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# Reconfiguration of DNA methylation in aging

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ABSTRACT

A complex interplay between multiple biological effects shapes the aging process. The advent of genome-wide quantitative approaches in the epigenetic field has highlighted the effective impact of epigenetic deregulation, particularly of DNA methylation, on aging. Age-associated alterations in DNA methylation are commonly grouped in the phenomenon known as "epigenetic drift" which is characterized by gradual extensive demethylation of genome and hypermethylation of a number of promoter-associated CpG islands. Surprisingly, specific DNA regions show directional epigenetic changes in aged individuals suggesting the importance of these events for the aging process. However, the epigenetic information obtained until now in aging needs a re-consideration due to the recent discovery of 5-hydroxymethylcytosine, a new DNA epigenetic mark present on genome. A recapitulation of the factors involved in the regulation of DNA methylation and the changes occurring in aging will be described in this review also considering the data available on 5hmC.



**Contents**



*Abbreviations:* DNMTs, DNA methyltransferases; SAM, S-adenosyl-methionine; CGIs, CpG islands; 5mC, 5-methylcytosine; LTR, long-terminal repeat; LINE, long interspersed nuclear element; SINE, short interspersed nuclear element; DMRs, differentially methylated regions; 5hmC, 5-hydroxymethylcytosine; TET, ten-eleven translocation; 5fC, 5-formylcytosine; 5caC, 5-carboxylcytosine; BER, base excision repair; SAH, S-adenosyl homocysteine; ROS, reactive oxygen species; WGBS, whole-genome bisulfite sequencing.

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## 1. Introduction

Longevity is not uniform throughout humans and it is largely influenced by environment and lifestyle choices. There is an overwhelming scientific consensus that directional epigenetic changes contribute to aging and alter the lifespan (Huidobro et al., 2013; Issa, 2014; Vlaming and van Leeuwen, 2012). In fact, only about 20–30% of human lifespan seems to be attributable to genetic effects, which implies that the major part of the variation is due to non-genetic factors (Herskind et al., 1996; Mitchell et al., 2001; Poulsen et al., 2007). It is becoming increasingly clear that some aging-associated diseases have an epigenetic origin (Huidobro et al., 2013; Issa, 2014; Vlaming and van Leeuwen, 2012). Undoubtedly, considering that epigenetic regulation is indispensable for many aspects of genome functions, the alteration of epigenetic landscapes could potentially account for the complex nature of aging by explaining the perturbation of the multitude of gene families and cellular pathways that are known to contribute to this process (De Carvalho et al., 2010; Kirkwood, 2005).Therefore, expectations are running high for insights into the epigenetic basis of the aging process.

## 2. Genomic DNA methylation patterns

DNA methylation is the first epigenetic modification identified on DNA. The enzymes deputed to the DNA methylation reaction belong to the family of the DNA methyltransferases (DNMTs) which introduce onto the C5 position of cytosine residue a methyl group deriving from S-adenosyl-methionine (SAM) (Fig. 1A). In mammals, the three active DNMT enzymes, namely DNMT1, DNMT3A and DNMT3B, preferentially modify cytosine followed by a guanine residue, commonly known as CpG dinucleotide (Bird, 2002; Jurkowska et al., 2011; Miranda and Jones, 2007; Suzuki and Bird, 2008). However, in embryonic stem cells methylated cytosine can also be present in non-CpG positions, mainly in CpA context, thanks to the action of DNMT3A/3B enzymes in concert with DNMT3L (Arand et al., 2012; Ziller et al., 2011).

CpG dinucleotide density is generally low in the bulk of genome but largely increasing in specific regions referred to as CpG islands (CGIs). Notably, mammalian genome is dominated by methylated DNA while CGIs, which account for only 1–2% of total CpG dinucleotides, are generally depleted of DNA methylation mark. Methylated cytosine, known as 5-methylcytosine (5mC), is considered the fifth base of DNA due to its non-random distribution in genome which introduces an epigenetic code (Bird, 2002; Jones and Takai, 2001; Suzuki and Bird, 2008; Takai and Jones, 2002).

### 2.1. DNA repetitive elements

CpG methylation of the bulk of genome assures genomic stability through the control of DNA repetitive elements, such as interspersed and tandem repeats, which comprise at least half of the human DNA (Jones and Takai, 2001; Lander et al., 2001). The interspersed repeats, including long-terminal repeats (LTR), long interspersed nuclear elements (LINE), short interspersed nuclear elements (SINE), are retrotransposable elements maintained inactive through DNA methylation to avoid the risk of insertional mutagenesis (Robertson and Wolffe, 2000; Yoder et al., 1997). Tandem repeats are present in centromeric, pericentromeric, and subtelomeric regions and are also constitutively methylated (Jurkowska et al., 2011; Suzuki and Bird, 2008) (Fig. 1B). DNA methylation of centromeric regions has been demonstrated to avoid centromere recombination and abnormal centromere length (Jaco et al., 2008). In the same way, DNA methylation is an important repressor of DNA recombination at telomeres and a negative regulator of telomere length (Blasco, 2007; Gonzalo et al., 2006). In addition, CpG methylation is fundamental for the inactivation of the X chromosome and the establishment and maintenance of mono-allelic expression of imprinted genes (Chaligne and Heard, 2014; Chang et al., 2006; Miranda and Jones, 2007; Weaver and Bartolomei, 2014).

