total mtDNA, depending on the specific mutation and tissue [25,27]. Hence, decreasing the mutant load below a critical threshold is expected to restore respiration, thereby alleviating the molecular basis of the disease and consequently the disease manifestations (Figure 1B).

## Selection and counterselection of mutant mtDNA

Following the identification of the first heteroplasmic mtDNA mutations, it became evident that the level of mutant DNA differed among tissues. Although a high proportion of deletions and

Given that protons both bear an electric charge and mediate acid/base equilibria, a steep electrochemical potential is formed across the inner mitochondrial membrane termed the proton motive force. Proton re-entry into the mitochondrial matrix via ATP synthase drives ATP synthesis from ADP and inorganic phosphate in a manner that can be conceived as a molecular water mill.

**Random genetic drift:** the random stochastic segregation or transmission of mtDNA molecules over time without the participation of active forces. For example, a cell with 50% mutant and 50% wild-type mtDNA organized as two segregating units will reach homoplasmy (all mtDNA copies are identical) after a single cell division; whereas one with 10,000 segregating units (each mtDNA molecule acting independently of every other) will take innumerable generations to reach the same point.

**Replication pausing:** mitochondrial transcription termination factor (MTERF) binds to a tridecamer sequence of mtDNA, which results in replication pausing [85]; the pathological variant m.3243A>G reduces MTERF binding to DNA [86], and is therefore predicted to relieve replication pausing, which will confer a replicative advantage to the mutant molecules.

**Replicative advantage:** describes a form of biased selection stemming from a variant that can replicate faster or more frequently than other variants.

**Selection:** the natural process that governs the propagation of genetic traits. Phenotypic selection or purifying selection will favor mitochondria with a functional respiratory chain, whereas those with reduced fitness will be lost.

**Selfish mtDNA variants:** mutated mtDNA that has a direct replicative advantage over the wild-type molecules even though its product may be harmful. For example, a mutation in a gene encoding a subunit of complex I may be positively selected, even if it leaves the mitochondria with low complex I activity.

m.3243A>G were found in the skeletal muscle of patients with neuromuscular disease [1,24], the level of mutant mtDNA was lower or undetectable in the blood [24,28,29]. These observations were also *prima facie* evidence that mutant genomes, despite producing dysfunctional products, can thrive. However, the fact that female patients almost never transmit mtDNA deletions to their offspring [30] indicated that severe mtDNA variants are strongly disadvantaged in the germline. Since then a key challenge for researchers has been to understand whether the mutants are maintained or eliminated via active selection, and, if so, whether this operates at the level of the genome, organelle, or cell.

## Selfish mechanisms: lessons from yeasts and cultured cells

To put the abstract concept of a selfish mechanism simply, DNA variants that replicate faster or more frequently than their counterparts (**selfish mtDNAs**) gain an advantage, even if they are detrimental to the host [31]. Consider a hypothetical point mutation in the gene encoding subunit I of complex IV that both impairs cytochrome *c* oxidase activity and creates a highly active origin of replication: the latter enables the mutant to replicate more frequently than the wild-type mtDNA, so that, without phenotypic selection, such a variant will be positively selected, and the cells will display complex IV deficiency.

Striking examples of this principle first arose from studies in the budding yeast *Saccharomyces cerevisiae* where some deleterious mtDNAs have an enormous selective advantage over the wild-type molecule. In the most pernicious cases, mutants that retain only a small fragment of the original yeast mitochondrial genome are transmitted to up to 98% of the progeny, even though they are unable to respire [32]. This selfish behavior can be ascribed to the residual mtDNA serving as an origin of replication and sequestering a large majority of the replication licensing factors. Similar selfish conduct is observed in partially duplicated and triplicated human mtDNAs where their additional origins of replication enable them to outcompete the wild-type mtDNAs (Figure 2A) [33]. Partially deleted molecules and m.3243A>G mutants can also increase in abundance at the expense of the wild-type mtDNA [34–36]. Deleted molecules will simply complete the replication cycle faster owing to their shorter length (Figure 2B), whereas the replicative advantage of the m.3243A>G point mutant can be attributed to the fact that the altered DNA sequence reduces the binding of the mitochondrial transcription termination factor, MTERF, which has the effect of relieving **replication pausing** [37] (Figure 2C). All these instances of the active selection of mutants, in yeast and human cells, can be explained by a voucher system model of mtDNA replication in which the voucher represents a rate-limiting factor required for the initiation of replication (Figure 2).

## Selfish mechanisms and phenotypic selection *in vivo*

Despite the evidence that defective mitochondrial genomes can propagate by selfish mechanisms [33–36], there was considerable doubt about the role of active selection in modulating mtDNA heteroplasmy *in vivo* [16,17] because random genetic drift offered a credible explanation for the transmission and segregation of mtDNA variants [14–16,18]. However, data from animal models have gradually built an overwhelming case in support of selection for and against mutant mtDNA in both somatic cells and the germline. In worms, large mtDNA deletions accumulate, likely through a mechanism akin to human deletions (as per Figure 2B), and are transmitted across multiple generations. This occurs despite the compromised mitochondrial and host function, indicating weaker phenotypic selection than in humans, perhaps owing to activation of the mitochondrial unfolded protein response (mtUPR) [13,38]. In a heteroplasmic fly, a deleterious mutation in the cytochrome *c* oxidase subunit I gene, mt:CoIT300I (hereafter *mt:Col*$^{ts}$), is transmitted through the germline unless the fly is maintained at a high temperature [39,40]. The restrictive temperature impedes transmission of the deleterious genome, presumably by enhancing the phenotypic pressure acting on the mutant; in other words it aggravates the respiratory chain

