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Cytosine methylation of mitochondrial DNA at CpG sequences impacts Transcription Factor A DNA binding and transcription

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Abstract

In eukaryotes, cytosine methylation of nuclear DNA at CpG sequences (^{5m}CpG) regulates epigenetic inheritance through alterations in chromatin structure. However, mitochondria lack nucleosomal chromatin, therefore the molecular mechanisms by which ^{5m}CpG influences mitochondria must be different and are as yet unknown. Mitochondrial Transcription Factor A (TFAM) is both the primary DNA-compacting protein in the mitochondrial DNA (mtDNA) nucleoid and a transcription-initiation factor. TFAM must encounter hundreds of CpGs in mtDNA, so the occurrence of ^{5m}CpG has the potential to impact TFAM-DNA recognition. We used biophysical approaches to determine whether ^{5m}CpG alters any TFAM-dependent activities. ^{5m}CpG in the heavy strand promoter (HSP1) increased the binding affinity of TFAM and induced TFAM multimerization with increased cooperativity compared to nonmethylated DNA. However, ^{5m}CpG had no apparent effect on TFAM-dependent DNA compaction. Additionally, ^{5m}CpG had a clear and context-dependent effect on transcription initiating from the three mitochondrial promoters. Taken together, our findings demonstrate that ^{5m}CpG in the mitochondrial promoter region does impact TFAM-dependent activities in vitro.

Keywords

mitochondrial DNA; CpG methylation; transcription; TFAM; DNA binding

INTRODUCTION

Reversible methylation at CpG sequences (^{5m}CpG) plays a crucial role in regulating nuclear gene expression via a variety of mechanisms, but the role ^{5m}CpG plays in mitochondria remains unclear. The existence of reversible methylation of mitochondrial DNA (mtDNA)

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SUPPLEMENTARY DATA

Supplementary data are available on BBA Online.

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has been a highly debated topic for five decades. In recent models of cancer, neurodegenerative diseases, diabetes and aging, reports consistently describe modest percentages of ^{5m}CpG (between 2–37%), in the mtDNA, in conjunction with altered levels of mitochondrial RNA transcripts and or mtDNA copy number [1–8]. These and other studies using bisulfite sequencing, restriction enzyme digestion, chromatographic analyses, ELISA and Methyl-DNA-immunoprecipitation support the existence of low levels of ^{5m}CpG in mtDNA (reviewed in [9–11]). However, other studies have raised concerns about the validity of the bisulfite sequencing approach, as only low levels of ^{5m}CpG were detected in mtDNA [12–16]. Yet the same studies that also used approaches other than bisulfite sequencing generally found low levels of ^{5m}CpG (between 2–10 %) [13, 16], which correspond to the levels of ^{5m}CpG in the literature supporting the existence of ^{5m}CpG in mtDNA. Additional evidence in support of mtDNA methylation comes from the identification of human DNA methyltransferases, DNMT1 [17], DNMT3A [18], DNMT3B [19], and human cytosine demethylation enzymes, including members of the Ten-Eleven Translocation (TET) family of methylcytosine dioxygenases, TET1 and TET2 [7, 20], in mitochondria or associated with the mitochondrial outer membrane. Thus, there is the need to consider the effects of ^{5m}CpG in the DNA-dependent processes of mtDNA.

CpG methylation in the nucleus participates in the formation of condensed nucleosomal arrays that create heterochromatin states that are important during development and epigenetic inheritance [21]. CpG methylation also has varied effects on direct protein-DNA recognition [22], with protein-DNA interactions that are either inhibited, promoted or unaffected [23]. Unlike nuclear chromatin, the mitochondria lack nucleosomes. Instead, the mitochondrial Transcription Factor A (TFAM, mtTFA) is the primary protective protein component of the mitochondrial nucleoid complex [24–30]. Additionally, TFAM is important for mtDNA maintenance [31], the mtDNA copy number [32, 33], as well as for coordinating mtDNA transcription with mtDNA replication (reviewed in [34]). In its role as a transcription factor, TFAM binds to each of three promoters, heavy strand promoters 1 and 2 (HSP1, 2) and the light strand promoter (LSP), which are located in the noncoding control region of the mtDNA known as the displacement loop (D-loop) (reviewed in [24]).

TFAM binds site-specifically to the mtDNA promoters and non-sequence specifically to the mtDNA, through interactions with the DNA minor groove, to produce a stable 180° bend, “U-turn” structure in the DNA (reviewed in [35]). As a transcription factor, TFAM binds to the three promoters LSP, HSP1 and HSP2, referred to here as the tri-promoter region (Figure 1), and aids in recruitment of the bacteriophage-related RNA polymerase (POLRMT) and the rRNA methyltransferase-related transcription factor 2 (TFB2M) to initiate transcription [11, 36]. TFB1M is an rRNA dimethyltransferase and has a primary role in modulating translation [37–39]. There are 435 CpG sites within the mtDNA including 5 CpGs in the tri-promoter region (Figure 1). ^{5m}CpG levels in the mtDNA, including the promoter region of the D-loop have been reported to be as little as >2% and as much 35% in various human cells lines and tissue samples [20, 40, 41]. Moreover, ^{5m}CpG has been observed in the promoter region, including in the HSP1 TFAM binding site as well the LSP and HSP2 promoters [8, 20, 42].

Due to the extensive direct DNA interactions of TFAM throughout the mtDNA, we hypothesized that ^{5m}CpG would directly impact TFAM-dependent molecular processes. To test this hypothesis, we investigated the effects of ^{5m}CpG in mtDNA on the DNA binding and transcription-initiation properties of TFAM in vitro. Using fluorescence resonance energy transfer (FRET) assays and electrophoretic mobility shift assays (EMSA), we observed increased affinity and cooperativity of TFAM for the ^{5m}CpG-HSP1 DNA. ^{5m}CpG did not significantly influence the ability of TFAM to compact plasmid-length DNA. However, run-off transcription assays revealed altered levels of transcription initiation for both the tri-promoter template and LSP-only templates. Thus, ^{5m}CpG has effects on TFAM DNA binding and transcription activities, but not DNA compaction, indicating that it impacts site-dependent binding properties of TFAM.

MATERIALS AND METHODS

Preparation of TFAM and DNA

Recombinant human TFAM (aa 42–246) was purified as previously described (Supplementary Figure S1A) [43].

Synthetic DNA was purchased from Integrated DNA Technologies (IDT) (See Supplementary Materials and Methods for sequences). EMSA experiments used HSP1 33 base pair (bp) DNA and 33 bp non-site specific (NS) DNA. For FRET assays HSP1 DNA included donor or acceptor fluorophores at the 3' end. For the LSP run-off transcription experiments, an 88 bp template (LSP88) comprised the DNA sequence 390–477 from human mtDNA. The oligonucleotides were synthesized with and without the ^{5m}CpG modification (IDT). Each oligonucleotide was purified using C18 Sep-Pak cartridges (Waters). To produce the 33 bp and 88 bp dsDNA fragments, with and without ^{5m}CpG, the complementary ssDNA strands were annealed by heating 95°C for 5 minutes and then cooling slowly to room temperature. The DNA was then purified using a DEAE anion exchange column (Tosoh Bioscience) with a gradient of 0 to 1 M NaCl in buffer (50 mM Tris-HCl (pH8.0) and 1 mM EDTA). The purity of each fraction was confirmed on nondenaturing gels (Supplementary Figure S1B–D).

For the compaction assays (EMSA and EM), the pGEMPTZLSP3 plasmid (pLSP3) containing human mitochondrial DNA sequence 242–825 [44] was expressed in Stellar Competent dam-/dcm- cells purchased from Clontech. The plasmid was methylated at the 5-position of cytosine of CpG sites using M.SssI methyltransferase (New England Biolabs) following the manufacturers protocol. The pLSP3 and ^{5m}CpG pLSP3 plasmids were then linearized using NdeI restriction enzyme (New England Biotech). The DNA was then purified by phenol and ether extractions followed by ethanol precipitation. The efficiency of CpG methylation was verified by digestion of pLSP3 with restriction enzymes HpaII and MspI (Supplementary Figure S2A).

