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Mitochondrial epigenetics: an overlooked layer of regulation?

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Despite decades of research, mitochondrial epigenetics remains a controversial notion. Recent findings, however, indicate that dysfunctional mitochondrial DNA (mtDNA) methylation could underlie aging and disease. Unraveling such a level of regulation will be essential in the understanding of and in interfering with the role of mitochondria in many physiological and pathophysiological processes.

Mitochondrial epigenetics

Human mtDNA is a 16 569-bp circular DNA containing a heavy (H) and a light (L) strand. It encodes 37 genes: 13 protein-coding genes, all involved in oxidative phosphorylation; two rRNAs; and 22 tRNAs. In contrast to nuclear DNA, mtDNA is intronless, is maternally inherited, and lacks histones [1]. Despite these differences, emerging evidence suggests that mtDNA may also be regulated at the epigenetic level in the form of mtDNA methylation.

Although the presence of mtDNA methylation has been the subject of controversy for many decades [1], accumulating evidence now firmly suggests that this is a real phenomenon [2–7]. Here we highlight these findings and discuss how mtDNA methylation may function. Moreover, we present currently used (Box 1 and Table 1) and new experimental approaches that could provide more insight into the functional relevance of this phenomenon.

DNA methyltransferases (DNMTs) and hydroxylases localize to the mitochondria

In 2011, the field of mitochondrial epigenetics was revitalized by the discovery of a mitochondrially targeted DNMT1 transcript variant (mtDNMT1) that uses an upstream alternative translation start site leading to the inclusion of a mitochondrial targeting sequence [6]. This variant comprises about 1–2% of total DNMT1 transcripts and is upregulated by the hypoxia-responsive transcription factors peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α) and nuclear respiratory factor 1 (NRF1) and via the release of p53 from the DNMT1 promoter (J. Balinang, PhD thesis, Virginia Commonwealth University, 2012) [6]. This finding suggests that mtDNMT1 plays a regulatory role during oxidative stress, confirming the link between oxidative stress and mitochondrial function. Similar capacities for mtDNMT1 and its nuclear counterpart were indicated by the finding that mtDNMT1 shows clear CpG-dependent mtDNA interactions proportional to the amount of CpGs in the target amplicons [6].

Besides mtDNMT1, no other specific mitochondrially targeted isoforms of enzymes involved in DNA methylation or hydroxymethylation are known. Nevertheless, several such enzymes have been detected in the mitochondrial protein fraction: DNMT3B (albeit at very low levels) and ten–eleven translocation (TET) 1 and 2 were found in mouse fibroblast and HeLa cells [2] and TET1 and TET2 were detected in primary neuronal cultures of 5-day-old mice [3]. Neither study detected DNMT3A or TET3 in the mitochondria [2,3]. Interestingly, the presence of DNMT1, DNMT3A, and DNMT3B in the mitochondria is tissue-type dependent: DNMT3A localizes predominantly inside the mitochondria of 'excitable tissues' (heart, skeletal muscle, and adult neurological tissues). In these tissues, DNMT1 was bound to the outer mitochondrial membrane only and DNMT3B was undetectable [7]. Artifacts from subcellular contamination were excluded by confirming the purity of the mitochondrial fraction using western blotting and/or electron microscopy [2,3,7].

Indications for involvement of mtDNA methylation in mitochondrial gene expression regulation

The three main players in mitochondrial transcription are mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factor B2 (TFB2M), and mitochondrial transcription factor A (TFAM) [8]. These factors regulate transcription from the L-strand promoter (LSP) and H-strand promoter (HSP) 1 and 2. Transcription from LSP produces a near-genome-length polycistronic transcript and the RNA primers required for initiation of mtDNA replication of the H-strand. Transcription from HSP1 generates a short transcript containing both mitochondrial rRNAs whereas HSP2 generates a near-genome-length polycistronic transcript [8].

Despite this increasing body of knowledge, the possibility that mtDNA methylation contributes to mitochondrial transcriptional regulation remains largely unexplored. Dysfunctional mtDNA methylation might help in our understanding of mitochondrial diseases, as only a minority (~15%) can be explained by alterations in the mtDNA sequence [9].

Several findings indicate the functionality of mtDNA methylation. First, the patterns of mtDNA methylation

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Box 1. Current methods used to detect mitochondrial DNA methylation

