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Mitochondrial DNA and epigenetics: unexpected complex interactions

Candidate

Marco Giordano

Supervisor

Prof. Dina Bellizzi

Co-ordinator

Prof. Lucio Grandinetti

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A mia moglie

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SOMMARIO

Nell'ambito della ricerca sulle basi dell'invecchiamento e della longevità, un campo emergente è rappresentato dall' "epigenetica dell'invecchiamento". Molti studi hanno infatti dimostrato che i meccanismi epigenetici, quali la metilazione del DNA, hanno un ruolo importante nella determinazione di diversi fenotipi complessi incluso l'invecchiamento, che, in quanto tale, è causato dalla complessa interazione tra fattori genetici, epigenetici ed ambientali.

Il termine "epigenetica" si riferisce a quei cambiamenti che, pur non causando una variazione della sequenza del DNA, sono in grado di garantire l'espressione di un genotipo in fenotipo.

La metilazione del DNA è tra i processi epigenetici più studiati e si verifica in tutti gli organismi procarioti ed eucarioti. Sebbene la presenza di tale processo ed i suoi effetti nei meccanismi intracellulari è stato ampiamente documentato per il genoma nucleare, la presenza della metilazione anche nel DNA mitocondriale (mtDNA) è ampiamente dibattuta. Inoltre, nonostante nel processo di metilazione sia stato ipotizzato un ruolo giocato dalla disponibilità di ATP, poco è noto su un eventuale ruolo della variabilità del DNA mitocondriale nella regolazione dello stato di metilazione del genoma nucleare.

Il lavoro qui presentato, ha affrontato le complesse interazioni che intercorrono tra DNA mitocondriale e cambiamenti epigenetici, sia determinando la presenza di metilazione nel genoma mitocondriale, sia analizzando gli effetti delle variazioni del mtDNA sulla metilazione globale del genoma nucleare. Pertanto, una serie di esperimenti *in vivo* e *in vitro* sono riportati nel presente lavoro suddiviso in tre sezioni.

Nelle prime due sezioni, sono riportate le evidenze sperimentali da noi ottenute circa la presenza di citosine metilate sia all'interno della regione di controllo mitocondriale (D-loop), contenente elementi regolatori per la replicazione e trascrizione del mtDNA, che all'interno del gene codificante per l'RNA ribosomiale 12S (12S rRNA).

Lo stato di metilazione del D-loop è stata analizzata su DNA estratto sia da sangue di soggetti umani che in DNA estratto da cellule in coltura. Dopo trattamento con sodio bisolfito il DNA è stato sequenziato e, successivamente, analizzato mediante saggi di immunoprecipitazione. In tutti i campioni analizzati abbiamo riscontrato la presenza sia di citosine metilate (5mC) che di citosine idrossimetilate (5hmC). Sorprendentemente la metilazione del mtDNA si verifica in particolare nei dinucleotidi non-Cp. È altresì emerso che il pattern di metilazione del D-loop sono strettamente dipendenti dal tipo cellulare e ciò

potrebbe essere un esempio di metilazione diretta da RNA (RdDM). La metilazione del D-loop si verifica soprattutto nella regione del promotore del filamento pesante e nei blocchi di sequenza conservata (CSBI-III), indicando il coinvolgimento delle modificazioni epigenetiche nella regolazione della replicazione del mtDNA e/o della propria trascrizione.
Il sequenziamento della regione 12S rRNA in campioni precedentemente trattati con sodio bisofito, ha evidenziato la presenza di metilazione in un sito CpG posizionato al nucleotide 931. Questo sito è stato poi analizzato mediante la real-time PCR MGB probe-based. Il DNA è stato estratto da sangue venoso periferico di soggetti con diversa età e classificati per fenotipo *frailty* d'invecchiamento. Abbiamo rilevato la co-presenza sia di citosine non metilate che citosine metilate in gran parte del campione. L'analisi statistica ha rivelato che la metilazione del 12S rRNA è correlata al sesso, all'età ed allo stato di salute degli individui analizzati.
Nella terza sezione, sono riportati i risultati che hanno evidenziato come i livelli di metilazione globale del DNA sono influenzati dalla variante del mtDNA ereditato, probabilmente attraverso una diversa regolazione del sistema delle OXPHOS. Abbiamo quindi dimostrato, avvalendoci dell'utilizzo della tecnologia dei cibridi, un metodo utile per rivelare possibili vie di comunicazione tra i genomi mitocondriale e nucleare, che i processi epigenetici sono modulati in risposta ai suddetti processi.

In appendice è riportato un articolo pubblicato (Montesanto et al., *The genetic component of human longevity: analysis of the survival advantage of parents and siblings of Italian nonagenarians*, 2011, Eur J Hum Genet 19: 882-886.), sul quale ho lavorato durante il mio percorso di dottorato.

SUMMARY

In the research of fundamental processes underlying aging and longevity, an emerging field is represented by "aging epigenetics". In fact, different experimental evidences demonstrate as the rate and quality of human aging depend on a complex interplay among genetic, epigenetic and environmental factors.

Epigenetics refers to the programmed changes, not involving alteration of DNA sequence, leading different genotypes to phenotypes.

DNA methylation is the most studied epigenetic modification occurring in all prokaryotic and eukaryotic organisms. Although the occurrence of this modification and its effects in intracellular processes has been extensively documented for the nuclear genome, conflicting reports regarding the possible presence of methylated cytosines within the mitochondrial DNA (mtDNA) have emerged. In addition, in spite of the hypothesized role of ATP availability on the methylation process , little is known about the role of mitochondrial DNA variability on the methylation status of nuclear genome.

The work presented here, has addressed the complex interactions between mitochondrial DNA and epigenetic changes, both investigating the methylation of mitochondrial genome and analyzing the effects of mtDNA variation on the Global methylation of nuclear genome. In fact, a series of *in vivo* and *in vitro* investigations are here reported in three sections.

In the first two sections, experimental evidences about the presence of methylated cytosines within the mitochondrial control region (D-loop), containing regulator elements for replication and transcription of mtDNA, and within the gene encoding for ribosomal RNA 12S (12S rRNA), are reported.

The methylation status of the D-loop was analyzed in both blood DNA collected from human subjects and in DNA from cultured cells by bisulfite sequencing and, consecutively, by methylated/hydroxymethylated DNA immunoprecipitation assays. We found the presence of methylated (5mC) and hydroxymethylated (5hmC) cytosines in all the samples analyzed. MtDNA methylation especially occurs within non-CpG sites. It also emerged that the methylation pattern of the D-loop is strictly cell type-dependent and that it might be an example of RNA-directed DNA methylation (RdDM). The methylation of D-loop occurred mainly in the promoter region of the heavy strand and in conserved sequence

blocks (CSBI-III), indicating the involvement of epigenetic modifications in regulating mtDNA replication and/or transcription.

Bisulfite sequencing of 12S rRNA region showed the methylation of one CpG site located at 931nt. This site was then analyzed by real-time MGB Probe-based PCR reactions in bisulfite-treated DNA extracted from peripheral venous blood collected from subjects of different age and classified for frailty phenotype. We detected the co-presence of both unmethylated and methylated cytosines in most sample analyzed. Statistical analyses revealed that 12S rRNA methylation displays sex- and age-specific differences, and it is correlated with the health status.

In the third section, it is reported that global DNA methylation levels are influenced by mitochondrial DNA inherited variants, probably via the different regulation of OXPHOS machinery. So that, we prove, through cybrid technology, which is an useful approach to reveal possible pathways of communication between mitochondrial and nuclear genomes, as epigenetics processes are modulated in response to the above pathways.

In the appendix an additional published paper (Montesanto et al., *The genetic component of human longevity: analysis of the survival advantage of parents and siblings of Italian nonagenarians*, 2011, Eur J Hum Genet 19: 882-886.), which I worked to during my PhD appointment is reported.

CHAPTER 1

Introduction

1. DNA methylation: an epigenetic process

The term epigenetics was initially referred to the study of how genotypes lead to phenotypes through programmed changes, not involving DNA sequence, during development [1,2,3]. Today, "epigenetics" refers to both hereditable changes in gene activity and expression and to stable not necessarily heritable alterations in transcriptional potential of a cell. Therefore, epigenetics include all mechanisms involved in performing the genetic program of many biological processes such as development, differentiation, stress response. Epigenetic modification can be affected by different factors, including physiological and pathological circumstances, as well as by environment. Constant progress are being made in the identification of epigenetic marks, i.e., the molecular marks on the chromosome that influence genome function, and while DNA methylation remains the most extensively studied, the importance of histone modifications as well as the contribution of ncRNAs has become increasingly clear.

In the genomes of vertebrates, one of the key mechanisms of epigenetic modification is the methylation of cytosine residues in DNA sequences, first discovered in calf thymus DNA by Hotchkiss in 1948 [4]. DNA methylation consists of the addition of a methyl group at 5-carbon position of deoxycytosines thus forming 5-deoxymethylcytosine (5mC). In particular, in humans this process accounts for 3-6% of the total cytosines [5]. The methylation of DNA in mammals occurs on cytosine residues located 5' to a guanosine, the so called CpG dinucleotide. CpGs are not evenly distributed across the genome but are concentrated in short CpG-rich sequences, named CpG islands (CGI), usually located in the promoter regions of genes [6]. A CpG island refers to a region composed by at least 550 bp, having an observed CG/expected CG ratio>0.65 (in the whole genome it is around 0.1–0.2) [7]. CGI are found in the promoter regions of about 70% of all human genes, including most housekeeping and tissue-specific genes [8]. Normally, the amount of methylated cytosine in a gene control region is inversely correlated with gene activation [9,10]. As a consequence, active genes usually show hypomethylation in the promoter region around the transcriptional start site (TSS), and high levels of methylation in the gene body, which increase with gene expression [11,12,13]. The presence of methylation in gene body has an important functional role. Indeed, it blocks aberrant transcription initiation inside the gene and therefore contribute to avoiding the assembly of cut mRNAs and proteins. In addition, exons show an higher DNA methylation levels than introns suggesting a role of DNA methylation also in the splicing process [12,13].

DNA methylation is involved in regulating many cellular processes as: i) development and differentiation. In fact, it was demonstrated that the homozygous loss of DNMT1, 3a and 3b genes in the early stage of embryonal development results in embryonic death. Furthermore, somatic DNA methylation contributes to differentiation by repressing key genes in the germline and irreversibly forging the cell to differentiate; ii) X chromosome inactivation. During the embryogenesis of females, the inactivation of one of the X chromosomes is realized by DNA methylation in order to lead to the dosage compensation and thus to equalize gene expression between the sexes; iii) genomic imprinting. Through DNA methylation one copy of a gene is preferentially silenced and retains the same mono-allelic expression of its parental origin. Some studies demonstrated as DNMT3 family is implicated in maternal imprinting; iv) parasitic DNA suppression. Transposable sequences, including retrotrasposons and retroviral elements, are neutralized by DNA methylation, thus suggesting that this process can act as defense mechanism.

The regulation of the above processes is realized by DNA methylation acting on chromatin structure that induces gene silencing. This process takes place either inhibiting the transcription factor binding to DNA target sequences or facilitating the recruitment of methyl-binding proteins.

2. Structural and functional characteristics of DNA (cytosine-5-)-methyltransferases (DNMTs)

DNA (cytosine-5-)-DNA methyltransferases (DNMTs) catalyze the transfer of the methyl group from a cofactor molecule S-adenosyl-L-methionine (SAM) to the C5 position of the cytosine residues. As a result, 5-methylcytosine is produced, and S-adenosylhomocysteine (AdoHcy, SAH) is released from the enzyme (**Figure 1**).

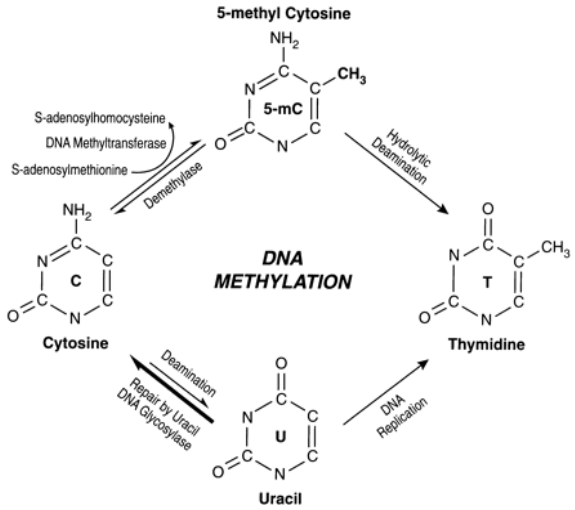

FIGURE 1. DNA methylation cycle.

The Watson and Crick pairing is not altered after the addition of the methyl group to cytosines because it is located in the major groove of DNA, a perfect place in which DNA-interacting proteins can identify it.

The human genome contains three active enzymes, called DNMT1, DNMT3a, and DNMT3b, and one related protein without any catalytic activity, called DNMT3L. After embryonic fertilization cells need a primary establishment of methylation patterns. This establishment is carried out by the *de novo* methyltransferases DNMT3a and DNMT3b, which have high affinity for unmethylated DNA [14,15]. The two enzymes are crucial for organism life and there is no other enzyme in the genome that can substitute their activity as demonstrated by knock out organisms in which the non expression of the above enzymes is fatal at the embryonic stage [16,17]. DNMT1, the most abundant form of DNMTs in mammalian cells, is highly expressed during the S phase and it works after each replication cycle acting as *maintenance* DNA methyltransferase [18].

In mammals, the initial methylation pattern (**Figure 2**) is decided by DNMT3a and DNMT3b enzymes. This pattern is perpetuated (with a fine tissue-specific regulation) by a mechanism first proposed by Riggs and Holliday in 1975 [19,20]. After each round of DNA replication, DNA is hemimethylated since the parental strand carries methylation marks meanwhile these marks are absent in de novo synthesized daughter strand. The unmethylated de novo strand is then methylated by DNMT1 recognizing specifically hemimethylated DNA. In case of altered activity of the maintenance methyltransferase and/or its suppression, some errors in the maintenance of methylation patterns take place

(i.e. passive demethylation). This generate a molecule of unmethylated DNA after another cycle of replication equivalent to loosing the 50% of the methylation per replication phase.

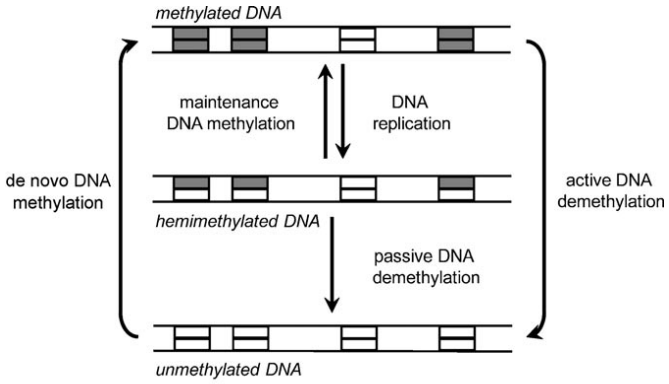

FIGURE 2. Dynamics and roles of DNA methylation in mammals.

Significant cooperation between the DNMTs is required to maintain DNA methylation patterns during the post-replication phase [21]. However, it seems that for maintenance of DNA methylation is necessary not only the identification of hemimethylated DNA by DNMT1 but also the recognition of the DNMT3a and DNMT3b enzymes of specific chromatin regions containing methylated DNA [22,23]. According to this hypotesis, errors made by DNMT1 can be corrected by DNMT3a and DNMT3b that complete the methylation process thereby assuring high-fidelity replication of DNA methylation patterns.

Mammalian DNA methyltransferases consist of two elements, a large multidomain N-terminal of variable size with regulatory functions, and a C-terminal catalytic domain (**Figure 3**). The N-terminal directs the nuclear localization of the enzymes and mediates their interactions with other proteins, DNA and chromatin. The smaller C-terminal part, conserved between eukaryotic and prokaryotic organisms, contain the active centre of the enzyme and ten amino acids motif diagnostic for all DNA C-5 cytosine methyltransferases [24]. A common core structure is shared by the catalytic domains of all enzymes, called the AdoMet-dependent MTase fold. It consists of a mixed seven-stranded β-sheet, formed by six parallel β strands and one strand in an antiparallel orientation, inserted into the sheet between strands 5 and 6. Six helices are folded around the central β-sheet [25]. This domain is involved both in cofactor binding and catalysis. The so called target recognition domain (TRD), a non-conserved region of the enzyme, is involved in DNA recognition and specificity [24,26].

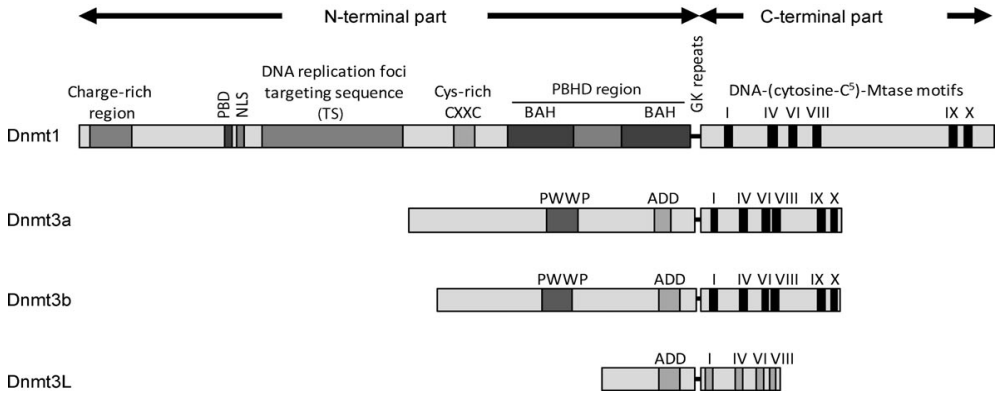

FIGURE 3. Domain architecture of mammalian DNA methyltransferases.

2.1 DNMT1 (DNA nucleotide methyltransferase1)

DNMT1 was the first mammalian DNA methyltransferase enzyme to be cloned and biochemically characterized [27]. As mentioned above DNMT1 shows a preference for hemimethylated DNA over unmethylated DNA and is localized at DNA replication foci during the S phase, properties that reflect its role as maintenance methyltransferase.

Human DNMT1 contains 1616 amino acid residues. The first three quarters of the protein contain regulatory domains; the DNA methyltransferase-associated protein 1 (DMAP1)-binding domain, the Replication Foci Targeting Sequence (RFTS) domain, the CxxC zinc binding domain and two Bromo-Adjacent Homology (BAH) domains (**Figure 3**). The remaining C-terminal region corresponds to the catalytic methyltransferase domain [28].

DNMT1 is highly expressed in proliferating cells. It has the major catalytic methyltransferase activity in somatic tissues all over mammalian development [29].

Ten motifs of the catalytic domain of eukaryotic DNMT1 are highly conserved also in prokaryotes. DNMT1 catalyzes methyl transfer according to the Michael addition reaction pathway as shown in **Figure 4** [30].

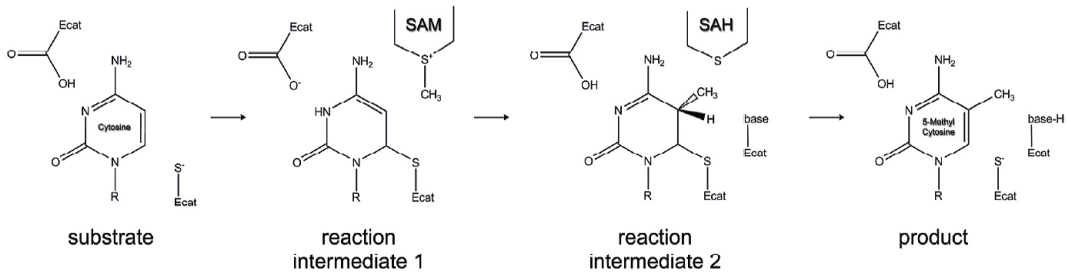

FIGURE 4. DNMTs reaction mechanism.

Some post-translational modification like phosphorylation, methylation and sumoylation have DNMT1 as a target. Although initially the serine 515 was identified as the main site of the phosphorylation in insect cells, three phosphorylated serine residues (Ser717, Ser958 and Ser1108) have been then successively observed, through high-throughput proteomics approaches, within DNMT1 purified from human cells [31,32,33,34]. In addition, sumoylation of DNMT1 has been shown to activate the enzyme [31,32,33,34]. In addition, sumoylation of DNMT1 has been shown to activate the enzyme [31,32,33,34]. In addition, sumoylation of DNMT1 has been shown to activate the enzyme [35]. Moreover, it was recently reported that DNMT1 is degraded by proteasome system when is methylated by SET7/9 at level of Lys142 [36].

2.2 DNMT3 (DNA nucleotide methyltransferase3)

The mammalian DNMT3 family of DNA methyltransferases includes three members: DNMT3a, DNMT3b and DNMT3L. DNMT3a and DNMT3b are responsible for the establishment of DNA methylation patterns early in mammalian development and in germ cells. DNMT3L is catalytically inactive and acts as regulatory factor in germ cell. [21,37,38,39].

Because of their *de novo* activity DNMT3a and DNMT3b are highly expressed in tissues that still need to differentiate, like embryonic tissues and undifferentiated embryonic stem cells (ESC). On the contrary, these enzymes are under-represented in differentiated cells. As DNMT1, DNMT3a is ubiquitously expressed and can be detected in different tissues. Nevertheless, a shorter isoform of the enzyme exists, called DNMT3a2, tightly regulated. It is predominant in embryonic stem cells, germ cells and embryonal carcinoma cells and is also detectable in spleen and thymus, but silenced in adult tissues [40].

Similarly, DNMT3b isoforms show distinct expression profiles and localization patterns during development, raising the possibility that they might contribute to the methylation of different sets of sequences in the genome [41].

Similarly to DNMT1, the DNMT3 enzymes possess an N-terminal regulatory part and a C-terminal catalytic part harboring the conserved C-5 DNA methyltransferase motif. The catalytic domains of DNMT3a and DNMT3b share approximately 85% sequence similarity but, in contrast to the catalytic domain of DNMT1, they are enzymatically active in isolated form [42].

The third member of the mammalian DNMT3 family, DNMT3L, is catalytically inactive, even though is homologue to the DNMT3a and DNMT3b enzymes. In addition, it is not capable to bind the cofactor SAM and binds to DNA only in a very weakly way [43,44]. DNMT3L is expressed specifically in germ cells during gametogenesis and embryonic stages [45,46]. DNMT3L acts as a positive regulator of *de novo* methyltransferases. Indeed, it co-localizes with both DNMT3a and DNMT3b, and is able to directly interact with catalytic domains of the enzymes stimulating their activity both in vivo and in vitro. Likely, this is due to a conformational change induced by DNMT3L that favors DNA and AdoMet binding and catalysis [45,47,48,49].

2.3 TET proteins are responsible for DNA demethylation

Beside enzymes involved in DNA methylation, cells also have an antithetical family of enzymes prone to demethylate DNA.

As already discussed, CpG islands are often in transcriptional sites and 60–70% of known gene promoters, including most housekeeping genes, as well as tissue-specific and developmental genes. For these reasons, cytosine methylation can be considered the major regulation mechanism of gene expression. This epigenetic process reveals its importance along cells differentiation controlling the differential expression of specific genes in diverse kind of tissues. Hence, it emerges the importance to finely regulate DNA methylation patterns in order to have a correct embryogenesis and to prevent a pathological development. To date, mechanisms regulating methylation levels and avoiding the accumulation of DNA methylation at CpG islands are yet not understood. Notably, despite of intense searches, none direct DNA demethylase has been identified. However, Rao and colleagues recently found that the ten-eleven translocation 1 (TET1) protein can promote

the conversion of 5mC into 5-hydroxymethylcytosine (5hmC) suggesting a potential mechanism for active demethylation (see also **Figure 2**) [50].

The TET protein family members TET1, TET2 and TET3 are 2-oxoglutarate and Fe-dependent dioxygenases [50,51,52]. The founding member of the TET family of DNA hydroxylases, the TET1 gene, was initially identified in acute myeloid leukemia (AML) as a fusion partner of the histone H3 Lys 4 (H3K4) methyltransferase MLL (mixed-lineage leukemia) [53,54]. TET proteins contain several conserved domains (**Figure 5**), including a CXXC domain that has high affinity for clustered unmethylated CpG dinucleotides, a cysteine rich region followed by the double-stranded β-helix (DSBH) fold required for catalytic activity [50]. In agreement with the known reaction mechanism of dioxygenases, mutation of putative iron-binding sites of TET proteins abolishes their enzymatic activities [50,51,55].

FIGURE 5. The domain structure of human TET proteins.

Contrarily to 5mC, 5hmC levels fluctuate significantly between tissues, with the highest levels reported in specific cell types of the brain and in ESCs, although these levels decrease during differentiation [50,56,57,58].

TET-mediated conversion of 5mC into 5hmC (**Figure 6**) could be realized by a passive mechanism but, in contrast to 5mC, 5hmC is not maintained through DNA replication. Alternatively, production of 5hmC could be determined, in non-dividing cells, by an active demethylation pathway during the repletion of 5mC into cytosine [59]. This hypothesis was recently supported by studies demonstrating the existence of formylcytosine and carboxylcytosine in mammalian DNA (**Figure 6**) [51,60,61]. These cytosine modifications can be generated by two successive oxidation reactions of 5hmC catalysed by the TET proteins, raising the possibility that the TET proteins might be involved in several steps in converting 5mC to cytosine [51,60]. As the TET proteins cannot convert carboxylcytosine to cytosine, a decarboxylase or a glycosylase might be involved in this step. In agreement with this, depletion of thymidine-DNA glycosylase (TDG) leads to accumulation of carboxylcytosine in mouse ESCs, and other studies have shown that TDG is required for DNA demethylation [60,62].

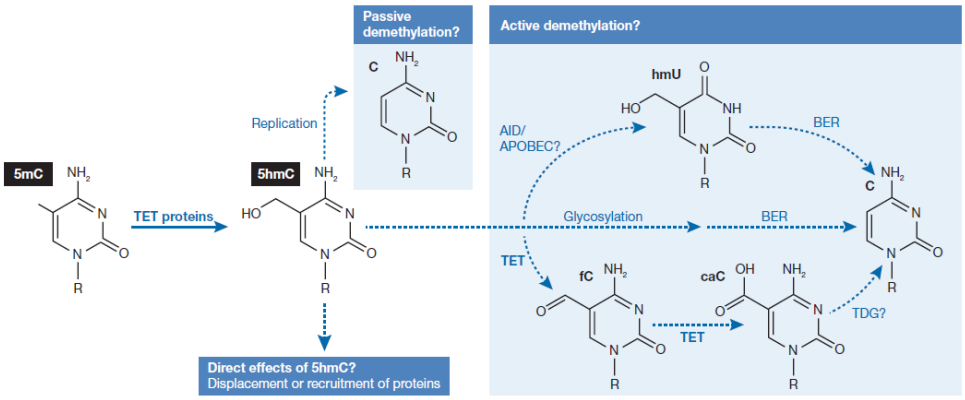

FIGURE 6. Different biological mechanisms of TET-mediated conversion of 5mC to 5hmC.