### 2.2. CpG islands

CGIs are DNA regions of more than 500 base pairs characterized by at least 50% of GC content. 60% of CGIs are associated with annotated gene promoters and are unmethylated allowing the transcription of the correlated gene (Jones, 2012; Suzuki and

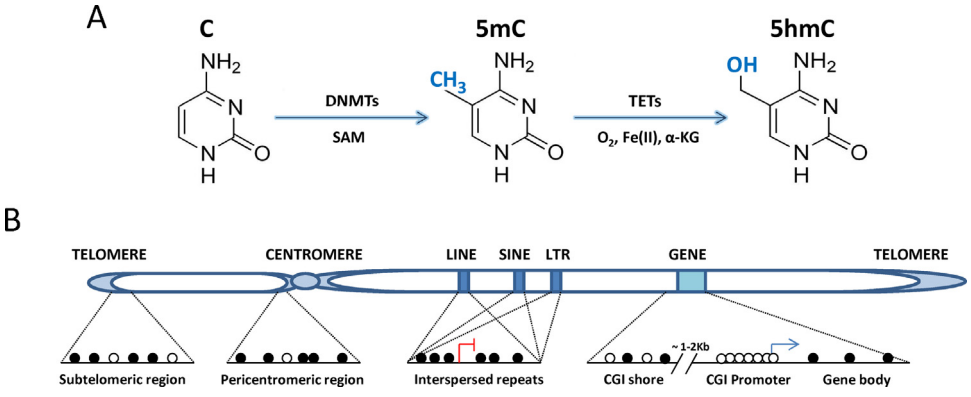


**Fig. 1.** DNA methylation reaction and patterns. A. Conversion of cytosine (C) to 5mC by DNMT enzymes which catalyze the transfer of a methyl group (CH3) from SAM to the 5-carbon position of cytosine. Oxidation of 5mC and formation of 5hmC by the Fe(II) and α-ketoglutarate(α-KG)-dependent TET enzymes. B. Schematic representation of DNA methylation patterns throughout a chromosome, showing repetitive elements and an example of gene with CGI-promoter. Arrows indicate transcription start sites, with blue color indicating active transcription, while red color indicates repressed transcription. White circles, unmethylated CpGs; black circles, methylated CpGs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Bird, 2008; Takai and Jones, 2002). On the contrary, the methylation state of CpG dinucleotides localized in gene body does not affect gene transcription but probably impedes the activation of alternative promoters (Maunakea et al., 2010). The remaining 40% of CGIs is localized in intergenic and intragenic regions and are defined as "orphan CGIs" because they are not associated with annotated promoters although presenting promoter-like features (Illingworth et al., 2010). Up to 2 Kb distant from promoter CGIs, particular regions characterized by a lower density of CpGs (around 1/10 of CGI) and defined as CGI shores are also involved in regulation of gene expression. CGI shores often overlap with tissue specific-differentially methylated regions (DMRs) in normal tissues (Doi et al., 2009; Irizarry et al., 2009) (Fig. 1B).

## 3. Introduction and removal of DNA methylation mark

DNA methylation patterns – characterized by widespread genomic methylation punctuated with unmethylated regulative regions, such as CGIs – need to be preserved. The loss of the bulk of genome methylation and/or the introduction of anomalous methyl groups onto generally unmethylated regions occur physiologically in aging but also in several pathological states (Bacalini et al., 2014; Calabrese et al., 2012; Kulis and Esteller, 2010; Lu et al., 2013).

### 3.1. Establishment and maintenance of DNA methylation patterns

In mammals, DNA methylation patterns are established during embryonic development after a wave of genome-wide demethylation occurring in preimplantation embryo soon after fertilization aimed at restoring totipotency (Feng et al., 2010; Santos et al., 2002). A second wave of DNA demethylation followed by remethylation occurs during germline development in primordial germ cells for the generation of totipotent gametes with proper specific genomic imprints (Ciccarone et al., 2012; Feng et al., 2010; Hajkova, 2011). The establishment of DNA methylation seems to be preferentially due to the action of the *de novo* DNMT3A and DNMT3B enzymes, also in cooperation with DNMT3L as observed in gametogenesis for imprinted genes and repetitive elements (Jia et al., 2007; Kato et al., 2007). Once introduced, DNA methylation patterns must be maintained during cell division in order to preserve cell identity. DNMT1 is considered the maintenance DNA methyltransferase being recruited during replication on the neosynthetized DNA filament by PCNA and UHRF1/NP95 to copy the methylation patterns (Bostick et al., 2007; Jurkowska et al., 2011; Schneider et al., 2013). However, recent evidence has clarified that DNMT3A and DNMT3B are also involved in maintenance of methylation patterns across specific DNA regions while DNMT1 can also function as *de novo* methyltransferase (Feltus et al., 2003; Jair et al., 2006; Liang et al., 2002; Sharma et al., 2011).

Together with the maintenance of DNA methylation across cell division, the preservation of the unmethylated state of DNA regulative regions is also necessary. In particular, several mechanisms have been identified for the control of unmethylated CGIs and unmethylated alleles of imprinted DMRs, such as intrinsic DNA sequences and structures (Ginno et al., 2012; Hori et al., 2002), the presence of epigenetic marks associated with active chromatin conformation (Jin et al., 2014; Ooi et al., 2007; Williams et al., 2011) and the ability of post-translational modifications and transcription factors to avoid the action of DNMT enzymes (Brandeis et al., 1994; Ciccarone et al., 2014; Fedoriw et al., 2004; Guastafierro et al., 2013; Zampieri et al., 2012).

