Exploration of the role of chromatin modification in human ageing

The dream of immortality

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Summary

Ageing affects all humanity and has caused immeasurable suffering through the ages, becoming an important part of culture and mythology. Men and women throughout history dreamt of ways to slow-down or even reverse the process, and modern science has finally begun to understand the process enough to attempt to make this dream a reality. In this thesis the role of chromatin modification in ageing has been explored and potential ways to curtail or reverse ageing are described.

The ageing process can be characterized by 9 major hallmarks, including dysfunction of the regulation of gene expression due to the decline of epigenetic mechanisms. These epigenetic mechanisms regulate gene expression without altering genetic sequences directly, instead modulating the ability of transcription mechanisms to interact with- and read DNA. In ageing the epigenetic patterns established during differentiation may become aberrant through failures of epigenetic maintenance mechanisms and decline of stable cellular metabolic activity. These aberrations will accumulate and stimulate further erosion of transcription regulation mechanisms like proper chromatin structure modulation, leading to improper transcription of random groups of proteins and eventual apoptosis. Such a state of cellular senescence[1] can be transmitted to neighbouring cells as well, leading to increasingly dysfunctional tissues and organs with subsequent age-related diseases emerging.

Experimentation has shown that in murine *in-vivo* models and *in-vitro* human cell lines the epigenetic patterns that degrade during ageing may be reset to rejuvenate cells. Cellular proliferation is not affected though, which ties in to the decline of stem-cell proliferation due to ageing, another hallmark of ageing. This interconnectedness of all aspects of ageing underlines the need for more research into all 9 hallmarks of ageing to fully understand in which ways ageing affects the cells, tissues, organs and the organism. It also suggests that hybrid therapies may be needed to slowdown or even reverse ageing.

Table of Contents

1. The nine hallmarks of ageing 2
    1.1. Primary hallmarks of ageing 2
    1.2. Antagonistic hallmarks of ageing 3
    1.3. Integrative hallmarks of ageing 3
2. The role of epigenetics in ageing 4
3. The role of nucleosome modification in ageing 5
    3.1. DNA modification: 6
        3.1.1. DNA methylation 6
        3.1.2. DNA methyltransferases 8
        3.1.3. DNA demethylation 11
        3.1.4. The effects of ageing on DNA methylation mechanics 11
    3.2. Histone modification 13
        3.2.1. Histone acetylation/deacetylation 14
        3.2.2. Histone methylation/demethylation 14
        3.2.3. Histone phosphorylation/dephosphorylation 17
        3.2.4. Histone ubiquitination/deubiquitination 17
        3.2.5. Collective influence of histone modification patterns on chromatin structure 18
4. Degeneration of epigenetic modification patterns during ageing 22
5. Rejuvenation of cells and tissues to slow and reverse ageing 24

Conclusion 25

References 27

Glossary: 35

Introduction

Ageing is the process most organisms undergo in which their physiology develops to maturity and subsequent senescence where the physiological and cellular mechanisms degrade leading to various symptoms and eventually to death. Humans are no exception as globally, age-related complications and diseases have claimed 4.3 million human lives in the European Union in 2016, accounting for 83.1% of deaths of seniors (over 65 years of age) (European-Union, 2020). Nevertheless, death due to old age does not come easily. After reaching maturity humans slowly start to "erode", gaining small chronic pains and symptoms over decades that make life more difficult. Physical and mental faculties fade leaving people unable to live their lives as they desire and as they once did. At a certain point it forces the individuals to seek aid from family or enter care-homes; both are generally not considered dignified fates. Such a loss of faculty and of subsequent self-reliance as well as social isolation, and an increasingly looming fear of their own death and that of their spouse, often leads to depression. It should come as no surprise then that in 2016 the suicide rates for the elderly (between 50-56 years and over 85 years of age) was between 15- and 22 per 100.000 in the European Union respectively, which is in stark contrast to the 4 suicides per 100.000 of teens (15-19 years of age) (European-Union, 2019).

The ageing individual is not only affected by his or her own declining health but also often wrestle with seeing loved ones, their father, mother, sister, brother or friend they care about, waste away. The most striking examples are elderly dementia patients who eventually even lose the ability to recognize the faces and names of their children. This painful process of decline has been an inevitable fate that all humans face, but that has not stopped scientists from trying to achieve the long dreamt-of goal of biological immortality, or at least of extension of the human life- and health span. The latter is of note because it is not age itself that leads to an organism's expiration but rather various age-related diseases like dementia, heart disease and diabetes.

One searing question remains then: Is it possible to mitigate or even reverse human ageing?

The processes involved in ageing are complicated however, so due to time- and space constraints this thesis will first describe the general characteristics of ageing, followed by a focus on the role of epigenetics in ageing and a more in-depth analysis of the roles of DNA methylation and chromatin modification in the ageing process. To conclude, different proposed therapies to treat ageing will be described.

1. The nine hallmarks of ageing

The characteristics of ageing have been organized by (Blasco et al., 2013) into 9 major hallmarks that define senescent cells and tissues. These hallmarks, in turn, have also been divided into 3 functional groups (Blasco et al., 2013; see Figure 1). The primary hallmarks actively cause damage, the antagonistic hallmarks designate the loss of cellular damage response while the integrative hallmarks are the causes and results of damage on an intracellular scale. The primary hallmarks of ageing lead to- and amplify the dysfunction described in the antagonistic hallmarks, which in turn lead to the integrative hallmarks of ageing. In this way, as cellular damage accumulates during an organism's lifetime the symptoms of ageing will worsen.

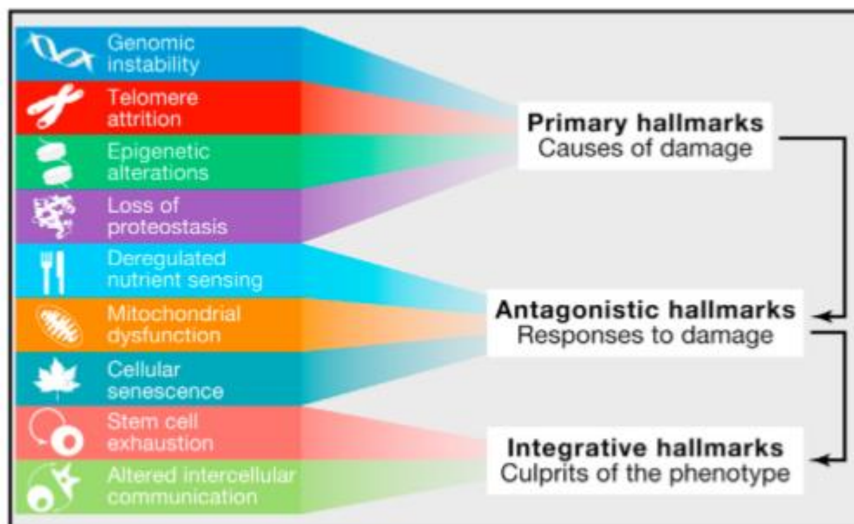

Figure 1: The 9 hallmarks of ageing: Altered intracellular communication, genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence and stem cell exhaustion. Taken from (Blasco et al., 2013).

1.1. Primary hallmarks of ageing

The first of the primary hallmarks is genomic instability caused by nuclear and mitochondrial DNA damage accumulated throughout the lifespan of an organism, as well as defects in nuclear lamina[2] that lead to a loss of chromosomal organization and subsequent alterations in gene expression (Moskalev et al., 2013; Shah et al., 2013; Bohr, 2015). Such genetic damage is accompanied by telomere erosion, the second primary hallmark of ageing. This is the more famous hallmark of ageing, where oxidative damage and the "end replicative problem" may cause the gradual shortening of telomeres. The end replicative problem describes the phenomenon of telomere shortening where during the replication of double-stranded DNA a short region of the lagging strand cannot bind a primer and can therefore not be replicated. Telomerase can extend this end and resolve this problem, but this enzyme's activity is downregulated in most human somatic cells (Saretzki, 2018). While telomeres of average length prevent DNA repair mechanisms from recognizing the ends of chromosomes as double-stranded breaks, shortened telomeres may not offer such protection, leading to genomic structural alterations and instabilities (Saretzki, 2018). Not only the genetic makeup itself is damaged as the chronological age of an organism increases, but subsequent epigenetic alterations may lead to changes in gene expression regulation with various, often detrimental consequences. Alterations in this regulatory system may lead to impaired functionality of stem cells, but also other symptoms that will be explored in more detail in the following chapters (Pollina and Brunet, 2011). All these genetic and epigenetic alterations may lead to changes in the expression of proteins, leading to loss of proteostasis[3] and resulting in various symptoms such as protein aggregation[4] and general deterioration of cellular biochemistry (Hipp, 2019).

1.2. Antagonistic hallmarks of ageing

Loss of proteostasis may also lead to weakened cellular maintenance and cellular repair mechanisms, which may cause cellular- and tissue damage and are described collectively as the antagonistic hallmarks of ageing. The first characteristic feature of this category is a deregulation of nutrient sensing of a cell, the signalling pathways that regulate the intake of nutrients by a cell, and other mechanisms related to growth and ageing on both a cellular and multicellular level. In summary, an abundance of nutrients upregulates cellular growth while downregulating cellular maintenance resulting in greater cellular damage over time and therefore more rapid ageing (Aiello et al., 2017). Nutrient restriction is therefore thought to slow ageing, as was supported by experiments in yeast, *C. elegans*, *Drosophila* (fruit fly), murine models but also in a long-term experiment with rhesus monkeys. The latter experiment involved a caloric reduction of 30%, resulting in a significant reduction in age-related deaths as compared to a control group. The health- and lifespan of calorie-restricted monkeys was also significantly increased (Colman et al., 2010). Besides deregulated nutrient sensing, ageing cells also display mitochondrial dysfunction manifested as gradually diminishing efficacy of the respiratory chain, as well as a decrease in effective removal of reactive oxygen species and alteration in other mitochondrial components/mechanisms such as a steady state of mutation of mitochondrial (mt)-DNA (Collins, 2012; Hekimi et al., 2014; Ross et al., 2014). The last antagonistic hallmark is cellular senescence, the state in which a cell can no longer divide as a result of its age. Cessation of the mitotic cycle in aged cells can have many causes, as described above, and is accompanied by the release of inflammatory factors. These factors allow for clearance of senescent cells to prevent potentially oncogenic cells from arising. However, the mechanisms that facilitate senescent cell clearance and replacement may become inefficient in aged organisms, leading to the accumulation of senescent cells in tissues. Such an accretion of ageing cells was found to increase secretion of pro-inflammatory molecules that accelerate ageing, effectively altering the senescence-associated secretory phenotype of the ageing organism (Sturmlechner et al., 2016; Malaquin and Rodier, 2016).

