Unravelling the molecular mechanisms underlying mitochondrial dysfunction in metabolic diseases

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CHAPTER TWO

Regulation of mitochondrial gene expression, the epigenetic enigma

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1.0 ABSTRACT

Epigenetics provides an important layer of information on top of the DNA sequence and is essential for establishing gene expression profiles. Extensive studies have shown that nuclear DNA methylation and histone modifications are associated with nuclear gene expression levels. However, it remains unclear whether mitochondrial DNA (mtDNA) undergoes similar epigenetic changes to regulate the expression of mitochondrial genes. Recently, it has been shown that mtDNA is differentially methylated in various diseases such as diabetes, colorectal cancer and non-alcoholic steatohepatitis. Interestingly, this differential methylation was often associated with altered mitochondrial gene expression. However, the direct role of mtDNA methylation on mitochondrial gene expression is yet to be ascertained. Alternatively, the activity of the mitochondrial transcription factor A (TFAM), a protein involved in mtDNA packaging, might influence mitochondrial gene expression. In this review we discuss the role of mtDNA methylation and potential epigenetic-like modifications of TFAM with respect to mtDNA transcription and replication. We suggest three mechanisms: (1) methylation within the non-coding D-loop, (2) methylation at gene start sites (GSS) or within gene bodies and (3) post-translational modifications (PTMs) of TFAM. Unraveling mitochondrial gene expression regulation could open new therapeutic avenues for diseases associated with dysfunctional mitochondria.

2.0 INTRODUCTION

2.1.1 Mitochondrial DNA

Mitochondria are vital in driving the cell's metabolic activity as they are responsible for producing the bulk of the cell's energy requirements in the form of ATP, maintaining calcium homeostasis and inducing apoptosis [1-3]. In the mitochondria, ATP is generated through the process of oxidative phosphorylation (OXPHOS), which occurs via the electron transport chain (ETC). Interestingly, with the exception of chloroplasts in plants, mitochondria are the only organelles that contain their own genome (mitochondrial DNA [mtDNA]). Each mitochondrion contains about 1-10 copies of mtDNA [4,5]. MtDNA is distinctly different from the nuclear DNA (nDNA) (Table 1)[6-8]. In part, this can be explained by the endosymbiotic theory, which states that mitochondria evolved from alphaproteobacterium that invaded eukaryotic cells [9-11]. Indeed, similar to DNA of prokaryotic cells such as bacteria, mtDNA is a circular, double-stranded DNA molecule of approximately 16 kb in size [12]. The mtDNA comprises a heavy (H) strand and a light (L) strand which encode 13 of the polypeptides that constitute the Complexes I, III, IV and V of the ETC [13]. MtDNA also encodes some of its own transcriptional and translational machinery, which includes 22 tRNAs and 2 rRNA [11,13,14]. However, it is important to note that, despite harboring their own genetic material mitochondria are heavily dependent on the expression of nDNA which encodes the bulk of mitochondrially localized proteins [14]. Not much is known about the complex coordination that exists between the nucleus and the mitochondria. It is known that gene expression in the nDNA is meticulously regulated via different mechanisms, including epigenetic modifications, transcriptional and post-transcriptional regulation. However, it remains elusive whether mtDNA adheres to the same principles.

In this review, we start with an overview of the current evidence supporting the presence and functionality of mtDNA methylation and another epigenetic-like modification: the PTMs of TFAM. Subsequently, we will highlight studies in which differential mtDNA methylation was reported to occur in diseases. Finally, based on the literature reviewed, we put forward hypotheses on how these phenomena may contribute to mtDNA replication and transcription.

Table 1. Differences between human nuclear DNA and mitochondrial DNA

Feature	Nuclear DNA	Mitochondrial DNA
Size (in bp)	~3 x 10^9	16,569
Shape	Linear double helix	Circular double helix
Inheritance	Both parents	Maternal
DNA copies/cell	2	~10-50,000
Number of genes	~20,000 protein coding	13 protein-coding + 24 non-protein coding
Gene density	~1 in 40,000 bp	1 in 450 bp
Introns	Found in almost every gene	Absent
% coding DNA	~3%	~93%
Histones	Associated with the DNA	Not associated with the DNA
CpG islands	24,000-27,000	None
CpG density	1%	2.6%
Methylation	Present (3-4% of all Cs [~70-80% of all CpGs]) (mainly CpG)	Present (~1.5-5% of all Cs) (both CpG and CnonG)
Hydroxymethylation	Present (0.03-0.69% of all Cs)	Present [6-8]