### 3.2. DNA demethylation

Failure of DNA methyltransferase action during DNA replication induces a loss of 5mC mark known as passive DNA demethylation process (Feng et al., 2010; Jurkowska et al., 2011). Multiple mechanisms can be involved in passive DNA demethylation including down-regulation of DNMT enzymes (Oda et al., 2013; Reichard et al., 2007; Zampieri et al., 2009), cytosolic localization of DNMT (Cardoso and Leonhardt, 1999; Jurkowska et al., 2011), impairment of DNMT recruitment on DNA (Bostick et al., 2007; Oda et al., 2013), decrease of the DNMT substrate SAM (Ulrey et al., 2005), inhibition of DNMT enzymatic activity (Caiafa et al., 2009; Fang et al., 2007) (Fig. 2A).

Only recently, several pieces of evidence have sustained the existence of molecular mechanisms involved in the active removal of 5mC through the formation of 5-hydroxymethylcytosine (5hmC). 5hmC is catalyzed by three Fe(II) and α-ketoglutarate-dependent DNA hydroxylases, the ten-eleven translocation (TET) family enzymes (Fig. 1), and it is a pivotal intermediate of active DNA demethylation (Delatte et al., 2014; Pastor et al., 2013). DNMT3A and DNMT3B are possibly able to convert 5hmC directly into cytosine (Chen et al., 2012a), although much more evidence suggests the need of further modifications of 5mC and 5hmC in order to trigger DNA damage response for the reintroduction of unmethylated cytosynes (Pastor et al., 2013). Consistently, iterative modifications of 5hmC by TET proteins into 5-formylcytosine (5fC) and then 5-carboxylcytosine (5caC) can be actively removed by the base excision repair (BER) pathway (Hashimoto et al., 2012; Ito et al., 2011). An additional mechanism, although still controversial, involves the deamination of 5mC and 5hmC by the deaminase enzymes AID/APOBEC (Hashimoto et al., 2012; Nabel et al., 2012; Pastor et al., 2013) (Fig. 2B). Notably, 5hmC is not recognized by DNMT1 enzyme leading also to a passive loss of 5mC. In addition, 5fC and 5caC can undergo replication-dependent dilution (Inoue et al., 2011; Pastor et al., 2013; Valinluck and Sowers, 2007) (Fig. 2A).

## 4. Effects of environmental exposure and lifestyle on DNA methylation

DNA methylation studies carried out on monozygotic twins highlighted that genome of young pairs is epigenetically similar while the patterns of aged ones are clearly dissimilar (Fraga et al., 2005). Notably, aging-associated DNA methylation changes are particularly evident in monozygotic twins who had spent a long period of their lives apart, suggesting why their medical histories are different (Feil and Fraga, 2012; Poulsen et al., 2007). DNA methylation changes can be mediated by several extrinsic factors deriving from lifestyle, diet and environmental exposure.

Factors that can positively or negatively affect lifespan are known to influence DNA methylation patterns. For example, smoking attitude may have pro-aging effects inducing DNA methylation changes of genes involved in age-associated diseases such as cardiovascular pathologies and cancer (Besingi and Johansson, 2014; Breitling et al., 2011; Lee and Pausova, 2013; Noreen et al., 2014). On the contrary, physical activity, antioxidant intake and caloric restriction may exert anti-aging action also by counteracting detrimental DNA methylation changes (Fang et al., 2007; Ions et al., 2013; Li and Tollefsbol, 2010; Miyamura et al., 1993; Rönn et al., 2013).

Part of these factors influences DNA methylation patterns altering the availability of the DNMT cofactor SAM or directly interfering with the regulation of DNMT enzymes (Fang et al., 2007; Lee and Pausova, 2013; Martinez-Zamudio and Ha, 2011; Ulrey et al., 2005).

### 4.1. SAM/SAH ratio

SAM is the methyl donor in the reaction of DNMT enzymes. As the final product of reaction is the S-adenosyl homocysteine

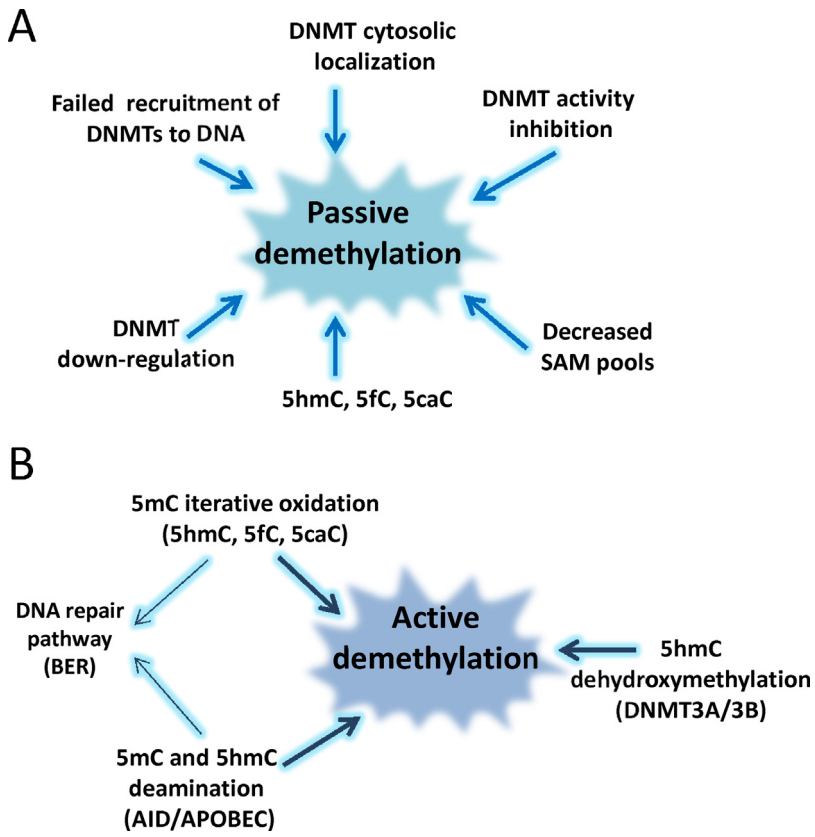


**Fig. 2.** DNA demethylation processes. A. Mechanisms affecting DNMT action during DNA replication and thus inducing passive DNA demethylation. B. Mechanisms involved in the active removal of 5mC via enzymatic modifications and activation of DNA repair pathways.