1.3. Integrative hallmarks of ageing

While the mechanisms of ageing of individual cells may result in damage to that particular cell, a dysfunction in the interaction between multiple cells within the tissues of an ageing organism may also occur. This phenomenon of altered intracellular communication is described as the first integrative hallmark of ageing. It is characterized by so-called 'inflammageing', the gradual increase over time due to various factors of inflammation of tissues. The most notable causes are the accumulation of pro-inflammatory cellular damage as well as an increasingly dysfunctional immune-system and secretion of pro-inflammatory molecules by senescent cells (Franceschi et al., 2016). Besides inflammation there is also evidence that reactive oxygen- and nitrogen species are shared between senescent cells and neighbouring cells through gap-mediated cell-cell contacts, accelerating ageing (Nelson et al., 2012). This underlines the importance of the last hallmark of ageing in the exhaustion of stem cells, which are vital in the clearing of senescent cells in ageing organisms. In time stem cells lose their ability to divide, so in ageing organisms their quantity will drop resulting in not only a loss of efficient tissue regeneration but also the decline of haematopoiesis[5]. This in turn results in decreased adaptive immune-cell generation for example, meaning that ageing organism will experience a weakened immune-system (Montgomery and Shaw, 2015).

2. The role of epigenetics in ageing

As detailed above, the ageing process of an organism is very complex, involving many different mechanisms ranging from genetic alterations to dysfunctional intracellular interactions. While studying the antagonistic and integrative hallmarks of ageing is of significant interest in the quest to achieve biological immortality and combat human suffering, the primary hallmarks of ageing are of special interest as these are the roots of many of the symptoms of an ageing organism. Additionally, when comparing the primary hallmarks that cause cellular damage it becomes clear that all components concern the loss of information, for example where the information stored in genes as patterns of nucleotides is degraded by mutation and genetic instability (Pal and Tyler, 2016). This leads to improper transcription and subsequent alteration of biochemical mechanisms of a cell. It is not only genomic degradation that affects cellular proteomics however, as epigenetics also plays a significant role by regulating gene expression through various means. Proper epigenetic organisation allows for the differentiation of stem cells into the different cell-types, allows existing cells to adapt to environmental factors like nutrient availability, and regulatory functions (Etchegaray et al., 2017). The information stored in epigenetics is different from genomic information however, as it manifests as the structure of chromatin and the methylation patterns of its subcomponents rather than the order of nucleotides. The loss of information due to age therefore takes a different form as well and can be divided into 8 characteristic changes: The loss of histones, an imbalance of activating and repressive histone modifications, transcriptional changes, changes in chromatin condensation (heterochromatin loss and gain), the breakdown of nuclear lamina which participates in chromatin organization, changes in DNA methylation patterns and changes in chromatin structure (Sen et al., 2016). The characteristics of an ageing cell are illustrated in Figure 2 below.

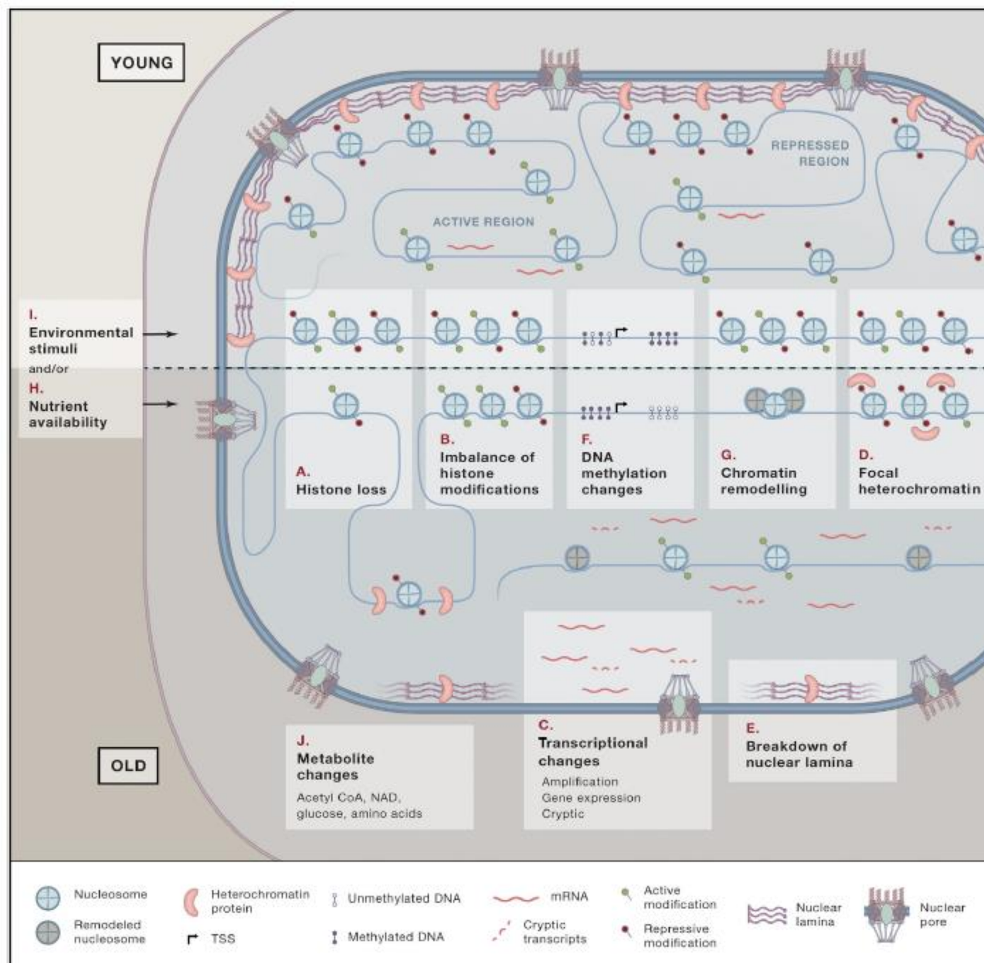

Figure 2: The differences in the epigenetic mechanisms between young and old cells. Taken from (Sen et al., 2016).

3. The role of nucleosome modification in ageing

Every cell of the human body holds approximately 2 meters of DNA, which leads to two questions: How does all that DNA fit in the nucleus of a tiny cell, and how is it possible that different types of cells (like brain cells, skin cells and muscle cells) all possess the same DNA but have widely different structures and functions? The answer to the first question is that the DNA of a cell is wrapped around histone octamers forming nucleosomes that are arranged into tightly packaged chromatin fibres (Alberts et al., 2002). See Figure 3 for an illustration of the packaging of DNA into chromosomes.

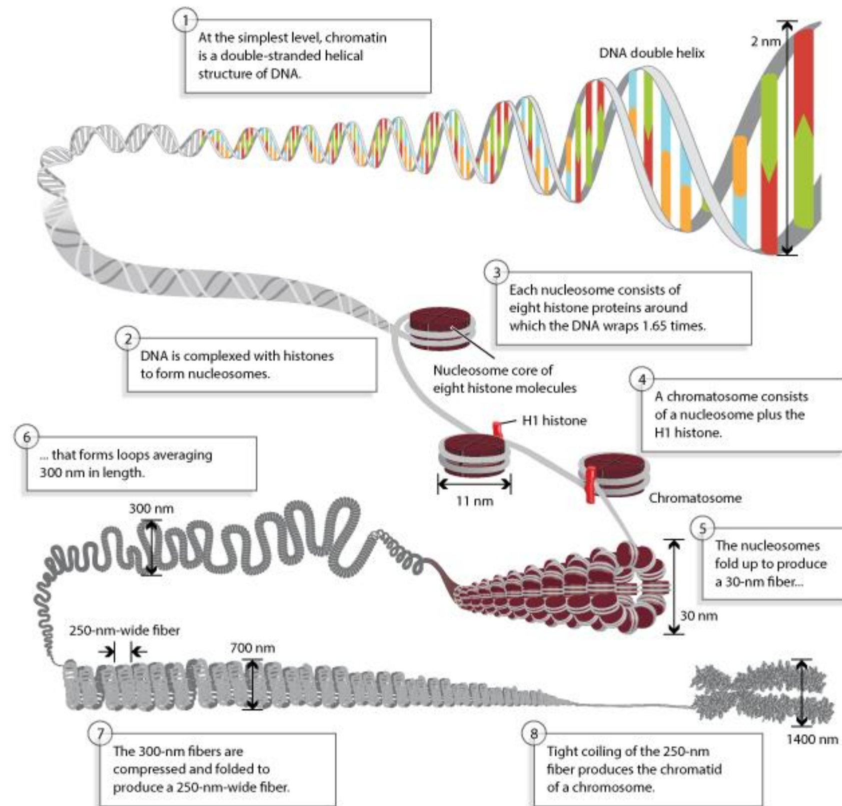

Figure 3: The organization of DNA (1) into nucleosomes (2 & 3), chromatosomes (4), 30-nm fibres (5), compacted into 300 nm fibres (6), then in 250 nm fibres (7) and eventually into the chromatids of a chromosome 8). Taken from (Pierce, 2014).

The second question is related to the first as, when the DNA is concentrated in chromatin fibres, the transcriptional machinery of the cell may be unable to access the DNA, preventing gene expression in the 'blocked' regions. Cells use epigenetic mechanisms to control what parts of the genome are blocked and unblocked however, by modifying specific regions of the genome and histones in various ways. For example, regulatory mechanisms may spool or unspool DNA from the histone octamers, revealing or blocking transcriptional access in that way (Becker and Workman, 2013). They do this based on environmental cues so that the epigenetics of a cell can determine which genes are expressed or not, and on a larger scale, what function and structure a cell will have in a particular environment (Benayoun et al., 2015; Etchegaray et al., 2017).

The epigenetic modifications of histones and DNA are not stagnant throughout a cell's or an organism's lifetime however, as enzymes constantly modify the epigenetic makeup of a cell. Modifications are constantly made and undone. If a cell ages and the biochemistry of that cell becomes unbalanced, this might potentially lead to hyper- or hypomethylation of DNA and to the inactivation of tumor-suppressor genes or other age-related genes (Benayoun et al., 2015).

3.1. DNA modification:

The inactivation of a gene can occur as a consequence of the modification of DNA itself. More specifically, the methylation of cytosine residues in CpG islands[6] close to- or within the promoter of a gene determines whether transcription mechanisms can bind to these regions. CpG islands are regions of DNA with many CpG sequences, cytosine residues followed by a guanine residue (5'-CG-3'). In double-stranded DNA this means that a CpG sequence is symmetrical (5'-CG-3' on both the forward and reverse strands of the DNA), and a methyl-group can be added to the cytosine residue on both strands of the DNA (Moore et al., 2012).