For the run-off transcription assays, the pLSP3 plasmid was digested with EcoRI-HF (New England Biolabs) to generate the 603 bp “tri-promoter” template (human mtDNA sequence 242–825). The DNA was treated as above to generate the ^{5m}CpG 603 bp template. The 603

bp DNA fragments were then gel purified and treated as above. The efficiency of ^{5m}CpG incorporation was verified as above (Supplementary Figure S2B).

Fluorescence resonance energy transfer assays

FRET assays were performed essentially as previously described [45]. Briefly, we used a Horiba Fluorolog 3 spectrofluorimeter that was thermostated to 20 °C. Experiments were conducted with the fluorophore-labeled HSP1 and ^{5m}CpG-HSP1 DNA each at a concentration of 3.4 nM in FRET Buffer (50 mM HEPES-Na, pH 7.4, 150 mM NaCl, and 1 mM DTT). TFAM was added to the DNA samples at final concentrations of 0 nM, 0.63 nM, 1.25 nM, 1.88 nM, 2.5 nM, 3.13 nM, 3.75 nM, 4.38 nM, 5.00 nM, 5.63 nM, 6.25 nM, 6.88 nM, 7.50 nM, 8.13 nM, 8.75 nM, 10.0 nM, 12.5 nM, 15.0 nM, 20.0 nM, 25.0 nM, 30.0 nM, 35.0 nM, 40.0 nM, 45.0 nM and 50.0 nM. To measure the FRET induced by protein binding to the fluorophore-labeled DNA, we employed the enhanced sensitization of the acceptor method described in detail by Clegg [46]. The FRET effect (FE) = I490/I560, where I490 is the fluorescence emission of TAMRA at 580 nm when excited at 490 nm, and I560 is the fluorescence emission of TAMRA at 580 nm when excited at 560 nm. A plot of normalized FE vs. [TFAM] was used to calculate the TFAM-DNA apparent dissociation constants (K_{Dapp}) using the ligand-depletion binding model (Equation 1), where DNA is the DNA concentration and x is the total TFAM concentration:

$$Y = (B_{max}) * \left(\frac{(DNA + x + KD) - \sqrt{((DNA + x + KD)^2 - (4 * x * DNA))}}{2 * DNA} \right) \quad \text{Equation (1)}$$

Electrophoretic mobility shift assays

Quantitative EMSA was performed using the HSP1, ^{5m}CpG-HSP1, NS and ^{5m}CpG-NS DNA, described above. TFAM was added to the DNA samples (10 nM) in Binding Buffer (20 mM HEPES-Na (pH 7.5), 50 mM KCl, 2 mM MgCl₂ and 2 µg/ml BSA) to final concentrations of 0 nM, 1.0 nM, 2.0 nM, 3.0 nM, 4.0 nM, 5.0 nM, 6.0 nM, 7.0 nM, 8.0 nM, 9.0 nM, 10.0 nM, 15.0 nM, 20.0 nM, 25.0 nM and 30.0 nM and incubated for 45 minutes on ice. The EMSA used nondenaturing PAGE (8% acrylamide; 37.5:1 acrylamide:N,N-methylene-bis-acrylamide (BioRad)) with 1X TBE buffer (100 mM Tris, 100 mM boric acid, 2.5 mM EDTA). The gels were prerun at 70 V for 30 min prior to sample loading and then electrophoresed at 70 V for 1 h. The DNA was visualized using Sybr Green I dye (Invitrogen) on a Typhoon 9400 Imager (Molecular Dynamics). The intensity of each band was measured using ImageQuant Software, and after background subtraction, the fraction of DNA bound was calculated, fitted with the Hill equation (Equation 1) and graphed using Prism software.

Stoichiometric EMSA was performed using 100 nM 33 bp DNA (HSP1, ^{5m}CpG-HSP1, NS or ^{5m}CpG-NS DNA) and a range of concentrations of TFAM (0 nM, 50 nM, 100 nM, 200 nM, 300 nM, 400 nM, 500 nM, 600 nM, 700 nM and 800 nM). The reactions were

incubated on ice for 45 minutes and then loaded onto a pre-run 8% nondenaturing polyacrylamide gel (37.5:1, acrylamide:N,N-methylene-bis-acrylamide, (BioRad)) and electrophoresed for 45 minutes on ice. The DNA was visualized using Sybr Green I dye (Invitrogen) on a Typhoon 8400 Imager (Molecular Dynamics). The intensity of each band was measured using ImageQuant Software, and after background subtraction, the fraction bound to DNA was calculated and graphed using Prism software.

Electron microscopy and DNA compaction assays

Compaction reactions were conducted with 100 nM linearized pLSP3 or ^{5m}CpG-pLSP3 DNA. The 10 µL reactions in Binding Buffer contained TFAM at concentrations (0 nM, 60 nM, 90 nM, 120 nM or 240 nM) and were incubated at room temperature for 30 minutes. Each sample was split for use both in EMSA and EM. For electron microscopy, each sample was diluted 1:1 with 4 mM Mg-acetate and applied to glow discharged EM grids. Samples were then washed with 4 mM Mg-acetate before staining with 0.02% (wt/vol) uranyl acetate. Grids were imaged on a JEOL 1230 (or JEOL 1200) transmission electron microscope with a Gatan- digital camera at a magnification of 120,000x. For the compaction assays, each sample was electrophoresed on a 0.7 % agarose gel and then visualized using Sybr Green I dye (Invitrogen) on a Typhoon 9400 Imager (Molecular Dynamics). The migration distance of each band was measured using ImageJ software. These values were analyzed by subtracting the migration distance of the free DNA from those of the TFAM:DNA complexes.

In vitro run-off transcription assays

Transcription reactions were performed essentially as in [44, 45, 47]. The samples contained the tri-promoter 603 bp template DNA (3.4 nM) in Reaction Buffer (10 mM Tris-Cl pH8.0, 10 mM MgCl₂, 100 µM DTT, 100 µg/mL BSA), 400 µM ATP, 150 µM CTP, 150 µM GTP, 10 µM UTP, 0.2 µM ³²P UTP and 4 units of RNAseOut (Invitrogen). The final concentration of NaCl in the transcription reactions is approximately 50 mM, due to the presence of NaCl in the stock solutions of the DNA, NTPs, TFAM, TFB2M, POLRMT. This concentration is in the optimal range for achieving TFAM-dependent mtDNA transcription [48, 49]. The reactions were incubated with 16 nM POLRMT (Enzymax, LLC), 16 nM TFB2M (Enzymax, LLC) and varying concentrations of TFAM (0 nM, 6.8 nM, 16 nM, 34 nM, 51 nM, 68 nM or 136 nM) at 32 °C for 3 hours. All reactions were stopped with the addition of 100 µL 10 mM Tris-HCl pH 8.0, 0.2 M NaCl, 0.5% SDS and 22.5 µg proteinase K. Transcription reactions were then ethanol precipitated and resuspended in 98% formamide, 10 mM EDTA pH 8.0, 0.025% xylene cyanol and 0.025% bromophenol blue. Samples were electrophoresed on an 8% polyacrylamide-7M urea gel. Visualization of transcripts was performed by phosphorimaging using the Typhoon 9400 Imager (Molecular Dynamics). An RNA ladder (RiboRuler Low Range, Thermo Fisher) was used to verify transcription sizes (270 bp for the HSP1, 173 bp for LSP and 186 bp for HSP2). For an internal loading control, the DNA template was quantitated from SybrGreen II-staining of the same gel. Loading was consistent and essentially no corrections were needed. The analyses of the data are described in the Supplementary Materials. Two-way ANOVA analysis was used to determine the statistical significance of the effect of ^{5m}CpG on transcription.

Transcription reactions for the LSP88 template used the same concentrations and reaction conditions as the tri-promoter reactions. Samples were electrophoresed on a 20% polyacrylamide-7M urea gel. An RNA ladder (Decade Marker, Thermo Fisher) was used to verify transcript sizes. The transcripts were visualized by phosphorimaging as above. They were quantitated and scaled as described in the Supplementary Materials.

Analyses of published structures

The crystal structures of both TFAM bound to the HSP1 (PDB ID 4NOD) [50] and the complex containing the LSP DNA, TFAM, TFB2M and POLRMT (PDB ID 6ERP) [51] were used to map the HSP1 CpG sites. The models were generated using the programs PyMOL [52] for alignments and coloration and Photoshop (Adobe) for final presentation.

