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Review

Rewiring cell signalling pathways in pathogenic mtDNA mutations

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Mitochondria generate the energy to sustain cell viability and serve as a hub for cell signalling. Their own genome (mtDNA) encodes genes critical for oxidative phosphorylation. Mutations of mtDNA cause major disease and disability with a wide range of presentations and severity. We review here an emerging body of data suggesting that changes in cell metabolism and signalling pathways in response to the presence of mtDNA mutations play a key role in shaping disease presentation and progression. Understanding the impact of mtDNA mutations on cellular energy homeostasis and signalling pathways seems fundamental to identify novel therapeutic interventions with the potential to improve the prognosis for patients with primary mitochondrial disease.

Highlights

Mutations of mitochondrial DNA (mtDNA) cause disease with a wide range of presentations and severity. The relationships between genotype and phenotype are not understood.

Mitochondrial heteroplasmy is the presence of more than one type of mitochondrial DNA within cells and tissues: notably mtDNA with a mutation and wild-type mtDNA.

Disease severity broadly correlates with the burden of mutant mtDNA (mutant load). A biochemical 'threshold effect' dictates that people with lower mutant burden may even be asymptomatic.

Individual mtDNA mutations lead to distinct metabolic remodelling associated with or driven by alterations in cell signalling pathways.

Understanding the interactions between mtDNA mutations, cell signalling pathways and energy homeostasis opens new horizons for potential therapeutic interventions to improve the outlook for patients with these diseases.

Mitochondrial biology

Mitochondria perform a central role in bioenergetic homeostasis in eukaryotic cells and are also involved in extensive diverse functions, acting as gatekeepers for apoptosis, as biosynthetic machines, and as a hub for cell signalling pathways [1]. Human mitochondria contain a circular plasmid-like DNA (**mtDNA**) (see Glossary) encoding 13 peptides of the **oxidative phosphorylation (OxPhos)** system subunits and 24 RNAs essential for mitochondrial protein synthesis [1–8]. Unlike **nuclear DNA (nDNA)**, mtDNA is maternally inherited and there are multiple copies (up to thousands) in each cell, packaged into '**nucleoids**' and distributed throughout the mitochondrial network [7]. Mitochondria provide the bulk of **adenosine triphosphate (ATP)**, which is required to maintain cellular energetic homeostasis through OxPhos. Subunit peptides of OxPhos, translated from genes encoded either in nDNA or mtDNA, assemble in the mitochondrial inner membrane and perform respiratory function, during which electrons from NADH and $FADH_2$, generated mainly by the **tricarboxylic acid (TCA) cycle**, are transferred along a series of four multi-polypeptide respiratory complexes [1–5,9]. This oxygen-dependent process is coupled with the generation of the mitochondrial membrane potential ($\Delta\Psi m$) by actively pumping protons from the **mitochondrial matrix** into the **intermembrane space**, providing the energy that ultimately drives the synthesis of ATP by the **F_1F_o-ATP synthase**. The fundamental importance of energy homeostasis for cellular, tissue, and organismal health is reflected by a wealth of processes that have evolved to ensure mitochondrial quality control. This umbrella term encompasses mitochondrial dynamics (fusion, fission, trafficking), the balance of biogenesis and mitophagy, and expression of mitochondrial chaperones, which are shaped by the mitochondrial **unfolded protein response** [**UPR^{mt}**; or **integrated stress response (ISR)**] [1–5,9,10]. Notably, a low level of mtDNA mutations can be found in healthy humans and can accumulate with age, possibly causing biochemical defects associated with ageing and age-related disease: metabolic disorders, cancer, and neurodegenerative diseases [5,7,9,11–13]. Therefore, it is important to understand the underlying cellular mechanisms that maintain mitochondrial homeostasis and mtDNA stability. In this review, we focus on how mutations of mtDNA interact with cell signalling pathways, influencing mitochondrial quality control mechanisms and shaping the presentation and progression of the associated primary mitochondrial diseases.

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Primary mitochondrial disease and mtDNA mutations

Mitochondrial diseases, a primary mitochondrial pathophysiology, result from mutations of genes for the organelle components encoded by either mtDNA or nDNA [2–4]. Among them, inherited mtDNA mutations, such as point mutations or large-scale mtDNA deletions/rearrangements (Table 1) [4,5,8], affect roughly 1 in 4300 of the population and there is still no effective treatment

Table 1. Clinical disorders caused by common mtDNA mutations

mtDNA-associated clinical disorders[a]	Associated pathogenic mtDNA mutations and gene name	Common clinical characteristics and features	Refs
MELAS	m.3243A>G (MT-TL1) m.3271T>C (MT-TL1) m.3251A>G (MT-TL1) m.13042G>A (MT-ND5)	• Myopathy, encephalopathy, lactic acidosis, stroke-like episodes, diabetes, and sensorineural deafness • Expressed with variable severity • Frequently heteroplasmic	[9–11,16,17]
Leigh syndrome	m.8993T>G/C (MT-ATP6) m.9176T>C (MT-ATP6) m.12706T>C (MT-ND5)	• Lactic acidosis, cardiomyopathy, progressive neurologic deterioration, seizures, hypotonia, loss of appetite, vomiting, and irritability • Apparent during infancy/childhood • Expressed with variable severity • Frequently heteroplasmic	[12,13,18,19]
KSS	mtDNA large-scale deletion	• Progressive ophthalmoplegia, pigmentary retinopathy • Onset before 20 years of age • Sporadic condition and not inherited • Frequently heteroplasmic	[4,7,20]
MERRF	m.8344A>G (MT-TK) m.13042G>A (MT-ND5)	• Myoclonic epilepsy, ataxia, and lipoma developments around the neck area characterised by 'reg ragged fibres' on muscle biopsy • Expressed with variable severity • Frequently heteroplasmic	[4,17,21]
Pearson syndrome	mtDNA large-scale deletion	• Equivalent to KSS presenting in individuals that survive past infancy • Expressed with variable severity • Frequently heteroplasmic	[15]
CPEO	mtDNA large-scale rearrangements	• Ophthalmoplegia, myopathy, central nervous system dysfunction, and diabetes • Expressed with variable severity • Frequently heteroplasmic	[4,7,15,21]
NARP	m.11778G>A (MT-ND4) m.8993T>G (MT-ATP6) m.9176T>G/C (MT-ATP6)	• Neurogenic weakness, ataxia, retinitis pigmentosa, sensory neuropathy, seizures • Frequently heteroplasmic	[7,15,22]
LHON	m.11778G>A (MT-ND4) m.14484T>C (MT-ND6) m.3460G>A (MT-ND1)	• Optic atrophy-loss of vision • More prevalent in males than females • Frequently homoplasmic	[23,24]
MIDD	m.1555A>G (MT-RNR1) m.3243A>G (MT-TL1)	• Sensorineural hearing loss and diabetes • Childhood onset • Expressed with variable severity • Frequently heteroplasmic	[25,26]
Exercise intolerance	m.14849T>C (MT-CYB)	• Muscle weakness, fatigue • Frequently heteroplasmic	[27]
Sensorineural hearing loss	m.1555A>G (MT-RNR1)	• Deafness • Frequently homoplasmic	[7,28]
Myopathy and diabetes	m.14709T>C (TRNE)	• Myopathy, diabetes, weakness • Frequently heteroplasmic and homoplasmic	[28]