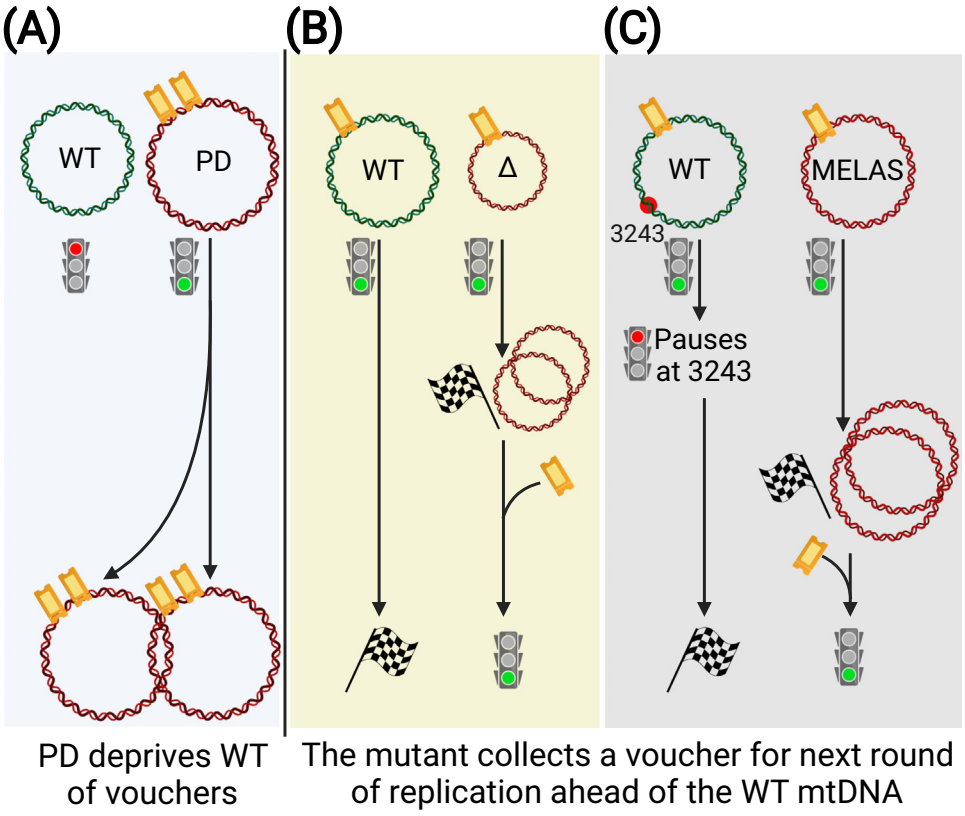


Figure 2. A voucher system for mitochondrial DNA (mtDNA) replication explains how the replicative advantage of deleterious mutants favors their selection. (A) Compared to WT mtDNAs, partially duplicated molecules have additional origins of replication. These enable them to collect more 'vouchers' (replication initiation factors; golden tickets) and begin replication (green traffic light), leaving the WT molecules voucherless and unable to start the replication cycle (red traffic light). Partially triplicated mtDNAs (not shown) have an even greater replicative advantage because of the still more origins, and they therefore accumulate even though the rearrangement confers severe respiratory chain deficiency. (B) Partial deletions reduce the length of the mitochondrial genome; as a result, they reach the end of the replication cycle (chequered flag) ahead of their WT counterparts, enabling them to be first to collect a voucher for the subsequent round of replication (second green traffic light). (C) The replicative advantage conferred by m.3243A>G is attributable to it reducing the binding affinity of the mitochondrial transcription termination factor (MTERF) at the tridecamer sequence that encompasses nt 3243, which relieves replication pausing [37]; this too enables the mutant to complete the replication cycle ahead of WT mtDNAs. Symbol and abbreviations: Δ, partial deletion; MELAS, Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes (one of the clinical phenotypes associated with m.3243A>G); PD, partial duplication; WT, wild type.

deficiency, and several mechanisms have been proposed ranging from inhibition of mtDNA replication to selective organelle clearance (discussed below). However, when *Drosophila mt:Col*$^{ts}$ is paired with a diverged but functional mitochondrial genome, *ATP6*, the deleterious mtDNA is transmitted at the higher temperature, albeit at a heavy cost, as most of the fly population dies out [41]. The few survivors carried *ATP6* mtDNA recombined with a portion of the major non-coding region of the *mt:Col*$^{ts}$, indicating that the selective advantage of the latter over the former stems from sequence elements in this region. Selfish behavior was also observed when mtDNAs were exchanged between different *Drosophila* species. *Drosophila melanogaster* flies carrying mtDNA of *Drosophila yakuba* were as healthy as wild-type flies; however, when *D. yakuba* mtDNA was in competition with *D. melanogaster* dysfunctional mtDNA, it was always outcompeted, despite providing better function [41].

Mammals also display active mtDNA selection. In heteroplasmic mice carrying the non-pathological mtDNA haplotypes C57 and NZB mtDNA (which differ at 90 nucleotide positions [42]), selection occurs in the germline [43] and postnatally in a cell/tissue-specific manner [44], and the heteroplasmy dynamics increases throughout the lifetime and across generations [45]. However, the majority of pathogenic variants are filtered out such that the transmission is largely restricted to the variants that either do not affect the sequence of the protein products [46], or are associated with less severe phenotypes [47]. Nevertheless, in some cases, such as the m.5024C>T, preferential selfish propagation occurs during oogenesis [48]. Finally, in humans, analysis of several cohorts also suggests selection against deleterious mtDNA variants [49,50] that possibly occurs during early embryogenesis [51]. Hence, in humans, as in mice, pathological variants can circumvent the purifying selection, with known mutations more likely to be transmitted to the offspring at higher levels than previously unidentified variants. Regarding the genomic location, some non-coding region variants are preferentially transmitted in humans [5], indicating a selfish drive linked to this region, as occurs in flies [41]. Together these findings provide compelling evidence that competing forces shape heteroplasmy levels, the nature of which, although complex, is now being elucidated.

## Nuclear and environmental control of mtDNA heteroplasmy

Although physically separate from the nucleus, mtDNA relies on hundreds of nucleus-encoded factors for its maintenance, replication, and expression [52,53]. In addition, signaling pathways between the nucleus and the mitochondria regulate the latter's function and adapt it to metabolic and environmental cues to maintain homeostasis [54–56]. Consequently, the crosstalk between the two organelles has long been considered to be a potential regulator of mtDNA heteroplasmy, for which there is burgeoning supporting evidence across multiple species.

### Genetic control: nuclear DNA–mtDNA interactions

A major genetic contribution to mtDNA selection was demonstrated as far back as the 1990s when, in cultured cells, wild-type mtDNA was found to be preferred over the pathological m.3243A>G on one nuclear background, whereas another background favored the mutant [57]. Subsequent studies in heteroplasmic (hybrid) mice carrying BALB and NZB mtDNA haplotypes revealed tissue-specific and age-dependent mtDNA selection. Specifically, the BALB mitochondrial genotype was selected in spleen and peripheral blood, whereas NBZ was favored in liver and kidney [15,58]. Both the mouse and the cultured cell studies suggested that the fate of mtDNA variants is ultimately determined by the nucleus, at least in some contexts. Although the regulatory mechanisms remained unknown, mtDNA selection appeared to be independent of the phenotypic consequences of the variants (thus named 'neutral' heteroplasmy), cell proliferation, or mtDNA replication. Later, the discovery that the endoplasmic reticulum protein GIMAP3 and its related lysosomal protein GIMAP5 play a role in the unbiased segregation of two different mtDNA genotypes in mouse hematopoietic cells suggested that organelle interactions and, possibly, an immune-related response might modulate such selection [59].