2.1.2 MtDNA transcription and replication

Before we discuss how mtDNA methylation may influence mtDNA transcription and replication, we will first explain what is already known about these processes in mitochondria. Unlike the nDNA, which contains at least one promoter region per gene, the mtDNA contains only three promoter regions that transcribe multiple genes at once to produce polycistronic transcripts. The L-strand is transcribed from the L-strand promoter (LSP), whereas the H-strand is transcribed from the H-strand promoters 1 and 2 (HSP1, HSP2) (Figure 1). The HSP1 enables the transcription of 12S and 16S ribosomal RNAs while the HSP2 promotes transcription of the entire H-strand as a polycistronic transcript (see Figure 1) [15]. For transcription the mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factor A (TFAM) and dimethyl

adenosine transferase 2, mitochondrial protein (TFB2M) assemble at the promoters to initiate the synthesis of polycistronic RNAs that are later processed into single mRNAs [16,17]. These promoters are located within or in the vicinity of a 1 kb locus, known as the mitochondrial displacement loop (D-loop) or mtDNA non-coding region (NCR).Besides the promoters, the D-loop also contains the origin of replication of the H-strand (O_H). The L-strand origin of replication (O_L) lies outside the D-loop (see Figure 1). The D-loop has a peculiar triple helix structure consisting of the L- and H-strand plus an additional 7S DNA primer, which forms the third nascent DNA strand [13]. The replication of mtDNA is a rather complex process and three models have been proposed to explain the mechanism of mtDNA replication (Reviewed by Holt *et al.,* and Nicholls, *et al.,*) [13,17]. In one of the models, it is hypothesized that the 7S DNA may play a pivotal role in mtDNA replication. Herein, according to this model, H-strand replication is initiated at the LSP leading to the synthesis of 7S RNA [13,18]. In the presence of the mitochondrial DNA polymerase-gamma (POLG), the newly-formed 7S RNA then primes the synthesis of the H-strand [13,18]. Even though the functions of the D-loop are still under debate, it is widely accepted that this structure facilitates mtDNA replication by maintaining an open structure [18]. This makes the D-loop a likely candidate for epigenetic modifications, which can also have a major impact on the expression of mitochondrial genes (See 3.2).

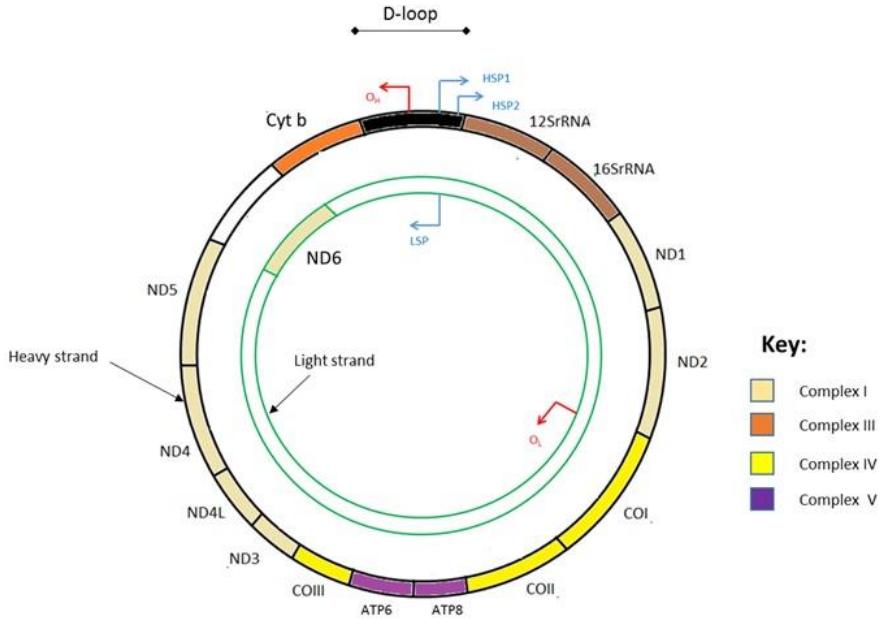

Figure 1: Simplified diagram of human mitochondrial DNA

2.2. Epigenetics and DNA methylation

Epigenetics refers to the heritable changes in gene expression that do not involve alteration of the DNA sequence itself. As such, epigenetics provides the basis explaining why different cell types have different gene expression profiles have despite having the same genetic material. There are various mechanisms by which epigenetic gene expression regulation can be achieved and these involve covalent modifications of either the DNA or proteins associated with the DNA, that is, histones in the case of nDNA, which may influence chromatin remodeling. The field of epigenetics has rapidly evolved over the past 50 years with the development of robust, single base-pair resolution techniques, such as whole genome sequencing, to study this phenomenon [19].

DNA methylation involves the addition of a methyl group on the cytosine base giving rise to 5-methylcytosine (5mC). In mammalian nDNA, 5mC frequently occurs on CpG sites (cytosine base preceding a guanine base). Furthermore, these CpG sites frequently occur in clusters of about 1 kb, often surrounding transcription start sites,

known as “CpG islands” [20]. Interestingly, while hypermethylation within promoter regions has been shown to correlate with low gene expression, methylation within gene bodies is associated with actively transcribed genes [21]. During early embryonic development, CpG methylation patterns are formed by the *de novo* methyltransferases, DNMT3A and DNMT3B, whereas maintenance of these methylation patterns is carried out by the maintenance methyltransferase, DNMT1. Moreover, in some cases, DNA also undergoes demethylation, which can occur either passively during DNA replication when maintenance methylation by DNMT1 fails or actively by the action of Ten-eleven translocation (TET) enzymes [22]. TET-induced DNA demethylation occurs through the interesting, yet less well studied, DNA modifications, 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC).