As to the regulatory role of TET proteins in determining gene transcription, many functions were postulated and some of them demonstrated. Both TET1 and 5hmC localize to TSSs supporting the TET proteins regulative role. However, in TET1-depleted ESCs was found that less than 10% of TET1 target genes change expression after TET1 depletion. Unexpectedly, it was also found that the number of genes down-regulated was similar to, or even lower than, the number of genes upregulated after TET1 depletion, indicating that TET1 could also have repressive effects on gene transcription. In agreement with this, TET1 shares many TET1-bound promoters with the Polycomb repression complex 2 (PRC2) [63,64].

The chromatin-binding ability of PRC2 members was decreased in TET1-depleted cells, suggesting a role of TET1 in PRC2 recruitment. TET1 might indirectly facilitate PRC2 chromatin binding by decreasing DNA methylation levels at PRC2 target genes (**Figure 7A**) [63,64].

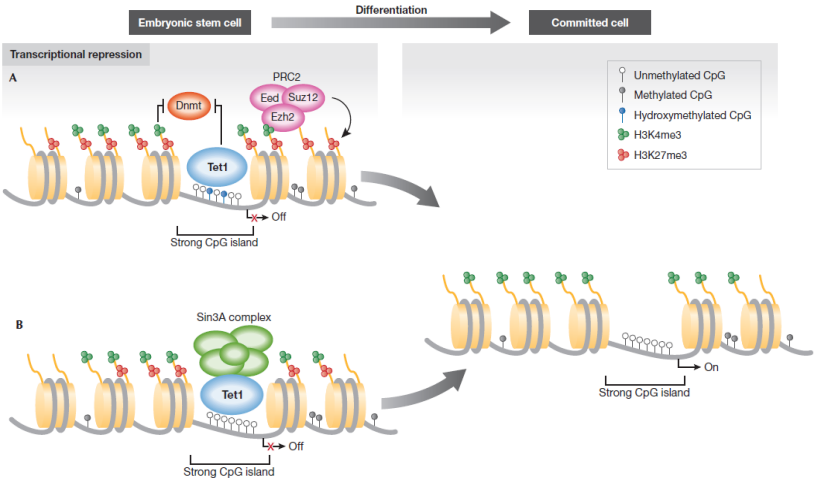

FIGURE 7. Potential roles of TET1 in transcriptional repression.

TET1 transcriptional repression could be explained by its association with the Sin3A co-repressor complex (**Figure 7B**). Indeed, TET1 has been shown to physically interact with the Sin3A complex and dysplay a significant overlap with Sin3A on target genes. Moreover, the recruitment of Sin3A to a subset of these genes was dependent on TET1 expression [63].

Even if the transcriptional activator effects of TET1 are enough weak, the repressive functions are much effective. It seems that 5hmC is enriched at several promoters with intermediate CpG content (**Fig 8a**). These "weak CpG islands" have a major possibility to undergo methylation during differentiation, suggesting that TET1 ensures that these promoters remain unmethylated in the undifferentiated ESCs [65,66]. Thus, besides its main objective to prevent casual and useless methylation of housekeeping genes, TET1 also protects numerous weak CpG island genes that are predisposed to undergo methylation. Weak CpG islands have been reported to become *de novo* DNA-methylated during differentiation. They often display high levels of 5hmC, indicating that TET1, by converting 5mC into 5hmC, ensures the opportune methylation and silencing of these target genes during differentiation (**Fig 8b**).

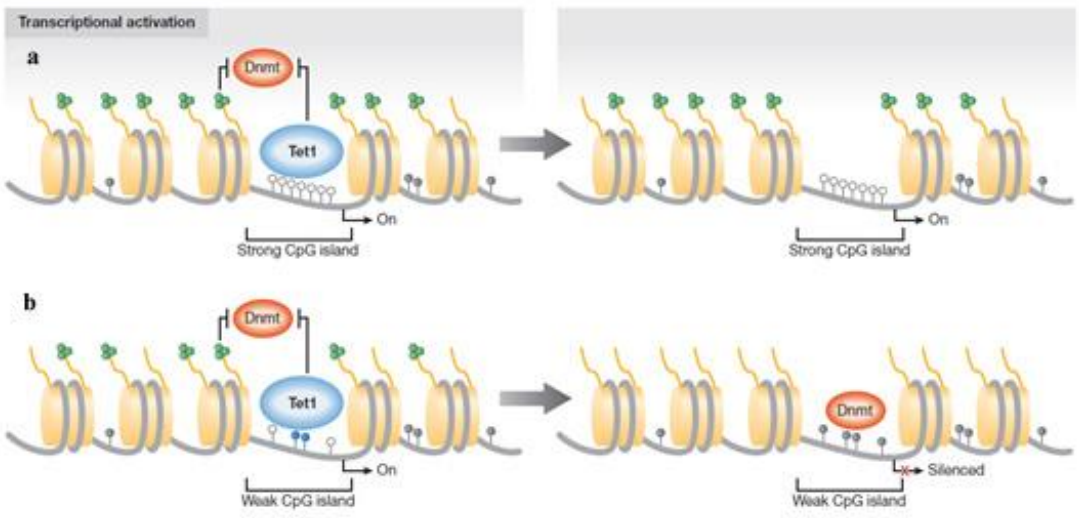

FIGURE 8. TET1 down-regulation, allows for the repressed genes activation.

Unlike the 5hmC, the pattern of 5mC can be maintained permitting the heritability of DNA methylation from a mother cell to its daughter cells.

A rapid decrease of global DNA methylation has been observed in developing Primordial Germ Cells (PGCs) as well as zygotes [67,68,69]. In the one-cell zygotes state, the 5mC mark in the paternal pronucleus quickly vanishes (in orange in the top panel of **Figure 9**)

while the same phenomenon is not observed in the maternal pronucleus. TET3 is highly expressed in the oocyte and one-cell zygote. Instantly after fertilization, TET3 may potentially translocate from the cytoplasm into the paternal nucleus converting 5mC to 5hmC. Then, in paternal and maternal genomes a reduction of 5hmC and 5mC levels occurs due to the initiation of the replication process. Although the exact mechanism is currently unclear, either knockout of TET3 from the female germ cells or siRNA specific for TET3 in the zygote prevent the conversion of 5mC to 5hmC in the paternal genome supporting the hypothesis that TET3 could be responsible for 5mC oxidation in the paternal pronucleus [70,71]. Since the different marking of 5hmC in the paternal pronucleus is observed also in bovine and rabbit zygotes, a conserved role for oxidation of 5mC in the erasure of paternal 5mC after fertilization is suggested [71].

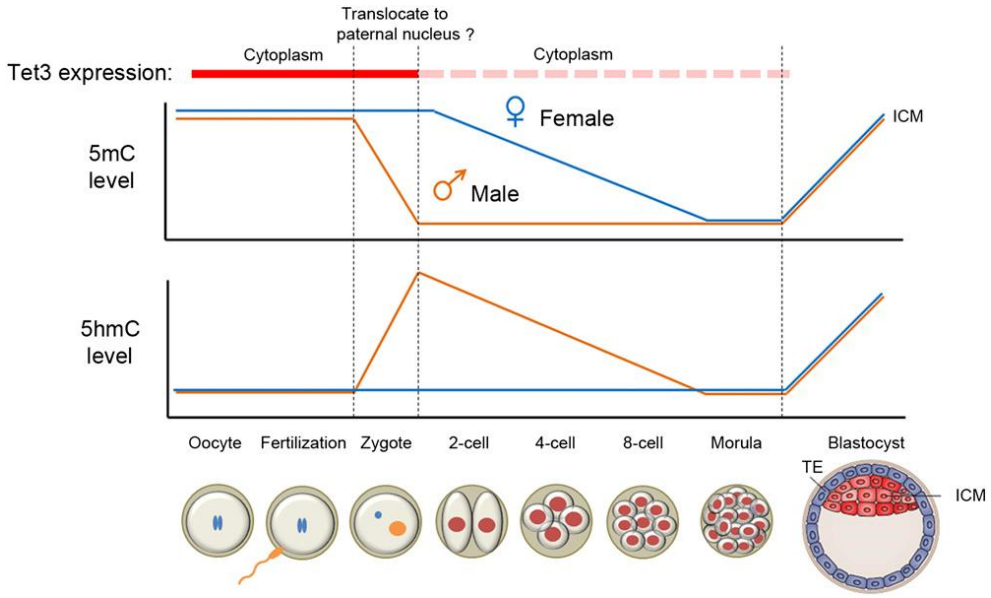

FIGURE 9. Dynamic changes of 5mC and 5hmC levels in the paternal and maternal genomes during pre-implantation development.

3. Non-CpG methylation

In plants, DNA methylation occurs in both CpG and CpNpG contexts permitting a normal plant development, regulation of transcription and transposition [72]. Plants have two classes of DNA methyltransferases (DNMTs): the MET1 family of methyltransferases and chromomethylases (CMTs). MET1s preferentially methylate cytosine in CpG sites with *de*

novo and maintenance functions. CMTs are unique to plants and methylate cytosine in CpNpG sites [72,73].

More recently, non-CpG methylation was found also in mammals, initially in embryonic stem cells then in the promoter regions of different genes. It was largely demonstrated that the amount of this methylation type within exons, introns and 3' UTRs of H1 embrionic stem cells was double that the CpG one, although gene expression in the H1 cells seem not to be correlated with CG methylation density. The uniqueness of non-CpG methylation in stem cells (it is no longer observed in differentiated cells) is likely to be maintained by continual *de novo* methyltransferase activity, suggesting that it may play a key role in the origin and maintenance the pluripotent state [11]. BMP4-induced H1 cells, in fact, maintain canonical CpG methylation pattern but misses non-CpG methylation at several loci indicating that, at the time of differentiation, the widespread non-CG methylation is lost [11]. Recently, Ziller and colleagues, through a comprehensive analysis of non-CpG methylation in 76 genome-scale DNA methylation maps across pluripotent and differentiated human cell types, confirmed non-CpG methylation to be predominant in pluripotent cell types observing also a decrease upon differentiation and near complete absence in various somatic cell types [74].

As to non-CpG methylation locus-specific, three independent studies revealed the presence of methylated cytosines within CCWGG sequence (W= A or T) located in the promoter of the murine *Sry* gene (sex determining region on the Y chromosome) [75]. Barres and coll., using a whole-genome promoter methylation analysis of skeletal muscle from normal glucose-tolerant and type 2 diabetic subjects, identified cytosine hypermethylation of peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1 α (PGC-1α) in diabetic subjects, in which the highest proportion of the above cytosines was found within non-CpG nucleotides. Methylation levels were negatively correlated with PGC-1α mRNA and mitochondrial DNA content [76].

Regarding the enzyme involved in non-CpG methylation, the *de novo* methyltransferase DNMT3a has been considered to be responsible for the establishment of non-CpG methylation patterns. Indeed, DNMT3a methylates to a lesser extent than CpG sites, both CpA and CpT dinucelotides in vivo after DNMT3a expression in *Drosophila*. Probably due to the high DNMT3a expression in ES cells rather than in differentiated tissues, the occurrence of non-CpG methylation in ES cells but not in tissues may be explained [77].

4. The heritability of DNA methylation changes

The definition provided by Conrad Waddington in the 1940 did not take into account the molecular aspects of the stable nature of epigenetics, but the inheritance of environmentally induced phenotypes. These aspects were reconsidered successively by Robin Holliday and Art Riggs in two independent studies [78,79]. The first coined "epimutation" term to distinguish heritable changes due to DNA modifications from classical gene mutations. The second discussed the stable nature of epigenetic inheritance following cell proliferation or mitosis. Hence, it was derived the concept that the epigenetic marks are mitotically and meiotically stable [80].

"Mitotically stable" implies that the epigenetic patterns, during a cell division or proliferation, are replicated. If an epigenetic mark is mitotically stable, then all cells coming from the initial cell will have the same epigenome. On the contrary, an epigenetic mark not mitotically stable would only be relevant in the individual cell and would not be important outside that cell's function. Mechanisms underlining the above stability are directly correlated to the replication of DNA, associated with mitosis, in which the methyltrasferase methylates the hemimethylated DNA to make the original strand epigenetic mark.

Mitotic stability of the epigenome highlights molecular mechanisms which allow to better understand somatic cell differentiation and function as well as the influence of environment on disease etiology and phenotypic variation.

The epigenome is programmed and maintained in a cell population as it further differentiates and is associated with the development of any tissue or organism.

An environmental factor occurring early in life could modify the epigenome of a somatic cell during a critical window of development and this, as is mitotically stable, would be replicated and influence the somatic cell differentiation and function throughout lifetime.

Concerning meiotically stability of epigenetic marks, or better known as epigenetic transgenerational inheritance, the basic mechanisms of this aspect involves the action of an environmental factor (nutrition or chemical) during germline remethylation to permanently alter epigenome of this line, that becomes permanently programmed. Therefore, altered epigenome and phenotype become transgenerational and appear in subsequent progeny and generations in the absence of any further environmental exposure.

Of course, the majority of exposures at other times of development or altering somatic cells, previously described, does not have the capability to become transgenerational. Epigenetic transgenerational inheritance through the permanently altered epigenome of the germ line dramatically influences developmental biology, disease etiology as well as other areas of biology including evolutionary biology. One the best known examples of specific environmentally dependent epigenetic marks transmitted between generations is probable the agouti mice. Morgan et al. studied epigenetic inheritance at the agouti locus. In viable yellow (A(vy)/a) mice, transcription originating in an intracisternal A particle (IAP) retrotransposon, inserted upstream of the agouti gene (A), causes ectopic expression of agouti protein, resulting in yellow fur, obesity, diabetes and increased susceptibility to tumors. Avy mice exhibits variable phenotypes because they are epigenetic mosaics for activity of the retrotransposon: inbred Avy mice, presumably isogenic, have coats that vary in a continuous spectrum from full yellow, through variegated yellow/agouti, to full agouti (pseudoagouti). It was found that the phenotype of a mouse dam with the Avy allele was related to the phenotypes of the offspring, whereby yellow dams produced yellow and mottled offspring, but not pseudoagouti offspring. Interestingly, the passage of the allele through two generations of pseudoagouti females produced significantly more pseudoagouti offspring than through only one generation of pseudoagouti dams [81]. Wolff et al. described how the maternal diet enriched in methyl donors could affect the expression of the agouti gene in the offspring [82].

In humans, the first report of a possible epimutation was published by Buiting et al., who found that epimutations at the SNURF-SNRPN locus correlated with loss of imprinting in patients with Prader–Willi syndrome [83]. Subsequently, Suter et al. reported that germ line epimutation at the DNA mismatch repair gene MLH1 was associated with a greater risk of nonpolyposis colorectal cancer [84]. However, further work by the same group questioned the transmission of this epigenetic mark between generations [85]. Chan et al. also reported a stably inherited, allele-specific, mosaic methylation in the promoter of another DNA mismatch-repair gene (MSH2) in a family affected with nonpolyposis colorectal cancer, although, the presence of the epimutation was not studied in germinal cells [86].

Importantly, two recent studies in monozygotic and dizygotic twins show that specific DNA methylation signatures can be trans-generationally heritable by either genetic or epigenetic mechanisms [87,88].

5. DNA methylation and aging

A growing number of human pathological (such as cancer, muscular dystrophy, lupus) and physiological (such as aging) phenotypes have been found to be associated with peculiar DNA methylation [89,90].

Aging is a process of slow and gradual deterioration of the functional capacities that makes the individual particularly susceptible to environmental challenges and more prone to a variety of illnesses, leading to a dramatic reduction of the individual survival probability and, ultimately, to death. It was largely demonstrated that it is the result of concurrent oxidative stress damage, ion homeostasis deregulation, chromosomal instability and of the accumulation of nuclear and mitochondrial DNA mutations. More recently, evidence about the involvement of epigenomic alterations on inter-individual susceptibility to functional decline and vulnerability to diseases in the elderly people.

5.1 General features of DNA methylation throughout aging

DNA methylation patterns are not fixed; during various stages of mammalian development they are reprogrammed to ensure the normal mammalian embryogenesis and cell differentiation. In particular, in mammalian germ cells and in pre-implantation embryos two waves of genome-wide epigenetic reprogramming occur, generating cells with a broad developmental potential [91].

Historically, the role of epigenetic changes in aging emerges from two independent studies of Berdyshev and Vanyushin, demonstrating as genomic global DNA methylation decreases in spawning humpbacked salmon and in rat brain and heart during aging [92,93]. Subsequent studies have revealed that two specific alterations of DNA methylation occur during aging: a robust and progressive rise in DNA methylation levels across lifespan for several loci and an hypomethylation of ALU and other repetitive elements [94]. Among the above mentioned studies a significant contribution has been provided by the study of mono- (MZ) and dizygotic (DZ) twins, in which gene-specific and global epigenetic differences in monozygotic twins increase overtime within different tissue and cells. These differences are ascribed to stochastic or systematic events, in response to external factor and environment changes. DNA methylation changes in aging exhibit familiar clustering in

individuals that do not share house-holds, suggesting that the DNA methylation stability is genetically determined [95].

A number of specific loci have been described to become hypermethylated with aging. The first gene showing an association between aging and promoter DNA methylation was the one encoding for estrogen receptor (ER). Subsequently, hypermethylation was found in genes encoding for ribosomal DNA clusters as well as in those involved in DNA binding and regulation of transcription, thus affecting a large spectrum of intracellular pathways [96,97]. Genes for tumor suppression (*COX7A1, LOX, RUNX3, TIG1, p16INK4A, RASSF1, DUSP22*), development and growth (*IGF2, cFos*), cell-cell adhesion (*CDH1*), metabolism (*ELOVL2, SLC38A4, SLC22A18,MGC3207, ECRG4, ATP13A4, AGPAT2, LEP*), DNA repair (*MLH1*) and control of signal transmission (*FZD1, FZD7*) exhibited altered DNA methylation patterns in aging, displaying sometimes tissue- and cell type-specific features with consequent different functional outcomes [98,99,100]. On the whole, these studies demonstrated a loss of the epigenetic control in aging, suggesting its correlation with age-related pathological phenotypes and physiological processes of aging itself including psycophysical and immune decline, sarcopenia and frailty [101,102,103]. In particular, recently Bellizzi et al. the reported that global DNA methylation levels were correlated to the frailty status in middle/advanced-aged subjects but not with age. Consistently, 7-year follow-up study revealed that a worsening in the frailty status was associated to a significant decrease in the above levels [103]. A gradual loss of DNA methylation with age occurs in most vertebrate tissues and in humans as well [95,104,105,106]. This hypomethylation predominantly affects non island-CpGs and interspersed repetitive sequences (IRSs), such as Alu and human endogenous retrovirus K (HERV-K). More recently, Heyn et al. corroborated and extended the above findings demonstrating that the age-associated hypomethylation is present in all genomic compartments, including promoters, exonic, intronic and intergenic regions [107].

Epigenetic alterations during aging are also attributed to modifications in expression and/or functions of DNA methyltransferases. Indeed, an under-expression of DNMT1 and DNMT3A in human lymphocytes T from elderly individuals and in senescent fibroblasts as well as an increase of DNMT3B in fibroblast was described [108,109]. Moreover, insufficient DNA methylation in Dnmt1+/- mice has been reported to cause immunosenescence, autoimmunity thus affecting healthy aging.

5.2 Factors affecting DNA methylation changes during aging

Non-random mechanisms such as stochastic errors in maintaining established patterns of DNA methylation or environmental stimuli are able to induce changes in epigenetic profiles at both early and late life stages, being in most cases responsible for many processes occurring during the lifespan, including development, differentiation, stress response, and pathological conditions. [110,111]. However, the above mechanisms normally do not mutate DNA sequence nor alter genetic processes indelibly.

The human life expectancy experienced a remarkable increase during the last century with an unprecedented gain in the developed world [112]. At the same time, researches have shown even larger improvement in health expectancy than in lifespan [113,114]. These phenomena suggest the important role of improving environment in determining individual health status and survival. Social-economic development and consequent advances in biomedical technology together with improved healthcare and disease treatment can be considered in explaining these changes. Recent works in scientific literature have shown that the epigenome could acquire and undergoes the impact of environmental factors [115,116,117,118]. As previously described, studies on monozygotic twins have highlighted the contribution of environmental factors to inter-individual susceptibility to disease as well as to determine lifespan, independently from genetic components of aging [119,120,121,122,123] [122,123]. A recent study demonstrated that in Calabrian population there is a greater genetic component in longevity in males than in females [124]. So genetic and environmental factors work together to determine many phenotypes in which the latter could have a major importance than the former and vice versa depending on the considered phenotype.

Several evidence, in keeping with the so-called “the fetal basis of origins of adult-onset disease” theory, indicate the mother's behavior or diet can affect epigenetic patterns in her offspring. A study carried out on Agouti pregnant mice showed that feeding the mice with a diet rich in methyl donors influences coat color, body weight, and health of their progeny [125]. Other studies conducted in rats showed that demethylation of both glucocorticoid (GR) and estradiol (ER) receptors, in the absence of appropriate nurturing and following exposure to a high-fate diet, respectively, induced increased expression of the receptor genes later in life, thereby leading to an increased stress sensitivity and a higher incidence of cancer in offspring. A diet supplemented with antioxidants like folate, choline and vitamins B6 and B12 have been demonstrated very effective for aging prevention, through different mechanisms, in several animal models. Their activity could reduce

overproduction of free radicals, which are responsible of the conversion of 5mC into 5hmC, with consequent loss of DNA methylation.

Calorie restriction (CR) is by far the most effective environmental manipulation that can extend maximum lifespan in many different species [126,127]. Although it was reported that CR acts via reduction of oxidative stress or regulation of metabolic pathways, precise mechanisms are not very well understood. Recent data suggest that DNA methylation modifications involving specific genes play an important role in CR-dependent aging and longevity. Evidences suggest that the biological effects of CR are closely related to chromatin function [128]. In fact, acting as an important environmental intervention, CR is speculated to exert its aging-delaying effect through its capacity to increase genomic stability. Reversal of aberrant DNA methylation patterns during aging is believed to be much effective for CR to maintain chromatin function and thus to influence aging processes. Interestingly, CR is likely to recover the above aberrant patterns by specific gene control rather than globally [129]. Although the majority of CR research has been based on experimental animal studies, Li et al. have generated an *in vitro* mammalian cellular system to mimic CR-controlled longevity by the reduction of glucose, the main caloric resource in cell culture medium [130]. DNA hypermethylation was found in the promoter of the *p16INK4a* gene, an important tumor suppressor and aging-associated gene, in correspondence of an E2F-1 binding site. This DNA hypermethylation blocks access of E2F-1, an active transcription factor of p16INK4a, to the p16INK4a promoter, resulting in p16INK4a down regulation, which contributes to CR-induced lifespan extension. Similar mechanisms were described for *Ras* gene. In this regard, there is a strong tendency for the DNA methylation pathway to predominately control key cancer-related genes during CR, suggesting a close connection between aging and cancer.

What is more, CR leads to aberrant DNA methylation patterns likely by modulating DNMT function. Indeed, DNMT1 activity is significantly elevated in response to CR, likely to correct the decreased methylation level during aging [130]. Evidence indicate also that Dnmt3a level changes induced by CR in the mouse hippocampus may promote brain function during aging [131].

Lastly, elements including chromium, zinc, selenium or arsenic have been demonstrated to affect DNA methylation during the lifetime. These elements are capable of reducing methylation levels at genetic loci inhibiting the activity of DNA methyltransferases. Many chemical compounds and xenobiotics, such as diethylstilbestrol (used to prevent miscarriages), bisphenol A (used in plastic industry) and vinclozolin (a fungicide used in

vineyards), alter DNA methylation at a global and/or locus-specific level, acting as endocrine disruptors with consequent developmental disorders and tumourigenesis [132].

6. Mitochondria, mitochondrial DNA replication and transcription

Mitochondria are essential cell organelles whose primary function consists in converting dietary calories in ATP via the electron transport chain (ETC) and the oxidative phosphorylation process (OXPHOS), thus implying that mitochondria have a central position between energy uptake and energy production [133]. Mitochondria are emerging to be involved in several cellular processes, including ion homeostasis, cell proliferation and differentiation, stress response and apoptosis.

Mitochondria are the sole cellular organelles that carry an own DNA (mtDNA). The human mtDNA is a 16569 bp closed-circular and double-stranded molecule, which sequence has been entirely determined in 1981 [134,135]. It has a very compact structure lacking of introns and intergenic spacing and with overlapping genes (ATPase 6 and 8, and ND4 and ND4L subunits).

MtDNA contains 37 genes encoding two ribosomal RNAs (rRNA 12S and 16S), 22 transfer RNAs and 13 polypeptides, all of which are components of the OXPHOS system (**Figure 10**).

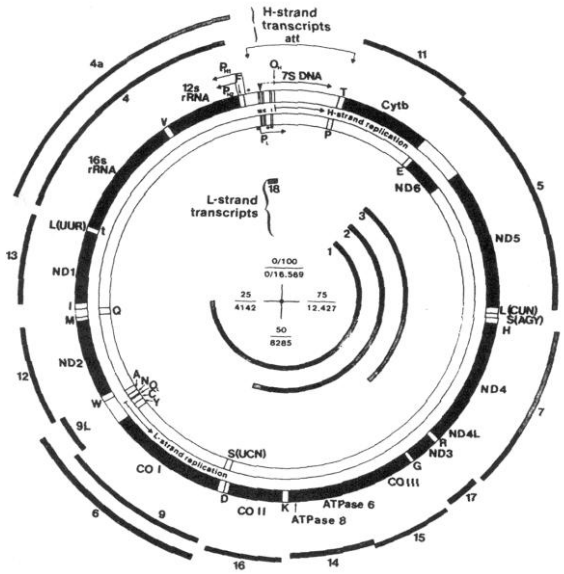

FIGURE 10. The gene products encoded by the L-strand are shown in the inner complete circle and the gene products of the H-strand in the outer complete circle.

Seven of the OXPHOS genes (MTND1, MTND2, MTND4L, MTND4, MTND5, and MTND6) encode subunits of respiratory Complex I (NADH dehydrogenase or NADH:ubiquinone oxidoreductase); one gene (MTCYB) encodes a component of Complex III (ubiquinol:cytochrome c oxidoreductase); three genes (MTCO1, MTCO2 and MTCO3) encode constituents of Complex IV (cytochrome c oxidase or COX); and two genes (MTATP6 and MTATP8) encode subunits of respiratory Complex V (ATP synthase). The remaining OXPHOS subunits, as well as all the factors required for maintenance, replication and expression of mtDNA, are nuclear-encoded, translated on cytoplasmic ribosomes and imported to their final mitochondrial compartment.

A series of distinctive features of mtDNA make it particularly interesting for genetic studies: i) cells are polyploid with respect to mtDNA: most mammalian cells contain hundreds of mitochondria and each mitochondrion contains several copies of mtDNA [136,137]. In a given individual, all mtDNA copies can be identical, a condition known as homoplasmy, although mutations can arise, be maintained or amplified to different levels and coexist with wild-type mtDNA, so called heteroplasmy; ii) the mitochondrial genome is maternally inherited, and does not undergo recombination; the few mitochondria from the sperm cell that could enter the oocyte during fertilization are actively eliminated by ubiquitin-dependent mechanisms [138,139]; iii) the evolution rate of mtDNA is much faster than that of the nuclear genome [140]. This aspect is attributable to several reasons: mtDNA is less protected by proteins, it is physically associated with the mitochondrial inner membrane where ROS are generated, and it appears to have less-efficient repair mechanisms than the nucleus [141]. This high mutation rate and the maternal inheritance pattern have made mtDNA sequence analysis an interesting tool in human population genetics and evolutionary studies [142]; iv) Mitochondrial genes are translated using a genetic code with some differences from the universal genetic code. In fact, in mammals UGA specifies tryptophan instead of a termination codon, AUA, AUC and AUU are used as initiation codons and AGA and AGG are termination codons instead of encoding arginine. In addition, a specific codon–anticodon pairing system allows translation to proceed with only 22 tRNAs. [134,135,143,144,145,146].