(SAH), the SAM/SAH ratio is fundamental for proper DNA methylation reactions and is guaranteed by the efficiency of one-carbon metabolism comprising enzymes involved in the synthesis of SAM, *e.g.*, methionine synthase (MTR), and catabolism of SAH, *e.g.*, cystathionine synthase (CS). Inappropriate intake of dietary molecules involved in SAM synthesis or SAH degradation, such as methionine, choline, betaine, folic acid, B6 and B12 vitamins, can influence DNA methylation patterns (Ulrey et al., 2005). Moreover, several environmental and nutritional compounds are also able to affect SAM/SAH ratio by becoming methylated themselves (*e.g.*, arsenic) (Reichard and Puga, 2010) or by inhibiting one-carbon metabolism enzymes (*e.g.*, the effect of alcohol on MTR enzyme) (Varela-Rey et al., 2013).

Arsenic is a common toxic environmental contaminant of water, soil and food. Apart from ubiquitous presence in earth's crust, human activities, mainly massive industrialization, have contributed to increase arsenic contamination (Sharma and Sohn, 2009). After entering the cells, arsenic undergoes methylation reaction by the arsenite methyltransferase (AS3MT) enzyme producing several methylated arsenic compounds (Kojima et al., 2009). Arsenic methylation was initially believed to be a detoxification reaction (Gebel, 2002), but more recent evidence invalidated such an hypothesis showing an increased toxicity of specific methylated intermediate metabolites (Kojima et al., 2009; Sun et al., 2014). Considering that SAM is the methyl donor cofactor also for arsenic methylation, excessive arsenic levels in a cell can lower the SAM/SAH ratio inducing DNA hypomethylation (Reichard and Puga, 2010). Besides arsenic and other metals such as bismuth and selenium (Hirner and Rettenmeier, 2010), also dietary constituents can undergo enzymatic methylation in the cell. This is the case of catechol structure-containing compounds such as the most abundant polyphenol in green tea, the (−)-epigallocatechin-3-gallate (EGCG), and the coffee polyphenols caffeic acid and chlorogenic acid, which are readily methylated by the catechol-*O*-methyltransferase (COMT) (Fang et al., 2007; Lee and Zhu, 2006) (Fig. 3).

Cigarette smoking is also deeply involved in alteration of DNA methylation patterns and it is an environmental risk factor for chronic diseases (Lee and Pausova, 2013; Zeilinger et al., 2013). Events of DNA hypomethylation caused by cigarette smoke can partially depend on arsenic, which is one of the carcinogens deriving from smoking. Demethylation of specific genes has been significantly associated with cigarette smoking attitude so that *F2RL3* gene is considered a promising biomarker of current and lifetime smoking exposure (Lee and Pausova, 2013; Zhang et al., 2014). An association between tobacco smoking and an increased incidence of aberrant promoter methylation of the *p16INK4A* and *MGMT* genes has been also described (Liu et al., 2006). It is interesting that the same genes have been shown to undergo hypomethylation and re-expression after treatment with the polyphenol EGCG (Fang et al., 2003; Li and Tollefsbol, 2010) suggesting that different compounds may have different outcomes on the methylation state of a specific DNA *locus*. These events are relevant in the case of *p16INK4A* and *MGMT* considering that they are frequently hypermethylated in cancer but also in aging (Liu et al., 2006; Matsubayashi et al., 2005; So et al., 2006)

### 4.2. DNMT regulation

Besides an indirect regulation of DNA methylation patterns through modulation of SAM pools, several compounds can also directly influence the expression or activity of DNMTs. Evidence of a competitive inhibition of DNMT activity affecting the entry of cytosine into active site has been demonstrated for EGCG, curcumin and the soy-bean isoflavon genistein (Fang et al., 2005, 2003; Lee et al., 2005; Liu et al., 2009; Xie et al., 2014). Nutritional (*e.g.*, cur-