More recent studies in similar models have revealed a more complex picture. Enriquez and colleagues have shown that the NZB and BALB/C57 mtDNA variants, although non-pathogenic, do not behave as neutral variants, and profoundly impact on cell metabolic fitness including OXPHOS function and signaling [42]. These metabolic effects influence mtDNA selection when the variants are in a heteroplasmic state, both in the germline [43] and in postnatal cells and tissues [44]. The direction and strength of the selection are influenced by interventions that alter OXPHOS performance, including genetic modification of nuclear factors involved in fuel utilization (SCAF1), mitochondrial dynamics (OMA1), and reactive oxygen species regulation (NNT), suggesting that suboptimal interaction between nuclear and mtDNA influences mitochondrial function and mtDNA selection [43,44]. Parallel studies in human

populations also suggest that the selection of heteroplasmic mtDNA variants depends on the nuclear background, leading to the proposal that heteroplasmy changes are frequently a consequence of mitochondrial–nuclear (in)compatibility issues [5,41]. In any case, the findings from the aforementioned studies indicate that interactions between the nuclear and mitochondrial genomes have phenotypic consequences that can shape mtDNA heteroplasmy. This framework also includes factors involved in mtDNA replication, degradation, and organelle recycling [60–64] [discussed in the section 'Molecular mechanism(s) of selection'].

## Mutant mtDNA is reliant on metabolic rewiring

### *Limiting resources to hamper mutant mtDNA propagation*

As well as providing insights into the importance of nuclear DNA–mtDNA compatibility, the study of hybrid mice highlighted flagged metabolism and fuel preference as potential drivers of mtDNA selection [43,44]. Hence, a potential route to influencing mtDNA segregation is to create the environmental and metabolic conditions that exert a selective pressure such that functional mtDNA and healthy mitochondria are favored over mutants and dysfunctional organelles. Given that whenever the respiratory chain is compromised there is a concomitant increase in glycolytic **ATP** production terminating in lactose, restricting substrate availability for energy production could promote the propagation of healthy mitochondria with functional mtDNAs. Indeed, replacing glucose with galactose (which reduces glycolytic flux) caused extensive cell death in cells with high levels of m.3243A>G [65], and galactose was even used as a 'death-screen' to identify genes whose products are essential for the maintenance of OXPHOS [66]. Although killing cells with mutant mtDNA (selection at the cell level) is not a rational therapy for mitochondrial diseases, a switch from glycolytic to oxidative metabolism has been proposed to select against deleterious variants in the human germline [51]. In recent years it has also become apparent that defective OXPHOS increases the consumption of glutamine as well as glucose (Figure 3) [67,68]. This led us to infer that restricting both glucose and glutamine utilization could exercise a selective pressure based on functionality. The glucose analog 2-Deoxy-D-glucose (2DG) was a strong candidate small molecule because it restricts glutamine utilization [69] as well as inhibiting glycolysis [70]. 2DG, and the related compound 5-thioglucose (5TG), proved to be effective at reducing the level of mutant mtDNA in a range of cell lines and, as expected, the increase in wild-type molecules restored mitochondrial function [71]. Restricting the amount of glutamine and glucose in the culture medium had the same effect, demonstrating that nutrient availability can determine the fate of mtDNA variants, and suggesting the potential use of these small molecules for the treatment of heteroplasmic mtDNA disorders [71]. The full array of druggable targets related to glucose and glutamine metabolism remains to be discovered, but it is expected to include transporters and metabolite sensors, as well as anabolic and catabolic enzymes. The importance of nutrient status in shaping mtDNA heteroplasmy is buttressed by studies in worms carrying mtDNA deletions. Nutrient restriction reduces the selective advantage of the deleted mtDNA molecules within individual worms, whereas a combination of food scarcity and inhibition of DAF-16-dependent nutrient sensing was important for selection at the level of dysfunctional mitochondria and at the population level [72]. The authors speculate that DAF-16, by alleviating stress, favors underperforming organelles under stress conditions shielding them from selective pressure and promoting their propagation [72].

Delving into the mechanism of selection revealed that, in heteroplasmic human cells, 2DG and 5TG selectively inhibit the replication of mutant mtDNAs [71]. In flies, loss of a deleterious variant at high temperature was also attributed to inhibition of mtDNA synthesis [39,40], indicating that this mechanism of selection is conserved. In human cells, glutamine provides the crucial support for mutant mtDNA replication, whereas respiratory complex I sustains the replication of wild-type mtDNA under nutrient-restricted conditions because replication is blocked by the addition of the complex I inhibitor rotenone [71]. In summary, plentiful glucose allows dysfunctional

