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The little big genome: the organization of mitochondrial DNA

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1. ABSTRACT

The small (16,569 base pair) human mitochondrial genome plays a significant role in cell metabolism and homeostasis. Mitochondrial DNA (mtDNA) contributes to the generation of complexes which are essential to oxidative phosphorylation (OXPHOS). As such, mtDNA is directly integrated into mitochondrial biogenesis and signaling and regulates mitochondrial metabolism in concert with nuclear-encoded mitochondrial factors. Mitochondria are a highly dynamic, pleiomorphic network that undergoes fission and fusion events. Within this network, mtDNAs are packaged into structures called nucleoids which are actively distributed in discrete foci within the network. This sensitive organelle is frequently disrupted by insults such as oxidants and inflammatory cytokines, and undergoes genomic damage with double- and single-strand breaks that impair its function. Collectively, mtDNA is emerging as a highly sensitive indicator of cellular stress, which is directly integrated into the mitochondrial network as a contributor of a wide range of critical signaling pathways.

2. mtDNA IN MITOCHONDRIAL BIOENERGETICS AND BIOGENESIS

Our understanding of mitochondria has undergone profound revision over recent years. While these endosymbiont-derived organelles had previously been thought of as a collection of battery-like organelles, current evidence reveals that mitochondria are an incredibly dynamic organellar network, of dual genetic origin, that is crucial to bioenergetics and metabolism, as well as a range of vital cellular processes.

As endosymbiotic organelles, mitochondria originated two billion years ago from the engulfment of a α-proteobacterium by an ancestor of a modern eukaryotic cell (1, 2). As such, human mitochondria closely resemble bacteria in both their membrane structure and maintenance of DNA. Mitochondria are membrane-bound organelles containing both an outer and inner mitochondrial membrane. The inner membrane contains the five complexes of oxidative phosphorylation (OXPHOS), required for mitochondrial ATP production. Unique among human organelles, mitochondria contain their own genetic material, mitochondrial DNA (mtDNA). MtDNA is circular, similar to bacterial plasmid DNA, and encodes factors that combine with those produced from nuclear DNA to comprise the OXPHOS complexes of the inner membrane, providing the cell with the necessary ATP. The two strands of mtDNA are differentiated into heavy (H-strand) and light strands (L-strand), with the H-strand being primarily composed of guanine and the light strand rich in cytosine (3). The mitochondrial genome in humans contains 16,569 base pairs that code for 37 genes: 13 polypeptides, 22 tRNAs, and the small and large rRNA subunits (4). MtDNA is maternally inherited through the oocyte during conception (5-7). During early embryonic development, paternal mtDNA in the mitochondria of spermatocytes is selected for destruction. Paternal mtDNA inheritance is exceedingly rare in humans (8).

Mitochondria also contain the nuclear-encoded factors necessary for the replication and transcription of this extra-nuclear genetic material. MtDNA replication requires proteins such as DNA polymerase γ (POLG), the mitochondrial single-stranded DNA binding protein (mtSSB), mitochondrial DNA helicase (Twinkle), and a number of accessory proteins and transcription factors. The role of mtSSB in replication is to stimulate the activity of the mitochondrial replicative helicase Twinkle and polymerase (9). Recent studies have shown Twinkle

helicase to be essential for mtDNA replication, as depletion of *Twinkle* causes severe mtDNA depletion (10). Pif1 is another helicase that has also been shown to cause mitochondrial instability: depletion of Pif1 causes deficiency in repairing oxidative stress-induced mtDNA damage and mitochondrial myopathy (11). POLG is crucial as it is the only replicative, highly-processive polymerase in the mitochondria, and also is necessary in the repair of mtDNA (12). The initiation of mtDNA transcription requires the binding of mitochondria transcription factor (mtTFB) to the mtRNA polymerase (POLRMT). MtDNA typically is present at ~1,000 copies per cell, varying between cell and tissue type (13). Transcription factor A, mitochondrial (TFAM) was originally identified for its key role in the activation of mtDNA transcription (14), but more recently has been shown to play a critical role in modulating mtDNA content within mammalian cells: exogenous expression TFAM is able to increase mtDNA content, independent of changes in transcription (15-17). The level of TFAM within the cell is directly modulated by the Lon protease, allowing for Lon-dependent changes in mtDNA copy number (18), using phosphorylation of TFAM in the HMG-1 domain to promote proteolytic degradation by Lon (19).

