REVIEW ARTICLE

Mitochondrial DNA copy number in human disease: the more the better?

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Most of the genetic information has been lost or transferred to the nucleus during the evolution of mitochondria. Nevertheless, mitochondria have retained their own genome that is essential for oxidative phosphorylation (OXPHOS). In mammals, a gene-dense circular mitochondrial DNA (mtDNA) of about 16.5 kb encodes 13 proteins, which constitute only 1% of the mitochondrial proteome. Mammalian mtDNA is present in thousands of copies per cell and mutations often affect only a fraction of them. Most pathogenic human mtDNA mutations are recessive and only cause OXPHOS defects if present above a certain critical threshold. However, emerging evidence strongly suggests that the proportion of mutated mtDNA copies is not the only determinant of disease but that also the absolute copy number matters. In this review, we critically discuss current knowledge of the role of mtDNA copy number regulation in various types of human diseases, including mitochondrial disorders, neurodegenerative disorders and cancer, and during ageing. We also provide an overview of new exciting therapeutic strategies to directly manipulate mtDNA to restore OXPHOS in mitochondrial diseases.

Keywords: ageing; Alzheimer's disease; cancer; mitochondria; mitochondrial diseases; mtDNA; mtDNA copy number; neurodegenerative disorders; Parkinson's disease; TFAM

Mitochondrial DNA: genetics, packaging and maintenance

Mitochondria are double-membrane organelles present in almost all eukaryotic cells and regulate a wide variety of cellular processes, including ATP production by oxidative phosphorylation (OXPHOS), apoptosis, β-oxidation of fatty acids and biogenesis of iron–sulfur clusters. Mitochondria house their own genome, the mitochondrial DNA (mtDNA) and the molecular machinery responsible for its maintenance and expression, that is several hundred factors controlling replication, transcription, transcript maturation and mitochondrial translation [1]. The mammalian mtDNA is a circular double-stranded DNA (dsDNA) molecule present in multiple copies per cell. In the mitochondrial matrix, mtDNA molecules are not naked but packaged into slightly elongated DNA–protein structures known as mitochondrial nucleoids (Fig. 1A) [2]. Superresolution microscopy techniques have revealed that mitochondrial nucleoids have a diameter of ~ 100 nm and mainly contain one single copy of mtDNA [3–5]. The most abundant structural component of nucleoids is the mitochondrial transcription factor A (TFAM) (Box 1) [6,7]. When bound to mtDNA, the high-mobility group (HMG) box domains of TFAM facilitate mtDNA compaction [8], thereby regulating the accessibility of the genome to the replication and transcription machineries [9].

In mammals, mtDNA is an intronless molecule of ~ 16.5 kb containing 37 genes encoding 13 protein subunits of the OXPHOS system, two rRNAs (12S and 16S) and a complete set of 22 tRNAs for mitochondrial translation [19,20]. Unlike the nuclear chromosomes, mtDNA replication is independent of the cell cycle and also occurs in postmitotic cells, a process often referred to as relaxed replication [21]. A minimal mtDNA replisome has been defined and consists of the hexameric helicase TWINKLE, the heterotrimeric DNA polymerase gamma (POLγ) and the tetrameric mitochondrial single-stranded DNA-binding protein (mtSSB), in addition to the replication primer forming mitochondrial RNA polymerase (POLRMT) [22] (Fig. 1A). During replication of the leading strand of mtDNA, TWINKLE moves in a 5′ to 3′ direction while unwinding the dsDNA [23]. The single-stranded DNA (ssDNA) is protected through mtSSB binding, which also further stimulates the dsDNA unwinding by TWINKLE and the DNA synthesis by POLγ [23,24]. The RNA primers required for the initiation of the synthesis of both DNA strands are produced by POLRMT [25]. It has been recognised for more than 45 years that replication of mammalian mtDNA occurs asymmetrically [26–28]. The replication process is understood in great detail, although not completely, and depends on dedicated origins on each DNA strand, the leading (heavy-) and the lagging (light-) strand origin of replication (O_H and O_L), respectively [1,29]. According to this strand-displacement mode, the replication of the leading strand initiates at O_H and proceeds unidirectionally until about two-thirds of the genome has been replicated. At this point, the replication of the leading strand proceeds beyond O_L, which is activated to form a stem–loop structure [24,30] that recruits POLRMT for RNA primer synthesis to initiate the replication of the lagging strand [31]. The synthesis of the two strands then proceeds continuously until two full-length, dsDNA molecules are formed. To complete mtDNA replication, the mitochondrial genome maintenance exonuclease 1 and other nucleases help forming ligatable DNA ends and the concatenated newly replicated mtDNA molecules are released from each other by the action of topoisomerase 3α [32].

Given that mtDNA expression is a prerequisite for biogenesis of the OXPHOS system [33], mtDNA content in cells and tissues must be adjusted according to metabolic needs [34]. Therefore, mtDNA levels are tissue- and developmental-stage-specific and finely regulated by a balance between replication and turnover. Studies on human samples have revealed that mtDNA copy number per cell can vary by several orders of magnitude, ranging from ~ 1×10^5 mtDNA copies in oocytes [35], ~ 4–6×10^3 in heart to 0.5–2×10^3 in lungs, liver and kidney [36]. Consistent with its key role in packaging mtDNA into nucleoids, TFAM also serves as a key regulator of mtDNA copy number (Box 1). Heterozygous disruption of *Tfam* causes a decrease in mtDNA levels of ~ 50% [15,16], whereas a moderate TFAM overexpression increases the amount of mtDNA by ~ 50–100% [16–18].