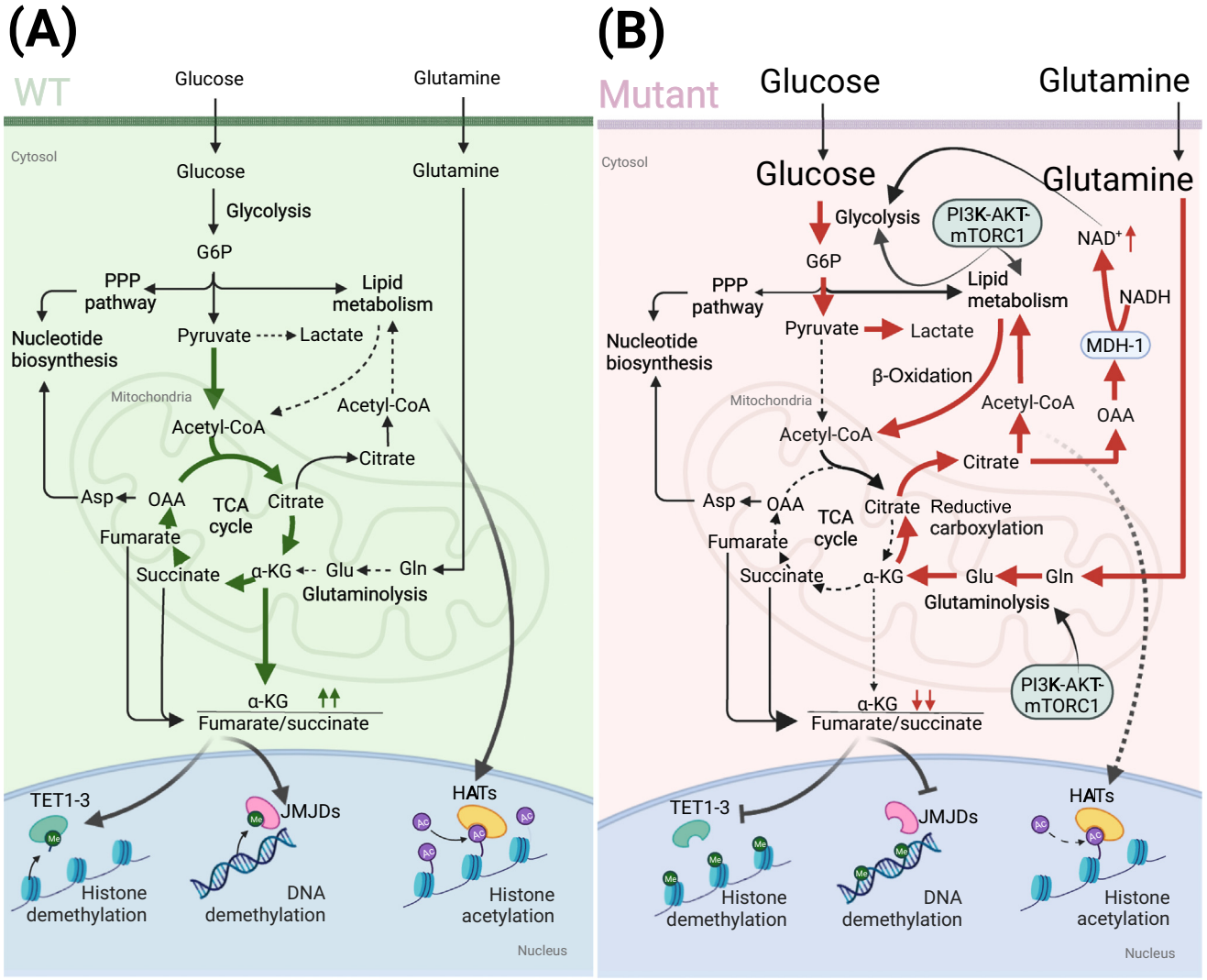


Figure 3. Mutant mitochondrial DNA (mtDNA) induces metabolic rewiring and an altered epigenome that in turn remodels cell metabolism. (A) Under conditions of normal oxidative phosphorylation (OXPHOS) function, glucose is mostly converted to pyruvate and then acetyl-coenzyme A (CoA), which is fed into the tricarboxylic acid (TCA) cycle to provide reducing equivalents for the respiratory chain and intermediates for biosynthesis. The TCA cycle also receives inputs from glutamine catabolism. Part of the citrate pool in the TCA cycle is exported from mitochondria and converted into acetyl-CoA in the cytoplasm from where it is directed to the nucleus to be used as substrate for histone acetylation to regulate gene expression. Furthermore, the usage of TCA cycle intermediates and their relative abundance (e.g., the α-KG:succinate ratio) plays a pivotal role in regulating the activity of DNA (TET1–3) and histone (JmjC) demethylases. (B) Impaired mitochondrial respiration, such as that caused by high levels of m.3243A>G, limits mitochondrial utilization of pyruvate, which is instead converted to lactate. The consequent decrease in glucose-derived acetyl-CoA reduces TCA flux, which affects the levels of metabolites that regulate gene expression. By contrast, the enzymes of the TCA cycle do not always follow the standard direction or complete cycle. Glutamine feeds the reductive carboxylation of α-KG to citrate to produce cytosolic acetyl-CoA. Although this can be utilized for lipid synthesis, as well as to provide the substrate for histone acetylation, in the case of marked respiratory (complex I) dysfunction the critical value of cytosolic citrate is to recycle NADH to $NAD^+$ via oxaloacetate (OAA) and the activity of malate dehydrogenase 1 (MDH-1). Thus, the steady-state level of cytosolic acetyl CoA, and its effects, including modulation of gene expression, are difficult to predict in the context of mitochondrial dysfunction, and may be prone to considerable fluctuations according to precise mutant load and immediate energy needs, as well as nutrient availability. These considerations highlight the importance of the nutrient-sensing PI3K–AKT–mTORC1 pathway in mitochondrial dysfunction: it enhances both glucose and glutamine consumption, and is activated in m.3243A>G cells at high mutant load, and inhibitors of the pathway reduce the mutant load [77]. Abbreviations: Ac, acetylation; G6P, glucose 6-phosphate; HAT, histone acetyltransferase; JMJDs, Jumonji C domain-containing demethylases; α-KG, α-ketoglutarate; Me, methylation; PPP, pentose phosphate pathway; TET1–3, ten-eleven translocation proteins 1–3.

mitochondria to obtain the ATP they need from aerobic glycolysis; as such, mitochondria with mutant mtDNA are parasitic. In addition, increased glutamine consumption via the TCA cycle and reductive carboxylation supports the replication of mutant mtDNAs (Figure 3). Intriguingly, nutrient availability has emerged as a pivotal factor in viral replication because 2DG represses the replication of HIV [73,74] and potentially severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [75,76]. Hence, there is effectively little difference between defective mtDNAs and viral genomes, with both utilizing host resources for their propagation.

*Targeting nutrient signaling pathways to undermine mutant mtDNA*

The metabolic regulation of mtDNA selection is supported by another recent study in which m.3243A>G was found to be associated with activation of the PI3K–AKT–mTORC1 pathway, repression of which, via inhibition of mTOR, AKT, or PI3K (with rapamycin, MK2206, or LY294002, respectively), selected the wild-type over the mutant [77]. Given that PI3K–AKT–mTORC1 activity is regulated by nutrient availability and in turn impacts on nutrient metabolism and related processes [78–80], these inhibitors may ultimately affect glucose and glutamine utilization similarly to 2DG, and thus can select wild-type mtDNA by a similar mechanism. Hence, the two studies [71,77] reinforce one another and strengthen the idea that nutrient and metabolite availability are central to the survival of mutant mtDNAs and can therefore be targeted to undermine them (Figure 3).

*Metabolites: mitochondrial signals aid the mutants*

The rewiring of cell metabolism that supports deleterious mtDNAs is tied to numerous changes in gene expression [81]. Chromatin remodeling is a major means to modulate gene expression, and this process is heavily influenced by a variety of mitochondrial metabolites that serve as cofactors, substrates, or donors for **epigenetic modification** [55,56]. Given that mitochondrial function affects the levels of these metabolites, signals originating from dysfunctional mitochondria will instigate epigenetic changes in the nucleus that alter the expression of a host of both mitochondrial and non-mitochondrial genes. For example, in the case of m.3243A>G, an increase in the mutant load alters the succinate:α-ketoglutarate ratio, and this correlates with changes in the activity of histone-modifying enzymes that remodel the epigenetic landscape [81,82].

Metabolite levels are also dependent on the availability and utilization of glucose, glutamine, and fatty acids, all of which in turn are linked to mitochondrial function. Studies from Frezza and colleagues showed that decreased glutamine oxidation and increased reductive carboxylation strongly correlate with the severe respiratory chain impairment caused by high levels of another mutant mtDNA [68]. Reductive glutamine carboxylation, by recycling NADH and enhancing GAPDH activity, leads to glycolytic turnover that supports ATP production in the cytosol, and these metabolic alterations likely lead to epigenetic changes that remodel cell migration and cytoskeleton dynamics (Figure 3). Hence, the reconfigured nutrient metabolism that occurs in several models of mitochondrial disease will inevitably arise from, and contribute to, altered gene expression via mitochondrial metabolites [67,83–85]. The reach of altered mitochondrial metabolites extends beyond histone proteins and epigenetics to the acetylation and acylation of a swathe of factors, among which lysine acetylation is one of the most important modifications that regulate enzyme activities throughout the cell.