3.1.1. DNA methylation

In the process of cytosine methylation a methyl-group (CH_3) is added directly to the 5th carbon of a cytosine residue by methyltransferases, forming 5-methylcytosine (5-mC). s-Adenylmethionine (SAM) is used as the methyl-donor in this reaction. This process involves association of the methyltransferase with the DNA target site followed by the twisting of the cytosine out of the DNA duplex into the catalytic site of the enzyme (Reinisch et al., 1995; Mandal et al., 2017). The SAM molecule is introduced to the catalytic site of the enzyme upon which its methyl-group is transferred to the 'naked' cytosine, converting SAM into adenosylhomocysteine (SAH). The 5cM is then twisted back into the DNA duplex and the methyltransferase dissociates. See Figure 4 for a schematic representation of the process (Mandal et al., 2017).

It should be noted that recent experimentation has demonstrated that other residues can also be methylated in mammalian cells on 5'-CpHpG-3' sequences where 'H' can be cytosine, adenine or thymine, though in this thesis only cytosine methylation of CpG islands will be explored (T. Wang et al., 2016).

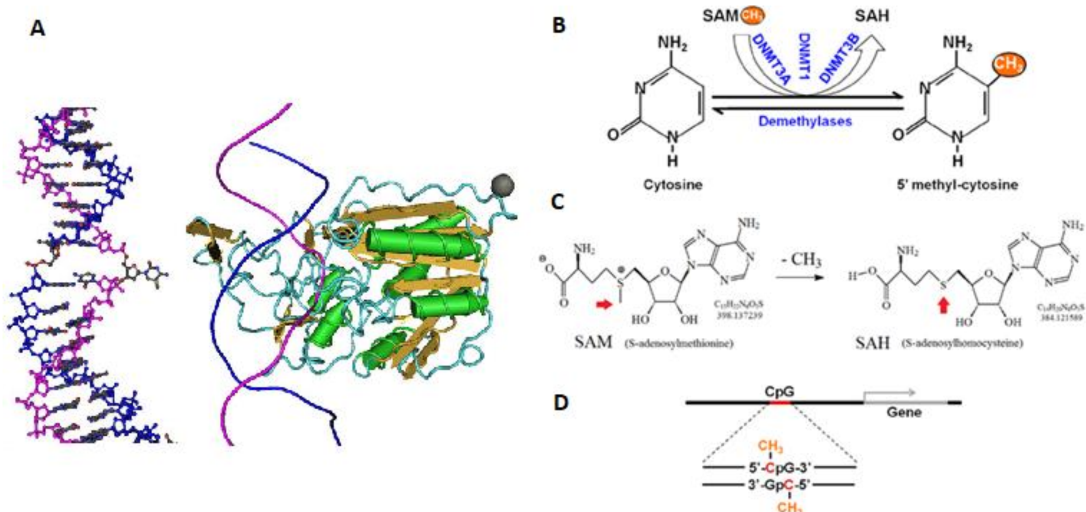

Figure 4: (A) Twisting of cytosine out of the DNA duplex into the catalytic site of a methyltransferase (HaeIII in this case). (B) Methylation of cytosine into 5-methylcytosine by methyl transferase using SAM. (C) A potential location of the CpG island in relation to the start of the affected gene and the symmetrical nature of the methylation. (D) The methylation reaction in relation to the SAM molecule, where CH_3 is transferred from the sulphur atoms (red arrows). Adapted from Mandal et al., 2017.

After methylation the added methyl-group will be oriented into the major groove of DNA so it does not interfere with base-pairing. However, the addition of this chemical group does change the functional group signatures in the major groove of DNA, which was visualized *in-silico* (Machado et al., 2014). This analysis displayed alterations in electrostatic potential of the methylated region as seen in Figure 5.

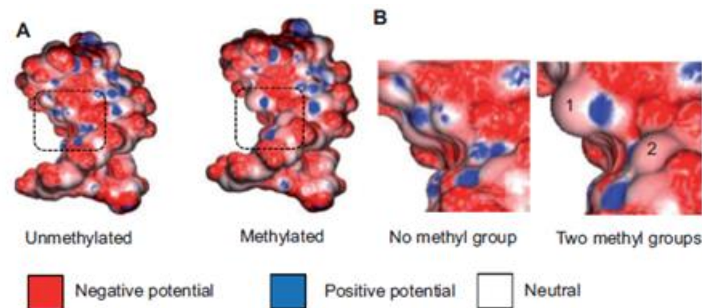

Figure 5: Effect of methylation of a CpG sequence on the electrostatic potential of a DNA double-helix. Section A displays the large-scale effects of the introduction of two methyl-groups while section B is zoomed in on the two added methyl-groups. Adapted from (Machado et al., 2014).

An *in-silico* simulation by the same group as well as a crystal structure analysis of DNA containing CpG sequence methylation also found that the local structure of DNA and chromatin itself were altered, most likely by steric hindrance from the bulkier methyl group (Tippin and Sundaralingam, 1997; Machado et al., 2014). This change appeared to widen the major groove of DNA while subsequently narrowing of the minor groove, potentially altering the accessibility of that region of DNA to proteins such as transcription factors. Figure 6 gives some illustrations of the alterations in functional group signatures and DNA structure due to CpG sequence methylation (Machado et al., 2014).
These changes in molecular interactions and DNA structure suggest that when CpG sites either within

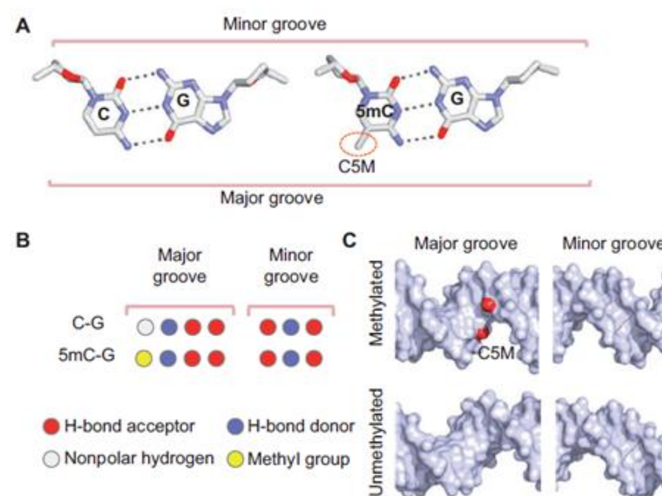

Figure 6: (A) Methylation of cytosine in respect to the minor- and major groove of DNA. (B) The effects of the methylation of cytosine on the functional group signature of the major- and the minor groove of DNA. (C) The effects of the methylation of a CpG sequence on the structure of the DNA double helix. The major- and minor grooves are both tightened due to the addition of two methyl groups. Adapted from (Machado et al., 2014).

the promoter of a gene or close to that gene are methylated, transcription initiation proteins may become unable to reach and/or bind to DNA. If that means that transcription cannot be initiated, this would lead to silencing the gene (Machado et al., 2014).

3.1.2. DNA methyltransferases

The methylation of DNA is not performed by just one type of DNA methyltransferase. There are three major types in humans that function during different stages of cellular development: DNMT1, DNMT3a and DNMT3b (Uysal, 2018). The RNA methyltransferase TRDMT1 (also known as DNMT2) is also part of the DNA modification system. This enzyme is involved in RNA methylation, but a description of this protein is beyond the scope of this thesis (Grace et al., 2014).

In the earliest stages of cellular development stem cells (like embryonic stem cells) will begin to differentiate into specific cell types, altering their structure and functionality in radical ways. What changes will take place depends on a complex network of signalling pathways being activated by environmental signalling molecules. After particular signalling pathways have been activated the epigenetic mechanisms of the cell will regulate gene expression to restructure the cell (Dimmeler et al., 2011).

The DNMT3 family

DNA methylation is one of the primary mechanisms by which stem cell differentiation is mediated and is carried out by the DNMT3 family of DNA methyltransferases. This family is composed of three proteins: DNMT3a, DNMT3b and DNMT3L. The former two are highly homologous in their catalytic sites but differ in protein complexing and function, the latter has no catalytic power on its own (Suetake et al., 2004).

Both DNMT3a and DNMT3b consist of a C-terminal catalytic methyltransferase domain and two N-terminal regulatory and targeting domains ADD and PWWP (see Figure 7 for an illustration of the structure of DNMT3a). The ADD domain is thought to allow the proteins to interact with histone tails and other proteins within the cell. It is also suggested that this domain may directly regulate the catalytic domain of the protein. The PWWP domain on the other hand is thought to bind to both modified and unmodified histone tails as well as to DNA, so it is thought this domain is important for DNA targeting (Jeltsch and Jurkowska, 2016).

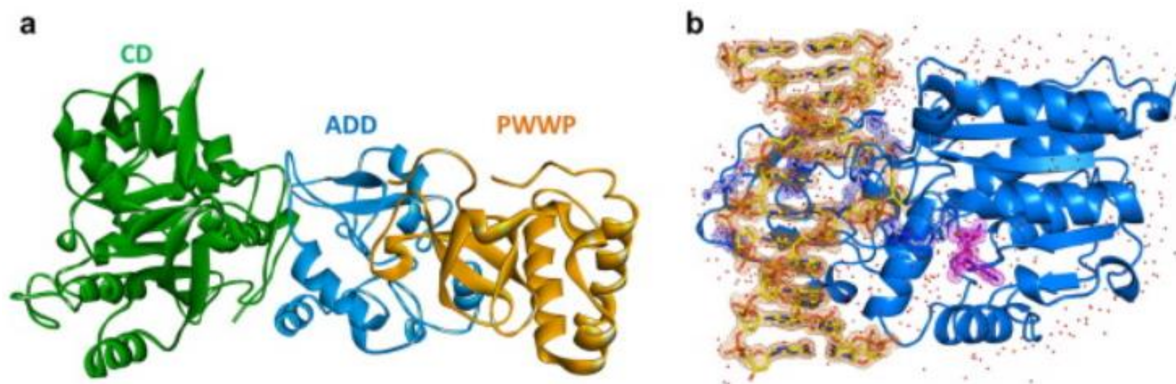

Figure 7: Structure of DNMT3a based on 3D crystallography data. (A) The CD (catalytic domain)-ADD-PWWP arrangement of the subunits is displayed. (B) The binding of DNMT3a to DNA without DNMT3L. Adapted from Rondelet et al., 2016.