RESULTS

^{5m}CpG methylation of the HSP1 increases the DNA binding affinity of TFAM

^{5m}CpG has been observed throughout the mitochondrial genome (mtDNA), including in the D-loop, which includes the sequences that direct transcriptional initiation (Figure 1) [1]. The 1,124 nt D-loop contains the three mitochondrial promoters, LSP, HSP1 and HSP2 (Figure 1). HSP1 directs the transcription of the 12S and 16S rRNAs and HSP2 directs all of the protein-encoding genes except for ND6 and 5 tRNA genes, which are transcribed from the LSP [24, 53]. Cytosine methylation was known to directly inhibit [54] and alter the specificity of transcription factor DNA binding [55]. Methylation of both of the CpG sites within the HSP1 TFAM binding site (Figures 1 and 2A) has the potential to influence TFAM-DNA binding.

To determine whether the DNA binding affinity of TFAM for HSP1 DNA was altered by the presence of ^{5m}CpG, we used a fluorescence resonance energy transfer (FRET) assay. The 33 bp DNA fragments of non-methylated HSP1 DNA and ^{5m}CpG-HSP1 were each labeled with a fluorescein (FAM) donor fluorophore on the 3' end of one strand and a tetramethylrhodamine (TAMRA) acceptor fluorophore on the other strand [43, 45]. As TFAM was titrated into these samples (Figure 2A), the donor signal at 520 nm decreased and the acceptor signal at 580 nm increased. This strong FRET effect was due to the TFAM-induced bending of the DNA, which reduces the distance between the fluorophores (Figure 2, B). From these data, the apparent binding affinity (K_{Dapp}) of TFAM for HSP1 was found to be 3.9 ± 0.3 nM and for ^{5m}CpG-HSP1 it was 1.2 ± 0.3 nM (Figure 2C), revealing a 3-fold increase in the affinity caused by the presence ^{5m}CpG (Cohen's d-value of 0.85, indicates a medium effect size). The magnitude of the FRET effect at full protein-DNA occupancy was similar for both templates, indicating no major change in the final DNA end-to-end distance.

Next, we carried out electrophoretic mobility shift assays (EMSA) using the same DNA sequence, but without fluorophore labeling. With addition of increasing concentrations of TFAM, distinct shifted bands were observed for both HSP1 and ^{5m}CpG-HSP1 DNA (Supplementary Figure S3A and B). The data were quantitated and the binding affinities (K_{Dapp}) were 5.7 ± 1.8 nM and 2.4 ± 0.7 nM, for the HSP1 and ^{5m}CpG-HSP1 DNA, respectively (Supplementary Figure S3C). This 2-fold difference is statistically significant

and has a Cohen's d -value of 0.67, indicating a medium effect size. Interestingly, a second shifted band appeared in the EMSA at lower concentrations of TFAM for the ^{5m}CpG-HSP1 lanes compared to HSP1. Together these results show that TFAM binds with a higher affinity to HSP1 when it is methylated at CpG sites, and points to a difference in the formation of higher order TFAM-DNA complexes.

Oligomerization properties of TFAM are influenced by ^{5m}CpG

TFAM is known to oligomerize on DNA as part of its mechanism for compacting DNA. TFAM binds to single DNA binding sites inducing a "U-turn" shaped 180° bend [35, 45, 56, 57] which promotes protein-protein interactions that stabilize the higher order complexes [50]. Therefore, we first examined the oligomerization properties of TFAM using stoichiometric EMSA with the HSP1 DNA (Figure 3). At concentrations of DNA above the K_{Dapp} , TFAM binding to HSP1 DNA gives rise to a population of higher order complexes. The stoichiometric EMSA with DNA:TFAM ratios from 1:0 to 1:8 revealed three major complexes 1–3 (Figure 3A and B). A plot of the fraction bound of each complex as a function of TFAM concentration showed a clear preference of TFAM to form the higher-order complexes at lower DNA:TFAM ratios for the ^{5m}CpG-HSP1 DNA (compare Figure 3A and B blue, red and green lines). However, as seen from the fraction of free DNA remaining at each concentration (black line), as expected the overall fraction bound was the same for both HSP1 and ^{5m}CpG-HSP1. These results show that ^{5m}CpG methylation of the HSP1 provides an environment which promotes the multimerization of TFAM on DNA.

In addition to the recognition of specific promoter sequences, TFAM condenses mtDNA to form the mitochondrial nucleoid [50, 58]. To accomplish this, TFAM coats the mtDNA using a non-sequence specific DNA binding mode that is highly cooperative [45, 50, 58, 59]. Therefore we used a stoichiometric EMSA to determine whether ^{5m}CpG in a nonspecific (NS) DNA sequence would similarly influence the formation of higher order TFAM-DNA complexes. The designed 33 bp sequence from the mitochondrial 16S RNA gene has CpG sites located at identical positions to that of HSP1. The EMSA with NS and ^{5m}CpG-NS DNA showed no difference in the formation of higher order TFAM-DNA complexes (Figure 3C and D). In addition, the complexes were not as well defined as they were for the HSP1 DNA. These results suggest that ^{5m}CpG in short non-sequence specific DNA does not alter TFAM binding, unlike the HSP1 for which higher order complexes are influenced by the CpG methylation state of the DNA.

CpG methylation has no significant impact on TFAM-induced DNA condensation

TFAM condenses the 16,569 bp mtDNA to form nucleoids [27, 57, 58]. Assuming a TFAM binding site size of 30 bp, based on footprinting [60], 552 TFAM molecules would bind to a single mtDNA. However, the TFAM-DNA ratio varies in cells; for example, in HeLa cells between 900–1700 molecules of TFAM can associate with a single mtDNA [26, 61]. Thus, oligomerization of TFAM on a short 33 bp DNA fragment does not mimic the physiologically relevant scenario of mtDNA nucleoid formation. Therefore, we investigated DNA compaction using an established model system for mtDNA nucleoid formation [27] using EMSA compaction assays and electron microscopy. These experiments used a 3,600 bp linearized plasmid (pLSP3) containing 583 bp of the mtDNA D-loop region [44]. The

pLSP3 plasmid was purified from a *dam⁻ dcm⁻* strain of *E. coli* and then linearized and methylated to completion at CpG sites using SssI methyltransferase (Supplementary Figure S2A). Assays were performed over a range of TFAM:DNA bp ratios. At increasing ratios of TFAM:DNA bp, the complexes exhibit decreased electrophoretic mobilities (Figure 4A). Quantitative analyses of the EMSA (Figure 4B) revealed no statistically significant difference between the CpG methylated and nonmethylated DNA in the distance the complexes migrated through the gel. We also examined the samples from the same EMSA experiments using transmission electronic microscopy to illustrate the degree of DNA compaction at each TFAM:DNA ratio (Supplementary Figure S4A and B). These results demonstrate that CpG methylation of DNA promotes TFAM-dependent condensation of DNA into model nucleoid structures as efficiently as nonmethylated DNA.

CpG methylation influences mitochondrial transcription *in vitro*

Mitochondrial transcription initiation requires three proteins, TFAM, POLRMT and TFB2M (reviewed in [24]). TFAM binds to the specific LSP, HSP1 and HSP2 sites, which facilitates the recruitment of the POLRMT and TFB2M to form transcription initiation complexes (Figure 5A) [62]. To test whether ^{5m}CpG in the promoter regions of LSP, HSP1 and HSP2 alters mitochondrial gene expression, we performed *in vitro* run-off transcription assays. The pLSP3 plasmid includes the promoter region of the mitochondrial DNA D-loop, corresponding to nucleotides 242–825 of the mtDNA. After excision from pLSP3, the resulting DNA (Figure 5A) is an established tri-promoter template for *in vitro* run-off transcription assays [44, 45]. Run-off transcription assays were performed with tri-promoter templates that were either nonmethylated or completely methylated at CpG sites (Supplementary Figure S2B).

