Endangered species: mitochondrial DNA loss as a mechanism of human disease

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1. ABSTRACT

Human mitochondrial DNA (mtDNA) is a small maternally inherited DNA, typically present in hundreds of copies in a single human cell. Thus, despite its small size, the mitochondrial genome plays a crucial role in the metabolic homeostasis of the cell. Our understanding of mtDNA genotype-phenotype relationships is derived largely from studies of the classical mitochondrial neuromuscular diseases, in which mutations of mtDNA lead to compromised mitochondrial bioenergetic function, with devastating pathological consequences. Emerging research suggests that *loss,* rather than *mutation,* of mtDNA plays a major role across a range of prevalent human diseases, including diabetes mellitus, cardiovascular disease, and aging. Here, we examine the 'rules' of mitochondrial genetics and function, the clinical settings in which loss of mtDNA is an emerging pathogenic mechanism, and explore mtDNA damage and its consequences for the organellar network and cell at large. As extranuclear genetic material arrayed throughout the cell to support metabolism, mtDNA is increasingly implicated in a host of disease conditions, opening a range of exciting questions regarding mtDNA and its role in cellular homeostasis.

2. mtDNA: COMPOSITION, COPY NUMBER AND ORGANIZATION

While the vast majority of the thousands of proteins present in mitochondria are encoded by nuclear genes, a small, yet crucial, number are produced from mitochondrial DNA (mtDNA). Human mtDNA is a small, circular 16,569 bp DNA (1) encoding 13 polypeptides, 2 ribosomal RNAs, and 22 tRNAs that are essential to the assembly and function of the mitochondrial respiratory chain, responsible for oxidative phosphorylation (2). Rapidly-dividing cells such as HeLas and fibroblasts typically have 1,000-2,000 copies per cell (3), while more energetically-demanding tissues such as myocardium and skeletal muscle have higher mtDNA content per diploid nuclear genome, consistent with their heightened mitochondrial content and energetic demand (4). Due to this high copy number, mtDNA comprises ~1% of all total cellular DNA, despite its small genomic size. In accordance with its endosymbiontic origin (5), mtDNA's organization and genetics are very different from chromosomal DNA. Its circular structure, maintenance within a double membrane-bound organelle, and distinct genetic code are attributes that echo its prokaryotic background, while the abundance of mtDNA within the cell indicates that mitochondrial genotype-phenotype relationships are really 'intracellular population' genetics, rather than Mendelian inheritance patterns. Taken as a whole, then, mtDNA is crucial cellular genetic content with a very different set of 'rules' than chromosomal nuclear DNA.

The vast majority of ATP production in a mammalian cell occurs via oxidative phosphorylation
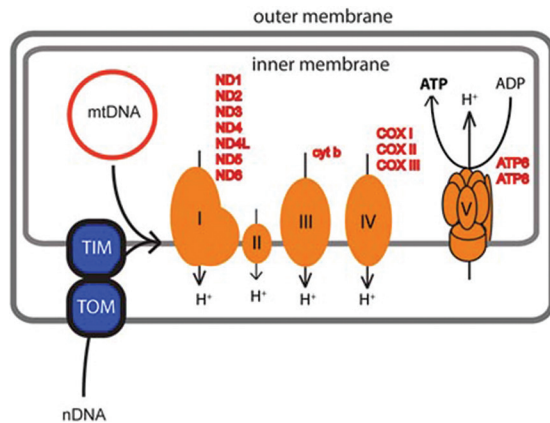
Figure 1. Nuclear and mitochondrial genomes contribute to the biogenesis of the OxPhos complexes. Complexes I-V of the OxPhos chain are composed of protein subunits derived from both mtDNA and chromosomal nuclear DNA. Nuclear-encoded polypeptides are targeted to the mitochondria and imported via the translocase of the outer mitochondrial membrane (TOM) complex. MtDNA (red circle) encodes 13 polypeptide subunits of the OxPhos complexes. The translocase of the inner mitochondrial membrane (16) proteins work to combine both nuclear- and mtDNA-encoded polypeptides into functional complexes which are assembled and inserted into the mitochondrial inner membrane. With the exception of Complex II, each of the OxPhos complexes contains at least one mtDNA-encoded polypeptide subunits (indicated in red next to each complex). The presence of the mtDNA-encoded subunits is essential to the full assembly and functioning of these complexes in mitochondrial ATP production.