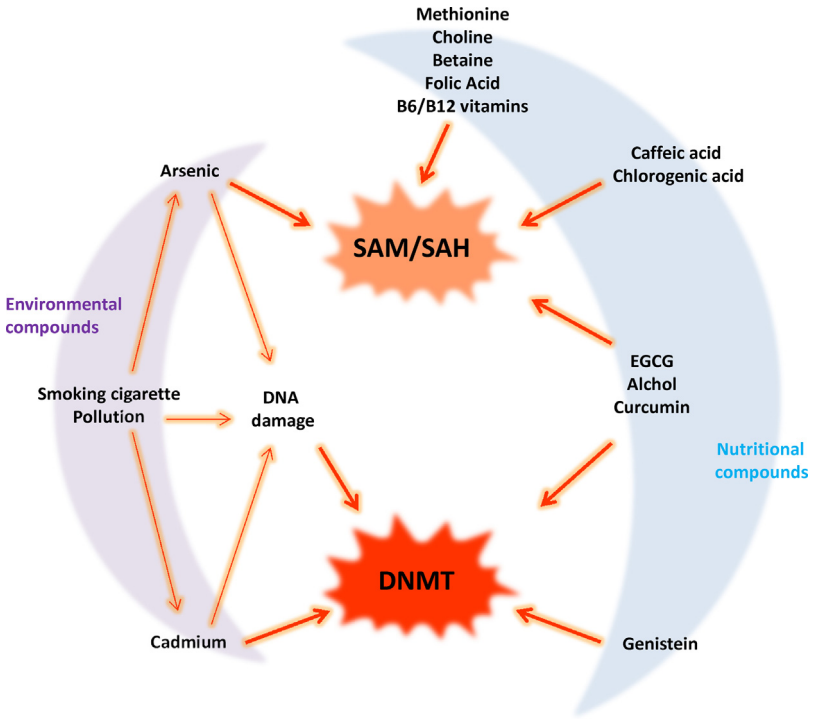


**Fig. 3.** Environment, diet and lifestyle influence DNA methylation. Nutritional and environmental compounds are able to affect DNA methylation reaction diminishing SAM/SAH ratio or regulating expression, activity, recruitment of DNMT enzymes.

cumin, alchol) and environmental factors (*e.g.*, arsenic, cadmium) have also been demonstrated to deregulate the expression of *DNMT* genes with unknown mechanism for most of them. According to time and dose of exposure to specific compounds, the effects on DNA methylation patterns can vary from extensive DNA hypomethylation to localized hypermethylation (Martinez-Zamudio and Ha, 2011; Reichard et al., 2007; Varela-Rey et al., 2013; Yu et al., 2013). Among the mechanisms able to induce DNA hypermethylation, the effect of DNA damage mediated by genotoxic agents and reactive oxygen species (ROS) has to be considered. Accordingly, it is well accepted that DNMTs are recruited to DNA repair sites inducing methylation of CpGs neighboring DNA breaks (Cuozzo et al., 2007; Morano et al., 2014; Mortusewicz et al., 2005). In this context, several DNA damaging carcinogens also present in cigarette smoking may induce aberrant DNA hypermethylation (Huang et al., 2013; Lee and Pausova, 2013). An indirect contribution to this mechanism can be mediated by cadmium, a weak genotoxic carcinogen highly accumulated in tobacco leaves (Satarug and Moore, 2004). Cadmium exposure has been associated with hypermethylation-dependent silencing of DNA repair enzymes (Zhou et al., 2012) and cadmium can also compete with zinc binding of DNA repair enzymes delaying in this way their turnover/activity (Fatur et al., 2003; Lutzen et al., 2004) and possibly favoring DNMT action at DNA breaks (Fig. 3).

## 5. Changes of DNA methylation patterns in aging

Several studies from the last three decades have clearly demonstrated that changes in DNA methylation patterns associate with chronological aging across nearly the entire human lifespan (Alisch et al., 2012; Bell et al., 2012; Bocklandt et al., 2011; Boks et al., 2009; Bollati et al., 2009; Christensen et al., 2009; Florath et al., 2013; Garagnani et al., 2012; Gentilini et al., 2013; Hannum et al., 2013; Heyn et al., 2012; McClay et al., 2014; Rakyan et al., 2010). This phenomenon, commonly defined as “epigenetic drift”, accounts for the evidence that epigenetic similarities between young individuals are lost over time leading to divergent methylomes in elderly population (Boks et al., 2009; Fraga et al., 2005; Heyn et al., 2012). DNA methylation drift is a non-directional change of methylome as it involves both hypermethylation and hypomethylation events and it has been associated with the progressive accumulation of epigenetic damage due to environmental factors or to spontaneous stochastic errors in the process of transmission of the epigenetic information. This phenomenon leads to basically unpredictable differences in the methylome among aging individuals. These observations seem to deny the possibility that the drift may reflect a programmed change of the methylation code. However, part of methylation changes that are observed with age involve specific regions of the genome and are directional. In fact, several studies indicate the existence of aging-associated differentially methylated regions (a-DMRs), clusters of consecutive CpG sites which exhibit change over time in the same direction. The existence of a-DMRs indicates that part of methylation changes is not stochastic, but instead they could be associated with biological mechanisms closely involved in the aging process or longevity.

Most of the initial analyses in aging have been focused on candidate *loci* with potential relevance for age-related diseases. Collectively, these studies showed that aging, similar to cancer, associates with gradual but profound changes in DNA methylation where epigenome is marked by genome-wide hypomethylation together with site-specific hypermethylation preferentially occurring at CGI promoters. Notably, although the functional importance of both events in aging progression and outcome remains to be ascertained, these observations support the notion that these changes may concomitantly contribute to development of aging-associated diseases.