[a]Abbreviations: CPEO, chronic progressive external ophthalmoplegia; KSS, Kearns-Sayre syndrome; LHON, Leber's hereditary optic neuropathy; MELAS, mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy with red ragged fibres; MIDD, maternally inherited diabetes and deafness; NARP, neuropathy, ataxia, and retinitis pigmentosa.

Glossary

Adenosine triphosphate (ATP): a central metabolite in cellular metabolism, consisting of a nitrogenous base, a ribose sugar, and three phosphate groups.
Cybrid cells: a cell line generated from fusion of one cell, which has had the mitochondrial DNA depleted but still retains nuclear DNA, with another enucleated cell that still retains mitochondrial DNA. The resulting cell line can be used as a mitochondrial disease model.
Electron transport chain (ETC): a series of complexes through which electrons are transferred to drive the generation of ATP.
F_1F_o-ATP synthase: a protein that catalyses the formation of ATP from adenosine di-phosphate (ADP).
Glycolysis: metabolic pathway that converts glucose into pyruvate, producing a net gain of two ATP.
Heat shock proteins (HSPs): a family of proteins that are induced upon conditions of stress.
Hypoxia-inducible factors (HIFs): dimeric regulatory protein and transcription factor that responds to low levels of oxygen in the cellular environment.
Integrated stress response (ISR): a signalling pathway found in eukaryotic cells that responds to stress stimuli.
Intermembrane space: the space occurring between two membrane regions.
Metabolomics: the study of metabolites that are present within cells and tissues of an organism.
Mitochondrial matrix: the space within the inner mitochondrial membrane.
Mitochondrial DNA (mtDNA): a maternally inherited DNA located in the mitochondria of eukaryotic cells.
Nuclear DNA (nDNA): DNA contained within the nucleus of eukaryotic cells.
Nucleoids: the region that contains most of the genetic material within a prokaryotic cell.
Oxidative phosphorylation (OxPhos): an electron transfer chain driven by substrate oxidation that is coupled to the synthesis of ATP through an electrochemical transmembrane gradient.
Oxygen consumption rate (OCR): the amount of oxygen consumed in a given time.

[2,5]. Numerous pathogenic mtDNA mutations have been identified since the discovery of mtDNA in the 1960s (Table 1). More comprehensive information regarding mtDNA mutation-related mitochondrial diseases can be found on https://www.mitomap.org/foswiki/bin/view/MITOMAP/WebHome. Disorders of mtDNA show unique variability in clinical phenotypes and can present across any age in any organ, with highly variable severity [5], and with remarkable heterogeneity in patient symptoms even associated with a single pathogenic mtDNA mutation [3–5]. It is often stated that mitochondrial dysfunction primarily affects the central nervous system and neuromuscular systems as tissues with a high dependence on OxPhos and a high energy demand [5–7]. However, if the fundamental problem was only bioenergetic insufficiency, all mitochondrial diseases would appear similar, and so the underlying mechanisms that define the phenotype of these disorders must be more complex. Furthermore, the pathophysiology associated with mtDNA mutations in many mitochondrial diseases is complicated by the multiple copies of mtDNA found in all cells, giving rise to heteroplasmy: tissues contain some normal (or so-called wild-type) copies of mtDNA and some that carry the mutation. The net resulting mitochondrial dysfunction is itself a complex function of the nature of the mutation and of the mutant to wild-type mtDNA ratio, referred to as the mutant load or burden [5,14,15]. Therefore, the challenge remains to understand the mechanisms that define the variability in clinical presentations caused by different pathogenic mtDNA mutations and within the same mtDNA mutation but with varying heteroplasmic mutant burden. Further understanding this relationship between phenotype and genotype is integral in identifying potential therapeutic strategies for these devastating diseases (Table 1) for which at present there is no cure.

Reactive oxygen species (ROS): highly reactive signalling molecules that can provoke cellular damage.
Tricarboxylic acid cycle (TCA cycle): a series of chemical reactions that allow energy to be released.
Unfolded protein response (UPR^{mt}): a transcriptional response that increases mitochondrial localised molecular chaperones and proteases to promote the recovery of organellar protein homeostasis (proteostasis).

The role of mtDNA heteroplasmy as a determinant of disease pathophysiology

The presence of heteroplasmy associated with most (but not all) pathogenic mtDNA mutations further complicates research efforts to investigate the pathophysiological mechanisms associated with these mutations, because different ratios of mutant and wild-type mtDNA substantially affect disease expression and severity, even between patients with the same mtDNA mutation. Similarly, a biochemical 'threshold effect' has been identified with regards to levels of mtDNA heteroplasmy [3,5,7,14,29], so that people with a low mutant burden may even be asymptomatic.