(OxPhos) in the mitochondrial inner membrane. The OxPhos system is composed of five multi-subunit complexes and two electron carriers (6). The five complexes are embedded within the inner membrane and consist of complexes I- IV and complex V, the ATP synthase. Complexes I, II, III, and IV utilize NADH and FADH$_2$, the energy-rich molecules derived from the citric acid cycle, to pump protons out of the matrix, generating a proton-motive transmembrane potential ($\Delta\psi_m$) across the mitochondrial membrane. This gradient is used by Complex V, the F_1F_0 ATP synthase, which permits a single H$^+$ to return to the matrix, thus driving the synthesis of ATP from ADP and P$_i$ (6). Strikingly, while the mitochondrial proteome is comprised of thousands of proteins (7), only 13 are encoded by mtDNA. The polypeptides encoded by mtDNA are all subunit components of the OxPhos system, and are in fact essential to the proper assembly and functioning of these complexes in mitochondrial bioenergetics. While nuclear-encoded subunits are synthesized on cytoplasmic ribosomes and targeted for mitochondrial import via the mitochondrial outer membrane protein translocase (TOM) and mitochondrial inner membrane protein translocase complexes (8), mtDNA-encoded OxPhos subunits are produced at the mitochondrial ribosome, combined with the nDNA-encoded subunits, and inserted into the inner membrane (Figure 1). Thus, mitochondrial ATP production is dependent on contributions from both chromosomal and mitochondrial

genomes. As such, defects in mtDNA-encoded subunits result in incomplete OxPhos complex assembly, causing mitochondrial dysfunction. Loss of mtDNA content is emerging as a common form of mitochondrial dysfunction across a range of highly prevalent human pathologies.

3. LOSS OF mtDNA ACROSS HUMAN DISEASE

Consistent with the central role of mitochondria in cellular metabolism, particularly in energetically demanding tissues, mitochondrial dysfunction has long been associated with pervasive human conditions such as diabetes, cardiovascular disease, and aging. Despite an abundance of correlative data, however, it has remained mechanistically unclear how mitochondria are defective in these prevalent conditions. Recently, *loss* of mtDNA content, rather than inherited or sporadic *mutation* of mtDNA, has emerged as a pervasive driving force in the pathogenesis of these prevalent degenerative conditions. Loss of mtDNA represents a 'sleeper' mitochondrial genetic defect, which has until recently gone unnoticed as a mediator of mitochondrial dysfunction in conditions affecting large portions of the global population.

3.1. Diabetes and metabolic disease

Given the shockingly high prevalence of diabetes in the U.S. (6.4.% of Americans, Centers for Disease Control), as well as the extensive co-morbid overlap of diabetes with cardiovascular disease and obesity, a massive amount of effort has been directed at determining the factors that drive insulin resistance and the pathogenesis of Type 2 diabetes. Increased inflammation has emerged as a major causative mechanism of insulin resistance in Type 2 diabetes, stemming from the seminal observations of Hotamisligil *et al*. that tumor necrosis factor alpha (TNF-α) is expressed at high levels in mouse models of both obesity and diabetes (9). Pro-inflammatory cytokines including TNF-α and IL-6 circulate in the blood and activate inflammation, thereby inducing insulin resistance (9-11). Elevated inflammation, mediated by increased secretion of adipose-expressed cytokines, combines with lipid metabolism and changes in gut biota to mediate insulin resistance (12).

Mitochondria appear to be a major intracellular target of cytokine-mediated inflammation, providing a mechanism to explain a long history of data correlating mitochondrial dysfunction with diabetes and metabolic disease. Decreased mitochondrial content and function have been shown in skeletal muscle from Type 2 diabetics (13), while genomic and expression profile studies have found broad decreases in mitochondrial (both nuclear- and mtDNA-derived) gene expression in Type 2 diabetes (14). Patients with pathogenic mtDNA mutations frequently present with maternally inherited diabetes (15) and insulin resistance (16), demonstrating

that mtDNA-mediated mitochondrial dysfunction can be causative in the pathogenesis of insulin resistance. Consistent with this, mtDNA is normally present at ~1,000 copies per cell (17), but is significantly decreased in blood (18,19), skeletal muscle (13,19,20), and adipose tissue (20,21) of Type 2 diabetics, as well as in blood of pre-diabetics (22). Collectively, these studies indicate a broad decrease in mtDNA copy number in diabetics, and suggest that cytokine-mediated mtDNA damage plays a strong role in insulin resistance.

3.2. Cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of death worldwide (23). In the U.S., 11.8.% of adults have been diagnosed with heart disease (National Center for Health Statistics, 2010), and is the cause of one in four deaths (24). As in diabetes, mitochondrial dysfunction has long been implicated in the pathogenesis of cardiovascular disease. Patients with end-stage heart failure have broad decreases in OxPhos activity (25), regardless of whether they are afflicted with dilated, hypertrophic, or ischemic cardiomyopathy (26). Decreased OxPhos enzymatic function and mitochondrial biogenesis are found in rodent hypertension models (27). The myocardium has the highest mitochondrial content of any human tissue due to the enormous bioenergetic demands of continuous cardiac muscle contraction (28). As such, it is logical that mitochondrial dysfunction should be highly correlated with cardiac disease. But how?