Our knowledge of mtDNA replication and maintenance has dramatically increased in recent years, but how the cellular mtDNA copy number is adjusted to and maintained at a certain level is currently not completely understood. Several models, based on ATP requirements, nucleotide availability and replication origin regulation, have been proposed to control mtDNA content [37]. In addition, genetic screens in *Saccharomyces cerevisiae* have been performed to identify novel regulators of mtDNA levels [38,39]. Unfortunately, these studies have provided little new mechanistic insights as loss of mtDNA is a common secondary effect of mitochondrial dysfunction in budding yeast. Genetic studies in humans have identified a region on chromosome 10, which contains *TFAM* and other genes, to have a notable impact on mtDNA levels [40].
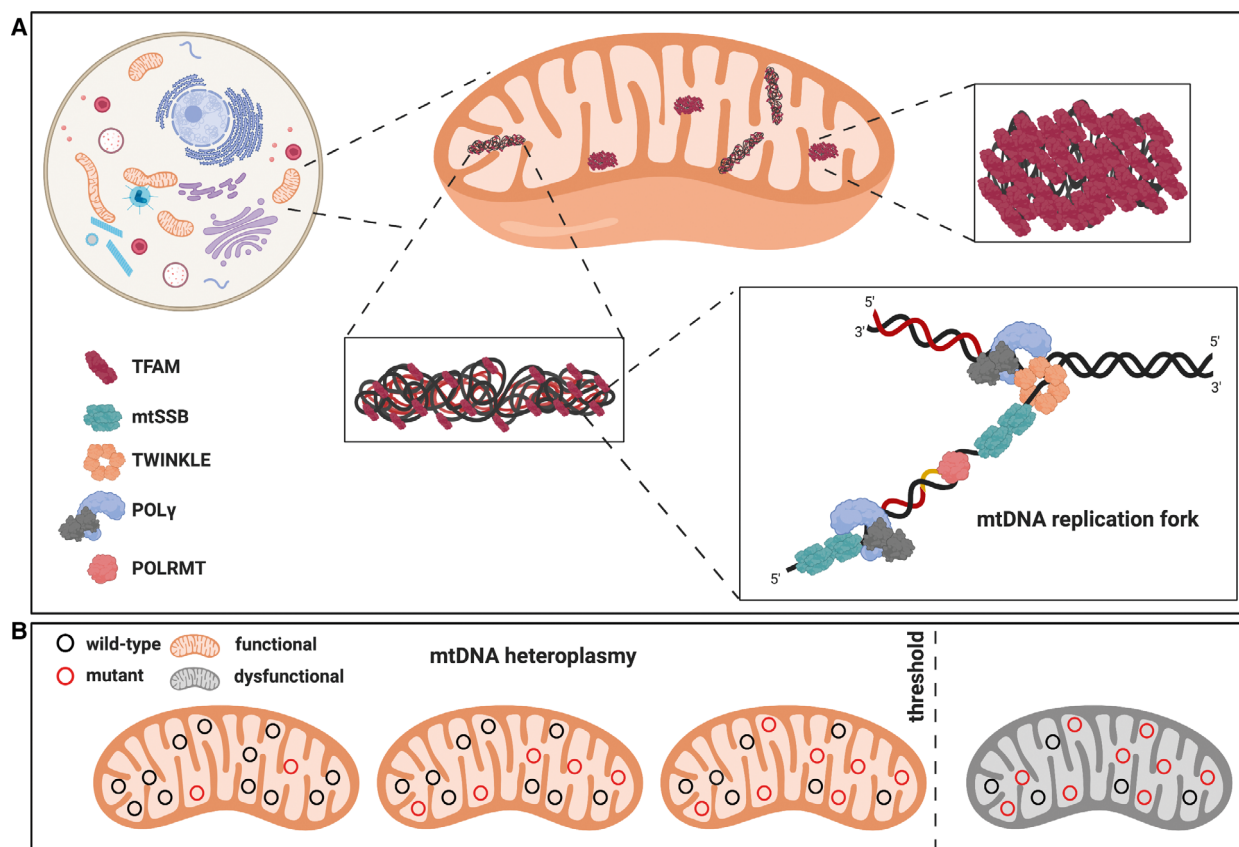
Fig. 1. Schematic representation of mtDNA maintenance, packaging and genetics. (A) mtDNA nucleoids and the replisome. mtDNA is present in multiple copies within the cell. It is compacted by TFAM into structures known as nucleoids. Mitochondrial nucleoids can be found in a compacted or relaxed state depending on the local TFAM concentration. In the relaxed state, mtDNA is accessible for replication by the mitochondrial replisome which is formed by the mitochondrial RNA polymerase POLRMT, the hexameric DNA helicase TWINKLE, the tetrameric mtSSB and the mtDNA polymerase gamma POLγ. POLγ is a heterotrimer formed by a catalytic subunit with DNA polymerase and 3′-5′ exonuclease activities (encoded by *POLGA*), and by two accessory subunits (encoded by *POLGB*) required for the tight DNA-binding and processive DNA synthesis. During replication, TWINKLE unwinds and proceeds on the DNA in a 5′ to 3′ direction, and the single-stranded DNA generated by TWINKLE activity is protected by the mtSSB binding. The RNA primers for replication initiation are generated by POLRMT. (B) The concept of heteroplasmy. Mutations affecting the mtDNA can coexist with the wild-type molecules, a condition known as heteroplasmy. mtDNA mutations are tolerated until they exceed a certain level (threshold). Therefore, defects in the OXPHOS system will manifest only when the proportion of mutated mtDNA molecules exceeds the biochemical threshold, which is known to be tissue- and mutation-specific.

Causes and consequences of mtDNA mutations

Mutations of mtDNA can in principle be generated either by spontaneous errors during DNA replication or by incorrect repair of damaged DNA bases. In spite of being very popular, the free radical theory of ageing, which proposes that oxidative stress is the main cause for mtDNA mutations [41,42], is much questioned. In fact, sequence analyses of mutations in a large number of mtDNA genomes and experimental studies of mice argue that the vast majority of mtDNA mutations are the result of spontaneous replication errors introduced by POLγ during mtDNA synthesis [43–48]. The explanation for the paucity of damage-induced mtDNA mutations may reside in the compacted nature of the mitochondrial nucleoid, which provides a protected environment that makes the mtDNA less accessible to chemical damage, including oxidation.

Pathogenic mtDNA mutations have been identified in mitochondrial tRNA, rRNA and protein-coding genes, and they invariably compromise mitochondrial gene expression causing various degrees of OXPHOS deficiency. Due to the multicopy nature of the mitochondrial genome, mtDNA mutations may be present either in all (homoplasmy) or only in a subset of all (heteroplasmy) copies of mtDNA. Many severe

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Box 1. Many faces of TFAM: one protein with multiple functions

TFAM is a highly abundant mitochondrial protein, present in about 1000 copies per mtDNA molecule or 1 TFAM molecule per 15–20 bp of mtDNA [5], and it is essential for mtDNA transcription initiation, nucleoid formation and mtDNA maintenance.

TFAM is a member of the HMG-box protein superfamily, contains two HMG-box domains, HMGA and HMGB, separated by a linker, and a charged C-terminal tail [10]. The crystal structures of TFAM have shown that each of the two HMG boxes bend the DNA nearly 90° inducing U turn in the DNA minor groove and bending it towards the major groove [8,11].

TFAM modulates the interactions with mtDNA thanks to a fine-tuning of its DNA-binding function. TFAM can bind, unwind and bend DNA without sequence specificity. It coats and packages individual mtDNA molecules to form mitochondrial nucleoids. In addition, TFAM can also bind DNA in a sequence-specific manner to facilitate unwinding of the promoter regions during transcription initiation [12]. Notably, the C-terminal tail of TFAM has been shown to prime the interaction with the transcription machinery [13], whereas the HMGB domain is responsible for the interaction with POLRMT to anchor it to the promoter [14].

TFAM is also essential for mtDNA maintenance and homozygous disruption of *Tfam* is embryonically lethal in the mouse [15]. Several genetic models have demonstrated a direct link between TFAM protein levels and mtDNA copy number *in vitro* and *in vivo*. Heterozygous ablation of *Tfam* causes ~ 50% decrease in mtDNA levels [15,16], whereas the overexpression of Tfam results in ~ 50% increase of mtDNA copy number [16–18]. TFAM is therefore a multifunctional protein, which is necessary for mtDNA genome maintenance and expression. To date, our knowledge related to the regulation of TFAM activity in different processes remains very limited.

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mtDNA mutations cannot be tolerated in the homoplasmic state and are therefore only found in the heteroplasmic state in patients. Heteroplasmic pathogenic mtDNA mutations are typically functionally recessive [49], meaning that they cause respiratory-chain deficiency only when present above a certain threshold level [50] (Fig. 1B). This implies that only cells containing levels of mutated mtDNA that exceeds a critical threshold will develop respiratory-chain dysfunction, whereas adjacent cells with a lower mutation load can sustain normal respiratory-chain function. The threshold level is highly dependent on the affected tissue and the type of mtDNA mutation present. For single large deletions of mtDNA, the threshold is ~ 50–60% [50], whereas some point mutations in tRNA genes have thresholds exceeding 90% mutated mtDNA [51–53]. During the life of an individual, the level of heteroplasmy can shift either up or down due to mitotic segregation, which is a consequence of relaxed replication and random partitioning of mitochondria between daughter cells. Furthermore, deleterious mtDNA alleles may be actively eliminated in proliferating tissues by purifying selection, which has been seen in blood for some types of mtDNA mutations [54–56].

Besides being the direct cause of primary mitochondrial diseases, typically characterised by severe mitochondrial impairment in multiple tissues [57], mtDNA mutations have also been implicated in the pathophysiology of common age-associated human diseases [57–61] and in the naturally occurring ageing process [62]. It is well established that point mutations in tRNA genes or single large deletions of mtDNA lead to functional impairment or lack of one or several tRNAs. However, these types of pathogenic mtDNA mutations have no dominant effects and high levels of mutated mtDNA can therefore be tolerated. The recessive nature of most human pathogenic mtDNA mutations leads to a deficiency of wild-type gene products, which, in turn, impairs OXPHOS and causes the disease phenotypes.

Almost 25 years ago, Attardi and colleagues hypothesised that both the fraction of wild-type mtDNA and the mtDNA copy number play a role in the phenotypic manifestations of mtDNA mutations. The authors demonstrated a correlation between mtDNA copy number and oxygen consumption rate and also experimentally proved that low levels of wild-type mtDNA can protect cybrid cell lines from the deleterious effects of a pathogenic mtDNA mutation in the tRNA^{Leu} gene [63]. In recent years, increasing evidence based on human correlative data and experimental mouse genetics has confirmed that high absolute levels of wild-type mtDNA genomes indeed may counteract the consequences of pathogenic mtDNA mutations [64–67].