Although the exact mechanisms that determine the mutant mtDNA levels are not fully understood, the positive correlation between mutant mtDNA burden in a heteroplasmic population and disease severity has been extensively documented [3,5,15,30]. In the case of the m.3243A>G mtDNA mutation, a mutant load of 20–30% measured in cord blood is associated with mild clinical manifestations such as diabetes [31]. Conversely, the substantial impairment of complex I of the **electron transport chain (ETC)** and the onset of more severe symptoms, such as cardiomyopathy, has been associated with a higher mutant load of 50–80% [31]. Similarly, in a family with the m.8993T>G mtDNA mutation, one member of the family carried an 85% mutant load in peripheral blood and suffered from severe symptoms, including ataxia, retinitis pigmentosa, and severe mental retardation, while two other family members who had greater than 90% mutant load died in infancy [32,33]. Other members of the same family who had a mutant load less than 60%, showed milder symptoms, including migraines and visual impairment and some were even asymptomatic [33]. These observations highlight the importance of the burden of heteroplasmic mutant mtDNA as a determinant of disease pathophysiology and presentation. It is also important to note that the level of heteroplasmy alone is insufficient to completely account for the phenotypes and progression of mtDNA disorders. The remaining proportion of wild-type mtDNA also plays an important role in determining the severity of mtDNA disease, as altering levels of wild-type mtDNA can influence disease severity and presentation [34]. Thus, understanding the mechanisms that determine the mutant burden and the ratio of wild-type to mutant mtDNA may have therapeutic potential, because even a

modest reduction in mutant load and increasing the copy number of wild-type mtDNA could have a disproportionate benefit for patients.

Metabolic phenotypes of diseases caused by mtDNA mutations

Pathogenic mtDNA mutations in genes encoding respiratory chain proteins disturb function of the complexes, while mutations of genes encoding mitochondrial tRNAs and rRNAs cause defects in mitochondrial translation and may therefore affect multiple components of the OxPhos machinery [8,35]. Intuitively, mtDNA mutations affect key variables of OxPhos, such as mitochondrial **oxygen consumption rate (OCR)**, $\Delta\Psi m$, NADH:NAD^+ ratio, ATP synthesis capacity and generation of **reactive oxygen species (ROS)**, as well as altering the concentrations of intermediate metabolites of the TCA cycle [8,35]. In consequence, changes in these factors, as potential signal transducers, may further alter the cell dependence on carbon sources, promote epigenetic modifications, and influence nutrient-related cell signalling pathways and mitochondrial quality control process [8,35].

Systematic characterisation of the metabolic phenotypes of cells with specific mtDNA mutations are limited in the literature. Therefore, we have reviewed the metabolic phenotypes of mtDNA mutations described across the literature (Table 2), including changes in OCR, $\Delta\Psi m$, NADH:NAD^+ ratio, ATP level, ROS production, cell dependence on carbon sources, and some features of metabolomic profiles. Decreased OCR and ATP synthesis, as well as increased ROS production, although to different extents, appear to be universal features among different mtDNA mutations, while changes in $\Delta\Psi m$ and metabolic remodelling vary hugely. However, even the same phenomenon could have different origins. For example, either mitochondrial hyperpolarisation or dysfunction of individual ETC subunits may increase ROS production [35]. Recently, another example of differential activation of cell signalling pathways due to different metabolic states and nature of the mitochondrial defect has been seen in healthy myoblasts and myotubes exposed to different mitochondrial inhibitors [36]. Moreover, the primary defects caused by specific mtDNA mutations and secondary cell adaptations to the mutations complicate the metabolic phenotypes. For example, enhanced ROS production is accompanied by an upregulation of antioxidant defence in cells with the m.3243A>G mutation [37,38], while in cells with mutations causing LHON, it is associated with a reduction in expression of antioxidant enzymes [39,40].

Recently, **metabolomics** analyses have been applied to mouse models and patient samples, such as urine, plasma, and muscle, in a search for biomarkers of mitochondrial diseases [41–44]. Nonetheless, detailed studies of the metabolic remodelling with specific mtDNA mutations in molecular and cell biology are still limited. Two reports applying metabolomics have shown that reductive carboxylation of glutamine supports cell survival and maintains redox balance in the m.8993T>G mutation cell models [45,46]. In contrast, glucose anabolism is increased in the m.3243A>G mutant cells and these cells are dependent on glucose (and not glutamine) for cell survival and proliferation [38]. These distinct profiles of metabolites argue that mtDNA mutations cannot be treated as one disease. Thus, applying metabolomics to characterise metabolic remodelling caused by mtDNA mutations should be encouraged and may provide insight into the biochemical basis of pathogenesis of these diseases.

Altered cell signalling and mitochondrial quality control in cells with mtDNA mutations

Changes in key parameters of metabolism, such as ADP:ATP and NADH:NAD^+ ratio, ROS, redox status, and intermediates in the TCA cycle, are known to serve as signalling transducers, affecting cell pathophysiology [70]. The mechanisms by which different pathogenic mtDNA mutations cause distinct metabolic and cell signalling remodelling, resulting in different clinical

presentations, remain elusive (Figure 1). Manipulating altered cell signalling pathways in animal models of primary mitochondrial diseases with nDNA mutations alleviates or even rescues disease phenotypes, even though the mutation is (obviously) still present [38,60,71–73]. These data highlight the principle that the disease phenotype may be largely determined by the activity of signalling pathways over and above (or in addition to) the impact of the

Table 2. Metabolic phenotypes of cells with common heteroplasmic mtDNA mutations[a,b]