### 5.1. DNA hypomethylation events

Global decline of genomic CpG methylation is the predominant event in aging. This change is widespread as it typically occurs at repetitive sequences dispersed throughout the genome such as

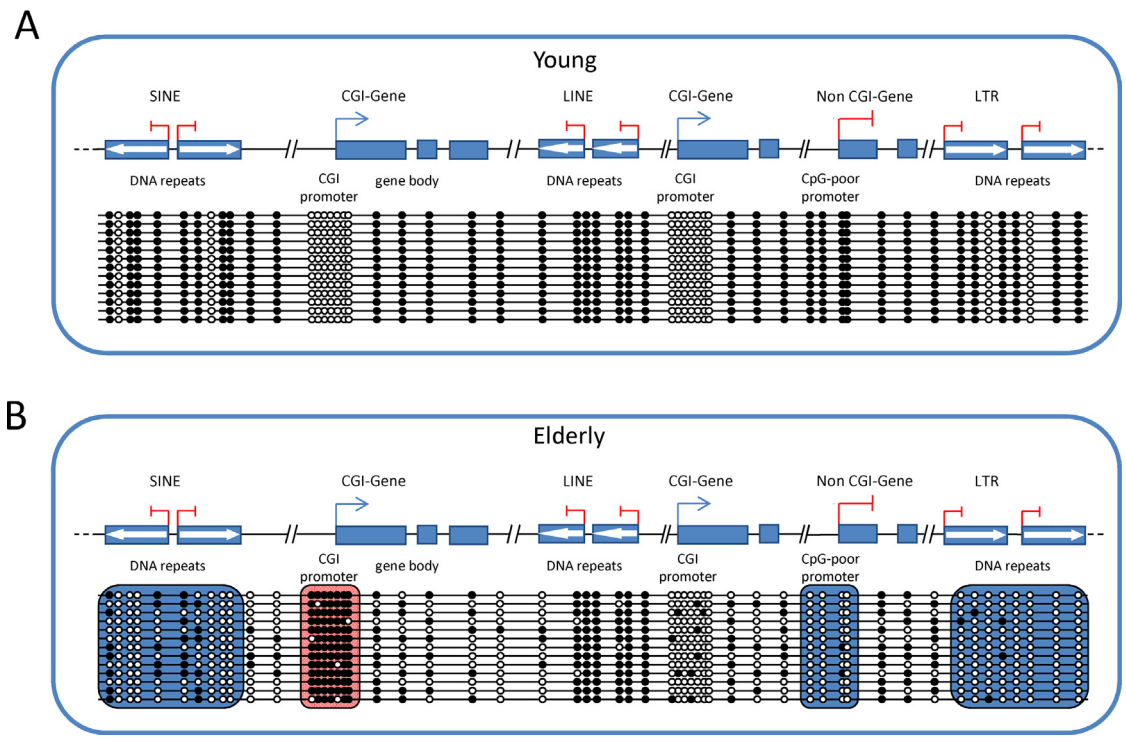


**Fig. 4.** Age-associated changes of DNA methylome. Schematic representation of DNA methylation patterns in young (A) *vs* older (B) people. The top line represents DNA containing three genes and three classes of interspersed repeats. Arrows indicate transcription start sites, with blue color indicating active transcription, while red color indicates repressed transcription. Exons are shown in dark blue. Each bottom line represents the methylation state of DNA as detected for a single individual. White circles, unmethylated CpGs; black circles, methylated CpGs. Hypomethylated and hypermethylated aging-associated DMRs are highlighted in blue and red, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

SINEs (*i.e.*, Alu elements) and LTR (*i.e.*, HERV-K) (Bollati et al., 2009; Christensen et al., 2009; Jintaridth and Mutirangura, 2010). However, age-related loss of methylation does not affect repetitive DNA equally. Hypomethylation of Alu and HERV-K sequences occurs at different ages while it does not affect LINE-1 repeats significantly (Fig. 4) (Bollati et al., 2009; Jintaridth and Mutirangura, 2010). It is reasonable to assume that this event accounts for the increased genome instability observed in the elderly (Vijg and Dolle, 2007). However, progressive age-dependent loss of methylation also concerns specific gene promoters, including *ITGAL* and *IL17RC* whose demethylation-dependent transcriptional activation has been proposed to trigger autoimmune responses (Wei et al., 2012; Zhang et al., 2002).

More recently, the application of novel next-generation sequencing technologies for genome-wide assessment of DNA methylation levels in aging research has permitted the confirmation of these earlier observations. Studies investigating DNA methylation on the whole have been particularly instructive for DNA hypomethylation events (Heyn et al., 2012; McClay et al., 2014). In fact, by whole-genome bisulfite sequencing (WGBS), Heyn et al. (2012) compared the DNA methylation state of more than 90% of all CpGs present in the genome between newborn and nonagenarian/centenarian samples. A significant loss of methylated CpGs was found in the centenarian *vs* newborn DNAs. This was observed for all chromosomes and concerned all genomic compartments such as promoters, exonic, intronic and intergenic regions. Most of these changes were focal and the aged genome was thus less homogeneously methylated with respect to the newborn according to the age-dependent epigenetic drift.

However, a part of methylation changes involved several neighboring CpGs. A more refined search of domains containing consecutive CpG units showing unidirectional methylation changes actually revealed that about 2.2% of total genomic CG sites were located in regions that were differentially methylated in the centenarian with respect to the newborn. In agreement with previous observations, most of these DMRs were located in intronic and intergenic regions and commonly colocalized with interspersed repetitive DNA elements. Interestingly, DMRs often corresponded to lamina associated domains (LADs), where cancer-specific methylation changes had been observed (Berman et al., 2011). The functional significance of LADs' hypomethylation in cancer and in aging remains to be defined. However, a far-reaching impact of this event on the epigenetic regulation of genome can be envisaged. In fact, the LADs define large heterochromatin compartments of the genome which embed key developmental and cell-type specific genes that are maintained in an epigenetically repressed state (Guelen et al., 2008; Harr et al., 2015; Peric-Hupkes et al., 2010; Reddy et al., 2008). Hence, an event of hypomethylation of these regions may cause or reflect a fault of the mechanisms that are central to the development and conservation of normal states of differentiation and tissue-specific patterns of gene expression.

