Charting the dynamic methylome across the human lifespan

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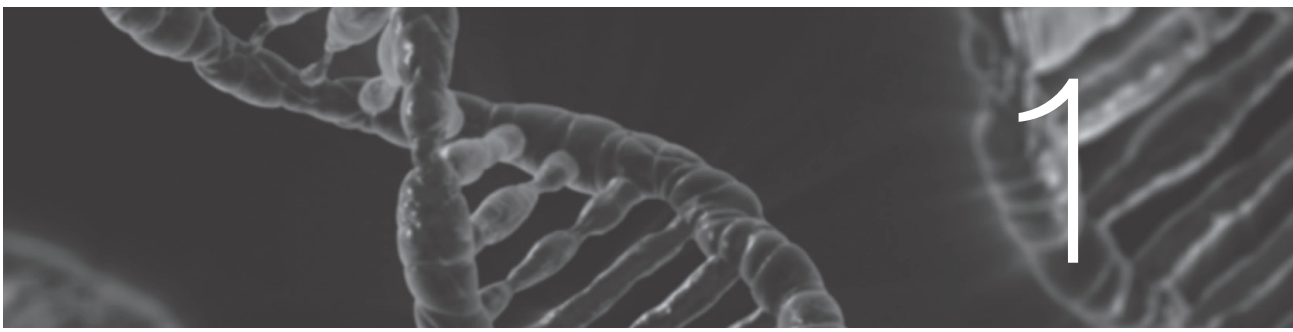
Introduction



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In the past years, insight into the processes that underlie ageing– the nine 'hallmarks of ageing' – has greatly increased, particularly in model organisms (**Figure 1**) (López-Otín et al., 2013). At the cellular level, the ageing process is characterized by accrual of DNA damage, shorter telomeres and epigenetic alterations, loss of proteostatis and mitochondrial dysfunction (López-Otín et al., 2013; Rossi et al., 2007). At the tissue level, stem cell exhaustion, cellular senescence and altered intercellular communication are seen (Campisi, 2013; Chandler and Peters, 2013; López-Otín et al., 2013). As a result of the impaired self-renewal of tissues and increased number of senescent cells, tissues show an age-related loss of function (Campisi, 2013). Lastly, at the organism level nutrient sensing pathways are deregulated, such as the insulin and IGF-1 signaling pathway (IIS) and the mTOR pathway (Gems and Partridge, 2001; Johnson et al., 2013; Zoncu et al., 2011). Yet, how these 'hallmarks of ageing' apply to humans is less clear.

Epigenetic alterations are increasingly being studied, as there is a particular interest to what extent epigenetic regulation as laid down in development is lost in age. For example, several epigenetic marks have been shown to closely track the chronological age (Garagnani et al., 2012). What intrinsic or extrinsic factors drive these age-related epigenetic changes is not

Figure 1. The nine hallmarks of ageing. Modified with permission from Elsevier, originally from (López-Otín et al., 2013).

clear. It is, however, known that the extrinsic environment has influence on the epigenome, as associations have been found with for example smoking (Zeilinger et al., 2013), prenatal undernutrition (Heijmans et al., 2008; Tobi et al., 2014) and overnutrition (Huang et al., 2015). So, the epigenome has two sides: on the one hand it regulates the normal functioning of different cell types in the body, while on the other hand the epigenome loses control during ageing and under influence of extrinsic factors. In this thesis we investigated these two sides of the epigenome, the normal epigenome that marks tissue differences and the changes to the epigenome that occur during the ageing process.