Mutations of mtDNA (diseases)	Mitochondrial oxygen consumption rate (OCR)	Mitochondrial membrane potential ($\Delta\Psi m$)	NADH: NAD^+ ratio	ATP synthesis	ROS production	Cell dependence on carbon sources and features of cell metabolomic profiles	Experimental models
m.3243A>G (MT-TL1, $tRNA^{Leu}$; MELAS, MIDD, CPEO)	↓↓↓ [38,47,48]	↓~↓↓↓ [38,48–50]	↑ [38,49]	↓↓↓ [37,47,51]	↑↑~↑↑↑↑ [37,38,50,52]	Increased glycolysis and glucose dependence [38]; lactate ↑↑↑↑ [38,47,48]; glucose uptake ↑↑ [38]; alanine and serine ↑↑ [38,42]; NADH reductive stress [43,44]	Cybrid cell lines [38,47,48]; patient fibroblasts [38,47,48]; patient blood [43,44] and urine samples [43,44]; patient-derived iPSCs [50]
m.8993T>G/C (MT-ATP6, complex V; NARP, Leigh syndrome)	↓↓ (T>G) [46,47] ↓ (T>C) [47]	↑↑ [32,53,54]	↑ [45,46]	↓↓~↓↓↓↓ (T>G) [32,47,53,54] ↓ (T>C) [32,47]	↑↑~↑↑↑↑ [32,54]	Increased glutaminolysis and glutamine dependence [45,46]; alanine and lactate ↑↑ [45,46,55]; no difference in lactate and pyruvate [47]	Cybrid cell lines [46,47]; patient lymphocytes [32,53,54] and platelets [32,53,54]; patient-derived iPSC and iPSC-derived neural progenitor cells and neurons [55]
m.8344A>G (MT-TK, $tRNA^{Leu}$; MERRF)	↓~↓↓↓ [47,48,56,57]	↓↓↓ [48,49]	↑↑↑ [49]	↓↓↓↓ [47,51,56]	↑↑~↑↑↑↑ [56,57]	Increased glycolysis [57]; lactate ↑↑~↑↑↑↑ [47,57]	Cybrid cell lines [46,47]; patient fibroblasts [38,47,48]; iPSCs and iPSC-derived cardiomyocytes and neural progenitor cells [46,47]
Large-scale deletion of mtDNA (KSS, CPEO, Pearson Syndrome)	↓↓↓↓ [47]	↓↓↓ [58,59]	NF	↓↓↓↓ [47]	↑↑↑↑ [58]	Decreased utilization of fatty acids [41]; lactate ↑↑↑↑ [47]; altered one-carbon metabolism [41,60]; changes in the methyl cycle and glutathione metabolism [42]	Cybrid cell lines [46,47]; Deletor mice [46,47,60,61]; patient muscle biopsies [46,47] and blood samples [43,44]
m.11778G>A (MT-ND4, complex I; NARP, LHON)	↓↓ [62,63]	No difference [40]; ↓↓ [62,64]	↑ [61]	↓↓~↓↓↓ [62,64]	No difference [62]; ↑↑↑↑ [40,65]	Decreased glycolysis [65]; increased glucose dependence [66]; glutamate uptake ↓↓↓ [61]; no difference in lactate [61,67]; decreased alanine, serine, aspartate, and glutamine and increased phosphatidylcholine [67,68]	Cybrid cell lines [46,47]; patient lymphoblasts [46,47], peripheral blood mononuclear cells (PBMCs) [46,47] and fibroblasts [46,47]
m.3406G>A (MT-ND1, complex I; LHON)	↓↓ [39,63]	No difference [40]; ↓↓ [64]	NF	↓↓ [64]	↑↑↑↑ [40,65];	Increased glucose dependence [66]; glutamate uptake ↓↓↓↓ [65]; no difference in lactate [67]; decreased alanine, serine, aspartate, and glutamine and increased phosphatidylcholine [67,68]	Cybrid cell lines [46,47]; patient lymphoblasts [46,47], PBMCs [46,47], and fibroblasts [46,47]
m.14484T>C (MT-CYB, complex III; LHON)	No difference [39,63]; ↓↓	↓↓~↓↓↓↓ [64,69]	NF	↓↓ [64]	↑↑↑↑ [65,69]	Increased glucose dependence [66]; glutamate uptake ↓↓↓ [65]; no difference in lactate [67]; decreased alanine, serine, aspartate, and glutamine and increased phosphatidylcholine [67,68]	Cybrid cell lines [46,47]; patient lymphoblasts [46,47], PBMCs [46,47] and fibroblasts [46,47]

[a]Symbols: ↓, decrease <25%; ↓↓, decrease 25–50%; ↓↓↓, decrease 50–75%; ↓↓↓↓, decrease 75–100%; ↑, increase <25%; ↑↑, increase 25–50%; ↑↑↑, 50–75%; ↑↑↑↑, >75%.
[b]Abbreviation: NF, information not found in literature.