Concerning regulatory or coding sequences, such as promoters and exons, only about 20% of the DMRs colocalized with these regions and more than 80% of them underwent hypomethylation in the elderly. This event, however, was largely dependent on promoter CpG content being much more common in CpG-poor/tissue-specific gene promoters than in CGI/housekeeping gene promoters. Significantly, some of these DMRs colocalized with promoters of genes involved in the aging process such as Sirtuins and IGF signaling pathway components (Heyn et al., 2012).

Recently, this finding has broadly been validated in a cohort of more than 700 individuals aged 25–92 years. In fact, also in this case, aging was predominantly found associated with methylation deficit. About 60% of age-associated DMRs actually showed age-related hypomethylation and colocalized with binding sites for chromatin regulatory proteins, such as CTCF and Polycomb proteins, or specific histone modifications typically associated with active chromatin (McClay et al., 2014). These findings indicate

that age-related hypomethylation may lead to global chromatin changes of potential functional relevance for transcriptional regulation.

### 5.2. DNA hypermethylation events

Besides extensive genome-wide hypomethylation, aging involves a progressive gain of DNA methylation that marks the loss of expression of specific genes. In fact, the majority of age-related hypermethylated sites corresponds to CGI-promoters, where methylation often correlates with suppression of transcription. This aspect is of particular interest as it confers to methylation changes, which accompany aging, the features of an epigenetic reprogramming.

Driven by the accidental observation that cancer-related hypermethylation of the CGI-promoter of estrogen receptor (*ER*) gene arises as a direct function of age in normal tissues (Issa et al., 1994), the majority of initial investigations into CGI methylation in aging focused on specific genes selected on the basis of their involvement in the pathogenesis of cancer or other age-related diseases. This strategy highlighted that aging violates, in a time-dependent manner, the unmethylated state of CGIs of genes involved in tumor suppression (*p16INK4A* (So et al., 2006), *CHD1* (Waki et al., 2003), *RASSF1* (Waki et al., 2003), *LOX* (So et al., 2006), *RUNX3* (So et al., 2006), *N33* (Ahuja et al., 1998) and *TIG1* (So et al., 2006)), genome stability and repair (*hTERT* (Silva et al., 2008), *MLH1* (Nakagawa et al., 2001), *MGMT* (Matsubayashi et al., 2005) and *OGG* (Madrigano et al., 2012)), metabolism (*e.g.*, *COX7A1* (Ronn et al., 2008) and *CRAT* (Madrigano et al., 2012)), differentiation and growth (*MYOD1* (Ahuja et al., 1998), *c-Fos* (Choi et al., 1996), *IGF-2* (Issa et al., 1996)), regulation of immune response (*INFG* (Madrigano et al., 2012)), coagulation (*F3* (Madrigano et al., 2012)) and connective tissue homeostasis (*Collagen I* (Takatsu et al., 1999)). Taken together, these studies also suggested that physiological aging could predispose one to age-related pathological phenotypes *via* a methylation-related gene silencing. It is interesting to observe that hypermethylation in age-related diseases affects essentially all CpG sites within a CGI, whereas the methylation pattern in physiologically-aged normal individuals is partial and heterogeneous. Therefore, partial methylation changes accumulating in the aging genome may predispose one to the development of age-related disease, in which the methylation changes are thereafter aggravated by a methylation spreading mechanism (Wong et al., 1999). Examples of such a precursor-product relationship of aging and age-related diseases have been proposed for prostate, bladder, colon cancers and Alzheimer's disease (Florl et al., 2004; Neuhausen et al., 2006; Shen et al., 2005; Siegmund et al., 2007).

Later genome-wide investigations further confirmed that age-associated hypermethylation happens preferentially at CGI promoters (Alisch et al., 2012; Bell et al., 2012; Bocklandt et al., 2011; Christensen et al., 2009; Florath et al., 2013; Garagnani et al., 2012; Gentilini et al., 2013; Hannum et al., 2013; Heyn et al., 2012; McClay et al., 2014; Rakyan et al., 2010; Xu and Taylor, 2014). Of note, this change does not equally impact CGI shore regions, which instead undergo similar proportions of hyper- and hypomethylation events (Florath et al., 2013; McClay et al., 2014).

Although the use of array-based techniques focusing mainly on gene promoters and CGIs indicated hypermethylation as prevalent event in aging (Alisch et al., 2012; Bell et al., 2012; Bocklandt et al., 2011; Boks et al., 2009; Christensen et al., 2009; Florath et al., 2013; Hannum et al., 2013; Rakyan et al., 2010; Xu and Taylor, 2014), age-associated hypermethylation seems a phenomenon of relatively low magnitude. Fewer than a hundred CpGs have actually been observed to methylate with age over about a total amount of 37,000 CGIs in the haploid human genome, confirming that CGI hypermethylation in aging is relatively uncommon. This clearly emerges from the studies that interrogated the genome as a whole (Heyn et al., 2012; McClay et al., 2014). For example, a study based on WGBS showed that only about 13% of age associated DMRs were hypermethylated in centenarians compared to newborns, thus suggesting that hypomethylation is predominant in aging (Heyn et al., 2012).

Despite this, aging-associated DMRs are more likely to be in CGIs undergoing methylation with age (McClay et al., 2014). Consistently, hypermethylation frequently occurs at genes with potential relevance for age-related phenotypes/diseases including genes involved in development (protocadherins, homeobox genes) and signaling (MAPK pathways' members, ryanodine receptors) associated with cancer, longevity, senescence and neurodegeneration (Bell et al., 2012; Hannum et al., 2013; McClay et al., 2014; Rakyan et al., 2010; Xu and Taylor, 2014). It is interesting to observe that, although most analyses have been performed on blood cells, hypermethylated aging-related DMRs seem to be largely shared by multiple tissues (Horvath et al., 2012; Rakyan et al., 2010; Teschendorff et al., 2010). This supports the view that DNA methylation changes do not occur randomly in the context of the human genome but are directed to regions sharing common features.