The genetic music is played different in each tissue

An organism is an extraordinary symphony of cells which occur in many forms with a wide variety of functions. While white blood cells defense the organism against infections, cone cells in the eye detect light and adipose cells store fat. Yet, each cell in the body has the same genetic code – its DNA – that remains the same during life. To get different cell types, the cells have instructions when, where and to what extent to use the DNA. Like sheet music, the genes can be played softly, loud or not at all, without changing the bases (or notes in case of music (Williams et al., 2014)). Differences in instructions are made possible by a layer on the DNA, epigenetic mechanisms, which is a collection of regulatory mechanisms that define and maintain the function of a cell. Epigenetic regulation occurs at the DNA level (DNA methylation and hydroxymethylation), at the chromatin level (histone modifications) and at the transcription level (non-coding RNAs).

Histone modifications

To keep or set the DNA to an active or repressed state, the DNA is packed around large proteins, the nucleosomes (**Figure 2**). Each nucleosome consists of eight histones of four types (H2A, H2B, H3, and H4) and a linker protein H1. The histone proteins have tails that can have different modifications at different positions. The tails on the histones influence the accessibility of the DNA and thereby the transcription of genes. Depending on the position on the tail and the type of modification, the DNA becomes more (euchromatin) or less accessible (heterochromatin) (Barski et al., 2007). For example, mono- and trimethylation of histone 3 at lysine 4 (H3K4me1 and H3K4me3 respectively) are associated with open chromatin and high gene expression, while trimethylation at histone 3 lysine 9 (H3K9me3) and trimethylation at histone 3 lysine 27 (H3K27me3) are associated with a heterochromatin and low expression (**Table 1**) (Barski et al., 2007; Wang et al., 2014). Histones can, in addition to methylation, also

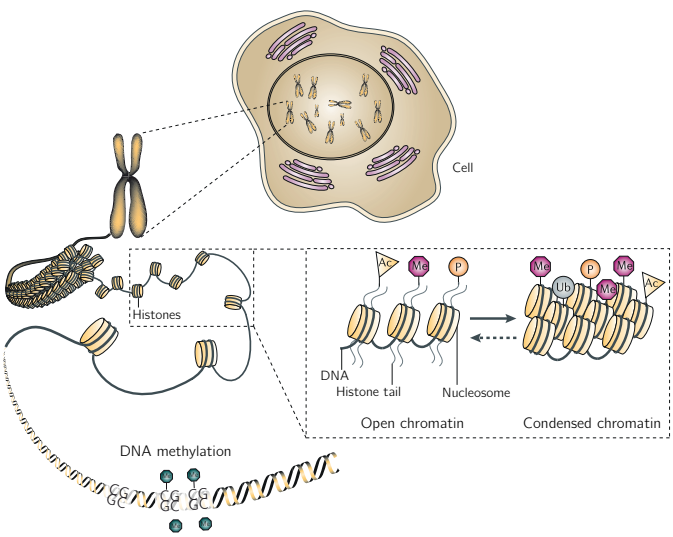

Figure 2. Epigenetic modifications. Modified with permission from Nature Publishing Group, originally from (Sparmann and van Lohuizen, 2006).

Type of modification	*H3K4*	*H3K9*	*H3K27*	*H3K36*	*H3K79*
Mono-methylation	Activation	Activation	Activation		Activation
Di-methylation	Activation	Repression	Repression		Repression
Tri-methylation	Activation	Repression	Repression	Activation	Repression
Acetylation	Activation	Activation	Activation		

Table 1. Effect of different histone modifications

be acetylated, which is found on histone 3 at lysine 4, 9, 14 and 27 and mark an active genome (**Table 1**) (Guillemette et al., 2011).

Histones do often not contain one histone modification, but rather combinations of active and/or repressive modifications. Previously it has been shown that combination of histone modifications mark the function of the genomic region (Day et al., 2007) and the Roadmap Epigenomics Consortium has refined this method and applied it to many primary tissues (Roadmap Epigenomics Consortium et al., 2015) (**Figure 3**). For example active transcription start sites (TSSs) are marked by the histone modification H3K4me3, while regions flanking active TSSs are marked by H3K4me1 and H3K4me4. Polycomb repressed regions are marked by H3K27me3 only, while bivalent regions also have an active mark in addition to the repressive mark H3K27me3 (**Figure 3**).

