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DNA methylation dynamics in aging: how far are we from understanding the mechanisms?

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ABSTRACT

DNA methylation is currently the most promising molecular marker for monitoring aging and predicting life expectancy. However, the mechanisms underlying age-related DNA methylation changes remain mostly undiscovered.

Here we discuss the current knowledge of the dynamic nature of DNA epigenome landscape in mammals, and propose putative molecular mechanisms for aging-associated DNA epigenetic changes. Specifically, we describe age-related variations of methylcytosine and its oxidative derivatives in relation to the dynamics of chromatin structure, histone post-translational modifications and their modulators.

Finally, we are proposing a conceptual framework that could explain the complex nature of the effects of age on DNA methylation patterns. This combines the accumulation of DNA methylation noise and also all of the predictable, site-specific DNA methylation changes.

Gathering information in this area would pave the way for future investigation aimed at establishing a possible causative role of epigenetic mechanisms in aging.

1. Epigenetic DNA modifications

1.1. Origin and function of 5-methylcytosine

DNA methylation is one of the heritable epigenetic marks of the genome linked to gene expression/regulation and developmental processes in various eukaryotes.

This DNA modification is achieved through the addition of a methyl group to cytosine, resulting in the formation of 5-methylcytosine (5mC) (Schubeler, 2015) (Fig. 1).

In mammals, methylated cytosines are mainly formed on CpG dinucleotides (CG) by the activity of the DNA methyltransferase enzymes DNMT1, 3A and 3B (DNMTs). CG sites are under-represented in mammals and tend to cluster in regions that are frequently located next to gene promoters and show atypically high CG frequency. These regions are known as CpG islands (CGIs) (Deaton and Bird, 2011; Schubeler, 2015). Of the approximately 28 million CG sites present in the human genome, 20–40% are generally unmethylated in contrast to the hypermethylated remainder of the genome (Deaton and Bird, 2011; Schubeler, 2015; Smith and Meissner, 2013).

This specific DNA methylation patterning in mammals relates to specific functions. Global genomic hypermethylation mainly concerns non-genic regions corresponding to heterochromatin. The 5mC role in such context is to preserve whole genome integrity at different levels as it avoids aberrant X-chromosome activation, transcription of endoparasitic repeated DNA and prevents aberrant centromeres recombination (Blasco, 2007; Jaco et al., 2008; Yoder et al., 1997).

Conversely, non-methylated CGIs allow the expression of almost 60% of genes in vertebrates, generally involved in housekeeping roles. It is rare for CGIs to undergo methylation. But when this occurs it ensures a stable transcriptional repression of genes that must remain silent, i. e. for tissue specific genes and oncogenes (Deaton and Bird, 2011). This precise partitioning does not concern regions right beside the CGIs and with lower CG dinucleotides content known as the CGI shores and shelves. These regions present variable 5mC levels and patterns that possibly govern tissue specific gene expression (Irizarry et al., 2009).

Beyond the CGIs, 5mC positioning on genes occurs preferentially on exons over introns (Jones, 2012; Lister et al., 2009), which is likely to modulate alternative splicing (Maunakea et al., 2010).

Furthermore, DNA methylation brings into play gene expression modulation even away from promoters as it influences the activity of distal regulatory elements such as enhancers, silencers and insulators (Weaver and Bartolomei, 2014).

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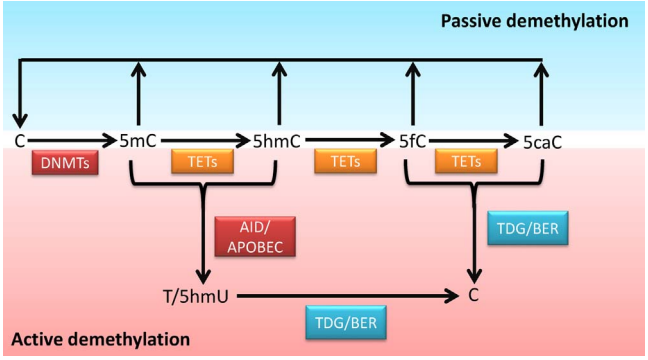

Fig. 1. Model of DNA methylation/demethylation pathways. DNA methyltransferases (DNMTs) form 5-methylcytosine (5mC) from cytosine (C). Iterative oxidations by TET dioxygenases produce 5- hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Passive DNA demethylation can occur by a reduction in activity, failed recruitment onto DNA or absence of DNMTs during cell divisions. 5hmC, 5fC and 5caC are no longer recognized by DNMT enzymes and can induce passive DNA demethylation as well. Active demethylation can be achieved through the removal of 5fC and 5caC by the Thymine DNA Glycosylase (TDG) followed by the base excision repair (BER) pathway. Alternatively, 5mC and 5hmC can be deaminated by AID/APOBEC enzymes to form thymine (T) and 5-hydroxymethyluracil (5hmU), respectively. Both T and 5hmU can be excised by the TDG/BER pathway.

The initial establishment of DNA methylation patterns takes place during development and differentiation and is likely determined by the activity of the so called "*de novo*" methyltransferases DNMT3A and DNMT3B (Clark, 2015). DNMT1 manages their maintenance over the course of DNA replication, thus ensuring DNA methylation pattern propagation. DNMT1 performs re-methylation of hemi-methylated CG sites generated during DNA replication after binding to the adaptor proteins Proliferating Cell Nuclear Antigen (PCNA) and Ubiquitin Like With PHD And Ring Finger Domains 1 (UHRF1) (Schubeler, 2015).

Despite the fact that DNMTs have well partitioned activities, there is some evidence of their functional redundancy. Indeed, methylation maintenance seems to involve DNMT3A and 3B, while "*de novo*" methylation might be carried out by DNMT1 (Sharma et al., 2011).

Therefore, DNA methylation patterns are the combinatorial result of both methylation establishment and maintenance.

However, the molecular events that govern regional and temporal evolution of DNA methylation patterns are not completely understood, despite the fact that some evidence indicates that DNA methylation processes may rely on pre-established chromatin modifications such as histone post-translational modifications (PTMs) (Rose and Klose, 2014).

In fact, studies conducted on lower eukaryotes reveal that pre-existing H3K9 histone methylation is crucial for the recruitment of DNMT3A and 3B on DNA, thus suggesting a mutual relationship between DNA methylation and histone PTMs (Lindroth et al., 2004; Tamaru et al., 2003). Similar evidence indicates that in several mammalian cell lines the SuUV39H1/2 histone methyltransferase is required for DNA methylation at pericentric heterochromatin, through the placement of H3K9 trimethylation (H3K9me3) (Fuks et al., 2003). In addition, a direct interaction between DNMT3A/3B and SUV39H1, SETDB1 and G9a/GLP H3K9 methyltransferases would appear to be crucial for carrying out *de novo* DNA methylation (Chang et al., 2011; Lehnertz et al., 2003; Li et al., 2006).

The synergic interaction between these two classes of enzymes also extends to DNMT1 for carrying out gene repression (Smallwood et al., 2007).

Similarly, H3K36 trimethylation (H3K36me3), catalysed by the histone methyltransferase SETD2, acts as a recruitment mark for DNMT3A/B (Dhayalan et al., 2010) and correlates with DNA methylation enrichment on gene bodies, crucially involved in the splicing machinery modulation (Brown et al., 2012).

Several observations suggest that histone methylation may play a role in contrasting the recruitment of DNA methylation enzymes to certain genomic regions. As such studies describe, H3K4 trimethylation (H3K4me3) seems to exclude DNMTs' action, thus preventing DNA methylation of the CGIs (Ooi et al., 2007).

Another chromatin epigenetic modification that inhibits DNA methylation occurs on lysine 27 of histone H3 (H3K27me) and is catalysed by the EZH1/2 components of the Polycomb repressive complex 2 (PRC2) (Statham et al., 2012). Curiously, H3K27me3 as well as other molecular events and histone PTMs have been found to provoke "*de novo*" methylation of CGIs in cancer (Schlesinger et al., 2007).

Such interplay between DNA methylation and chromatin configuration also concerns maintenance pathways since DNMT1 activity depends on several molecular factors in the resolution of CG dinucleotides hemi-methylation (Jeltsch, 2006). UHRF1 seems to carry out these tasks on behalf of DNMT1 as it recognises specific histone PTMs. The C-terminal Ring domain of UHRF1 works as an E3 ligase and performs histone H3K23 ubiquitylation, which favours DNMT1 recruitment on hemi-methylated CG. H3K23 ubiquitylation antagonises the auto-inhibitory effect of the DNMT1's RFTS domain (The Replication Focus Targeting Sequence) (Liu et al., 2013b; Nishiyama et al., 2013).