In this review, we provide a comprehensive overview of the current knowledge on the contribution of mtDNA copy number regulation to clinical manifestations of mitochondrial disorders, neurodegenerative

diseases, cancer and ageing. We critically assess available data on mtDNA copy number regulation and experimental challenges. Finally, we discuss the rationale and the applications of current therapeutic strategies to improve mitochondrial function.

mtDNA copy number in human disease

Quantification of mtDNA copy number

To understand the implication of mtDNA variations in human diseases, mtDNA copy number must be accurately assessed to avoid biases that confound interpretation of results.

Measurements of mtDNA levels can be performed with a variety of techniques. For a long time, southern blot (SB) hybridisation was used as the gold standard method to assess mtDNA levels and integrity in patient samples [68–70] and in model organisms [71–74]. Despite being highly reliable in assessing mtDNA content, SB techniques are time-consuming, only semi-quantitative and require a relatively large amount of DNA, which combined represent a major disadvantage when studying human tissues.

Fluorescent in situ hybridisation approaches have also been employed to spatially visualise mtDNA content with single-cell resolution but have proven to be only partially informative [75–77]. In fact, this multi-step protocol can be quite laborious and it provides only a rough estimate of changes in mtDNA amount.

The most popular current standard techniques to measure mtDNA copies are PCR-based methods. Due to its simplicity, quantitative real-time PCR (qPCR) is the most widely used approach to assess relative or absolute mtDNA levels. However, the large majority of protocols assess the relative mtDNA copy number as a ratio between mtDNA levels and levels of a selected nuclear gene, which makes comparison between studies very difficult. It is also possible to engage qPCR-based assays to determine the absolute mtDNA copy number by using either a standard curve based on the mtDNA region of interest inserted into a reference plasmid [78,79] or droplet digital PCR (ddPCR). Although the limited effective range of the ddPCR method might affect precision when measuring high copy number, this technology can provide a rigorous quantification of mtDNA copies without the use of external standards [80–82].

Recent studies have shown that the quantification of mtDNA copy number can be inferred from next-generation sequencing (NGS) data, including whole-exome sequencing (WES) and whole-genome sequencing (WGS) [83–86]. The output from NGS datasets relies on the ratio of sequencing reads from nuclear DNA and mtDNA and allows analysis of hundreds of thousands of available data sets shared by research consortia. Although a series of normalisations are needed to correct for counts, ploidy, purity and batch biases [36,87,88], the NGS technology enables high-throughput, high-sensitivity and accurate assessment of mtDNA levels. Thus, WES/WGS approaches will likely be increasingly employed as gold standard tools to assess mtDNA content and heteroplasmy levels in the future.

mtDNA copy number in mitochondrial diseases

Mitochondrial diseases are a group of heterogenous hereditary disorders characterised by OXPHOS deficiency. These disorders can affect different cell types and organs, and can therefore cause a wide range of symptoms. Mitochondrial diseases have been classified according to their clinical manifestations and can be caused either by mutations in nuclear genes, leading to reduced mtDNA expression, or by primary mtDNA mutations directly impairing the function or abundance of mtDNA-encoded gene products [59,60].

mtDNA depletion syndromes (MDSs) [89] are autosomal recessive disorders characterised by a strong tissue-specific reduction in mtDNA levels due to mutations in genes involved in various mtDNA maintenance processes, ranging from mitochondrial nucleotide metabolism, mtDNA replication, mitochondrial dynamics and quality control (Table 1). Thus, MDSs establish a causal and direct link between low mtDNA content and pathological conditions. However, the importance of mtDNA copy number in the pathophysiology of other mitochondrial diseases often remains enigmatic.

The *POLGA* gene, encoding the catalytic subunit of mitochondrial polymerase POLγ, is one of the several nuclear genes associated with MDS. However, the over 300 mutations identified so far in the *POLGA* gene can cause a variety of mtDNA defects leading to disparate symptoms, disease severity and age of onset of disease (https://tools.niehs.nih.gov/polg/) [90]. Patients affected by *POLGA*-related disorders can manifest either severe reduction in mtDNA copy number or accumulation of mtDNA mutations and deletions with no effect on mtDNA levels [91]. Even though the crystal structure of POLγ has shed some light on how different mutations can impact enzyme activity and replication fidelity [92], the relation between genotype and phenotype is not always straightforward. The strong phenotypic variability in *POLGA* patients can even occur between subjects of the same family [93].

Table 1. mtDNA copy number variation among mitochondrial diseases. Top panel: mitochondrial diseases caused by mutations in nuclear genes. n.a. not available in the original work. Bottom panel: diseases caused by primary mtDNA mutations.

Disease	Gene (nuclear)	Sample type	mtDNA levels	Quantification method	Reference
MDS	*ANT1*	Skeletal muscle	Down	qPCR	[232]
	TK2	Skeletal muscle	Down	SB; qPCR	[91,233]
	DGUOK	Liver	Down	SB; qPCR	[91,234]
	TYMP	Gastrointestinal tract	Down	qPCR	[235]
	MPV17	Liver	Down	SB; qPCR	[91,236]
	SUCLA2	Skeletal muscle	Down	qPCR	[91,237]
	SUCLG1	Liver/Skeletal muscle	Down	qPCR	[91,238]
	MFN2	Skeletal muscle	Unchanged/down	n.a	[239,240]
	FBXL4	Skeletal muscle	Down	qPCR	[241,242]
	POLGA	Liver/blood	Unchanged/ down	qPCR	[91,243]
	POLGB	Liver/skeletal muscle/blood	Down	qPCR	[244]
	TWNK	Liver	Down	qPCR	[245,246]
	mtSSB	Muscle/blood/kidney	Down	qPCR	[247]

Disease	mtDNA mutation	Sample type	mtDNA levels	Quantification method	Reference
Pearson's syndrome	Deletion	Blood	Up	qPCR	[95]
KSS	Deletion	Blood/muscle	Up	qPCR	[95]
MELAS	m.3243A>G	Leucocytes	Up/unchanged/down[a]	qPCR	[96]
MERRF	m.8344A>G	Leucocytes	Up/unchanged/down[a]	qPCR	[96]
LHON	m.11778G>A	Peripheral blood cells	Up	qPCR	[65]
LHON	m.11778G>A	Blood	Up/unchanged	qPCR	[97,98]
LHON	m.3460G>A	Blood	Up/unchanged	qPCR	[97,98]

[a]Copy number found to be increased in younger patients but unchanged or even decreased in older patients.

Interestingly, a mutation markedly impairing the proof-reading activity of DNA polymerase gamma, subunit A (POLGA) in mice resulted in numerous mtDNA mutations, but had no impact on copy number [94].

Homoplasmic and heteroplasmic pathogenic mtDNA mutations are responsible for diverse mitochondrial syndromes, including mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS), Leber's hereditary optic neuropathy (LHON), myoclonus epilepsy with ragged-red fibres (MERRF), Pearson's syndrome or Kearns–Sayre syndrome (KSS). mtDNA copy number has been assessed only in small cohorts of patients affected by either Pearson's or KSS, caused by single large heteroplasmic mtDNA deletions. The results of these investigations showed that most patients presented an increased mtDNA copy number compared with the control individuals, although mtDNA levels did not correlate with the size or position of the deletion [95].

The copy number variations in patients with mtDNA point mutations have been more extensively investigated. A recent study of a large group of MELAS patients carrying the heteroplasmic m.3243A>G mutation showed that high mtDNA copy number and low heteroplasmy levels correlated with less severe disease [66]. Other studies with smaller patient cohorts reported that MELAS and MERRF patients with moderately high mtDNA copy number showed milder phenotypes

[96]. Also, in LHON patients, that mostly carry homoplasmic mutations, mtDNA copy number was reported to play a role in the penetrance of the disease, as the asymptomatic carriers of the m.11778G>A or m.3460G>A mutations had higher mtDNA copy number than visually impaired patients [65,97,98].