Effective mitochondrial ATP production requires the transcription and translation of mtDNA-encoded genes, along with the import of nuclear-encoded polypeptides and the coordinate assembly of proteins from both genomes into the multisubunit OXPHOS complexes. The biogenesis of OXPHOS complexes and mitochondrial content as a whole is controlled by cell signaling pathways that can upregulate mitochondrial content and gene expression in response to cellular metabolic demand. The OXPHOS complexes are large, multisubunit protein complexes located in the mitochondrial inner membrane. Complexes I-IV catalyze electron transfer from NADH and $FADH_2$, providing the energy for the proton pumping that generates the transmembrane potential across the inner membrane ($\Delta\psi_m$). This $\Delta\psi_m$ drives ATP synthesis by the F_1F_0 ATP synthase, in which the rotation of the gamma and epsilon subunits causes ADP and P_i to coalesce to form ATP (20). With the exception of Complex II (succinate dehydrogenase), each of the complexes contains one or more mtDNA-encoded polypeptides that are required for assembly and function. Complex I (NADH dehydrogenase), the largest of the five complexes, is composed of more than 40 proteins, as well as iron-sulfur clusters and flavin prosthetic groups, and is the entry point for electrons from NADH. Of the numerous polypeptides that make up Complex I, seven (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6) are encoded by mtDNA. Complex III (coenzyme Q: cytochrome c oxidoreductase) is comprised of 11 subunits, including 1 mtDNA-encoded subunit (cytochrome b), while Complex IV (cytochrome c oxidase) contains 14 polypeptide subunits, of which CO1, CO2, and CO3 are mtDNA-encoded. The F_1F_0 ATP synthase contains two mtDNA-encoded proteins, ATP6 and ATP8, as part of its complement of 16 subunits (21). Assembly of these complexes requires the transcription and translation of mtDNA-encoded factors and the import of nuclear-encoded proteins, followed by assembly and insertion of the finished complex into the mitochondrial inner membrane. Loss or mutation of the genes for any of these mtDNA-encoded subunits can disrupt the assembly and function of the complex affected: a host of point mutations, partial deletions (Δ-mtDNAs), and depletion of mtDNA cause loss of specific complexes or OXPHOS activity as a whole (22), illustrating the crucial role of mtDNA in cellular bioenergetics, in spite of the small handful of gene products derived from mtDNA.