Recent findings strongly suggest that mitochondrial dysfunction in cardiovascular disease is frequently mediated by loss of mtDNA content. *In vivo* models of cardiac-specific mtDNA depletion display massive apoptosis (29), while models of myocardial infarction (30) and high fat diet (31), as well as patients with end-stage heart failure (32, 33) show dramatic decreases in cardiac mtDNA content. Cardiovascular disease has strong co-morbidity with diabetes, as diabetics are twice as likely to die of heart disease relative to non-diabetics (34). Many of the mechanisms of mitochondrial dysfunction found in diabetes are likely to result in cardiac mitochondrial damage as well, causing or contributing to the co-morbidity of these two prevalent human conditions. These combined basic and clinical findings suggest strongly that damage to mtDNA and the concomitant mitochondrial dysfunction plays a profound role in the 'vicious cycle' of cardiovascular damage.

3.3. Aging

Diminished mitochondrial function and oxidative capacity have long been appreciated as one of the leading manifestations of cellular aging. Denham Harman first proposed that oxidative radicals damage vital cellular processes in the free radical theory of aging (35,36), which he later amended to postulate that free radicals, a by-product of oxidative phosphorylation, directly damage mitochondrial lipids, proteins, and mtDNA, thus leading to metabolic dysfunction and diminished cellular lifespan (37). Experimental findings have revealed that oxidants, alkylating agents, and γ-irradiation damage mtDNA more frequently than nuclear DNA (35,38,39). Whole-organism studies reveal age-related decreases in mtDNA copy number and mitochondrial function in skeletal muscle, liver, and heart (40,41). These model system studies are in agreement with findings of decreased mtDNA copy number in human aging across a range of disparate tissues, including pancreatic beta cells (42) and skeletal muscle (43), as well as age-related decreases in mitochondrial protein synthesis and bioenergetic function in skeletal muscle (44).

Taken together, these studies show *loss* of mtDNA, rather than *mutation*, represents a 'sleeper' form of mitochondrial damage that appears to be much more widespread than previously appreciated, and is a likely underlying mechanism of mitochondrial involvement in aging and other prevalent human diseases. The 'ground rules' of mtDNA genotype-phenotype relationships observed in the classical mitochondrial neuromuscular diseases provide crucial insights into mechanisms by which loss of mtDNA may negatively impact mitochondrial function, organization, and the homeostasis of the cell at large.

4. mtDNA MUTATIONS: HETEROPLASMY THRESHOLD EFFECTS, AND BIOENERGETIC FUNCTION

MtDNA, although small, is crucial to the ability of the organelle to produce ATP. The first pathogenic mtDNA mutations demonstrated this in the pathology of the classical mitochondrial neuromuscular diseases. As a result, much of our current understanding of mtDNA genotype-phenotype relationships is derived from studying diseases such as Kearns-Sayre Syndrome and Leigh disease, providing key concepts to apply towards understanding the contribution of mtDNA to the pathogenesis of conditions such as diabetes and cardiovascular disease.

4.1. MtDNA mutations

The first pathogenic mtDNA mutations were first revealed in 1988, when it was shown that a single nucleotide change at nt11178 caused Leber's Hereditary Optic Neuropathy (LHON) (45). Concurrently, Holt *et al*. showed that patients with mitochondrial myopathy carried mtDNAs that had deleted regions of ~7 kb, resulting in a smaller, ~9 kb circular mtDNA (46). These studies revealed the two major classes of pathogenic mtDNA mutations: point mutations (either in protein-coding or tRNA-coding regions) or large-scale deletions (Δ-mtDNAs). This opened the door to further exploration of the mitochondrial genome in neuromuscular disease, which to date have described 260 pathogenic mutations and 120 rearrangements of the mitochondrial genome.

Point mutations in protein-coding regions result in loss of function only to the individual complex containing the mutated polypeptide. For example, the T8993G point mutation in *ATP6* causes defective activity of the F_1F_0 ATP synthase, but leaves complexes I-IV unaffected. Conversely, point mutations of mitochondrial tRNAs, as well as large-scale deletions encompassing one or more mtDNA-encoded tRNAs, result in global defects in production of mtDNA-encoded polypeptides, crippling the OxPhos machinery as a whole. For example, the A3243G point mutation in *tRNA^Leu* causes MELAS, while Kearns-Sayre Syndrome (KSS) is caused by Δ-mtDNAs, in which a large (usually several kb) region is missing, causing a smaller circular mtDNA. While the size and exact breakpoints of Δ-mtDNAs are highly variable, any deletion that eliminates a tRNA causes the same inability to produce mtDNA-encoded polypeptides.

4.2. Heteroplasmy and threshold

Mutations of these types have devastating effects on energetically demanding tissues such as skeletal muscle, nervous tissue, heart, and liver. Heteroplasmy, the presence of both wildtype (WT) and mutant mtDNAs together in the same organelle, cell, and tissue, is a hallmark of the classical mitochondrial neuromuscular disorders, and is a critical determinant of mitochondrial function (2). Specifically, the relative proportion of WT versus mutant mtDNA determines whether or not mitochondria are functional in heteroplasmic cells (the mitochondrial threshold effect) (47). While each cell carries ~1,000 copies of mtDNA, studies of mtDNA heteroplasmy reveal that well-defined thresholds exist for the WT mtDNA content required for function. The threshold ratio of WT mtDNA and Δ-mtDNA is of extreme importance determining mitochondrial function. Cells usually can withstand a high proportion of mutant mtDNA, but when the proportion of mutant mtDNA surpasses the threshold, OxPhos defects occur. For example, the overall mutation load for the 8993 T>G mtDNA mutation must exceed 95% before mitochondrial function is compromised, causing Leigh disease pathology (48). For Δ-mtDNAs, the threshold for loss of function is lower: the proportion of Δ-mtDNA must typically exceed 80% of total mtDNA before mitochondrial function is compromised in KSS patients (49-51). Thus, in both point mutations and Δ-mtDNAs, a relatively small overall proportion of WT mtDNA content is required to maintain normal OxPhos function.