The two strands of the mtDNA have an asymmetric distribution of guanines and cytosines generating heavy (H) and light (L) strands, respectively. The H-strand encodes for the majority of the genes; the L-strand encodes only eight genes for tRNAs and the gene of the ND6 OXPHOS subunit. Each strand is transcribed from one predominant promoter, HSP (high strand promoter) and LSP (light strand promoter) both having a bipartite structure.

One element contains a consensus sequence motif of 15 bp surrounding the initiation points and is essential for transcription. A second element, located immediately upstream of the initiation point is required for optimal transcription and can be considered as an enhancer. In this element it is included the binding site for mitochondrial transcription factor A (mtTFA), main regulator of mtDNA expression. The two promoters are located in the control region which includes the displacement loop (so called D-loop). The D-loop is the sole non coding region, with a triple-stranded structure generated from a template-directed termination of H-strand DNA synthesis soon after initiation carrying out to the synthesis of a short piece of DNA, the 7S DNA. This fragment remains stably hybridized to the parental molecule, thus forming the triple-strand structure characterized by the displaced parental H-strand.

Transcription occurs from the HSP or the LSP in opposite directions around the entire genome circle. The H-strand is transcribed by two overlapping units. One starts at the initiation site H1, located upstream of the $tRNA^{Phe}$ gene and ends at the 16S rRNA 3' end. This unit operates much more frequently than the second one and is responsible for the synthesis of the two ribosomal RNAs, $tRNA^{Phe}$ and $tRNA^{Val}$. The activity of this unit is linked to a transcription termination event taking place immediately downstream from 16S rRNA, inside the gene for tRNA Leu. The second transcription unit, operating with a frequency about 20 times lower, starts at the initiation site H2, close to the 12S rRNA 5' end and originates a polycistronic molecule covering almost the whole H-strand [147,148]. Therefore, a differential regulation of rRNA *versus* mRNA transcription can be operated through the initiation of H-strand transcription at the two alternative sites.

The L-strand gives rise to a single polycistron starting at the 5' end of 7S RNA, about 150 bp away from the H1 initiation point, from which the eight tRNAs and the ND6 mRNA are derived. To date it has not been clearly established whether transcription occur from both promoters at the same time on the same molecule or two independent events take place.

A single-subunit mitochondrial RNA polymerase (mtRNAP), that is distantly related to the RNAP of bacteriophage T7, the pol I family of DNA polymerases, and single-subunit RNAPs from chloroplasts, is responsible of both the transcription for RNA synthesis and for initiation of replication. mtRNAP, which X-structure was recently characterized, requires the factors mtTFA and mtTFB2 for binding and melting promoter DNA [149].

Long polycistronic RNA chains are produced and subsequently cleaved on both sides at tRNA level into distinct mature RNA molecules. Additional transcript processing intermediates have been observed in selected tissues and in association with various

pathogenic mtDNA mutations [150,151,152,153]. Polyadenylation of mRNAs occurs during or immediately after cleavage.

mtDNA replication starts at O_H (origin of H-strand), located in the D-loop region downstream of the LSP, and proceeds along the parental L-strand to produce a daughter H-strand circle. When H-strand replication reaches the origin of L-strand replication (O_L), approximately located at two thirds of the genome, within a cluster of five tRNA genes, the parental H-strand is displaced, O_L is exposed and its replication starts and proceeds in the opposite direction producing a daughter L-strand. Initiation of H-strand replication needs short RNA primers, which are originated by the processing of L-strand promoter transcripts. Therefore, replication of mammalian mtDNA is functionally linked to mitochondrial transcription and, hence, depends on the activity of regulatory factors required for L-strand transcription. Briefly, during the replication initiation mtRNA polymerase starts transcription from the L-strand promoter producing the primer precursor. This RNA remains hybridized with a region of DNA upstream of O_H containing the CSBs, highly conserved sequences, forming a stable R-loop structure. A mitochondrial RNA-processing endoribonuclease (RNase MRP) cuts the RNA primer precursor generating the mature primer(s) for replication. Finally, the mitochondrial DNA polymerase starts H-strand replication through the extension of an RNA primer. Then, H-strand synthesis events or are arrested around the TAS sequences, short (15 bp) sequences conserved in vertebrates, creating the triplex D-loop structure or replication proceeds over the entire length of the genome. The precise mechanisms regulating the two events are not yet fully understood. This initiation of replication from O_L requires a specific primase capable of generating short RNA primer molecules. Once initiated, L-strand replication proceeds over the entire length of the strand and ends after the H-strand.

Both transcription and replication of mtDNA are regulated mainly by mtTFA transcription factor, that it is not only required for transcription initiation, but also seems to have a direct role in mtDNA maintenance.

mtTFA binds throughout the D-loop with a 40 to 50 base periodicity, with MTCSB2 and MTCSB3 being unbound and MTCSB1 being strongly bound. The mtTFA phasing downstream from MTCSB1 corresponds to DNA synthesis initiation sites, thus suggesting that mtTFA play a role in defining the transition from RNA to DNA [154]. Results from *in vitro* transcription assays suggested that the amount of TFAM bound to DNA dictates whether LSP or H1 is activated; this promoter-switching activity was recently confirmed quantitatively with an assay utilizing recombinant mitochondrial polymerase and

mitochondrial transcription factor 2B. At low TFAM concentration LSP is preferentially activated, and as TFAM concentration increases, transcription activity switches to H1. In addition, it was demonstrated that TFAM alone is sufficient to compact DNA *in vitro*, likely through DNA looping to reduce contour length, and supercoiling to impart structural rigidity. The rules governing TFAM–mtDNA interactions should allow for a fine-tuning of TFAM activity that integrates specific and non-specific DNA binding functions to ensure proper maintenance of mtDNA [155]. However, controversial data emerged about the activity of mtTFA, since it was demonstrated that the reduction in its levels in heterozygous knock-out mice was accompanied in all tissues by a drop in mtDNA levels but not in mtRNA levels [156].

7. Mitochondria and aging

The role of mitochondria in the aging process has been a topic of intense interest for many years; in fact age-related changes in mitochondrial content, structure and function, as well as in mitochondrial DNA have been extensively documented. A series of studies have demonstrated a decline of the mitochondrial respiration efficiency with age in human and primate and that this decline can be attributed either to a progressive down regulation of genes encoding for mitochondrial proteins such as several subunits of cytochrome-c oxidase, NADH dehydrogenase and ATP synthase, or to the decline of mitochondrial biogenesis with aging [157,158,159,160,161,162].

It was also documented that the decline in OXPHOS activity, and in turn in the overall mitochondrial function, is correlated with an accumulation, in post-mitotic tissues during human aging, of mitochondrial DNA mutations, including point mutations, large scale deletions and duplications. Among the variety of mtDNA alterations, the most prevalent age-associated point mutations of mtDNA are A3243G and A8344G transition, while the most common mtDNA deletion in aging human tissues is the 4977 bp deletion [163,164]. The creation of mtDNA-mutator mice, that are knock-in mutant mice expressing a proofreading-deficient version of the mitochondrial DNA polymerase γ gene (POLG), has provided the first direct evidence that accelerating the mtDNA mutation rate can result in premature ageing, consistent with the view that loss of mitochondrial function is a major causal factor in ageing. Mitochondria accomplish numerous tasks crucial for cellular and

organism good health status in eukaryotes. Many cell lines that are genetically modified and constructed to study pathological alterations show an altered energetic metabolism or a defective mitochondria. For this reason mitochondrion is an important cellular organelle that permit to maintain a subtle regulation of cellular energy metabolism and so has a significant role in some complex phenotype or disease. As a complex phenotype, during aging some mitochondrial functions are altered. However, for many years if is mitochondrial dysfunction that cause aging or if the natural process of aging that altered normal mitochondria remained an unsolved question.

Since mitochondria is the main source of reactive oxygen species (ROS) and free radicals, molecules both involved in cellular age-dependent damage, researchers proposed the connection between mitochondria and aging [165]. Mitochondrial role in aging was refined by Miquel and colleagues that in 1980 stated that these organelles begin cellular processes leading to aging since they are in the main source of ROS and the principal target of their injury [166]. In fact, the age-related accumulation of free radicals in the mitochondrion has heavy effects on the integrity of the organelle itself that are indeed oxidized by these reactive species [133].

A healthy mitochondrion has the ability to produce the amount of ATP proportional to the energy demand. Thus, the equilibrium between mitochondrial functions and dysfunction is necessary for retarding or avoiding cellular senescence and extend an organism survival probability.

Mitochondrial theory of cellular aging relies on the fact that mitochondrial DNA (mtDNA) has a high rate of mutation and limited capacity for repair. Essential for mitochondrial function is the integrity of the mtDNA. Nonetheless, over time an accumulation of mtDNA mutations compromises mitochondrial genome [167]. Thus, while continue to release higher quantity of ROS the organelle progressively lose their ability to generate energy [168]. During the aging of tissues the amount of cells containing mitochondria whit altered functions raises. This caused cellular injure and/or death that induce degeneration of tissues and organism disorders, which are present in age-related diseases that could lead to the individual death. Interesting is the definition of the “aging clock” that considers mtDNA and its mutation like a clock that marks the organism’s time and initiate the aging events determining the organism lifespan [169].

Rates of mtDNA mutation have been reported to be 15-fold higher than that of nuclear DNA (nDNA). This induce abnormal expression of electron transport chain (ETC) proteins unbalancing the oxidative phosphorylation process [162,170]. ETC dysfunction decreases

ATP production and raises ROS production that lead to impair nucleotide synthesis, that, in turn, affects nDNA genes [171].

Human mtDNA contain 37 genes encoding 13 polypeptides that compose subunits within the OXPHOS complexes (see paragraph 7). Histone are not present in mtDNA and so mitochondrial nucleic acid does not has its protection. In addition, mtDNA and has a less effective repair mechanisms than nDNA [171,172]. As mentioned above, mutations in mtDNA lead to mitochondrial dysfunction and increased ROS production. As its location in the mitochondrial matrix, mtDNA is directly involved into harmful consequence of ROS production making it highly prone to oxidative damage [171]. Indeed, in rat liver cells was found that the total dosage of 8-hydroxydeoxyguanosine, a marker of DNA oxidative damage, was 16 times higher than that of nDNA [172]. Together, this damage results in numerous pathological anomalies, which are related to the degree of mtDNA alterations, quantity of altered mitochondria and the kind of tissue involved [173,174]. The age-related tissues deterioration in heart, in nervous system and in kidney has been associated with the gradual accumulation of mtDNA mutations [175].

The employment of mice with a proof-reading-deficient version of mtDNA polymerase γ (POLG) permitted to shed light on the connection between mutated mtDNA and aging. This defective POLG lead to an increase three to five folds of point mutations along with broken linear mtDNA and deletions. Up to their early adolescence these “mutant mice” were in a good status of health, but in the course of time they showed the onset of aging phenotypes such as osteoporosis, weight loss, heart enlargement, anemia, curvature of the spine, hair loss, and reduced fertility, associated with significant reduction of life span [176].

Miquel and colleagues in 1980 stated that mitochondria are involved in regulating cellular processes leading to aging since they are the main source of Reactive Oxygen Species (ROS) as well as principal targets of their injury [166]. A progressive and irreversible accumulation of the above species is realized during aging with consequent heavy effects on the integrity of the organelle itself that are indeed oxidized by these molecules, as emerged in the well known theory of aging [133]. Mitochondrial DNA is especially susceptible to attack by ROS, for several reasons: i) its close proximity to the electron transport chain, the major site of ROS production; ii) the size and the compactness of the genome; iii) the lack of protective histones; iv) the absence of adequate mitochondrial DNA repair systems.

As largely reported, mtDNA mutations accumulate progressively during lifetime and influence directly the cellular oxidative phosphorylation activity, thus leading to an enhanced ROS production. In turn, increased ROS production results in an increased rate of mtDNA damage and mutagenesis, thus causing a "vicious cycle" which ultimately culminates in cell death. Interesting it is the definition of the "aging clock" that considers mtDNA and its mutations like a clock that marks the organism's time and initiate aging events.

In liver cells of rat it was found that the total dosage of 8-hydroxydeoxyguanosine, a marker of DNA oxidative damage, was 16 times higher than that of nDNA and that an inverse correlation between the steady-state concentration of 8-OH-dG in mitochondrial DNA and the maximum lifespan (MLSP) of a wide range of mammalian species occurring [177,178,179]. Since high concentrations of ROS cause irreversible oxidative damage to proteins, nucleic acids and membrane lipids, cellular antioxidant enzymes and ROS scavengers provide protection by maintaining their levels within beneficial ranges [171,180]. Transgenic mice overexpressing the mitochondrial antioxidant enzyme manganese superoxide dismutase (MnSOD) or catalase showed an increase lifespan [181,182]. On the contrary, early perinatal death associated with severe mitochondrial damage was observed in mice deficient in MnSOD showing dilated cardiomyopathy and neurodegeneration [183,184]. Although these studies demonstrate a link between ROS levels, POLG efficiency and longevity, they also indicate the importance of mitochondria as a primary target for oxidative damage. Nevertheless, not only these conditions trigger the aging process but it is really important that mitochondrion is able to maintain a balance in its dynamics via the harmonious regulation of some processes like autophagy, fusion, fission, biogenesis and apoptosis.

7.1 Mitochondrial dynamics and aging: autophagy, fusion, fission, biogenesis and apoptosis

Due to their essential function in energy production, an efficient quality control system is essential to remove dysfunctional mitochondria and maintain their overall integrity. Two processes make up the mitochondrial quality control: the remove of malfunctioning mitochondria via autophagy, and the biogenesis of new mitochondria.

In autophagy, the cell intentionally recycles damaged components such as proteins and organelles to increase survivability [185,186]. Indeed, the autophagy process is utilized by

the cell for those mitochondria which have a defective respiratory chain and depolarized membrane. As consequence they are unable to fuse with other healthy mitochondria [187]. Autophagy of mitochondria occurs by opening of the mitochondrial permeability transition pore and the depolarization of the mitochondrial membrane. An important reduction of lifespan has been demonstrated when a mutation, causing a loss of function, occurs in genes encoding for autophagic proteins such as Atg5 following a decrease in basal autophagy [188,189,190].

On the other hand, constant renewal of mitochondria is crucial for maintaining healthy organelles with age. So happens that mitochondria incurs in the process of biogenesis, that can occur in response to several physiological stimuli, including muscle myogenesis, exercise, cold exposure and calorie restriction [191,192,193,194,195].

Mitochondria can alter their organization, form and dimension, following intracellular and extracellular signals [196,197]. Mitochondria undergo a continuous cycle of fusion (**Figure 11a**) and fission (**Figure 11b**), and the morphology of the organelle is determined by the equilibrium between these opposite events [198]. On the one hand, reduced fission can produce long mitochondrion characterized by an elevated number of connections, the other, decreased fusion can lead to mitochondrial fragmentation because of excessive fission [199].

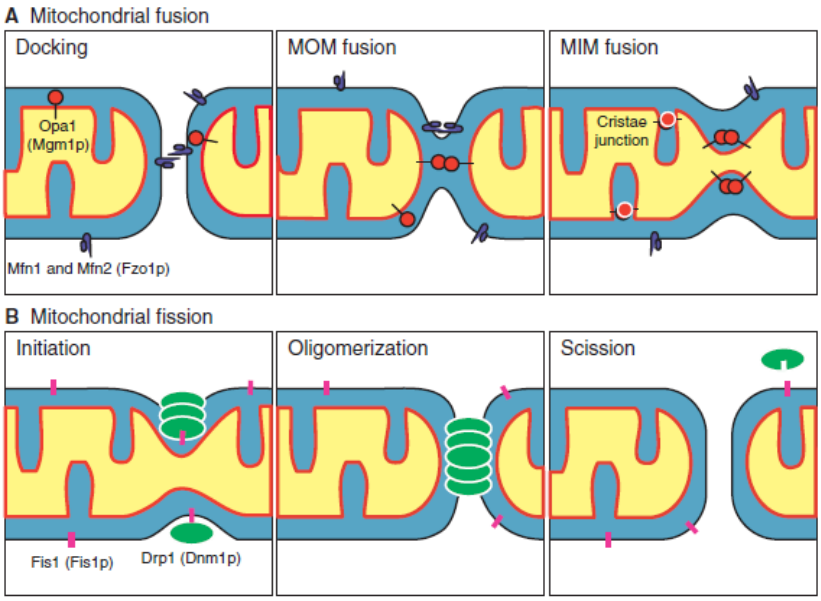

FIGURE 11. Mitochondrial fusion and fission processes.

Twig *et al*. observed that fission events often generated uneven daughter units: one daughter exhibited increased membrane potential (Δψm) and a high probability of

subsequent fusion, while the other had decreased membrane potential and a reduced probability for a fusion event. Together, this pattern generated a subpopulation of non-fusing mitochondria having reduced $\Delta\psi m$ and decreased levels of the fusion protein OPA1.

Twig *et al*. observed that if a mitochondrion after a fission process divided into two daughter mitochondria producing a functionally active polarized mitochondrion and a non-functional depolarized mitochondrion, the latter is subsequently removed by autophagy selecting healthy mitochondria with more efficient energy production and better resistance to cellular stress [200].

Most cellular components are conserved in mammals both from a structurally and a functionally point of view indicating the importance of the omnipresence of fusion and fission.

A reduced mitochondrial biogenesis is a common feature of the aging process as well as an inefficient mitochondrial degradation. Both cause a reduced turnover of mitochondria [201]. It was demonstrated that mitochondrial fusion provides to move metabolites during biogenesis while fission permits to distribute evenly mitochondria into daughter cells during mitosis in healthy cells. Moreover, this processes allow the selective degradation of non-functional mitochondria via autophagy [202,203]. Nonetheless, the flexibility of these organelles decreases with age. In fact, in aging cells has been demonstrated the presence of giant mitochondria indicating altered degradation pathways.

As regards autophagy, in yeast this process is required for chronological longevity and for preventing damage to mitochondria in chronologically old cells [204]. It is possible that the inclusion of dysfunctional mitochondria by autophagosomes blocks the release of pro-apoptotic proteins from mitochondria into the cytosol, thereby inhibiting DNA fragmentation and hampering irreversible cell death. However, an open question is what determines the fate of a mitochondrion.

An hypothesis is that mitochondrion, during aging, loses the capability to regulate its dynamic in a right and fine manner avoiding to maintain efficiently and functional mitochondria, resulting in cellular senescence. This leads to oxidative damage that could exceed a critical threshold above which many destroying signals may be triggered, thus resulting in cell apoptosis. (**Figure 12**).

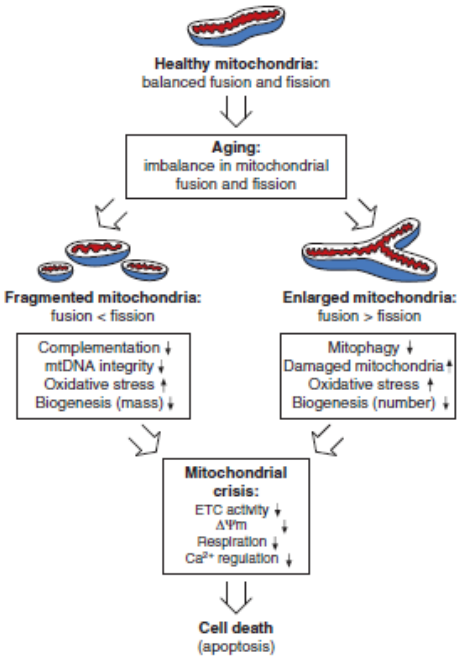

FIGURE 12. Model of the influence of mitochondrial dynamics on aging.

8. Methylation of mitochondrial DNA

Methylation status of mitochondrial DNA has been largely debated and controversial. The first data about this methylation were reported by Nass and coll. in 1973 [205]. They found in various cell lines 5mC residues within mtDNA at lower levels (one-fourth to one-fourteenth) with respect to those determined in nuclear DNA.

A year later, these findings were swiftly challenged by Dawid in a brief methodological report, that suggested that the observed 5mC presence in mtDNA might be an artifact [206]. In spite of this, a DNMT activity was found first in a mitochondrial fraction from loach embryos then in mammalian mitochondria thus leading to idea that mtDNA may be methylated [207]. This idea was confirmed successively by the same authors after revealing the presence of 5mC in mtDNA from beef heart as well as various species , including fish, birds, and mammals. [208]. Due to the methodological issues of the above researches and, at the same time, to the release of many discoveries demonstrating that *Drosophila* had no proven DNA methylation, mtDNA epigenetic research slowed for years.

Successively, Shmookler *et al*, in 1983 demonstrated that the methylation status of mtDNA extracted from human fibroblasts decreased with culture age, but only in fibroblasts obtained from young donors and not in fibroblasts of old donors [209].

In 1984, Pollack and colleagues first reported in detail patterns of mtDNA methylation in the mouse [210]. The level of methylation observed in mtDNA were 15 to 25 fold lower than that of mammalian DNA. The methylated cytosine appeared exclusively in the dinucleotide sequence CpG at an extremely low extent of 3 to 5% and different sites showed different levels of methylation, implying that the methylation of mtDNA is non random.

For more than thirty years mitochondrial epigenetics disappeared from scientific literature, but in 2011 a new intriguing discovery reopened the unsolved question about mtDNA methylation.

More recently, a mtDNA–protein interaction study carried out in living cells suggested that this genome may be methylated and DNA methyltransferases targeting the mitochondria have access to different sites on the mtDNA depending on the levels of protein occupancy [211].

Shock and colleagues demonstrated an enrichment of mtDNA sequences by immunoprecipitation against 5mC and 5-hydroxymethylcytosine (5hmC) and the translocation of DNA (cytosine-5-)-methyltransferase 1 (DNMT1) into the mitochondria. In fact, upstream of the mature peptide a mitochondrial targeting pre-peptide sequence is present enabling direct access of the enzyme into the organelle [212]. Once inside the mitochondrial matrix, mtDNMT1 binds to the mtDNA. Genes known to regulate mitochondrial biogenesis, like NRF1 and PGC1α, up-regulate the level of intra-mitochondrial DNMT1 [212].

More recently, it has been reported that aging decreased mtDNA 5hmC levels in the frontal cortex enzymes as well as regulated the expression of enzymes involved in 5-methylcytosine and 5-hydroxymethylcytosine synthesis, namely mtDNMT1 and TET1-TET3 (TET methylcytosine dioxygenases 1-3), respectively [213].

Finally, a recent study reported that the 12S rRNA mitochondrial gene, encoding the RNA component of the small (28S) mitochondrial ribosome subunit, seems to be methylated in patients affected by maternal hearing loss. Nevertheless, the methylation not occurs in a CpG site but into two tandem adenine residues and is carried out by a site-specific rRNA adenine N6-di-methyltransferase, the h-mtTFB1/TFB1M. These residues are in close proximity to an evolutionarily conserved stem-loop because of the pathogenic mutation

may raises the probability for the adenine residues of being methylated [214,215]. This methylation is essential in mice, the lack of which disrupts mitochondrial 28S ribosome subunit assembly [216].

9. Plan of the thesis

The present thesis is organized in 3 sections.
The first two sections report two manuscripts, still unpublished, aimed at investigating the methylation status of mitochondrial DNA. In particular, in the manuscript "The control region of mitochondrial DNA shows an unusual CpG and non-CpG methylation pattern" we report results obtained by analyzing the presence of methylated cytosines within the regulative mitochondrial D-loop region as well as the probable molecular mechanisms involved in this methylation. In the manuscript "Age and gender related pattern of Methylation in the 12S rRNA mitochondrial gene", we determined methylation levels of one CpG site located within the mitochondrial gene encoding the ribosomal RNA 12S.
The third section of the thesis, reports the integral version of the manuscript "Global DNA methylation levels are modulated by mitochondrial DNA variants" published on *Epigenomics* (2012). In this paper we describe the influence of mtDNA variation on Global DNA methylation.

In the appendix an additional published paper (Montesanto et al. (2011), *The genetic component of human longevity: analysis of the survival advantage of parents and siblings of Italian nonagenarians*, Eur J Hum Genet 19: 882-886.), which I worked to during my PhD appointment is reported.

CHAPTER 2

The control region of mitochondrial DNA shows an unusual CpG and non-CpG methylation pattern

*Dina Bellizzi,Patrizia D'Aquila,Teresa Scafone, Marco Giordano, Giuseppe Passarino (*MS. *Submitted to BMC Genomics*

Department of Cell Biology, University of Calabria,87036 Rende, Italy

Abstract

Background

DNA methylation is one of the most common epigenetic modification taking place in the mammalian genome. Although the occurrence of this modification has been extensively documented for the nuclear genome, conflicting reports regarding the possible presence of methylated cytosines within the mitochondrial DNA (mtDNA) have been emerged. To clarify this point, we analyzed the methylation status of the mtDNA control region (D-loop), containing the main regulatory elements for replication and expression of the mitochondrial genome. In particular, this study was carried out on human and murine DNA samples from blood and cultured cells by bisulfite sequencing and methylated/hydroxymethylated DNA immunoprecipitation assays.

Results

Here we report the presence of methylated (5mC) and hydroxymethylated (5hmC) cytosines in the mitochondrial control region (D-loop) of all samples analyzed. MtDNA methylation especially occurs within non-CpG nucleotides, mainly in the promoter region of the heavy filament and in conserved sequence blocks (CSBI-III), thus indicating its involvement in regulating mtDNA replication and/or transcription. The presence of DNA methyltransferases (DNMT1 and DNMT3B) and tet methylcytosine dioxygeneases (TET1 and TET2) we observed within mitochondria suggests that these enzymes may direct methylation of the D-loop.

Conclusions

Taken as a whole, our data show that DNA methylation occurs in the mitochondrial DNA control region (D-loop) of mammals, not only at symmetrical CpG dinucleotides, typical of the nuclear genome, but in a peculiar non-CpG pattern so far reported for plants and fungi.

Keywords

mitochondrial DNA, D-loop region, bisulfite sequencing, 5-methylcytosine immunoprecipitation, 5-hydroxymethylcytosine immunoprecipitation, 5-methylcytosine, 5-hydromethylcytosine, CpG methylation, non-CpG methylation

Background

DNA methylation is the most studied epigenetic modification that occurs in all prokaryotic and eukaryotic organisms, with rare exception for yeast, roundworm, and fruit fly. In mammals, it is a post-replication modification in which a methyl group is covalently added to the 5-position of cytosines (5-methylcytosine, 5mC) that are part of symmetrical CpG dinucleotides. In plant genomes, DNA methylation can occur symmetrically at cytosines in both CG and CHG (H = A, T, or C) contexts, or asymmetrically in a CHH context [2]. On the contrary, non-CpG methylation in mammals is quite a rare event, although it has been recently described in embryonic stem cells as well as in promoter regions of different genes [3-6]. Both CpG and non-CpG methylation are determined and maintained by a family of conserved DNA methyltransferases [7-10]. It has been widely demonstrated that CpG methylation status influences chromatin structure thus regulating the accessibility of transcription factors to their DNA target sequences [11-13]. Conversely, the biological significance of non-CpG methylation is currently unknown.