The data discussed above and additional data summarised in Table 1 strongly suggest a direct relation between the absolute mtDNA copy number and mitochondrial diseases onset and progression. Particularly intriguing is the idea that an increase in the absolute mtDNA copy number could be a compensatory mechanism aimed at sustaining OXHPOS activity. This endogenous upregulation of mtDNA content might be able to efficiently overcome the bioenergetic defects caused by some mtDNA mutations. In a well-documented mouse model harbouring a pathogenic heteroplasmic tRNA gene mutation in mtDNA [99], affected animals spontaneously upregulate mtDNA copy number in several tissues, which may delay onset of disease manifestations [64].

mtDNA levels in ageing and age-related neurodegenerative disorders

Ageing is a biological process characterised by the slow and progressive decline of the physiological

functions of an organism, which ultimately determines multimorbidity and lethality. This process includes a decay of mitochondrial function, concomitant with alterations in mitochondrial morphology [100,101], mitochondrial content (number and protein levels) [102] and OXPHOS capacity [103]. As previously mentioned, pathogenic mtDNA mutations, both large deletions and point mutations, have been identified in postmitotic and proliferating tissues of aged individuals [62,104–106]. There is experimental evidence suggesting that the somatic mtDNA mutations seen in ageing humans and other mammals have the capacity to cause at least some ageing phenotypes. In mice, proofreading-deficient POLγ caused a progressive accumulation of mtDNA mutations and a premature ageing syndrome with reduced lifespan, decreased fertility, anaemia, hair greying, hair loss, hearing impairment and stem cell dysfunction [94,107]. Interestingly, increasing evidence suggests that somatic mtDNA mutations may contribute to age-related neurodegenerative disorders, such as Parkinson's disease (PD) and Alzheimer's disease (AD). The accumulation of mtDNA mutations was observed in the Substantia nigra (SN) of PD patients [108] and in frontal cortex and hippocampus of AD patients [109]. Furthermore, in these brain regions the presence of high levels of mtDNA point mutations or deletions correlated with severe mitochondrial biochemical defects, for example in complex I [110] or complex IV [111].

Correlative studies in humans with age-associated degenerative disease and ageing have thus strongly implicated mtDNA mutations and OXPHOS dysfunction in these conditions, but a possible role for mtDNA copy number is less clear. Multiple studies have assessed the mtDNA content in tissues of individuals at different ages (Table 2). The majority of these investigations showed a reduction in mtDNA copy number in aged individuals. For instance, mtDNA levels measured in lymphocytes from over 2000 Sardinians were found modestly, but significantly, decreased with age [112]. A more pronounced reduction in mtDNA copy number was found in blood samples and was first observed in individuals in their 50s. The decline in mtDNA levels was even more dramatic in older subjects [113,114], and a loss of a few per cent of all copies per decade has been estimated [115]. Remarkably, in the old group (> 58 years of age), low mtDNA copy number in peripheral blood was associated with high mortality and poor health, including a decline in cognitive and physical performance [114]. Studies performed in long-lived families comprising nonagenarian and centenarian individuals have often shown puzzling and contradictory outcomes. mtDNA

copy number in blood samples was reported to be clearly reduced in individuals between 50 and 70 years of age, whereas mtDNA levels were either lower [116] or higher [117] in nonagenarians and centenarians in comparison with middle-aged controls. Therefore, these results do not clarify whether low mtDNA copy number correlates with a negative or positive impact on longevity.

A series of experiments performed on samples other than blood and lymphocytes also yielded mixed results. For instance, age-related reduction in mtDNA levels were not observed in skeletal muscle and heart [118,119], whereas a more recent study reported decreased mtDNA copy number in skeletal muscle and increased copy number in liver of aged subjects [120].

Significant differences in mtDNA copy number have been reported across different brain regions, and these variations were more pronounced in patients affected by neurodegenerative disorders. For instance, no alterations in mtDNA levels were identified in samples from caudate nucleus, frontal lobe and cerebellar cortex of aged individuals [118,121]. Interestingly, microdissected dopaminergic (DA) neurons from SN of healthy aged subjects contained increased levels of mtDNA deletions and increased total mtDNA levels [121]. In contrast, DA neurons of PD patients had no increase in total mtDNA copy number despite having increased levels of deleted mtDNA [121]. It is possible that this selective depletion of functional wild-type mtDNA molecules caused a bioenergetic deficiency in DA neurons of PD patients [121]. Other studies that analysed both total SN homogenates and microdissected DA neurons found a general reduction in mtDNA content in PD patients [122,123]. Notably, mtDNA levels were unaltered in brain regions that were only mildly affected or not compromised in PD patients [123,124].

Measurements of mtDNA levels were also performed in brains of subjects affected by AD. These investigations revealed that mtDNA levels were decreased by 30–50% in the frontal cortex of AD patients when compared to controls [109,125]. This mtDNA depletion was also present in microdissected pyramidal neurons from the hippocampus and was concomitant with a disruption in mitochondrial biogenesis signalling pathways [126]. The correlation between reduction in mtDNA levels and neurodegeneration in AD has been further supported by a comprehensive in-depth analysis of mtDNA sequence variation and abundance in over 1000 human brains [124].

The need for novel diagnostic and prognostic biomarkers for PD and AD has promoted significant research efforts to assess mtDNA copy number in

Table 2. mtDNA copy number variation in ageing and neurodegenerative disorders.

Disease	Sample type	mtDNA levels	Quantification method	References
Ageing	Lymphocytes	Down	WGS	[112]
	Blood	Down	qPCR; qPCR; WGS; qPCR; qPCR	[113–117]
	Heart	Unchanged	qPCR; SB	[118,119]
	Skeletal muscle	Unchanged	qPCR; SB	[118,119]
	Skeletal muscle	Down	NGS and ddPCR	[120]
	Liver	Up	NGS and ddPCR	[120]
	Caudate nucleus	Unchanged	SB	[118]
	Frontal lobe cortex	Unchanged	SB	[118]
	Cerebellar cortex	Unchanged	SB	[118]
	SN[a]	Up	qPCR	[121]
PD	SN[a]	Down	qPCR; qPCR	[121,122]
	Cerebellum	Unchanged	WES	[124]
	Cerebellar cortex	Unchanged	WES	[124]
	Frontal cortex	Unchanged	qPCR	[123]
	SN	Down	qPCR	[123]
	Blood	Down	qPCR; qPCR	[123,127]
	CSF	Down	qPCR	[130]
AD	Frontal cortex	Down	qPCR	[109]
	Cerebellum	Unchanged	qPCR	[125]
	Cerebellum	Down	WES	[124]
	Hippocampus	Unchanged	qPCR	[125]
	Hippocampus[a]	Down	qPCR	[126]
	Cerebellar cortex	Down	WES	[124]
	Blood	Unchanged	qPCR	[125,128]
	CSF	Down	qPCR	[129]

[a]Analyses were performed on microdissected neurons from these specific brain regions.

peripheral blood and cerebrospinal fluid (CSF). In blood samples from PD patients, mtDNA levels were decreased when compared to healthy controls [123,127]. Lower mtDNA copy number was more frequently observed in elderly PD subjects, suggesting that mtDNA content might even have a prognostic relevance in PD progression [127]. Different results were found in AD subjects, where mtDNA levels were unchanged [125,128] in spite of altered mitochondrial gene expression [128]. Analyses performed in CSF, which often mirrors the pathological changes in brain metabolism, revealed a reduction in cell-free mtDNA in both AD and PD samples, suggesting that the levels of cell-free mtDNA may be used as biomarker for the early detection of both of these neurodegenerative diseases [129,130].