4.3. MtDNA depletion syndromes

In addition to neuromuscular diseases caused by mutations of mtDNA, a major category of mitochondrial disease is caused by mutations in nuclear genes encoding factors involved in mtDNA maintenance and assembly of mitochondrial protein complexes. These conditions cause loss of mtDNA, leading to similar diverse phenotypes of ptosis, exercise intolerance, optic pathology, and diabetes. Mutations in genes encoding mitochondrial polymerase gamma (POLG), the Twinkle mitochondrial helicase, and thymidine kinase 2 (TK2) have been shown to cause loss of mtDNA content, leading to disease phenotypes similar to those found in patients with mutations of mtDNA (52,53). Cell-level studies recapitulate these findings, as cells depleted of all mtDNA (ρ^0 cells) have similar fragmented morphology, loss of $\Delta\psi_m$, and loss of bioenergetic function as cells carrying Δ-mtDNAs (54,55), suggesting that a similar threshold may exist for the loss of mtDNA. However, it remains unclear what the threshold is for mitochondrial function in cells undergoing *loss,* rather than *mutation*, of mtDNA.

5. MECHANISMS OF mtDNA DAMAGE AND CELL-WIDE EFFECTS

Crucially, pathogenic mtDNA mutations cause the classical mitochondrial diseases, which affect ~1 in 5,000 individuals (56). These diseases, caused by rare inherited or sporadic mtDNA mutations, have systemic and tissue-specific pathologies including maternally inherited diabetes and heart block, demonstrating a causative role for mtDNA-derived mitochondrial dysfunction in human disease. Mutations of mtDNA are an established causal mechanism of disease, yet are not highly prevalent (although less rare than previously thought, at 1:200 (57)).

The free radical theory of mitochondrial damage has frequently been interpreted to mean that oxidative stress causes an accumulation of different base-change mtDNA mutations, leading to diminished mitochondrial function. Richter *et al*. showed that gamma irradiation and oxidant agents induce 8-hydroxydeoxyguanosine lesions in both mtDNA and nDNA, but at a higher frequency in mtDNA (58). Transgenic mice with an exogenous mitochondrial polymerase gamma (POLG) mutation resulting in an increased error rate produce high rates of mutation and concomitant acceleration of aging (53,59). However, mtDNA genotype studies show that a single pathogenic mtDNA mutation must comprise a majority of the cell's mtDNA population before function is lost: a single oxidatively-introduced base change mutation would need to be preferentially propagated and comprise ~60-90% of total cellular mtDNA before mitochondrial dysfunction would be negatively affected. The mitochondrial threshold effect has been consistently demonstrated in human neuromuscular disorders. Δ-mtDNA's require a 60-80% threshold before mitochondrial function is compromised, while for point mutations, the threshold level is even higher (90% or greater!) (49). Critically, Shokolenko *et al*. found that there was no significant difference in base-change mutations in mtDNA from young versus old individuals (60). The notable exception to this is the clonal expansion of Δ-mtDNA species in the substantia nigra (61,62) and choroid plexus of the aging brain (63). Again, however, it must be noted that these examples
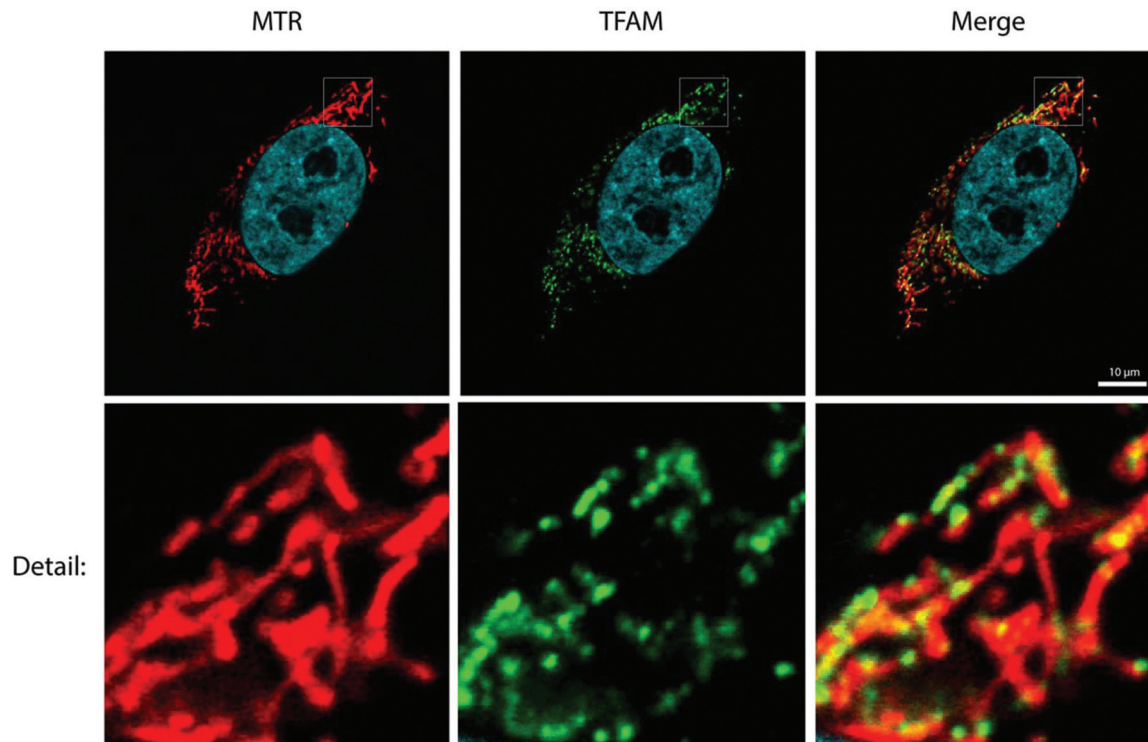
Figure 2. MtDNA nucleoids are distributed throughout the mitochondrial network. Confocal microscopy of 143B osteosarcoma cells stained for mitochondria with MitoTracker (red) and immunolabeled for the mtDNA-binding protein TFAM (green). Nuclei were visualized with DAPI (blue).