The detection of 5-hydroxymethylcytosine (5hmC) residues in different tissues and cells (mainly neurons, brain and embryonic stem cells) has recently been reported [14-16]. It was previously proposed that 5hmC is generated through oxidation or deamination of 5mC by the TET family of methylcytosine dioxygenases or via the base excision repair pathway, respectively [17-19]. However, a role in 5hmC production was recently also ascribed to DNMT enzymes suggesting that this species may not be exclusively considered an intermediate of the 5mC demethylation process, but also an important epigenetic marker regulating the pluripotency of stem cells, cellular development, aging and carcinogenesis [20-21].

Previous studies have reported the complete absence of 5mC in mitochondrial DNA (mtDNA) from *Paramecium aurelia*, *Xenopus leavis*, *Neurospora crassa* and other species, mainly based on the identical restriction patterns obtained with the methyl-sensitive isoschizomers HpaII and MspI [22-24]. More recently, the lack of 5mC residues was confirmed by Maekawa et al. by analyzing cancer cell lines and tissues from patients with gastric and colorectal cancer [25]. Conversely, different amounts of 5mC were observed in mtDNA of many other organisms, such as mouse, hamster, plant, fish and bird, although the distribution of the methylated cytosines has not been determined in any of these species [26-29]. More recently, a mtDNA–protein interaction study carried out in living cells suggested that this genome may be methylated and DNA methyltransferases targeting the mitochondria have access to different sites on the mtDNA depending on the

levels of protein occupancy [30]. Lastly, Shock et al. demonstrated an enrichment of mtDNA sequences by immunoprecipitation against 5mC and 5hmC and the translocation of DNA (cytosine-5-)-methyltransferase 1 (DNMT1) into the mitochondria [31].

Aim of this study was to investigate the presence of methylated residues of cytosines within mtDNA. In particular, the methylation status of the mtDNA control region (D-loop) was analyzed in both blood DNA collected from human subjects and in DNA from cultured cells by bisulfite sequencing and, consecutively, by methylated/hydroxymethylated DNA immunoprecipitation assays. We focused on the above region because it is the sole control region of the mtDNA, it contains the main regulatory elements for its replication and expression and it is the most rapidly evolving region of this genome [32]. The same analysis was also applied to DNA samples extracted from mouse blood and fibroblast cells. In addition, immunoblotting analyses were carried out to identify which of the three DNA (cytosine-5-)-methyltransferases (DNMT1, DNMT3A and DNMT3B) and tet methylcytosine dioxygenases (TET1, TET2 and TET3) were located within mitochondria thus being potentially responsible of the maintenance of 5mC and 5hmC, respectively, within the D-loop region of both human and mouse mtDNA.

Results

CpG and non-CpG methylation patterns in human mitochondrial D-loop

Bisulfite sequencing was used to investigate the presence of methylated cytosine residues in the human mitochondrial control region DNA (D-loop).

Results revealed the presence of unconverted cytosines, thus indicating the existence of methylation in the D-loop (Fig.1A and 1B). In particular, the majority of the methylated cytosines were located outside of CpG nucleotides, where the percentage of methylation was around 35% compared to that at CpG sites equal to 17% in DNA samples extracted from human bloods. Similar patterns were observed also in tumor (HeLa and osteosarcoma 143B.TK$^-$) and primary (skin fibroblasts) cell cultures, although they exhibit significant differences in methylation levels at CpG (ANOVA test, $p=0.002$) and non-CpG sites (ANOVA test, $p=0.013$). In fact, HeLa cells showed higher overall percentage of methylated cytosines (23% and 27%) compared to fibroblasts (10% and 21%) and osteosarcoma 143B.TK$^-$ (10% and 17%) cells, thus suggesting as the observed methylation pattern might be cell-type specific.

CpG and non-CpG methylation patterns in the mitochondrial D-loop of mouse

In order to outline a broader framework of the mitochondrial D-loop methylation pattern we extended the analysis to the mouse homologous region. Then, we applied bisulfite sequencing to two DNA genomic samples, the first isolated from whole blood, the second extracted from 3T3-L1 fibroblast cell cultures. As for humans, the analysis demonstrated not only the presence of methylated residues within the murine mitochondrial D-loop, but also that these residues are preferentially located in non-CpG nucleotides (Fig. 2A and 2B). However, for both the CpG (ANOVA test, $p=0.029$) and the non-CpG (ANOVA test, $p=0.005$) sites, the overall percentage of methylated cytosines appeared to be higher in the blood DNA samples (58.8% and 70.6%, respectively) than in the fibroblasts (17.7% and 12.5%, respectively).

Overall, the overlapping data obtained in both human and mouse seems to indicate that the methylation of the mitochondrial control region could be a general phenomenon and is established across species, at least in the higher eukaryotes, according to similar patterns.

5-methylcytosine and 5-hydromethylcytosine presence in mtDNA

To confirm the methylation status of the mitochondrial D-loop, we determined the presence of 5mC and 5hmC within mtDNA specific fragments by immunoprecipitation assays, because sodium bisulfite treatment does not distinguish between these two modified bases. In both human and mouse a significant enrichment for the two bases was observed in those fragments detected by bisulfite sequencing as methylated. In particular, immunoprecipitated DNA fragments were enriched approximately 3.5- to 5- fold in human and 3- to 4- fold in mouse for 5mC relative to the non-immune IgG control (Fig. 3A). MtDNA immunoprecipitated using anti-5hmC were enriched approximately 4- to 6-fold in human and 3- to 8- fold in mouse (Fig. 3B) relative to IgG controls. Noteworthy, the human mitochondrial DNA fragment 16037-16477 was found to be composed primarily of 5hmC residues. Consistently, with both antibodies, no enrichment was observed for those fragments detected as unmethylated by bisulfite sequencing.

Taken as a whole, the results we obtained demonstrated the presence of both 5mC and 5hmC modifications within the D-loop region with a prevalence of 5hmC.

DNA methyltransferases and tet methylcytosine dioxygenases presence within mitochondrial protein fractions

We then questioned whether the enzymes involved in the maintenance and *de novo* methylation of the nuclear DNA could localize within mitochondria and, consequently be potentially responsible for the establishment of the methylation pattern observed above. In addition, we investigated the mitochondrial localization of methylcytosine dioxigenases, reported to be involved in the conversion of 5mC to 5hmC. For this purpose, using as model both human HeLa and murine 3T3-L1 cultured cells, we performed immunoblotting assays on mitochondrial protein sub-fractions to detect the presence of DNMT1, DNMT3A, DNMT3B as well as of TET1, TET2 and TET3.

As shown in Fig. 4, DNMT1 and very low levels of DNMT3B were observed in the mitochondrial fraction in both human and mouse. As for TET proteins, we found TET1 and TET2 in the mitochondrial fraction of HeLa cells, meanwhile in 3T3-L1 cells only TET1 was detected in the same fraction. On the contrary, we did not find DNMT3A and TET3 in the mitochondrial fraction of the analyzed samples (data not shown).

These findings suggest that enzymes involved in nuclear DNA methylation processes can cross the mitochondrial membrane to their final destination inside the mitochondria and, therefore, they might have a potential role in determining the methylation pattern observed in the D-loop.

Discussion

The presence of methylated cytosines within the nuclear genome of mammals has been extensively investigated and validated in the last 20 years by several lines of evidences, especially in relation to their role in the gene expression silencing that explains the inverse correlation between density of methylation within the regulatory sequences of a gene and its transcriptional levels [12, 33-35].

On the contrary, methylation of the mitochondrial genome is largely debated and is far from being elucidated. Indeed, conflicting reports are currently in disagreement over the possible presence of methylated cytosines within mtDNA [23-31]. Nevertheless, a CpG under-representation in mtDNA, first observed by Pollack et al. in mouse and successively by Cardon and McClelland in humans, has always suggested a susceptibility to mutation of this dinucleotide also in the mitochondrial genome and, consequently, to methylation [28, 36, 37].

Here, we provide evidence for epigenetic modifications in mtDNA as methylated cytosines were detected within the mitochondrial D-loop in DNA samples extracted from blood and cultured cells of both humans and mouse. A peculiar aspect of our data is that the majority of the methylated cytosines was located outside of CpG nucleotides. The presence of non-CpG methylated cytosines might suggest that the limited available data about mtDNA methylation can be attributable to the techniques that have been used to date aimed prevalently at identifying methylated CpG dinucleotides located within recognized sequences of methylation-sensitive restriction enzymes [23, 24].

Of note is also the existence of intercellular variability for both CpG and non-CpG methylation, with higher methylation levels in human blood and HeLa cells with respect to fibroblasts and osteosarcoma cells. The same pattern was observed in mouse, supporting the notion of tissue specificity of DNA methylation. On the other hand no clear age related differences were observed.

Although recent findings have shown the presence of methylation and hydroxymethylation marks (5mC and 5hmC) in mtDNA by immunological and alternative methods, no data have been documented so far about this presence in DNA samples extracted from blood and cultured cells (HeLa, osteosarcoma cells and fibroblasts) of human and mouse. The results of our study provide the first evidence of the exact localization and the relative abundance of 5hmC relative to 5mC in the mtDNA from these tissues and cells. Our findings provide also further support to what has already been reported by Shock et al., specifically, that epigenetic modifications of cytosines in the mtDNA is likely much more frequent than previously believed [31, 39, 40]. It would be interesting to identify the 5hmC residues selectively, but at present the only method for quantitative mapping of 5hmC in genomic DNA at single-nucleotide resolution (oxidative bisulfite sequencing) is able to determine the above residues only if located in CpG dinucleotides [41].

It is important to underline the many control experiments we carried out in order to accurately confirm results we obtained. First, we performed different bisulfite treatments, also repeated many months later, to ensure that cytosine conversion was complete. Second, we adopted alternative DNA extraction procedures and extended proteinase K treatment for removing any residual protein detrimental to the above conversion. Using both strategies, the observed methylation patterns were similar. Third, the presence within the analyzed region of unconverted cytosines directly adjacent to converted cytosines represents a good marker of a successful conversion. Finally, both bisulfite sequencing and MeDIP/hMeDIP-PCR assays were carried out also on DNA extracted from osteosarcoma

143B.TK$^-$ Rho^0 cells, completely lacking of mtDNA, in order to exclude spurious amplification of nuclear mitochondrial pseudogenes (NUMTs), with consistent results [38].

The presence of DNMT1 and much lower levels of DNMT3B enzymes within the mitochondria of the HeLa and 3T3-L1 cells, used as cellular model systems in this study, might well correlate with the methylation patterns we observed in the regulatory D-loop. In fact, previous studies have demonstrated the involvement of DNMT methyltransferases in the establishment of methylation in both canonical and non-canonical sites [8, 9]. However, the translocation of the DNMTs within mitochondria is emerging to be tissue-specific, thus we cannot assume that the results observed in HeLa and 3T3-L1 cells is a general phenomenon. It is likely that in other tissues a role in mtDNA methylation might be also ascribed to DNMT3A [42].

Moreover, the intra-mitochondrial presence of TET1 and TET2 might imply that the presence of 5hmC within the D-loop could represent an intermediate step in the 5mC demethylation mediated by the above enzymes, thus indirectly regulating the replication or transcription machinery of mtDNA. Alternatively, it is possible that the two mitochondrial DNMTs may add a 5-hydroxymethyl group to DNA [20]. This process may be plausible especially for mouse where TET enzymes are totally absent or expressed at very low levels. On the whole, functional properties of DNMT and TET enzymes within mitochondria represent interesting points that deserve further investigation.

Our findings lead us to question on the biological significance of the D-loop methylation. In both human and mouse samples we identified methylated cytosines in the promoter region of the heavy filament (P_H) and within conserved sequence blocks (CSBI-III), which are highly conserved sequences located at the 5' end of the D-loop and considered to be implicated in the processing of the RNA primer during the replication of the H filament [43]. Therefore, it is possible to hypothesize that D-loop methylation might play an important role in modulating either replication or transcription of mtDNA, two processes widely described as functionally correlated.

Finally, it is interesting to notice that the dense methylation of symmetrical (CpG methylation) and non-symmetrical (non-CpG methylation) sites we observed within mitochondrial D-loop is a typical features of RNA-directed DNA methylation (RdDM), so far described only in plants and in fungi [35, 44]. In fact, RdDM occurs specifically along DNA regions that are complementary to RNA which directs the formation of a putative RNA-DNA duplex. It does not seem a coincidence to have detected the methylation

patterns described above within the D-loop, which is a stable triple-helical structure where a RNA sequence forms a hybrid structure with the L-filament. In this respect, Wassenegger observed a correlation between specific antisense RNAs and transcriptional and post-transcriptional gene silencing, thus the RdDM is beginning to be considered to be involved in epigenetic gene regulation throughout eukaryotes.

Conclusions

Taken together, our data provide unequivocal evidence supporting the presence of both methylated and hydroxymethylated cytosines within the human mitochondrial DNA control region (D-loop), with an unexpected prevalence in non-CpG moieties. Since both CpG and non-CpG methylated sites were located within the promoter region of the heavy filament and in conserved sequence blocks, it is plausible that epigenetic modifications can regulate replication and/or transcription of the mtDNA. From our data it also emerges that the peculiar methylation patterns we observed were strictly cell type-dependent and that the methylation of mitochondrial genome mighty be an example of RdDM, so far described only in plants and in fungi.

Methods

Population sample

A total of 30 unrelated adult individuals (14 men and 16 women), 41–102 years old, participated in the present study. The Ethics committee of the University of Calabria approved the recruitment and the use of the information gathered, as well as the use of the biological specimens collected on the 9th of September 2004. All subjects lived in Calabria (South of Italy) and their origin in the area was ascertained up to the grandparents' generation. Health status was ascertained by medical visit and at that time peripheral blood samples were also obtained. Before the interview each subject provided informed consent to permit her/his phenotypic and genetic data to be used anonymously for genetic studies.

Cell cultures

Human skin fibroblasts, HeLa, osteosarcoma 143B.TK- and murine 3T3-L1 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing 4.5 g/l glucose and 110 µg/ml pyruvate, supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen) and 50 µg/ml gentamycin (Invitrogen). The Rho^0 cell line, obtained by

culturing 143B.TK$^-$ in routine growth medium containing 50 ng/ml ethidium bromide with regular replenishment of medium for about 2 months, was maintained in DMEM supplemented with 10% FBS and 0.2 mM uridine (Sigma). Cells were cultured in a water-humidified incubator at 37°C in 5% CO_2/ 95% air.

DNA samples

Six milliliters of venous blood were drawn from each subject. Plasma/sera were used for routine laboratory analyses, while DNA was extracted from blood buffy coats following standard procedures.

DNA samples from human skin fibroblasts, HeLa, osteosarcoma 143B.TK$^-$, Rho^0 and murine 3T3-L1 cells were obtained by phenol/chloroform purification.

Mouse genomic DNA, isolated from whole blood of disease-free mice, was purchased from Promega Corporation.

Bisulfite treatment

Bisulfite conversion of each DNA sample was performed by using EZ DNA Methylation-Direct kit (Zymo Research), according to the manufacturer's protocol. Briefly, 1 µg of genomic DNA, previously incubated for 20 minutes at 50°C in Proteinase K and purified by centrifugation, was added to 130 µl of CT Conversion Reagent in a final volume of 150 µl. The mix was incubated at 98°C for 10 minutes and, successively, at 64°C for 3.5 hours. After adding 600 µl of M-Binding Buffer into a Zymo-Spin IC Column, each sample was loaded into the column and centrifuged at 16,000 g for 30 seconds. After adding of 100 µl of M-Wash Buffer to the column and a centrifugation at 16,000 g for 30 seconds, 200 µl of M-Desulphonation Buffer were added to the column and incubated at room temperature for 20 minutes. Then, the solution was removed by centrifugation at 16,000 g for 30 seconds and the columns were washed twice with 200 µl of M-Wash Buffer. Deaminated DNA was eluted in 10 µl of M-Elution Buffer.

As control, all samples was also analyzed by using EZ DNA Methylation-Gold kit (Zymo Research), and Qiagen's EpiTect Bisulfite Kit according to the manufacturer's protocol.

Primer design for PCR reactions

Six and 4 sub-regions, covering the entire D-loop of human (nt 16024-576) and mouse (nt 15423-16299) respectively, were isolated by PCR carried out on each bisulfite-converted DNA sample. In particular, as DNA strands are no longer complementary after sodium

bisulfite treatment, we designed primers specifically amplifying the top strand (Heavy filament) of the bisulfite-converted DNA (Additional file 1: Table S1). Moreover, some precautions were taken for primer design: i) cytosines in forward primers and guanines in the reverse primers were replaced with thymines and adenines, respectively; ii) cytosines within CpG sites were avoided; iii) when possible, DNA regions characterized by low polymorphism content were preferred; iv) short size of the amplicons was defined (range: 150-350 bps); v) a 10-bp tag was added to the 5'- ends of some primers in order to increase the annealing temperature of the A-T enriched primer sequences. In addition, primer specificity for mtDNA target sequences was tested on DNA extracted from osteosarcoma 143B.TK$^-$ Rho^0 cells, completely lacking of mtDNA.

Bisulfite sequencing

The PCR mixture (20 µl) contained 2 µl of bisulfite-treated DNA, Reaction Buffer 1X, TaqMaster PC Enhancer 1X, 0.5 µM of each primer, 0.2 mM dNTP mix (Promega) and 0.05 U of PCR Enzyme (5 Prime). The thermal profile used for the reaction included a 4-minute heat activation of the enzyme at 95°C, followed by 45 cycles of denaturation at 94°C for 20 seconds, different annealing temperature (see Table S1) for 30 seconds, extension at 72°C for 1 minute, then one cycle at 72°C for 3 minutes.

The obtained PCR products, previously purified by DNA Clean & Concentrator-5 Kit (Zymo Research), were cloned into pGEM-T Easy Vector (Promega) according to the manufacturer's protocol. Individual clones were grown and plasmids were purified using ZR Plasmid Miniprep Classic (Zymo Research). For each sub-region, 5 positive clones were analyzed by automated sequencing in a ABI PRISM 310 with BigDye Terminator Cycle Sequencing Ready reaction Kit (Applied Biosystems).

5-methylcytosine immunoprecipitation (MeDIP)

A total of 4 µg DNA extracted from both blood and cell lines were incubated with 40 U of AluI restriction endonucleases in a total volume of 20 µl overnight at 37°C and subsequently at 65°C for 20 min to inactivate the endonuclease.

5-methylcytosine immunoprecipitation was carried out using EpiQuikTM Methylated DNA Immunoprecipitation (MeDIP) Kit (Epigentek, NY, USA) according to the manufacturer's specifications. First, wells were washed once with Wash buffer (CP1) and then incubated at room temperature (RT) for 60 minutes in presence of 100 µl of Antibody

Buffer (CP2) supplemented with 1 µl of 5-methylcytosine antibody (or 1µl of Normal Mouse IgG, as negative control).

After three consecutive washes of the wells with 150 µl CP2, AluI digested DNA samples, diluted with ChIP Dilution Buffer (CP4), were added into the assay wells. The solution was incubated at RT for 90 minutes on an orbital shaker to allow DNA binding onto the assay wells. Therefore, the wells were first washed 6 times with 150 µl of the 1X WB, allowing 2 minutes on a rocking platform for each wash, followed by the addition of 150 µl 1X TE Buffer. Afterwards, 40 µl of the DNA Release Buffer (DRB) containing Proteinase K were added to each well and samples were incubated at 65°C for 15 minutes. Next, samples were incubated in 40 µl of Reverse Buffer (CP6) at 65°C for 30 minutes, 150 µl of Binding Buffer (CP7) were subsequently added to the wells and the released samples, transferred to the F-Spin column, were centrifuged at 14,000 g for 20 seconds. After a centrifugation in presence of 200 µl of 70% ethanol and two consecutive centrifugations in 90% ethanol, at 14,000 g for 30 seconds, purified DNA was eluted in 15 µl of Elution Buffer (CP8).

CpGenome Universal Unmethylated DNA (Chemicon) and CpGenome Universal Methylated DNA (Chemicon) were used as negative and positive standard control, respectively.

5-hydroxymethylcytosine immunoprecipitation (hMeDIP)

5-hydroxymethylcytosine immunoprecipitation was carried out using EpiQuikTM Hydroxymethylated DNA Immunoprecipitation (hMeDIP) Kit (Epigentek, NY, USA) according to the manufacturer's recommendations. Briefly, 100 µl of Antibody Buffer (AB) were added to each well of the microplates, followed by the adding of 1 µl of 5hmC antibody (or 1µl of Non-Immune IgG, as negative control) and incubation at RT for 60 minutes. After removing AB and washing of the wells with 200 µl 1X Wash Buffer (WB), 1 □ g of AluI digested DNA samples were diluted to 10 ng/µl with hMeDIP Solution (HS) and added into the assay wells to be incubated at RT for 90 minutes on an orbital shaker. Therefore, the wells were first washed 5 times with 200 µl of 1X WB, and, then, with 200 µl of DNA Release Buffer (DRB). Afterwards, 40 µl of the DRB containing Proteinase K were added to each well and samples were incubated at 60°C for 15 minutes followed by an incubation at 95°C for 3 minutes.

The reference DNA fragments containing 5hmC was used as the positive standard control.

MeDIP/hMeDIP-PCR

Immunoprecipitated methylated and hydroxymethylated DNAs were then used as a template for realtime PCRs carried out using the SYBR Green qPCR Master Mix (Promega) in a StepOne Plus machine (Applied Biosystems). In these reactions, PCR primers specifically amplifying D-loop fragments previously detected by bisulfite sequencing as unmethylated and methylated were used (Additional file 2: Table S2).

The final PCR mixture (20 µl) contained 1 µl of immunoprecipitated DNA, 1X GoTaq® qPCR Master Mix, 0.2 µM of each primer and 1X CXR Reference Dye. The thermal profile used for the reaction included a 2-minutes heat activation of the enzyme at 95°C, followed by 35 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 58°C for 60 seconds, followed by melt analysis ramping at 58°C to 95°C. All measurements were taken in the log phase of amplification.

Specific PCR primers were used for verifying the enrichment efficiency of methylated and hydroxymethylated positive control DNAs.

Fold enrichment was calculated as ratio of amplification efficiency of the MeDIP/hMeDIP sample over that of non-immune IgG.

Isolation of mitochondrial protein fractions

Mitochondrial extracts were prepared using Mitochondrial Fractionation Kit (Active Motif). 1.5 x 10^7 HeLa and 3T3-L1 cells were scraped on ice after the addition of 10 ml of ice-cold 1X phosphate-buffered saline (PBS) and then centrifuged at 600 g for 5 minutes at 4°C. Cell pellets were re-suspended in 5 ml of ice-cold PBS and centrifuged at 600 g for 5 minutes at 4°C. Then, cell pellets were resuspended in 250 µl of 1X cytosolic buffer included in the kit and incubated on ice for 15 minutes. Successively, cell pellets were homogenized and the resulting supernatant was centrifuged at 850 g for 20 minutes at 4°C. After centrifugation, the supernatant, containing the cytosol and the mitochondria, was removed and centrifuged a second time at 800 g for 10 minutes at 4°C. Then, the supernatant was again removed and centrifuged at 11,000 g for 20 minutes at 4°C to pellet the mitochondria. Mitochondrial pellets were washed with 100 µl of 1X cytosolic buffer and then centrifuged at 11,000 g for 10 minutes at 4°C. Finally, mitochondrial pellets were lysed by adding 35 µl of complete mitochondria buffer, prepared by adding mitochondria buffer, protease inhibitor cocktail, and dithiothreitol and incubating on ice for 15 minutes.

As control, whole protein extracts were obtained according standard procedure.

Western Blotting of DNMT methyltransferases and TET methylcitosine dioxygenases

Eighty micrograms of whole protein extracts and 30 µg of cytosolic and mitochondrial protein fractions were resolved on a 7% SDS-PAGE and transferred into Hibond-P membranes at 30 V for 2 hours at 4°C. Membranes were washed with TBST 1X (0.3 mM Tris–HCl, pH 7.5, 2.5 mM NaCl, 0.05% Tween 20) for 10 minutes and then incubated at room temperature for 1 hour with 5% non-fat dried milk in TBST 1X. Then, blots were washed three times with TBST 1X for 10 minutes and incubated overnight, in TBST containing 1% milk, with anti-DNMT1, DNMT3A and DNMT3B monoclonal mouse antibodies (1:200), anti-TET1 policlonal goat antibody (1:200) and anti-TET2 and anti-TET3 policlonal rabbit antibodies (1:200) Then, anti-mouse, anti-goat and anti-rabbit (1:5000) antibodies conjugated with horseradish peroxidase (HRP, GE Healthcare) were used as secondary antibodies. Immunoreactivity was determined by means of the ECL chemiluminescence reaction (GE Healthcare). Tubulin antibody (1:500) and cytochrome c oxidase subunit IV isoform 1 (CoxIV) antibody (1:200) were used as internal control of the total and cytosolic and mitochondrial fraction, respectively. All primary antibodies were purchased from Santa Cruz Biotechnology, except for DNMT3A and CoxIV which were from Abcam.

Statistical analyses

Statistical analyses were performed using SPSS 15.0 statistical software (SPSS Inc., Chicago, Illinois). One-way analysis of variance (ANOVA) and Student's t-test were adopted, with a significance level defined as $\alpha=0.05$.

Authors' contributions

PDA, DB and GP ideated and planned the study; PDA: performed the experiments; TS: carried out replicate of bisulfate sequencing after one year; MG: analyzed data; DB and GP directed the work, discussed results and wrote the manuscript. All authors have read and approved the manuscript.

Acknowledgments

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Figure legends

Figure 1. Methylation patterns of the human mitochondrial D-loop in DNA samples from blood and cultured cells. The graphical representation of bisulfite sequencing results was generated by MethTools software (version 1.2). Bisulfite generated sequence of each sample (black line) was compared to the untreated sequence of the mitochondrial D-loop, reported here in a base pair scale. In **(A)** methylation of cytosine residues located within CpG nucleotides (CpG methylation) is shown. Hollow circles represent unmethylated CpG, full black circles represent methylated CpG. The variability among samples is due to reported polymorphisms (http://www.mitomap.org) which insert or delete cytosines or guanines based on the revised Cambridge reference sequence (rCRS), thus creating/suppressing CpGs. In **(B)** methylation of cytosine residues located outside CpG nucleotides (non-CpG methylation) is shown. Red, blue and green circles represent methylated CpA, CpC and CpT, respectively.

Figure 2. Methylation patterns of the mouse mitochondrial D-loop in DNA samples from blood and cultured cells. In **(A)** methylation of cytosine residues located within

CpG nucleotides (CpG methylation) is shown. Hollow circles represent unmethylated CpG, full black circles represent methylated CpG. In **(B)** methylation of cytosine residues located outside CpG nucleotides (non-CpG methylation) is shown. Red, blue and green circles represent methylated CpA, CpC and CpT, respectively.