3. IS MITOCHONDRIAL GENE EXPRESSION REGULATED BY A MITOCHONDRIAL EPIGENETIC LAYER?

3.1. The presence of mtDNA methylation

One of the leading questions with respect to the regulation of mitochondrial gene expression is whether epigenetic-like phenomena are present on the mtDNA and if such mechanisms indeed affect mitochondrial gene expression. To date, controversy still surrounds the notion of mtDNA methylation (Table 2). The discovery of the mitochondrial-localized DNA methyltransferase 1 (mtDNMT1) and the presence of mtDNA methylation by Shock, *et al.,* [23] sparked the debate on the role of methylation and epigenetic-like mechanisms in regulating mitochondrial gene expression. Indeed, the discovery of the mtDNMT1 suggests that mtDNA undergoes methylation, contrary to earlier studies that disputed mtDNA methylation (see Table 2). The debate on the existence of mtDNA methylation stems from the early studies, which observed only low levels (<5%) of methylation [24-27], if any at all [28-30]. It was unclear whether the observed methylation was merely an artefact, arising from the incomplete separation of the mitochondrial DNA from the nuclear DNA or whether the absence of methylation was a consequence of the use of insensitive detection techniques [19]. Interestingly, recent advanced single base pair resolution sequencing studies showed compelling evidence that mtDNA can be modified by methylation and even hydroxymethylation of

mtDNA (see section 4, Table 2 and reviews by Manev *et al.,* 2012 and van der Wijst *et al.,* 2015 [19,31]).

Table 2. Summary of mtDNA (hydroxy) methylation

mtDNA methylation?	**Source of mtDNA**	**Detection technique**	**Investigated area: Specific region vs whole mtDNA**	**Differentially methylated locus**	**Ref**
Yes: 10-23% CpG 17-35% non-CpG,	-Human and mouse blood samples - Human skin fibroblasts, - HeLa, -143B.TK2 cells -3T3-L1 cells -Mouse embryonic stem cells	- BS-seq - Me/hMeDIP	Specific regions: D-loop	**D-loop**: -Only L-strand CpH methylation	7
Yes: 0.03-0.07% 5hmC, 0.1-0.3% 5mC	-primary murine cerebellar granule neurons - Murine purkinje cells	-5mC/5hmC ELISA -DNA glucosylation + MSRE + qPCR	Whole mtDNA + Specific regions: D-loop, *MT-ND2, 4, 5, MT-CO1, 3,* tRNAs	**Gene body and D-loop:** - *MT-ND2, MT-ND5* -D-loop	8
Yes:	-HUVEC (senescent vs replicative ECs)	-BS-seq	Specific regions: D-loop and *MT-CO1*	**D-loop:** -D-loop	18
Yes: 10-20 fold enrichment of 5mC and 85-580 fold enrichment of 5hmC	-primary embryonic fibroblasts (mouse) -HCT116	- Me/hMeDIP -DNA glucosylation + GlaI-seq	Specific regions: D-loop, *12S* and 16S rRNA, MT-*CO2, ATP6*	**Gene body**: - 12S rRNA	23
Yes:	- Beef heart	- TLC-UV/Vis	Whole mtDNA	ND	24

3.15% CpG methylation					
Yes: 2-5% of the CpG sequences is methylated	-Skin fibroblasts	- MSRE	Specific regions: (CCGG sites)	ND	[25]
Yes: 3-5% of the CpG sequences is methylated	- Ltk$^-$aprt$^-$ mouse cells (fibroblastoid cell line)	- TLC-RAD - MSRE	Whole mtDNA + Specific regions: (CCGG sites)	**Gene body**: -12S and 16S rRNA region (*MT-RNR1* and *MT-RNR2)*	[26]
Yes: 0.2-0.6% of all Cs is methylated	- Mouse fibroblasts - BHK21/C13, C13/B4, PvY cells	- TLC-RAD	Whole mtDNA	ND	[27]
No:	-Frog ovary -HeLa cells	- TLC-RAD	Whole mtDNA	NA	[28]
No	- Gastric cancer tissue -Colorectal cancer tissue	- BS-seq -BS-PCR-SSCP	Specific regions: *16S* rRNA, *MT-CO1, 2*	NA	[29]
No:	- Yeast - *Neurospora crassa* - Calf cells - Rat cells	- MSRE	Specific regions: (CCGG sites)	NA	[30]
Yes:	-human liver samples (from NASH patients)	-MSP	Specific regions: *MT-ND6, MT-CO1* and D-loop	**Gene body**: *-MT-ND6*	[33]
Yes:	-Colorectal cancer tissue (Human and rat)	-MSP	Specific regions: D-loop	**D-loop** - hypomethylated	[35]
Yes:	-Human blood samples	-MSP	Specific regions: D-loop	**D-loop** - hypermethyla	[36]