are a single Δ-mtDNA expanding over time via replicative advantage, due to their smaller size (64), rather than an accumulation of many disparate mutations.

Thus, while mitochondrial damage has been extensively demonstrated in aging and disease, the accumulation of oxidative mtDNA base-change mutations faces major conceptual hurdles as a general mechanism of mitochondrial dysfunction in aging and disease. Alternatively, damage and loss of mtDNA copy number appears to be a major contributor to metabolic dysfunction in a wide range of human diseases. While individual point mutations must clonally accumulate to very high levels to produce mitochondrial dysfunction, damage that results in strand breakage and loss of mtDNA may have rapid, negative impacts on mitochondrial bioenergetic capacity. In the following section, we explore the macromolecular packaging of mtDNA and mechanisms of damage, as well as the organellar impacts and cell-wide consequences of decreased mtDNA content.

In another similarity to prokaryotic genetic organization, mtDNA is maintained in DNA-protein complexes called nucleoids. MtDNA is present at ~1,000 copies per cell. Mitochondrial nucleoids package one or more mtDNA molecules into punctate assemblies through interaction with a variety of DNA-binding and accessory proteins, providing an efficient integration of replication, transcription, and translation, while simultaneously ensuring that mtDNA is distributed throughout the mitochondrial network of the cell. The mitochondrial nucleoid is thought to be composed of both 'core' and 'peripheral' components. Chief among the core components is Transcription Factor A, Mitochondrial (TFAM), which, although originally identified as a transcription factor, is equally invaluable as an mtDNA-packaging protein. TFAM has been shown to actively bind DNA (65) and accurately mirrors the abundance of mtDNA within the cell. Thus, while mtDNA has traditionally been described as naked due to its high mutation rate (relative to nuclear DNA), mtDNA is highly packaged by TFAM, with ~100 TFAM molecules binding per mtDNA molecule (66). Other core nucleoid components include the mitochondrial polymerase γ (POLG), the helicase Twinkle (17), and the Lon protease, which appears to regulate mtDNA copy number by proteolytically modulating TFAM content (67). Peripheral nucleoid factors are more transiently associated; several of these (including prohibitin and ATAD3) have major signaling functions elsewhere in the cell and may mediate mtDNA's involvement in cell signaling. These factors collectively organize mtDNA into a tightly-packaged segregating unit that is distributed throughout the mitochondrial network of human cells, appearing as punctate foci (Figure 2). However, the impact of cellular stress and genomic insults on the mitochondrial nucleoid remains a pathogenic mechanism in need of exploration.

Recent studies demonstrate that disruption of mtDNA's integrity, rather than base-change mutation, is much more common than previously appreciated, and likely to lead to a rapid decrease in bioenergetic function. Shokolenko *et al*. found that oxidants such as hydrogen peroxide (H_2O_2) introduced significant levels of strand breakage, rather than base-change mutations (60). Strikingly, cytokine-mediated inflammation directly attacks mitochondria and mtDNA, resulting in loss of mtDNA copy number (68, 69). These studies strongly indicate that mitochondria and mtDNA are major cell-intrinsic targets of inflammatory and oxidative insults. These findings open a host of questions in need of answers. What are the impacts of strand breakage on nucleoid organization? What are the dynamics of mtDNA-associated factors when mtDNA content is lost? When mtDNA is damaged and 'lost' how does this occur? EndoG is the only known mitochondrial endonuclease and is chiefly famous for degrading DNA upon release to the cytosol during apoptosis. Recent findings suggest that mtDNA is actually released both from the organelle and the cell, and can have pro-inflammatory effects (70,71). The molecular pathobiology of mtDNA damage thus presents a host of dynamic, unexplored questions.