However, there is a mutual relationship existing between DNA methylation and chromatin configuration. In fact, 5mC is recognised by some methylated-CG DNA binding proteins, such as the members of the MBD (MeCP2 and Mbd1–4) and BTB/POZ (Kaiso and ZBTB4/38) families. Several methylated-CG DNA binding proteins seem to act as a recruiter for histone deacetylases and histone lysine methyltransferases. This leads to chemical and structural remodelling of chromatin by introducing epigenetic marks functionally linked with transcriptional repression and heterochromatin formation (Bogdanovic and Veenstra, 2009).

1.2. 5-Methylcytosine removal and oxidation

To reverse DNA methylation, both passive and active mechanisms take place in the cell (Fig. 1). More specifically, passive demethylation occurs when DNA replication is uncoupled from DNA methylation maintenance. The outcome of such non-catalytic events leads to 5mC removal over the course of cellular replication (Clark, 2015; Schubeler, 2015).

The catalytic 5mC replacement with cytosine is the active demethylation process and involves iterative 5mC oxidative steps. This chain of reactions involves a group of enzymes, the Ten-Eleven Translocation (TET) dioxygenases (TET1, TET2 and TET3). The first oxidative step is the 5mC conversion to 5-hydroxymethylcytosine (5 hmC). Following this first event, demethylation occurs when 5hmC is oxidized further by TET proteins, subsequently forming 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Unmethylated cytosine replaces these two compounds when the thymine DNA glycosylase (TDG) triggers the base excision repair (BER) process (Delatte et al., 2014). An additional mechanism might involve 5mC and 5hmC deamination by the AID/APOBEC (Activation-induced deaminase/apolipoprotein B editing complex) enzymes. Deamination forms thymine and 5-hydroxymethyluracil (5hmU), which are then removed from DNA by the BER/TDG activity (Delatte et al., 2014).

However, 5hmC is not merely a demethylation intermediate. A growing body of evidence highlights that 5hmC may act as a stable epigenetic signal. Thus, on a par with 5mC, 5hmC is recognised as an additional base of DNA (Shen and Zhang, 2013).

However, 5hmC levels very dramatically vary among mammalian cell types and tissues, with the highest recorded content in the central nervous system (CNS) (Globisch et al., 2010; Munzel et al., 2010). In the brain, 5hmC is enriched on transcriptional starting sites as well as gene bodies of actively transcribed genes. More specifically, on gene bodies 5hmC localizes at exon-intron boundaries. 5hmC locates also around flanking regions of transcriptional regulatory elements, such as

enhancers/silencers and transcription factor binding sites. Similar pattern occurs on CGIs, where 5hmC is positioned at their borders. In contrast, a reduced 5hmC presence is observed in repeated sequences such as SINE, LINE, LTR and satellite DNA (see (Shen and Zhang, 2013) and references therein).

The mechanisms behind 5hmC patterning haven't been characterized as yet. However, evidence suggests that 5hmC positioning is influenced by DNA sequence as well as chromatin epigenetic protein factors such as PRC2 (Neri et al., 2013; Williams et al., 2011b).

It is plausible to assume that this epigenetic signal influences chromatin structure and function. For example, 5hmC recognition and binding by MeCP2 in the brain is functionally associated with transcription (Mellen et al., 2012).

In regards to 5fC and 5caC, their extreme scarcity in mammalian genomes limits an understanding of their roles. However, several studies suggest that these demethylation intermediates might be stable on mammalian genome, thus paving the way for further studies to better understand their functions beyond demethylation (Bachman et al., 2015).

1.3. DNA epigenetic modifications as changeable components of the epigenome

Genomic DNA methylation landscape is highly dynamic, undergoing both physiological changes and disease-associated alterations.

Large scale reprogramming occurs during early stages of development. In fact, the genome of pre-implantation embryos undergoes massive demethylation in order to re-establish cell totipotency (Feng et al., 2010). Formation of totipotent gametes also requires a radical genome-wide DNA demethylation (Ciccarone et al., 2012; Feng et al., 2010). If we combine these observations, demethylated genomes are the starting point to reconstruct cell specific epigenetic patterns during further developmental and differentiation stages.

Outside of these physiological events, DNA methylation patterns have long been considered static except for pathological conditions, most notably in cancer.

One of the most crucial factors that modifies DNA methylation patterns is the environment (Feil and Fraga, 2012; Leenen et al., 2016), but the mechanisms behind this influence still do not point to a clear mechanism. A groundbreaking study on the association between methylome and environment was conducted by Fraga et al. on monozygotic twin pairs. Despite these individuals having the same genetic backgrounds, their methylation status gradually diverges with increasing age as a consequence of different environments and lifestyles (Fraga et al., 2005). A possible interaction between environment and age-related methylation divergence was confirmed by a longitudinal study (Martino et al., 2013).

A number of environmental cues have been associated with DNA methylation changes. These events include chemical (e.g. pollutants and food additives), physical (e.g. UV radiations and temperature), biological (e.g. viral infections, microbiota and genetic effects), psychological (e.g. mood disorders) and behavioural (lifestyle, overeating, high-fat diet, famine, caloric restriction, physical activity and circadian rhythms) factors (Feil and Fraga, 2012; Leenen et al., 2016).

Some of the above mentioned environmental factors are strongly associated with a higher risk of disease. For example, exposure to arsenic has been linked to an increased cancer risk, possibly due to DNA hypermethylation of oncosuppressor genes (Marsit et al., 2006). The same phenomenon has been associated with environmental pollution by asbestos and chronic exposure to sun or UV rays (Chanda et al., 2006; Gronniger et al., 2010).

Recently, it was also discovered that the genetic variation might influence the epigenome, as a number of single nucleotide polymorphisms have been associated with a deregulated DNA methylation maintenance at several smoking-associated CGs (van Dongen et al., 2016).

It is interesting to note that mammalian methylome dynamicity extends not only to postnatal life but also prenatally as dietary habits during pregnancy strongly determine the genome methylation status of offspring (Aagaard-Tillery et al., 2008).

An important natural case study in the field of prenatal epigenomics is the 'Dutch Hunger Winter' study. Individuals that experienced *in utero* exposure to famine had an increased incidence of several health problems such as obesity, hypertension, cardiovascular diseases and impaired glucose homeostasis. With regard to epigenetic variations, the genomes from the same group of people showed hypomethylation and hypermethylation of several *loci* associated with specific pathways (Heijmans et al., 2008; Tobi et al., 2009).

Similarly, individuals that experienced *in utero* depression of their mothers also had altered methylation levels at imprinted genes, likely predisposing them to a higher risk of chronic diseases during adulthood (Liu et al., 2012).

Along with the environmental influences exercised on prenatal epigenome, it would be interesting to prove trans-generational inheritance of epigenetic changes in mammals as already described in plants and nematodes (Heard and Martienssen, 2014). Despite the fact that germline methylome resetting in mammalian embryos might exclude this possibility, some sequences in mouse genome, such as intracisternal A particle (IAP) retrotransposons, are pretty much resistant to such DNA methylation reprogramming. This relevant discovery opens up the possibility that some postnatally acquired epigenetic changes might be passed from one generation to another (Lane et al., 2003; Morgan et al., 1999).

As the epigenome is dynamic by nature, it is reasonable to speculate that some environmental or behavioural factors might induce beneficial epigenetic outcomes via changes of DNA methylation patterns. For instance, long-term physical activity is associated with lower cancer risk and mortality via methylation changes at a number of genes involved in metabolism, muscle growth, haematopoiesis and inflammation (Aoi et al., 2010; Keller et al., 1985; Nakajima et al., 2010; Smith et al., 2008). Even the methylation status of the mitochondrially encoded gene NADH dehydrogenase 6 (MT-ND6) changes as a result of regular physical activity (Pirola et al., 2013).

Recently, it has been discovered that natural patterns of sleep modulate the methylome in the brain. In fact, a regular 24-hour rhythm of DNA methylation reprogramming positively affects gene expression in the human brain and its disruption is associated with Alzheimer's disease (Lim et al., 2014).

An advantageous modulation of DNA methylation patterns has also been suggested in the case of nutritional factors. This relationship is well known in many organisms, but it still remains a subject of study in humans (Bacalini et al., 2014). Indeed, a diet rich in vegetables and plant oil influences methylation on CG sites functionally related to genomic integrity maintenance in humans (Switzeny et al., 2012). These epigenetic changes are adaptive and they could prolong lifespan, promoting better health.