DNA methylation

In this thesis we primarily investigated DNA methylation (**Figure 2**). DNA methylation is the covalent bond of a methyl group at the dinucleotide CG, referred to as CpG, where the 'p' denotes the phosphate backbone of the DNA strand. The human genome contains 28 million CpG sites and the majority (60-80%) are methylated (Smith and Meissner, 2013). In addition to methylation of CpGs, other modifications have been identified, including 5-carboxylcytosine (5caC), 5-formylcytosine (5fC), 3-methylcytosine and 5-hydroxycytosine (5hmC). The latter has lately received more attention, as gene bodies in the brain are rich of hydroxymethylation that is associated with the regulation of transcription (Branco et al., 2012; Jin et al., 2011; Lunnon et al., 2016; Wen et al., 2014).

Early studies on DNA methylation have focused on CpG dense regions, the CpG islands (CGIs), that contain approximately 10% of the CpGs (Smith and Meissner, 2013). About half of the CpG islands overlap with TSSs and the other half – 'orphan CGIs' – are found throughout the genome (Illingworth et al., 2010). Genes harboring a CGI-promoter are often associated with housekeeping genes and developmental genes (Deaton et al., 2011). Promoter CGIs are often hypomethylated, suggestive of active transcription, but the activity of these genes is repressed via other mechanisms, such as H3K27me3 and polycomb binding (PcG) (Aloia et al., 2013).

In addition to CpG-dense regions, the relevance of DNA methylation in a non-CGI context has been increasingly recognized. Low density of CpGs is found in promoters, but also intergenic and intragenic. Intragenic DNA methylation, also in a CpG island context, has been suggested to play a role in alternative splicing (Kornblihtt, 2006) and alternative promoter

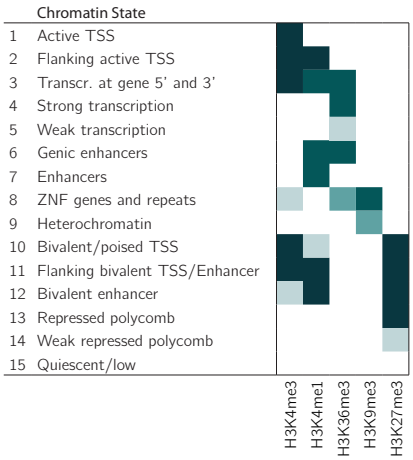

Figure 3. Combinations of histone modifications mark different genomic functions (Roadmap Epigenomics Consortium et al., 2015).

usage (Maunakea et al., 2010). More distal from genes other non-CGI regulatory elements are found, enhancers (marked by H3K4me1 and H3K27ac), that can influence expression over a long distance (Shlyueva et al., 2014). DNA methylation at enhancers may mediate or be a reflection of regulation of the distal target gene. Differential DNA methylation at enhancers has been particularly found to be associated with tissue-specification and normal tissue functioning (Andersson et al., 2014; Hon et al., 2013; Wiench et al., 2011).

The link between DNA methylation and transcription remains complex. A mechanism via which DNA methylation may have effect on transcription is the competition of DNA methylation with transcription factor binding sites (Brandeis et al., 1994). Although CGI-promoters do not contain core promoter elements, transcription is regulated via the transcription factor SP1 (Deaton et al., 2011; Smith and Meissner, 2013). Moreover, CTCF and DNA methylation are negatively correlated, that is lower transcription factor binding is associated with higher DNA methylation. The latter is either the cause or the consequence of the decreased binding of CTCF to its binding site (Wang et al., 2012). However, not all CTCF binding sites are methylation-sensitive and those that are methylation-sensitive are variable across cell types (Maurano et al., 2015). Another methylation-sensitive transcription factor is NRF1, that in murine cells can only bind to unmethylated DNA (Domcke et al., 2015). To bind methylated DNA, NRF1 requires the binding of methyl-insensitive transcription factors to adjacent sequences to demethylate the DNA, allowing the NRF1 to bind (Domcke et al., 2015). Other methylation-sensitive transcription factors may also dependent on this cooperation of transcription factors, where one transcription factor makes the DNA accessible ('pioneer TF') allowing other transcription factors to bind (Slattery et al., 2014).