4.6- 5.2 fold increase in 5mC	(obese vs lean subjects)			ted in obese subjects	
Yes: CpG and non-CpG methylation	-Human brain tissue (AD patients) -Mouse brain tissue (AD and PD)	- 454 GS FLX Titanium pyrosequencer -hMeDIP	Specific regions: D-loop	**D-loop:** - hypermethylated in AD -not methylated in PD	[37]
Yes: 0-95% methylation at specific positions	-embryonic stem cells -Primary breast cells -PBMCs, $CD4^+$ and $CD8^+$ -Brain/neuronal cells -Penis cells	- WGBS -MeDIP-Seq	Whole mtDNA	**Gene body/GSS and D-loop**: - *MT-ND2, MT-ND4, MT-ND5, MT-ND6, CYB* genes, -P_H region -Spatio-temporal differences in gene start sites	[38]
Yes: 25% 5mCs of all Cs in healthy controls (vs 13% in Down Syndrome patients).	- Epstein-Barr virus-immortalized lymphoblastoid cell	-LC-ESI-MS/MS	Whole mtDNA	ND	[58]
Yes	Tumour samples from colorectal cancer patients	-MSP	Specific regions: D-loop	**D-loop:** - hypomethylated compared	[61]

				to non-cancerous tissue	
Yes: 0.92-18.53% CpG	Human platelets (from CVD patients)	-Pyro-seq	Specific regions: *MT-CO1, 2 & 3, MT-TL1 , MT-ATP6 & 8, MT-ND-5*	**Gene body**: *MT-CO1, 2 & 3, MT-TL1*	[62]
Yes: 2-6% 5mC	Human blood samples (18-91 year old female subjects)	-Illumina NGS	Specific region: *MT-RNR1*	**Gene body:** *MT-RNR1*	[63]
Yes: >10% 5mC	Human blood samples (38- 107 year old male and female subjects)	-BS-seq	Specific regions: *MT-RNR1* and *MT-RNR2*	**Gene body:** *MT-RNR1*	[64]
Yes <2% CpG methylation	-Human blood samples from people exposed to metal-rich paticulates	-Pyro-seq	Specific regions: D-loop, 12S rRNA, tRNA-F	**D-loop** -D-loop	[68]
Yes: ~1%-14% CpG	- Mouse blastocysts - Embryonic stem cells	- BS-seq	Whole mtDNA	ND	[69]
Yes: 1.6%-6.5% CpG	- White blood cells	- Pyro-seq	Specific regions: 12S rRNA, tRNA-F, D-loop	**Gene body**: -12S rRNA and tRNA-F region	[70]
Yes: 5mC (0.2-0.4% of the input) 5hmC (0.05-0.15% of the input)	- Liver neonatal pigs	- Me/hMeDIP	Specific regions: D-loop	ND	[71]
No: 0.2-0.8% CpG,	- HEK293 (embryonic kidney) cells	- WGBS	Whole mtDNA + Specific regions	NA	[72]

0.08-1.01% non-CpG	- HCT116 (colon cancer) cells - Leukemia - Healthy blood cells (whole blood, PBMCs, B-cells, $CD4^+$ or $CD34^+$) - Healthy and cancerous breast cancer cells				

Abbreviations:

HUVEC, Human Umbilical Vein Endothelial Cells**; 5hmC**, 5-hydroxymethylcytosine**; 5mC**, 5-methylcytosine; **GlaI -Seq**, restriction endonuclease GlaI coupled with sequencing; **ATP6**, ATP synthase F0 subunit 6; **BS-seq**, bisulfite sequencing; **WGBS**, Whole genome bisulfite sequencing; **BS-PCR-SSCP**, Bisulfite-PCR-single-stranded DNA conformation polymorphism (SSCP) analysis; **NGS,** Next generation sequencing; **COII**, cytochrome *c* oxidase subunit II; **CYB**, cytochrome B; **CpG**, C-phosphate-G dinucleotide; **CpH**, C-phosphate-(A/C/T) dinucleotide; **CSB**, conserved sequence block; **Me/hMeDIP**, 5mC/5hmC DNA immunoprecipitation; **LC-ESI-MS/MS**, liquid chromatography-electrospray ionization-tandem mass spectrometry; **NA**, not applicable; **ND**, not determined; **ND2/4/5/6**, NADH-ubiquinone oxidoreductase chain 2/4/5/6; **PBMC**, peripheral blood mononuclear cells; $\mathbf{P_H}$, promoter region H-strand; **rRNA**, ribosomal RNA; **TLC-UV/Vis/RAD**, thin-layer chromatography/ultra violet spectrometry/radioactivity detection. **MSP,** Methylation specific PCR. **MSRE**, Methylation-sensitive restriction enzymes; **CVD**, cardiovascular disease, **AD,** Alzheimer's disease; **PD,** Parkinson's disease

The absence of CpG islands in mtDNA is a strong argument that has been used to refute the idea that mtDNA undergoes functional methylation. Intriguingly, studies have shown that despite the absence of CpG islands, methylation within the D-loop and other loci within the mtDNA correlates with the expression of mitochondrial genes [32,33]. Besides CpG methylation, it has also been shown that mtDNA exhibits a peculiar non-CpG methylation pattern (CpC, CpA and CpT) which is characteristic of prokaryotic genomes such as those of bacteria [7,12]. In essence, this points to the fact that methylation (5mC and 5hmC, CpG and non-CpG) occurs within the mtDNA.