All these observations point towards the intriguing hypothesis that the epigenome might be the molecular interface between the environment and DNA, where their interactions are recorded as a type of epigenetic memory.

2. The DNA methylation landscape of the aging genome

2.1. The impact of aging on DNA epigenetic modification patterns

Aging is a complex physiological process characterised by a progressive loss of tissue functionality and an increased risk of death. Many intrinsic and extrinsic cues, both genetic and stochastic factors, are linked to progressive physiological, mechanical and cognitive age-associated decline. Moreover, age represents one of the main risk factors of a number of diseases, including cancer, atherosclerosis as well as Alzheimer's disease (Niccoli and Partridge, 2012).

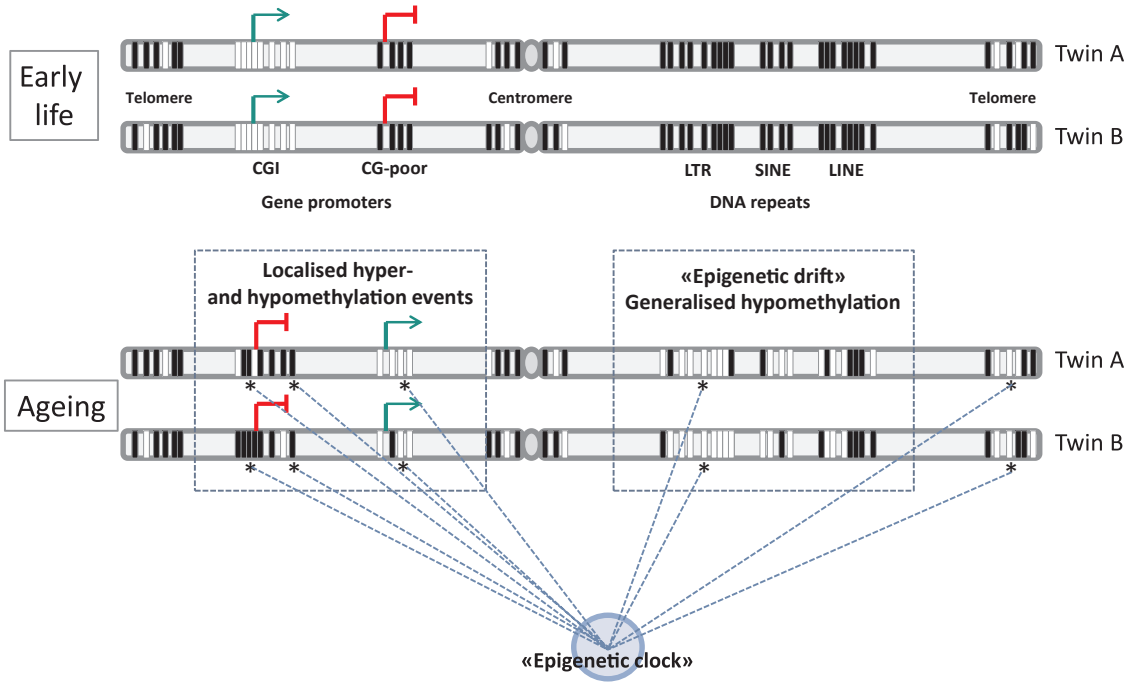

Fig. 2. Schematics of DNA methylation patterns in early life and ageing. A representative chromosome of monozygotic twins was shown to highlight epigenetic alterations in aging occurring independently of genetic background. Interspersed, telomere and centromere associated DNA repeats as well as CGIs and CG poor promoters, associated with altered gene expression in aging are reported. In early life (upper panel), DNA methylation distribution of the twins is comparable. In fact, the CGI promoters of housekeeping genes are protected from DNA methylation and are generally associated with active transcription. In contrast, repetitive elements (LTR, SINE and LINE), telomeric and centromeric heterochromatin regions and CG-poor promoters of repressed genes are typically methylated. In aging (lower panel), epigenetic drift alters the DNA methylation patterns leading to divergences despite identical genetic backgrounds. In particular, DNA repeats undergo a generalised hypomethylation, while *locus* specific epigenetic changes are observed in gene-associated regulatory regions. Moreover, a number of CG sites (*) exhibit reproducible DNA methylation changes across individuals culminating in the epigenetic clock. Arrows indicate transcription start sites. The colour green indicates active transcription, while the colour red indicates repressed transcription.

Many efforts in the last years have been spent in identifying age-associated deregulation of the epigenetic landscape, which is now recognised as a hallmark of the aging process. Changes of epigenetic patterns occur practically all through the entire lifetime in all species, tissues and cell types (Christiansen et al., 2016; Maegawa et al., 2010). In humans, DNA methylation changes start early in life, as demonstrated by a longitudinal study (five years duration) of infants' blood where increased levels of 5mC were shown to have already occurred during the first year of life (Herbstman et al., 2013; Martino et al., 2011). These early changes have also been documented in monozygotic twins. Notably, their epigenetic profiles continue to accumulate changes with the advancement of age causing diverging patterns, even more so in twins that did not share the same habits and/or environments (Fraga et al., 2005; Tan et al., 2016). This would indicate that aging-associated DNA methylation changes are caused in part by environmental factors.

Early studies investigating the relationship between DNA methylation and aging in mammals date back to the 1980's, showing that senescent normal fibroblasts and various tissues in aged mice presented low levels of 5mC (Wilson and Jones, 1983; Wilson et al., 1987). Since these pioneering studies, knowledge of age-associated epigenetics has grown enormously, thanks mainly to developments in the area of genome-wide technologies. A lot of new data now proves that DNA methylation patterns are altered during aging in a wide range of tissues and species (Ben-Avraham et al., 2012; Day et al., 2013). This emerging scenario indicates that during the aging process mammalian genome undergoes an erosion of DNA methylation patterns along with directional, *locus*-specific hypermethylation and hypomethylation (Day et al., 2013; Johansson et al., 2013; Jones et al., 2015; Zampieri et al., 2015).

Age-associated hypomethylation occurs in heterochromatic regions of the genome, such as repetitive elements and transposons, which contain the majority of methylated CG dinucleotides in human DNA (Bollati et al., 2009; Heyn et al., 2012). In addition to repetitive elements, loss of methylation with aging was also demonstrated in CG-poor regions located next to genes (Day et al., 2013; Heyn et al., 2012) together with "open sea regions". These are regions of megabase extension characterised by low CG content and are also demethylated in cancer (Yuan et al., 2015).

Conversely, focal hypermethylation in aging mainly occurs at gene-associated CGIs (Madrigano et al., 2012), which are normally unmethylated. A number of genes hypermethylated with age also undergo methylation events in cancer cells and in other age-related diseases (Post et al., 1999; So et al., 2006; Zampieri et al., 2015).

These age-dependent methylation changes at promoter regions may have an impact on transcriptional programmes. In fact, impaired immunocompetence in the older generation has been linked to repression of immune cell differentiation genes along with activation of autoimmunity genes dependent upon alterations of DNA methylation patterns (Marttila et al., 2015; Tserel et al., 2015; Wei et al., 2012; Zhang et al., 2002; Zhao et al., 2016). However, aging-related DNA methylation changes are not necessarily associated with transcriptional changes (Horvath, 2013; Peters et al., 2015; Yuan et al., 2015). This is probably because other layers of epigenetic regulation, such as histone PTMs, may subsist (Johansson et al., 2013; Rakyan et al., 2010; Teschendorff et al., 2010; Yuan et al., 2015).

Most of the conclusions drawn so far about DNA methylation in aging may be biased by the fact that the methods used for detecting 5mC are not capable of excluding 5hmC contribution. This is particularly true for analyses performed in the brain where the 5hmC levels are abundant and increase with aging both in mice (Chen et al., 2012; Szulwach et al., 2011) and humans (Wagner et al., 2015). However, several reports have demonstrated a global 5hmC decrease with age in non-neural tissues such as blood (Buscarlet et al., 2016; Valentini et al., 2016; Xiong et al., 2015).

The first genome-wide analysis focusing on 5hmC in aging has been

performed on human mesenchymal stem cells (hMSCs). It showed that aging is associated with equal amounts of both 5hmC gain and loss. In particular, hypo-hydroxymethylated sites were largely enriched at CG-poor regions while hyper-hydroxymethylated sites tended to occur at CGIs and gene bodies (Torano et al., 2016).