Crystal structure analyses revealed that DNMT3a forms heterotetramers[7] with DNMT3L where the DNMT3a subunits form the centre and DNMT3L flanks this core (Jia et al., 2007). In this arrangement a DNMT3a heterotetramer is capable of interfacing with another DNMT3a heterotetramer when both are bound to DNA. This arrangement would potentially allow for the formation of large multimers (see Figure 8) with a predicted gap of 8-10 bp between the active sites, thus perhaps allowing for significant efficiency in methylation (see Figure 8, panels B and C). If no DNMT3L is bound, however, multimers can still form out of the catalytic subunits alone (see Figure 8, panels D and E). It was found that in this case a 2 dimensional plane of multiple DNA-protein strands can form (Jurkowska et al., 2011). In such an structure there may also be significant rearrangement of chromatin, forming densely packed heterochromatin[8] (Jeltsch and Jurkowska, 2016).

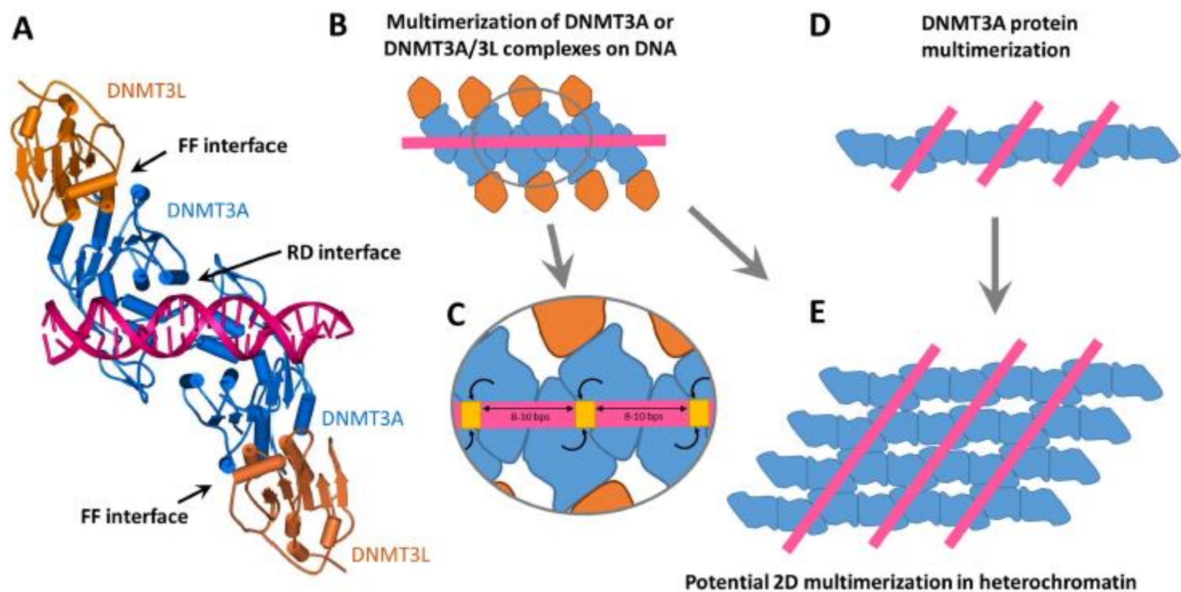

Figure 8: (A) A DNMT3a-DNMT3L heterotetramer bound to DNA. (B and C) The multimerization of DNMT3a-DNMT3L heterotetramers. (D and E) The multimerization of DNMT3a dimers. Taken from Jeltsch & Jurkowska, 2016.

Unlike DNMT3a, DNMT3b has not been found to form such complex arrangements, despite significant similarities between the tertiary structures and sequences of both proteins. As the quaternary structure of this protein is not known yet, future research should elucidate the exact mechanistics and activities of this protein.

Besides difference and similarities in structure between DNMT3a and DNMT3b there are also differences and similarities in function. DNMT3a is especially important in the downregulation of multipotency genes, which maintain the state of multipotency of progenitor cells, and the enzyme is involved in the upregulation of differentiation genes. This was shown in knock-out strains of haematopoietic stem cells (Challen et al., 2011). These mutant cells had an impaired ability to differentiate while multiplying more rapidly. Interestingly, the study found a general decrease in methylation but also hypermethylation at different regions of the DNA suggesting that the methylation capacity of DNMT3a and DNMT3b is contextual and depends on other epigenetic cues such as histone modification (Challen et al., 2014)

DNMT3b appears to have a similar function, as it was observed that the differentiation of haematopoietic stem cells was significantly impaired when DNMT3a and DNMT3b were both knocked-out. However, DNMT3a knock-out strains were significantly less capable of differentiation while DNMT3b knock-out variants were much less affected. This suggests that DNMt3a is able to cover for a lack of DNMT3b functionality while DNMT3b is unable to fully cover for a lack of DNMT3a (Challen et al., 2014).

Further study is required to determine the exact mechanisms of both DNMT3a and DNMT3b in relation to other epigenetic mechanisms.

DNMT1

After cells have matured they will begin to duplicate. During the process of chromosome replication the two parent strands of DNA are separated by the replication mechanism and each strand is copied. The parent strands contain all original methylations as established originally by DNMT3a and DNMT3b when the cell was differentiating, but the daughter-strands do not possess this methylation pattern yet. The DNMT1 enzyme restores proper methylation of the daughter strands using the parent-strand as a template, essentially copying the methylation pattern. DNMT1 is able to copy the methylation-pattern from one strand of DNA to the other due to its unique structure. (Song et al., 2011)
Just like its cousins within the DNMT3 family, the protein consists of a C-terminal methyltransferase domain and an N-terminal regulatory region. The latter determines the target of the protein and is completely different from the regulatory regions of the DNMT3a and DNMT3b. It contains a CXXC domain, two different tandem bromo-adjacent homology (BAH) domains (BAH1 and BAH2) and a target recognition domain (TRD). See Figure 9 for a representation of DNMT1 (Song et al., 2011).

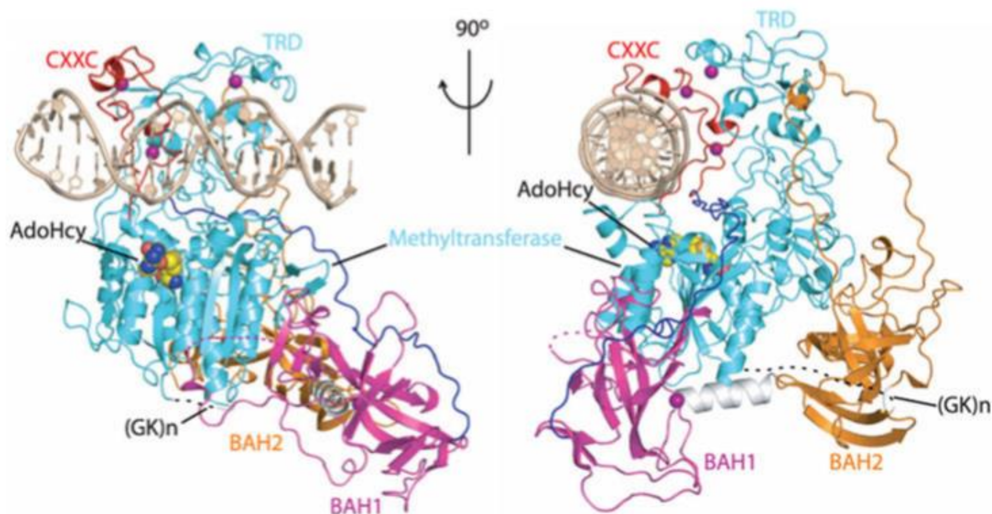

Figure 9: DNMT1 bound to DNA. Two angles are displayed. Colour coding: Green = MTase domain, pink = CXXC domain, blue = TRD domain, orange = BAH-2 domain, and yellow = BAH-1 domain. Adapted from J. Song et al., 2011*.*

The regulatory region allows the copying of DNA methylation patterns mainly through the cooperation between the TRD and the CXXC domains. In this process, the CXXC domain allows binding to unmethylated residues in the DNA (most commonly the cytosine of a CpG island) while the TRD domain assists in DNA binding, possibly only to DNA with one methylated strand. The two BAH domains are associated with the methyltransferase domain and influence targeting, mostly through long chains that span the rest of the protein. Thus, BAH1 is connected to the CXXC domain with a long linker shown in dark blue in Figure 9, which is thought to have an autoinhibitory function on CXXC. *In-silico* simulation suggests that BAH2 plays a role in the specificity of DNMT1, as it is intimately bound to the TRD domain. Both BAH domains may also serve as binding platforms for other cellular proteins, allowing DNMT1 to both specifically methylate the unmodified DNA strands and potentially bind regulatory proteins to modulate DNMT1 activity (Song et al., 2011).

3.1.3. DNA demethylation

While DNMTs may actively methylate DNA, it is also possible for such modifications to be lost. For example, mistakes in the maintenance methylation by DNMT1 may lead to the loss of DNA modifications after successive rounds of DNA replication. Besides such passive demethylation there are also mechanisms that actively remove the methyl-groups from DNA residues (Trerotola et al., 2015).

This route of active demethylation of cytosine first involves the oxidation of the methyl group of 5-mC by a TET enzyme (Ten-eleven translocation methyl-cytosine dioxygenase). Once one alcohol group has been attached to form to 5-hydroxymethylcytosine (5-hmC) the same TET enzyme may remove hydrogen atoms from the hydroxymethyl group of the cytosine, leading to the formation of a formyl group and thus converting 5-hmc into formyl-cytosine (5-fC). After this reaction the entire 5-fC residue can be excised by thymine DNA glycosylase (TDG) to form a DNA lesion that can then be repaired by the base excision repair (BER) system of the cell to insert an unmethylated cytosine using the sister-strand of DNA as a template. Alternatively, before excision of the 5-fC moiety, a TET enzyme may further oxidize the 5-fC into 5-carboxylcytosine (5-caC) which can then also be excised and restored by BER (Pastor et al., 2013; Trerotola et al., 2015; See Figure 10).

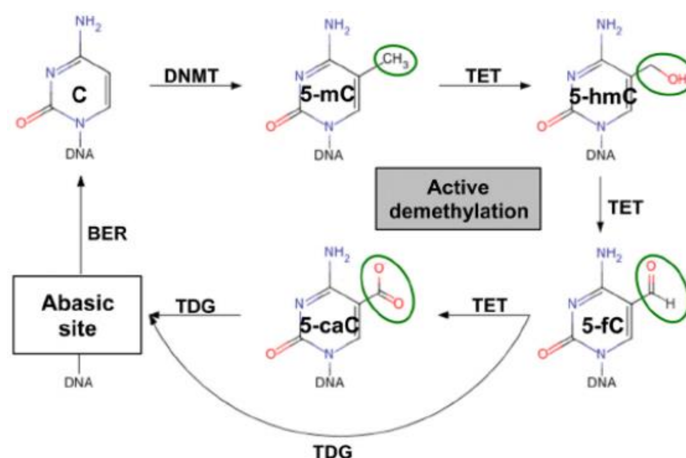

Figure 10: The demethylation of 5-mC through active demethylation. Adapted from (Trerotola et al., 2015).