The majority of the data discussed here suggest that the decline in mitochondrial function observed in age-related disorders and ageing correlates with a progressive reduction in mtDNA copy number. Even if it is completely plausible that a gradual loss of functional mtDNA may have harmful effects on the brain, the results of these investigations might be deeply biased by the quality and the composition of the specimens (see further discussion below). Therefore, the possible

existence of a causal relationship between mtDNA levels and age-related disorders needs further experimental validation.

mtDNA variation in human cancer

Historically, the existence of a relationship between mitochondrial metabolism and cancer was first proposed by Otto Warburg in the early 1920s based on his observation that cancer cells can fulfil their energetic needs almost exclusively through aerobic glycolysis [131]. Warburg postulated that an impairment in mitochondrial respiration could drive tumorigenesis. This view became a milestone in cancer research but gradually faded away when the focus was shifted to oncogenes and the regulation of the cell cycle. However, during the last decades new evidence suggests important roles for mitochondria in cancer initiation and progression [132]. A recent *in vivo* characterisation of metabolic needs in tumours showed for instance that the conversion of pyruvate to lactate (aerobic glycolysis) was not essential for melanoma tumour growth [133]. Similarly, lung adenocarcinomas displayed high dependency on substrates for glucose oxidation and the tricarboxylic acid cycle to harvest

energy [134] and were also reported to retain a high mitochondrial membrane potential [135]. In contrast, in the intestine, the ablation of the mitochondrial pyruvate carrier was sufficient to initiate tumour formation [136]. Therefore, the extent of the contribution of mitochondrial metabolism to tumour onset and progression is heterogeneous and it is likely to depend on both the cancer type and the tumour stage.

Mutations in the mtDNA can be used as a measure of mitochondrial fitness, and the accumulation of mtDNA mutations is well known to occur in multiple cancers [137,138]. Several groups have characterised the mutational pattern of tumours by exploiting the recent high availability of NGS and WGS data [139–142]. Although mutations in mtDNA seem to accumulate in virtually all cancer types tested [139,142], the pathophysiological relevance, that is whether mtDNA mutations are causative or simply by-products generated by rapid mtDNA replication in fast-dividing cancer cells, is typically unclear. Mathematical models suggest that mtDNA mutations may accumulate due to the expansion of pre-existing heteroplasmic mutations or polymorphisms and that these undergo passive clonal expansion during the multiple cell divisions in tumours [143]. There are also other arguments against a positive role for mtDNA mutations in tumour development because mtDNA mutations that truncate proteins are counterselected in the majority of tumours [142,144,145], with the exception of kidney, and colorectal and thyroid cancers [142]. Interestingly, in these tumours the levels of mtDNA were found upregulated, suggesting a compensatory mechanism to sustain mitochondrial function [142]. The study also assessed the mutational landscape of over 2000 cancer patients and identified a strong strand bias for mtDNA mutations, which strongly argues for a replication-related mechanism in the generation of mutations [142]. A likely scenario for mtDNA mutations accumulation in tumours is that pre-existing mutations or mutations created by POLγ replication errors can clonally expand to high levels in fast-dividing cancer cells and become fixed in tumour subpopulations. Oncocytomas may represent an interesting confirmation of this hypothesis, as they are characterised by high levels of mtDNA mutations that result in a strong OXPHOS dysfunction and a compensatory increase in mitochondrial mass. Although pre-existing mtDNA mutations may drive oncocytoma formation, these tumours are typically benign lesions, characterised by low-invasiveness and a non-aggressive phenotype [146–149]. Mitochondrial dysfunction might represent a major boundary to cancer progression, but it is also possible that the accumulation of mtDNA mutations in some types of cancer provides a selective advantage to transformed cells and that mtDNA mutations thereby directly contribute to cancer development. Along these lines, a recent report has shown that metabolic alterations induced by mtDNA mutations may facilitate tumour formation in colon [150].

Besides the mutation load, mtDNA abundance is an important indicator of the reliance of a cell on OXPHOS. However, not many groups have looked at these two parameters at the same time and most studies only explore one of the two aspects. The levels of mtDNA have been associated with cancer, for either diagnostic or prognostic purposes. For diagnostic purposes, many attempts were directed towards finding a correlation between mtDNA copy number and disease onset, pursuing the idea that mtDNA levels in blood could be a marker for cancer risk. High mtDNA levels in blood were reported to correlate with an increased risk of developing lymphomas [151], breast [152,153], skin [154], lung [155] and pancreatic tumours [156], whereas other studies reported a protective effect of high mtDNA copy number against bone, kidney and other cancer types [157]. The risk of developing colorectal carcinoma was associated with both higher [158] or lower [159] amount of mtDNA in peripheral blood.

The levels of mtDNA have also been assessed as a prognostic factor by comparing matched normal and tumour tissue samples in several cancer types. The data from such studies, summarised in Table 3, are highly heterogeneous, and the reports suggest that both increased and decreased mtDNA levels correlate with disease severity. Colorectal carcinoma progression was associated with both an increase [160,161] and a decrease [162–164] in mtDNA copy number. Similar findings were reported for lung, and gastric and renal cell carcinomas, where some research groups found a correlation between high mtDNA levels and disease severity [142,165,166], whereas some earlier studies found the inverse relationship [163]. Data addressing multiple cancer types are not really clarifying the situation, as some tumours seem to upregulate mtDNA levels, including lymphomas, pancreatic, thyroid [142] and prostate cancers [141,142] and renal oncocytomas [147], whereas tumours affecting the breast [166–169], the brain [170], bones [171], the oral tract [166] and the liver [142,166,172] have been associated with a reduction in mtDNA levels.

Generally, it is important to be careful when comparing data from different sources as it is possible that at least part of the above discrepancies could be explained by methodological and study design biases

Table 3. mtDNA copy number variation in cancer.

Tissue affected	Sample type	MtDNA levels	Cancer risk	Quantification method	References
Lymphocytes	Peripheral blood lymphocytes	Up	Increased risk	Meta-analysis of literature search (qPCR)	[151]
Bone	Peripheral blood lymphocytes	Up	Decreased risk	Meta-analysis of literature search (qPCR)	[151]
Brain (glioma)	Blood	Up	Increased risk	qPCR	[248]
Breast	Blood	Up	Increased risk	qPCR; qPCR	[152,153]
Colon/rectum	Peripheral blood lymphocytes	Down	Increased risk	qPCR	[159]
	Peripheral blood lymphocytes	Up; down	Increased risk	qPCR	[158]
Kidney	Peripheral blood lymphocytes	Down	Increased risk	qPCR	[157]
Lung	Blood	Up	Increased risk	qPCR	[155]
Pancreas	Blood	Up	Increased risk	qPCR	[156]
Skin	Blood	Up	Increased risk	qPCR	[154]

Tissue affected	Sample type (matched)	mtDNA levels	Disease severity	Quantification method	References
Bladder	Bladder	Down	Increased	WGS and WES	[166]
Bone	Ewing sarcoma	Down	Increased	qPCR	[171]
Brain	Glioma	Down	Increased	qPCR	[170]
Breast	Primary breast tumours	Down	Increased	WGS; Other[a]qPCR; WGS and WES	[166–169]
Colon/rectum	Colorectal carcinoma	Up	Increased	qPCR; qPCR; qPCR	[160–162]
	Colorectal adenoma	Up	Increased	qPCR	[164,249]
	Advanced colorectal carcinoma	Down	Increased	qPCR; qPCR	[163,164]
Head and neck	Squamous cells carcinoma	Down	Increased	WGS and WES	[166]
Kidney	Chromophobe renal cell carcinoma	Up	Increased	WGS and WES	[165]
	Renal carcinoma (TCGA)	Down	Decreased	WGS; WGS and WES	[142,166]
	Renal oncocytomas	Up	Decreased[c]	WES; qPCR	[147]
Liver	Hepatocellular carcinoma	Down	Increased	qPCR; WGS and WES; WGS	[142,166,172]
Lung	Non-small cell lung cancer	Down	Increased	Other[b]; qPCR	[249,250]
	Adenocarcinoma, small cells lung cancer	Up	Increased	qPCR; WGS and WES; WGS	[142,166,251]
Lymphocytes	Lymphocytic leukaemia	Up	Increased	WGS	[142]
Oral / digestive tract	Oesophagus	Down	Increased	WGS and WES	[166]
	Stomach	Down	Increased	Other[b]	[252]
		Up	Increased	qPCR	[253]
Pancreas	Pancreas tumour (endocrine)	Up	Increased	WGS	[142]
Prostate	Primary prostate cancer	Up	Increased	qPCR; WGS	[141,142]
Thyroid	Adenocarcinoma	Up	Increased	WGS	[142]

[a]Microarray data.; [b]Competitive PCR method.; [c]mtDNA presents high levels of mutations.