Currently, the epigenetic theory of aging is the result of two opposing phenomena. On one hand, baseline DNA methylation levels progressively diverge during aging. This process, known as "epigenetic drift", also occurs in individuals with identical genetic backgrounds such as monozygotic twins (Fraga et al., 2005). On the other hand, some *locus*-specific DNA methylation changes are highly reproducible across aged people, independently of gender and tissue type. This phenomenon could point to the existence of a programmed epigenetic reconfiguration during aging that has given rise to the "epigenetic clock" theory (Horvath, 2013) (Fig. 2).

Epigenetic drift leads to inter-individual epigenetic divergence as age increases and is likely determined by a life-long epimutation accumulation (Fraga et al., 2005; Jones et al., 2015) due to environmental factors and/or intrinsic stochastic factors (Fraga et al., 2005; Tan et al., 2016). Curiously, recent studies suggest that in later stages of life the whole process paradoxically culminates in an inter-individual convergence of epigenomes, a phenomenon termed as "epigenetic assimilation". In fact, reduced epigenetic variability was observed in very old twin pairs (Talens et al., 2012), even when they were discordant for the aging-associated disease of Alzheimer (Oh et al., 2016). Notably, even the inter-tissue epigenetic variability of cerebral cortex and cerebellum was affected (Oh et al., 2016), suggesting that the reduced epigenetic complexity of tissues may lead to cell de-differentiation and loss of cell identity in very old people.

Together with the accumulation of stochastic, age-dependent epigenetic variability due to the drift, part of the aging-associated DNA methylation changes are definite and probably programmed. In fact, a number of studies have broadly proved the existence of an age-associated epigenetic reprogramming consisting of DNA methylation changes at specific CG sites that are reproducible across individuals and tissues (Bysani et al., 2017; Garagnani et al., 2012; Steegenga et al., 2014). Of greater significance, in 2013, two predictive mathematical models of biological age, based on aging-associated site-specific DNA methylation changes, highlighted the existence of the "epigenetic clock"(Hannum et al., 2013; Horvath, 2013). These age prediction tools interrogate the DNA methylation state of a number of CG sites. Specifically, the Hannum's clock assesses 71 CGs and is mainly applicable to whole blood, while the multi-tissue Horvath's clock assesses 353 CGs, of which those hypermethylated with aging are highly compatible with a broad spectrum of tissues (Horvath, 2013). Additional mathematical models estimating DNA methylation age have been proposed to achieve the following aims: i) to restrict the number of CGs under investigation, thus setting up cost effective methodologies suitable for both clinical or forensic purposes (Durso et al., 2017; Weidner et al., 2014); ii) to expand the number of analyzable target tissues in order to use them in specific research fields as in the case of teeth for anthropological applications (Giuliani et al., 2016); iii) to extend epigenetic age predictors to other species (Stubbs et al., 2017).

DNA methylation predicts biological age more efficiently than chronological age. In fact, the rates of epigenetic age acceleration, representing the gap between DNA methylation age and the chronological one, has been associated with symptoms of aging, such as frailty and menopause (Breitling et al., 2016; Levine et al., 2016), as well as to several aging-associated pathologies including cancer and neurodegenerative diseases (Ambatipudi et al., 2017; Horvath, 2013; Levine et al., 2015). Moreover, the DNA methylation age can predict life expectancy because it was shown to predict all-cause mortality independently of common risk factors (Chen et al., 2016; Christiansen et al., 2016; Lin et al., 2016; Marioni et al., 2015).

2.2. DNA methylation changes in cellular and disease models of aging

Aging is accompanied by a debilitating loss of cellular integrity and function, which induces age-related degeneration of tissues and organs, manifesting in age-related pathologies, such as sarcopenia, osteoporosis and neurodegeneration. At the cellular level, senescence is a process that can actively contribute to aging phenotypes as the number of senescent cells in the body increases with age and it has been suggested that senescence underlies many aging-related diseases. Cellular senescence refers to a cell proliferation arrest which is essentially irreversible and occurs when cells experience various types of stresses including the replication-associated shortening of telomeres (replicative senescence), activation of oncogenes and a permanently maintained DNA damage response state (Campisi, 2013).

Cellular senescence shares epigenetic similarities with aging. Early studies showed that the replicative senescence process is associated with loss of DNA methylation similar to what is observed in aging (Nilsson et al., 2005; Wilson and Jones, 1983). In fact, senescence can be induced by treating cells with DNA methylation inhibitors (Vogt et al., 1998).

More recent analyses confirmed that aging and cell senescence, induced by long-term culture conditions, share similar methylation changes at specific CGIs and may lead to transcriptional deregulation (Bork et al., 2010; Koch et al., 2011; Wagner et al., 2009). In addition, epigenetic changes observed both in aging and in senescence can be reversed when cells are reprogrammed to form induced pluripotent stem cells (iPSCs) (Frobel et al., 2014; Horvath, 2013; Koch et al., 2013; Weidner et al., 2014).

Recent epigenetic clock analyses of different *in vitro* senescence models of primary endothelial cells, demonstrated that both replicative and oncogene-driven senescence are actually associated with an increased epigenetic age, suggesting that senescent cells alter their DNA methylation state in an age-dependent manner (Lowe et al., 2016). However, the relationship between age- and senescence-associated DNA methylation changes seems to be less extensive than was previously believed. In fact, in contrast to replicative and oncogene-induced senescence, DNA damage-dependent senescence was not accompanied by epigenetic aging. Moreover, progression of DNA methylation age in long-term cultures of cells, maintaining telomere integrity, occurs in the absence of any typical signs of senescence (Lowe et al., 2016). This indicates that cellular epigenetic aging is not associated with senescence, even though the epigenetic clock speed can be affected by some senescence-associated factors. Similar conclusions can be drawn from studies showing that senescent cells are characterised by specific epigenetic signatures (Franzen et al., 2017; Koch et al., 2013) able to predict the number of replication rounds of both fibroblasts and hMSCs independently from the age of donors (Koch et al., 2012; Schellenberg et al., 2014).

Premature aging-related phenotypes characterise a number of rare genetic disorders known as progeroid syndromes, which are mainly caused by defects in lamin proteins or in DNA repair factors. In some progeroid syndrome patients, accelerated aging has not been associated with any genetic aberration, thus suggesting a possible contribution by epimutations.

In this context, a relevant epigenetic study was performed in immortalised B-cells from Werner syndrome (WS) and Hutchinson–Gilford progeria syndrome (HGPS) patients who were harbouring, or not harbouring, mutations in the RecQ helicase like (WRN) and Lamin A (LMNA) genes. Genome-wide analysis of DNA methylation patterns revealed that mutated patients share some epigenetic similarities and differ from controls. On the contrary, non-mutated samples showed barely distinguishable DNA methylation patterns when compared to controls. This indicates that genetic mutations causing progeroid syndromes might have a causal role in the establishment of disease-specific methylomes. Indeed, WS/HGPS mutated samples and naturally aged individuals shared various differentially methylated CGs mostly in

inflammation-related genes putatively relevant in aging (Heyn et al., 2013). In addition, investigations on whole blood from WS patients revealed that methylation changes are recurrent in specific loci associated with sphingolipid metabolism and signalling pathways that might explain the scleroderma-like changes occurring in WS (Guastafierro et al., 2017). Moreover, recent evidence shows that WS holds an accelerated epigenetic age (Maierhofer et al., 2017).

Besides typical progeroid syndromes, further evidence comes from epigenetic studies of Down Syndrome (DS). DS is actually considered a premature aging disease with aging-related features that particularly affect CNS and the immune system. DS is the most common chromosomal disorder originating from the presence of all or part of an extra chromosome 21.

Regarding DNA methylation, DS-related changes are already detected in foetal and early postnatal developmental stages as well as in extra-embryonic tissues (El Hajj et al., 2016; Jin et al., 2013; Mendioroz et al., 2015). Changes extend to post-natal life as suggested by the identification of several DS-associated differentially methylated regions in adult somatic tissues (Bacalini et al., 2015; Jones et al., 2013; Kerkel et al., 2010; Mendioroz et al., 2015). DNA methylation variations were not limited to chromosome 21 and include both 5mC gains and losses, the latter highly represented in brain and placenta (Bacalini et al., 2015; Jin et al., 2013; Mendioroz et al., 2015). As observed in WS, some tissues from DS patients exhibit epigenetic age acceleration (Horvath et al., 2015).

Emerging evidence indicates that DNA epigenetic variations in DS patients also involve 5hmC.

In fact, 5hmC changes contribute to 5mC pattern alterations at several *loci* in the DS cerebellum. However, in other genomic regions changes relate only to 5hmC and, in most cases, a 5hmC loss was observed (Mendioroz et al., 2015). Moreover, reduced 5hmC global levels have been detected in DS peripheral blood cells (Ciccarone et al., 2017a), as well as in physiologically aged individuals (Buscarlet et al., 2016; Valentini et al., 2016; Xiong et al., 2015).