Figure 3. Methylated and hydroxymethylated mtDNA immunoprecipitation. AluI digested DNA extracted from blood and cultured cells of human and mouse were immunoprecipitated with anti-5mC **(A)** and anti-5hmC **(B)**. Samples were amplified with primers specific for DNA fragments detected as unmethylated and methylated by bisulfite sequencing. The nucleotide position of these fragments is indicated. Fold enrichment was calculated as ratio of amplification efficiency of the immunoprecipitated sample over that of non-immune IgG. Data represent the means of six triplicate experiments with standard errors of the mean.

Figure 4. Representative Western blot electrophoresis patterns of DNMTs and TETs. Whole cell lisate (W), cytosolic (C) and mitochondrial (M) protein fractions of human HeLa and murine 3T3-L1 cells. Cytochrome c oxidase subunit IV isoform 1 (CoxIV) and tubulin were analyzed as mitochondrial and cytosolic specific markers, respectively.

Additional file

Additional file 1: Supplemental Material file.

Table S1. Sequences, localization and annealing temperature of primers used in bisulfite-sequencing procedure. Size of the resulting amplicons is also shown. The 10-bp tags added to primers are indicated in bold.

Additional file 2: Supplemental Material file.

Table S2. Sequences and localization of primers used in MeDIP/hMeDIP-PCRs. Location within mtDNA of fragments obtained by AluI digestion and size of the resulting amplicons are also shown.

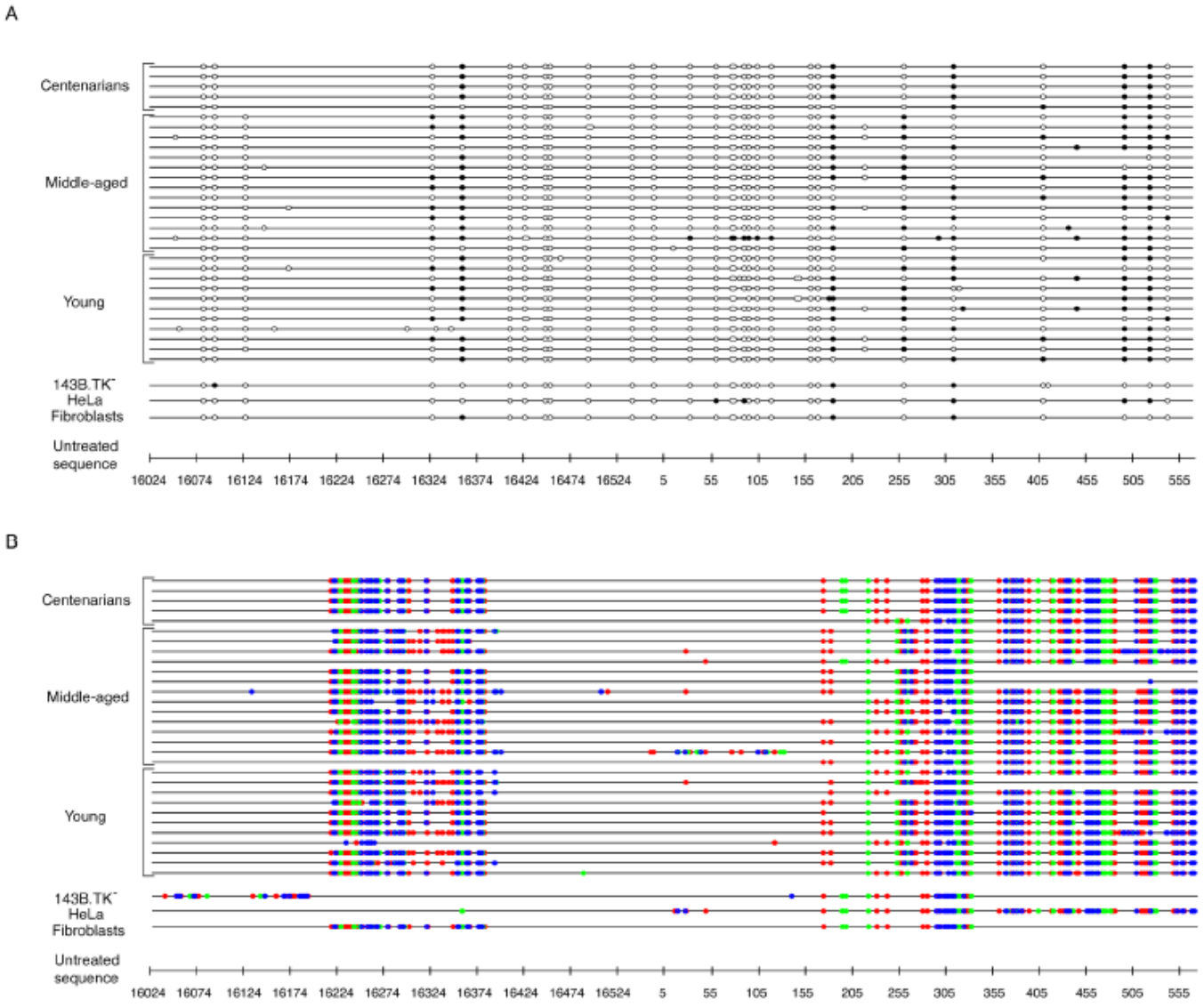
A
Centenarians
Middle-aged
Young
143B.TK⁻
HeLa
Fibroblasts
Untreated
sequence
16024 16074 16124 16174 16224 16274 16324 16374 16424 16474 16524 5 55 105 155 205 255 305 355 405 455 505 555
B
Centenarians
Middle-aged
Young
143B.TK⁻
HeLa
Fibroblasts
Untreated
sequence
16024 16074 16124 16174 16224 16274 16324 16374 16424 16474 16524 5 55 105 155 205 255 305 355 405 455 505 555

Figure 1

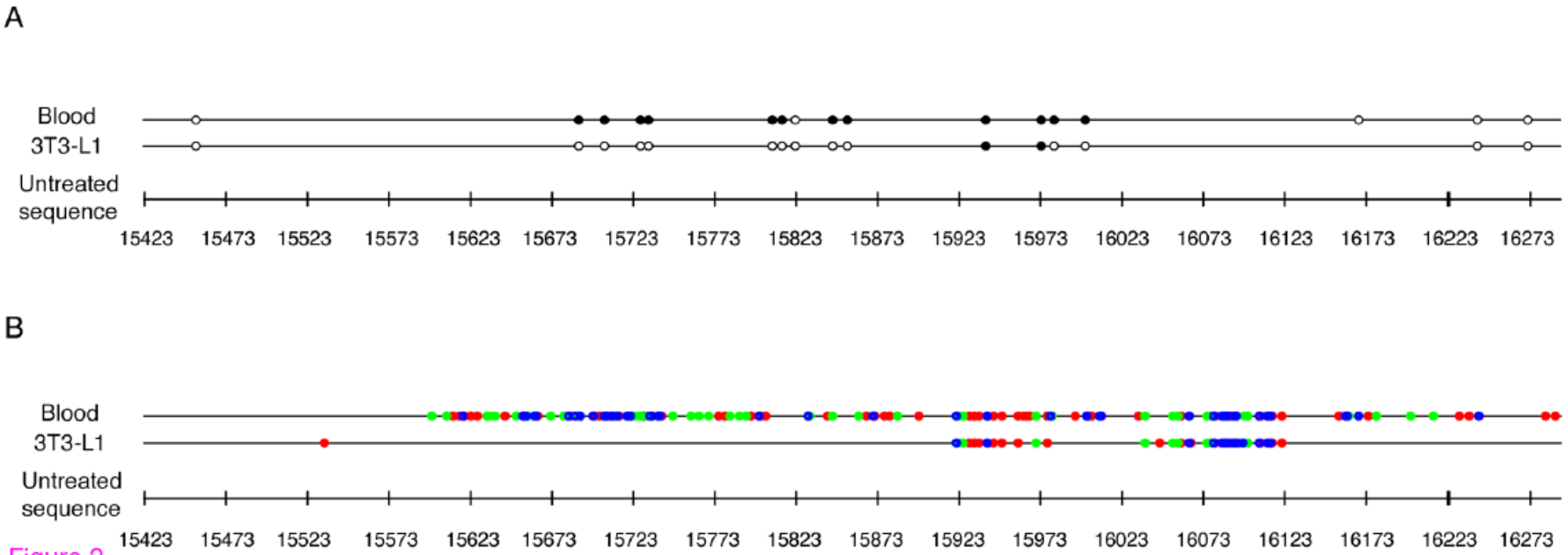
A
Blood
3T3-L1
Untreated sequence
15423 15473 15523 15573 15623 15673 15723 15773 15823 15873 15923 15973 16023 16073 16123 16173 16223 16273
B
Blood
3T3-L1
Untreated sequence
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Figure 2

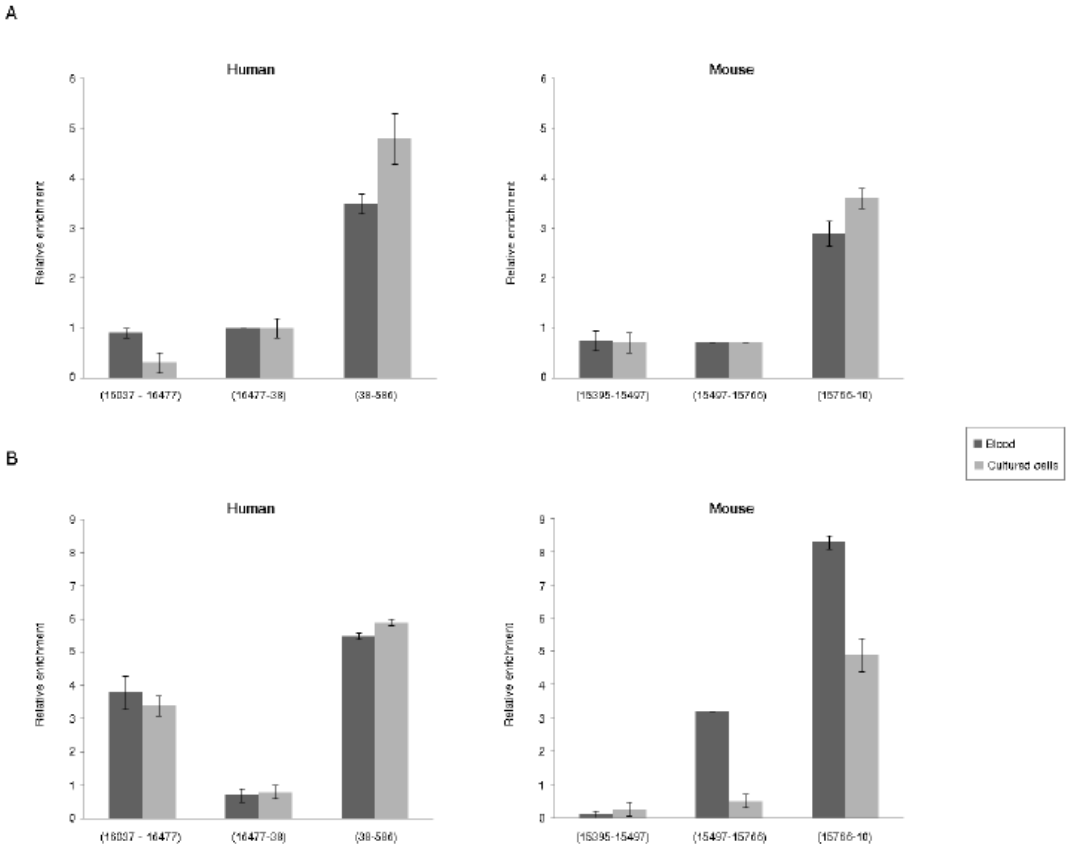
A
Human
Mouse
Relative enrichment
(16037 - 16477)
(16477-38)
(38-586)
(15395-15497)
(15497-15765)
(15765-16)
B
Human
Mouse
Blood
Cultured cells

Figure 3

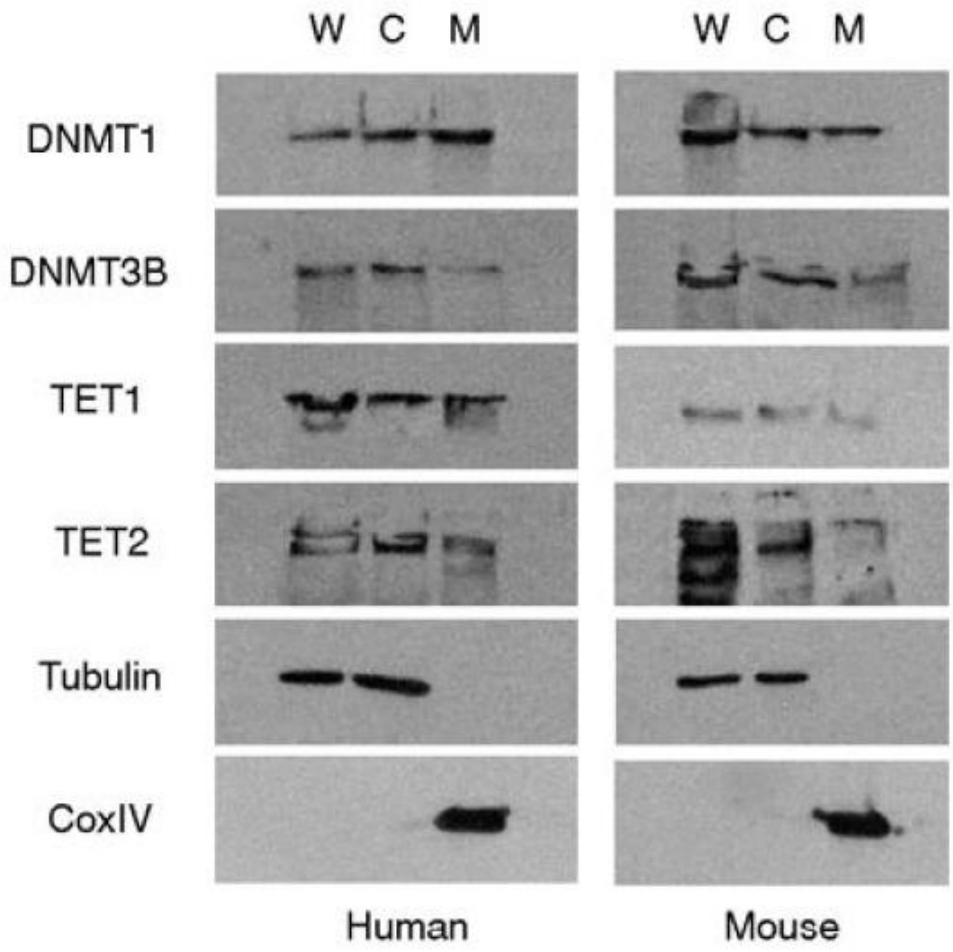

Figure 4

Additional files provided with this submission:
Additional file 1: Table S1.pdf, 61K
http://www.biomedcentral.com/imedia/2019438064823118/supp1.pdf
Additional file 2: Table S2.pdf, 14K
http://www.biomedcentral.com/imedia/1862978705823118/supp2.pdf

CHAPTER 3

Age and gender related pattern of methylation in the 12s rRNA mitochondrial gene

Marco Giordano, Patrizia D'Aquila, Alberto Montesanto, Teresa Scafone, Giuseppe Passarino, Dina Bellizzi. (*draft to be submitted*)

Department of Cell Biology, University of Calabria,87036 Rende, Italy

Abstract

Although the occurrence of methylation has been extensively documented for the nuclear genome, conflicting reports regarding the possible presence of methylated cytosines within the mitochondrial DNA (mtDNA) have emerged. To clarify this point, we analyzed the methylation of CpG sites of mitochondrial 12S rRNA gene.

Bisulfite sequencing of 12S rRNA region showed the methylation of one CpG site located at 931nt. This site was then analyzed by real-time MGB Probe-based PCR reactions in bisulfite-treated DNA extracted from peripheral venous blood collected from 513 subjects (19- to 107-year-old subjects). We detected the co-presence of both unmethylated and methylated cytosines in most sample analyzed. Statistical analyses revealed that 12S rRNA methylation displays sex- and age-specific differences, and, in elderly females, it is correlated with the health status.

Taken as a whole, our data provide unequivocal evidence supporting the presence of methylated cytosines within the human mitochondrial DNA. Moreover, the correlation of mitochondrial methylation with gender, age and aging phenotypes suggests that the above modifications are modulated overtime.

Introduction

The presence of 5mC residues within the nuclear genome of mammals has been extensively investigated, especially in relation to the role of methylation in gene expression silencing [1,2,3]. On the contrary, methylation of the mitochondrial genome (mtDNA) is largely debated and is far from being elucidated. In fact, controversial evidences have been reported about epigenetic modifications of mitochondrial DNA. Several studies have reported the complete absence of 5mC in mitochondrial mtDNA from *Paramecium aurelia*, *Xenopus leavis*, *Neurospora crassa* and, more recently, from cancer cell lines and tissues of patients with gastric and colorectal cancer [4,5,6,7]. Nevertheless, different amounts of 5mC were observed in mtDNA of many other organisms, such as mouse, hamster, plant, fish and bird [8,9,10]. Recently, a mtDNA–protein interaction study carried out in living cells suggested this genome may be methylated and that DNA methyltransferases targeting the mitochondria have access to different sites on the mtDNA depending on the levels of protein occupancy [11]. In addition, Shock and coll. demonstrated an enrichment of mtDNA sequences by immunoprecipitation against 5mC and 5hmC and the translocation of DNA methyltransferase 1 (DNMT1) into the mitochondria[12].

Since previous studies found methylated CCGG sites in rRNAs mouse mtDNA genes [9] we investigated if cytosines methylation also occurs in 12S rRNA human mitochondrial gene. Indeed, we found methylated site which was then analyzed through real-time MGB Probe-based PCR reactions in a population sample of Calabria (Southern Italy), composed by 513 individuals aged from 19 to 107 years in order to verify if the methylation of this site changes along the lifetime and if it is correlated with age and/or with the health status.

Materials and Methods

Sample

A total sample of 513 individuals (222 men and 291 women) aged between 19 and 107 years belonging to three different age groups was analyzed: group 1 (G1) included 168 unrelated subjects younger than 60 years; group 2 (G2) included 264 unrelated subjects aged from 60 to 90 years; group 3 (G3) included 81 unrelated subjects older than 90 years. The features of the analyzed sample are reported in Table 1.

	N	Age range	Males	Females
Group 1	168	< 60 years	70	98
Group 2	264	63-89 years	114	150
Group 3	81	90-107 years	38	43
Total	513	19-107 years	222	291

Table 1. Characteristics of the analyzed sample by age groups.

Out of the 264 subjects in G2, 45 had at least one living centenarian parent included in G3. Out of these 45 pairs, 17 were same-sex pairs (12 mother-daughter and 5 father-child), while the 28 remaining pairs were discordant for sex. All subjects lived in Calabria (southern Italy) and the Calabrian origin of all the subjects had been ascertained up to the grandparents' generation. Health status was ascertained by medical visit carried out by a geriatrician who also conducted a structured interview including questions on common diseases occurred in the past. At the same time of the visit, peripheral venous blood samples were also obtained. In particular, each subject had 6 ml of venous blood drawn. Plasma/sera were used for routine laboratory analyses, while DNA was extracted from blood buffy coats following standard procedures. Through a hierarchical cluster analysis, which availed of specific geriatric parameters, this sample was classified in three aging phenotype: nonfrail (the cluster with subjects showing the best scores for the classification variables), frail (the clusters with subjects showing the worst scores for the classification variables), and prefrail (the cluster with subjects showing intermediate scores for the classification variables). The diagnostic and predictive soundness of this classification was confirmed by a 3-year longitudinal study that allowed the authors to formulate a frailty index [13]. Before the interview, each subject consented to her/his phenotypic and genetic data to be used anonymously for genetic studies on aging (informed consent).

Construction of the standard curve

Samples were treated with sodium bisulphite using the EZ DNA Methylation-Direct Kit (Zymo Research).To a 1 µgr of DNA was added 1 µl of Proteinase K and 13 µl of M-digestion Buffer per sample with a final volume of 26 µl. An incubation at 50°C for 20 minutes was performed and then the samples were centrifuged at 13,000 rpm for 5

minutes. 20 µl of the supernatant were collected and then, to each sample, 130 µl of CT Conversion Reagent previously prepared was added together with 900 µl of water, 300 µl of M-dilution Buffer and 50 µl of M-dissolving Buffer supplied with the kit to a lyophilized vials of CT Conversion Reagent, reaching a final volume of 150 µl. All samples were incubated at 98°C for 8 minutes and subsequently at 64°C for 3.5 hours. After the incubation, samples were loaded on a Zymo-Spin IC column, previously hydrated with 600 µl of M-Binding Buffer, mixed several times and then centrifuged at 13,000 rpm for 30 seconds. After an addition of 200 µl of M-Wash Buffer, and a centrifugation at 13,000 rpm for 30 seconds, 200 µl of M-Desulphonation Buffer were added to each sample incubating them for 20 minutes. Then, a centrifugation at 13,000 rpm for 30 seconds and a wash with 200 µl of M-Wash Buffer twice were performed, each followed by a centrifugation at 13,000 rpm for 30 seconds. Then samples were eluted from the column using 10 µl of M-Elution Buffer per sample followed by a centrifugation at 13,000 rpm for 30 seconds. Finally, samples were stored at a temperature of -80°C to preserve their stability.

In order to construct the standard curve, two plasmids were constructed using the pGEM-T Easy Vector System I (Promega). Two fragments of the 12S rRNA mtDNA gene were cloned in these plasmids using the TA cloning method. This procedure allowed to obtain the methylated (p931M) and unmethylated (p931U) plasmid respectively.

In order to construct both the plasmids, DNA completely methylated and DNA completely unmethylated (EMD Millipore Corp.) were firstly amplified and subsequently treated with sodium bisulphite as previously described. The primers used were: 931RTFor 5'-CGCGCTTAGTAATAAATGAAAGTT -3' and 931RTRev5'-CGCGTCAAATAAATTTTAACTTTATTA -3'.

PCR reactions were conducted in a final volume of 20 µl containing 2 µl of bisulfite DNA, Reaction Buffer 1X, 0.5 µM of each primer, dNTP mix 0.2 mM and PCR Enzyme 0.05 U (Roche). A 15 minutes UV-ray exposition was also executed to eliminate any possible contaminant. The PCR profile used starts with a pre-denaturating phase at 95°C for 4 minutes, follows by 45 cycles at; 94°C for 20 seconds, 46°C for 30 seconds and 72°C for 1 minute and a final extension phase at 72°C for 3 minutes. 5 µl of PCR product were analyzed with an electrophoresis on agarose gel 2% in TAE Buffer 1X (Tris 0.04 M, EDTA 0.001 M, Acetic Glacial Acid 0.04 M) and then visualized using an UV lamp, previous coloration with Ethidium Bromide (EtBr, 0.5 mg/ml). 15 µl of the remaining PCR product were purified using DNA Clean & Concentrator - 5 kit (Zymo Research).

The two fragments obtained were ligated with the plasmid pGEM in a 10 µl final volume which contains: 3.5 µl of purified fragment, Rapid Ligation Buffer 1X (Promega) and 25 ng of pGEM–T Easy Vector System I. Samples were incubated at environment temperature for 30 minutes.2 µl of ligation mix were added to 20 µl of *E. coli* TOP10 electro-competent cells. 480 µl of LB medium culture were added to the electroporated cells of which, after an incubation at 37°C for 45 minutes, 100 µl were placed on LB plate contains Amp 50 µg/ml, Strep 50 µg/ml, X-Gal 20 mg/ml and IPTG 200 mg/ml.

To confirm the presence of the methylated or unmethylated fragment in the plasmids, 10 clones were randomly selected from each plate and then analyzed by a PCR reaction. Single transformed colonies were resuspended in 5 µl of sterile distilled water and the reaction was performed in a final volume of 20 µl in presence of F-514 Reaction Buffer 1X, dNTP mix 0.2 mM (Promega), 0.5 µM of each primer 931For and 931Rev, DyNAzyme 0.04 U (Finnzymes). The amplification profile consisted in a pre-denaturation phase at 93°C for 5 minutes followed by 35 cycles at 94°C for 30 seconds, 49°C for 30 seconds, and 72°C for 90 seconds. Then, a final extension phase at 72°C for 5 minutes. The product was analyzed via an electrophoresis assay on agarose gel 2% in TAE Buffer 1X (Tris 0.04 M, EDTA 0.001 M, Acetic Glacial Acid 0.04 M) and then visualized after coloration with Ethidium Bromide (EtBr, 0.5 mg/ml).

Colonies were isolated and the plasmid inside was extracted using the ZR Plasmid Mini PrepClassic Kit (Zymo Research). In order to confirm the ligation of the correct fragment, a sequence PCR was performed in a final volume of 20 µl which contains 1µg of extract plasmidic DNA, 3.2 pm of primer M13For, Big Dye Terminator Sequencing Buffer 1X and 2 µl of Big Dye Terminator Sequencing RR-100 (Applied Biosystems). The amplification profile used consisted in 25 cycles of the following steps: 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. The PCR product were purified with the ZR DNA-Sequencing Clean-Up Kit (Zymo Research). To the purified samples 10 µl of the loading Buffer Template Suppression Reagent (TSR) (Applied Biosystems) were added and then analyzed using the ABI PRISM 310 automatic sequencer(Applied Biosystems).

25 ng of each plasmid (p931M and p931U) were amplified with a PCR reaction in a final volume of 10 µl. The forward primer sequence was 5'-CGCGCTTAGTAATAAATGAAAGTT -3' and the reverse primer sequence was 5'–CGCGTCAAATAAATTTTAACTTTATTA –3'. The reaction mix contained both primers at a final concentration of 0.5 µM, 2 U of Taq DNA Polymerase (5 U/µl), 1XTaq Buffer Advanced containing 500 mMKCl, 100 mMTris-HClpH 8.3, 15 mM Mg^{2+}, dNTP Mix 1

mM. Then,a 15 minutes UV-ray exposition was performed to eliminate any possible contaminant. The PCR conditions were 94°C 5 minutes followed by 35 cycles at 95°C for 1 minute, 46°C for 20 seconds, 72°C for 30 seconds and a final extension phase at 72°C for 3 minutes.

5 µl of PCR product were analyzed with an electrophoresis on agarose gel 2% in TAE Buffer 1X (Tris 0.04 M, EDTA 0.001 M, Acetic Glacial Acid 0.04 M) and then visualized after coloration with Ethidium Bromide (EtBr, 0.5 mg/ml).

In order to build a reference curve for measuring the levels of methylated DNA in the biological samples, the previously amplified plasmids containing the fully methylated (p931M) and the fully unmethylated DNA (p931U)sequences were mixed in different proportions. Since the amplification efficiency of these two plasmids might be different, a densitometrica analysis was carried out to ensure that an equally amount of fully methylated/unmethylated DNAs was utilized in the serial dilutions. After the densitometrica analysis the following DNA ratio methylated DNA/unmethylated DNA (p931M/p931U) were analyzed: 0/100, 10/90, 20/80, 30/70, 40/60, 50/50, 60/40, 70/30, 80/20, 90/10 and 100/0.