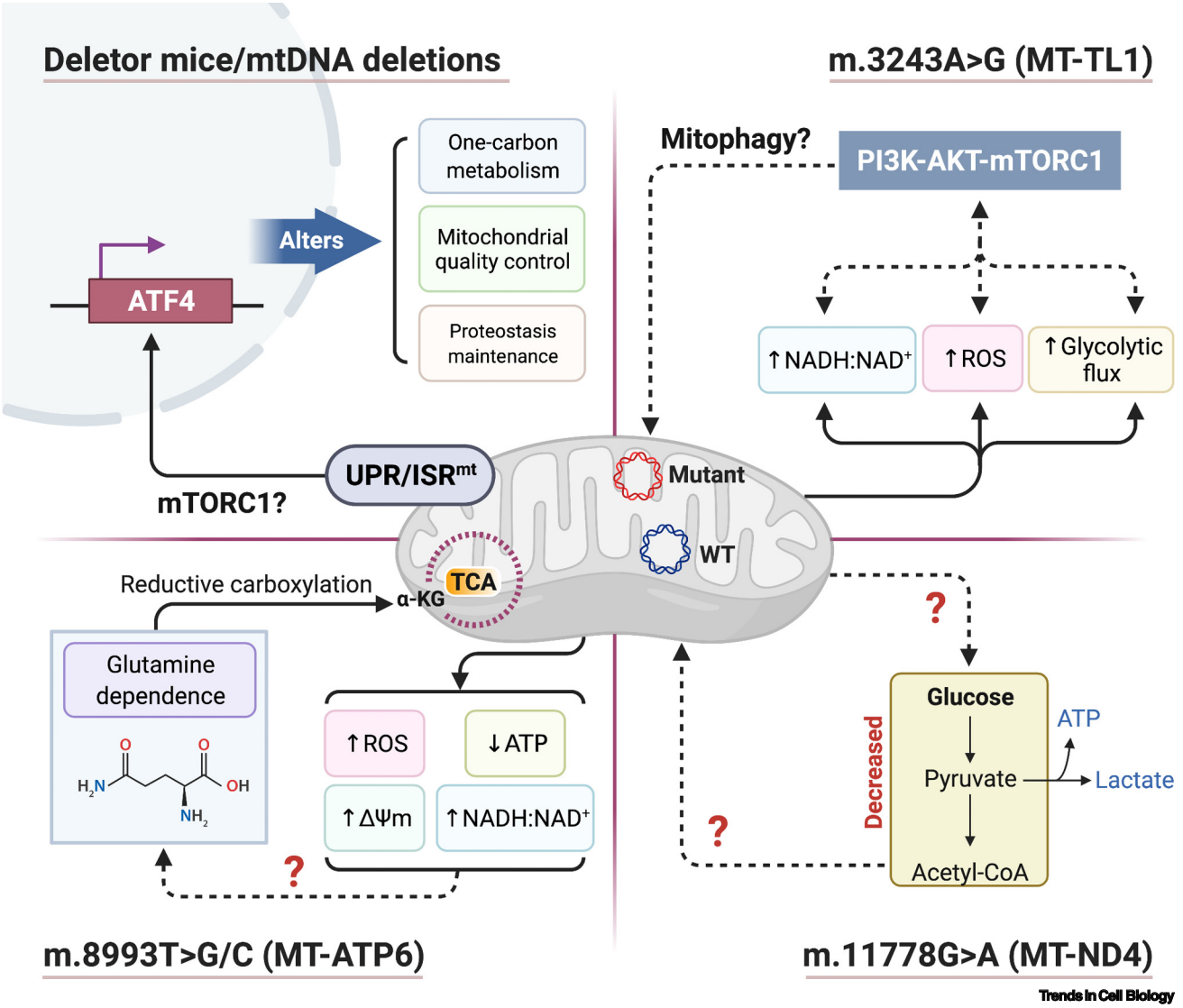

Figure 1. Overview of the main features from four different mtDNA mutations. The 'deletor' mice or patients carrying mtDNA deletions (top left) are represented by the enhanced ISR/UPR^{mt} activation, altering one-carbon metabolism, mitochondrial quality control, and proteostasis [41,42,58–60]. The constitutive activation of the PI3K-Akt-mTORC1 axis associated with NADH:NAD$^+$ imbalance, excess ROS generation, and upregulated glycolytic flux is shown as characteristics of the m.3243A>G point mutation (MT-TL1, tRNA^{Leu}); it is still unclear whether activation of the axis impairs mitochondrial quality control process, especially mitophagy (top right) [38,42–44,47–52]. Glutamine dependence is highlighted in the m.8993T>G/C mutation (MT-ATP6, complex V); however, the link between the respiratory defects led by the mutation (increased ROS production, ΔΨm and NADH:NAD$^+$ ratios, as well as decreased ATP synthesis) and increased glutaminolysis is missing (bottom left) [32,46,47,53–55]. The decrease in glycolysis is a feature of the m.11778G>A mutation (MT-ND4, complex I) but there have been few studies of the underlying mechanisms leading to the metabolic phenotype (bottom right) [40,46,47,61–68]. This figure was created using BioRender (https://biorender.com/). Abbreviations: ISR, integrated stress response; ROS, reactive oxygen species; TCA, tricarboxylic acid; UPR, unfolded protein response; WT, wild-type.

bioenergetic defect itself. Altered signalling pathways in cells or animals with mtDNA mutations play a critical role in sustaining levels of heteroplasmy [38,74,75], suggesting that these pathways may shape the progression of mtDNA diseases (Table 3). The specific roles of these pathways in primary mitochondrial diseases, the mechanisms that regulate these signalling pathways, or the time course over which their status is altered all remain very poorly

Table 3. Altered cell signalling pathways in mtDNA mutations

Signalling pathways[a]	Mutations of mtDNA
ISR^{mt} and UPR^{mt}	• Deleterious mtDNA (uaDf5) in a heteroplasmic *Caenorhabditis elegans* strain [74,75]. • Deletor mice and patient muscle biopsies with heteroplasmic mtDNA deletions [72].
The PI3K-Akt-mTORC1 axis	• Conplastic mouse strain with a low level of heteroplasmic mtDNA [76]. • Deletor C57BL/6 mice [72,73]. • Patient-derived fibroblasts, A549 cybrid cells, and muscle biopsies carrying the m.3243A>G heteroplasmic mutation [38]. • Patient muscle biopsies carrying the m.14674T>C homoplasmic mutation [77]. • Patient-derived induced pluripotent stem cells (iPSCs) and the iPSC-derived neurons carrying nearly homoplasmic the m.8993T>G mutation [55]
Mitochondrial dynamics and mitophagy	• Deleterious mtDNA in a heteroplasmic *C. elegans* strain [78,79]. • A heteroplasmic lethal mtDNA deletion in adult *Drosophila* muscle [80]. • Human cybrid cells carrying mtDNA deletions or depletion [59]. • Patient-derived fibroblasts, A549 cybrid cells, and muscle biopsies carrying the m.3243A>G heteroplasmic mutation [38]. • Human cybrid cell carrying the COXI heteroplasmic mutation [81]. • Patient-derived primary fibroblasts carrying the m.13514A>G heteroplasmic mutation [82]
Hypoxic pathway	• Mouse cybrid cells carrying the m.13997G>A heteroplasmic mutation [83]. • Human cybrid cells carrying the m.3243A>G heteroplasmic mutation or mtDNA depletion [79].

[a]Abbreviations: ISR^{mt}, mitochondrial integrated stress response; UPR^{mt}, mitochondrial unfolded protein response.

understood. Establishing links among specific mtDNA mutations, metabolic remodelling, and altered cell signalling advances our understanding of the pathophysiology of mtDNA mutation disorders. Therefore, in the following paragraphs, we discuss current knowledge about altered cell signalling pathways in common pathogenic mtDNA mutations and how this may help to identify novel therapeutic strategies.

UPR/ISR^{mt}

Mitochondrial function and proteostasis are maintained by the UPR^{mt}, a quality control system that is part of the first response of the ISR^{mt} [45]. Since only 13 mitochondrial proteins are encoded by the mtDNA, most mitochondrial proteins are imported into the organelle and are regulated by UPR^{mt}. Activation of the UPR^{mt} alters the expression of mitochondrial **heat shock proteins (HSPs)**, chaperones and proteases that ensure the integrity and function of mitochondrial proteins and increase degradation of misfolded proteins.