Altogether, DNA methylation is highly context specific, also in the sense how it is associated with the alteration of gene expression. Integration with other genomic information, such as histone modification and transcription factor binding data, gives better insight into the context of DNA methylation and enables better interpretation of DNA methylation.

Methylation profiling techniques

Several techniques are available to measure DNA methylation each with different number of CpGs interrogated, including whole genome bisulfite sequencing, RRBS, MeDIP and the Illumina array-based techniques, the 27k, 450k and 850k (EPIC) arrays. In this thesis we used the Illumina 450k array in all research chapters, because it covers relevant regions and it is an affordable reproducible high-throughput method. The 450k array interrogates over 450,000 CpGs in the genome (Bibikova et al., 2011). Although the number of CpGs interrogated is limited, it measures CpGs in relevant regions, such as CpG islands, shores, promoters, enhancers etc. (**Figure 4**).

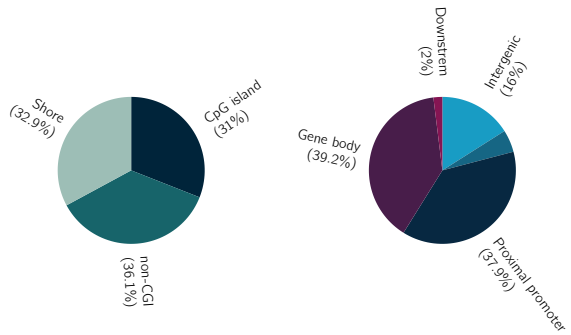

Figure 4. Percentage of CpGs covered on the 450k array in each annotation category.

Integrative genomics

To better understand the complexity of DNA methylation, we integrated DNA methylation data with other layers of regulation, such as gene expression data, histone modification data and transcription factor binding sites. This was enhanced by the publically availability of reference epigenomes, including the Encyclopedia of DNA elements (ENCODE), the Epigenomics Roadmap and BLUEPRINT. These projects contain multilevel omics data of the same tissue type or cell line, including gene expression data, DNA methylation, histone modifications, DNaseI hypersensitivity sites, transcription factor binding sites. In ENCODE data of (cancer) cell lines is available, in the Epigenomics Roadmap primary tissues (fetal, stem cells and adult) and in BLUEPRINT blood cell types. After all, DNA methylation cannot be studied on itself, it is dependent on the genomic sequence the CpG is in (transcription factor binding sites, distance to genes etc.). Only by integrating different layers of data, one can understand what functional consequences differential methylation has in a cell population.

The methylome during development

To understand loss of control over the methylome, one needs to understand both how the normal methylome in tissues is formed and how it is maintained during life. The formation tissue-specific methylation patterns occurs during the fetal development and is preceded by the embryonic development. After conception, the fusion of the oocyte and the sperm cell, the paternal and maternal methylomes are cleared from DNA methylation, with the

paternal methylome actively - and the maternal passively demethylated (Guo et al., 2014; Smith et al., 2014) (**Figure 5**). After implantation the levels of DNA methylation again rise to high levels (Guo et al., 2014; Smith et al., 2014; Smith et al., 2012). In the embryonic period, from the 3rd to the 8th week, the primordial organs are formed, that further develop into fully functional organs during the fetal period. Much of the knowledge of dynamics of the DNA methylation landscape during embryonic- and fetal development has been derived from *in vitro* differentiation experiments (Gifford et al., 2013; Laurent et al., 2010; Nazor et al., 2012; Xie et al., 2013). However, knowledge of the embryonic- and fetal period *in vivo* is largely lacking but is of interest for the changes to the methylome seen in late life.