These samples were then subjected to real-time PCR and a reference curve was then assembled.

Real-Time PCR TaqMan MGB Probe based

1 µl of each dilution was amplified in a Real-Time PCR reaction by means of the same forward and reverse primers used for the plasmids amplification. The MGB-probes used had the following sequences: methylated probe5'-VIC– ATAGAAGTCGGTGTAAAG-MGB-3' (931U) and unmethylatedprobe5'-6FAM–ATAGAAGTTGGTGTAAAG–MGB-3' (931M).

Each sample contained 1X of TaqMan Universal PCR Master Mix No AmpErase UNG (uracil-N-glycosylase), 0,05 U of GoTaq® Hot Start DNA Polymerase (Promega), 500 nM of each forward and reverse primers, 300 nM TaqMan MGB-probe. A 15 minutes UV-ray exposition was also executed to eliminate any possible contaminant. The following PCR conditions were used: 60°C for 30 seconds, 95°C for 4 minutes followed by 30 cycles at 94°C for 20 seconds, 60°C for 1 minute and a final phase at 60°C for 30 seconds.

Probes were characterized by two different fluorochromes: the probe marked with the *VIC* fluorochrome at 5'-ending detects the presence of methylated DNA, while the probe marked with the *FAM* fluorochrome at 5'-ending detects the presence of unmethylated

DNA. The 3'-ending of both probes was marked by the MGB quencher molecule. The experiments were carried out using a StepOne™ Real time PCR instrument and the results were analyzed with the StepOne™Software v. 2.2.1 (AppliedBiosystems).

Standard curve construction

The amount of FAM and VIC fluorescence released in each tube was measured as a function of the PCR cycle number at the end of each cycle. The cycle number at which the fluorescence signal crosses a detection threshold is referred to as CT. The threshold is the level of fluorescence at which the signal can be considered not to be background and, in our experiments, it has been automatically calculated by the StepOne™ Software. The difference of both CT values within a sample (ΔCT) is calculated as follows $\Delta_{CT} = C_{T(VIC)} - C_{T(FAM)}$.

In order to make a highly accurate standard methylation curve each standard ratio sample was analyzed in triplicate and its mean value (ΔC_{Tm}) was then used for further analyses. Standard curve was obtained by regression analysis of the ΔC_{Tm}valuesonthe corresponding predefined methylation ratio:

$$\Delta_{CT} = aC + b$$

whereΔ_{CT} is specific of the analyzed sample; *a* and *b* are two regression coefficients, whereas *C* is the methylation ratio. *a* and *b* are specific for the experimental condition used, that take into account the different probe's PCR efficiency and their different affinity for the template DNA. Using the regression coefficients the methylation levels in the biological samples were then estimated as:

$$C = (\Delta_{CT} - b)/a$$

Methylation analysis of the 12S rRNA gene in the population sample

Once the standard curve was obtained, samples could be analyzed in the same way as plasmids. After bisulfite treatment, PCR amplification, agarose gel visualization and dilution as previously described, samples were analyzed by using the real-time PCR assay in order to determine their 12 rRNA mtDNA gene methylation value. The methylation

values were determined using the regression equation of the standard curve as previously described.

mtDNA analysis

Haplogroup typing was carried out by restriction analyses of mtDNA according to Torroni*et al.* and De Benedictis *et al.*[14,15].

Statistical analysis

Descriptive statistics for continuous and categorical variables were used to describe the characteristics of the analyzed population samples. For continuous variables (methylation values, age, frailty index) measures of central tendency and dispersion, including mean, median and standard error of the mean were reported. Departures from normality assumption were tested using the Kolmogorov–Smirnov normality test. In case of evidence of non-normality, the nonparametric Kruskal–Wallis or Mann–Whitney tests (as appropriate) were used. Categorical variables were examined by analyzing the relevant frequency distributions.

The linear correlation coefficient (r) was used to measure the strength and the direction of the parent-child resemblance in the methylation levels. Permutation procedures were used to verify if the observed correlation in these pairs was statistically significant.

Statistical analyses were performed using SPSS 20.0 (IBM SPSS Statistics 20.0).

A significance level of 0.05 was chosen for all tests.

Results

Presence of methylation on Methylation 12S rRNA

The analysis of 12S rRNA showed the methylation of one CpG site located at 931nt. In particular, the co-presence of both unmethylated and methylated mtDNA molecules were obtained. As a consequence a new method to determine this co-presence was then used. So, this site was analyzed by real-time MGB Probe-based PCR reactions in bisulfite-treated DNA in a population sample.

Real-time PCR MGB-probe based and sensitivity of the method

In order to quantify the levels of methylation in the biological samples under study, we applied real-time PCR MGB-probe based assay to various known ratios of fully methylated

and fully unmethylated plasmids and assembled the reference curve. In Figure 1 we report the plotted ΔCT values (average value of three independent experiments) as a function of the predefined methylation ratios (Figure 1).

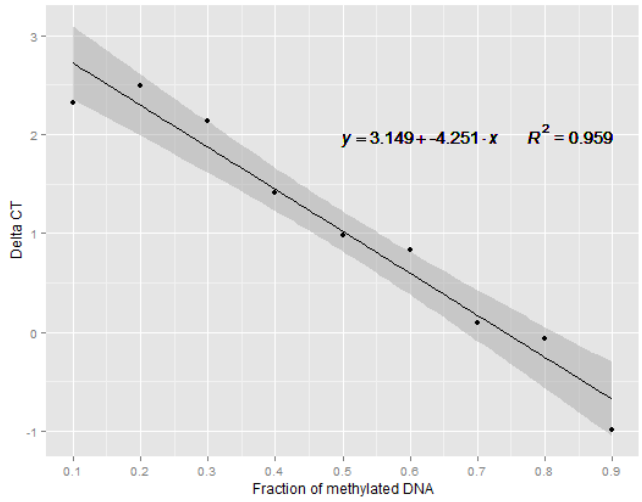

Figure 1. Standard curve as obtained by plotting the ΔCT values against the predefined methylation ratio of each sample. The CT value was set to 30 in the case that the respective fluorescence signal did not cross the threshold.

The curve exhibits a linear shape with a square R value equals to 0.96. This reference curve was used for estimating the methylation levels of the biological samples.

Methylation analysis in the population sample

By means of the reference curve, the obtained PCR results were used to determine the methylation values of 12S rRNA gene in the analyzed sample.

The frequency distribution of the methylation values in the whole sample is reported in Figure 2.

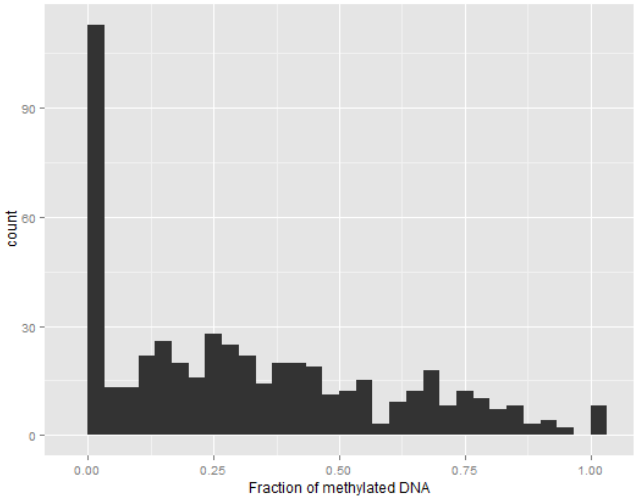

Figure 2. Frequency distribution of methylation values in the whole sample

Figure 2 shows that approximately the 40.7% (209/513 subjects) of the sample displayed levels of methylation lower than 20%. In addition, it can be observed that the distribution of these values was not normal with a median value of about 0.273.

A significant difference in methylation levels was observed between men and women both in the total sample (0.373 in men *vs* 0.214 in women, p<0.001) and in each of the age groups (0.231 in men *vs* 0.130 in women, p=0.023 for G1; 0.434 in men *vs* 0.264 in women, p=0.005 for G2; 0.377 in men *vs* 0.302 in women, p=0.077 for G3), as shown in Figure 3.

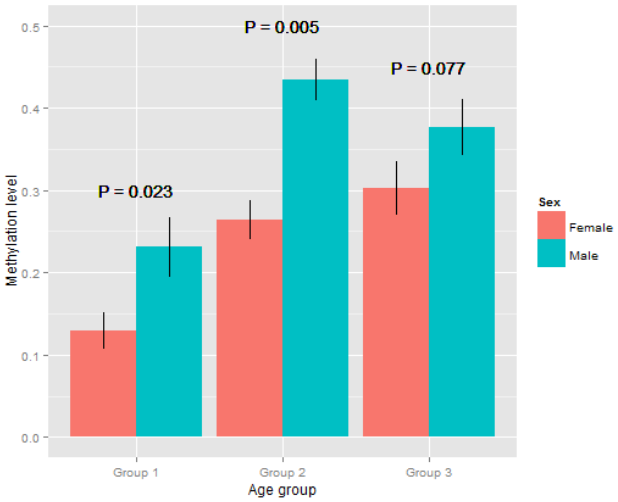

Figure 3. Median methylation values in males and females across the three age groups analyzed.

Figure 3 also shows that methylation levels increase with age. In fact, by comparing the pattern of methylation between pairs of samples (G1 *vs* G2, G1 *vs*G3 and G2 vs G3) we found that subjects belonging to the younger group exhibit lower methylation values than the others (0.150 in G1 *vs* 0.345 in G2, p<0.001; 0.150 in G1 *vs* 0.330 in G3, p<0.001). On the contrary, no significant difference was detected by comparing the methylation values of subjects belonging to G2 and G3 groups (0.345 in G2 *vs* 0.330 in G3, p = 0.369).

Methylation value of 12S rRNA gene and frailty status

As mentioned in Materials and Methods, for subjects belonging to both G2 and G3 groups on the basis of specific geriatric parameters a frailty classification and a frailty index have been previously formulated [13]. In G2 group we observed that the methylation values were correlated with the degree of frailty. In particular nonfrail females subjects exhibit higher methylation levels than prefrail and frail subjects (0.397 in nonfrail cluster, 0.180 in prefrail cluster and 0.122 in frail cluster, p<0.001).Conversely, in males no correlation with frailty was found (p=0.875). Consistently, we found that also the frailty index was significantly correlated to methylation levels in females (p<0.001), but not in males (p=0.117, Figure 4).

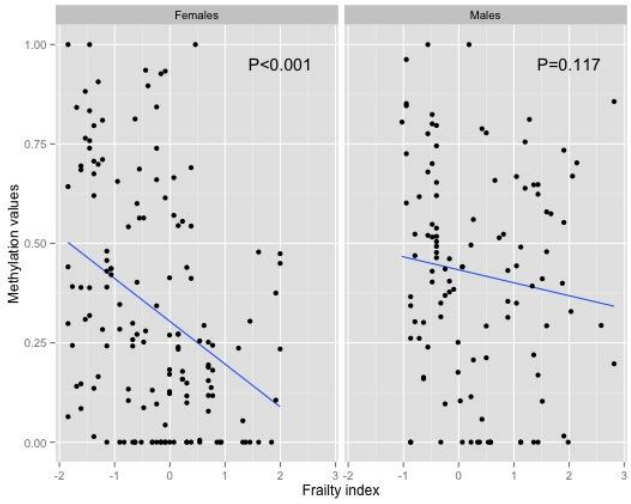

Figure 4. Linear regression analysis as obtained by plotting the methylation values against the frailty index of each sample for females individuals (left panel) and for the males one (right panel).

Figure 4a shows that in females methylation values decrease as the frailty index increase (i.e. females with a worst quality of aging exhibited lower methylation levels).

As it regards G3 group, no significant difference in methylation values was detected between the frailty phenotypes neither in males (p=0.973) nor in females (p=0.872).

Methylation value of 12S rRNA gene and mtDNA variability

In order to verify whether the mtDNA variability could influence methylation levels of mtDNA12S rRNA we compared the distributions of methylation values according to the mtDNA haplogroup classification and age groups membership (Figure 5 and Table S1).

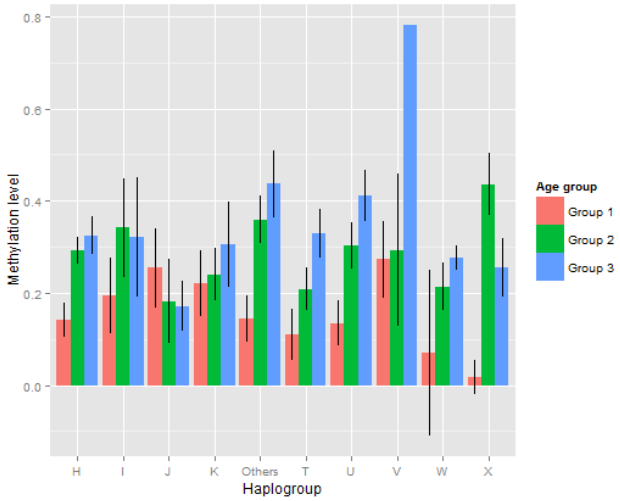

Figure 5. Distributions of methylation values according to the mtDNAhaplogroup classification and age groups membership.

Figure 5 indicates that methylation levels exhibit a modest degree of variability among mtDNA haplogroups in all age groups. However, no significant difference was found in methylation levels in each of these groups with respect the mtDNA variability. Since subjects carrying J haplogroup showed lower methylation levels than the other ones, we compared their methylation levels with those obtained grouping all non-J haplogroups. We found that only G3 individuals harboring J haplogroup had significant lower methylation levels than non-J carriers (0.171 *vs* 0.339, p=0.019). It is important to note that due to the small number of J haplogroup carriers a sex-stratified analysis in this case was not feasible.

Parent-child resemblance of methylationat 12S mtDNA

In order to understand whether the presence/accumulation of methylation at 12S mtDNA gene is a genetically controlled event we analyzed the family resemblance in the parent-child pairs with respect to these levels. As mentioned in Material and Methods, out of the

264 subjects in G2, 45 had at least one living centenarian parent included in G3. Out of these 45 pairs, 17 were same-sex pairs (12 mother-daughter and 5 father-child), while the 28 remaining pairs were discordant for sex.

Figure 6 shows the results of the linear regression analysis between methylation levels in parents(x axis, G3 group) and their offspring (y axis, G2 group) according to the concordance for sex.

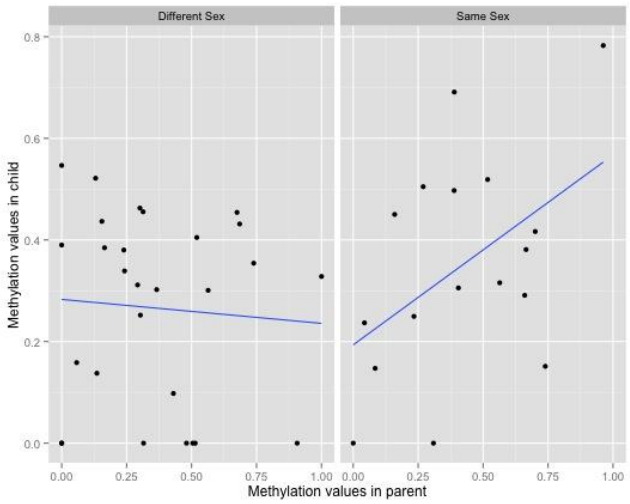

Figure 6. Linear regression analysis between methylation levels in parents(x axis, G3 group) and their offspring (y axis, G2 group) according to the concordance for sex.

We found that methylation levels were significantly correlated in same-sex parent-offspring pairs ($r = 0.472$; $p = 0.029$),but not in pairs discordant for sex ($r = -0.069$; $p = 0.642$).

Discussion

In the present study we have analyzed, for the first time, the methylation status of the human 12S rRNA mitochondrial gene. We found that the CpG site at position 931 can be methylated and that the level of methylation of this site is correlated with the gender (methylation is significantly higher in males than in females), with age (methylation increases with age) and, in elderly females, with the frailty status (methylation is higher in nonfrail females).

Our findings, showing a differential methylation of the mtDNA 12S rRNA gene, suggest that methylation of this region may regulate gene expression of the mitochondrial DNA. In fact, in human mtDNA, two transcription mechanisms take place and their starting sites overlap each other [16,17,18]. The first mechanism starts at the initiation site H1 and ends at the 16S rRNA 3'-end and is the most frequent. The second one (which is about 20 times less frequent) starts at the initiation site H2, close to the 12S rRNA 5'-end, originating a polycistronic molecule covering almost the whole H-strand. The mRNAs for the 12 H-strand encoded polypeptides and 12 tRNAs derives from the processing of this polycistron. This transcription model explains how a differential regulation of rRNA versus mRNA transcription can be operated through the initiation of H-strand transcription at the two alternative sites suggesting that the entire mitochondrial genome expression might be controlled by involving one of these sites on the mtDNA [147]. A methylation event occurring in the rRNA region might, in part, regulate this function. Thus, these two sites could serve as sites of controlling the expression of the mitochondrial genome.

It is interesting also to notice that the methylation level of the 931bp CpG site is correlated also with the variability of the mitochondrial molecule. In fact, molecules falling in J haplogroup, which was previously to negatively affect global nuclear methylation, were found to be associated to lower level of methylation of the 931bp CpG. This data, together with the strong correlation found in same-sex parent-offspring pairs suggested that mtDNA methylation levels has also a genetic component besides the environmental one that is at the base of epigenetic processes.

In conclusion, our results support the idea that methylation takes place in mtDNA. In particular, this is the first evidence that methylation occurs in 12S rRNA mitochondrial gene suggesting that methylation of this region may regulate 12S rRNA gene expression in the mitochondrion and all the mtDNA expression as one of the transcription start site is within this gene. In addition, our results show that methylation levels of 12S rRNA gene

change during the lifetime (they are correlated with sex, age and health status) but they are also affected by the genetic background.

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CHAPTER 4

Global DNA methylation levels are modulated by mitochondrial DNA variants

Dina Bellizzi, Patrizia D'Aquila, Marco Giordano, Alberto Montesanto, Giuseppe Passarino (*published on Epigenomics*)

Department of Cell Biology, University of Calabria,87036 Rende, Italy

Global DNA methylation levels are modulated by mitochondrial DNA variants

Aim: In the present study, we investigated whether global DNA methylation levels are affected by mitochondrial DNA (mtDNA) variants, which are known to modulate mitochondrial functions. **Materials & methods:** Global DNA methylation levels were evaluated in peripheral blood DNA collected from adult subjects and *in vitro* using the DNA of cybrid cells harboring mtDNAs of different haplogroups. In these cells, mRNA expression of genes involved in DNA methylation processes, and ATP and reactive oxygen species levels were also analyzed. **Results:** The analysis revealed that methylation levels were higher in the subjects carrying the J haplogroup than in non-J carriers. Consistently, cybrids with J haplogroup mtDNA showed higher methylation levels than other cybrids. Interestingly, we observed overexpression of the *MAT1A* gene and low ATP and ROS levels in J cybrids. **Conclusion:** Our findings indicate that mtDNA-specific interactions between mitochondria and the nucleus regulate epigenetic changes, possibly by affecting oxidative phosphorylation efficiency.

KEYWORDS: ATP ■ cybrid cell lines ■ global DNA methylation ■ *MAT1A* gene ■ mitochondrial DNA variability ■ ROS

Dina Bellizzi‡, Patrizia D'Aquila‡, Marco Giordano, Alberto Montesanto & Giuseppe Passarino*

Department of Cell Biology, University of Calabria, 87036 Rende, Italy
**Author for correspondence:*
Tel.: +39 0984492932
Fax: +39 0984492911
g.passarino@unical.it
‡Authors contributed equally

Mitochondria hold a central position between energy uptake and energy production. As a consequence, they play a key role in a wide variety of both pathological (e.g., aging, cancer, neurodegenerative diseases and diabetes) and nonpathological (e.g., heat production, reactive oxygen species [ROS] generation, apoptosis and cellular differentiation) traits [1–3]. Over the past two decades, many studies have focused their attention on the influence of variability of mitochondrial DNA (mtDNA), whose genes are responsible for the synthesis of 13 enzymatic subunits of the oxidative phosphorylation (OXPHOS) complex, on mitochondrial function and on the above-mentioned traits.

mtDNA variants define specific mtDNA haplotypes. Haplotypes with a common phylogenetic origin are categorized into haplogroups which display a continent-specific distribution. For example, approximately 99% of the mtDNA of the European population falls within nine different haplogroups (H, J, U, X, T, I, K, W and V) as identified by Torroni *et al.* [4]. More recently, sequencing of the complete mtDNA genome has allowed the subdivision of haplogroups into smaller subhaplogroups [5]. The mtDNA haplogroups were initially considered as being neutral and used only for phylogeny analysis and population studies [6–8]. Afterwards, a series of experimental evidence suggested that they are not in fact neutral, and an association between common mtDNA variants and physiological or pathological phenotypes has gradually emerged. The first strong evidence demonstrating such a functional consequence came from the association between the J haplogroup and Leber hereditary optic neuropathy (LHON) [9]. Subsequently, mtDNA variants have been found to affect the quality of aging, sperm motility and the susceptibility to late-onset pathologies, such as neurodegenerative and cardiovascular diseases, diabetes and cancer [10–17]. However, other studies have reported inconsistent results, suggesting the existence of complex interactions among mtDNA genotype, nuclear background and environment [18–22]. It has been widely reported that mtDNA molecules belonging to different haplogroups may differ in the degree of oxidative phosphorylation activity, and in turn, result in different percentages of oxygen consumption, ATP and mitochondrial ROS production or heat generation [23–30]. On the other hand, there is also evidence that different mtDNA haplogroups may maintain similar efficiency in OXPHOS performance through the fine-tuning of ROS production and mitochondrial biogenesis [31,32]. It has also been proposed that the variants which alter OXPHOS coupling efficiency, thus inducing less ATP and more heat production, are more frequent in cold areas where they purportedly confer an advantage, thus suggesting that climatic selection has played a role in shaping the present worldwide distribution of mtDNA variation [33–35].

Future Medicine part of fsg

Functional differences between haplogroups have also emerged from *in vitro* studies carried out in cybrid cell lines, which were first described by King and Attardi as sharing the same nuclear genome but as having different mitochondrial genomes [36]. These cells are generated by the fusion of mtDNA-depleted cells (Rho^0) with enucleated cells harboring particular types of mtDNA molecules [36,37]. In particular, in these cell models, intracellular calcium dynamics, mtDNA copy number, mitochondrial ROS production and the expression levels of several nuclear-encoded genes were demonstrated to be influenced by mtDNA variability [38–44]. For example, differences in mtDNA and mRNA levels, mitochondrial membrane potential, cytochrome oxydase activity, growth capacity and oxygen consumption were observed between cybrids harboring haplogroups H and UK [28]. By contrast, no difference in bioenergetic capacities and coupling efficiencies was identified between H and T cybrids [31,45].

More recently, the importance of the interactions between mitochondrial and nuclear genomes has also emerged from studies on epigenetic changes and, more specifically, on the DNA methylation of cytosines. In fact, the efficiency of mitochondrial energy producing machinery and mitochondrial metabolism can modulate the activity of MAT, the enzyme responsible for SAM synthesis from L-methionine and ATP [46–50]. It follows that dysfunctions in mitochondrial activity may have direct effects on epigenetic markers and, consequently, disrupt gene expression patterns and cellular and organism functioning [51–53]. Recently, Smiraglia *et al.* reported that the depletion of mtDNA (Rho^0 cells) induced changes in DNA methylation of different genes and that these changes were partially reversed by the re-introduction of mtDNA into these cells [54]. As mitochondrial functions strongly rely on proteins that are coded by the nuclear genome, epigenetic changes in the methylation status of nuclear genes, and thus in their expression, may affect mitochondrial function with the onset of a vicious cycle [55]. Although it has not been completely elucidated, this complex interplay between mitochondrial function and epigenetic modifications appears to have important consequences on many traits [48].

On the basis of these observations, we carried out population and *in vitro* studies to investigate the relationship between epigenetic modifications and mtDNA variability. Global DNA methylation levels were measured in peripheral blood DNA collected from adult subjects and in DNA samples extracted from cybrid cell lines which have identical nuclear DNA but harbor mtDNA molecules of different haplogroups (H, J, U, X and T). In addition, using these cell lines we analyzed the expression profiles of different genes involved in methylation processes as well as ATP and ROS levels, major products of mitochondrial activity and of the communication network between mitochondrial and nuclear genomes.

Materials & methods

Population sample

A total of 354 (163 men and 191 women) unrelated adult individuals participated in the present study. The Ethics committee of the University of Calabria, Italy approved the recruitment and the use of the information gathered, as well as the use of the biological specimens collected on 9 September 2004. All subjects lived in Calabria (south of Italy) and their origin in the area was ascertained up to the grandparents' generation. A more detailed sample description can be found elsewhere [56]. Health status was ascertained by medical visit and, at that time, peripheral blood samples were also obtained. Before the interview each subject provided informed consent for her/his phenotypic and genetic data to be used anonymously for genetic studies.

Cell lines & culture conditions

143B.TK$^-$ osteosarcoma and cybrid cell lines were grown, in a water-humidified incubator at 37°C in 5% CO_2/ 95% air, in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, CA, USA) containing 4.5 g/l glucose, 110 µg/ml pyruvate and supplemented with 10% fetal bovine serum (Invitrogen). Rho^0 cells, obtained by culturing 143B.TK$^-$ with 50 ng/ml ethidium bromide, were grown in the above medium supplemented with 50 µg/ml uridine (Sigma, MO, USA).

Cybrid production was carried out as described elsewhere [37,40,42]. Briefly, platelets were isolated through differential centrifugations from blood samples of five donors, previously characterized for their mtDNA haplogroup. 10^6 Rho^0 cells, collected by low-speed centrifugation and resuspended in DMEM, were mixed with an equal number of platelets and the culture medium was eliminated by centrifugation. Cells were resuspended in 100 µl of 42% polyethyleneglycol 1500 (PEG 1500) and seeded in standard DMEM for 48 h, and then in selective uridine-free DMEM supplemented with 10% fetal bovine serum. After 2–3 weeks,

distinct colonies emerged which were isolated by trypsinization in cloning rings and propagated.

We routinely assessed the cellular state of the native Rho^0 and cybrid cell lines by carrying out control experiments including proliferation, quantification of mtDNA (quantitative-competitive PCR) and mitochondrial membrane potential (MMP) assays (cytofluorimetric analysis of cells stained with MitoTracker® Green, Invitrogen and tetramethyl rhodamine methylesther), which have been fully described elsewhere [40,42].

DNA samples

Each subject had 6 ml of venous blood drawn. Plasma/sera were used for routine laboratory analyses, while DNA was extracted from blood buffy coats following standard procedures.

DNA samples from 143B.TK$^-$ osteosarcoma, Rho^0 and cybrid cell lines were obtained by phenol/chloroform purification. The DNA concentration and 260/280 absorbance ratio were determined spectrophotometrically.

Restriction analysis of DNA samples

A total of 100 ng of population sample DNA and DNA extracted from cell lines were incubated separately with 5 U of *Hpa*II and 5 U of *Msp*I restriction endonucleases in a total volume of 30 µl overnight at 37°C and subsequently at 65°C for 20 min to inactivate the two endonucleases.