The mitochondrial proteome is composed of over 1,000 proteins, varying across species (23-26). The cell's mitochondrial content, expression of OXPHOS components and other mitochondrial proteins, and mtDNA copy number varies dramatically between cell and tissue types, depending on metabolic demand and cellular signaling cues. For example, cardiac muscle has the highest mitochondrial content of human tissues, with 20-30% of cellular volume composed of mitochondria, consistent with the extreme bioenergetic demands of the heart (27). To respond to energetic demand, mtDNA copy number, OXPHOS capacity, and mitochondrial content are regulated by mitochondrial biogenesis factors controlling the coordinated expression of mtDNA- and nuclear-derived factors. The best-characterized of these is PPAR-gamma related cofactor-1 alpha (PGC-1α), which was first identified in brown fat cells, and was found to induce dramatic increases in mitochondrial content when expressed in white adipose tissue (28). PGC-1α directly activates the expression of nuclear respiratory factors-1 and -2 (NRF-1, NRF-2) (29). These transcription factors directly activate the expression of nuclear-encoded mitochondrial proteins including OXPHOS complex subunits and TFAM, the key mtDNA-packaging protein that modulates mtDNA copy number. Similar to nuclear-encoded OXPHOS components, TFAM expression is controlled by NRF-1 (30) and NRF-2 (31), placing mtDNA copy number control under PGC-1α-mediated mitochondrial biogenesis signaling. MtDNA copy number may also be regulated by DNA methylation of the nuclear encoded DNA polymerase gamma (32). MtDNA content is thus coordinately regulated along with overall mitochondrial content to provide bioenergetic capacity in response to cellular demand. PGC-1α has since emerged as a master regulator of mitochondrial biogenesis, and interacts with numerous signaling factors such as AMPK and SIRT3 that have dramatic impacts on cellular metabolism and energetics (33, 34). In addition, several nuclear transcription factors have been found to localize to the mitochondria, with the ability to activate transcription of mtDNA-encoded factors: cyclic AMP response binding element-binding protein (CREB) binds to the D-loop of mtDNA (35) and produces mtDNA-derived

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mRNA (36), while myocyte enhancer factor-2D (MEF2D) localizes to the mitochondria and activates expression of Complex I subunits (37). Conversely, nuclear factor kappa B (NFκB), a key cellular stress response factor, also localizes to the mitochondria, but appears to repress the expression of mtDNA-encoded genes (38, 39). Collectively, it is clear that multiple signaling pathways converge to modulate mitochondrial biogenesis, mtDNA copy number, and the expression of both nuclear- and mtDNA-encoded OXPHOS subunits.

3. INTEGRATION OF mtDNA INTO THE ORGANELLAR NETWORK

To maintain bioenergetic homeostasis, mtDNA nucleoids are distributed at regular intervals throughout the highly dynamic mitochondrial network. This organellar network continuously balances fusion and fission events to maintain structural homeostasis in response to cellular cues. To unite separate organelles into an interconnected reticulum, mitochondria employ distinct GTPase factors to establish continuity of both the outer and inner mitochondrial membranes. Fusion of the mitochondrial outer membrane is mediated by the mitofusins 1 and 2 (MFN1 and MFN2), which act both individually and in concert with each other to accomplish fusion (40). Inner membrane fusion is carried out by optic atrophy-1 (OPA1). As an inner membrane protein, OPA1 is present in multiple isoforms (41). Long isoforms of OPA1 (L-OPA1) are fusion-active (42), which are cleaved to short, fusion-inactive forms (S-OPA1) by several proteases including Yme1 (43) and OMA1 (44, 45). OPA1 interacts with MFN1 and 2, permitting coordination of both outer and inner membrane fusion (43). The opposing process, fission, is mediated by the cytosolic GTPase dynamin-related protein-1 (DRP1). The outer membrane proteins Fis1 and MFF1 act to recruit DRP1 to the organelle (46, 47), where they bind DRP1 and promote the formation of DRP1 multimers that wrap around the mitochondrial tubule, using GTP hydrolysis to constrict the organelle and carry out membrane scission (48, 49). Thus, mitochondrial dynamics are mediated by two opposing sets of factors, balancing these complex interactions to establish structural homeostasis.

These organellar dynamics are mechanistically tied to bioenergetic function. Based on their experiments using photobleaching and recovery of mitochondrial fluorescence, Skulachev and co-workers proposed that the fused, reticular organization of mitochondria mediates energetic connectivity (50), while subsequent work found that loss of bioenergetic function resulted in an inability to maintain mitochondrial interconnection (51). More recently, it has become clear that mitochondrial fusion requires an intact $\Delta\psi_m$ across the inner membrane (52). OPA1-mediated fusion of the inner membrane is $\Delta\psi_m$-dependent: loss of $\Delta\psi_m$ causes cleavage of L-OPA1 to fusion-inactive S-OPA1 isoforms (42). The groups of Langer and van der Bliek concurrently found that this $\Delta\psi_m$-sensitive cleavage of OPA1 is mediated by the OMA1 metalloprotease (44, 45). In addition, dissipation of $\Delta\psi_m$ activates fission (53), engaging calcium-dependent DRP1 dephosphorylation signaling mechanisms (54). Thus, loss of $\Delta\psi_m$ impacts both mitochondrial fusion and fission, causing the collapse of mitochondrial structure to the completely fragmented organization observed in genetic and pharmacological models of bioenergetic dysfunction.