In addition to the formation of the methylome during development, characterization of the regions that differ between tissues adds value to understanding the regions that change during life. Several studies have investigated DNA methylome in multiple adult tissues by identifying tissue-specific differentially methylated positions (tDMPs) in adult tissues (**Figure 5**). These studies either investigated a subset of the regions, such as CGIs (Illingworth et al., 2008) or were limited by the DNA methylation profiling technology used (Byun et al., 2009; Nagae et al., 2011; Rakyan et al., 2008). A genome-scale view would enhance the understanding of the tissue-specific differences in DNA methylation and which genomic features particularly harbor tissue-specific signatures.

The methylome during ageing

The other side of the epigenome, in addition to normal maintenance in tissues, is the loss of epigenetic control that occur with increasing age, which may contribute to the ageing process. DNA methylation at several loci closely tracks chronological age. A vast number of studies identified age-related differentially methylated positions (aDMPs, **Figure 5**) in blood (Bell et al., 2012; Florath et al., 2014; Garagnani et al., 2012; Johansson et al., 2013; Marttila et al., 2015; McClay et al., 2014; Rakyan et al., 2010; Reynolds et al., 2014; Steegenga et al., 2014). DNA methylation of *ELOVL2* has been found to be strongly associated with age and replicated in many studies (Florath et al., 2014; Garagnani et al., 2012; Steegenga et al., 2014).

The strong reproducibility of aDMPs in various tissues enables the accurate prediction of chronological age in blood, also irrespective of the tissue studied. Several studies have shown that chronological age in blood can be remarkably accurately predicted with a

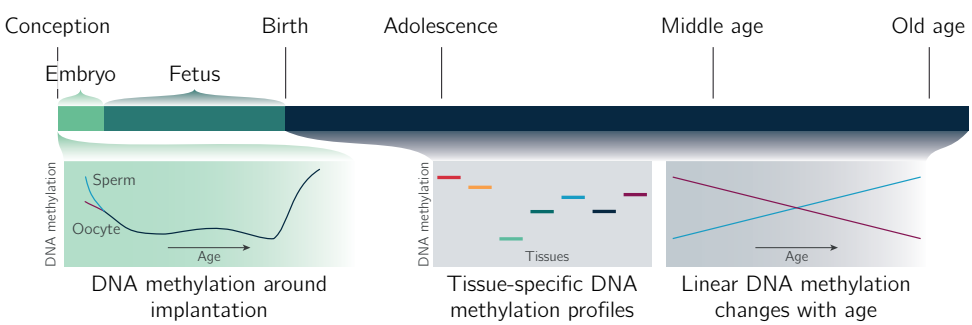

Figure 5. Current state of knowledge of DNA methylation during the human lifespan.

small subset of all CpGs even down to a few CpG sites (Bekaert et al., 2015; Hannum et al., 2013; Horvath, 2013; Weidner et al., 2014) and Horvath's age predictor is even independent of tissue (Horvath, 2013). In comparison, while telomere length also tracks chronological age, the variance among individuals is much higher compared to DNA methylation (Rode et al., 2015). The prediction of chronological age based on DNA methylation provides an interesting tool for forensics (Bekaert et al., 2015; Zbieć-Piekarska et al., 2015). Similarly, chronological age predictors have been developed using transcriptome data (Peters et al., 2015). Although clocks accurately predict chronological age, the ultimate goal would be to develop a clock that can predict the 'biological age' that marks the health of an individual. The DNA methylation and the transcriptomic age have been associated with biological age to some extent (Horvath et al., 2014; Marioni et al., 2015b; Peters et al., 2015) and the predicted age based on DNA methylation has been associated with all-cause mortality (Christiansen et al., 2015; Marioni et al., 2015a). This indicates that DNA methylation may be a reflection of an individual's health, but the mechanisms driving them remain unclear.