DS-associated 5hmC changes may affect transcriptional regulation. In fact, increased 5hmC of certain gene loci is positively associated with transcriptional upregulation in DS (Lu et al., 2016).

3. Putative mechanisms underlying changes of DNA epigenetic modifications in aging

In the wake of the growing awareness where epigenetic alterations form the basis for some aging related pathologies, one hypothesis may be that changes of DNA epigenetic modification signatures lie at the heart of the aging process.

However, this hypothesis remains tentative because of the current lack of detailed explanations as to how these changes arise and/or might relate to molecular events in the cell. Serious limitation is imposed by the complexity and diversity of mechanisms governing chromatin structure dynamics, which involve many other mechanisms over and above DNA epigenetic modifications. PTMs of histone tails, non-coding RNAs and histone positioning are included.

Despite the general lack of any exhaustive evidence, there is an emerging realisation that DNA epigenetic modifications are highly interrelated with other chromatin modifications such as histone PTMs and rely mechanistically on them (Rose and Klose, 2014).

That being said, to better understand DNA methylation changes that occur during aging, investigation of their relationship with chromatin context is therefore necessary. However, only a few recent studies have integrated DNA methylation data with information regarding other epigenetic modifications of chromatin (Avrahami et al., 2015; Sun et al., 2014; Zhang et al., 2015).

These findings indicate that aging-related DNA methylation signatures correlate with specific histone PTMs, thus suggesting that they are the result of molecular mechanisms operating within a broader language of epigenetic modifications of chromatin. This section outlines a possible molecular rationale for this hypothetical scenario.

3.1. Loss of 5mC

The loss of 5mC in aging is a complex, multifaceted phenomenon. It may be possible to distinguish two different events: a ‘global' hypomethylation involving non-genic repetitive elements of the genome and a ‘local' loss in gene regions.

It is reasonable to assume that global hypomethylation relates to the loss of constitutive heterochromatin integrity, a hallmark of aging invariably observed in various eukaryotes ranging from yeasts to humans (Tsurumi and Li, 2012). Notably, age-related loss of heterochromatin is also observed in organisms lacking DNA methylation (Tsurumi and Li, 2012). This suggests that where methylation is coupled with other epigenetic mechanisms to ensure heterochromatin condensation, the aging-related global 5mC loss is probably a consequence rather than a cause of heterochromatin loss.

In fact, the deposition of 5mC in vertebrates depends on H3K9me3, a prerequisite for the subsequent methylation of DNA in the constitutive heterochromatin. This has been attributed to the physical interaction of DNMT enzymes with components of the H3K9 methylation machinery, specifically the histone methyltransferase SUV39H1/2 (Chang et al., 2011; Fuks et al., 2003; Lehnertz et al., 2003; Li et al., 2006). In addition, H3K9me3 constitutes a platform for the HP1-mediated recruitment of the DNA methylation machinery to chromatin (Smallwood et al., 2007).

By means of apparently different mechanisms, H3K9me3 is also important for maintaining heterochromatin 5mC level. In fact, H3K9me3 at hemi-methylated CG dinucleotides is recognized by the UHRF1 protein, which recruits DNMT1 thereby favouring full-methylation of DNA immediately after the replication fork passage (Liu et al., 2013b; Nishiyama et al., 2013; Sharif et al., 2007).

Interplay between histones and DNA methylation machinery is possibly required for 5mC maintenance even outside the replication process. Nucleosomes containing CG-rich methylated DNA have the ability to bind DNMT3A and 3B. Apparently, this mechanism is required for proper propagation of 5mC in hyper-methylated domains of the genome. The aim might be to favour cytosine methylation in unwanted unmethylated CGs generated either by errors of the replication-associated maintenance methylation process or by spurious losses of 5mC (Jeong et al., 2009; Jones and Liang, 2009).

In view of this dependence of 5mC patterns on pre-existing histone PTMs for its establishment and maintenance, an aging-associated hypomethylation due to loss of heterochromatin is therefore a feasible hypothesis.

In fact, aging seems to undermine many of the elements involved in this cross talk. Specifically, the aging cells show nucleosome core histone depletion, which causes reduced occupancy and disordered positioning of residual nucleosomes along the chromatin fibres (Hu et al., 2014; O'Sullivan et al., 2010). Reduced levels of H3K9me3 are observed in tissues and cells of aged humans and rodents (Djeghloul et al., 2016; Scaffidi and Misteli, 2006; Sidler et al., 2013; Sidler et al., 2014; Zhang et al., 2015). The same event takes place in the cells of HGPS (Scaffidi and Misteli, 2006; Shumaker et al., 2006) or WS (Zhang et al., 2015) patients. It is likely that this reflects a down-regulation of SUV39H1/2 (Djeghloul et al., 2016; Sidler et al., 2014) that, in combination with an age-associated decrease of HP1 (Scaffidi and Misteli, 2006; Zhang et al., 2015) and of DNMTs (Casillas et al., 2003; Ciccarone et al., 2016; Ronn et al., 2015; Sun et al., 2014) expression levels, may possibly account for heterochromatin DNA demethylation.

Accordingly, factors responsible for DNA methylation maintenance may operate with reduced stringency due to suboptimal dosage over lifespan, which may result in an increase in inter-individual variability along with an overall decrease in DNA methylation (Fig. 3A).

The origin of this phenomenon still remains unknown. However, recent studies suggest the involvement of both transcriptional and post-