Transcription occurs in the context of the mitochondrial nucleoid, which is coated with TFAM. Therefore, transcription was monitored for reactions with increasing concentrations of TFAM. As a function of TFAM concentration, all of the templates showed a gradual increase of RNA products from the LSP, HSP1 and HSP2 promoters to a point at which additional TFAM results in decreased production of transcripts (Figure 5B). This is commonly observed and attributed to competition between TFAM binding and preinitiation complex formation *in vitro* [44, 63, 64]. In the comparison of transcription from the ^{5m}CpG to the nonmethylated template, there is a striking enhancement of 2.4-fold for HSP1 transcription, which was not observed for HSP2 (Figure 5C–E and Supplementary Figure S5). For the LSP there is approximately a 30% decrease in transcript levels for the ^{5m}CpG template, evident at lower concentrations of TFAM.

HSP1 is the only promoter that has CpG at well-defined positions in the TFAM binding site. In the LSP, the only CpG is located in the POLRMT/TFB2M binding region, not the TFAM binding site (Figure 1). To isolate this region from the tri-promoter context, we investigated the impact of ^{5m}CpG on transcription from the LSP in a shorter, 88 bp single promoter template (LSP88) (Supplementary Figure S6A). Interestingly, ^{5m}CpG enhanced transcription by 2-fold in this context (Supplementary Figures S6B and C). Therefore, the effect of ^{5m}CpG shows some context dependence *in vitro* and impacts transcription whether it is located in the TFAM binding site or the POLRMT/TFB2M binding region.

The crystal structures of mitochondrial transcription initiation complexes and TFAM-DNA complexes provides a context for the CpG sequences examined here. The structure of TFAM bound to the HSP1 shows the typical U-turn [50]. Strikingly, the CpG sites are located one base pair away from each of the sites where TFAM intercalates the DNA (Figure 6A). The structure of the mitochondrial transcription initiation complex containing TFAM, TFB2M and POLRMT [51] was produced using the LSP DNA with substitutions designed to promote the formation of an “open-bubble” at the transcription start site (Figure 6B). One cytosine of the CpG is in the double-stranded region 5' to the open bubble, whereas the other C has been replaced with a T, and is not base-paired. Thus, in the transcription initiation complex structure, this CpG is located precisely at the junction between the duplex DNA and the open-promoter (Figure 6B).

DISCUSSION

In this study we examined the effects of cytosine methylation of CpG on TFAM DNA binding, TFAM-dependent DNA compaction and transcription. Quantitative binding experiments showed that ^{5m}CpG at two sites within a single HSP1 33 bp TFAM binding site increases the binding affinity for TFAM by a factor of 2. Additionally, as observed by EMSA, there were ^{5m}CpG -induced differences in the formation of higher order TFAM-DNA complexes in this short HSP1 DNA fragment. However, this effect did not extend to a much longer DNA fragment (3,600 bp) to which TFAM binds primarily non-sequence-specifically. *In vitro*, ^{5m}CpG also had an impact on transcription in a promoter-context dependent manner. These findings provide new insights into the impact of ^{5m}CpG in mtDNA directly on two central functions of TFAM suggesting that ^{5m}CpG has the potential to influence mitochondrial function in a manner distinct from that in the nucleus.

Unique mechanism for the effects of ^{5m}CpG on TFAM-dependent activities

The main mechanism of action of ^{5m}CpG in the nucleus involves the binding of methyl-DNA specific proteins, which in turn regulates access to nucleosomal DNA, and ultimately regulates gene expression (reviewed in [65, 66]). Cytosine methylation is also known to influence the DNA-binding properties of transcription factors (reviewed in [22, 67, 68]). CpG methylation inhibits the DNA binding of methylation sensitive endonucleases and transcription factors, for example homeodomain, bHLH, bZIP, Ets, nuclear receptor and Zn-finger family members [55]. In contrast, some members of the same or different transcription factor families bind with an increased affinity for ^{5m}CpG DNA, including homeodomain, Zn-finger, bZIP and nuclear receptor proteins. Additionally, ^{5m}CpG can alter the DNA binding specificity of transcription factors [55]. The likely mechanisms by which ^{5m}CpG influences these major-groove binding proteins include direct steric hindrance or direct stabilizing hydrophobic interactions with the methyl group [55]. Indeed, structural studies using X-ray crystallography have revealed that the methyl-binding domains (MBDs) of MeCp2 (PDB IDs 3C2I and 5BT2) [69, 70] and MBDs (PDB ID 1IG4, 3VYQ and 6CNQ) [71–73] have stabilizing interactions that involve both hydrophobic as well as hydrophilic interactions, such as hydration at the 5-methyl group of the cytosine.

TFAM does not have the potential to interact directly with the 5-methyl group of cytosine, because it binds in the minor, not major, groove. Additionally, ^{5m}C is not associated with significant changes in the structure of B-DNA [74, 75]. However, methylation does alter other properties of DNA (reviewed in [22]). The 5-methyl group of thymine stabilizes base stacking relative to uracil as does the ^{5m}cytosine relative to cytosine [76], and the overall stabilization enthalpy can be strong at −1 kcal/mol [77]. DNA methylation also increases the rigidity of the DNA [78, 79]. For example, nucleosomes contain highly distorted and deformable DNA, but ^{5m}C reduces nucleosome stability in a manner that is correlated with base-stacking interactions [80]. Further, recent computational analyses provide additional support and indicate that the effects of ^{5m}C on stacking interactions could be greatly magnified in the context of highly deformed DNA (reviewed in [22]). Therefore, it is highly likely that ^{5m}C alters stacking and/or deformability of the DNA in the HSP1, which impacts the relative stability of the TFAM-DNA complex.

TFAM as an HMGB protein profoundly bends DNA toward the major groove. This compresses the major groove and the bending is also associated with amino acids that intercalate between adjacent base pairs (base steps) in two regions of the DNA, inducing distortions such as unusually high roll angles (blue in Figure 6A). The locations of the two CpGs in the HSP1 (red in Figure 6A) are both at the base step adjacent to the sites of intercalation (blue in Figure 6A). These segments of DNA are relatively undistorted in the TFAM-DNA crystal structures [35, 50, 57] (red in Figure 6A), but this flanking DNA makes further contacts with TFAM, including with the C-terminal tail. As such, stabilization of this flanking DNA through increased base stacking could lead to the increased affinity of TFAM for ^{5m}CpG DNA.

Even though there is no CpG in the TFAM binding site, we found that transcription from the LSP single-promoter template was also increased 2-fold. A single CpG is found at the −5 position relative to the transcription start site of the promoter. This dinucleotide interacts extensively with both POLRMT and TFB2M (Figure 6B). Alterations in the stacking of the −4C with −5A that increase the rigidity of the DNA could facilitate strand separation at the −3G position (Figure 6B). However, structural studies with the actual ^{5m}CpG sequence will be required to substantiate these ideas.

Interestingly, the impact of ^{5m}CpG does not appear to extend to the non-sequence specific DNA binding properties of TFAM that are important for DNA compaction. The EMSA results (Figure 4) show that TFAM does not adopt well-defined complexes with non-specific sequences of DNA. We previously showed that TFAM bends non-site-specific DNA to a lesser degree than the promoter DNA sequences and that TFAM lacking the C-terminal tail also was impaired in DNA bending [43, 45]. These results, suggest a decreased importance of the positioning of the flanking DNA in non-sequence-specific DNA binding. As such, we suggest that any ^{5m}CpG-induced DNA stabilization of DNA in that region might have little impact on TFAM. Therefore, it was not surprising that ^{5m}CpG had no apparent effect DNA compaction by TFAM, because the majority of the binding interactions in the nucleoid are non-sequence specific [58, 81].