Horvath's age predictor suggests that some CpGs change with age irrespective of tissue or that each CpG site within the predictor is specific for a tissue. Subsets of identified aDMPs have been found to be tissue independent (Day et al., 2013; Fernández et al., 2014; Hernandez et al., 2011), including the *ELOLV2* CpGs (Bekaert et al., 2015). Yet, these studies that investigated tissue-specific and tissue-independent age-related changes were often small studies or limited by the technology. Characterizing aDMPs in multiple tissues may give a better understanding of what and what not these age-related changes have in common between tissues.

Although aDMPs are an interesting tool to predict age, the trajectory of DNA methylation of aDMPs is similar for all individuals, that is, all individuals gain or lose DNA methylation at these loci in a linear fashion. Yet, individuals in an ageing population do neither age with the same speed, nor do they develop the same diseases if at all. Instead of linear changes in DNA methylation with age, one would expect a diverge in DNA methylation from chronological age. Age-related divergence may reflect the observed age-related increase in inter-individual variability. Investigating such age-related changes may give a better understanding of other age-related changes that exist and early studies indicate that age-related increase in variability of DNA methylation exists (Fernández et al., 2014; Talens et al., 2012).

Aims and outline of this thesis

In this thesis we aimed to study the two sides of the methylome: the formation and maintenance of the methylome and on the loss of epigenetic control occurring over the life course.

In the first two chapters we studied the formation and DNA methylation differences between tissues. In **Chapter 2**, we chartered the differences in DNA methylation that exist between peripheral and internal tissues in adults. Hereto, we used two datasets, consisting of isogenic pairs of internal - and peripheral tissues. Given that DNA methylation is context specific, we developed tools to interpret DNA methylation data: annotations for genomic features, alternative splicing and promoter usage and open chromatin and a tool to call regions from

single CpG sites. Next we investigated in what genomic features changes between tissues occur, to what extent they cluster to regions and to what groups of genes they are locate to.

In **Chapter 3** we investigated what regions are dynamic during fetal development. Many of the age-related changes may have a developmental origin. We generated DNA methylation profiles of four human fetal tissues of multiple isogenic replicates during first and second trimester of human gestation (**Chapter 3**). To study overall differences between tissues and time points, we compared the tissues and time points at the genome-scale level. Next, we sought to gain insight in an early tissue-specific signature present as early as week 9 by selecting for tissue-specific DNA methylation independent of time. Finally, we studied dynamic DNA methylation between week 9 and week 22, to gain insight into functional elements that drive or mediate fetal development and tissue-specification. Functionality of identified dynamic DNA methylation was further investigated using DNA gene expression data and external data of histone modifications.

In the last two chapters we investigated the age-related changes that occur to the methylome across the human lifespan. DNA methylation within a tissue is well maintained during life to preserve the integrity of the cell, but is lost under several circumstances at specific loci. In **Chapter 4**, we studied DNA methylation in blood with age. More specifically, we investigated what loci in the genome show an age-related increase in variability of DNA methylation, which may be a reflection of a diverging ageing population. CpGs with increased variability in DNA methylation were characterized using various annotations, such as chromatin state segmentations and data on transcription factor binding sites. Finally, to investigate functionality of identified loci, increased variability in DNA methylation was compared to gene expression in cis and in trans. Genes associated to DNA methylation in cis and in trans were further characterized by investigating enrichment for specific gene sets. In **Chapter 5**, we studied age-related changes that closely track chronological age in multiple tissues to investigate the tissue-independency of aDMPs (**Chapter 5**). Hereto, we identified age-related DNA methylation changes in 7 tissues using publically available datasets. aDMPs in tissues were compared to determine whether aDMPs are predominantly tissue-dependent or independent. Moreover, aDMPs were compared to multiple annotations to get a better insight in the type of genomic features that gain or lose DNA methylation with age.

Together, these studies give insight in the two sides of the methylome and how they connect: formation, maintenance and loss of control of the methylome during development and ageing.

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