Several methods have been exploited to detect mtDNA methylation (see Table 1 in main text). Currently, many of these methods are combined with genome-wide sequencing approaches [4]. Some methods require the purification of mitochondria [e.g., liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS), 5mC/5-hydroxymethylcytosine (5hmC) ELISA], whereas others do not (e.g., bisulfite sequencing, pyrosequencing). In the former, incomplete mitochondrial purification can be a problem, whereas in the latter nuclear integration of mtDNA sequences (NUMTs) can blur the outcome. Therefore, artifacts from subcellular contamination should be excluded by confirming the purity of the mitochondrial fraction using western blotting and/or electron microscopy [2,3,7]. To determine the contribution of NUMTs, cells depleted of mtDNA (ρ_0 cells) [2] or unique regions in the mtDNA can be selected for analysis [4]. Each method has advantages and drawbacks (see Table 1 in main text) [1] that should be taken into account. To distinguish cytosine (C) from 5mC and/or 5hmC, several approaches can be followed, including bisulfite treatment, antibody-based affinity enrichment, and methylation-/glucosyl-sensitive digestions. Bisulfite treatment, which converts all Cs to thymines (Ts) unless they are methylated or hydroxymethylated, distinguishes C from 5mC/5hmC [2,4,7]. To further distinguish between 5mC and 5hmC, other approaches are required, such as DNA glucosylation, whereby a glucosyl group is transferred to 5hmC only, combined with glucosyl-sensitive cleavage [3,6].

and hydroxymethylation are nonrandom [2,6] and show a peculiar arrangement in the D-loop region (noncoding mtDNA control region): methylation is observed on the L-strand only, mainly in the promoter regions (LSP, HSP1) and in conserved sequence blocks [2]. Second, upregulation of mtDNTM1 results in gene-specific effects on mitochondrial transcription: of the four genes determined, *ND6*, the only protein-coding gene on the L-strand, was repressed, whereas on the H-strand the *ND1* gene (but neither *ATP6* nor *COX1*) was significantly activated [6]. Last, several factors, including cell type [2–4,7], differentiation state [2], age [3], and disease state [5,7], have been correlated with the pattern or level of mtDNA methylation and/or hydroxymethylation. For example, in Down's syndrome patients the average mtDNA methylation level was found to be almost halved compared with healthy controls [13% versus 25% 5-methylcytosine (5mC)], a significant enough difference to justify further exploration of the involvement of mtDNA methylation in the mitochondrial dysfunction observed in these patients [5]. It is unclear, however, whether the reduced mtDNA methylation is the result or a consequence of this mitochondrial dysfunction.

mtDNA methylation: an overlooked layer of regulation?

As in the nucleus, DNMTs and DNA hydroxylases seem to be important in the regulation of mtDNA methylation [2,3,6,7]. DNMT1/3A/3B triple knockout embryonic stem cells show a decrease in CpG, and to a lesser extent non-CpG, methylation of the mtDNA, suggesting that additional enzymes are involved in mtDNA methylation [2]. Moreover, in the nucleus DNMT3L binds unmethylated histone 3 lysine 4, guiding DNMT3A/3B to the DNA [10]. However, this process is not expected to occur in the mitochondria for several reasons: mitochondria lack histones and mtDNA methylation levels remained unchanged in DNMT3L$^{-/-}$ knockout mouse oocytes [11]. Interestingly, on knock down of DNMT3L, the main nuclear target of DNMT3A and DNMT3B shifts from CpGs to non-CpGs, resulting in non-CpG hypermethylation [12]. In contrast to the low level of non-CpG methylation in the nucleus of wild type cells, bisulfite sequencing of the mitochondrial D-loop revealed high levels of non-CpG versus CpG methylation in human blood samples, tumor, and primary cell cultures [2]. This difference might be explained by the lack of DNMT3L in the mitochondria, which could result in DNMT3A/3B targeting non-CpG sites in the mitochondria, similar to the situation in the nucleus in the DNMT3L knockout.

To date, only associations support the functional relevance of mtDNA methylation [2–4,7] and no mechanistic studies have been reported to confirm causality. Here we propose mechanisms that might explain how mtDNA methylation can affect the regulation of mitochondrial transcription.

Depending on the ratio between TFAM and POLRMT/TFB2M, transcription preferentially occurs from LSP (intermediate ratio), HSP1 (high ratio), or HSP2 (low ratio) [8]. However, this information has been obtained in artificial systems and therefore may reflect the *in vivo* situation only for unmethylated mtDNA. Methylation of mtDNA might affect the binding of TFAM to the DNA either directly or indirectly (see below), impacting the relative activity of LSP, HSP1, and HSP2. Modulation of the TFAM-to-POLRMT/TFB2M ratio enables the cell to focus more on mitochondrial biogenesis (LSP, HSP1) or maintenance of the electron transport chain (HSP2).

Interestingly, TFAM is a member of the high-mobility group (HMG) proteins, a class of proteins that can be post-translationally modified, similar to histones [13]. In line with the effect of DNA methylation in the nucleus, mtDNA methylation may attract proteins that post-translationally modify TFAM [14], as observed for histones in the nucleus. As a result, these post-translationally modifications might modulate the DNA affinity of TFAM and its role in mtDNA compaction and bending [15]. As a consequence, the mtDNA might become more compact and less accessible for proteins such as POLRMT and TFB2M. Therefore, regional promoter methylation of the mtDNA might affect the outcome of the 'general rules' established from studies using artificial systems [8].