Relationship of mtDNA ^{5m}CpG to mitochondrial transcription

CpG methylation impacts transcription initiation of mtDNA *in vitro*. Transcripts originating from the HSP1 promoter were increased 2.4-fold, whereas those from LSP were decreased by 28% (Figure 5). This effectively changes the ratio of HSP1 to LSP transcription initiation by a factor of 3. These results are consistent with the increased affinity of TFAM for the ^{5m}CpG-HSP1 leading to the enhanced recruitment of POLRMT and TFB2M. Also consistent is the decrease of transcripts from the LSP, which occurs in the tri-promoter context. This could be because of a 'squenching effect' such that less TFAM is available for promoter recognition. Although there are no CpG sites within the LSP TFAM binding site, methylation of the CpG in the POLRMT/TFB2M binding region (Figure 1) in the LSP-single promoter template showed not repression of transcription, but increased transcript levels (Supplementary Figure S6). Therefore, the effect of ^{5m}CpG on transcription is context dependent and appears to alter not just TFAM binding, but also the transcription initiation complex. An explanation for such context-dependence of transcription from the LSP could derive from structural features of the promoter regions. For example, if the promoter region were to be occupied by two (or more) transcription complexes, there is the potential for them to interact with each other (Figure 5A). Moreover, there is a pattern of TFAM binding sites between the promoters [82], which could be altered by ^{5m}CpG creating an environment that is less or more favorable for transcription. We observe changes in TFAM-DNA activities *in vitro* with fully-methylated promoter DNA. However, the effects of both ^{5m}CpG elsewhere in the mtDNA and even low levels of ^{5m}CpG could impact the processes of transcription including elongation and termination, as well as replication, mtDNA copy number and compaction through changes to the relative ratio of utilization of the HSP1 relative to the LSP.

The function of the low ^{5m}CpG mtDNA in mitochondria *in vivo* is still unclear. Dozens of studies have correlated the levels of ^{5m}CpG in the mitochondrial genome to changes in mitochondrial gene expression in cells and *in vivo* (reviewed in [9, 10]). These studies used different experimental systems and approaches and there is no clear consensus in the literature. With enforced expression of CpG or GpC methyltransferases in mitochondria, a decrease of mitochondrial transcription for Gp^{5m}C, but not ^{5m}CpG, has been reported [83]. Other studies find that lower levels of ^{5m}CpG globally or in the D-loop mtDNA are accompanied by increases in mitochondrial RNA transcript levels [1, 2, 6, 7]. This anti-correlation between CpG mtDNA methylation and transcript levels was also observed when the ^{5m}CpG levels in the D-loop were increased in disease states [3–5]. In a recent study, both nuclear and mitochondrial DNA transcript levels, mtDNA copy number and cellular functions were quantitated and compared, as ^{5m}CpG levels were pharmacologically decreased. Here, lower ^{5m}CpG levels resulted in a two-fold decrease in HSP1/2 transcripts and a slight decrease in LSP ND6 transcripts, and not the anti-correlation previously observed. However, this study reported an increase in mtDNA copy number with reduced ^{5m}CpG levels [8]. Such an anti-correlation between ^{5m}CpG levels and mtDNA copy number has consistently been reported [1, 83–86]. Interestingly, a ^{5m}CpG-dependent shift to lower utilization of the LSP compared to HSP1, has the potential to lower mtDNA copy number, which is consistent with our findings *in vitro* (Figure 5). Although the function of mitochondrial ^{5m}CpG is not well understood, understanding how it impacts TFAM-

dependent activities *in vitro* provides a better understanding of ^{5m}CpG effects on DNA recognition and a foundation and direction for future research.

CONCLUSIONS

The results presented here reveal the impact of CpG methylation of mtDNA promoter DNA on the TFAM-dependent properties of DNA recognition, compaction and transcription initiation. The influence of ^{5m}CpG on DNA binding and transcription is both sequence and context selective. In contrast, there was no obvious effect of ^{5m}CpG on TFAM recognition of non-sequence-specific DNA or DNA compaction. Together these results lead to the conclusion that the impact of ^{5m}CpG on TFAM activities is related to its sequence-selective mode of DNA recognition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- [1]. Bianchessi V, Vinci MC, Nigro P, Rizzi V, Farina F, Capogrossi MC, Pompilio G, Gualdi V, Lauri A, Methylation profiling by bisulfite sequencing analysis of the mtDNA Non-Coding Region in replicative and senescent Endothelial Cells, *Mitochondrion*, 27 (2016) 40–47. [PubMed: 26910457]
- [2]. Yamazaki M, Munetsuna E, Yamada H, Ando Y, Mizuno G, Murase Y, Kondo K, Ishikawa H, Teradaira R, Suzuki K, Ohashi K, Fructose consumption induces hypomethylation of hepatic mitochondrial DNA in rats, *Life Sci*, 149 (2016) 146–152. [PubMed: 26869391]
- [3]. Blanch M, Mosquera JL, Ansoleaga B, Ferrer I, Barrachina M, Altered Mitochondrial DNA Methylation Pattern in Alzheimer Disease-Related Pathology and in Parkinson Disease, *Am J Pathol*, 186 (2016) 385–397. [PubMed: 26776077]
- [4]. Jia L, Li J, He B, Jia Y, Niu Y, Wang C, Zhao R, Abnormally activated one-carbon metabolic pathway is associated with mtDNA hypermethylation and mitochondrial malfunction in the oocytes of polycystic gilt ovaries, *Sci Rep*, 6 (2016) 19436. [PubMed: 26758245]
- [5]. Mishra M, Kowluru RA, Epigenetic Modification of Mitochondrial DNA in the Development of Diabetic Retinopathy, *Invest Ophthalmol Vis Sci*, 56 (2015) 5133–5142. [PubMed: 26241401]
- [6]. Gao J, Wen S, Zhou H, Feng S, De-methylation of displacement loop of mitochondrial DNA is associated with increased mitochondrial copy number and nicotinamide adenine dinucleotide subunit 2 expression in colorectal cancer, *Mol Med Rep*, 12 (2015) 7033–7038. [PubMed: 26323487]
- [7]. Dzitoyeva S, Chen H, Manev H, Effect of aging on 5-hydroxymethylcytosine in brain mitochondria, *Neurobiol Aging*, 33 (2012) 2881–2891. [PubMed: 22445327]