(see section below). Moreover, the composition of the samples from different tumour types and stages could well explain the differences reported by various research groups. In this respect, recent analyses of thousands of available NGS/WGS data from cancer patients with specific focus on mtDNA may unravel more robust correlations between mtDNA and onset and progression of different tumour types. For future studies along these lines, it is of crucial importance to simultaneously assess both mtDNA mutations and mtDNA copy number and to associate these parameters with assessment of mtDNA expression and OXPHOS capacity.

The challenges of the precise quantification of mtDNA in humans

An extensive review of the literature finds many attempts aimed at understanding the role of mtDNA copy number in human disease. These studies are correlative and descriptive in their nature and often limited by methodology, sample selection and study design. Herein, we discuss some potential issues that must be considered when interpreting the available literature and when designing novel studies.

Firstly, methodological bias can contribute to the variability emerging among studies. The accuracy of the measurements can be much influenced by DNA

extraction methods. Traditional approaches based either on organic solvent extraction (phenol–phenol–chloroform–isoamyl alcohol) or on silica-based solid-phase columns (DNA extraction kits) can influence the mtDNA:nuclear DNA ratio and can affect the experimental outcomes [84,173]. Likewise, different quantification methods, as discussed above in the section quantification of mtDNA copy number, may well account for differences between studies, laboratories or even among batches in the same study. The NGS technologies allow sensitive and high-throughput assessment of mtDNA levels in big data sets; thus, they will likely be more and more employed in the near future to overcome some of the methodological limitations.

Secondly, specimen heterogeneity represents a major constraint to reproducibility, as it is well known that mtDNA copy number often varies significantly between different cell types, and therefore, the cellular composition of the investigated tissue should be a main concern. For instance, the use of blood samples has obvious advantages, including the minimally invasive sampling procedure and the low cost. However, mtDNA levels in blood do not necessarily reflect levels in other tissues and the measurements can be strongly biased by differences in blood cell composition. Platelets are known to have high mtDNA content compared with white blood cells, and they also lack the nuclear genome. As consequence, mtDNA content can be overestimated in blood samples where platelets are more abundant, and mtDNA levels should be hence normalised to the platelets/leucocyte ratio [174,175].

Thirdly, the cell-type composition of a given tissue can change due to pathology or ageing. For example, in age-associated forms of neurodegeneration, the loss of certain neuronal populations is accompanied by reactive gliosis [176]. Thus, neurons will likely be underrepresented in tissue homogenates from patients with neurodegeneration. The naturally occurring ageing process can also severely influence tissue composition due to fibrotic remodelling of the heart or nerve cell loss in the brain. Similar problems are evident in cancer, because tumour tissues typically contain multiple cell types besides the cancer cells, for example fibroblasts and endothelial cells. To circumnavigate the heterogeneity bias, significant attempts to isolate specific cell types have been made with a certain degree of success by using laser-capture microdissection in defined organs. However, the availability and time engagement of such technology remain serious limiting factors for its application to a large number of samples.

Finally, it is important to point out that the study design has a strong impact on reliability. Genetic and environmental factors, such as age, gender and lifestyle, must be considered when selecting the study cohort. For instance, gender differences are important and it has been reported that females have higher mtDNA content than males [112,116]. Although most studies aim to use matched control groups, this is not always trivial, as in the case of studies addressing ageing, where controls are by definition nonaged individuals. Similar caution should be used for individuals undergoing long-term drug treatments, for example chemotherapy, which may well influence mtDNA levels. Likewise, the lifestyles should be taken into account, as regular endurance training is known to induce an adaptive metabolic response in skeletal muscle that leads to a general increase in mitochondrial mass and mtDNA [177,178]. Finally, another important limitation of many clinical studies is that they are by necessity mostly retrospective and do not include a longitudinal follow-up. Hence, their outcomes are correlative and must be validated by experimental investigations in animal models in order to establish cause-and-effect relationships.

Therapeutic approaches exploiting the modulation of mitochondrial fitness

As discussed above, there is good evidence that the absolute mtDNA levels can be a critical factor in human pathology and ageing. This has led to the development of strategies that directly or indirectly manipulate mtDNA levels to ameliorate or prevent disease progression. In essence, two strategies have been engaged to increase OXPHOS capacity and restore mitochondrial function, which employed either the manipulation of total mitochondrial mass or the selective manipulation of mtDNA to influence mtDNA copy number and/or heteroplasmy levels (Fig. 2). Although these therapeutic approaches have been mostly focused on treating primary mitochondrial disorders, if human trials prove successful, they might also be extended to treat various types of common age-associated human diseases. Here, we briefly describe the rationale for each strategy and discuss applications, benefits and limitations.

Manipulation of mitochondrial mass: autophagy

The selective degradation of impaired mitochondria certainly represents an appealing approach that could have the power of shifting the balance in favour of the functional mitochondria. Therefore, attempts to promote the mitochondrial turnover have been carried out by stimulation of autophagy, the cellular process
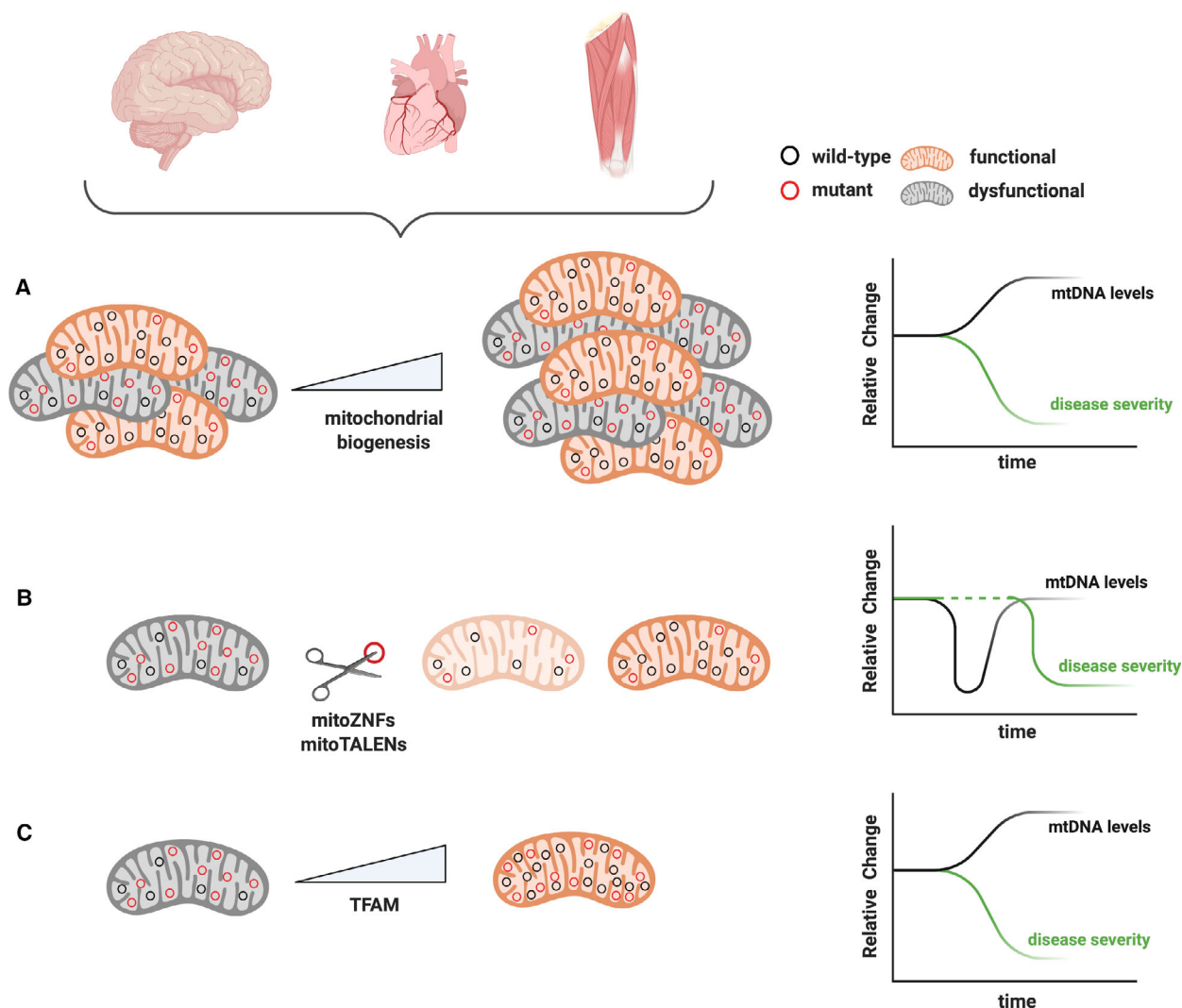
Fig. 2. Therapeutic strategies to manipulate mtDNA and disease severity. (A) Boosting mitochondrial biogenesis represents an unspecific approach to decrease disease severity. With this approach, a general increase in all mitochondrial components, as well as mtDNA levels, is accomplished in order to rescue biochemical OXPHOS defects. (B) Mitochondrially directed ZNFs and TALENs selectively cut mtDNA in a sequence-specific way. Mutant mtDNA is cleaved and the resulting linear molecules are quickly degraded generating a transient mtDNA reduction in the cell, which is restored by replication of the residual (wild-type) mtDNA. (C) A moderate and selective increase in mtDNA copy number can be achieved by modulating TFAM levels. By doing so, mitochondrial function can be partially restored due to absolute increase of the functional mtDNA copies. Schematic representations of mtDNA levels (black lines) and their correlation with disease severity (green lines) are represented for each approach on the right-hand side panels.