In addition to their integration with bioenergetics and metabolism, fission/fusion dynamics are critical to mitochondrial participation in key cellular signaling pathways including apoptosis (53), mitosis (55), autophagy (56), and stemness (57). For example, during nutrient starvation, functional mitochondria undergo fusion and elongation, protecting them from degradation (58), while mitochondria with low $\Delta\psi_m$ are selectively targeted for autophagy via PINK1/Parkin signaling (59). Similarly, DRP1-mediated mitochondrial fission is an integral component of apoptotic pathways (60), while OPA1 interacts with SIRT3 as part of cellular stress response signaling (61). Collectively, mitochondrial structural dynamics are critical to the organelle's role in both bioenergetics and crucial cell-wide signaling events, directly impacting the survival or death of the cell.

To ensure effective distribution of mtDNA within this pleiomorphic, dynamic organellar network, nucleoids are distributed at regular intervals throughout the mitochondrial reticulum. This distribution, when visualized microscopically, has a striking 'beads on a string' appearance (62, 63), allowing mtDNA-derived transcripts and gene products to diffuse efficiently throughout the mitochondrial network. Measurements of nucleoid foci using multiple visualization methods reveal that nucleoids are found at a frequency of one nucleoid every 0.8. μM of mitochondrial length (64). While nucleoids are somewhat constrained in their ability to diffuse via their association with the inner membrane (65), they are nevertheless sufficiently mobile to repopulate mitochondria lacking mtDNA and restore bioenergetic function, as shown through cell fusion experiments (13). Mitochondrial nucleoids do not appear to exchange genetic material (66), but they have been shown to divide, likely as a result of replication events followed by the partitioning of mtDNAs into daughter nucleoids (67). While a fused mitochondrial network allows for increased bioenergetic function (68) and an electrically-connected mitochondrial continuum (50), the spatial distribution of nucleoids also allows for efficient mtDNA partitioning during fission of the network. A variety of imaging methods, including *in situ* hybridization (63), ethidium bromide, and anti-DNA immunolabeling (64), reveal that when the mitochondrial network undergoes fission to exist as a population of individual organelles, each mitochondrion will contain at least one nucleoid.

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Moreover, nucleoids appear to be protected from fission events: DRP1-mediated fission occurs to either side, but not at, the site of nucleoids (Iborra *et al*., 2004); the MFF and FIS1 factors that recruit DRP1 appear to prevent fission at nucleoid sites. This is similar to prokaryotic mechanisms, in which bacterial nucleoids are protected from cell division by specific factors such as Noc to prevent loss of nucleoid genetic material (69, 70).

The arrangement of nucleoids throughout this highly dynamic network plays a major role in determining the bioenergetic status of the cell, and allows for both complementation between individual organelles and elimination of mitochondria carrying deleterious mtDNA mutations. Mitochondrial genetics are best thought of as population genetics on a cellular scale: the ~1,000 copies of mtDNA within a human cell frequently contain mixed populations of wildtype (WT) and mutant mtDNAs. These competing populations of WT and mutant mtDNAs, referred to as heteroplasmy (71), lead to heterogeneity of function within the mitochondrial network. Following the sequencing of the mitochondrial genome (4), it was shown that mutations of mtDNA cause loss of mitochondrial bioenergetics, via maternally-inherited base-change mutations in polypeptides and tRNAs, as well as through large-scale somatic Δ-mtDNAs (22). In evaluating mtDNA mutations and their phenotypic impact, it became quickly apparent that heteroplasmy was a crucial determinant of bioenergetic capacity: patients with pathogenic mtDNA mutations showed a sharp threshold effect of mtDNA mutation load (72), while complementation experiments by Attardi's group found that just 10% of WT mtDNA was sufficient to restore full mitochondrial function (73). Thus, the presence of WT and mutant mtDNAs within the cell creates a heterogeneous population of both functional and dysfunctional mitochondria.