Measurement of global DNA methylation levels

Global DNA methylation levels of population sample DNA and DNA extracted from cell lines were estimated using the CpGlobal method [57]. A total of 2 µM of both biotin-11-dCTP (Perkin Elmer, MA, USA) and biotin-11-dGTP (Perkin Elmer) were added to the digested DNA samples in 20 µl final volume containing biotynilation buffer (40 mM Tris-HCl pH 7.5, 20 mM Tris-HCl and 50 mM NaCl) and 2 U of Sequenase™ (USB Corporation, CA, USA). After incubation at 37°C for 30 min, the samples were incubated on an orbital platform at room temperature overnight in presence of 100 µl of Reacti-Bind™ DNA Coating Solution (Pierce, IL, USA). The solution was removed through three consecutive washes with Dulbecco's phosphate buffered saline (Sigma). Samples were then incubated at room temperature for 30 min in presence of 200 µl of the detector block solution (KPL, WA, USA). After the removal of the solution, 150 µl of the detector block solution containing 0.5 µg/ml of horseradish peroxidase (HRP) streptavidin (KPL) were added and the samples were incubated at room temperature for 30 min. The detector block solution was removed through four consecutive washes with biotin wash solution 1× (KPL). After a 2-min incubation at room temperature in presence of 150 µl of LumiGLO® Chemiluminescence substrate (KPL), the chemiluminescence emitted from each sample was quantified using a Lumat LB9507 luminometer (EG&G Bertold, Wildbad, Germany). Each sample was analyzed three independent times in triplicate. In order to determine the possible 'background effect' and to calculate the net luminescence for each sample, a control lacking enzyme was also analyzed. The data were calculated as global DNA methylation index (GDMI) by dividing the mean net luminescence values for the *Hpa*II enzyme by the mean net luminescence values for the *Msp*I enzyme. The GDMI values inversely correlate to the global DNA methylation levels, whereby high GDMI values indicated lower methylation level, while low GDMI values indicated hypermethylation.

A subsample (108 samples) of the entire study group was also analyzed by using Sigma's Imprint® Methylated DNA Quantification Kit according to the manufacturer's protocol.

mtDNA analysis

Haplogroup typing was carried out by restriction analyses of mtDNA according to Torroni *et al.* and De Benedictis *et al.* [4,10].

Gene-expression analysis

Expression levels of the following genes were analyzed: *DNMT1*, *DNMT3A*, *DNMT3B*, *MAT1A*, *MAT2B*, *MBD2* and *MBD4*. Total RNA was extracted from cells using RNeasy® Mini Kit (Qiagen, Hilden, Germany). The RNA concentration was measured for each sample using a spectrophotometer and purity of the sample evaluated using the 260/280 nm absorbance ratio. RNA samples were treated with DNA-free DNase to remove any residual genomic DNA contamination. Reverse transcription (RT) was carried out using the ImPromII Kit™ (Promega, WI, USA). A RT mix including 500 ng of total RNA and 0.5 µg of oligo-dT primers was preheated at 70°C for 5 min. The reaction was carried out in a 40-µl final volume containing 1× RT buffer, 0.5 mM of each dNTP, 3 mM $MgCl_2$, 20 U RNase inhibitor and 5 U reverse transcriptase. The mix was incubated at 25°C for 5 min, then at 37°C for 1 h and successively, at 95°C for 10 min to inactivate the reverse transcriptase. The cDNA obtained was

then used as a template for real-time PCR carried out with the SYBR® Green qPCR Master Mix (Promega) in a StepOne Plus™ machine (Applied Biosystems, CA, USA). Forward and reverse primers were as follows: DNMT1For 5′-AGAACGGTGCTCATGCTTACA-3′; DNMT1Rev 5′-GGGGCTAGGTGAAGG TCAG-3′; DNMT3AFor 5′-CCGATGCTG GGGACAAGAAT-3′; DNMT3ARev 5′-CCC GTCATCCACCAAGACAC-3′; MAT1AFor 5′-CAGTGTGCAAGACCGGCAT-3′; MAT1ARev 5′-TAGCCGATGTGCTTGATG GTG-3′; MAT2BFor 5′TCTGGGAATTT AGCAAAGGAAGC-3′; MAT2BRev 5′-GCT CCTAGATTGTTCTCCAGGAC-3′; MBD2For 5′-CCCACAACGAATGAAT GAACAGC-3′; MBD2Rev 5′-TGAAGACCT TTGGGTAGTTCCA-3′; MBD4For 5′-CC CCACCGTCACCTCTAGT-3′; MBD4Rev 5′-GTAGCACCAAACTGAGCAGAA-3′; GAPDHFor 5′-ATGGGGAAGGTGAAGGT CG-3′; GAPDHRev 5′-GGGGTCATTGATGG CAACAATA-3′.

The PCR mixture (10 µl) contained 1 µl of cDNA, 1× GoTaq® qPCR Master Mix, 0.2 µM of each primer and 1× CXR reference dye. The thermal profile used for the reaction included a 2-min heat activation of the enzyme at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s, followed by a melt analysis ramping at 60°C to 95°C. All measurements were taken in the log phase of amplification. Negative controls (in which water instead of cDNA was added) were also run in each plate. StepOne Software V 2.0 was used to analyze data. Gene expression values were normalized to *GAPDH* gene expression, used as internal control. In addition, the normalized values measured in the 143B.TK$^-$ cell line were used as reference values (relative quantification) for the other cell lines.

Measurement of ATP levels

ATP measurement was performed using ENLITEN® ATP assay system bioluminescence detection kit for ATP measurement (Promega), according to the manufacturer's protocol. Briefly, 1×10^6 143B.TK$^-$, Rho^0 and cybrid cells were seeded in 6-well plates. In the exponential growth phase, cells were washed with phosphate buffered saline, trypsinized and counted on a hemocytometer with an inverted light microscope. A total of 5×10^5 cells were collected and centrifuged at 400 g for 5 min at 4°C. Then, the intracellular ATP was extracted by resuspending pellets in 100 µl 0.5% trichloroacetic acid. After ATP extraction, the trichloroacetic acid in the samples was neutralized and diluted by adding tris-acetate buffer pH 7.75. Last, the bioluminescence emitted from samples was assessed using a luminometer. For all luminescence assays, blank values were subtracted from measurement values. Concentration values of ATP produced in our cell lines (nmoles of ATP) were obtained by fitting luminescence values to a standard ATP curve, constructed by analyzing series of dilutions ranging from 1×10^{-13} to 1×10^{-6} M of the ATP stock standard supplied in the kit.

Total ROS/superoxide detection

Intracellular ROS were quantified using two fluorescent dye reagents: oxidative stress detection reagent (green), which reacts directly with a wide range of ROS/reactive nitrogen species (RNS) species; and superoxide detection reagent (orange), which reacts specifically with superoxide (O^{2-}) (total ROS/Superoxide Detection Kit, Enzo Life Science, NY, USA).

143B.TK$^-$, Rho^0 and cybrid cells (5×10^5 each) were seeded in 6-well plates. In the exponential growth phase, cells were washed with 1× wash buffer (Enzo Life Science), collected and centrifuged at 400 g for 5 min. Pellets were washed with 1× wash buffer (Enzo Life Science), and the cell suspensions were centrifuged at 400 g for 5 min. The supernatant was discarded and the cell pellet was resuspended in 500 µl of ROS/superoxide detection mix (Enzo Life Science). The cell suspensions were incubated for 30 min at 37°C in the dark. In all analyses a minimum of 1×10^4 cells per sample were acquired and analyzed with Cell Quest Software (BD Bioscience, CA, USA).

Statistical analysis

Descriptive statistics for continuous and categorical variables were used to describe the characteristics of the analyzed samples. For continuous variables (GDMI, gene expression levels, ATP and ROS/superoxide quantification) measures of central tendency and dispersion, including mean, median and standard error of the mean were reported. Departures from normality assumption were tested using the Kolmogorov–Smirnov normality test. One-way analysis of variance for multiple comparisons and student's t-test for pair-wise comparisons were adopted to compare continuous normally distributed variables with respect to the mtDNA haplogroup classification. In case of evidence of non-normality, the corresponding nonparametric Kruskal–Wallis or Mann–Whitney tests (as appropriate) were used.

Categorical variables were examined by analyzing the relevant frequency distributions. Monte Carlo approximation to Fisher's exact test (based on 10,000 random permutations) was adopted to compare haplogroup frequency distributions between the sexes [58].

Statistical analyses were performed using SPSS 15.0 (SPSS Inc., IL, USA). A significance level of 0.05 was chosen for all tests.

Results

Global DNA methylation level analysis in population DNA samples

Global DNA methylation levels were determined as GDMI values by applying the CpGlobal assay developed by Anisowicz *et al.* to DNA collected from human individuals who were previously analyzed for their mtDNA variability [57]. As a quality control, in each assay we also evaluated the global methylation levels of unmethylated, fully and partially methylated samples of human genomic DNAs. Specifically, in the experimental conditions we adopted, we observed that fully methylated control human DNA was digested by *Msp*I but not by *Hpa*II, fully unmethylated control human DNA was totally digested by both enzymes, whereas the DNA sample obtained by mixing an equal ratio of unmethylated and methylated control human DNA showed an intermediate result. As expected, the GDMI values in the fully methylated, in the mix of methylated and unmethylated, and in the fully unmethylated control DNAs were about 0.1, 0.5 and 1.0, respectively. We maintained that the above results allowed us to validate effectiveness of the experimental conditions [59].The distribution of the GDMI values in our population sample was not normally distributed as indicated by the Kolmogorov–Smirnov test result ($p = 0.007$; Supplementary Figure 1, www.futuremedicine.com/doi/suppl/10.2217/epi.11.109). Table 1 reports for each gender the frequency distribution of mtDNA haplogroups with the relevant mean values of age and GDMI in the analyzed sample. The frequency distributions observed in the present study were in agreement with those previously reported for the Calabrian population [10]. We found no significant age and sex difference with respect to the haplogroup classification in the analyzed sample ($p = 0.783$ and $p = 0.534$, respectively). In addition, we observed that in both sexes subjects carrying mtDNA belonging to the J haplogroup exhibited lower GDMI values than those carrying mtDNA belonging to non-J haplogroup ($p = 0.019$ in males; $p = 0.002$ in females). As GDMI inversely correlates with the global DNA methylation levels, we can infer that the J haplogroup carriers have higher global DNA methylation levels with respect to the non-J haplogroup carriers.

A subset of the whole sample analyzed with Sigma's Imprint Methylated DNA Quantification Kit gave consistent results (data not shown).

Global DNA methylation level analysis in cybrid cell lines

In order to better clarify the influence of the mtDNA variability on global DNA methylation, we carried out an *in vitro* study by applying the CpGlobal assay to cybrid cell lines which were constructed by repopulating 143B.TK$^-$ Rho^0 cells with mitochondria derived from platelets of healthy human donors harboring mtDNA of H, J, U, X and T haplogroups. At the beginning of all experiments, these cells were carefully evaluated for proliferation rate, number of copies of mtDNA and MMP as previously reported [40,42]. We did not observe any significant difference in the above parameters among cybrids and between any of them and the parental 143B.TK$^-$ cell line (data not shown). As expected, MMP was lower in the Rho^0 cells.

Results we obtained indicated that global DNA methylation levels were different among cybrids (Figure 1; $p < 0.001$), with the J cybrids showing lower GDMI values and, thus, higher methylation levels than each of the other lines ($p < 0.05$ in all comparisons). Since mtDNA is the sole variant among the cybrid lines, these results demonstrate a correlation between mtDNA variability and global DNA methylation levels, thus confirming the evidence emerging from the population study. Moreover, the comparison between the global methylation levels of the 143B.TK$^-$ and Rho^0 cell lines demonstrated that these levels are also associated with the depletion of mtDNA as Rho^0 cells exhibited higher levels with respect to the native line ($p = 0.009$).

Methylation in cybrids was also tested with Sigma's Imprint Methylated DNA Quantification Kit and gave consistent results (data not shown).

Gene-expression analysis

Once we determined that the global DNA methylation status was increased in the J cybrid line, quantitative real-time PCR assays were carried out in order to identify which of the following genes, *DNMT1*, *DNMT3A*, *DNMT3B*, *MAT1A*, *MAT2B*, *MBD2* and *MBD4*, involved in DNA methylation processes, were responsible for the hypermethylation profile. By

Table 1. Global DNA methylation index values of the population samples according to the mitochondrial DNA haplogroup.

Haplogroup	Males				Females				Total			
	n	*Rel. Freq.*	*Age (years)*	*GDMI*	*n*	*Rel. Freq.*	*Age (years)*	*GDMI*	*n*	*Rel. Freq.*	*Age (years)*	*GDMI*
H	47	0.29 ± 0.04	83.15 ± 1.80	0.52 ± 0.03	73	0.38 ± 0.04	83.38 ± 1.52	0.51 ± 0.02	120	0.34 ± 0.03	83.29 ± 12.69	0.51 ± 0.19
I	5	0.03 ± 0.01	88.80 ± 5.03	0.42 ± 0.12	3	0.02 ± 0.01	81.33 ± 8.84	0.54 ± 0.11	8	0.02 ± 0.01	86.00 ± 12.42	0.47 ± 0.23
J	10	0.06 ± 0.02	85.50 ± 4.05	0.39 ± 0.08	15	0.08 ± 0.02	86.53 ± 3.95	0.36 ± 0.05	25	0.07 ± 0.01	86.12 ± 14.09	0.37 ± 0.21
K	18	0.11 ± 0.03	85.44 ± 3.28	0.55 ± 0.05	18	0.09 ± 0.02	84.06 ± 3.39	0.53 ± 0.05	36	0.10 ± 0.02	84.75 ± 13.95	0.54 ± 0.21
Others	21	0.13 ± 0.03	83.43 ± 2.74	0.53 ± 0.05	18	0.09 ± 0.02	90.00 ± 2.78	0.52 ± 0.03	39	0.11 ± 0.02	86.46 ± 12.49	0.53 ± 0.17
T	20	0.12 ± 0.03	86.55 ± 2.86	0.52 ± 0.05	18	0.09 ± 0.02	81.78 ± 2.88	0.53 ± 0.05	38	0.11 ± 0.02	84.29 ± 12.59	0.52 ± 0.23
U	23	0.14 ± 0.03	87.17 ± 2.91	0.52 ± 0.04	17	0.09 ± 0.02	81.94 ± 2.61	0.50 ± 0.04	40	0.11 ± 0.02	84.95 ± 12.80	0.51 ± 0.19
V	1	0.01 ± 0.01	96.00	0.99	4	0.02 ± 0.01	75.00 ± 2.80	0.56 ± 0.13	5	0.01 ± 0.01	79.20 ± 10.57	0.64 ± 0.30
W	7	0.04 ± 0.02	74.57 ± 2.74	0.54 ± 0.10	15	0.08 ± 0.02	84.73 ± 2.63	0.50 ± 0.06	22	0.06 ± 0.01	81.50 ± 10.36	0.51 ± 0.23
X	11	0.07 ± 0.02	90.64 ± 3.29	0.57 ± 0.05	10	0.05 ± 0.02	83.90 ± 4.18	0.55 ± 0.05	21	0.06 ± 0.01	87.43 ± 12.25	0.56 ± 0.17
Total	163	–	84.96 ± 0.99	0.52 ± 0.02	191	–	83.96 ± 0.92	0.51 ± 0.01	354	–	84.42 ± 12.67	0.51 ± 0.20

These values, which represent means of three independent triplicate experiments, are also reported by age and gender.
GDMI: Global DNA methylation index; mtDNA: Mitochondrial DNA; n: Absolute frequency of the individuals belonging to the haplogroup class ± standard error of the mean; Rel. Freq.: Relative frequency of the individuals belonging to the haplogroup class ± standard error of the mean.

comparing the mRNA levels of the above genes among cybrids, a significant difference was observed only for the *MAT1A* gene (Supplementary Figure 2 & Figure 2; $p < 0.001$). More specifically, the J cybrids showed higher mRNA levels of the *MAT1A* gene than each of the non-J cybrids ($p<0.05$ in all comparisons). These results suggested that the mtDNA variability may modulate the expression of *MAT1A* and that the overexpression of this gene might be involved in the hypermethylation status of the J cybrids.

The role of mtDNA in modulating the expression profiles of the above genes was also supported by the comparison of the Rho^0 cells with the parental cell line. Indeed, as the J cybrids, the Rho^0 cells showed higher *MAT1A* mRNA levels than the parental cell line (Figure 2; $p = 0.011$).

Measurement of ATP & ROS levels

To investigate the molecular mechanisms underlying the differences in the *MAT1A* expression profiles previously reported between J and non-J cybrids, we measured intracellular ATP and ROS levels, major products of mitochondrial activity and regulators of cross signaling between mitochondrial and nuclear genomes. The J cybrids showed lower intracellular ATP (Figure 3A) and lower ROS levels (Figure 3B; $p < 0.05$ in all comparisons) than each of the non-J cybrids. Interestingly, Rho^0 cells exhibited lower ATP levels but higher ROS levels with respect to the parental cell line.

Discussion

The aim of the present study was to investigate whether mtDNA variants could affect global DNA methylation profiles. Indeed, while it is quite clear that these variants are able to regulate several intracellular functions as well as to induce changes in nuclear gene expression, no data has been documented to date about their influence on epigenetic processes. From our population association study and *in vitro* analyses, it has emerged that the subjects and cybrid cells harboring mtDNA molecules belonging to the J haplogroup have higher global DNA

methylation levels than non-J carriers. These data indicate a clear correlation between mtDNA variability and global DNA methylation. The above correlation might be explained by a differential activation of signaling pathways involved in the crosstalk between nucleus and mitochondria, widely described in the literature. These pathways, by acting either directly on energy production efficiency or indirectly by interacting with nuclear genes, could be responsible for qualitative and quantitative differences in living cells in a mtDNA-specific manner [60,61]. What is more, the difference in DNA methylation levels between native and Rho^0 cells we observed are in line with data reported by Smiraglia *et al.* and provide additional evidence for the above interactions, thus confirming the existence of a retrograde response in human cells [54,62]. Interestingly, it has been repeatedly reported that the variants defining the J haplogroup have functional consequences on several complex traits, including LHON, multiple sclerosis and longevity [10,12,17,63–66]. The data we present here may highlight a novel function for this haplogroup implying that its phenotypic effects may partly be due to its effects on epigenetic changes.

We are aware that the CpGlobal method relies on measurements of luminescence following DNA digestion with *Hpa*II and *Msp*I enzymes, and thus is dependent on their cutting efficiency, and that a large debate exists regarding the technical manipulations leading to cybrid production and its effect on casual chromosomal rearrangements. Conversely, we are confident of the reliability of our results since: a series of control experiments demonstrated the effectiveness of the CpGlobal method [40,42]; the global DNA methylation levels determined by the CpGlobal assay were confirmed by adopting an independent method on a subset of the entire study group; all significant differences between cybrids were replicated three times on independent cybrid clones.

Gene-expression studies reported in this paper also demonstrate that the hypermethylation observed in the J cybrids might be due to the *MAT1A* gene which is overexpressed in these cells with respect to the other cybrids.

How could the mtDNA sequence-dependent genetic information be responsible for the regulation of the *MAT1A* gene, and thus, of the hypermethylation in the J cybrids? It is possible that such effects are mediated by the lower ATP levels we observed in the J cybrids than in non-J cybrids.

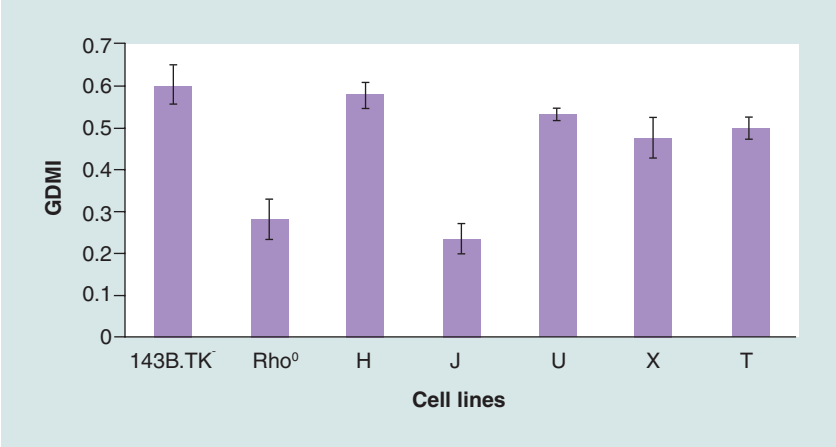

Figure 1. Global DNA methylation index values determined in 143B.TK$^-$, Rho^0 and cybrid cell lines. The values represent the mean of three independent triplicate experiments with standard errors of the mean.
Analysis of variance test $p < 0.001$; Student's t-test: 143B.TK$^-$ vs Rho^0 $p = 0.009$; J vs H $p = 0.011$; J vs U $p = 0.008$; J vs X $p = 0.002$; J vs T $p = 0.005$.
GDMI: Global DNA methylation index.

This hypothesis is also confirmed by observing that Rho^0 cells, which exhibit minimum levels of ATP, have a global methylation pattern similar to that displayed by J cybrids. We retain that the presence/absence of the mtDNA or the variability of its sequence affects the global methylation levels by regulating ATP levels. It is likely that the effect of the ATP on methylation may be threshold-dependent and that the ATP levels of both Rho^0 cells and J cybrids are below this threshold. Further studies are needed to evaluate this hypothesis.

The low ATP levels and the above-mentioned association with different cellular phenotypes of the J haplogroup have been

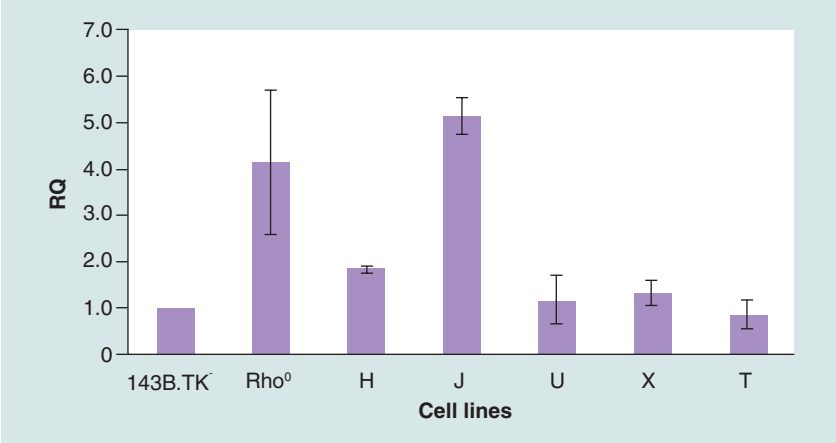

Figure 2. mRNA levels of *MAT1A* gene measured in 143B.TK$^-$, Rho^0 and cybrid cell lines. These levels are reported as the means of RQ values, measured in three independent triplicate experiments, with standard errors of the mean.
Analysis of variance test $p < 0.001$; Student's t-test: 143B.TK$^-$ vs Rho^0 $p = 0.011$; J vs H $p = 0.001$; J vs U $p = 0.002$; J vs X $p = 0.001$; J vs T $p = 0.005$.
RQ: Relative quantification.

extensively ascribed to the low OXPHOS efficiency, given that the variants clustered in J haplogroup mainly fall within mitochondrial complex I (ND1 subunit) and complex III (cytochrome b) [24,29,30,67–70]. In line with this hypothesis, oxygen consumption has been reported to be lower in individuals from the J haplogroup with respect to the H haplogroup, as well as the T4216C transition, characterizing the J haplogroup, has an effect on the structural integrity of the ND1 complex and, subsequently, on its activity [24–26]. In addition, Arning *et al.* reported that haplogroup J variations "partially uncouple OXPHOS" [29]. Here, the demonstration that J cybrids produce lower ATP levels than the non-J cybrids adds further experimental evidence of the uncoupling effect of this haplogroup on oxidative phosphorylation, although some considerations need to be outlined. First, we observed differences in OXPHOS efficiency in cybrids harboring the J and T haplogroups, although they share a common root in the mitochondrial tree, such as the 4216/ND1 and 15542/cytb variants. Second, we observed differences in OXPHOS efficiency between J and non-J cybrids, but not among non-J cybrids, all of them characterized by different clusters of mtDNA variants (Supplementary Table 1). Last, according to previously reports, we did not observe any difference in OXPHOS efficiency between H, 'tightly coupling OXPHOS' and U, X and T haplogroups, which harbor variants that are thought to be 'partially uncoupling' OXPHOS [29,45]. Scenarios we described induce us to also consider the role of nonsynonymous 'private' variants for which a functional role has yet to be determined. These variants are thought to contribute to overall phenotypic variance of each haplogroup through complex interactions with the environment and nuclear background. We retain that, in our study, the role of private variants on the functional effects of the J haplogroup is quite irrelevant. This assumption comes from both the consistence of results we obtained in population and *in vitro* studies and by the sequence analysis of the whole mtDNA of our J cybrids demonstrating that they do not harbor private variants [40].

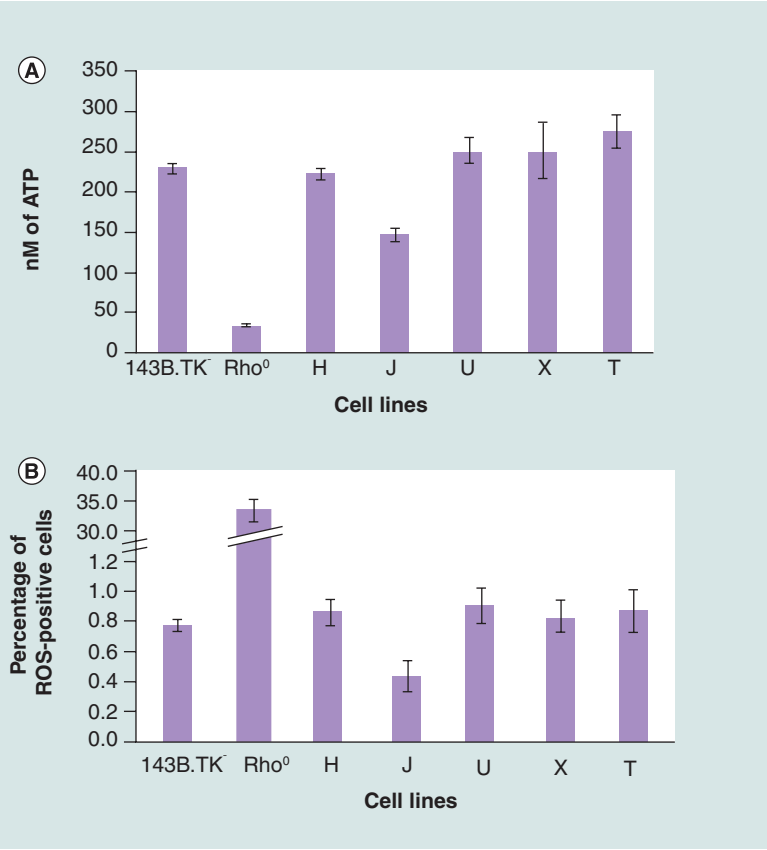

Figure 3. Measurement of ATP and reactive oxygen species levels in 143B.TK⁻, Rho⁰ and cybrid cell lines. (A) ATP levels are reported as nM determined in three independent triplicate experiments with standard errors of the mean. Analysis of variance test $p < 0.001$; Student's t-test: 143B.TK⁻ vs Rho⁰ $p < 0.001$; J vs H $p = 0.027$; J vs U $p = 0.003$; J vs X $p = 0.040$; J vs T $p = 0.046$. **(B)** ROS levels are represented as percentage of ROS-staining positive cells. Data represent the means of three independent experiments with standard errors of the mean. Analysis of variance test $p < 0.001$; Student's t-test: 143B.TK⁻ vs Rho⁰ $p < 0.001$; J vs H $p = 0.032$; J vs U $p = 0.028$; J vs X $p = 0.031$; J vs T $p = 0.038$.
ROS: Reactive oxygen species.