Although some of the changes that accompany high levels of mutant mtDNA serve to mitigate the loss of mitochondrial function, they can unwittingly support deleterious mutants. As revealed by the 2DG studies, increasing glycolysis and glutamine consumption aids the mitochondria with defective mtDNAs [71]. Hence, adaptations that are beneficial or represent a 'quick fix' may exacerbate the underlying problem by reducing competition between mutant and healthy mitochondria or mtDNAs. By the same token, epigenetic changes could mediate the effect of pharmacological

interventions that target nutrient availability. Therefore, metabolite-mediated mito-cellular communication has the potential to change mitochondrial homeostasis for good or ill. Although the field is some distance from understanding the full complexity of this regulation, mitochondrial–nuclear crosstalk represents a credible target of intervention for mtDNA disorders.

## Molecular mechanism(s) of selection

### Selection via mtDNA replication or degradation

Once 2DG and 5TG were found to promote the selection of wild-type over m.3243A>G mtDNA [71], there were two credible explanations: selective inhibition of replication or selective degradation of the mutant mtDNA/organelle clearance. Although nutrient restriction can trigger **autophagy** activation, and 2DG has been shown to induce autophagy [86], the preferential inhibition of mutant mtDNA replication by 2DG was coupled to strong inhibition of autophagy in cells with the highest mutant loads, without a change in the mtDNA copy number. Therefore, the selection mediated by 2DG appears to operate principally at the level of mtDNA synthesis rather than through mtDNA disposal via organelle clearance. Notably, 2DG's impact on autophagy in mutant cells is influenced by intracellular oxygen levels, such that, in cells treated with oligomycin, the compound inhibits instead of promoting autophagy [86]. Moreover, selection based on replication competition is supported by several other studies. In the aforementioned studies in flies, a deleterious variant persists unless countered by a decrease in mtDNA replication at high temperature [39,40,87], and, for distantly related genomes, selection is driven by the replicative advantage of the mutant rather than via OXPHOS function [41]. In the same model organism, a genome-wide screen identified the replicative mtDNA polymerase γ (POLG, *tam* in fly) as a modifier of the relative levels of mutant to wild-type mtDNA [62]. A reduced dose of *tam* led to the elimination of the defective mtDNAs, supporting the idea that restricted replication undermines the replicative advantage of the mutant mtDNA. Selection was again linked to the availability of replication factors in another study in the heteroplasmic *mt:Col*$^{ts}$ fly. A decrease in **mitochondrial membrane potential (MMP)** (Box 1) at restrictive temperature stabilizes PINK1 in the outer mitochondrial membrane (OMM), and this inhibits local protein synthesis mediated by the RNA-binding protein, LARP1. This results in a dearth of nucleus-encoded replication factors, which impairs the synthesis and propagation of *mt:Col*$^{ts}$ [88]. Finally, an extensive analysis of human mtDNA sequences points to replication-related factors as central to regulating heteroplasmy [60].

Notwithstanding all these pointers to replication, there is clear evidence that autophagy or allied processes can influence mtDNA segregation in some contexts. In a fly model harboring mtDNA deletions in muscle, overexpression of autophagy- or mitophagy-related factors (ATG8A, PINK1, or PARKIN) promoted the selection of the wild-type over the mutant genome [89], whereas in the germline mitophagy-related selection against the mutants appears to be mediated by ATG1–BNIP3 in concert with fragmentation of the mitochondrial network [63]. In the NZB/C57 heteroplasmic mouse lines autophagy favored the NZB mtDNA genotype because abrogation of the autophagy factor ATG7 annulled its selective advantage in the liver [90] and, in another study, NZB mtDNA levels increased relative to the C57 mtDNA following treatment with an mTOR inhibitor or a PERK activator, which stimulate autophagy, among other pathways [44]. Given that NZB mtDNA confers better mitochondrial and cell function than C57 mtDNA in some contexts [42], these findings are congruent with a study in which the same mTOR inhibitor also favored the mtDNA genotype that was associated with better mitochondrial fitness – namely wild-type mtDNA versus m.3243A>G in human cells [77]. However, here again it does not follow that autophagy, and thus selection at the level of the organelle, is the driver of the heteroplasmic shift. In yeast, repression of autophagy decreases mtDNA replication while promoting mtDNA degradation by the 3′–5′ exonuclease activity of the mtDNA polymerase (MIP1 in yeast), leading to a decrease in mtDNA copy number [91]. Exactly such selective degradation of NZB mtDNA