A secondary mechanism by which methylated cytosine can be demethylated is through the deamination of 5-mC or 5-hmC into thymine or 5-hydromethyluracil (5-hmU), respectively, by AID (Activation-induced cytidine deaminase) and APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) enzyme families. When either thymine or 5-hmU is formed TDG will excise the entire base so the BER system may restore the lesion (Pastor et al., 2013).

3.1.4. The effects of ageing on DNA methylation mechanics

Both methylation and demethylation are tightly regulated in developing cells and can be used to alter gene expression in those cells. Although both the de-novo methylation and demethylation of DNA is less common in somatic cells, this balance may be thwarted by mutation or other changes in cellular biochemistry that are caused by ageing. When methylation balance is lost certain regions of the DNA may undergo hypo- and hypermethylation (a lower- and higher than average amount of methylation, respectively). When a tumour-suppressor gene is hypermethylated for example, that gene would be silenced and the cell may become cancerous, which may be the cause of the observed increase in prevalence of cancer in senior humans (Song and He, 2012). Alterations in transcription may lead to further destabilization not only of the biochemistry of the individual affected cell but also the intracellular biochemistry of tissues, organs and the organism. This in turn may give rise to various age related diseases (Trerotola et al., 2015; Xu, 2019).

It was recently found that certain CpG islands, known as 'epigenetic age estimators' contain CpG sequences that had an especially strong link with an organism's age. These sequences are called 'clock CpGs'. Using DNA methylation arrays[9] the methylation patterns of these epigenetic age estimators could be determined to predict the age of the sampled tissue. Tissue age generally reflects the age of the organism, but this is not always the case. The DNA methylation based age (also known as DNAm age) of an organism is affected by its lifestyle, so an unhealthy way of life leads to accelerated ageing for example (Horvath and Raj, 2018). Nonetheless, epigenetic clocks were found to be exceptionally accurate and have even been used to determine the age of refugees without IDs attempting to enter Germany (Abbott, 2018). The epigenetic clock used to determine their age was developed by Horvath in 2013 and was the first of its kind to unify the methylation patterns of multiple tissues using 354 clock CpGs. The predicted age of 50% of the tested subjects differed less than 3,6 years from their actual age highlighting that, while Horvath's epigenetic clock is capable of predicting an organism's age quite accurately, there can still be a large difference between the chronological- and the DNAm based age of a subject (Horvath, 2013). This difference may be explained by the previously mentioned effect of lifestyle on the DNAm based age. For example, a study has shown that smoking significantly increased the DNAm based age, as determined with Levine's epigenetic clock (Levine et al., 2018; Yang et al., 2019).

3.2. Histone modification

As detailed above, DNA modification has profound effects on the expression of genes in a cell. In addition to these changes in the DNA, the histone proteins within a nucleosome may be post-translationally modified as well in various ways. A histone itself is formed as an octamer consisting of the 8 histone monomers H2A, H2B, H3 and H4, each of which is present in 2 copies. The globular cores of these monomers may be modified, but modifications are far more common within the long flexible N-terminal histone tails that can insert into the minor groove of DNA (see Figure 11; Richmond et al., 1997; Cutter and Hayes, 2015). Besides the 4 primary histones, monomer H1 is also associated with each nucleosome bead. Its function is to keep the DNA in place around the histone octamer. H1 does not seem to be modified (Watson et al., 2014; Cutter and Hayes, 2015).

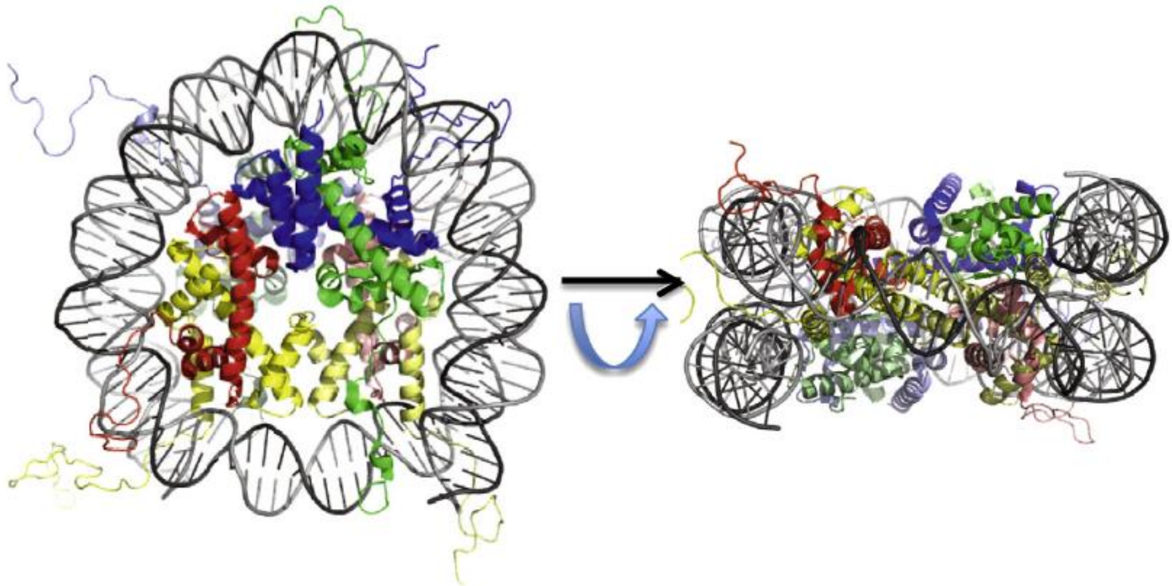

Figure 11: The structure of a nucleosome bead from two perspectives. H2A = green, H2B = blue, H3 = yellow and H4 = red. The second copies of the histone monomers are depicted in less saturated colours. Adapted from Cutter & Hayes, 2015.

There are 2 ways in which a cell may change the structure of chromatin: Through covalent chromatin remodelling complexes that covalently bind functional groups to residues in the histone proteins, and through ATP-dependent chromatin remodelling complexes. The former includes 4 major types of modifications that can be made to (most commonly) the N-terminal tails of histones: acetylation, methylation, phosphorylation and ubiquitination. Other less common types of modification that the enzymes from the family of covalent remodelling complex family can perform are sumoylation, citrullination, deamination, ADP ribosylation, propionylation and butyrylation, while more types are may also exist (Sterner & Berger, 2000; Zhang and Reinberg, 2001; Cuthbert et al., 2004; Nowak and Corces, 2004; Hassa et al., 2006; Nathan et al., 2006; C. J. Nelson et al., 2006; Pavri et al., 2006; Kouzarides, 2007). Due to time and space constraints only the 4 major types will be described in more detail.

3.2.1. Histone acetylation/deacetylation

The most common histone modification is acetylation, which involves the addition of an acetyl functional group (C_2H_3O) to lysine (Lys) residues in the histone molecule (most commonly in the

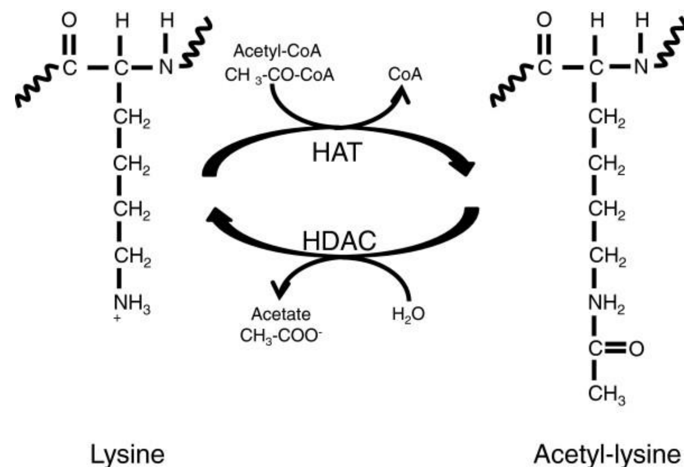

Figure 12: The acetylation and deacetylation of lysine by HATs and HDACs respectively. Taken from (Gutierrez & Romero-oliva, 2013).

histone tail) to form acetyl-lysine. This reaction is mediated by enzymes of the histone acetylase (HAT) family using the acetyl group from acetyl-coenzyme A (Acetyl-CoA). The modification can be reversed by enzymes from the histone deacetylase (HDAC) family using water (H_2O) to form acetate, as illustrated in Figure 12 (Gutierrez and Romero-oliva, 2013). While the acetylation of lysine always follows this reaction, the strategies employed by the enzymes of the different families of HAT and HDAC can vary.

The two major sub-families of HATs are GNATs (General Control Non-Derepressible 5 (Gcn5) –related N-Acetyltransferases) and MYSTs (named after the 4 founding proteins), though more HATs have been identified (Vetting et al., 2005; Berndsen et al., 2007; Lee & Workman, 2007). The HDAC family is divided into 4 sub-families named class I to IV based on sequence homology. The different sub-families often vary in functionality and may require cofactors like Zn^+ or NAD^+ (Seto and Yoshida, 2014).

3.2.2. Histone methylation/demethylation

Histone methylation involves the addition of one or more methyl groups (CH_3) to the side chains of lysine (Lys) or arginine (Arg) residues in the histone tail or -body. The mechanisms by which this addition is executed varies and depends on the target residue (Y. Wang and Jia, 2009).

Arginine methylation

The methylation of arginine residues is mediated by PRMTs (protein arginine N-methyltransferases) that are divided into 4 types, although only type I and type II are used by the cell to methylate histones. Both type I and type II are capable of methylating an arginine residue to monomethyl-arginine (MMA) using SAM (the also used in DNA methylation) as a cofactor which is converted into SAH. MMA can then be methylated again in one of two ways: Either by adding a methyl group to the already methylated nitrogen atom in the side-chain or the unmethylated NH_2^+ group. The former is mediated by PRMT type-I enzymes forming asymmetrical dimethyl-arginine (DMA), while the latter is mediated by PRMT type-II enzymes, forming symmetrical DMA. In both reaction, SAM is used as a cofactor (Y. Zhang and Reinberg, 2001; Gao et al., 2015).

Arginine demethylation

Demethylation of arginine is also thought to occur, but identification of enzymes capable of doing so has been difficult. Two proteins are of special interest. The first one is PAD4 (peptidyl arginine deaminase 4), which deaminates both MMA and unmethylated arginine, preventing further methylation by forming citrulline (Cit) or Cit-Met (Litt et al., 2009; J. Zhang et al., 2019; see Figure 13).