Notably, age-associated hypermethylated DMRs in differentiated tissues often overlap with promoters of genes that in stem cells have bivalent chromatin marks (H3K4me3 and H3K27me3) and are target of the polycomb repressive complex 2 (PRC2) (Hannum et al., 2013; Heyn et al., 2012; Rakyan et al., 2010; Teschendorff et al., 2010; Xu and Taylor, 2014). Many of these genes encode transcription factors necessary for differentiation and are already target of epigenetic deregulation in stem cells during aging, likely underlying the observed decline of stem cell function (Beerman et al., 2013; Bork et al., 2010; Brack and Rando, 2007). This would suggest that the age-associated methylation defects observed in differentiated tissues may reflect changes occurring in the aged stem cell population. Moreover, the same stem cell-like bivalent marks and PRC2 occupancy have been recognized to predispose tumor suppressor gene promoters to DNA hypermethylation in cancer. In fact, there is significant correspondence between those genes undergoing hypermethylation in aging and genes undergoing the same event in cancer (Teschendorff et al., 2010) and in cancer-associated conditions such as obesity, inflammation and smoking addiction (Issa, 2011; Issa et al., 2001; Selamat et al., 2012; Suzuki et al., 2009; Xu et al., 2013). These observations link stem cell aging to cancer risk and suggest that aging may elicit an epigenetic switch from less stable histone-based gene repression in stem cells to permanent DNA methylation-based gene repression in cancer cells (Xu and Taylor, 2014). Taking cancer as an example, this epigenetic mechanism could be a model explaining how the aging process could predispose one to age-specific phenotypes/diseases.

All in all, it is evident that some specific DNA regions directionally undergo DNA methylation changes across aged individuals probably as a consequence of shared chromatin features. However, these site-specific events co-exist with the epigenetic drift according to which deviation of inter-individual genomic methylation patterns occurs over time (Fig. 4). One possible explanation is that aging might primarily introduce a general disorder in the methylation patterns which could be then followed by the selection and clonal expansion of those cells bearing methylation defects in specific regions of the genome where these defects would be tolerated and give the cells advantages of survival or proliferation (Issa, 2014).

## 6. 5hmC and aging

The identification of the new DNA epigenetic mark 5hmC opens new perspectives for the study of epigenetic reprogramming in

aging. Our knowledge of DNA methylation patterns in both physiological and pathological conditions indeed needs a revaluation after the discovery of 5hmC. This is due to the fact that conventional bisulfite sequencing method, which has been generally considered the gold standard method for DNA methylation analysis, is not able to discriminate between 5mC and 5hmC. Therefore, studies based on conventional bisulfite modification actually mask the contribution of 5hmC (Huang et al., 2010).

Information regarding 5hmC in aging process is currently limited. Only few reports have addressed this issue focusing attention on 5hmC changes in aged mice. Mouse cerebellum and hippocampus show an increase of 5hmC levels with aging which can be prevented by caloric restriction, a well-known phenomenon associated with longevity (Chen et al., 2012b; Chouliaras et al., 2012; Szulwach et al., 2011). An increase in 5hmC signals was observed in genes activated in old mice with respect to young ones demonstrating that 5hmC is acquired in developmentally activated genes (Szulwach et al., 2011). Age-dependent increase of 5hmC in mouse hippocampus was also observed on the *5-LOX* gene, whose expression is known to increase during aging (Chen et al., 2012b).

The interest for 5hmC is now growing enormously considering its involvement not only in physiological states, such as development and aging, but also in pathological conditions including cancer, autoimmune and neurodegenerative disorders (Calabrese et al., 2014; Chen et al., 2012b; Cheng et al., 2014; Pfeifer et al., 2014; Putiri et al., 2014; Villar-Menendez et al., 2013).

## 7. Conclusions and perspectives

Over the last years, a growing body of research has led to the progressive and sharp description of the impact of aging on the methylome, especially as a result of the recent application of genome-wide analyses. From this work, it emerges that aging-related changes involve very different phenomena such as epigenetic drift together with directional methylation changes of specific genome regions, leading to the clear perception that the methylome is a dynamic landscape that reflects a variety of chronological complex changes. Relevant challenge for future research would be to determine if these changes can be modeled to trace underlying mechanisms. In this context, it would be extremely important to shed light on the relationships that link age-associated methylation changes with deficit of the methylation machinery (Casillas et al., 2003; Xiao et al., 2008) and environmental exposure. In this research framework, more attention should also be addressed to the contribution of DNA hydroxymethylation and TET enzymes. To understand the mechanistic basis of age-related methylation changes it would be relevant not only to clarify the molecular features of aging, but also to set the stage for the development of strategies to counteract its pathological phenotypes.

Another promising field of research sees DNA methylation as a marker of aging to be used to predict and monitor age-associated physiological decline and diseases. Consistently, pioneering studies revealed that methylation changes of certain genes can serve to detect different rates of human aging (Bocklandt et al., 2011; Garagnani et al., 2012; Hannum et al., 2013; Horvath, 2013). From this point of view, it will be interesting to test these biomarkers in relation to clinical and environmental variables that impact aging rate. This would allow for the application of DNA methylation-based markers to evaluate quality of life in the aging population.

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