5.1. The network is down: Impacts on organellar structure and bioenergetic function

In addition to the genetic material itself, damage and loss of mtDNA has severe, direct consequences for mitochondrial ultrastructure and bioenergetic function. As an organellar network, mitochondrial organization is increasingly revealed to be highly dynamic and responsive to cellular cues. The dynamics of the mitochondrial network are inextricably and directly linked with bioenergetic function, particularly the $\Delta\psi_m$ across the inner membrane. As such, loss of mtDNA causes severe disruption of this elegant balance of mitochondrial structure and function.

Rather than a static collection of organellar 'batteries', mitochondria are increasingly emerging as a highly dynamic organellar network distributed throughout the cell, providing ATP and integrating into cellular homeostasis by participation in a range of cell signaling pathways. As a metabolic network, mitochondria can exist in two states: as a united reticulum of threadlike organelles or as a fragmented population of individual organelles. These morphological transitions, described as the 'thread-grain' transition (72), are genetically regulated, maintaining a balance of organellar fission and fusion events. Mitochondrial fission and fusion are governed by different sets of interacting factors: fission involves recruitment of the cytosolic dynamin-related protein DRP1 to the mitochondrial outer membrane by the mitochondrial factors FIS1 (73) and MFF (74). Conversely, fusion of the mitochondrial outer membrane is mediated by mitofusins 1 and 2 (75), while inner membrane fusion is mediated by OPA1 (76,77).

Strikingly, mtDNA maintenance is elegantly coordinated with mitochondrial fission/fusion balance. Mitochondrial nucleoids are distributed at regular intervals throughout the mitochondrial network (Figure 2). The packaging of mtDNA into nucleoids is coordinated with mitochondrial fission and fusion to ensure that mtDNA is not lost. When the mitochondrial network undergoes fission, each individual mitochondrion will carry at least one mtDNA nucleoid (78), such that mitochondrial fission events take place on either side of, but not at, nucleoid foci (79), with DRP1 and MFF localizing to mitochondria immediately adjacent to nucleoid sites (80), allowing for distribution of mtDNA throughout the mitochondrial network and efficient transmission of mtDNA during cell division. Within the mitochondrial matrix, nucleoids are closely, albeit indirectly, tethered to the inner membrane through a series of protein-protein interactions that serve to coordinate mtDNA transcription and translation with the mitochondrial ribosome (81). Thus, mtDNA is efficiently integrated into the overall ultrastructure of the mitochondrial network, in which the molecular interactions responsible for fission/fusion balance and nucleoid maintenance act in concert to ensure that the organellar network has evenly distributed mtDNA content, regardless of the morphological state of the network. However, loss of mtDNA has profound effects on mitochondrial organization.

While mitochondrial dynamics are regulated as a balance of fission and fusion, they are also inextricably tied to function. Maintenance of a fused reticular network requires an intact $\Delta\psi_m$ across the inner membrane: both pharmacological uncoupling of $\Delta\psi_m$ (3) and depletion of mtDNA (82) results in fragmented mitochondrial morphology and inability to accomplish mitochondrial fusion. Dissipation of $\Delta\psi_m$ (via uncoupling agents such as CCCP and valinomycin) causes complete fragmentation of the mitochondrial network (3), as does inhibition of mitochondrial protein synthesis (83). $\Delta\psi_m$-dependent changes in mitochondrial morphology are a result of loss (76) or proteolytic cleavage (84) of the OPA1 fusion protein, in which the long isoforms of OPA1 (L-OPA1) are cleaved to short isoforms (S-OPA1) in response to decreased $\Delta\psi_m$. Head *et al*. (85) and Ehses *et al*. (86) concurrently identified OMA1, a mitochondrial metalloprotease, as the protease responsible for $\Delta\psi_m$–sensitive cleavage of the fusion-competent long isoforms of OPA1. OMA1 and OPA1 have thus emerged as crucial factors in mediating the interaction of mitochondrial fission/fusion balance and bioenergetic function.