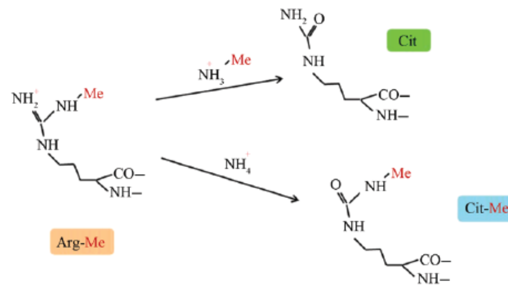

Figure 13: The proposed deamination reaction on methylated arginine by PAD4. Taken from J. Zhang et al., 2019.

The other protein is JMJD6 (jumonji domain-containing protein 6), an enzyme that was first tested for its demethylation capabilities in two separate studies, yielding opposing results. First it was found by Chang and co-workers, who determined the role of this protein in demethylation of arginine (Chang et al., 2007). Subsequently, a study by Webby and colleagues showed that JMJD6 in human cells catalyses the lysyl-hydroxylation of an RNA splicing related protein instead (Webby et al., 2009). Other more recent studies also provide conflicting results, shrouding the demethylation mechanism of arginine residues in histones in uncertainty (Webby et al., 2009; Liu et al., 2013; Lawrence et al., 2014; Poulard et al., 2014; J. Zhang et al., 2019).

Lysine methylation

Lysine methylation is more straightforward compared to arginine methylation. In a series of reactions members of the HMT family methylate the lysine residues in histones. This protein family is divided into 2 subfamilies: The SET-domain containing HMT enzymes are capable of methylating lysine residues in histone tails, while the non-SET domain-containing subfamily can methylate lysine residues in the histone cores. During the process of lysine methylation the hydrogen atoms of the amine group on the side chain are replaced step by step with methyl groups to form monomethyl-lysine (Me1-K), then dimethyl-lysine (Me2-K), and lastly trimethyl-lysine (Me3-K) *(Zhang and Reinberg, 2001*; see Figure 14).

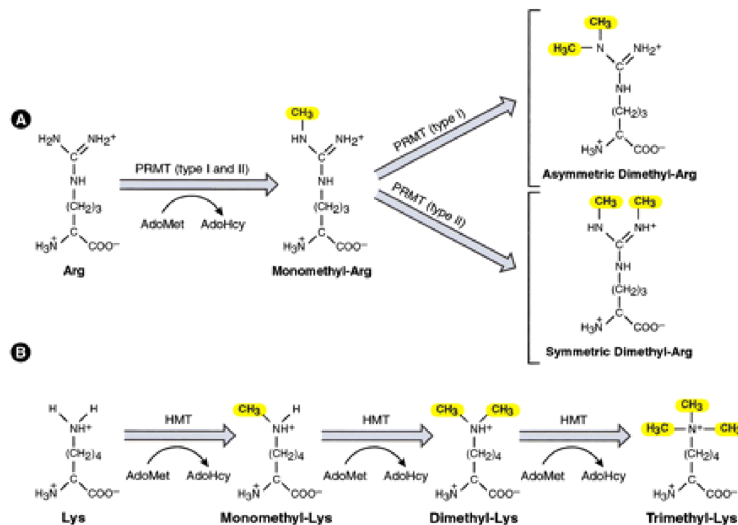

Figure 14: The methylation of arginine (A) and lysine (B) residues within a histone tail. SAM is named AdoMet and SAH is named AdoHcy. The added methyl groups are highlighted in yellow. Taken from (Y. Zhang & Reinberg, 2001).

Lysine demethylation

Unlike the demethylation of (methylated) arginine, the demethylation of (methylated) lysine has been explored more thoroughly and can occur either by oxidation or by oxygenation, using different proteins and reactions. The first method is oxidation, which is mediated by lysine demethylase 1 (LSM1), an amine oxidase that uses the cofactor FAD (Flavin Adenine Dinucleotide) to oxidise the methylated amine group of the lysine residue into an imine group. As this process is only possible with a protonated nitrogen within the group, only methyl-lysine or dimethyl-lysine can be demethylated in this manner. The oxidation reaction results in the reduction of 2 electrons from FAD, which is re-oxidized by O_2 to form a renewed FAD and H_2O_2. The imine group of the target residue is then hydrolysed to carbinolamine, which naturally degrades to release formaldehyde, leaving the lysine in a demethylated state or as a methyl-lysine. The methyl-lysine may then be demethylated as well, following the same process (Cloos et al., 2014; see Figure 15).

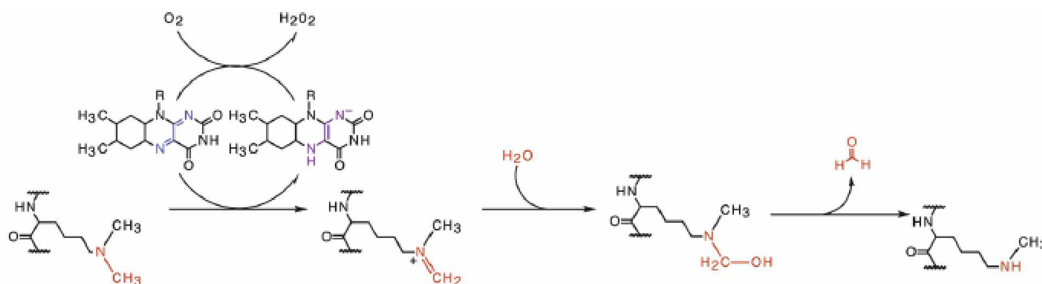

Figure 15: The demethylation of dimethyl-lysine by LSM1 using a FAD cofactor. Adopted from Cloos et al., 2014.

The second method is through oxygenation by members of the Jumonji C (JmjC) protein family, which includes a large amount of demethylating proteins of varying specificity. Such proteins may demethylate (methylated) lysine using the molecule αKg (shown in Figure 16 in the bottom-left of every frame) and an Fe(II) ion as cofactors. The first step of the reaction involves the binding of the Fe(II) ion to the enzyme using an iron-binding domain (shown as the amino-acid residues in the figure), followed by the binding of the αKG molecule to the bound Fe(II) ion and the oxygenation of the iron. The addition of the oxygen allows for the oxidative decarboxylation of the αKG molecule into succinate (as seen in step 2) which releases CO_2 and turns the Fe(III) ion into a ferryl (Fe(IV)). The ferryl ion is very reactive and will be able to oxidize one of the C-N bonds between the lysine side-chain and one of the methyl groups (see step 3). This oxidation will produce an unstable carbinolamine that degrades to release a formaldehyde molecule, leaving the lysine residue with one less methyl group. This process may be repeated to demethylate the lysine completely (Cloos et al., 2014).

It should be noted that the mechanisms of demethylation of lysine, just like the demethylation of

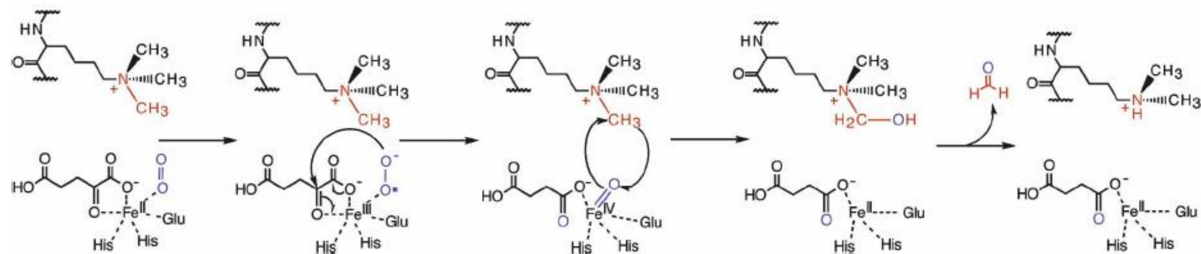

Figure 16: The demethylation of trimethyl-lysine by JmjC using αKG and iron (Fe) as cofactors. Adopted from Cloos et al., 2014.

arginine, has not been elucidated completely yet. The reaction described above is our current understanding of the mechanism, but different sub-families of JmjC may function differently and some even use different cofactors. Much is left to be discovered (Cloos et al., 2014).

3.2.3. Histone phosphorylation/dephosphorylation

Histone phosphorylation plays an important role in chromosome condensation during mitosis, is involved in DNA damage repair, and influences transcription in various ways. The phosphorylation of a histone involves the addition of a phosphate group (PO_4^{3-}) by kinases to serine (Ser) or threonine (Thr) residues in the histone tail using ATP. This reaction can be reversed by phosphatases, which remove phosphate groups from residues through a hydrolysis reaction involving H_2O. See Figure 17 for an illustration of both reactions.

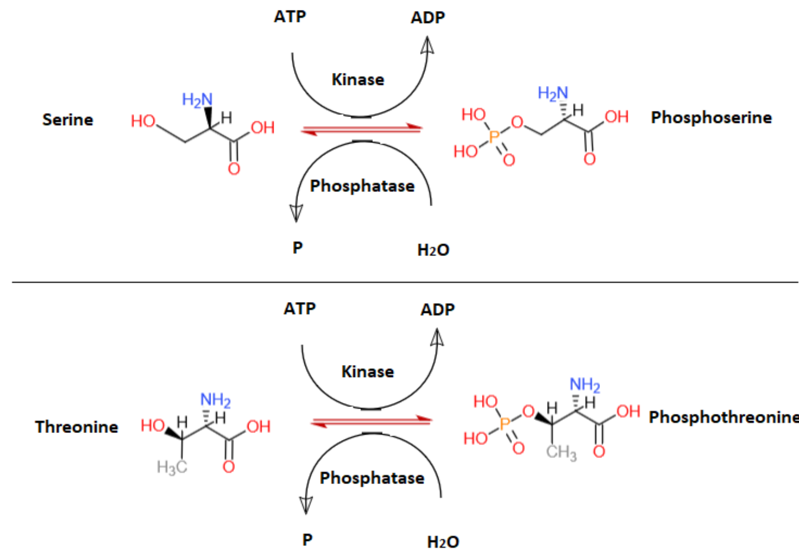

Figure 17: The phosphorylation and dephosphorylation of serine and threonine. Own work.