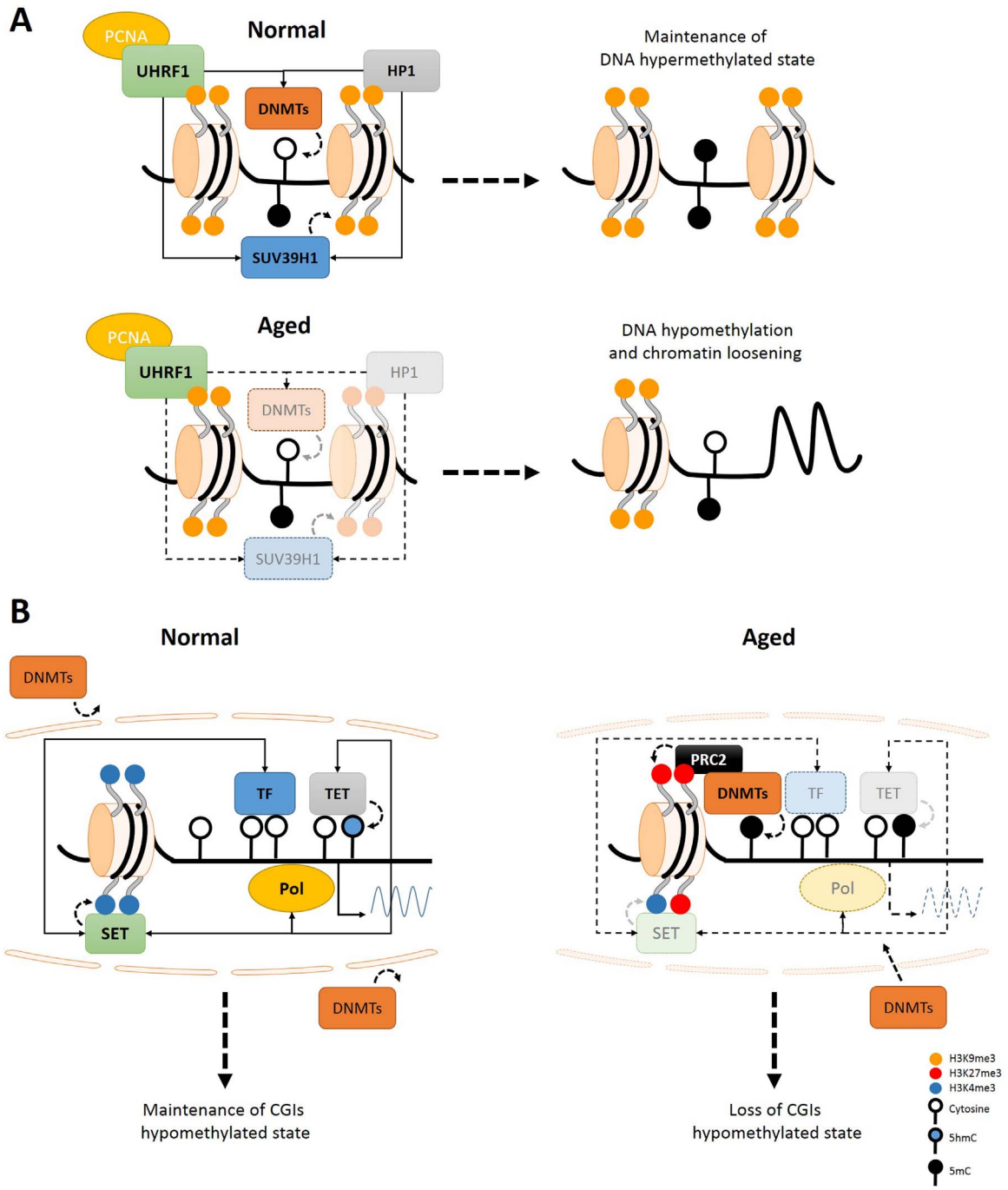

Fig. 3. Putative molecular mechanisms underlying age-related DNA methylation changes. A) DNA methylation maintenance processes (upper panel). Immediately after DNA synthesis, maintenance activity of methyltransferases methylates hemi-methylated CpG sites in a H3K9me3-dependent manner with the help of partner proteins including PCNA, UHRF1 and SUV39H1. Post-replication, the DNMTs complete the methylation process by correcting errors left by the replication-coupled process and/or due to stochastic loss of 5mC. The DNMTs are recruited to the chromatin regions containing methylated H3K9 via their interaction with HP1 and SUV39H1. During aging, both mechanisms are impaired (lower panel) due to defective SUV39H1, HP1, nucleosome core histones and DNMTs. This, in turn, would affect proper propagation of heterochromatin DNA methylation patterns. B) Maintenance of unmethylated state at CGI-promoters (right panel). CGIs associated with actively transcribed genes are protected from aberrant *de novo* methylation by multiple, interrelated mechanisms that collectively conflict with DNMTs' access and activity. These include action by active histone PTMs, such as H3K4me3, and CxxC protein factors. H3K4me3 is placed by histone methyltransferases (e.g. SET1A) with the help of specific CxxC transcription factors (TF). Other CxxC protein factors, such as TET enzymes, cause the removal of spurious, unwanted 5mC via DNA demethylation processes, initiated by 5mC to 5hmC conversion. The hypermethylation of CGIs with age (left panel) is shown as a process by which DNMTs gain access to DNA due to a weakening of one or more factors that contribute to CGIs protection. These include transcriptional silencing, H3K4me3 loss coupled with H3K27me3 deposition and defects of the active DNA demethylation processes governed by TET enzymes. The susceptibility of H3K27me3-marked chromatin to gain 5mC relies on the capability of components of the Polycomb Repressive Complex 2 (PRC2) to recruit DNMTs on chromatin. During aging, these processes would cause a gradual shift of some CGI chromatin configuration towards heterochromatinization with gain of DNA methylation.

transcriptional mechanisms.

A reduced expression is associated with the acquisition of the repressive mark H3K27me3 at the transcription start sites of histone genes in muscle stem cells from aged mice (Liu et al., 2013a). Similarly, a study in human adipose tissue from individuals with an age range of 20-80 years reported that age-related decrease of *DNMT* transcripts is associated with the hypermethylation of specific CG sites at the corresponding genes (Ronn et al., 2015). This evidence raises the possibility

that an altered chromatin state at specific gene *loci* may precede the global alteration of 5mC in aging.

A recent study has also shown that the expression of *DNMTs* is influenced by the growth hormone, suggesting that age-related growth hormone dysfunctions may contribute to *DNMTs* reduction in aging via the IGF-1/FOXO pathways, which play a profound role in aging (Armstrong et al., 2014). An additional regulatory mechanism to be explored in aging epigenetics is the role of p53, whose activation has been linked to heterochromatin relaxation and H3K9me3 loss via transcriptional silencing of SUV39H1 (Nicolai et al., 2015; Zheng et al., 2014).

In addition to transcriptional down-regulation, loss of heterochromatin key factors may also occur through post-translational mechanisms.

In human hematopoietic stem cells, the age-related global loss of H3K9me3 is associated with increased expression of miR-125b, which acts as a negative regulator of the SUV39H1 transcript level (Djeghloul et al., 2016). However, at the moment, the mechanism driving up-regulation of miR-125 with age in human hematopoietic stem cells remains undiscovered.

In addition, heterochromatin loss may be a derivative of defects of the nuclear envelope, which provides a special microenvironment for the establishment, propagation and maintenance of heterochromatin. This could involve a dysfunction of proteins which safeguard the integrity of the nuclear lamina and/or its function as a peripheral heterochromatin organiser. For example, Lamin A splice variants, that mimic the mutation of HGPS, increase with age and are associated with H3K9me3 loss (Scaffidi and Misteli, 2006). Similarly, aging-associated WRN protein down-regulation leads to destabilisation of heterochromatin by altering the interplay of SUV39H1 and HP1 with the LAP2β protein, which mediates the interaction of the nuclear lamina with heterochromatin (Zhang et al., 2015).

Aging-dependent remodelling of heterochromatin and nuclear envelope constituents also seems to occur through a more generalised process of piecemeal degradation of the nucleus, mediated by the autophagosome. It has been suggested that this mechanism participates in the senescence-induced heterochromatin loss in human fibroblasts (Dou et al., 2015; Ivanov et al., 2013). However, it is unclear whether it also contributes to heterochromatin and 5mC loss in physiologically aged human tissue. This is an important area for future investigation because it could provide a molecular explanation for the observation that hypomethylation associated with aging and photo-aging of the human skin involves large blocks of the genome corresponding to the lamina-associated domains (Vandiver et al., 2015).

An additional promising research area links aging-related chromatin defects, including heterochromatin loss, to the relocation of epigenetic machinery in response to DNA damage. In fact, DNA repair requires a significant amount of chromatin remodelling at damaged sites, which is achieved by re-positioning several chromatin-remodelling factors away from their normal housekeeping activities and positions. Consequently, chronic damage and/or a defective response to it could lead to epigenomic changes by titrating away epigenetic enzymes normally needed for accurate epigenome homeostasis.

This is of particular interest from an aging perspective, as elevated levels of persistent DNA damage are major aging culprits (Burgess et al., 2012) and the dominant part of any naturally occurring DNA damage seems to occur in DNA repeats (Wang et al., 2011).

A mechanism for such global heterochromatin misregulation in aging may involve the NAD^+-dependent histone deacetylase SIRT1 and the nucleosome remodelling and deacetylase (NuRD) complex. Both these factors are involved in heterochromatin homeostasis where they prevent de-repression and aberrant recombination of repeated sequences. In mouse embryonic stem cells, SIRT1 is redistributed to DNA damage sites leading to de-repression of several undamaged *loci* including repetitive DNA and deregulating transcription, in a similar way to organismal aging (Oberdoerffer et al., 2008).

This process would seem to involve a repressive multiprotein complex containing SIRT1, Polycomb factors and the DNMTs, which was shown to cause persistent epigenetic changes involving DNA methylation in response to oxidative stress (O'Hagan et al., 2011).

Similar conclusions can be drawn on the NURD complex, which is also relocated by DNA damage, favours DNA methylation and whose deficit has been linked to heterochromatin loss in both HGPS cells and normal aging (Pegoraro et al., 2009).

This mechanism appears to provide a likely explanation for the relationship between DNA damage and aging. However, recent findings suggest more complex molecular pathways behind this association. A further piece of the puzzle is the involvement of the poly(ADP-ribosyl)ation (PARylation) by PARP enzymes, responsible for NAD^+ molecule conversion to poly-ADP-ribose (PAR) upon binding to damaged DNA. PAR is conjugated to histones and other proteins in the vicinity of the DNA lesion and is believed to initiate DNA repair (Ray Chaudhuri and Nussenzweig, 2017).

PARP1 activation increases with age, probably because of an age-related accumulation of DNA damage (Fang et al., 2014; Mouchiroud et al., 2013). This condition may have harmful consequences and contributes to the aging process. In fact, over-activation of PARP enzymes results in NAD^+ depletion, which in turn affects SIRT1 activity.

In keeping with this picture, pharmacological inhibition of PARP enzymes extends lifespan in nematodes (Mouchiroud et al., 2013). In addition, a compromised balance between PARP1 and SIRT1 signalling activities contributes to premature aging syndromes (Fang et al., 2014; Scheibye-Knudsen et al., 2014).