- [8]. Sun X, Johnson J, St John JC, Global DNA methylation synergistically regulates the nuclear and mitochondrial genomes in glioblastoma cells, *Nucleic Acids Res*, (2018).
- [9]. Maresca A, Zaffagnini M, Caporali L, Carelli V, Zanna C, DNA methyltransferase 1 mutations and mitochondrial pathology: is mtDNA methylated?, *Front Genet*, 6 (2015) 90. [PubMed: 25815005]
- [10]. Mposhi A, Van der Wijst MG, Faber KN, Rots MG, Regulation of mitochondrial gene expression, the epigenetic enigma, *Front Biosci (Landmark Ed)*, 22 (2017) 1099–1113. [PubMed: 28199194]
- [11]. Barshad G, Marom S, Cohen T, Mishmar D, Mitochondrial DNA Transcription and Its Regulation: An Evolutionary Perspective, *Trends Genet*, (2018).
- [12]. Hong EE, Okitsu CY, Smith AD, Hsieh CL, Regionally specific and genome-wide analyses conclusively demonstrate the absence of CpG methylation in human mitochondrial DNA, *Mol Cell Biol*, 33 (2013) 2683–2690. [PubMed: 23671186]
- [13]. Matsuda S, Yasukawa T, Sakaguchi Y, Ichiyanagi K, Unoki M, Gotoh K, Fukuda K, Sasaki H, Suzuki T, Kang D, Accurate estimation of 5-methylcytosine in mammalian mitochondrial DNA, *Sci Rep*, 8 (2018) 5801. [PubMed: 29643477]
- [14]. Owa C, Poulin M, Yan L, Shioda T, Technical adequacy of bisulfite sequencing and pyrosequencing for detection of mitochondrial DNA methylation: Sources and avoidance of false-positive detection, *PLoS One*, 13 (2018) e0192722. [PubMed: 29420656]
- [15]. Mechta M, Ingerslev LR, Fabre O, Picard M, Barres R, Evidence Suggesting Absence of Mitochondrial DNA Methylation, *Front Genet*, 8 (2017) 166. [PubMed: 29163634]
- [16]. Pawar T, Eide L, Pitfalls in mitochondrial epigenetics, *Mitochondrial DNA A DNA Mapp Seq Anal*, 28 (2017) 762–768. [PubMed: 27246961]
- [17]. Shock LS, Thakkar PV, Peterson EJ, Moran RG, Taylor SM, DNA methyltransferase 1, cytosine methylation, and cytosine hydroxymethylation in mammalian mitochondria, *Proc Natl Acad Sci U S A*, 108 (2011) 3630–3635. [PubMed: 21321201]
- [18]. Wong M, Gertz B, Chestnut BA, Martin LJ, Mitochondrial DNMT3A and DNA methylation in skeletal muscle and CNS of transgenic mouse models of ALS, *Front Cell Neurosci*, 7 (2013) 279. [PubMed: 24399935]
- [19]. Chestnut BA, Chang Q, Price A, Lesuisse C, Wong M, Martin LJ, Epigenetic regulation of motor neuron cell death through DNA methylation, *J Neurosci*, 31 (2011) 16619–16636. [PubMed: 22090490]
- [20]. Bellizzi D, D'Aquila P, Scafione T, Giordano M, Riso V, Riccio A, Passarino G, The control region of mitochondrial DNA shows an unusual CpG and non-CpG methylation pattern, *DNA Res*, 20 (2013) 537–547. [PubMed: 23804556]
- [21]. Felsenfeld G, Groudine M, Controlling the double helix, *Nature*, 421 (2003) 448–453. [PubMed: 12540921]
- [22]. Dantas Machado AC, Zhou T, Rao S, Goel P, Rastogi C, Lazarovici A, Bussemaker HJ, Rohs R, Evolving insights on how cytosine methylation affects protein-DNA binding, *Brief Funct Genomics*, 14 (2015) 61–73. [PubMed: 25319759]
- [23]. Zhu H, Wang G, Qian J, Transcription factors as readers and effectors of DNA methylation, *Nat Rev Genet*, 17 (2016) 551–565. [PubMed: 27479905]
- [24]. Bonawitz ND, Clayton DA, Shadel GS, Initiation and beyond: multiple functions of the human mitochondrial transcription machinery, *Mol Cell*, 24 (2006) 813–825. [PubMed: 17189185]
- [25]. Bogenhagen DF, Wang Y, Shen EL, Kobayashi R, Protein components of mitochondrial DNA nucleoids in higher eukaryotes, *Mol Cell Proteomics*, 2 (2003) 1205–1216. [PubMed: 14514796]
- [26]. Alam TI, Kanki T, Muta T, Ukaji K, Abe Y, Nakayama H, Takio K, Hamasaki N, Kang D, Human mitochondrial DNA is packaged with TFAM, *Nucleic Acids Res*, 31 (2003) 1640–1645. [PubMed: 12626705]
- [27]. Kaufman BA, Durisic N, Mativetsky JM, Costantino S, Hancock MA, Grutter P, Shoubridge EA, The mitochondrial transcription factor TFAM coordinates the assembly of multiple DNA molecules into nucleoid-like structures, *Mol Biol Cell*, 18 (2007) 3225–3236. [PubMed: 17581862]

- [28]. Wang YE, Marinov GK, Wold BJ, Chan DC, Genome-wide analysis reveals coating of the mitochondrial genome by TFAM, PLoS One, 8 (2013) e74513. [PubMed: 23991223]
- [29]. Wang Y, Bogenhagen DF, Human mitochondrial DNA nucleoids are linked to protein folding machinery and metabolic enzymes at the mitochondrial inner membrane, J Biol Chem, 281 (2006) 25791–25802. [PubMed: 16825194]
- [30]. Legros F, Malka F, Frachon P, Lombes A, Rojo M, Organization J Cell Sci, 117 (2004) 2653–2662.
- [31]. Kanki T, Ohgaki K, Gaspari M, Gustafsson CM, Fukuoh A, Sasaki N, Hamasaki N, Kang D, Architectural role of mitochondrial transcription factor A in maintenance of human mitochondrial DNA, Mol Cell Biol, 24 (2004) 9823–9834. [PubMed: 15509786]
- [32]. Ikeda M, Ide T, Fujino T, Arai S, Saku K, Kakino T, Tynnismaa H, Yamasaki T, Yamada K, Kang D, Suomalainen A, Sunagawa K, Overexpression of TFAM or twinkle increases mtDNA copy number and facilitates cardioprotection associated with limited mitochondrial oxidative stress, PLoS One, 10 (2015) e0119687. [PubMed: 25822152]
- [33]. Ekstrand MI, Falkenberg M, Rantanen A, Park CB, Gaspari M, Hultenby K, Rustin P, Gustafsson CM, Larsson NG, Mitochondrial transcription factor A regulates mtDNA copy number in mammals, Hum Mol Genet, 13 (2004) 935–944. [PubMed: 15016765]
- [34]. Campbell CT, Kolesar JE, Kaufman BA, Mitochondrial transcription factor A regulates mitochondrial transcription initiation, DNA packaging, and genome copy number, Biochim Biophys Acta, 1819 (2012) 921–929. [PubMed: 22465614]
- [35]. Rubio-Cosials A, Sola M, U-turn DNA bending by human mitochondrial transcription factor A, Curr Opin Struct Biol, 23 (2013) 116–124. [PubMed: 23333034]
- [36]. Morozov YI, Temiakov D, Human Mitochondrial Transcription Initiation Complexes Have Similar Topology on the Light and Heavy Strand Promoters, J Biol Chem, 291 (2016) 13432–13435. [PubMed: 27226527]
- [37]. Seidel-Rogol BL, McCulloch V, Shadel GS, Human mitochondrial transcription factor B1 methylates ribosomal RNA at a conserved stem-loop, Nat Genet, 33 (2003) 23–24. [PubMed: 12496758]
- [38]. Metodiev MD, Lesko N, Park CB, Camara Y, Shi Y, Wibom R, Hultenby K, Gustafsson CM, Larsson NG, Methylation of 12S rRNA is necessary for in vivo stability of the small subunit of the mammalian mitochondrial ribosome, Cell Metab, 9 (2009) 386–397. [PubMed: 19356719]
- [39]. Litonin D, Sologub M, Shi Y, Savkina M, Anikin M, Falkenberg M, Gustafsson CM, Temiakov D, Human mitochondrial transcription revisited: only TFAM and TFB2M are required for transcription of the mitochondrial genes in vitro, J Biol Chem, 285 (2010) 18129–18133. [PubMed: 20410300]
- [40]. Ghosh S, Sengupta S, Scaria V, Comparative analysis of human mitochondrial methylomes shows distinct patterns of epigenetic regulation in mitochondria, Mitochondrion, 18 (2014) 58–62. [PubMed: 25058022]
- [41]. Shmookler Reis RJ, Goldstein S, Mitochondrial DNA in mortal and immortal human cells. Genome number, integrity, and methylation, J Biol Chem, 258 (1983) 9078–9085. [PubMed: 6307991]
- [42]. Armstrong DA, Green BB, Blair BA, Guerin DJ, Litzky JF, Chavan NR, Pearson KJ, Marsit CJ, Maternal smoking during pregnancy is associated with mitochondrial DNA methylation, Environ Epigenet, 2 (2016).
- [43]. Gangelhoff TA, Mungalachetty PS, Nix JC, Churchill ME, Structural analysis and DNA binding of the HMG domains of the human mitochondrial transcription factor A, Nucleic Acids Res, 37 (2009) 3153–3164. [PubMed: 19304746]
- [44]. Shutt TE, Lodeiro MF, Cotney J, Cameron CE, Shadel GS, Core human mitochondrial transcription apparatus is a regulated two-component system in vitro, Proc Natl Acad Sci U S A, 107 (2010) 12133–12138. [PubMed: 20562347]
- [45]. Malarkey CS, Bestwick M, Kuhlwillm JE, Shadel GS, Churchill ME, Transcriptional activation by mitochondrial transcription factor A involves preferential distortion of promoter DNA, Nucleic Acids Res, 40 (2012) 614–624. [PubMed: 21948790]