Loss of mtDNA can rapidly disrupt this balance of organellar structure and function, leading to severe consequences for the cell. King *et al*. (87) first showed that mtDNA could be depleted from live cells in culture by treating with ethidium bromide, which preferentially inhibits mitochondrial POLG, rather than nuclear-localized DNA polymerases, thus leading to decreased mitochondrial
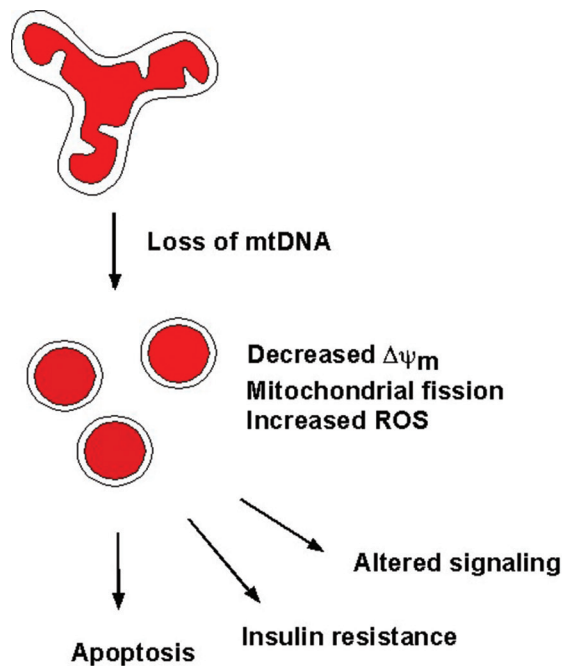
Figure 3. Cell-wide impacts of mtDNA loss. In response to loss of mtDNA copy number, a variety of cell types reveal decreased bioenergetic function, $\Delta\psi_m$, and ATP synthesis. In addition, the mitochondrial network loses the capability to maintain a united, networked morphology, instead assuming an obligately fragmented morphology, with concomitant increased production of mitochondrial reactive oxygen species. This collapse of mitochondrial homeostasis has profound effects on the cell at large, causing increased apoptosis, insulin resistance, and alterations in a variety of cell-wide signaling networks.

respiratory function (88). These mtDNA-depleted ρ^0 cells rely entirely on cytosolic glycolysis, but can nevertheless be passaged in cell culture. The inability to produce mtDNA-encoded polypeptides leads to incompletely-assembled OXPHOS complexes (89), resulting in electron transport deficiency (55) and decreased $\Delta\psi_m$ (90). ρ^0 cells maintain a minimal $\Delta\psi_m$, which is likely due to the presence of an intact F_1 ATPase, reversing H^+ transport to permit growth (91). This lack of bioenergetic function has dramatic effects on mitochondrial ultrastructure, causing a completely fragmented morphology, with a swollen matrix and loss of cristae (54, 82). This disruption of organellar homeostasis may activate mitochondria-to-nucleus 'retrograde' stress signaling, and predispose the cell to apoptosis.

5.2. Cell-wide impacts of mtDNA loss: apoptosis and signaling impacts

While the loss of mtDNA directly causes loss of OxPhos function, the consequences for the rest of the cell are also emerging. Crucially, loss of mtDNA appears to have serious effects on cell viability, insulin signaling, and apoptosis, as well as broadly altering cell-signaling pathways via 'retrograde' mitochondria-to-nucleus signaling (Figure 3).

Apoptosis, derived from the Greek meaning "falling off", was first described by Kerr *et al.* as the morphological features surrounding a distinct form of cell death, including cytoplasmic and nuclear compression followed by fragmentation and dispersal of the cell (92). Apoptotic cell death is essential to normal development and tissue homeostasis (93), and occurs in response to a range of stimuli, including cell stress, DNA damage, deprivation of growth factors, and other stimuli (94), and can function as a defense mechanism towards disease or cellular damage (95). Caspases are a family of genes that carry out proteolytic cleavage, and are activated by two major cellular pathways, the intrinsic and the extrinsic pathways (96), both of which activate caspase-3, resulting in DNA fragmentation and cell death (97). The extrinsic pathway is activated by cell surface death receptors such as tumor necrosis factor (TNF) related family (Fas) (98) The TNF superfamily includes TNFR1, Fas, APO-1, DR3, TRAIL-R1 and DR5. The extrinsic pathway is initiated by the binding of death receptor ligands, allowing procaspase-8 to be recruited to the death-inducing signaling complex, binding to a FAS-associated death domain (FADD). Dimerization then occurs in the procaspase-8 and caspase-8 is then activated (99). The intrinsic pathway involves the release of factors, particularly cytochrome c, from the mitochondria and can be activated by DNA damage, cytoskeletal disruption and other stimuli (100), initiating dimerization of caspase-9 (101). When cytochrome c is released from the mitochondria, it binds apoptotic protease-activating factor-1 (APAF1) (102) inducing activation of caspase-9 (103).

The discovery that mitochondria directly participate in apoptosis prompted an explosion of interest in mitochondrial cell death mechanisms (29, 104-108). Mitochondrial outer membrane permeabilization (MOMP) is a crucial step of early-stage apoptosis, allowing Bax and Bak from the Bcl 2 family to be translocated to the outer mitochondrial membrane (109) and concentrate into submitochondrial punctate foci (110), which then allows release of cytochrome c (111). In addition to the release of cytochrome c, Smac/Diablo, apoptosis-inducing factor (AIF) and Endo G (112-114) are pro-apoptotic factors that are released from mitochondria during apoptosis. Release of these mitochondrial factors leads to assembly of the apoptosome, activating procaspase-9, which in turn is then able to activate caspase-3 and caspase-7, triggering a cascade leading to oligonucleosomal DNA fragmentation (115).