whereby cells can self-degrade and recycle different cellular components, including mitochondria [179]. Stimulation of bulk autophagy has been pursued by the inhibition of the mammalian target of rapamycin complex 1 (mTORC1), a master regulator of nutrient sensing and metabolism [180]. Inhibition of mTORC1 by using rapamycin improved mitochondrial function in some mouse models, but had no effect in others [181–183]. This may be explained by the complexity of mTORC1 pathway, which engages many targets and

influences several cellular processes. In one study, it was reported that the beneficial effect of rapamycin treatment in a mouse model of the Leigh syndrome was related to the redirection of cellular metabolism towards amino acid catabolism, rather than through the activation of autophagy [183]. Thus, the therapeutic application of rapamycin and mTORC1 inhibitors might be strongly limited by possible off-target effects. More specific autophagy stimulators, such as urolithin A, extended life span in *Caenorhabditis elegans* and

increased muscle function of aged mice [184]. However, the mechanisms controlling bulk autophagy and the mitochondrial quality control are highly intricate and far from being understood, hence, their dysregulation might lead to an excessive mitochondrial clearance and potential long-term harmful consequences. Along this line, it has been recently reported that mutations in *FBXL4* in humans cause excessive mitochondrial turnover, which, at least partly, explains the severity of the disease in affected patients [185]. Therefore, the downstream effects of treatment strategies aiming to increase mitochondrial degradation must be carefully evaluated in preclinical studies before being safely translated into therapies for human diseases.

Manipulation of mitochondrial mass: boosting mitochondrial biogenesis

Increasing the overall amount of mitochondria by boosting mitochondrial biogenesis represents a valid rationale to counteract the bioenergetic defects caused by mutations in either mtDNA or the nuclear genome [186]. This hypothesis is supported by experimental evidence showing that an increase in mitochondrial mass was sufficient to restore the overall ATP production in skeletal muscle of mice with mitochondrial myopathy, although the individual mitochondria have a severely impaired function [187]. Mitochondrial biogenesis is a highly regulated physiological process controlled by both internal and external stimuli, such as exercise, calory intake, temperature, cell division and differentiation. The stimulation of mitochondrial biogenesis requires a synchronised expression of both nuclear and mtDNA-encoded gene products. The peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α) [188] is involved in making more mitochondria on demand and it interacts with several nuclear transcription factors, such as peroxisome proliferator-activated receptors (PPARs), to induce the expression of nucleus-encoded genes, including OXPHOS subunits [189,190], *Tfam* and other genes involved in mtDNA gene expression [191]. PGC1α is post-translationally activated *via* phosphorylation by the AMP-dependent kinase (AMPK) [192] and through deacetylation by the nuclear deacetylase Sirtuin 1 (Sirt1) [193].

Both genetic and pharmacological interventions have been used to target PGC1α [194]. Overexpression of PGC-1α ameliorated the myopathy phenotype in complex IV assembly-deficient mice [195] and partially rescued the premature ageing phenotypes in the mtDNA mutator mouse [196], consistent with the hypothesis that increased mitochondrial biogenesis can alleviate mitochondrial diseases severity. In support of this view, studies in families carrying homoplasmic LHON-causing mtDNA mutations have shown that asymptomatic carriers tend to have increased mitochondrial biogenesis and higher mtDNA copy number when compared with visually impaired maternal relatives [65].

PGC1α can also be activated by the pan-PPAR agonist bezafibrate, by the AMPK agonist 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and by Sirt1 activators and nicotinamide adenine dinucleotide (NAD)+ precursors, that is nicotinamide riboside (NR) and niacin. In spite of a few promising findings [197,198], mouse studies have shown that bezafibrate treatment had no effect or even a deleterious impact on mitochondrial dysfunction [195,199]. In contrast, the administration of AICAR or NR successfully rescued the pathological manifestations in different mouse models of mitochondrial diseases [195,200]. Mitochondrial biogenesis boosters, such as bezafibrate, NR and niacin, have also been tested in human trials. A clinical trial with bezafibrate was not very encouraging and suggested that the prolonged use of this drug may worsen the metabolic signature of mitochondrial dysfunction in patients [201]. A recently published study with niacin supplementation was more promising and revealed increased mitochondrial biogenesis and improved muscle strength in patients with mitochondrial myopathy and NAD$^+$ deficiency [202]. It should be pointed out that none of these clinical trials have yet been performed in the more rigorous double-blind format.

Boosting mitochondrial biogenesis may provide a therapeutic strategy to treat mitochondrial diseases. Unfortunately, pharmacological interventions have produced mixed results and raised questions about the consequences of long-term treatments [201]. PGC-1α seems to be involved in the regulation a plethora of cellular processes by influencing the expression of a large number genes involved in metabolic pathways, such as gluconeogenesis, fatty acid synthesis and oxidation and glycolysis [203], and its activation must be finely tuned to avoid deleterious side effects. Indeed, detrimental impacts on cell physiology due to the over-activation of PGC-1α have been reported to occur in different organs of the mouse, for example heart [204], skeletal muscle [205] and DA neurons [206]. In addition, several studies in mouse models have explored whether the overexpression of PGC-1α is a potential therapeutic strategy in PD, but the results of these investigations provided inconsistent and conflicting findings [207].

Selective manipulation of mtDNA: mitoTALENs and mitoZNFs

Currently, the most efficient strategy to shift the levels of heteroplasmy in favour of wild-type mtDNA is the expression of mitochondrially targeted endonucleases [208,209]. Unfortunately, the powerful CRISPR-Cas9 system, which has revolutionised nuclear genome editing, will likely not work in mammalian mitochondria. Although Cas9 can be easily imported after addition of a mitochondrial targeting sequence (MTS), the import of guide RNA into mitochondria seems improbable. RNA import occurs in some metazoans, but several lines of evidence suggest that mammalian mitochondria lack RNA import system. Importantly, mammalian mtDNA encodes the full set of tRNAs required for mitochondrial translation overcoming the need for tRNA import from the cytosol. Furthermore, mammalian mitochondrial RNaseP is composed of three protein subunits and lacks a catalytic RNA component [210,211]. Finally, the central protuberance of the large subunit of the mitochondrial ribosome contains a mtDNA-encoded tRNA, and there is therefore no need to import 5S rRNA from the cytosol for mitoribosomal biogenesis [212,213]. An additional hurdle, besides the lack of RNA import, preventing the use of the CRISPR-Cas9 system in mammalian mitochondria, is the lack of a machinery for double-strand break (DSB) repair and homologous recombination, whose activities are required to introduce a deletion or any other type of mutation after Cas9-mediated DNA cleavage.