- [46]. Clegg RM, Fluorescence resonance energy transfer and nucleic acids, *Methods Enzymol*, 211 (1992) 353–388. [PubMed: 1406315]
- [47]. Wang Z, Cotney J, Shadel GS, Human mitochondrial ribosomal protein MRPL12 interacts directly with mitochondrial RNA polymerase to modulate mitochondrial gene expression, *J Biol Chem*, 282 (2007) 12610–12618. [PubMed: 17337445]
- [48]. Shi Y, Dierckx A, Wanrooij PH, Wanrooij S, Larsson NG, Wilhelmsson LM, Falkenberg M, Gustafsson CM, Mammalian transcription factor A is a core component of the mitochondrial transcription machinery, *Proc Natl Acad Sci U S A*, 109 (2012) 16510–16515. [PubMed: 23012404]
- [49]. Bergbrede T, Hoberg E, Larsson NG, Falkenberg M, Gustafsson CM, An Adaptable High-Throughput Technology Enabling the Identification of Specific Transcription Modulators, *SLAS Discov*, 22 (2017) 378–386. [PubMed: 28328323]
- [50]. Ngo HB, Lovely GA, Phillips R, Chan DC, Distinct structural features of TFAM drive mitochondrial DNA packaging versus transcriptional activation, *Nat Commun*, 5 (2014) 3077. [PubMed: 24435062]
- [51]. Hillen HS, Morozov YI, Sarfallah A, Temiakov D, Cramer P, Structural Basis of Mitochondrial Transcription Initiation, *Cell*, 171 (2018) 1072–1081 e1010.
- [52]. DeLano W, The PyMOL Molecular Graphics System, in, DeLano Scientific, Palo Alto, CA, 2008.
- [53]. Gaspari M, Larsson NG, Gustafsson CM, The transcription machinery in mammalian mitochondria, *Biochim Biophys Acta*, 1659 (2004) 148–152. [PubMed: 15576046]
- [54]. Watt F, Molloy PL, Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter, *Genes Dev*, 2 (1988) 1136–1143. [PubMed: 3192075]
- [55]. Yin Y, Morgunova E, Jolma A, Kaasinen E, Sahu B, Khund-Sayeed S, Das PK, Kivioja T, Dave K, Zhong F, Nitta KR, Taipale M, Popov A, Ginno PA, Domcke S, Yan J, Schubeler D, Vinson C, Taipale J, Impact of cytosine methylation on DNA binding specificities of human transcription factors, *Science*, 356 (2017).
- [56]. Rubio-Cosials A, Sidow JF, Jimenez-Menendez N, Fernandez-Millan P, Montoya J, Jacobs HT, Coll M, Bernado P, Sola M, Human mitochondrial transcription factor A induces a U-turn structure in the light strand promoter, *Nat Struct Mol Biol*, 18 (2011) 1281–1289. [PubMed: 22037172]
- [57]. Ngo HB, Kaiser JT, Chan DC, The mitochondrial transcription and packaging factor Tfam imposes a U-turn on mitochondrial DNA, *Nat Struct Mol Biol*, 18 (2011) 1290–1296. [PubMed: 22037171]
- [58]. Kukat C, Davies KM, Wurm CA, Spahr H, Bonekamp NA, Kuhl I, Joos F, Polosa PL, Park CB, Posse V, Falkenberg M, Jakobs S, Kuhlbrandt W, Larsson NG, Cross-strand binding of TFAM to a single mtDNA molecule forms the mitochondrial nucleoid, *Proc Natl Acad Sci U S A*, 112 (2015) 11288–11293. [PubMed: 26305956]
- [59]. Wong TS, Rajagopalan S, Freund SM, Rutherford TJ, Andreeva A, Townsley FM, Petrovich M, Fersht AR, Biophysical characterizations of human mitochondrial transcription factor A and its binding to tumor suppressor p53, *Nucleic Acids Res*, 37 (2009) 6765–6783. [PubMed: 19755502]
- [60]. Farge G, Laurens N, Broekmans OD, van den Wildenberg SM, Dekker LC, Gaspari M, Gustafsson CM, Peterman EJ, Falkenberg M, Wuite GJ, Protein sliding and DNA denaturation are essential for DNA organization by human mitochondrial transcription factor A, *Nat Commun*, 3 (2012) 1013. [PubMed: 22910359]
- [61]. Fisher RP, Clayton DA, Purification and characterization of human mitochondrial transcription factor 1, *Mol Cell Biol*, 8 (1988) 3496–3509. [PubMed: 3211148]
- [62]. Morozov YI, Parshin AV, Agaronyan K, Cheung AC, Anikin M, Cramer P, Temiakov D, A model for transcription initiation in human mitochondria, *Nucleic Acids Res*, 43 (2015) 3726–3735. [PubMed: 25800739]

- [63]. Furukawa R, Yamada Y, Matsushima Y, Goto Y, Harashima H, The manner in which DNA is packaged with TFAM has an impact on transcription activation and inhibition, *FEBS Open Bio*, 2 (2012) 145–150.
- [64]. Farge G, Mehmedovic M, Baclayon M, van den Wildenberg SM, Roos WH, Gustafsson CM, Wuite GJ, Falkenberg M, In vitro-reconstituted nucleoids can block mitochondrial DNA replication and transcription, *Cell Rep*, 8 (2014) 66–74. [PubMed: 24981867]
- [65]. Almouzni G, Cedar H, Maintenance of Epigenetic Information, *Cold Spring Harb Perspect Biol*, 8 (2016).
- [66]. Edwards JR, Yarychivska O, Boulard M, Bestor TH, DNA methylation and DNA methyltransferases, *Epigenetics Chromatin*, 10 (2017) 23. [PubMed: 28503201]
- [67]. Liu Y, Zhang X, Blumenthal RM, Cheng X, A common mode of recognition for methylated CpG, *Trends Biochem Sci*, 38 (2013) 177–183. [PubMed: 23352388]
- [68]. Ren R, Horton JR, Zhang X, Blumenthal RM, Cheng X, Detecting and interpreting DNA methylation marks, *Curr Opin Struct Biol*, 53 (2018) 88–99. [PubMed: 30031306]
- [69]. Ho KL, McNae IW, Schmiedeberg L, Klose RJ, Bird AP, Walkinshaw MD, MeCP2 binding to DNA depends upon hydration at methyl-CpG, *Mol Cell*, 29 (2008) 525–531. [PubMed: 18313390]
- [70]. Chia JY, Tan WS, Ng CL, Hu NJ, Foo HL, Ho KL, A/T Run Geometry of B-form DNA Is Independent of Bound Methyl-CpG Binding Domain, Cytosine Methylation and Flanking Sequence, *Sci Rep*, 6 (2016) 31210. [PubMed: 27502833]
- [71]. Ohki I, Shimotake N, Fujita N, Jee J, Ikegami T, Nakao M, Shirakawa M, Solution structure of the methyl-CpG binding domain of human MBD1 in complex with methylated DNA, *Cell*, 105 (2001) 487–497. [PubMed: 11371345]
- [72]. Otani J, Arita K, Kato T, Kinoshita M, Kimura H, Suetake I, Tajima S, Ariyoshi M, Shirakawa M, Structural basis of the versatile DNA recognition ability of the methyl-CpG binding domain of methyl-CpG binding domain protein 4, *J Biol Chem*, 288 (2013) 6351–6362. [PubMed: 23316048]
- [73]. Liu K, Xu C, Lei M, Yang A, Loppnau P, Hughes TR, Min J, Structural basis for the ability of MBD domains to bind methyl-CG and TG sites in DNA, *J. Biol. Chem*, 293 (2018) 7344–7354. [PubMed: 29567833]
- [74]. Renciuik D, Blacque O, Vorlickova M, Spingler B, Crystal structures of B-DNA dodecamer containing the epigenetic modifications 5-hydroxymethylcytosine or 5-methylcytosine, *Nucleic Acids Res*, 41 (2013) 9891–9900. [PubMed: 23963698]
- [75]. Theruvathu JA, Yin YW, Pettitt BM, Sowers LC, Comparison of the structural and dynamic effects of 5-methylcytosine and 5-chlorocytosine in a CpG dinucleotide sequence, *Biochemistry*, 52 (2013) 8590–8598. [PubMed: 24147911]
- [76]. Sowers LC, Shaw BR, Sedwick WD, Base stacking and molecular polarizability: effect of a methyl group in the 5-position of pyrimidines, *Biochem Biophys Res Commun*, 148 (1987) 790–794. [PubMed: 3689373]
- [77]. Ehama R, Tsushima M, Yuzuri T, Suezawa H, Sakakibara K, Hirota M, Substituent Effect on the Enthalpies of Formation of Ch-Pi Complexes of Aromatic Pi-Bases, *B Chem Soc Jpn*, 66 (1993) 814–818.
- [78]. Umezawa Y, Nishio M, Thymine-methyl/pi interaction implicated in the sequence-dependent deformability of DNA, *Nucleic Acids Res*, 30 (2002) 2183–2192. [PubMed: 12000838]
- [79]. Mirsaidov U, Timp W, Zou X, Dimitrov V, Schulten K, Feinberg AP, Timp G, Nanoelectromechanics of methylated DNA in a synthetic nanopore, *Biophys J*, 96 (2009) L32–34. [PubMed: 19217843]
- [80]. Portella G, Battistini F, Orozco M, Understanding the connection between epigenetic DNA methylation and nucleosome positioning from computer simulations, *PLoS Comput Biol*, 9 (2013) e1003354. [PubMed: 24278005]
- [81]. Kukat C, Larsson NG, mtDNA makes a U-turn for the mitochondrial nucleoid, *Trends Cell Biol*, 23 (2013) 457–463. [PubMed: 23721879]