This heterogeneity reveals the role of fission/fusion dynamics in determining mitochondrial phenotypes. Hayashi's group demonstrated that two respiration-deficient cell lines, each carrying a different mtDNA point mutation, were able to transcomplement and restore mitochondrial function upon cell fusion (74). Similarly, mitochondria in mice carrying both WT and Δ-mtDNAs showed robust ability to fuse and maintain mitochondrial function unless the overall mutation load exceeded threshold, at which point bioenergetic function was lost (75). While nucleoids themselves do not appear to exchange mtDNAs, as discussed above, this complementation results from diffusion of gene products between individual organelles upon fusion of the outer and inner membranes (52, 66), as even mitochondria in cells lacking mtDNA have fusion machinery and can fuse with 'healthy' mtDNA-containing mitochondria to permit complementation to occur (76). Conversely, fission permits the isolation and degradation of dysfunctional mitochondria. Autophagy has emerged as a major cellular mechanism mediating the selective degradation of dysfunctional mitochondria. Collapse of $\Delta\psi_m$ causes recruitment of the Parkin E3 ubiquitin ligase and subsequent targeting of individual organelles to autophagosomes (59). This selective degradation requires both the loss of $\Delta\psi_m$ (59) and DRP1-mediated fission to isolate the dysfunctional organelle (58). Mitochondria carrying pathogenic mtDNA mutations that impact $\Delta\psi_m$ can thus be targeted for autophagic degradation (77).

4. MITOCHONDRIAL AND mtDNA DAMAGE

As a network that is directly integrated into crucial cell signaling pathways, with multiple $\Delta\psi_m$-dependent factors that sense cellular cues to alter organellar dynamics, mitochondria are a highly responsive indicator of cellular stress. While mitochondria have long been thought to be a major cellular target of oxidative damage, recent findings indicate that a wide range of cellular stresses cause damage to both the mitochondrial network and mtDNA.

In consuming oxygen as the final electron acceptor of the redox reactions catalyzed by Complexes I-IV, mitochondria frequently produce reactive oxygen species (ROS), such as superoxide and hydroxyl radical, as byproducts during oxidative phosphorylation. Denham Harman first proposed that these free radicals produced by the ETC accumulate to damage mitochondria, contributing to the free radical theory of aging (78). Subsequent examination of oxidative damage to mtDNA showed that oxidative lesions to mtDNA occur more frequently, and persist longer, than nuclear DNA damage (79, 80). While it had been postulated that mitochondria lack histone packaging, making its DNA more susceptible to damage (81), the extensive packaging of mtDNA by TFAM makes this much less plausible (82, 83). Moreover, while mitochondria contain less overall DNA repair machinery than the nucleus, they are not devoid of repair mechanisms, instead maintaining both base-excision and putative mismatch repair mechanisms (BER and MMR, respectively). The BER pathway has a robust ability to repair oxidative lesions in DNA (84, 85). As the major replicative DNA polymerase in mitochondria, POLG is a critical player in the mitochondrial BER pathway. When a lesion occurs, DNA glycosylases cleave the N-glycosidic bond between the damaged base and its deoxyribose. An abasic site is left allowing recognition by apurinic/apyrimidinic endonuclease 1 (APE1) which then removes the ribose (86). When ribose is removed final repair can occur by POLG and ligase III. POLG fills in the gaps while ligase III seals the backbone (87). In MMR, base pairs that are inserted or deleted during DNA replication are repaired (86). Bohr's group showed mismatch-binding activity in mitochondria involving the YB-1 repair factor (88), while Lightowlers' group also found low-level mismatch repair activity, but did not find the nuclear MMR factor Msh2 in mitochondria (89). Mlh1