Although interaction with TFAM offers a possible mechanism by which methylation could influence transcription, by definition epigenetic regulation involves heritable changes that affect genomic function. In the nucleus the function of DNMT1 is coupled to DNA replication and thus is mitotically stable, whereas in the mitochondria mitotic inheritance is unclear. In the mitochondria, L-strand-specific methylation was found in the mitochondrial D-loop [2], indicating that the methylation pattern might not be mitotically stable by similar mechanisms as in the nucleus. However, this could also indicate DNA replication-coupled gene regulation mediated by methylation whereby the hemimethylated DNA (during DNA replication) relieves the repression mediated by fully methylated DNA, as has been observed for certain promoters in bacteria. In line with this, TFAM might bind to the promoter regions within the D-loop and thereby prevent their fast remethylation,

Table 1. Current methods used to detect mtDNA methylation

Detection method	Resolution	Distinguishes	Mechanism to distinguish C/5mC/5hmC	Total DNA pool versus mitochondrial purification	Pitfalls	Solutions/ minimize drawbacks	Refs
Bisulfite sequencing	Single base pair, site specific (target amplicon), DNA strand specific, single DNA molecule	C versus 5mC/ 5hmC	Sodium bisulfite treatment	Total DNA pool	Nuclear DNA contamination, incomplete bisulfite conversion, DNA degradation, PCR jackpot effect[a], cloning preferences, underrepresentation of 5hmC regions[b]	Multiple independent experiments, distinguish random versus nonrandom events, select unique mtDNA regions or determine contribution of NUMTs (ρ_0 cells)	[2,4]
Pyrosequencing	Average all DNA molecules						[7]
5mC/5hmC DIP[c]	Average of target amplicon, site specific (target amplicon), average all DNA molecules	C versus 5mC versus 5hmC	Affinity enrichment	Total DNA pool (when combined with mitochondria-specific amplification; e.g., qPCR) or mitochondrial purification	Nuclear DNA contamination	Select unique mtDNA regions or determine contribution of NUMTs (ρ_0 cells), determine antibody background on unmethylated DNA	[2,4,6]
LC–ESI–MS/MS	Average whole mtDNA, average all DNA molecules	C versus 5mC versus 5hmC	Mass-to-charge ratio	Mitochondrial purification	Nuclear DNA contamination, incomplete DNA hydrolysis	Confirm purity of mitochondria, add abundance of endonucleases for a sufficient time	[5]
5mC/5hmC ELISA	Average whole mtDNA, average all DNA molecules	C versus 5mC versus 5hmC	Affinity enrichment	Mitochondrial purification	Nuclear DNA contamination	Confirm purity of mitochondria, determine background signal on unmethylated DNA	[3]
Methylation-sensitive/ dependent restriction	Single base pair, (restriction) site specific, average all DNA molecules, (potentially) DNA strand specific	C versus 5mC	Differential endonuclease digestion	Total DNA pool (when combined with mitochondria-specific amplification; e.g., qPCR) or mitochondrial purification	Nuclear DNA contamination, incomplete digestion	Add abundance of endonucleases for a sufficient time, determine background signal on unmethylated DNA	Pioneering studies (reviewed in [1]) [6]
Glucosyl-sensitive/ dependent restriction		C and 5mC versus 5hmC	DNA glucosylation followed by differential endonuclease digestion		Incomplete *in vitro* methylation[d] (when combined with methylation-dependent digestion)		[3,6]

[a]Errors that arise during the early stages of PCR are amplified exponentially and thus can make a large contribution to the end analysis.

[b]The product of bisulfite-converted 5hmC halts DNA polymerase, resulting in less amplification of 5hmC regions.

[c]DNA immunoprecipitation.

[d]Methylation-dependent restriction endonucleases specifically digest methylated sequences and can be used to distinguish between C and 5(h)mC. To further distinguish between 5mC and 5hmC, all DNA can be *in vitro* methylated and glucosylated. Subsequent digestion with methylation-dependent restriction endonucleases results in only 5hmC sites being undigested.

enabling transcription. Further work will be necessary to determine whether these mechanisms are at work in mitochondria.

Concluding remarks: the impact of mtDNA methylation

Based on the above, it is tempting to view mtDNA methylation as an unexplored mitochondrial response mechanism for a cell to cope with changing environments. Moreover, the reversible nature of epigenetic modifications opens new avenues to modulate the mitochondrial response in health and disease.

To truly understand the impact of mtDNA methylation, it will be essential to identify all of the players involved and disentangle cause from consequence for this modification, a debate that is ongoing for methylation of the nuclear genome. Innovative technologies such as epigenetic editing

provide tools to induce locus-directed mtDNA methylation and hydroxymethylation and such efforts will greatly aid our understanding of mitochondrial epigenetics and its role in many physiological and pathophysiological processes, ranging from aging to metabolism and from cancer to neurological disease [5,7,9].

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