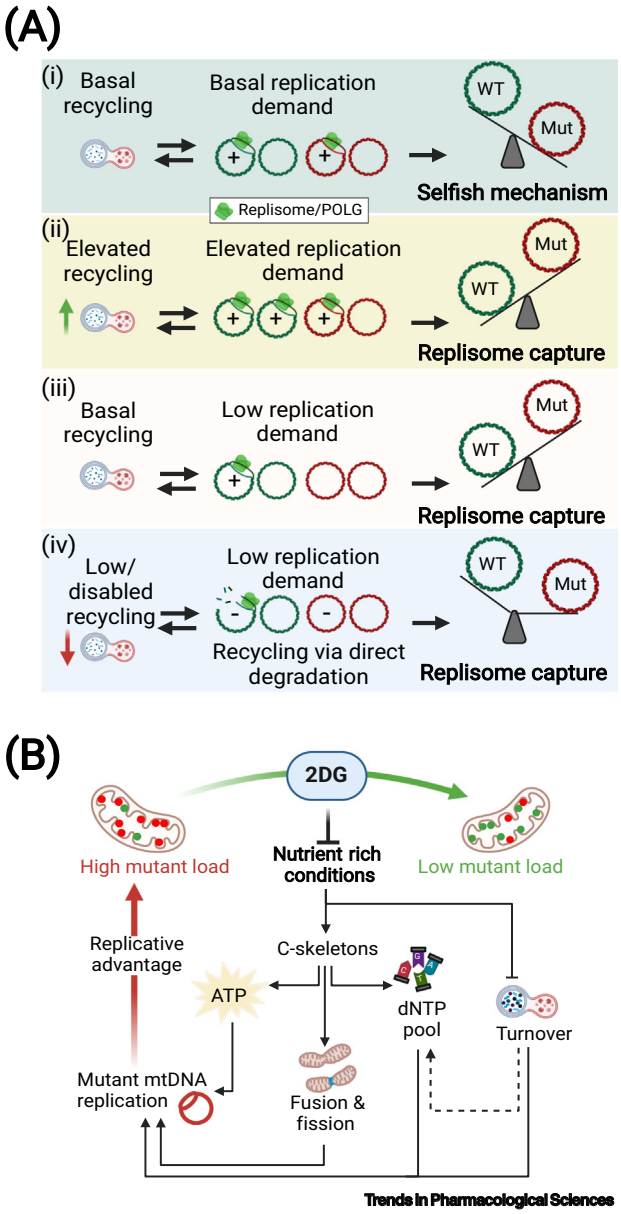


Figure 4. Mechanisms of mitochondrial DNA (mtDNA) selection: replication versus degradation and the influence of the nutrient status. (A) Deleterious mutants can be selected owing to their replicative advantage (a selfish mechanism) when there are basal levels of autophagy and replication (panel i and Figure 2). However, when there is a high demand for mtDNA replication (+ indicates a molecule seeking a replisome), POLG or other replisome factors become limiting, and these are sequestered principally by the wild-type molecules, giving them an advantage over the mutants (ii). POLG will also be limiting under conditions of basal autophagy and low replication demand, again, given the wild-type an advantage (iii). However, this advantage is reversed when there is a low demand for replication and autophagy is repressed. In such circumstances, POLG-mediated degradation of mtDNA occurs as an alternative to recyclying via autophagy (– indicates a molecule seeking POLG), leading to a shift in the ratio of wild-type to mutant mtDNA (iv). These considerations suggest that a complex interplay between mtDNA replication demand and the amount and method of recycling influences the selection of mtDNA variants. (B) Cells with deleterious mtDNAs have high nutrient consumption. These nutrients, especially glucose, glutamine, and their derivatives, support ATP and dNTP production, enabling their inherent replicative advantage (Figure 2) to reach high mutant loads [mitochondrion with predominantly mutant mtDNAs (red-filled circles)]. Nutrients also affect

*(Figure legend continued at the bottom of the next page.)*

Box 1. Mitochondrial depolarization and mtDNA selection

Because mitochondrial fitness is important for mtDNA selection, decreased MMP could be a key determinant of mtDNA heteroplasmy. A decline in the mutant load would occur through clearance of defective mitochondria carrying high levels of mutant mtDNA whenever increased reliance on mitochondrial energy production resulted in their depolarization. Most of the evidence linking mitochondrial depolarization to mitochondrial purging as a mechanism of mtDNA selection comes from cell culture experiments where chemical uncouplers were used to trigger PINK1/PARKIN-mediated mitophagy [129]. However, later studies showed that such harsh conditions poorly mimic the pathophysiology of mtDNA mutations, and the role of PINK1/PARKIN is variable across cells, tissues, and species [39,130,131]. Even without organelle removal (or fragments thereof), depolarization of mutant mitochondria can influence mtDNA heteroplasmy because it inhibits mitochondrial protein import, including that of many factors required for mtDNA replication. This mechanism of selection is proposed in the heteroplasmic *mt:Col*$^{ts}$ fly study [88].

Notwithstanding all of the above, replication is not directly proportional to MMP. Removing glucose, or replacing it with galactose, places much greater reliance on mitochondria to produce their own energy [66] and causes loss of MMP followed by extensive cell death in cells with impaired respiration, such as those with high levels of m.3243A>G, yet mtDNA replication remains active [71,132]. Conversely, mtDNA synthesis is blocked in the vast majority of mitochondria of cells with >90% m.3243A>G when treated with 2DG, whereas the proportion of depolarized mitochondria is much lower [71]. The idea that MMP is a key factor in the selection of wild-type mtDNA is also contradicted by experiments with oligomycin that inhibits ATP synthase. Oligomycin increased the numbers of depolarized mitochondria in adenocarcinoma cells with mutant mtDNA [133], but had the opposite effect to 2DG on mtDNA segregation; in other words, oligomycin selected the mutant rather than the wild-type mtDNA [71]. Finally, Enriquez and colleagues found that MMP did not correlate with mtDNA segregation in NZB/C57 murine cells, rather decreased respiration was accompanied by an increase in the MMP; moreover, the uncoupling agent CCCP (carbonyl cyanide m-chlorophenyl hydrazone) alone did not alter the segregation pattern in NZB/C57 congenic mouse tissues [44]. In conclusion, despite being intuitive and straightforward, mitochondrial depolarization has remarkably little effect on controlling rogue mitochondria with mutant mtDNA.

may underpin its decrease in response to inhibition of autophagy because its copy number decreased whereas the C57 mtDNA copy number remained constant in the liver [90]. Similarly, decreasing the expression of ATG1 or BNIP3 causes a drop in the wild-type copy number in fly *mt:Col*$^{ts}$ [63]. Integrating these findings [39–41,44,61–63,71,77,87–91] yields a model wherein wild-type mtDNAs have greater affinity for, or better access to, POLG than many deleterious mutants, whose consequences depend on replication demand and turnover. A basal level of turnover of mitochondria and mtDNA replication does not benefit the wild-type molecules because a surfeit of replisomes allows the mutants to thrive via selfish mechanisms (Figures 2A and 4A,i). Instead, elevated autophagy leads to increased mitochondrial biogenesis, thereby escalating the demand for mtDNA replication. In such circumstances wild-type mtDNAs sequester the majority of the POLG such that they outcompete the mutant (Figure 4A,ii). Replisomes may also become limiting when there is low replication demand (Figure 4A,iii). Conversely, when autophagy is repressed, POLG scarcity can promote mtDNA degradation, which will result in the preferential degradation of wild-type molecules (Figure 4A,iv).

## Mitochondrial dynamics and (mtDNA) quality control

mtDNA is organized into discrete protein–DNA complexes known as nucleoids [92]. These structures are distributed throughout the mitochondrial network, the dynamics of which is shaped by

mitochondrial dynamics, which influences mtDNA selection. Although recycling of mitochondria via autophagy helps to keep mutant mitochondria in check by removing overtly dysfunctional organelles, autophagy is repressed by plentiful nutrient availability and low autophagy decreases the demand for mitochondrial biogenesis, which will reduce the competition for replisomes that favors WT mtDNAs or promotes their degradation (i and iv, also Figure 2), and the ratio of mutant to WT mtDNA increases. By restricting nutrient availability, 2-Deoxy-D-glucose (2DG) remodels the cellular landscape such that there is much less non-mitochondrial ATP production and carbon skeletons for dNTP synthesis are scarce. Consequently, the replication of the mutants is inhibited in the presence of 2DG. Autophagy, whether this is basal or minimally activated (because of nutrient scarcity), could aid the clearance of mutant mitochondria whenever their dysfunction causes a loss of mitochondrial membrane potential or cristae alteration. Conversely, mitochondria with WT mtDNA (green-filled circles) can efficiently use the limited pyruvate from the reduced glycolytic flux to produce energy and intermediate metabolism to replicate. Thus, the new conditions created by 2DG disadvantage the mutants and favor the functional mitochondria, resulting in a shift of heteroplasmy towards a substantial lower mutant load [mitochondria with predominantly WT mtDNAs (green-filled circles)]. Abbreviations: dNTPs, deoxynucleotide triphosphates; Mut, mutant; WT, wild type.