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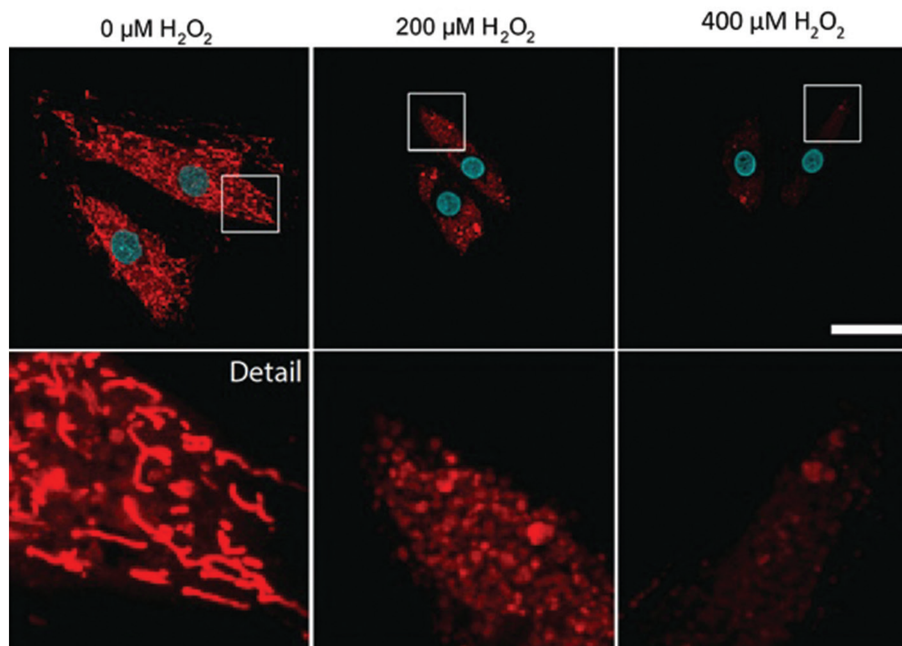

Figure 1. Impact of oxidative stress on mitochondrial morphology. Confocal microscopy of H9C2 cardiomyoblasts stained with MitoTracker (red) and DAPI (cyan) following treatment with H_2O_2 at concentrations of 0, 200, and 400 μm for 1 hour. Size bar = 10 μm.

overexpression may allow for MMR in the D-loop region to protect mitochondrial integrity (90). It is worth noting, however, that robust mismatch-specific repair has not been demonstrated in purified mitochondria.

Mitochondria thus have both active packaging of mtDNA by TFAM and functional base-excision repair machinery. In addition, the mitochondrial genetic threshold effect further predicts that oxidative base-change mutations are not a likely source of widespread mitochondrial dysfunction. For a single base-change mutation of mtDNA to cause a metabolic defect, even in a tRNA or similar highly-deleterious position, the mutated mtDNA variant must first accumulate to become a majority of the overall mtDNA content of the cell (typically 60-90%, depending on the type of mutation (22). This would seem to suggest that oxidative mutation of mtDNA is not a likely form of critical mitochondrial damage.

Alternately, however, oxidants are found to cause single- and double-strand breaks in mtDNA as the predominant form of mtDNA damage, directly causing loss of mtDNA content through degradation, with the potential for devastating impacts on mitochondrial function and the viability of the cell as a whole. Experiments treating cultured human cells with oxidants such as hydrogen peroxide reveal that while ROS do not elicit any significant increase in base-change *mutations* of mtDNA, the same treatment elicits a tenfold increase in *strand breakage* (91). Subsequently, this oxidative strand breakage of mtDNA was shown to decrease OXPHOS activity (92). This is consistent with the loss of mitochondrial metabolism in cells depleted of mtDNA (52), and demonstrates a clear link between oxidative of mtDNA and decreased mitochondrial bioenergetic function.