- [82]. Uchida A, Murugesapillai D, Kastner M, Wang Y, Lodeiro MF, Prabhakar S, Oliver GV, Arnold JJ, Maher LJ, Williams MC, Cameron CE, Unexpected sequences and structures of mtDNA required for efficient transcription from the first heavy-strand promoter, *Elife*, 6 (2017).
- [83]. van der Wijst MG, van Tilburg AY, Ruiters MH, Rots MG, Experimental mitochondria-targeted DNA methylation identifies GpC methylation, not CpG methylation, as potential regulator of mitochondrial gene expression, *Sci Rep*, 7 (2017) 177. [PubMed: 28282966]
- [84]. Sanyal T, Bhattacharjee P, Bhattacharjee S, Bhattacharjee P, Hypomethylation of mitochondrial D-loop and ND6 with increased mitochondrial DNA copy number in the arsenic-exposed population, *Toxicology*, 408 (2018) 54–61. [PubMed: 29940200]
- [85]. Stocco A, Siciliano G, Migliore L, Coppede F, Decreased Methylation of the Mitochondrial D-Loop Region in Late-Onset Alzheimer's Disease, *J Alzheimers Dis*, 59 (2017) 559–564. [PubMed: 28655136]
- [86]. Tong H, Zhang L, Gao J, Wen S, Zhou H, Feng S, Methylation of mitochondrial DNA displacement loop region regulates mitochondrial copy number in colorectal cancer, *Mol Med Rep*, 16 (2017) 5347–5353. [PubMed: 28849075]
- [87]. Martin M, Cho J, Cesare AJ, Griffith JD, Attardi G, Termination factor-mediated DNA loop between termination and initiation sites drives mitochondrial rRNA synthesis, *Cell*, 123 (2005) 1227–1240. [PubMed: 16377564]
- [88]. Zollo O, Sondheimer N, Topological requirements of the mitochondrial heavy-strand promoters, *Transcription*, (2017) e1331156.
- [89]. Lodeiro MF, Uchida A, Bestwick M, Moustafa IM, Arnold JJ, Shadel GS, Cameron CE, Transcription from the second heavy-strand promoter of human mtDNA is repressed by transcription factor A in vitro, *Proc Natl Acad Sci U S A*, 109 (2012) 6513–6518. [PubMed: 22493245]

Figure 1. Schematic diagram of the mitochondrial genome (mtDNA).

The circular genome illustrates the heavy strand and light strand gene products for transcripts originating from the LSP, HSP1 and HSP2 (colored in green, gold and blue, respectively). The tRNA genes are shown as black sticks. Promoters, origins of replication, consensus sequence blocks (CSBs) and termination-associated sequences (TAS) are shown in the expanded D-loop region. The expanded promoter region details the LSP (green), HSP1 (gold) and HSP2 (blue) promoters highlighting the interactions with TFAM, TFB2M (B2) and POLRMT (POL). Reported ^{5m}CpG sites within the promoter region are shown as red dots.

Figure 2. Binding properties of TFAM to HSP1 and 5mCpG-HSP1 DNA.

(A) The HSP1 sequence with the 5mCpG highlighted. Representative fluorescence emission spectra from FRET experiments with 3.4 nM FAM/TAMRA labeled HSP1 DNA and 3.4 nM FAM/TAMRA labeled 5mCpG-HSP1 DNA with added TFAM (0–50 nM). (C) Binding curves of normalized FRET effect were used to measure the K_{Dapp} values ($n=7$; error bars = $\pm$ S.D.)

Figure 3. Methylation of HSP1 promotes multimeric TFAM-DNA complexes.

Representative images of stoichiometric EMSA experiments. Increasing concentrations of TFAM (0–800 nM) were used in binding reactions with the (A) HSP1, (B) ^{5m}CpG-HSP1, (C) non-sequence specific (NS) and (D) ^{5m}CpG-NS DNA. Graphs show the fraction of free DNA (black line) plotted relative to the left-hand y axis and the fraction bound relative to the right-hand y axis. HSP1 and ^{5m}CpG-HSP1 were quantitated separately for each of the distinct multimers (from 1 to 3). The NS and ^{5m}CpG-NS bound products were quantitated as one ‘multimer’, as the complexes did not appear as distinct bands. (n=3; error bars = ± S.D.).

Figure 4. The effect of ^{5m}CpG DNA on the DNA-compaction properties of TFAM.

(A) Representative EMSA of the TFAM-induced compaction of linearized pLSP3 and ^{5m}CpG -pLSP3 DNA. The reactions included DNA alone (lane 1 and 6), and DNA bound to TFAM, at the following ratios: TFAM:DNA = 1:60 bp (lane 2 and 7), TFAM:DNA = 1:45 bp (lane 3 and 8), TFAM:DNA = 1:30 bp (lane 4 and 9), and TFAM:DNA = 1:15 bp (lane 5 and 10), which are equivalent to estimated DNA coverage by TFAM of 50%, 75%, 100% and 200%, respectively. CpG-LSP3 is shown in lanes 1–5 and ^{5m}CpG-LSP3 is shown in lanes 6–10. (B) Bar graph shows the quantitation of the difference in electrophoretic mobility of each band from (panel A) compared to the free DNA. (n=3; error bars = ± S.D.).

Figure 5. ^{5m}CpG influences transcription from a tri-promoter template.

(A) Schematic diagram showing the binding sites of the mitochondrial preinitiation complexes at the HSP1, HSP2 and LSP in the tri-promoter template. HSP2 has a predicted transcription start site, but no reported footprint for TFAM, POLRMT or TFB2M [87–89]. The DNA length and protected sites are to scale and the dashed lines indicate that these complexes are not expected to occupy the promoters simultaneously. Abbreviations for TFB2M and POLRMT are B2 and POL, respectively. The red stars indicate ^{5m}CpG sites. (B) Representative autoradiogram of *in vitro* run-off transcription reactions. The increasing TFAM concentration (0–136 nM) is indicated by the triangle. Arrows identify the three transcription products. Shown in panels (C) through (E) for the LSP, HSP1, and HSP2, respectively is the quantitation of transcripts levels for the combined data from raw images that have been scaled to the nonmethylated DNA (as in Supplementary Figure S5 and the Supplementary Materials and Methods). 2-way ANOVA analysis showed that the effect of ^{5m}CpG on transcript levels was significant for LSP ($p < .001$) and HSP1 ($p < .0001$), but not HSP2. ($n=3$; error bars = $\pm$ S.D.).

Figure 6. Model showing location of $5mCpG$ in HSP1-TFAM and LSP-TFAM-TFB2M-POLRMT structures.

(A) Schematic diagram of HSP1-TFAM structure (PDB ID 4NOD), showing the positions of the CpG sequences. (B) Schematic diagram of the structure of the LSP-TFAM-POLRMT/TFB2M complex (PDB ID 6ERP) to illustrate the CpG positions in a model for the initiation complex. As this structure used an engineered sequence in the position of the CpG, there is a T in place of one C of the CpG. Red spheres represent the DNA nucleotides corresponding to the $5mC$ (or T in panel B) and the green circles indicate the site of the TFAM C-terminal tail. (B) Amino acid residues that contact the $5mC$ are circled in black