fusion and fission events [93]. One widely adopted model suggests that mitochondrial fusion offers protection in the face of defective mitochondria/mutant mtDNA [93], whereas fission exposes the mutants to phenotypic selection. That is, fission will cause mutants to become isolated in individual organelles, leading to their clearance via autophagy or failure to propagate owing to loss of MMP [94]. In support of this model, decreasing fusion, by repressing mitofusin, lowered the level of mutant mtDNA in the skeletal muscle of a fly carrying mtDNA deletions [89]; similarly, decreased mitochondrial size and interconnectivity in the germline restricted mutant mtDNA mixing with wild-type mtDNA, and enabled the mutants to be removed via ATG1- and BNIP3-dependent mitophagy [63]. Finally, increased fusion owing to reduced expression of DRP1 and hFIS1, which support mitochondrial fission, increased the level of mutant mtDNA in human cultured cells [95]. However, in yeast, selection against mutants can occur within a continuous network, and does not require mitochondrial fission or Atg32-mediated mitophagy [96]. Instead, this selection depends on intact cristae because local remodeling or loss of cristae allows mutant mtDNAs to go undetected. This alternative framework might also explain the accumulation of mutant mtDNA in OPA1/mitofusin dysfunction [93,97,98]. Thus, a necessary refinement of the fusion/fission model entails the recognition that nucleoids are restricted in their mobility and have a limited sphere of influence. Maintaining the mtDNA and its products in close proximity (and in spatial confinement, such as in a cristae subdomain) ensures that the source of mutant products can be flagged for removal, thereby maintaining mtDNA fidelity. Conversely, untethered nucleoids would be unaccountable and might therefore escape the surveillance mechanisms designed for purging mutant elements.

All these various aspects of mitochondrial structure and ultrastructure could be accommodated in the replication factor availability model, allied to nutrient status (Figure 4B). Concordantly, impaired OMM fusion not only affects the stoichiometry of the mtDNA replication machinery, leading to mtDNA depletion, but also causes nucleoid clustering, indicating that OMM fusion is linked to mtDNA replication and segregation [99]. Regarding fission, the process of mitochondrial constriction and division, mediated by DRP1, follows directly on the heels of mtDNA replication [100]. This coordination of mtDNA replication with mitochondrial division suggests that the latter may influence selection at the level of the mitochondrial genome rather than the organelle. Crucially, mitochondrial dynamics is heavily influenced by nutrient availability and metabolic alterations [101–104], and changes in the mitochondrial network may therefore be incidental to changes in the mutant load, which may stem chiefly from phenotypic pressures related to nutrient availability. By contrast, DRP1 is intricately linked to nutrient metabolism, including through LETM1, a mitochondrial ion channel [84,102,105]. LETM1 insufficiency radically alters nutrient sensing and tolerance, as well as perturbing mtDNA organization and expression, and several mitochondrial abnormalities are attenuated by DRP1 repression [84]. Thus, the phenotypic pressure acting against mutant mtDNA, such as when cells are exposed to 2DG, may also depend on the activity of factors that regulate mitochondrial morphology, for example, DRP1. In this case, mitochondrial dynamics would offer additional drug targets.

## The clinical credentials of small-molecule therapies

While the transition from preclinical studies to the clinic is challenging for any new potential treatment, in the case of heteroplasmic mitochondrial disease the process is aggravated by the dearth of mouse models for testing emerging compounds (Figure 5) and gene therapy strategies (reviewed in [106,107]), and by the laborious and lengthy process to obtain the sufficient number of animals with a high mutant load [46,108]. On the positive side, some candidate small molecules have either gained approval for other purposes [109] or have undergone in *vivo* investigation in preclinical and clinical studies (discussed below). This provides a substantial body of information on their pharmacodynamics, safety, and tolerability, thus facilitating the clinical development of small-molecule therapies for mtDNA heteroplasmic disease.

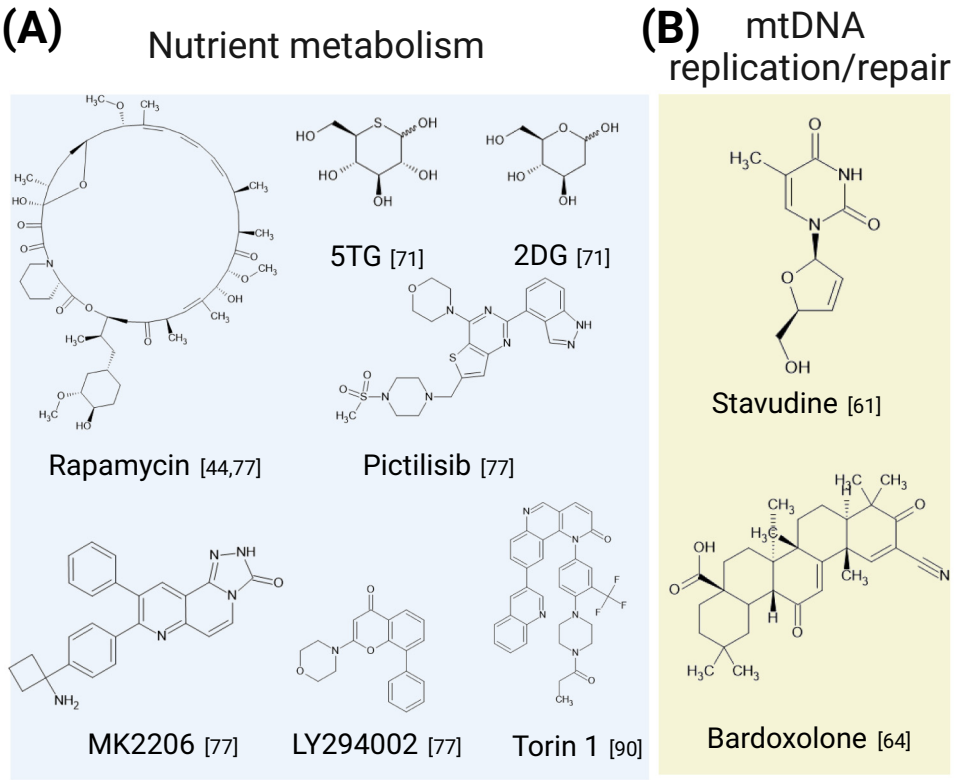


Figure 5. Small molecules that modify mitochondrial DNA (mtDNA) heteroplasmy. Several chemical and pharmacological compounds have been reported to influence the selection of pathological and benign mtDNA variants. We classify them into (A) compounds that target nutrient metabolism – either inhibitors of nutrient-sensing signaling pathways or nutrient utilization, and (B) compounds that target known factors affecting mtDNA replication or repair. Pictilisib and MK2206 are AKT inhibitors, rapamycin and Torin 1 inhibit TORC1, and LY294001 is a PI3K inhibitor, whereas 2-Deoxy-D-glucose (2DG) and 5-thioglucose (5TG) decrease glycolysis by inhibiting hexokinase and restricting glutamine consumption by an unknown mechanism. Bardoxolone (a CCDO methyl ester) inhibits Lonp1 protease activity, and the nucleoside analog stavudine is a DNA polymerase (reverse transcriptase) inhibitor. Thus, bardoxolone and stavudine may impact on mtDNA selection via replisome activity and replication demand analogously to the schemes in Figure 4. Bardoxolone has shown activity in heteroplasmic worm and in human cells, stavudine in heteroplasmic flies, Torin 1 and rapamycin in mouse and human cells, and all the others in heteroplasmic human cells. See [44,61,64,71,77,90]. Abbreviation: CCDO, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid.