By combining our results with published data, we hypothesize that ATP levels affect *MAT1A* gene transcription. Such effect may be obtained either by chromatin epigenomic remodeling or by modulating the methylation levels of its promoter region, which is known to be regulated by epigenetic changes [71–73]. Alternatively, ATP may regulate the activity of transcription factors involved in *MAT1A* gene expression. In this regard, it is important to note that factors, such as AP1, CRE-BP, E2F and CAAT, have several motif binding sites located within the *MAT1A* promoter region, exhibit activity correlated to ATP levels and are also involved in the cross-signaling between nucleus and mitochondria [74,75]. We are currently carrying out experiments which could clarify the involvement of *MAT1A* in the hypermethylation of the J cybrids and confirm one or the other hypothesis.

With regards to ROS levels, we found conflicting results: low levels of ROS correlated with high DNA methylation in J cybrids and high levels of ROS correlated with high DNA methylation in Rho⁰ cells. This might suggest that ROS are not predictors of DNA methylation status, although we need to highlight

that Rho^0 cells are in an 'artificial state' due to mitochondrial dysfunction caused by mtDNA depletion and to the consequent compensatory mechanisms [76].

Conclusion

On the whole, our data provide evidence that mtDNA variability modulates global DNA methylation levels, possibly via the regulation of OXPHOS efficiency. This may represent an alternative mechanism for the remodeling of gene expression that commonly occurs during lifetime. Conversely, whether the observed epigenomic differences due to mtDNA variability are epiphenomena or part of the causal pathways leading to biological functions is still largely to be determined. Future studies seeking to determine the methylation levels of specific mitochondrial genes will need to clarify this point.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Several published studies report evidence for mitochondrial functions affecting DNA epigenetic changes during a human's lifetime.
- Variants of mitochondrial DNA (mtDNA) contribute to the interindividual variability in mitochondrial function by a network of signaling between the nucleus and mitochondria.
- The results reported in this study aim to determine whether global DNA methylation levels are affected by mitochondrial DNA variability.
- Population and *in vitro* results we obtained demonstrate a clear correlation between J mtDNA haplogroup and global DNA methylation levels.
- The above correlation seems to be associated with differences in ATP levels and in *MAT1A* gene expression.
- This work indicates that mtDNA-specific interactions between mitochondria and the nucleus regulate epigenetic changes, possibly by changes in oxidative phosphorylation efficiency.

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Conclusive remarks

Many studies concerning the biological basis of human longevity outlined that both genetic and epigenetic factors, together with environmental and stochastic ones, contribute to determine successful aging. The methylation of mitochondrial DNA remains currently controversial and strongly debated.

In this context, the studies here presented contribute to shed a light on the occurrence of the above epigenetic modification as well as on its correlation with aging peculiarities.

In fact, our works demonstrate that both 5mC and 5hmC residues were present in the mitochondrial regulative region D-loop. Interestingly, it seems that mtDNA methylation especially occurs within non-CpG sites and that it is cell type-dependent. Taken as a whole, our data provide unequivocal *in vivo* evidence, for the first time in human DNA, supporting the presence of methylated cytosines within the human mitochondrial DNA, occurring not only at symmetrical CpG dinucleotides, typical of the nuclear genome, but in a peculiar non-CpG pattern so far reported for plants and fungi. Immunoblotting analyses revealed the presence of DNMT1, DNMT3B, TET1 and TET2 within mitochondria, thus suggesting that these enzymes may direct mtDNA methylation.

The correlation between the methylation of 12S rRNA gene with gender, age and aging phenotypes suggests that the above modifications are modulated along the lifetime.

Lastly, the evidence that DNA methylation levels are modulated by mtDNA variability through mtDNA-specific cross signaling between mitochondrial and nuclear genome, specifically influenced by OXPHOS efficiency, highlight the importance of the mitochondrial function in age-related processes of gene expression remodeling and in the decrease of the physiological and cellular homeostasis during aging.

Overall, our data demonstrate that the documented cross-talk between nucleus and mitochondrion, known to regulate many different cellular mechanisms, might also influence the epigenetic processes as mtDNA variability influences nuclear gene transcription and nuclear genes influence mtDNA methylation processes.

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APPENDIX

The genetic component of human longevity: analysis of the survival advantage of parents and siblings of Italian nonagenarians

Montesanto A, Latorre V, Giordano M, Martino C, Domma F, Passarino G. (*published on European Journal of Human Genetics)*

Department of Cell Biology, University of Calabria,87036 Rende, Italy

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ARTICLE

The genetic component of human longevity: analysis of the survival advantage of parents and siblings of Italian nonagenarians

Alberto Montesanto[1], Valeria Latorre[1], Marco Giordano[1], Cinzia Martino[1], Filippo Domma[2] and Giuseppe Passarino*[,1]

Many epidemiological studies have shown that parents, siblings and offspring of long-lived subjects have a significant survival advantage when compared with the general population. However, how much of this reported advantage is due to common genetic factors or to a shared environment remains to be resolved.We reconstructed 202 families of nonagenarians from a population of southern Italy. To estimate the familiarity of human longevity, we compared survival data of parents and siblings of long-lived subjects to that of appropriate Italian birth cohorts. Then, to estimate the genetic component of longevity while minimizing the variability due to environment factors, we compared the survival functions of nonagenarians' siblings with those of their spouses (intrafamily control group).We found that both parents and siblings of the probands had a significant survival advantage over their Italian birth cohort counterparts. On the other hand, although a substantial survival advantage was observed in male siblings of probands with respect to the male intrafamily control group, female siblings did not show a similar advantage. In addition, we observed that the presence of a male nonagenarians in a family significantly decreased the instant mortality rate throughout lifetime for all the siblings; in the case of a female nonagenarians such an advantage persisted only for her male siblings.The methodological approach used here allowed us to distinguish the effects of environmental and genetic factors on human longevity. Our results suggest that genetic factors in males have a higher impact than in females on attaining longevity.
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Keywords: human longevity; genetic component; inheritance; familial determinants

INTRODUCTION

In the last decade many epidemiological studies on human longevity have shown that parents, siblings and offspring of long-lived subjects have a significant survival advantage compared with the general population in attaining longevity.[1–11] Although these studies do not distinguish between shared environmental and genetic factors, twin data suggest that genes may have a modest role in achieving longevity.[12,13] In order to better distinguish the effect of genes from the effect of shared familial environment, Schoenmaker *et al*[3] analyzed the survival data of the spouses of long-lived subjects as an additional control group. They found that members of this control group, who shared most of their adult life with the long-lived partner, did not show any advantage/benefit in terms of survival, suggesting that a substantial contribution in the familiarity of human longevity is attributable to genetic factors. However, as a complex trait, the heritability of 'lifespan' may be influenced by an interplay of genetic, environmental and stochastic factors.[14,15] In addition, the influence of the genetic component on lifespan is expected to be stronger in populations of areas where environmental factors are harsher[16] as demonstrated in different studies.[9,17,18]

Calabria is one of the poorest Italian regions located in the southern part of the peninsula. In the present study we aimed (i) to estimate the familial component of human longevity in Calabrian population; (ii) to uncouple within such a familial component the genetic from the environmental component. For these purposes, we reconstructed 202 pedigrees of Calabrian families where at least one nonagenarian individual was present. In order to estimate the presence of a familial component of longevity, we compared the survival data of parents and siblings of long-lived subjects with appropriate Italian birth cohorts. Then, to minimize the variability of familial environmental factors, we compared the survival functions of long-lived siblings with those of their spouses (intrafamily control group). This approach allowed us to estimate how much of the familiarity of the analyzed phenotype is due to genetics.

MATERIALS AND METHODS

Our sample consisted of the members of 202 families identified in seven municipalities (Bisignano, Cariati, Cosenza, Luzzi, Montalto Uffugo, Rende, and Rose) of Calabria (southern Italy). Each municipality was contacted in 2006 and invited to send a list of subjects living in their territory born in 1916 or before (probands). In total, 1475 eligible probands were identified. In the present study, which started in October 2008, we reconstructed the family pedigree of 202 probands.

Age validation

For complete age validation of long-lived individuals, their parents, siblings and the long-lived spouses of siblings, the following documents were

[1]Department of Cell Biology, University of Calabria, Rende, Italy; [2]Department of Economics and Statistics, University of Calabria, Rende, Italy
*Correspondence: Professor G Passarino, Department of Cell Biology, University of Calabria, 87036, Rende, Italy. Tel: +39 0984 492932; Fax: +39 0984 492911; E-mail: g.passarino@unical.it
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examined: the birth certificate, marriage certificate(s), the population registry (*Anagrafe*) personal sheet, the birth certificate of both parents (except for non-related parents) and death certificates. In addition, in order to further confirm the completeness of the reconstructed pedigrees, specialized personnel contacted a relative (usually a child or nephew/niece) for each proband whose genealogical tree had been reconstructed to verify information regarding the name(s), places and dates of birth, marriage, death, and emigration of the parents, all siblings and their spouses of the long-living probands.

Statistical analyses

As we focused on the mortality and survivorship of their parents and siblings, the probands were not included in the analysis described here. Because of our interest in longevity, we examined the survival patterns of the parents and siblings of the probands conditional on survival to age 30. We chose age 30 as a cutoff because siblings who died at younger ages probably did so because of stochastic, non-heritable factors (eg, infectious diseases, accidents, violence).[4,9] This minimizes the effect of such errors on cumulative survival probability.

In order to verify whether parents of probands lived longer than expected, we compared their life span with that of their respective Italian birth cohort. We first estimated the mean age at death of proband's parents conditional on survival to age 30. All parents had died, and thus their survival experience was complete. Following Perls *et al*,[19] we then matched each participant by year of birth and sex with their respective Italian cohort to obtain life expectancies conditional on survival to age 30. For the Italian population, sex-specific life tables are available from the Human Mortality Database (HMD) with the percentages of death for each year of age in the range of 0–100 years and each birth year since 1872 (http://www.mortality.org). The weighted average of these cohort-specific estimates was then compared with the corresponding estimates obtained for the parents of the probands.

Death rates for siblings and their spouses were computed, separately, from tabulations by age of sibling deaths and censored observations. Both the death counts and exposure estimates were aggregated in 5-year age groups. Standard demographic methods were used to calculate the mortality rate and its variance. Death rates, d_x, were computed as the ratio of deaths, D_x, over the exposure-to-risk E_x in a given age group:

$$d_x = \frac{D_x}{E_x}$$

E_x was calculated as the number of sibling survivors at the beginning of an age interval, N_x, minus half of the deaths, D_x, and censorings, W_x, during the interval:

$$E_x = N_x - \frac{1}{2}(D_x + W_x)$$

The variance of the estimated mortality rate was calculated according to Poisson distribution.[20]

The survival rate for interval x was computed as following:

$$p_x = \frac{R_x - D_x}{R_x}$$

The risk-set R_x equaled the number of sibling survivors at the beginning of an age interval, minus half of the censorings over that interval:

$$R_x = N_x - \frac{1}{2}W_x$$

The survival curves, S_x, were computed as $S_x = p_0 p_1 \ldots p_{x-1}$.

Standard errors for sibling survival probabilities were calculated based on an assumption of binomial variability (conditional on the observed collection of R_x values) using Greenwood's formula.[21] The obtained survival curves were then compared by log-rank test.

In order to investigate whether proband siblings had lower mortality and higher probability of surviving at advanced ages, siblings survival curves were compared with (i) the corresponding survival curves of the 1910 birth cohort for the general Italian population (the average year of birth for siblings was 1911) and (ii) the survival curves of their spouses (intrafamily control group). In this case, as survival experiences of proband siblings were not complete (some were still alive at the time of the study) the approach used for the parents of the probands was not applicable. To bypass this problem, we used an approach widely applied in other studies[1,9] that is, to define a 'control group' by determining the mean year of birth of the siblings of the probands. Then, we compared their survival experience with respect to those of the Italian birth cohort of such year. Survival data from the 1910 cohort were derived from the HMD. As in the previous case, survival probabilities were conditional on survival to age 30. The siblings of the probands and their respective spouses who emigrated from Italy were excluded from the study and their immigration periods were used as censoring dates. The exclusion circumvented the introduction of a bias due to the effect exerted on the phenotype by the 'new' environment in which they went to live.

In order to quantify the survival advantage due to a presence of a long-lived individual in the reconstructed family, the siblings' hazard function was compared with those of their spouses using a Cox regression model.[22] In this model 'relationship to the proband', 'gender of the sibling/spouse' and their interaction were used as explanatory covariates.

RESULTS

Table 1 reports a descriptive analysis of the subjects analyzed for this study. Of the 202 probands (126 women and 76 men), 129 were deceased (63.9%) at the time of this analysis and 73 (36.1%) were alive. The probands had a median of six siblings with a range of 1–13. A total of 1160 siblings, 593 men and 567 women, were identified for the analysis. Of these, 90 (15.2%) males and 105 (18.5%) females died in childhood (0–10 years of age). Of the remaining, at the time of data collection 63 (12.5%) male and 68 (14.7%) female siblings were still alive. These and siblings who migrated produced a total of 179 (18.5%) censored observations. In addition, a total of 669 non-related individuals (spouses of siblings, 298 men and 371 women) were identified for the same analysis. At the time of data collection, 18 (6.0%) male spouses and 90 (24.3%) female spouses were still alive. These and siblings' spouses who migrated outside of Italy gave a total of 128 (19.1%) censored observations. In the case of the siblings, early childhood mortality was included, hence the relatively large difference in number of deceased *vs* deceased ≥ 30.

The average year of birth of the probands was 1910 and for their siblings the average was 1911. With regard to parent's data, for 43 mothers and 33 fathers information on age at death were unknown. The average year of birth for fathers was 1876 and for mothers 1882.

Median ages at death for fathers and mothers of the probands were 77.5 and 79 years, respectively. Excluding deaths which occurred before age 30, the median age at death of the siblings of probands was higher than those observed in the relevant spouses (78 years in male siblings of probands *vs* 75 of the male spouses; 81 years in female siblings of probands *vs* 79 of the female spouses).

Table 2 shows the results for comparisons of mean ages at death of the proband's parents with the corresponding estimates for Italian birth cohort conditioned on survival to the age of 30 years. The mean age at death of the father's of probands was about 75 years. These estimates were substantially higher than the corresponding estimates for the respective Italian birth cohorts. In fact, the mean age at death was about 11% higher (8.05 years, $P<0.001$) when compared with the relevant Italian birth cohorts.

Figure 1 shows the survival curves obtained for the siblings of the probands and the 1910 Italian birth cohort. Both curves are conditioned for survival to the age of 30 years, as reported in Materials and Methods. Although the 1910 Italian birth cohort is not totally extinguished, Figure 1 shows the presence of a substantial survival advantage, which is more evident in male siblings ($P<0.001$) than in females ($P=0.01$).

Table 1 Characteristics of the subjects (belonging to 202 families) analyzed in the study. Median age and interquartile range are displayed

	Men		*Women*	
	N	*Age*	*N*	*Age*
Parents of proband				
Deceased	167[a]	77.5 (68–85)	157[b]	79 (70–86)
Deceased (≥30 years)	166	78 (68–85)	157	79 (70–86)
Sibling				
Alive	63[c]	85 (81–89)	68[c]	86 (81–90)
Deceased	450	73 (33.75–82)	481	74 (22.5–85)
Deceased (≥30 years)	384	78 (68–84)	355	81 (71–86)
Proband sibling's spouses[d]				
Alive	18[c]	84 (82–87.5)	90[c]	82.5 (77–87)
Deceased	272	75 (64–82)	269	79 (71–85)
Deceased (≥30 years)	270	75 (64.75–82)	267	79 (71–85)
1910 Italian birth cohort				
Life expectancy at birth		49.33		54.52
Life expectancy conditional on survival to age 30 years		71.12		78.15

[a]33 fathers had unknown age at death.
[b]43 mothers had unknown age at death.
[c]Censored for immigration not included.
[d]Calculations include only the first spouse.

Table 2 Comparisons of mean ages at death (SE in parenthesis) conditioned on survival to age 30 of parents of probands with the respective Italian birth cohort, birth years 1876 for fathers, 1882 for mothers

Parents of probands	*Mean age at death by sex conditional on survival to age 30*	*Italian cohort life tables*[a]	*Excess years*	*P-value*[b]
Men (*N*=166)	75.49 (0.98)	67.44	8.05	<0.001
Women (*N*=157)	76.06 (1.10)	70.78	5.28	<0.001

Note: source, Human Mortality Database: http://www.mortality.org; calculations by the authors.
[a]Life expectancy at birth conditioned on survival to age 30 for the Italian birth cohort. Cohort life table estimates were assumed to have zero variance.
[b]*P*-value refers to *t*-Student's test.

Figure 2 compares the survival curves of the siblings of probands with those of their spouses. A substantial survival advantage is observed in male siblings of probands with respect to the male spouses ($P<0.001$). This is not true for women ($P=0.950$). In both genders the chances of survival for the two groups does not differ substantially during early adulthood. However, after the age of 50, the survival patterns begin to diverge in favor of male siblings with respect to the intrafamily control group, revealing a significant gap, which becomes more evident at very old ages.

In order to quantify the survival advantage due to a presence of a long-lived subject in the family, the siblings' hazard function was compared with those of their spouses by means of a Cox regression model. In this model, 'relationship to the proband', 'gender of the sibling/spouse' and their interaction were used as explanatory covariates. In Table 3, the maximum likelihood estimation of the parameters of this model and the hazard ratio (HR) for mortality risk are reported. From this model, a significant survival advantage for male siblings of probands is shown. In fact, they have a substantial mortality reduction of about 28% ($e^{-0.005-0.325}$) when compared with the spouses of female siblings (HR=0.719). Also adjusting for cohort effect (by inserting the year of birth of siblings/spouses as adjunctive covariate in the model) this reduction remained almost constant (data not shown).

In order to further investigate whether the sex of the proband had an effect on the survival probabilities of their siblings, we split the data set according to the sex of the proband. In 76 out of 202 families the sex of the proband was male. Figures 1 and 2 of the Supplementary Material show the survival curves of the siblings of probands and those of their spouses according to the sex of the proband. When the sex of the proband was male (Supplementary Figure 1), both male and female siblings had a survival advantage with respect to their spouses ($P=0.029$ for males; $P=0.037$ for females). When only families with a female proband were analyzed (Supplementary Figure 2), only male siblings showed a survival advantage with respect to the intrafamily control group ($P=0.007$). Parallel results were obtained by Cox regression analysis (see Table 4). In fact, siblings of male probands had a mortality reduction of about 23% with respect to their spouses (HR=0.772; $P=0.004$). On the contrary, when the sex of the proband was female, only male siblings showed such a survival advantage. In fact the interaction term of the correspondent model indicated that male siblings had a significant mortality reduction of about 30% ($e^{0.144-0.494}$).

It is of note that, although life tables show that women live longer than males (about 5 years), none of the results obtained here differed if we considered different cut offs (between 91 and 99) to define female probands.

DISCUSSION

For years, the reduced mortality of family members of centenarians has suggested the presence of a genetic component in the longevity trait. However it has always been very clear to scientists studying this issue that environmental and familiar factors (such as economic and social status) could influence the probability of attaining longevity together with genetics. In addition, it is well known that the heritability of a trait is population specific, as it may be influenced by different factors acting differently on certain traits in different populations. This is probably particularly true for longevity, which is increasing due to environmental factors (better food, better medical assistance and so on) across western countries but at different speeds. It is then likely that the importance of genetics on longevity may be higher in areas with slower yet more recent progress (such as Calabria and Sardinia) than in other areas of Western Countries.[17,18] Finally, many cues support the hypothesis that the heritability of longevity might be higher in males than in females.

The present study has confirmed the presence of a strong familiar component on longevity. In fact both the parents and the siblings (either females and males) of long-lived probands were found to live longer than the general contemporary population. On the other hand, the comparison of survival curves of the siblings of nonagenarians with those of their spouses (which are genetically unrelated but share a great part of their environment) shows a slightly different picture. In fact, we found that brothers of nonagenarians lived significantly longer than the husbands of their sisters. By contrast, no difference could be detected between survival curves of sisters of nonagenarians and the survival curves of the wives of their brothers, suggesting that the heritability of longevity is higher in males than in females. This is further reinforced by the subsequent observation that the siblings of

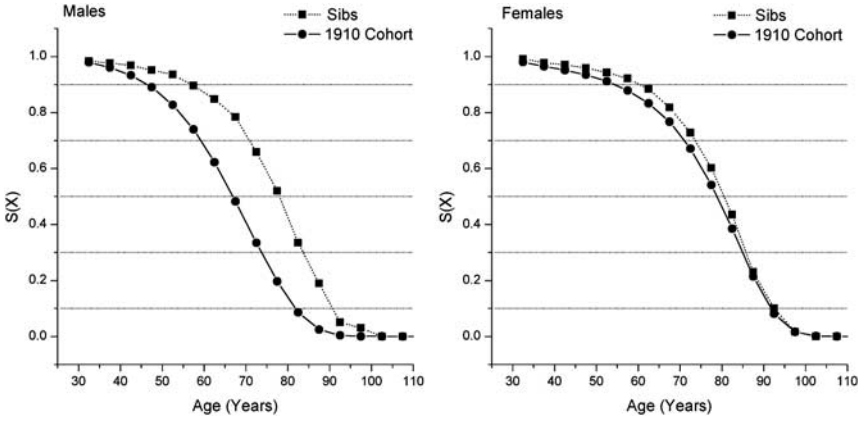

Figure 1 Survival probabilities from age 30 for siblings of probands with respect to the Italian 1910 cohort by gender.

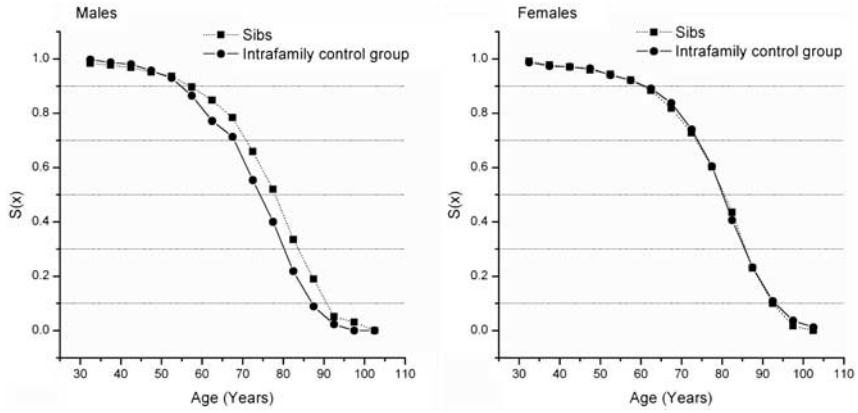

Figure 2 Survival probabilities from age 30 for siblings of probands with respect to the relevant intrafamily control group.

Table 3 Maximum likelihood estimation of the parameters of the fitted Cox proportional hazards model

						95% CI for HR	
Variables	*Coefficient (β)*	*SE*	*Wald*	*P-value**	*HR*	*Lower*	*Upper*
Relation to the proband=sibling	−0.005	0.081	0.003	0.953	0.995	0.849	1.167
Gender of the sibling/spouse=female	0.521	0.087	36.260	0.000	1.684	1.422	1.996
Relation to the proband*, gender of the sibling/spouse	−0.325	0.114	8.182	0.004	0.723	0.578	0.903

Abbreviations: CI, confidence interval; HR, hazard ratio.
SE of the estimated coefficients with the relevant HR and CI of the model are reported.
**P*-values refer to the Wald tests.

male probands (either males or females) show a reduced mortality than their spouses. By contrast, when we analyzed the siblings of female probands we found that only their brothers had a lower mortality when compared with the male spouses. This result suggests that, independently of gender, family members with a male proband share, on average, a significant genetic advantage. On the other hand, in the sibships with a female proband, the genetic share of the familial advantage is on average lower, and the female spouses of brothers of nonagenarians benefit most from the familial advantage. These results confirm that longevity has a genetic component, and suggest that such a component is stronger in males than in females. On the other hand, they also suggest that females can take advantage of a favorable environment more than males. In fact, we may state that, according to our data, being the sister of a long-lived subject or marrying one of the brothers of this subject provides a woman almost with the same survival advantage.

It is certainly important to outline some limitations of the study. First of all it is important to point out that our results may be in part specific to a largely rural and underdeveloped society where social differences are very strong, especially until a few decades ago.[23] In fact, in contrast to the study of Shoenmaker *et al*,[3] spouses of proband's siblings also live longer than the corresponding birth cohort. It is also worth mentioning that males in these cohorts may have taken advantages of their families more than their sisters in terms of wealth and social benefits. Indeed, we previously showed that only a very small percentage of women born around the beginning of the XX

Table 4 Maximum likelihood estimation of the parameters for the Cox regression models with respect to the sex of the proband

Variables	*Coefficient (β)*	*SE*	*Wald*	P-*value**	*HR*	*95% CI for HR*
(a) Sex of the proband=female						
Relation to the proband=sibling	0.144	0.104	1.902	0.168	1.155	0.941–1.417
Gender of the sibling/spouse=female	0.691	0.113	37.178	<0.001	1.996	1.598–2.492
Relation to the proband *, gender of the sibling/spouse	−0.494	0.147	11.305	0.001	0.610	0.458–0.814
(b) Sex of the proband=male[a]						
Relation to the proband=sibling	−0.258	0.090	8.165	0.004	0.772	0.647–0.922
Gender of the sibling/spouse=female	0.254	0.090	8.035	0.005	1.289	1.082–1.537

Abbreviations: CI, confidence interval; HR, hazard ratio.
SE of the estimated coefficients with the relevant HR and CI of the model are reported.
[a]The interaction term was not significant (β=−0.092; P=0.607; HR=0.912 with a CI=0.641–1.297).
**P*-values refer to the Wald tests.

century were properly scholarized.[23] This may partly explain the small excess in survival of sisters over the wives of the brothers or over the birth cohort as compared with the same groups in men.

In addition we need to point out that we used life tables referring to the 1910 Italian birth cohort as for that period they are not available for the Calabrian population alone. Calabrian life tables from 1940s onward do not show significant differences with respect to the average Italian mortality data. However, we may suppose that, based on its socio economic conditions,[24] life expectancy in Calabria at the beginning of the XX century was lower than in the rest of Italy, where, on turn, it was lower than in northern European countries.[25] Therefore we can expect that this point does not affect our results or led to an underestimate of survival advantage with respect to the general population cohorts. On the other hand, our results are in agreement with numerous demographic reports showing that in the last decades, where medical and social conditions have greatly improved, the increase in the number of female centenarians in Europe has been by far faster than the increase of male centenarians.[26]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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