The UPR^{mt} was initially described in mammalian cells but the discovery of this pathway in *Caenorhabditis elegans* led to the identification of genes involved in sensing mitochondrial dysfunction [84]. Recent evidence suggests that defects in mitochondrial proteostasis in *C. elegans* with a heteroplasmic mtDNA deletion induces constitutive activation of the UPR^{mt} mediated by ATFS-1 [74,75]. Notably, prolonged UPR^{mt} activation maintains and propagates the mutant mtDNA [74,75] and further exacerbates mitochondrial dysfunction [73]. It is possible that the UPR^{mt} activation, in an attempt to maintain proteostasis and promote recovery of mitochondrial dysfunction, also gives a replicative advantage to mutant mtDNA molecules and thus results in its accumulation. However, in a *C. elegans* model with mtDNA instability, upregulation of the UPR^{mt} ameliorates the mtDNA disease phenotype [78].

In mammals, studies in the 'deletor' mouse model, generated by a mutation of the mitochondrial helicase (TWINKLE) that leads to accumulation of mtDNA deletions, have highlighted the importance of the ISR^{mt}, which regulates one carbon metabolism, nucleotide synthesis, and dNTP pools. The ISR^{mt} induction is also found in human patients with single or multiple mtDNA mutations [73]. Inhibition of mTORC1 by rapamycin in the 'deletor' mouse downregulated the ISR^{mt}, reverting progression and curing hallmarks of mitochondrial myopathy [73]. However, in

mice with astrocyte-specific TWINKLE knockout, activation of ISR^{mt} is independent of mTORC1 activity, suggesting that mTORC1 was not a direct regulator of ISR^{mt} in this model [85]. The induction of ISR^{mt} has also been documented in human patients with single or multiple mtDNA mutations [73]. Indeed, a recent report suggests that different mitochondrial defects trigger the ISR^{mt}, depending on the metabolic state of the cellular model, showing that there is no common mechanism linking mitochondrial dysfunction to ISR^{mt} activation [36]. Inhibition of Eukaryotic Initiation Factor 2 (EIF2) pathway, a major mediator of ISR^{mt}, and activation of the PI3K-Akt-mTORC1 pathway were both identified using transcriptomic analysis of patient-derived fibroblasts bearing the m.3243A>G mutation [38]. Although the crosstalk between the PI3K-Akt-mTORC1 axis and ISR^{mt} are still unclear, these two pathways seem to communicate and to determine cell fate under stress [73].

AMPK and PI3K-Akt-mTORC1 pathways

The AMPK and PI3K-Akt-mTORC1 signalling are two major nutrient-sensing pathways displaying antagonist roles. AMPK is activated by increased AMP:ATP or ADP:ATP ratios. Once activated by energy stress, AMPK activates pathways that restore cellular energy balance by switching on ATP-generating pathways (e.g., **glycolysis** and amino acid oxidation) while switching off ATP-consuming pathways (e.g., fatty acid synthesis and gluconeogenesis) [57]. This effect on metabolic pathways has been documented in primary cultures of skin fibroblasts carrying the m.8344A>G mtDNA mutation (MERRF), where energy metabolism shifts to anaerobic glycolysis as an adaptive response to oxidative stress [57].

In contrast, the PI3K-Akt-mTORC1 signalling pathway regulates anabolism and plays an important role in cell proliferation, apoptosis, and metabolism [86]. As aforementioned, mtDNA deletions in patients and mouse models elicit the ISR^{mt} and perturb Akt and mTORC1 activity [73]. Similarly, chronic activation of mTORC1 was found in iPSC-derived neurons carrying nearly homoplasmic m.8993T>G mutation [55], although other conflicting reports found no change or even inhibition of the PI3K-Akt-mTORC1 axis in **cybrid cells** bearing the same mutation [38,87]. Transcriptomic analysis and immunoblotting of patient fibroblasts carrying the heteroplasmic m.3243A>G mutation revealed activation of the PI3K-Akt-mTORC1 axis, which was further confirmed in patient muscle biopsies [38]. Although the mechanisms by which the PI3K-Akt-mTORC1 pathway is activated in this disease are still elusive, inhibition of the PI3K-Akt-mTORC1 pathway over several weeks reduced the mtDNA mutant burden and alleviated the bioenergetic defects and increased cell proliferation rate [38,88].

The PI3K-Akt-mTORC1 pathway indeed seems to be a promising therapeutic target [59,89]. However, in some models, mTORC1 inhibition has shown limited benefit and may even exacerbate the pathology. For example, in a mouse model of Leigh syndrome (NDUFS4 knockout), mTORC1 inhibition by rapamycin, whilst delaying the progression of the disease, failed to improve OxPhos [60]. Similarly, in a mouse model of mitochondrial encephalomyopathy ($Coq9^{R239X}$), mTORC1 inhibition failed to improve either mitochondrial bioenergetics or survival [90]. Moreover, mTOR may play a protective role in the reversible infantile respiratory chain deficiency (RIRCD) caused by a homoplasmic m.14674T>C mutation in healthy carriers, where the initial activation of ISR modulates lipid and one carbon metabolism as well as amino acid availability and the following activation of mTOR leads to increased mitochondrial biogenesis in muscle; while in the majority of affected carriers, additional heterozygous mutations in nuclear genes interacting with $mt\text{-}tRNA^{Glu}$, including EARS2 and TRMU, were found [77]. Therefore, nDNA genotypes may further complicate the penetrance and presentations of mtDNA mutations and the therapeutic efficacy of the PI3K-Akt-mTORC1 axis inhibition may be distinct and limited to different diseases.

However, several studies suggest that AMPK plays a role in the progression and manifestation of clinical phenotype [57,82,91]. Considering the antagonism between the PI3K-AKT-mTORC1 axis and AMPK, inhibition of the axis may lead to an increase of AMPK activity [38]. Therefore, although some studies suggest that prolonged AMPK activation triggers apoptosis, promoting AMPK activity seems to be beneficial in some mtDNA mutation models and may further induce catabolic processes such as autophagy.

It is well established that the AMPK and PI3K-AKT-mTORC1 pathways regulate autophagy, which maintains cellular homeostasis through the degradation of dysfunctional cellular components. In general, these two pathways are also crucial for mitophagy, an autophagy process that targets dysfunctional mitochondria. The impact of mtDNA mutations or heteroplasmy on mitophagy will be discussed in the next section.

Mitochondrial quality control: dynamics, mitophagy, and biogenesis

The mitochondrial quality control process maintains mitochondrial function and bioenergetic cellular homeostasis, at least including mitophagy, fusion–fission dynamics, and biogenesis [92]. Here, we discuss these mitochondrial quality control mechanisms in the context of various mtDNA mutations and how they influence the presentation and progression of primary mitochondrial disease [93].