However, whether the PARP-mediated inhibition of SIRT1 translates into epigenomic changes remains to be seen as yet. In this context, it would be useful to consider that deregulation of PARP activity may cause epigenetic changes through alternative pathways. In fact, PARylation regulates the expression, the catalytic activity and recruitment to chromatin of the DNA modifying enzymes DNMT1 e TET1 (Ciccarone et al., 2014; Ciccarone et al., 2015; Fujiki et al., 2013; Zampieri et al., 2012; Zampieri et al., 2009). Consistent with this notion, over-activation of PARP enzymes may cause global hypomethylation of the genome (Ciccarone et al., 2012; Guastafierro et al., 2008). Furthermore, PARP enzymes are thought to be multifunctional modulators of chromatin structure and some of their functions may be very relevant in aging. For example, PARylation plays a crucial role in the establishment and maintenance of heterochromatin (Ciccarone et al., 2017b; Dantzer and Santoro, 2013).

Putting this knowledge together, it is reasonable to assume that the 5mC global loss is a pernicious consequence of a non-programmed inefficiency of the heterochromatin epigenetic maintenance systems. However, it cannot be ruled out that it may also represent the result of a regulated process, which could therefore accomplish specific outcomes. For instance, even though the aging-related loss of DNA repeats methylation can be defined as 'global', it actually is not a generalised phenomenon. In fact, Alu repeats seem to be preferentially targeted compared to SINE repeats (Bollati et al., 2009; Jintaridth and Mutirangura, 2010). Moreover, 5mC changes in Alu repeats mainly occur at specific ages (Jintaridth and Mutirangura, 2010). Whether this proves that DNA repeats hypomethylation in aging is the outcome of a regulatory programme still remains to be seen. This seems to be the case in simple eukaryotes, where activation of transposon elements plays a major role in the response to stressful environmental conditions (Rey et al., 2016). Nevertheless, even in vertebrates, the hypermethylated state of DNA repeats is not an inviolable state either. In fact, in the physiological setting of mammalian development and differentiation, Alu repeats demethylation has been shown to be dynamically modulated to regulate transcriptional networks (Garcia-Perez et al., 2016).

Compared to global 5mC loss, the aging-associated site-specific hypomethylation is even more obscure and probably underestimated. Indeed, many genome-wide methodologies for DNA methylation analysis focus on specific tissues, if not on a whole organ. However, most of

the site-specific changes involve differentially methylated regions specific to cell types and therefore they may be largely overlooked by techniques that do not have a population-level cellular resolution. In fact, site-specific hypomethylation in aging mainly concerns regulatory regions enriched in H3K4me1, which typically characterises enhancers that govern tissue-specific genes expression (Fernandez et al., 2015; Rada-Iglesias et al., 2011). This suggests that aging-associated hypomethylation of these regions could result in deregulation of differentiation-related gene expression programmes. Accordingly, a recent genome-wide analysis of highly purified mice β cells has shown that genomic regions undergoing age-associated loss of DNA methylation are distal to promoters and they probably function as enhancers by binding β cell-specific transcription factors. Significantly, demethylation of these regions is anticipated by the acquisition of activating histone marks including H3K4me1 (Avrahami et al., 2015). Parallel transcriptomic analyses showed that this epigenetic remodelling is associated with transcriptional upregulation of genes essential for β-cell identity and function. As a result of that, older mice experience a dramatic improvement in β cell function (Avrahami et al., 2015). This observation is very significant as it suggests that aging can promote adaptive responses via epigenetic mechanisms.

The fact that site-specific 5mC loss and local H3K4me1 enrichment regularly correspond indicates that site-specific hypomethylation probably has an origin distinct from the global hypomethylation. This may relate to the impairment that all forms of H3K4 methylation cause to the recruitment of DNMTs on chromatin (Rose and Klose, 2014; Schubeler, 2015). Alternatively, site-specific hypomethylation may follow the age-related loss of histone H4 arginine 3 (H4R3) symmetric dimethylation (H4R3me2s), which specifically recruits the DNA methyltransferase DNMT3A to chromatin in order to suppress gene expression (Hong et al., 2012; Zhao et al., 2009). Significantly, an H4R3me2s to H3K4 methylation switch at specific genes is required to promote differentiation (Dhar et al., 2012). Another possibility is the intervention of site-specific active demethylation mechanisms. In fact, it has been proposed that activation of enhancer regions involved in establishing cell identity and differentiation requires TET-mediated demethylation (Baumann, 2014). Indeed, some of the binding sites that are recurrent in aging-associated site-specific hypomethylated regions are specific to transcription factors capable of causing demethylation via TET1 recruitment (Ancey et al., 2017; Avrahami et al., 2015).

3.2. Gain of 5mC

The aging-associated hypermethylation is a significantly less frequent phenomenon in the genome but much more specific for gene sequences than the hypomethylation (Johansson et al., 2013; Li et al., 2017; Marttila et al., 2015).

CGIs are mainly affected by an increased DNA methylation status, while it does not immediately influence adjacent regions such as the CGI-shores and shelves (Li et al., 2017). In fact, the CGIs involved share distinctive features: i) they are *cis*-associated to genes with specific functions (Sun et al., 2014; Zampieri et al., 2015), ii) they are targeted by repressive chromatin modifications (i.e., H3K27me3) and their related epigenetic factors (i.e. SUZ12, EZH2) (Beerman et al., 2013; Dozmorov, 2015; Rakyan et al., 2010; Sun et al., 2014); iii) they share specific transcription factor sequence motifs (Sun et al., 2014; Yuan et al., 2015). Surprisingly, for some CGIs the aging-associated hypermethylation is a phenomenon that appears to be largely shared across tissues and species and the degree of their hypermethylation correlates with the species-specific lifespan (Maegawa et al., 2017). This suggests that aging interferes with mechanisms that preserve the non-methylated state of a specific subset of the CGIs. However, drawing a complete mechanistic picture of these associations is difficult as what prevents the CGIs from accumulating methyl groups is still unknown.

The first point that needs clarification is the origin of *de novo* methylation in somatic cells. In fact, this process has long been considered to be confined to early development. However, recent evidence suggests the existence of a continuous *de novo* activity also outside early developmental stages. Allegedly, this is required for maintaining global hypermethylation of the genome as an auxiliary mechanism to maintenance methylation processes (Jeltsch and Jurkowska, 2014; Jones and Liang, 2009). However, *de novo* methylation activity must be counteracted in the CGIs to preserve their unmethylated state. Such hypothesis is supported by new findings showing that *de novo* methylation of some CGIs is the natural outcome when two distinct conditions occur simultaneously: the interruption of a methylation-blocking machinery and the repression of transcriptional activity (Takahashi et al., 2017).

Protection machinery may rely on proteins harbouring the CXXC (Cys-X-X-Cys, where X is any amino acid) domain, since it has been proposed that they safeguard the CG clusters within the CGIs from *de novo* methylation. One of these proteins is TET1, which localises at CGIs via its CXXC domain to counteract stochastic DNA methylation events by promoting active demethylation processes (Williams et al., 2011a). In addition, it has been proposed that the CXXC finger protein 1 (CFP1) prevents the recruitment of *de novo* DNA methyltransferase to CGIs by promoting the SET1 complex-mediated accumulation of the DNA methylation-blocking histone modification H3K4me3 (Clouaire et al., 2014; Ooi et al., 2007; Thomson et al., 2010). Of particular importance is the recent discovery that the F-Box And Leucine-Rich Repeat Protein 10 (FBXL10), which binds nearly all CG-dense promoters via a CXXC domain, protects Polycomb-occupied CGI promoters from DNA hypermethylation (Boulard et al., 2015).

Transcriptional activity also plays a part via the binding of transcription factors (e.g. SP1 (Brandeis et al., 1994)), transcription cofactors (E.g. PARP1 (Ciccarone et al., 2017b; Krishnakumar et al., 2008)), Pol2 (Takeshima et al., 2009) and transcripts themselves (Ginno et al., 2012).

Although there is a lack of direct evidence, the tendency of aging-related hypermethylation to occur in CGI promoters, targeted by an H3K27me3-mediated silencing, indicates that aging may interfere with both mechanisms.

The whole process seemingly give rise to an “epigenetic switch” that changes the chromatin structure and activity of certain CGI-promoters from a less stable histone-based transcriptional repression to permanent DNA methylation silencing, resembling differentiation-linked regulatory mechanisms that control pluripotency and germ line-specific genes (Mohn et al., 2008). In aging, this process may involve the well-known capability of the Polycomb repressor complex to recruit the *de novo* DNMTs to CGIs (Vire et al., 2006) coupled with the documented aging-associated down-regulation of components of the CGI methylation-blocking machinery, such as TET1 and TDG (Ronn et al., 2015; Valentini et al., 2016), FBXL10 (Tzatsos et al., 2011) and SP1 (Kim et al., 2012) (Fig. 3B).