3.2. Specific subregions with importance for functional methylation

The mitochondrial D-loop is unarguably one of the most important regions on the mtDNA due to its central role in transcription and replication. Apart from housing the promoters (LSP, HSP1 and HSP2), the D-loop also contains three conserved sequence blocks (CSB I, CSBII and CSBIII). Of these three CSBs, it has been reported that CBSII is particularly important for transcription termination and 7S DNA primer formation [34]. Differential methylation within the D-loop has been reported in many studies now although none have been able to ascribe a precise function for it [7,18,35,36]. Since the D-loop plays an important role in mtDNA replication and transcription, it is likely that methylation of this region would influence mtDNA gene expression, either directly or indirectly, via modulation of the mtDNA copy number (Figure 2). A recent study by Bianchessi *et al.,* [18] described D-loop methylation in replicative and senescent endothelial cells (ECs) and identified an uneven distribution of 5mC (both CpG and non-CpG) between the L-strand and the H-strand. Moreover, they found that methylated sites within the D-loop have a tendency to form clusters. Interestingly, on average, the frequency of methylation on the H-strand was found double compared to the L-strand. These findings concur with an earlier study by Bellizzi *et al.,* [7] demonstrating that non-CpG methylation frequently occurs within CSBs. However, these two studies disagree as Bianchessi observed that CpA methylation occurs most frequently and CpC methylation occurs the least whereas Bellizzi reported that CpC methylation is dominant. Moreover, Bellizzi only detected methylation at the L-strand and not on the H-strand. In contrast, several studies have shown that both strands are methylated within the D-loop [18,32,35,37]. In retrospect, these differences may have arisen from the different species or cell types that have been used for the various studies. It is interesting to see whether the presence of non-CpG methylation within CSBs affects 7S DNA formation, which in turn may affect mtDNA replication.

Next to 5mC, also 5hmC was reported to be present in the mitochondrial DNA [6,7,23,38]. In two recent studies by Ghosh *et al.,* it was shown that 5mC marks are enriched in regions upstream of the gene start site (GSS) and within gene bodies while 5hmC marks cluster near the GSS rather than in the coding regions (gene bodies) of mitochondrial encoded genes [6,38]. Furthermore, in one of the studies they analyzed data sets from human brain mtDNA and showed that progressive reduction in 5mC

across GSSs correlates with the development stage of the brain [38]. Indeed, this spells out a possible epigenetic function of GSS methylation. However, the presence of 5hmC around the GSS did not correlate with gene expression [6]. This leaves 5mC as the most likely candidate to test whether GSS methylation influences gene expression. Apart from D-loop and GSSs, gene bodies might be important subregions where methylation may have a profound effect on gene expression. In the nDNA, it is known that 5mC in gene bodies is associated with actively transcribed genes [21]. In mtDNA, differential gene body methylation, mostly 5mC, has indeed been shown to correlate with changes in mitochondrial gene expression and the progression of diseases such as non-alcoholic steatohepatitis (NASH) (see 4.1).

3.3. The role of TFAM and other proteins directly associated with mtDNA

For nDNA, histone modifications are important epigenetic mechanisms that influence gene expression. However, in the mitochondria, histones are not associated with mtDNA and therefore mtDNA cannot undergo histone-mediated gene expression regulation [39]. Interestingly, despite lacking histone proteins, the mtDNA is not naked; the mtDNA is clustered in protein-DNA complexes called nucleoids, of which the main constituent, TFAM, is thought to entirely coat the mtDNA [5,40]. TFAM is a versatile protein located in the mitochondrial matrix and it is thought to have a histone-like function [40]. It is responsible for packaging and organizing the protein-mtDNA complex [5,40,41], a role that is played by histones in the nucleus. In addition to packaging the mtDNA, experimental evidence shows that TFAM promotes the replication, transcription and general maintenance of mtDNA [42]. It binds mtDNA to initiate the transcription at the LSP and HSP1. On the other hand, it has been shown that HSP2 transcription is independent of TFAM, but rather depends on POLRMT and TFB2M [43]. However, it has been described that TFAM has a dual function on HSP2 whereby it can activate or repress HSP2 transcription depending on the TFAM: TFB2M/POLRMT ratio [43,44]. TFAM appears to competitively repress HSP2, but its activity is diminished when the concentrations of TFB2M and POLRMT are high[44]. In addition, Ngo and colleagues observed that the binding of TFAM to mtDNA generated a U-shaped bend, which is essential for both mtDNA compaction and transcriptional activation [40]. Moreover, it has been shown that the accessibility of different sites on the mtDNA depends on the levels of TFAM occupancy, that is, regions with high TFAM occupancy

are less accessible to DNMTs and hence they are difficult to methylate [45]. This suggests that TFAM activity plays a role in determining the methylation pattern of mtDNA. Therefore, it is plausible that PTMs of TFAM may alter its binding to mtDNA, and thus, indirectly alter the methylation status of the mtDNA.

In the nDNA, histone PTMs, such as acetylation and methylation, are important in epigenetic regulation of gene expression by changing the chromatin state (open versus closed). Interestingly, PTMs such as acetylation, phosphorylation and ubiquitination have been reported for TFAM [46-48]. Of these three PTMs, TFAM phosphorylation has indeed been shown to impair TFAM binding activity. Unfortunately, the study did not assess the effects of TFAM phosphorylation on mitochondrial gene expression [48].This brings into question, whether TFAM phosphorylation or any other PTM actually has an influence on TFAM activity, translating to modulation of gene expression. Besides TFAM, other important factors that are associated with nucleoids include the POLG, the mitochondrial DNA helicase Twinkle and the mitochondrial single-stranded DNA binding protein (mtSSB). While TFAM is involved in both mtDNA transcription and replication, POLG, Twinkle and mtSSB are thought to be only involved in mtDNA replication [49-51]. Due to its versatile functions, TFAM appears to be the most prominent mitochondrial protein whose activity may greatly influence mitochondrial gene expression.