Mitophagy eliminates damaged components of the mitochondrial network. However, this pathway fails to eliminate mitochondria containing pathogenic mutations in patients with primary mitochondrial disease [50]. Theoretically, malfunctioning mitochondria with mutant mtDNA would be eliminated by mitophagy, which would eventually decrease the mutation [50]. Nonetheless, the diseases persist and experimental work confirms that mitophagy is not necessarily upregulated in cells with mtDNA mutations, signifying that the presence of pathogenic mtDNA itself is not sufficient to drive selection against the mutation by activation of mitophagy [50]. Although the increased activation of mitophagy was scarce in iPSCs generated from fibroblasts harbouring the m.3243A>G mutation, the iPSCs showed increased markers of autophagy (i.e., LC3B-II) [50], which seems to be induced as a response to oxidative insults. Alternatively, the activation of mitophagy to remove pathological mutant mtDNA may operate in a cell-dependent manner [94]. Investigations of two cybrid cell lines demonstrated differences in levels of mitophagy between the cell lines, where muscle-derived RD.Myo cells sustained the deleterious mtDNA compared with the lung carcinoma-derived A540.B2 cells [94]. The mechanisms that dictate these differences remain obscure, but the work emphasises the importance of studying these processes in relevant cell types wherever possible. In a mtDNA disease model using cybrid cells carrying either large-scale partial deletions or complete depletion of mtDNA, where loss of $\Delta\Psi m$ was insufficient to activate mitophagy, mitophagy could nevertheless still be induced through the inhibition of mTORC1 with rapamycin [59]. Other data also point to a key role for the activity of mTORC1 and Parkin in driving mitochondrial quality control processes to alter mutant burden. Parkin overexpression selected against a deleterious COXI mutation in heteroplasmic cybrid cells, reducing the mtDNA mutant burden possibly through activation of selective mitophagy [81]. When Parkin was no longer overexpressed in the cells, mutant mtDNA reaccumulated [81].

Mitochondrial fusion–fission dynamics is also influenced by mtDNA mutations. In a study of adult *Drosophila* muscle, the activation of the PINK1/Parkin pathway, stimulation of autophagy, or decreasing the expression of *mitofusin*, thereby increasing mitochondrial fragmentation, all promoted the selective decrease of a heteroplasmic mtDNA deletion by facilitating mitophagic

clearance [81]. In this study, the role of ATPIF1, which regulates the function of the F_1F_o ATP synthase, was explored for its role in mitochondrial quality control. Genetic knockdown of ATPIF1 reduced the mtDNA mutant burden, presumably by limiting the ability of pathogenic mtDNA to maintain membrane potential by reversal of the ATP synthase [80,81]. Certainly, mitochondrial fission plays an important role in mitophagy. In *Drosophila*, pro-fission factors, such as Drp1, drive the selective removal of mutant mtDNA in the germline [95,96]. These fission events isolate mutant mtDNA, prevent complementation, and render these mitochondria accessible to removal by mitophagy [95,96]. In a muscle-derived rhabdomyosarcoma heteroplasmic cell line harbouring the m.3243A>G mutation, silencing of Drp1, thereby inhibiting mitochondrial fission, was able to influence and favour the propagation of mutant mtDNA [97].

Regulation of mitochondrial biogenesis is a further process that is influenced by the interaction between mtDNA mutations and signalling pathways. The biogenesis is induced through the transcriptional coactivator PGC-1α, which operates by activating a number of transcription factors, promoting the expression of mitochondrial transcription factor A (TFAM), NRF1, and NRF2 [98]. Activation of the AMPK pathway has emerged as a key player in regulating the balance between mitochondrial biogenesis and mitophagy in fibroblasts with the m.3243A>G mutation, thereby playing a role in defining the severity of disease by improving the pathophysiological defects [98]. In a genome-wide screen, the reduction of 'tam', a catalytic subunit of the mtDNA polymerase gene (PLOG), reduced the propagation of pathogenic mtDNA, potentially by increasing the copy number of wild-type mtDNA and promoting the elimination of mutant mtDNA in a heteroplasmic *Drosophila* line [99]. Again, in a mouse model with a heteroplasmic m.5024C>T mutation, the induction of biogenesis by increasing TFAM levels, increased the mtDNA copy number and ameliorated the pathological phenotypes [100]. These findings suggest that the total mtDNA copy number may determine the severity of pathological manifestations through the modulation of biogenesis, despite persistence of the mutant mtDNA [34,100,101].

Taken together, it is evident that the presence of mtDNA mutations and associated alterations in signalling pathways define the mutant burden and contribute to shaping mitochondrial disease through the modulation of mitochondrial quality control processes.

Hypoxia or oxygen-sensing pathways

The mitochondrial OxPhos system is the major consumer of oxygen for generating ATP. The hypoxia response pathway promotes adaptation to low ambient oxygen availability, mediated primarily by **hypoxia-inducible factors (HIFs)** but also other pathways (e.g., nutrient-sensing, inflammation, and proteostasis), sensing changes in metabolite levels and ROS production by mitochondria [102]. For example, a dysfunctional TCA cycle caused by mutations of nDNA encoded mitochondrial proteins, such as fumarate hydratase or succinate dehydrogenase, induces accumulation of succinate or fumarate, which activate the HIF1 pathway under normoxic conditions [102]. Similarly, mouse cybrid cells bearing the m.13997G>A mutation (ND6) generated excess ROS and increased HIF-1α expression [83,103]. In contrast, in human cybrid cells, the m.3243A>G mutation and mtDNA depletion led to a decreased HIF-1α stabilisation [79]. Moreover, oxygen tension modulates levels of mtDNA heteroplasmy in a mouse embryonic stem cell model for germline cell formation, which likely operates through reduction of mtDNA replication but not activation of mitophagy [104]. Interestingly, mtDNA sequencing revealed that pathogenic mutations occurred far more frequently in Tibetan highlanders who live at low ambient oxygen tensions [105]. It could be either that these pathogenic mtDNA mutations help hosts adapt to a hypoxic environment or that the environment allows the mutations to exist by relieving the selection pressure. However, it is still unclear how the change in hypoxia response and the mtDNA

mutations or heteroplasmy affect each other and how the pathway is involved in the manifestation and progression of mtDNA disorders.