It is very likely that H3K4me3 also plays a part in this process. Indeed, it has been suggested that hypermethylation of some CGIs in cancer can be anticipated by an epigenetic silencing, mediated not only by a gain of H3K27me3 but also by a loss of H3K4me3. Notably, at every level of H3K27me3 enrichment, CGIs with higher amounts of H3K4me3 are less methylated than those with lower amounts, indicating that the presence of H3K4me3 has a marked positive effect on the degree of local resistance to *de novo* methylation. However, since the hypermethylated pattern in cancer is initially formed by a slow *de novo* methylation process that occurs gradually in normal tissues with increasing age, both H3K27me3 enrichment and H3K4me3 loss may represent an epigenetic remodelling that anticipates *de novo* methylation in aging (Nejman et al., 2014). This suggests the need to enhance our knowledge of the importance of H3K4me3 and its regulators in mammalian aging, which is also indicated by evidence that H3K4me3 modifiers affect longevity in *C. elegans* and *D. melanogaster* (Han and Brunet, 2012). Actually, some findings in human neurons suggest that the H3K4me3 genomic landscape is altered as a function of age towards

a predominant loss of H3K4me3 peaks (Cheung et al., 2010).

Site-specific hypermethylation events might also be the consequence of aging-dependent regulatory pathways that use CG methylation as the final step of a transcriptional silencing process. For example, cumulative lifetime pro-inflammatory stimuli and stress-related activation of the glucocorticoid signalling pathway may cause hypermethylation of specific *loci* as a function of age by activating the STAT3 transcription factor, which is capable of recruiting the DNMTs on chromatin (Chazaud and Mouchiroud, 2014; Lee et al., 2012). Significantly, some CG sites that are part of the epigenetic clock are located in the glucocorticoids response elements (Zannas et al., 2015).

However, the general age-dependent dynamics of *de novo* methylation are largely incompatible with a programmed transition of CGIs chromatin towards a condensed configuration. In fact, accumulation of methylation on CGIs resembles a gradual process that apparently proceeds by a series of stochastic CG methylation events causing a progressive increase in variance and in average DNA methylation of certain CGIs with increasing age (Nejman et al., 2014; Slieker et al., 2016). These dynamics of CGIs methylation in aging better fits with an age-related functional decline of specific epigenetic maintenance systems that would expose some unmethylated CGIs to an asymmetric, stochastic flux of de novo methylation leading to gradual hypermethylation.

Nevertheless, the affected regions are well defined and reproducible across tissues and individuals. This may relate to the existence of a universal hierarchy of methylation sites in the genome, with every CGI having its own methylation susceptibility due a specific chromatin configuration and environment.

Interestingly, this mechanism might open up relevant new research paths and scenarios for unravelling the link between oxidative stress/DNA damage with epigenome deregulation in aging. In fact, oxidative stress has been associated with abnormal methylation due to the aberrant recruitment of DNMT-containing complexes to low/basal expression CGI-promoters marked by Polycomb-associated H3K27me3. Downstream restoration of the unmethylated state requires active demethylation involving TET enzymes and the BER mechanisms (Zhang et al., 2017). In aging, this editing process may be less efficient due to a defective TET enzymes expression and BER activity (Ronn et al., 2015; Valentini et al., 2016; Xu et al., 2008). This could result in hypermethylation of H3K27me3-marked CG-rich DNA regions as a result of oxidative stress and may explain the finding that aging-related CGI hypermethylation, although it is largely driven by tissue-independent factors, is accelerated by environmental hazards that induce oxidative stress (Slieker et al., 2016).

The correspondence between different tissues of the genomic sites which undergo a gradual and age-dependant hypermethylation, may be explained by a phenomenon of positive selection concerning adult stem cells and/or progenitor cells. These are multipotent cells involved in replacing terminally differentiated cells lost by normal tissue attrition. These cells present H3K27me3-repressed chromatin at common developmental genes, which tend to resolve upon further differentiation to grant transcriptional activation and which are required for proper lineage progression. This epigenetic reprogramming concerns genes involved in development, differentiation, repression of pluripotency and self-renewal (Voigt et al., 2013). In this cellular context, aging-related abnormal methylation of H3K27me3-marked DNA may impair differentiation, thus resulting in the formation of an abnormal stem cell/progenitor cell and/or mal-differentiated cells that may hold some advantageous traits such as cell death resistance and senescence. In an aged selective tissue microenvironment, these traits would lead to the expansion of this cell population over time. Such a mechanism has been proposed as an explanation of how aging may lead to an increased risk of developing cancer (Nejman et al., 2014).

3.3. From mechanism to patterns: how to integrate molecular models with the observed methylome dynamics in aging

While it is possible to correlate both aging-associated methylation losses and gains with multiple, complex chromatin-based mechanisms, it may also be possible to integrate them into a dynamic scenario that takes into account the opposing processes of the epigenetic drift and the epigenetic clock. However, this creates a further level of complexity that can't easily be clarified. One possibility is that both the epigenetic drift and clock share some parts of the same mechanisms.

Indeed, both gain and loss of methylation have a predominant stochastic component that could relate to a certain lack of efficiency of the epigenetic maintenance mechanisms. This lack of efficiency could be intrinsic to the system, as in many other biological processes. Alternatively, it could be caused by aging itself, via deregulation of key factors involved in the control of epigenetic homeostasis of the genome. In any case, the result would be an accumulation of DNA methylation errors over time, which would be consistent with the epigenetic drift observed in aging.

Conversely, site-specific changes might be the major contributors to the epigenetic clock. This component of the aging methylome could easily be derived from programmed or semi-programmed regulatory processes that ultimately provoke cellular responses to aging-related stimuli through epigenetic mechanisms. However, the scenario could be much more complex. On the one hand, it cannot be ruled out that some site-specific changes may also be involved in the epigenetic drift since the setting of these processes is the single cell rather than entire organs and tissues. On the other hand, site-specific changes may also be caused by stochastic processes due to a differential susceptibility of specific chromatin regions to either be resistant or be prone to change. Some changes may be neutral, with no apparent functional consequences, and therefore be retained passively throughout the life of the cell. For such events, the reproducibility across cells and tissues could emanate from the presence in the genome of common sites where the accumulation of methylation errors can be tolerated within certain limits. Conversely, other changes may have functional consequences and may undergo selective mechanisms because they influence cell survival/proliferation. As a result, these changes may become over-represented in the aged epigenome. In fact, it is highly likely that the site-specific reproducible changes observed with aging are happening in adult stem cells/progenitor cells and are carried into differentiated somatic cells (Beerman et al., 2013; Issa, 2014; Maegawa et al., 2017). Conversely, changes that are restricted to differentiated cells would likely be lost after cell death due to normal turnover of tissues. They would indeed contribute to an increase in epigenetic noise with age, and also to the highly reproducible site–specific methylation changes, but to a lesser extent. Moreover, given that epigenetic programmes are largely modulated during cellular replication (Gonzalez and Li, 2012), it is likely that methylation drift also reflects random epigenetic errors accumulated during stem cell divisions.

More exploration of the "stemness" origin hypothesis of aging-associated methylation changes will be required to further elucidate this scenario and clarify how the epigenetic drift and the epigenetic clock can coexist in the methylomic profile of aged cells and tissues.

4. Conclusions

Epigenetic control of genome functions may have a central role in aging. However, notwithstanding the evidence that the epigenetic clock is an excellent lifespan biomarker with potential predictive powers for mortality, we cannot, as yet, be certain whether epigenetic changes are "drivers" or whether they are "passengers" in the aging process. Actually, little is known about which specific cellular dysfunction and phenotype depend on epigenetic events in aging. Indeed, there is evidence that in some tissue contexts, aging is not accompanied by any evident DNA methylation drift (Hadad et al., 2016). This raises

reasonable doubt about the universality of this association. The identification of molecular mechanisms causally linked to the methylome changes is an approach that could address the problem because it would identify molecular processes to be targeted in order to contrast these changes and to evaluate their impact on the progression of aging. In this context, it is important to continue with the study of the relationship between DNA methylation and other epigenetic modifications of chromatin in aging. The causal-mechanistic view we outline here could be helpful towards achieving a hierarchical understanding of chromatin processes underlying methylomic changes in aging. The final goal would be to trace aging-associated epigenetic changes back to central regulatory mechanisms, which take control of functions necessary for the proper orchestration of epigenetic events. This would facilitate the development of experimental approaches, such as the use of specific inhibitors of epigenetic enzymes, which can be tested for anti-aging properties.

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