4. DISEASES AND CONDITIONS ASSOCIATED WITH DIFFERENTIAL MTDNA METHYLATION

Recent studies, as described in more detail below (4.1-4.5), report an increasing number of diseases and conditions associated with changes in mtDNA methylation at various loci. This brings about the important question whether changes in mtDNA methylation are the cause for a disease or are a mere consequence of these diseases and whether this acts through gene expression regulation. At this stage it appears prudent to ask the question: does mtDNA methylation serve any purpose? In this respect, it is of importance to note that some studies have not been able to observe any correlation between differential mtDNA methylation and disease (see Table 2). Below, we highlight the diseases for which differential mtDNA methylation was reported and which thus might serve as model diseases to unravel the functional role of mtDNA methylation.

4.1. Metabolic disorders

Obesity, a leading metabolic disorder in developed countries, is associated with a higher risk of developing type II diabetes [52,53]. Understanding the different mechanisms that drive this intricate relationship is important to shed light on how metabolic disorders develop. Recently, a study showed that insulin signaling influences mtDNA methylation in obese human subjects [36]. Increases in mtDNA methylation at the D-loop were strongly associated with obesity (5.2-fold increase compared to lean controls) and insulin resistance (4.6-fold increase compared to insulin sensitive controls). Interestingly, the level of methylation increased at the D-loop region only and, importantly, this correlated with a decrease in mtDNA copy number. A general assumption with regard to the decrease in mtDNA copy number is that it can have an overall effect on mitochondrial gene expression and therefore lead to mitochondrial dysfunction [54]. From this study, it thus appears that D-loop methylation correlates with a decrease in mtDNA copy number, which may result in an overall cellular decrease of mitochondrial gene expression.

In addition, obese individuals are prone to liver diseases such as NASH [55,56]. NASH is characterized by triglyceride accumulation, hepatocellular damage and inflammation. Mitochondrial dysfunction contributes to the development of NASH due to disruption of lipid metabolism in the mitochondria. Recently it has been reported that mtDNA aggravates inflammation in NASH patients by activating the toll-like receptor 9 (TLR9) pathway [57]. Interestingly, a study by Pirola and colleagues showed that the *MT-ND6* region is about 20% more methylated in NASH patients compared to the patients who are at the initial stages of the disease. Importantly, an increase in *MT-ND6* methylation correlated with a decrease (>50%) in *MT-ND6* mRNA and protein expression [33]. Methylation was also measured in the D-loop and *MT-COI* but, although methylation could be detected in both diseased and healthy samples, these profiles did not correlate with NASH. The increase in methylation within the *MT-ND6* gene body was associated with progression of the disease condition. The ND6 protein is a subunit of the mitochondrial complex I, which is a vital component of the electron transport chain (ETC) during ATP production. As such, a change in *MT-ND6* expression may negatively impact on mitochondrial function, which includes lipid metabolism, and thus, contribute to the disease pathogenesis. This study presents an interesting notion with

respect to gene body methylation and gene expression regulation whereby methylation prevents gene transcription. This is contrary to the widely accepted notion for nDNA where gene body methylation is associated with actively transcribed genes [21]. However, it is important to note that the effect of methylation on gene expression might have been an indirect effect.

4.2. Neurodegenerative diseases (Down Syndrome, Alzheimer and Parkinson's disease)

In Down syndrome (DS) patients, the DNA methylome is known to be disturbed; in the nuclear genomes of DS subjects, global hypermethylation is observed [58-60], whereas the mitochondrial genomes are hypomethylated [58]. Interestingly, DS patients have been shown to harbor a higher risk of developing early onset Alzheimer's disease (AD). Mitochondrial dysfunction has been shown to correlate with the development of neurodegenerative disorders, such as AD and Parkinson's disease (PD). A recent study was carried out using postmortem brain tissue from AD patients [37]. In this study, only the D-loop was analyzed for methylation and it was observed that both CpG and non-CpG sites in the entorhinal cortex and substantia nigra of patients are methylated, whereas the authors could not detect methylation for healthy controls. In mouse models of AD, these dynamic methylation patterns were also observed. Interestingly, contrary to the observations in AD, the authors detected that the D-loop in a mouse PD model was not methylated in nearly all CpG and non-CpG sites. From these two contrasting observations it appears that mtDNA is differentially methylated depending on the disease. Mitochondrial dysfunction in neuronal tissue is associated with neurodegenerative diseases, hence, any factor that contributes to this dysfunction might have a key role in the disease initiation and progression. If mtDNA methylation has an effect on mitochondrial function then this propels it up as a potential candidate in the etiology and therapy of neurodegenerative diseases.