3.2.4. Histone ubiquitination/deubiquitination

Unlike histone acetylation, methylation and phosphorylation, histone ubiquitination does not simply add a small functional group to a residue in a histone. Instead, usually one ubiquitin peptide is bound directly to a histone lysine residue through the formation of an isopeptide bond (see Figure 18). This process is mediated by a family of ubiquitin ligases (ULs), whose members all specifically ubiquitinate certain residues of specific histone monomers. In this way, cellular regulatory mechanisms will be able to specifically determine what residues to ubiquitinate by only expressing a certain ubiquitin ligase (Cao and Yan, 2012).

Just like all previously described histone modifications, ubiquitination is also reversible. Intuitively named histone de-ubiquitinases (DUBs) are a family of peptidases capable of recognizing and hydrolysing the isopeptide bonds between the ubiquitin and the lysine (Cao and Yan, 2012).

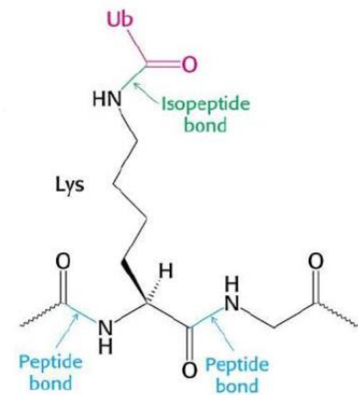

Figure 18: The ubiquitination of a lysine residue of a protein (like a histone tail). The ubiquitin is shown in purple. Taken from Kerrigan, n.d..

Collective influence of histone modification patterns on chromatin structure

When histone modifications are made and unmade by chromatin remodelling complexes, the expression of DNA may be changed in different ways. It could be that the addition of a functional group alters the properties of the histone in such a way that the organization of chromatin is altered directly, but the modifications themselves may also allow for the binding of protein complexes to the DNA or histones to alter expression indirectly (Izzo and Schneider, 2011).

Acetylation

Deacetylated lysine residues in histones correlate with tightly wound euchromatin[10] while acetylated lysine is associated with loose heterochromatin. This is probably caused by a change in charge in the histone, as lysine is a positively charged residue due to the NH_3^+ group at the end of its side chain that is electrostatically attracted to negatively charged DNA. The presence of many positively-charged lysine residues therefore strengthens the winding of DNA around the histones. The acetylation of lysine residues neutralizes the charge, which weakens the attraction between the modified histone and DNA. The weakened interaction results in the DNA wrapping more loosely around the histone. The euchromatin formed this way increases the availability of DNA to transcription mechanisms. Histone acetylation is therefore generally associated with an increase in gene expression, which is supported by the discovery that acetylated lysine residues are commonly present at the promoter regions of active genes (Biology et al., 2009; Zentner and Henikoff, 2013).

This direct effect of histone acetylation is complemented by the ability of bromodomain proteins to bind to acetylated lysine residues in histones. These proteins have various functions. For example, many of the proteins containing such a bromodomain are capable of acetylating other lysine residues after binding to an already acetylated lysine, while others directly alter chromatin structure or are able to recruit transcription-related proteins such as transcription factors (Josling et al., 2012).

Methylation

The regulation of transcription and chromatin restructuring by histone methylation is far more complex however, as lysine residues can be methylated either as Me1-K, Me2-K, Me3-K, and arginine residues can be methylated as MMA, as symmetrical DMA or as asymmetrical DMA. It was found that not only the location, but also the type of methylation determines the result of the modification, whether certain genes are enhanced or repressed.

Histone arginine methylation was found to directly affect chromatin structure, as while it does not change the charge of the residue the number of hydrogen bonds arginine can form will be decreased as more methyl groups are attached. This can significantly alter the interaction of this residue with other proteins and molecules. It was also found that the addition of bulky methyl groups changes the shape of the residue and results in a difference in steric hindrance between the methylated arginine and other proteins (Fuhrmann et al., 2015).

Unlike arginine it is not known if and how lysine methylation affects gene expression directly, but part of the observed enhancing and suppressive effects on certain genes is thought to be caused the binding of specialized protein complexes that affect transcription in particular ways (Izzo and Schneider, 2011). The domains capable of binding methylated lysine and arginine present in such proteins are called chromo- tudor- and PHD domains. One example of such proteins is the polycomb (Pc) group proteins, which are a family of proteins that contain a chromodomain as well as repressor motifs capable of repressing the expression of specific genes (Hublitz et al., 2009; Golbabapour, 2013; Hassler et al., 2016).

Phosphorylation

Serine and threonine phosphorylation have been associated with chromatin condensation, despite the fact that the addition of a negatively charged phosphate group to a histone would cause repulsion between the group and the negatively charged DNA. While such interactions are not unheard of, the phosphorylation of specific residues was found to serve as the recruitment platform for a HDAC. As detailed above, these proteins deacetylate histones, resulting in a strengthened bond between DNA and the histone, leading to condensation (Wilkins et al., 2014) In this way, histone phosphorylation is thought to function as an anchor-point for various proteins, therefore influencing expression and chromatin organization on a larger scale (Sawicka and Seiser, 2014).

Ubiquitination

Histone ubiquitination on the other hand is most commonly associated with gene expression, but also with repression depending on the ubiquitinated residues. It is thought that adding ubiquitin to histone residues affects expression in various ways, for example by serving as recruitment platforms for transcription-related proteins, as well as by influencing other histone modification mechanisms. In this way ubiquitination does not necessarily affects chromatin structure directly, but does serve as a signal for other nuclear mechanisms to activate or be repressed, potentially altering gene expression and chromatin structure indirectly (Cao and Yan, 2012).

The histone code hypothesis

As detailed above, some protein complexes are capable of binding to specific modified residues to have various effects on chromatin structure and transcription. It was found however, that certain complexes did not simply have one histone binding domain, but sometimes contained several binding domains with different specificities and/or capable of targeting different modifications simultaneously. An example is a murine homolog of TAF1, which contains a bromodomain that can bind to the acetylated lysine 5 and 8 in histone monomer 4 but wasn't found to bind to the modification individually (Morinière et al., 2009).

The discovery of specific binding like this gave rise to the hypothesis that particular combinations of different histone modifications function as a kind of code, the so-called histone code, which was postulated to define transcription on a larger scale, similar to how transcription mechanisms read the nucleotides of DNA (Chi et al., 2010; Rando, 2013). There is still uncertainty about the existence of such a histone code reading mechanism however, as multiple combinations of histone modifications can allow the same proteins to bind for example. This lack of combinatorial complexity suggests that while there may be specific proteins capable of more readily binding to particular combinations of histone modifications, these are not the foundation of an intricate network of code dependent proteins and biochemical mechanisms (Rando, 2013).

ATP dependent chromatin remodelling

Another layer of complexity in the epigenetic regulation of gene expression is found beyond the mechanisms of covalent-modifying chromatin remodelling complexes, as there is a family of protein complexes capable of altering the structure of chromatin using ATP as an energy source. In eukaryotes these ATP-dependent chromatin remodelling complexes have been divided into 5 sub-families: SWI/SNF, ISWI, CHD, INO80 and SWR1 (Clapier et al., 2017).

The SWI/SNF family is one of the most studied sub-families of ATP dependent chromatin remodelling complexes and was found to be vital for chromatin organization and transcription. In humans it is called the BAF complex. As detailed above, chromatin is organized as DNA wrapped around histone octamers like beads on a string with open internucleosomal space in between the beads. While the DNA wrapped around the histone octamers is generally inaccessible to transcription, the internucleosomal spaces are fully accessible. The promoter region of a gene may therefore be wrapped around a histone preventing expression of the related gene causing it to be repressed, or the promoter region may be located in the linker region allowing for expression. BAF complexes use this principle by binding to

acetylated lysine of histones and then moving those histones to a different position on the DNA, like pulling a pulley along a rope, to change the length of the internucleosomal space. This in turn may reveal the promoter regions of specific genes allowing for expression of those genes while resulting in the repression of others. BAF complexes may also play a role in the manipulation of histone octamers by either switching monomers to histone variants[11] or by removing histone octamers from DNA in its entirety, affecting chromatin structure and revealing longer regions of DNA respectively. It is not clear yet what the exact mechanisms of nucleosome sliding are yet, but several concepts have been proposed (Whitehouse et al., 1999; Clapier et al., 2017). SWI/SNF complexes like BAF complexes were also found to inhibit transcription repression complexes like polycomb complexes. See Figure 19 (Giles et al., 2014).

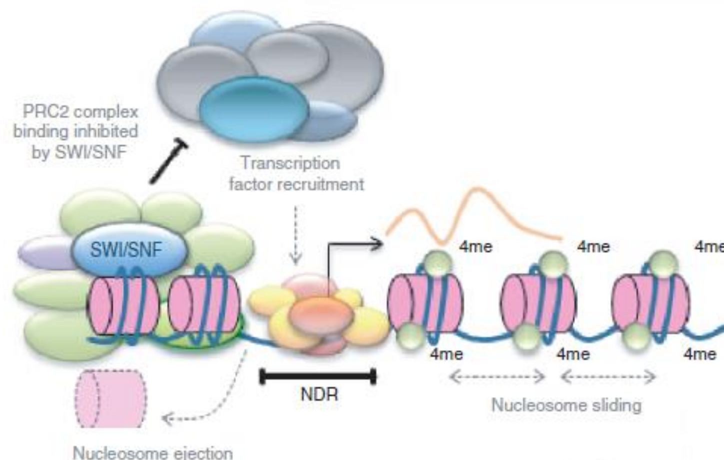

Figure 19: The function of SWI/SNF complexes. The internucleosomal space is named NDR (Nucleosome Depleted Region) Adapted from Giles et al., 2014.

The ISWI sub-family is somewhat similar to the SWI/SNF subfamily, but is only able to regulate nucleosome spacing and are mostly associated with gene repression, though there are exceptions like the NURF complex (Clapier et al., 2017).

The CHD sub-family contains many proteins with various structures, mechanisms and functions, but were generally found to be able to bind both methylated DNA and methylated histone residues to either repress or activate transcription of particular genes. It was found that *in-vitro* CHD proteins were able to slide nucleosomes like the previously described families, displayed deacetylase activity and were able to manipulate the structure of histone N-terminal tails to allow for deacetylation. CHD proteins are thought to also be involved in the transcription process itself by opening up promoters from chromatin, removing nucleosomes during the elongation step of transcription, may be involved in transcription termination and in RNA splicing by affecting nucleosome positions (Murawska and Brehm, 2011; Clapier et al., 2017).

The INO80- and SWR1 families are involved in transcription regulation mostly in DNA repair and in various aspects of mitosis like DNA replication, chromosome structuring and telomere maintenance. Much is yet to be discovered about these last two sub-families however (Morrison et al., 2009; Falbo and Shen, 2014).