In the case of 2DG, the compound has been tested on control animals [110–112], as well as in models of epilepsy [113,114] or model of autosomal dominant polycystic kidney disease (ADPKD) [115]. In human trials, 2DG has been investigated in patients with cancer or coronavirus disease 2019 (COVID-19), and it is currently being tested in subjects with epilepsy ([116–118]; https://clinicaltrials.gov/study/NCT05605301). Although reports of cardiotoxicity in rodents and of QT interval prolongation in human (indicative of prolonged ventricular repolarization) have raised some concerns that 2DG might be harmful, a detailed analysis of these preclinical and clinical studies indicates that 2DG is bioactive and well tolerated, and in humans is non-toxic at doses up to 64 mg/kg/day, when administrated intermittently, whereas lower doses (up to 45 mg/kg/day) are well tolerated even when given continuously [110,111,116–118]. Together these studies suggest that the cumulative average weekly dose and the dosing interval are key parameters for optimizing 2DG safety and tolerability.

It is important to recognize that the therapeutic goal in mtDNA heteroplasmic disorders is to gradually lower the mutant load without inducing a crisis or cell death. Furthermore, cells with

high levels of mutant mtDNA exhibit higher sensitivity to compounds such as 2DG [71]. Therefore, the optimal approach is not to achieve the highest possible tolerated dose of 2DG; instead, it is to promote metabolic remodeling that both undermines the mutant and stimulates the mitochondria with fewer mutant genomes, leading to a gradual shift to wild-type mtDNA via exposure to a minimal (likely ascending) amount of 2DG. In this respect, the observed molecular effects of 2DG on mice are encouraging. The small molecule is well tolerated over extended periods of time, and in the heart of control mice increases the respiratory chain capacity [112], and it reduces the cyst number in a murine model of the ADPKD [115]. Importantly, ADPKD exhibits metabolic alterations akin to human cells with mutant mtDNA, including perturbed glucose and glutamine metabolism alongside mitochondrial dysfunction [119,120]. The noteworthy aspect of the ADPKD study is the remarkably low effective dose, equivalent to 8 mg/kg/day in humans, with no observed adverse effects [115]. Hence, the promising outcomes from these mouse studies suggest a favorable trajectory for transitioning to human trials.

Among other compounds demonstrating selection towards wild-type mtDNA, the approved drug rapamycin has yielded mixed results in mouse models of mitochondrial disease associated with mutations in nuclear genes required for mtDNA maintenance [121,122]. Nevertheless, its capacity to alter nutrient metabolism and influence mtDNA selection in human cells [77] and hybrid mice [44] suggests that further investigation is warranted. Finally, the triterpenoid CDDO [2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid] and its derivative (Figure 5), which, by inhibiting mitochondrial LON protease prevents mtUPR-mediated propagation of the mtDNA deletion in worms [64], have been investigated in both subjects with cancer and individuals with diabetic nephropathy [123], with some apparent benefits. However, the observed toxic effects may limit their clinical use [124].

## Concluding remarks and future perspectives

Over the past decade, it has become clear that competing forces shape mtDNA heteroplasmy. Leading among them are the recent discoveries that nutrient availability and substrate preference can drive the selection of mtDNA variants [43,44,71,72,77]. These advances not only provide important mechanistic insight into a fundamental biological problem but also open up a wealth of pharmacological opportunities to tackle mtDNA disorders. Moreover, the myriad changes in nutrient and energy metabolism that accompany, if not drive, late-onset neurodegenerative disorders and aging [7–11] mean that the application of these new drugs could extend far beyond inborn errors of metabolism caused by mtDNA defects.

Looking ahead, several issues remain to be resolved (see Outstanding questions), and addressing them could expedite translation to the clinic. Pivotal challenges include the identification of the key metabolic changes that drive selection against the full range of deleterious mtDNA variants, and gaining a deeper understanding of how these configurations impact on the diverse mechanisms of selection (Figures 2, 3, and 4), as well as the biological level at which selection acts. The mechanisms of selection are likely to differ among different tissues and cell types owing to their distinct nutrient preferences and metabolic demands [43,44], and may also vary depending on the specific mutation and its interaction with the host nuclear DNA. Thus, the application of metabolic flux analysis [67,68] and other omic approaches [108], alongside image-based cell profiling and high-resolution microscopy including induced pluripotent stem cells differentiated to disease-relevant cell types [125] and 3D organoids [126], would be highly beneficial. Such studies have the potential to reveal effectors – from metabolites to proteins – that are necessary and sufficient for selecting functional mtDNAs, and could lead to the identification of novel drug targets, in turn facilitating the development of tailored, refined, and more effective treatments. While aside

### Outstanding questions

What are the key factors affected by restricted glutamine and glucose utilization that promote the selection of wild-type over deleterious mtDNA variants?

Can we identify tissue- and cell type-specific features that regulate mtDNA heteroplasmy?

How many more small molecules favor wild-type over mutant mtDNA?

Does mtDNA polymerase and ancillary protein availability influence mtDNA selection according to replication demand and turnover?

To what extent can we manipulate the mtDNA partitioning and packaging processes via nutrient metabolism, and to what degree does this impact on mtDNA selection?

How do other consequences of altered nutrient availability and sensing influence mtDNA heteroplasmy, such as interorganelle contacts, mitochondrial dynamics, and redox (dys)homeostasis?

from hybrid models [43,44], mice have only played a limited role in understanding mtDNA segregation to date, further progress will be aided by the growing number of heteroplasmic mice with deleterious mtDNA variants. These already include two tRNA variants that can reach high mutant loads and that recapitulate several molecular and histopathological features of human mtDNA disorders [108,127], and the use of mtDNA base editing is enabling the generation of a much broader set of mutants [128]. These emerging models are expected to be informative for preclinical assessment by helping to determine the efficacy of candidate drugs or genetic modification procedures across various cell types. After many years of dim prospects, the potential for manipulating the levels of mutant mtDNA has significantly brightened and holds out the exciting opportunity of the first pharmaceutical treatments for mtDNA disorders.

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## Declaration of interests

The use of 2DG to regulate mtDNA selection is protected in the patent GB2116499.1 in which A.S. and I.J.H. are named as inventors. The other authors declare no conflicts of interest.

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