At the same time that oxidative radicals are damaging mtDNA, they are also attacking the mitochondrial network as a whole. While cardiomyoblasts maintain a balance of fusion and fission, mitochondria become completely fragmented upon exposure to concentrations of H_2O_2 equivalent to those described above (Figure 1), indicating that oxidative damage has a profound effect on the mitochondrial network, disrupting steady-state fusion/fission balance. Moreover, it has been shown that defects in either fission or fusion can also disrupt mtDNA: decreased mitochondrial fission through downregulation of DRP1 causes decreased mtDNA content (93) likely through replication stress (94), while deletion of mitofusins 1 and 2 causes massive loss of mtDNA content (95). Thus, in addition to direct damage to mtDNA, oxidant-mediated disruption of fission and fusion factors also are likely to contribute to the loss of mtDNA that occurs as part of ROS-mediated damage.

While mitochondrial OXPHOS is a significant source of ROS production, other cellular stresses are emerging as potential mediators of mtDNA and mitochondrial damage, indicating that the mitochondrial network is both a target and participant in cytokine-mediated inflammation. Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine, requiring Toll-like receptors to initiate inflammation, producing a burst of ROS within the cell. Upon incubation with

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increasing concentrations of TNF-α, significant increases in ROS and concomitant decreases in mtDNA copy number were clearly evident in cardiac myocytes (96). Subsequent work showed that TNF-α-mediated mtDNA damage requires TNF receptors and ROS production; moreover, this mtDNA damage involves binding of p53 to TFAM (97). Screening for novel mtDNA repair factors has identified the novel mitochondrial DNA polymerase theta (POLθ) that is recruited to the mitochondria when under oxidative stress (98). Moreover, damaged mtDNA appears to be released from the organelle, where it acts as a pro-inflammatory signal in a variety of contexts (99). The mitochondrial network, and mtDNA specifically, are thus a highly sensitive indicator of cellular stress, directly integrated into critical stress-signaling pathways.

5. CONCLUDING REMARKS

Our understanding of the mitochondrial genome and its maintenance within a highly dynamic organellar network has evolved to show that mtDNA is actively packaged and dynamically regulated, interacting with an increasing number of cellular pathways to coordinately participate in mitochondrial responses to a wide range of cellular stimuli. As a critical component of the pleiomorphic, responsive mitochondrial network, mtDNA is emerging as a critical indicator of cell stress, with new insights revealing this small genome to have an outsized impact on metabolism and cellular homeostasis.

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Abbreviations: mitochondrial DNA, mtDNA; oxidative phosphorylation, OXPHOS; mitochondrial transmembrane potential, $\Delta\psi_m$; Transcription factor A mitochondrial, TFAM; polymerase γ, POLG; mitochondria transcription factor, mtTFB; mitochondrial single-stranded DNA binding protein, mtSSB; optic atrophy-1, OPA1; dynamin-related protein-1, DRP1; PPAR-gamma related cofactor-1 alpha, PGC-1α; reactive oxygen species, ROS; mtRNA polymerase, POLRMT; nuclear respiratory factors-1 and -2, NRF-1, NRF-2; cyclic AMP response binding element-binding protein, CREB; myocyte enhancer factor-2D, MEF2D; nuclear factor kappa B, NFκB; mitofusins 1 and 2, MFN1 and MFN2; deletions of mtDNA, Δ-mtDNAs; base excision repair BER; mismatch repair, MMR; polymerase theta, POLθ; tumor necrosis factor-alpha, TNF-α; apurinic/apyrimidinic endonuclease 1, APE1

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