4.3. Colorectal cancer

Differential mtDNA methylation was recently reported for colorectal cancer in two studies by Feng *et al.,* [35,61]. In both studies, progressive hypomethylation of the D-loop was observed in colorectal cancer patients and this corresponded with an increase in mitochondrial *MT-ND2* expression. Intriguingly, in both cohorts, the D-loop methylation was reported much higher in healthy controls (80% and 81.5%) compared to colorectal

cancer patients (11.4% and 13.8%). In both studies, the demethylation of the D-loop was associated with increased expression of *MT-ND2* and an increased mtDNA copy number during both the initial stages of colorectal cancer as well as during its progression [35,61]. In a letter to the editor, Maekawa and colleagues reported absence of mtDNA methylation in the *MT-RNR2, MT-COI* and *MT-COII* loci in 15 cancer cell lines and in tissues (both malignant and healthy) from 32 patients with gastric cancer and 25 patients with colorectal cancer [29]. However, it is important to note that in this study, methylation within the D-loop was not assessed, nor mitochondrial gene expression levels. The data provided in these studies suggests that colorectal cancer may be associated with differential D-loop methylation, which affects gene expression similarly as in the nDNA. However, it still leaves some questions unanswered with regard to whether the changes in gene expression are a direct or indirect effect of D-loop methylation. As described earlier, the D-loop acts as the control region of the mtDNA where polycistronic transcription of the mtDNA is initiated. Based on this polycistronic transcription, altered gene expression would have been expected to occur on all the H-strand encoded genes. Unfortunately, in these studies, expression of other mitochondrial genes was not measured.

4.4. Cardio-vascular diseases

Recently it was shown that platelet-derived mtDNA is hypermethylated in cardiovascular disease (CVD) patients compared the healthy controls, regardless of their age, race or BMI [62]. Unlike most studies where researchers focus on the methylation state of the D-loop, the CVD study looked at methylation patterns within gene bodies. Significantly high levels of mtDNA methylation were only observed for *MT-COI* (18.53%), *MT-COII* (3.33%), *MT-COIII* (0.92%), and *MT-TL1* (1.67%) in patients with CVD compared to the healthy controls [62]. The occurrence of methylation within gene bodies suggests a potential mechanism of regulating gene expression in mtDNA, as was observed before in the *MT-ND6* locus of NASH patients. Based on the existing evidence, methylation of the mtDNA appears to somehow affect the expression of mitochondrial genes, but not in a clear manner.

4.5. Aging

Mitochondria have been implicated in many studies as major drivers of the aging process . In light of this notion, hypotheses such as the free radical theory, rate of living theory and mtDNA mutations hypothesis have been proposed to explain the role of mitochondria in aging. In a recent study, it was observed that two CpG sites located within the 12S ribosomal RNA gene (*MT-RNR1*) are differentially methylated and this correlates with aging [63]. This study confirms a previous study by D'Aquila et al, where they checked for methylation in *MT-RNR1* and *MT-RNR2*. In this study, mtDNA methylation levels up to 10% were observed within the *MT-RNR1* and this correlated with the age of the patients. Based on differential methylation within this gene, the age of 64.5% of patient samples was correctly predicted using a linear regression prediction model [64].Apart from being used as a marker for aging, methylation of mtDNA may provide new insights into the mechanisms that drive the aging process. However, it can be argued that as people age there is a manifestation of various diseases and conditions that may influence the mtDNA methylation state. Therefore, it is important to always include age-matched controls for disease association studies to avoid bias.

5. MTDNA METHYLATION AND DISEASES, A CAUSE OR CONSEQUENCE?

The examples described in section 4 indicate interesting associations between mtDNA methylation and disease, but the functional role of mtDNA methylation in the development of diseases remains an open question. The underlying question is whether mtDNA methylation contributes to gene expression dysregulation? While some studies open exciting options for mtDNA to be further explored as mechanisms underlying the "cause" of a disease, it is also possible that mtDNA methylation might be a mere consequence of the disease arising from the dysregulation of many vital metabolic pathways. According to the few epigenome association studies that have been reported on mtDNA methylation to date, the correlation between metabolic diseases (e.g., diabetes, NASH) and mtDNA methylation highlights its probable impact on metabolic pathways and/or *vice versa*. One of the pitfalls in studies aimed at deciphering the functional relevance of mtDNA methylation is the heteroplasmic nature of mtDNA. While methylation may be present at a particular location on a single mtDNA molecule, the probability that all the mtDNA molecules in the cell will have the same modification is uncertain. However, this may point to a possible physiological

thresh-hold above which methylation has a noticeable effect on mitochondrial function and or gene expression. Existing data suggests that mtDNA methylation may be linked to mitochondrial gene expression via one of at least three mechanisms: methylation within the D-loop, methylation at the GSSs or within gene bodies and post-translational modifications of TFAM.

Differential methylation within the mitochondrial D-loop correlates with different diseases and is associated with changes in mtDNA copy number [35-37,61]. It is possible that D-loop methylation may influence mitochondrial gene expression either directly by altering mtDNA transcription, or indirectly by modulating mtDNA copy number. It can be hypothesized that via the regulation of TFAM binding to promoter regions, D-loop methylation may alter transcription. Moreover, any factor that modulates mtDNA copy number might indirectly influence gene expression (Figure 2) [54]. An example of such a factor that may be influenced by D-loop methylation and may alter mtDNA copy number is the 7S DNA primer, as suggested by Bianchessi *et al.,* [18]. On the other hand, methylation on GSSs and gene bodies may potentially influence gene expression by affecting gene splicing. For instance, in nDNA, it has been reported that methylation within exons and splice sites regulates alternative splicing of mRNA precursors [65]. This may be an alternative function for GSSs and gene body methylation, although the absence of introns in mtDNA makes it less probable to undergo alternative splicing. However, based on evidence showing that mtDNA gene body methylation in some diseases correlates with altered gene expression it is possible that the polycistronic mitochondrial mRNA precursor undergoes unknown post-transcriptional modifications to influence gene expression.