Modulating the hypoxia response appears to be a promising strategy in treating primary mitochondrial diseases with nDNA mutations. While genetic or pharmacological activation of the HIF response is protective in cultured cells and zebrafish models with dysfunctional mitochondria, in the mouse model of Leigh syndrome (NDUFS4 knockout), exposure to a hypoxic environment but not activation of HIFs proved to be beneficial [106,107]. Therefore, HIF activation was insufficient to explain the disease rescue in response to hypoxia. On the contrary, HIF-1α was found to be upregulated in *Drosophila* models of mitochondrial dysfunction (TFAM overexpression), Leigh syndrome and Parkinson's disease; remarkably disease phenotype was rescued by knockdown of HIF-1α [71]. Indeed, the hypoxia response has been linked to the UPR/ISR and mitochondrial quality control [102]. Furthermore, CRISPR screening identified 109 gene knockouts with relative fitness defects at low ambient oxygen tension, most without a known connection to HIF or ROS [108]. Further studies are therefore needed to explore how these genes and pathways are coordinated. These distinct roles of HIFs in a variety of mitochondrial disease models point to varied mechanisms underlying the pathology of mitochondrial diseases and again emphasise the complexity driving the consequences of mitochondrial dysfunction.

Other cell signalling pathways

Changes in epigenetic regulation, calcium, and ROS signalling and associated changes in redox state have been investigated as potential signal transducers, with the ability to alter cell pathophysiology and mitochondrial function [109–112]. However, studies exploring their role in mtDNA mutation disorders are few and remain largely unexplored. This review has mainly focussed on the pathways that have been more intensively studied in the context of mtDNA mutations (Figure 1); nonetheless, here we detail the involvement of some other signalling pathways in mtDNA mutation disorders. A recent study showed that a decrease in mitochondrial calcium uptake may be important in remodelling metabolism in myofibroblasts in a manner that drives epigenetic changes important in differentiation [110], suggesting that changes in intermediary metabolites (notably αKG) provide a mechanism that links changes in mitochondrial calcium to cell fate and function. Alternatively, cybrid cells expressing the m.3243A>G mutation showed changes in levels of acetyl-CoA and α-KG that correlated with histone modifications [31]. In mouse embryonic fibroblasts, ROS production was shown to be elevated in all mtDNA mutation carrying lines investigated, regardless of the level of heteroplasmy present, and was shown to reduce the efficiency of reprogramming cells into iPSCs, as pluripotent cells negatively selected against pathogenic mtDNA [113]. Furthermore, although in most reports the decline in mitochondrial bioenergetics is positively correlated with mutant load [32,46,53,54], some reports suggest that different levels of heteroplasmy of the m.3243A>G mutation in cybrid cells result in different gene expression patterns and even in discrete changes in metabolism [114,115]. From these studies, the potential rewiring of signalling pathways in response to the presence of mtDNA mutations are further emphasised.

Concluding remarks

Primary mitochondrial diseases are a heterogeneous group of diseases that can manifest at any age, affect almost any tissue, and vary radically in clinical presentation and severity. Recently, there has been growing interest in pharmacological strategies to target primary mitochondrial diseases; the development of small molecules that improve mitochondrial function and repurposing drugs, especially using compounds that influence mitochondrial quality control pathways (Box 1).

Outstanding questions

To what extent do genetics, metabolism, and cell signalling pathways regulate levels of mtDNA heteroplasmy and what are the detailed mechanisms that drive this process?

How do metabolic remodelling and cell signalling pathways affect each other in cells bearing mtDNA mutations?

What are the underlying mechanisms that define the phenotype associated with a given mtDNA mutation?

Can differences in cellular signalling pathways associated with specific mtDNA mutations be harnessed as pharmacological targets to reduce the burden of mutant mtDNA for patient benefit?

Box 1. Therapies and clinical trials

At present, therapeutic strategies available for most patients with primary mitochondrial disease are limited to supportive care and symptomatic management, and licenced treatments that rescue mitochondrial biochemical function are lacking [116]. As many primary mitochondrial diseases affect multiple organs and body systems, symptomatic management may require combinations of medication to target different systems, presenting an additional challenge for clinicians. Pharmacologic approaches using antioxidants like Coenzyme Q 10 are often implemented in the care plan for primary mitochondrial disease affected patients. The rationale for the use of antioxidants is to counteract the excessive generation of ROS associated (or assumed to be associated) with the dysfunction of the respiratory chain. However, there is still considerable disagreement over whether antioxidants provide useful therapeutic strategies for primary mitochondrial disease and therefore the need for alternative and more effective therapeutic strategies, specific to the mtDNA mutations, is still evident. Recently, there has been growing interest in the development of pharmacological strategies to target primary mitochondrial diseases [3,117,118]. Research efforts have primarily focussed on finding and developing small molecules that improve mitochondrial function in these patients. Similarly, focus has also shifted to the prospect of drug repurposing, especially using compounds that influence pathways of mitochondrial quality control, such as rapamycin and antioxidants like idebenone, EPI1743 and sonlicromanol [116]. Currently, there are a number of small molecule compounds that are moving from laboratory to clinical trials as well as therapeutic strategies, including gene therapy and enzyme bypass, which are moving along the translational pipeline [116].

While these therapeutic strategies offer exciting promise, we suggest the added understanding of the interplay between mtDNA mutations and cell signalling pathways can further accelerate research efforts in this field. Traditionally, disease manifestations have, not unreasonably, been perceived as a direct consequence of impaired energy metabolism. However, an increasing body of data points to major roles of cell signalling pathways in shaping disease presentation and progression. These pathways may contribute directly to altered cell function in shaping manifestations of disease but may also feedback through mitochondrial quality control pathways and so influence heteroplasmic mutant load. While introducing complexity to our current understanding of the pathophysiology of mitochondrial disease, these considerations also signpost the potential of pharmacological manipulation of mitochondrial quality control pathways and/or of the altered signalling pathways as novel therapeutic strategies. This opens a wealth of new possibilities in the way that we think about management strategies for people with these awful diseases (see Outstanding questions).

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Declaration of interests

The authors declare no competing interests.

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