Due to mitochondrial control of cell death, there has been widespread interest in potential roles of mtDNA-derived mitochondrial dysfunction in apoptosis. *In vivo*, cardiac-specific *TFAM* knockout mice have mtDNA depletion, and show both mitochondrial dysfunction and massive apoptosis (29). Decreased OxPhos activity and increased fission have been proposed as mechanisms

by which mitochondrial dysfunction may cause increased apoptosis, as both have been mechanistically connected with apoptosis (94, 106). Strikingly, strand breakage of mtDNA itself has been implicated as a trigger for apoptosis (116). As an electron carrier, cytochrome c is crucial for a stable membrane potential. Thus, removal of cytochrome c from electron transport to the cytoplasm results in decreased membrane potential and ATP synthesis (106). HeLa cells undergo apoptosis with little change in $\Delta\psi_m$, suggesting that membrane potential is decreased in late-stage apoptosis, but is not a crucial step of early apoptosis (106). ρ^0 cells maintain a minimal membrane potential and undergo apoptosis (117), but this $\Delta\psi_m$ is due to reversal of ATP synthase proton flux, rather than electron transport (91). Thus, cytochrome *c* is available for apoptotic signaling and is not required for electron transport in ρ^0 cells. Kwong *et al.* suggested that OxPhos activity may regulate apoptosis in a context-dependent manner: ρ^0 cells are protected against apoptosis because of the complete *absence* of electron transport, while cells with *decreased* electron transport have *heightened* sensitivity to apoptosis (118).

Alternatively, the increased organellar fission associated with loss of mtDNA may mechanistically activate apoptosis. Recent studies have sparked controversy whether fission is required for apoptosis. Several apoptotic stimuli cause mitochondrial fragmentation mitochondria via DRP1 and FIS1 (119). Down-regulation of DRP1 has been reported to prevent release of cytochrome c, but not Bax/Bak-dependent apoptosis, suggesting that fission is not important in Bax/Bak-dependent apoptosis (119). On the other hand, mitochondrial recruitment of DRP1 may be concurrent with Bax activation during early apoptosis (120). Stable DRP1 is involved in apoptotic events such as cristae remodeling (121) which leads to the release of cytochrome c and eventually to the complete loss of $\Delta\psi$ (120). Staurosporine (STS) causes translocation of DRP1 to the mitochondrial outer membrane during apoptotic induction, while DRP1-negative cells can block apoptosis (122), suggesting that mitochondrial fission is necessary in order for apoptosis to occur. More recently, mitochondrial DRP1 has been shown to stimulate oligomerization of Bax and cytochrome c release (123), while decreased OPA1 fusion leads to apoptotic sensitivity (124). These findings strongly indicate that disruption of mitochondrial structural dynamics in mtDNA-depleted cells leads to increased apoptotic sensitivity.

In addition, mitochondrial dysfunction appears to disrupt the delicate web of cellular signaling events via 'retrograde' mitochondria-to-nucleus signaling. While the nuclear signaling pathways regulating mitochondrial biogenesis have become increasingly well characterized, centering on the PGC-1α master regulator of mitochondrial biogenesis and related factors, mitochondria appear to feedback to the nucleus,

modulating key pathways. Nuclear-encoded signaling factors tightly regulate mitochondrial content within the cell, modulating expression of nuclear-encoded mitochondrial proteins, as well as mtDNA copy number (via TFAM expression) and expression of mtDNA-encoded genes (29). Strikingly, however, it is also becoming clear that mitochondria can communicate with the rest of the cell via 'retrograde' signaling pathways. The loss of mtDNA has profound effects on the rest of the cell through retrograde modulation of signaling pathways and apoptotic modulation (116), and appears to act via modulation of both AMP kinase, ROS-mediated NFκB signaling pathways (125). Thus, while loss of mtDNA causes an immediate bioenergetic deficit, the effects on cell signaling reverberate throughout the cell, impacting a wide variety of pathways crucial to homeostasis, yet not directly involved in energy metabolism.

6. CONCLUDING REMARKS

Originally, the idea of pathogenic mtDNA defects was highly controversial. How could a defect in something so crucial as ATP production not be intrinsically lethal? Following the sequencing of the human mitochondrial genome, it became clear that mutations of mtDNA are causative in an enormous range of disease pathologies, particularly concentrated in neuromuscular disorders. The evolving face of mitochondrial biology is revealing that loss of mtDNA is equally serious (and in many cases phenotypically identical), playing a strong role in many of the most prevalent diseases facing public health. The complex interplay of mtDNA with mitochondrial ultrastructure, metabolic functions, and cellular signaling paints an increasingly dynamic picture of the integral role of the mitochondrial network in cellular homeostasis. As such, many of the mechanistic and pathology-based questions raised here will provide crucial information for preventive and therapeutic approaches.

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