TFAM binding to the mtDNA appears to play a crucial role in determining the accessibility of mitochondrial genes for other proteins by modulating mtDNA compaction. In the nDNA, compact heterochromatin is associated with repressed genes. This may be true for the mtDNA as well, whereby high levels of TFAM binding to the mtDNA are characteristic of compact mtDNA. There is indeed evidence that higher levels of TFAM increase mtDNA compaction and prevent methylation of CpG sites [40,45]. PTMs of TFAM may influence its binding to the mtDNA and as a consequence may modulate mtDNA compaction, mtDNA gene expression and

replication. Together with TFB2M and POLRMT, TFAM is an important player in mtDNA transcription. Therefore, proteomic studies aiming at assessing the interaction between post-translationally modified TFAM and these other proteins may reveal undiscovered pathways by which TFAM may influence mitochondrial gene expression.

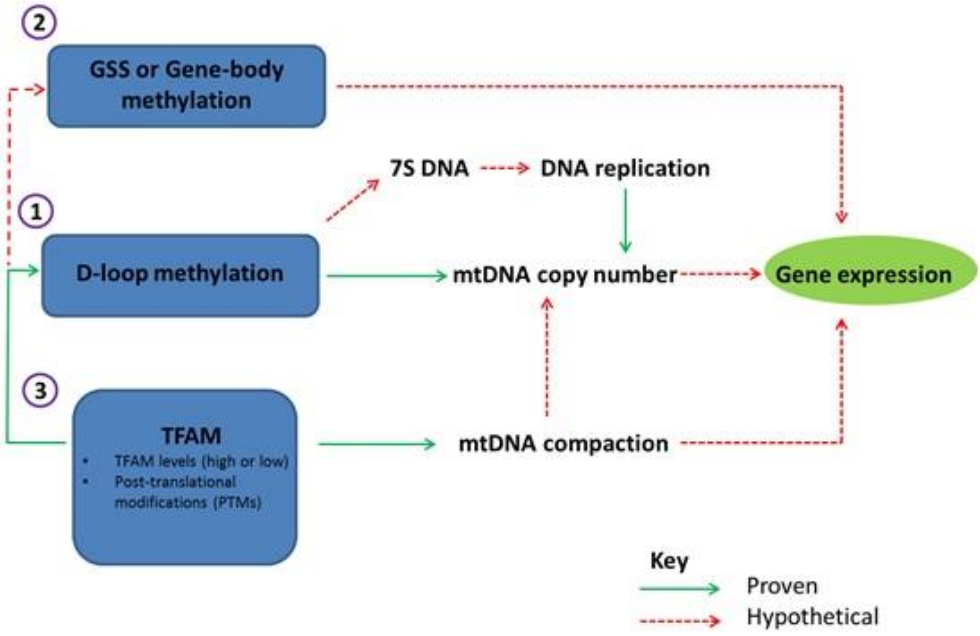

Figure 2: Proposed mechanisms of epigenetic regulation of mitochondrial gene expression

6. FUTURE PERSPECTIVES

To date, we have only scratched the surface of the topic on mitochondrial epigenetics and mitochondrial gene expression regulation. Whether mtDNA methylation contributes to disease pathogenesis by altering gene expression directly or indirectly remains a biological enigma that resembles the “chicken and egg” conundrum. As the debate rages on, it is important to understand the mechanisms that regulate transcription of mitochondrial genes. This assessment should take into account all epigenetic-like modifications on the mtDNA and its associated proteins. At present, even with the increasing number of studies on mtDNA methylation, it is difficult to attest that mtDNA methylation has a direct effect on gene expression. There is no clear mechanism that explains how this regulation on gene expression is achieved apart

from the association studies that have been carried out. However, taking into account the increasing evidence that supports the notion that mtDNA methylation correlates with gene expression, there is need to further test this hypothesis. In this respect, epigenetic editing provides potent tools to test whether mtDNA methylation influences gene expression [66,67]. Indeed, the mitochondrial targeting of epigenetic enzymes fused to DNA binding domains designed to bind a mtDNA locus of interest could unravel the actual effects of methylation on gene expression. If indeed mtDNA methylation has a profound effect on the expression of mitochondrial genes then its role in the development of different diseases could be clearly defined. Moreover, the reversibility of DNA methylation presents an opportunity for the discovery of novel drug interventions to treat diseases associated with mtDNA methylation. Unravelling the function of mtDNA methylation and other epigenetic-like phenomena, such as TFAM phosphorylation and acetylation, may open new insights in the field of mitochondrial epigenetics, which could further aid in drug discovery directed to prevent or even treat the wide variety of diseases, known to be associated with dysfunctional mitochondria.

7. ACKNOWLEDGEMENTS

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