The import into mitochondria of the transcription activator-like effector nucleases (mitoTALENs) and zinc finger nucleases (mitoZFNs) is straightforward, as well-characterised machineries for protein import are engaged. Manipulation of mtDNA with mitoTALENs or mitoZFNs exploits distinctive features of mitochondrial genetics: (a) heteroplasmic pathogenic mtDNA mutations are typically functionally recessive [214], (b) mitochondria lack a DSB repair machinery [215], but have an efficient mechanism for degradation of linear mtDNA molecules [216], and (c) the mtDNA copy number is tightly regulated and after digestion of mutant molecules, the cells quickly recover from depletion by stimulating the replication of the residual mtDNA copies [37]. The earliest attempts to degrade mtDNA in cells were based on the use of bacterial restriction endonucleases targeted to mitochondria (mitoREs) with the addition of an amino-terminal MTS. Once imported, the mitoREs shifted the levels of heteroplasmy both in cells [217,218] and in mice [219,220] by selectively binding and cleaving one of the

mtDNA variants. However, the use of these enzymes for a wider range of mutations was hampered by their need of a unique restriction site in the mtDNA sequence, which only rarely is generated by pathogenic variants, and by the fact that restriction endonucleases (REs) cannot be engineered to recognise chosen DNA sites. These limitations were overcome by the use of the programmable mitoTALENs and mitoZFNs. Unlike REs, these endonucleases rely on sequence-specific DNA-binding domains coupled to a *Fok*I endonuclease domain, which is active only as a dimer and creates a DSB when both monomers are bound to the target allele and interact. MitoTALENs and mitoZNFs have proved efficient in targeting numerous disease-causing mtDNA variants in cells, where they cleave mutant genomes and thereby rescue the biochemical defect [221–223]. The *in vivo* feasibility of these technologies was recently demonstrated in a heteroplasmic mouse model carrying a pathogenic point mutation in the mitochondrial tRNA^{Ala} gene [99]. In these mice, either mitoTALENs or mitoZFNs, delivered by injection of adeno-associated virus (AAV) vectors, shifted the heteroplasmy levels below the threshold leading to amelioration of the molecular phenotypes in heart and skeletal muscle [224,225]. These data represent important proof of concept that mitoZFNs and mtTALENs have the potential to serve as therapeutic tools for heteroplasmic mitochondrial diseases. It is important to mention that the possible success of this strategy depends on vectors that efficiently can target cells in brain, skeletal muscle, heart and other organs. Some relevant AAV vectors already exist, and this field is under rapid development giving hopes for more efficient targeting of the brain and other tissues and for potential translation into the clinic. Other issues to be addressed are common limitations for any gene therapy approach, that is selectivity, efficiency, cost and potential off-target effects. Lastly, it should be pointed out that the use of mitoTALENs and mitoZFNs is limited to heterosplamic mtDNA mutations, for example point mutations and large deletions, and cannot be used to treat some of the common homoplasmic pathogenic mutations, for example those causing LHON.

Selective manipulation of mtDNA: modulation of the absolute mtDNA levels

An increase in the absolute mtDNA copy number represents another possibility of treatment for human disease caused by heteroplasmic mtDNA mutations. In these pathological conditions, affected individuals always carry perfectly functional wild-type mtDNA

molecules, in spite of high levels of mutant mtDNA. It is widely recognised that the mutation load is an important determinant for disease severity, whereas the role of the absolute levels of mtDNA often has often been neglected. Over the last 15 years, only few studies have directly measured total mtDNA copy number in patients with pathogenic mtDNA mutations.

Although the molecular mechanisms underlying this regulation remain unclear, it is well established that the amount of mtDNA is directly proportional to TFAM protein levels (Box 1). As consequence, manipulation of mtDNA copy number can be genetically achieved through modulation of TFAM expression [17]. Notably, a moderate overexpression of human TFAM in wild-type mice caused nearly 50% increase in mtDNA levels, without interfering with mtDNA transcription, mitochondrial respiratory-chain function or mitochondrial mass [17]. Thus, the upregulation in mtDNA copy number can be dissociated from the mitochondrial biogenesis process, that is it is possible to selectively increase mtDNA copy number without increasing the amount of mitochondria.

Experimental studies have shown that an increase in mtDNA copy number, through TFAM overexpression, ameliorated phenotypes in mouse models with mitochondrial diseases caused by heteroplasmic mtDNA deletions [226] or a pathogenic point mutation in the tRNA^{ALA} gene [64]. These results were further supported by the observations that increasing total mtDNA levels partially rescued the male infertility in the mtDNA mutator mice, which carry a large number of different mtDNA point mutations and a linear deletion [67]. Remarkably, the results of these investigations also demonstrated that the increased mtDNA levels did not affect the proportion of pathogenic mtDNA mutations, that is the mutational load was unchanged. However, in absolute terms, the levels of wild-type mtDNA segments were higher, which partly restored mitochondrial function despite the mutant mtDNA still being the predominant species [64,67,226].

Other studies have reported beneficial effects of an increase in mtDNA copy number in various types of pathology, primarily not of mitochondrial origin, for example in a mouse model of myocardial infarction [227]. TFAM overexpression has also been reported to improve cognitive function in an AD mouse model [228] and to reduce the memory impairment seen in aged mice [229]. The modulation of mtDNA levels seems to be a more selective way to improve OXPHOS compared with boosting the whole process of mitochondrial biogenesis. However, the degree of TFAM expression must be carefully considered. We and others have shown that moderate TFAM

overexpression had beneficial effects on cellular functions *in vivo* [64,67,226]. In contrast, a marked TFAM overexpression can have detrimental consequences by impairing mtDNA transcription and causing a progressive OXPHOS dysfunction [230]. Similar negative outcomes have been reported in *S. cerevisiae* where high levels of ABF2, the yeast ortholog of TFAM, led to a dramatic loss of mtDNA, possibly due to hypercompaction of the genome preventing proper replication and distribution of genomes [231]. Modulation of mtDNA copy number through manipulation of TFAM levels may provide a future avenue to treat not only primary mitochondrial diseases but also to intervene in other human diseases characterised by mitochondrial impairment.

Concluding Remarks

Mitochondrial dysfunction is heavily implicated in variety of human pathological conditions and ageing, and it is therefore not surprising that a large number of studies have aimed to correlate the presence of mutations and the levels of mtDNA with decline of organ function. Although a key role for mtDNA mutations in inherited mitochondrial diseases is firmly established, the evidence for the involvement of mtDNA levels in common diseases and ageing remains merely correlative. In mitochondrial diseases, high mtDNA copy number mostly correlates with decreased disease severity, or even with incomplete disease penetrance [65,66]. In contrast, the interpretation of the existing data related to mtDNA variations in neurodegenerative disorders, cancer and ageing is not equally straightforward, partly due to method-, specimen- and study design-related issues. Recent advances in NGS enable assessment of mtDNA mutation load and copy number in large-scale data sets, which will much expand our knowledge about the impact of these factors on disease burden. Approaches exploiting the isolation and analysis of large numbers of selected cell types will also be required, for instance in studies on neurogenerative disorders, where dysfunctional cells driving the disease only represent a minority of the affected brain region.

In humans with mitochondrial diseases, the upregulation of mtDNA copy number, mostly concomitant with an overall increase in mitochondrial biogenesis, is frequently occurring and typically regarded as a compensatory mechanism to sustain cellular bioenergetics. Studies of animal models have provided an experimental proof of concept for selective increase in total mtDNA copy number induced by genetic manipulation of TFAM expression ameliorating pathogenic effects of heteroplasmic mtDNA mutations [64,67] and

the mitochondrial impairment associated with other diseases or with the physiological age-related decline [227–229]. Unfortunately, the same rationale might not be applicable to cancer, or at least not for all forms of cancer. The available studies have indeed highlighted that the scenario in cancer is even more complex and varied, as mtDNA copy number can correlate with both increased and decreased disease burden. Future studies will be necessary to more exactly define the impact of mtDNA copy number on human health. Importantly, future development of therapies aimed at moderately increasing mtDNA copy number may provide a strategy to rescue the mitochondrial dysfunction not only in primary mitochondrial diseases but also in age-associated degenerative diseases and ageing.

Acknowledgements

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