Epigenetic crosstalk between DNA methylation, histone modification and related processes

The epigenetic mechanisms described above were shown to be highly dynamic and complex, utilizing a balance between proteins that make modifications to DNA and histones (writers) and those that remove modifications (erasers), as well as proteins that alter transcription based on combinations of these modifications (readers).

Writers, erasers and readers are also able to influence the activity of other aspects of the epigenetic machinery as already described above. For example, the phosphorylation of serine and threonine by kinases may lead to the binding of a member of the CHD sub-family of ATP-dependent chromatin remodelling complexes, which in turn may induce deacetylation of specific residues around the phosphorylated site. Deacetylation of acetylated lysine residues then results in the formation of heterochromatin and removes the binding sites for transcription activators that contain chromodomains, resulting in a decrease of expression of certain genes (Wang et al., 2018).

This interconnectedness of epigenetic mechanisms could be taken advantage of by cells to be able to respond to environmental changes on either a small scale (changes in the microenvironment), or on larger scales in response to the release of specific hormones (Mohammad and Baylin, 2010). On the most basic level however, the availability of metabolites that function like cofactors for epigenetic mechanisms like ATP (for phosphorylation), NAD^+ (for certain histone deacetylases) and Acetyl-CoA (for acetylation) will affect transcription of certain genes. For example, starvation of the organism may decrease the availability of ATP, resulting in decreased phosphorylation and decrease deacetylation with subsequent increased transcription of certain genes that may be of use in preserving energy and may increase cellular maintenance related gene expression for example. Such a signalling cascade that stimulates cellular maintenance may be what caused the increased longevity of the rhesus monkeys subjected by caloric restrictions in the study by (Colman et al., 2010). The exact mechanisms by which epigenetic modifications exactly interact with extracellular influences is yet to be fully elucidated however (Wang et al., 2018).

4. Degeneration of epigenetic modification patterns during ageing

What has become clear about the epigenetic mechanisms described in this thesis is that the regulation of transcription in even a single cell depends on an extraordinarily complex network of balanced biochemical processes and modulation of numerous proteins and enzymes. The question remains however, how these mechanisms are affected by age and how these effects lead to cellular senescence.

The primary reason for cellular senescence is the loss of transcriptional control. This was shown in murine models for example, where the transcription levels in heart cells from young and old mice were compared. This study revealed that large differences in gene expression exist even between cells in the same tissue (Bahar et al., 2006). It also showed that a parallel exists between the accumulation of genetic mutations leading to altered gene expression and the observed differences in transcription levels in young and old mice. Other studies suggest that loss of heterochromatin has a profound impact on the longevity of an organism. *Drosophila* (fruit fly) displayed an increased lifespan when heterochromatin formation was increased, while a decrease in heterochromatin resulted in a decreased lifespan (Larson et al., 2012). This effect may be caused by the loss of repression of transposable elements[12] related to non-coding RNAs like miRNAs and siRNAs that induce post-transcriptional silencing (Larson et al., 2012; Peleg, et al., 2016).

While in senescent cells a decrease in repressive heterochromatin was observed, this decrease was accompanied by the formation of new regions of heterochromatin. These senescence-associated heterochromatin foci (SAHFs) are function in a beneficial way by repressing proliferation-related genes of ageing cells. The mechanism of SAHF formation is not yet fully understood, but it was found that SAHFs contain an abundance of macroH2A, a histone variant of H2A that represses transcription. It is thought that this histone variant is integrated into chromatin through the use of the chromatin regulator ASF1a and a complex containing the HIRA protein. The exact mechanism by which the latter protein functions is unknown, though HIRA cellular localization, *in-vitro* modulation (where the gene was controlled by an inducible promoter) and coimmunoprecipitation experiments suggests that interaction between ASF1a and HIRA is vital in macroH2A integration into chromatin (Zhang et al., 2005). Other differences between the chromatin of SAHFs and regular heterochromatin are specific methylation and acetylation patterns, eluding to the involvement of covalent-modifying chromatin remodelling complexes (Aird and Zhang, 2013).

Another notable difference between young and senescent cells is an alteration in alternative splicing and a loss of precision in transcription. It was found that different chromatin modifications and modification related proteins influenced pre-mRNA splicing. The coactivator-associated arginine methyltransferase CARM1, an enzyme which can methylate arginine residues within histones can also promote exon skipping[13] for example (Doi et al., 2005). Histone modifications themselves can affect splicing as well. For example, in human mesenchymal stem cells the histone residues H3K36 and H3-K4me1 were found to be methylated thrice and once respectively (so H3K36me3 and H3K4me1) in the FGFR2 gene when exon IIIb is spliced out of the pre-mRNA (Luco et al., 2010).

Any dysfunctions in histone- and DNA modification writers and erasers (enzymes that add and remove modifications to and from histones and DNA), as well as imbalance between these two types of enzymes, would therefore significantly affect both transcription levels directly and alter the translated proteins. Random alterations in DNA- and histone modification patterns will therefore alter cellular biochemistry and result in decreased lifespan, as was demonstrated in the yeast *Saccharomyces cerevisiae* where the demethylation of H3K36me3 results in a significant decrease in lifespan due to the activation of normally repressed genes (Sen et al., 2015). In humans, a similar effect was found, as ageing cells showed an increased chance of becoming cancerous. This effect may be explained by changes in chromatin modification patterns as well, as H4K16 deacetylation and H4K20 trimethylation were found to result in hypomethylation of DNA and subsequent activation of genes that are normally repressed, leading to cancer (Fraga et al., 2005).

The latter example where altered acetylation due to ageing leads to a pathology shows that a connection exists between metabolism and epigenetic mechanisms. After all, it was observed that

ageing is accompanied with metabolic dysfunction through e.g., decreased concentrations of NAD^+, which results in a drop in HDAC activity and subsequent increases in acetylated histones and decreased longevity (Bonkowski and Sinclair, 2016; Clement et al., 2019). Another example is the metabolism of acetyl-CoA, which is a cofactor for HATs. Increased cytosolic acetyl-CoA concentrations are seen in ageing organisms. One study showed that a reduction of cytosolic acetyl-CoA renewal resulted in decreased histone acetylation and an increased lifespan in *Drosophila* (Peleg et al., 2016). These findings provide evidence for a strong link between metabolism and ageing.

In summary, ageing is strongly linked to dysfunction in epigenetic modification patterns, chromatin structure, and the resulting alterations in transcription and splicing, as well as to changes in metabolism. All evidence points to a highly complex system of interacting mechanisms that collectively begins to slowly deteriorate during ageing. The consequent dysfunctioning leads to increasingly severe cellular senescence which itself can have destabilizing influences on neighbouring cells (Peleg et al., 2016).

5. Rejuvenation of cells and tissues to slow and reverse ageing

Despite the apparent complexity of the progression of cellular senescence, the knowledge that even the concentration of a single metabolite in a cell may have significant effects on the lifespans of model organisms suggests that there might be ways to slow down or even reverse cellular ageing. Several studies using model organisms have already been performed with the purpose to explore these possibilities. Due to time constraints only one promising example will be described in detail in this thesis.

A recent study, which has not been peer-reviewed yet, has restored the gene expression of ageing and damaged cells in a murine model using the TET-based restoration of cellular methylation patterns (Lu et al., 2019). The study is based on the idea that aberrant transcription in ageing cells is caused by altered DNA methylation patterns, and that the removal of those patterns would allow cells to re-methylate the DNA to their youthful state using an unidentified template of signal.

To determine whether this was possible, the group used 3 Yamanaka factor genes[14] known to be able to return somatic cells to their pluripotent state if activated through the removal of DNA methylation patterns: Oct4, Sox2, and Klf4 (together called OSK). These were integrated into a viral vector (adeno-associated vector (AAV)), where a tetracycline inducible promoter controls the OSK genes. The first experiment was successful in returning fibroblast cells to a state of young DNAm based age *in-vitro*, so the method was then applied to mice *in-vivo* to test toxicity, with discernible symptoms being recorded. The next step was to determine the regenerative properties in damaged tissue, specifically targeting retinal ganglion cells (RGCs), which are a part of the central nervous system. The central nervous system loses regenerative properties first out of all tissues during ageing, so these tissues are important candidates for regeneration in ageing organisms.

The most successful attempt at cellular restoration involved the intravenous injection in mice of the AAV, followed by the surgical crushing of the optical nerve. The mice were then fed with tetracycline to induce OSK expression for 12-16 weeks, resulting in the RGC nerve fibres extending up to 5 mm into the crushed region of the nerve. While individual cells were restored, no new cells were produced, suggesting that cellular proliferation is not induced by this procedure.

It was also found through knock-out experiments that TET1 and TET2 are vital in the restoration of RGCs through the described method. These results suggest that DNA demethylation returns the RGCs to pluripotent stem cells by resetting DNA methylation patterns. The cells may then differentiate back into RGCs with restored epigenetic modification patterns. This was analysed using the epigenetic clock by Wang and Lemos, which showed a decrease in DNAm based age, indicative of the rejuvenation of the treated cells (Wang and Lemos, 2019). All experiments were also done *in-vitro* with human RGCs, and similar effects on cellular rejuvenation were observed.

Though peer-review is needed, these results suggest that reprogramming of epigenetic patterns in cells may very well be a way to combat cellular senescence on a tissue-wide scale(Lu et al., 2019).

Conclusion

This thesis aimed to explore the role of chromatin modification in ageing and to investigate potential methods to slow- or even reverse ageing in humans. Indeed, recent experimentation has shown that during ageing epigenetic modification patterns that regulate transcription directly or through chromatin remodelling may become aberrant by failures in modification maintenance, as wells as through dysfunction in the balances between modification writer and eraser proteins.

The 5 major types of DNA- and histone modifications are DNA methylation, histone acetylation, histone methylation, histone phosphorylation and histone ubiquitination, though several minor types of histone modifications also exist. Such modifications modulate the accessibility of DNA for the transcription machinery either directly by preventing binding of proteins to DNA, or by inducing steric clashes or electrostatic repulsion between the added group and other parts of the chromatin leading to changes in chromatin structure. A number of proteins are capable of binding to modified DNA- and histone residues, sometimes even specifically to certain combinations of modifications. This has given rise to the histone code hypothesis, which postulates that histone modification is organized in sequences that can be identified and "read" by the cell. Evidence for this hypothesis is lacking, although some protein families have been identified of which members are capable of altering transcription on a larger scale based on DNA- and histone modification patterns. These ATP-dependent chromatin-remodelling complexes alter chromatin structure by using ATP to introduce histone monomer variants into nucleosomes, change nucleosome spacing on DNA, or by interacting with histone modification writers and erasers.
The complexity of interactions between histone/DNA modifications and cellular machinery is deepened by the influence of cellular metabolism of cofactors on modification writer and eraser activity. Acetyl-CoA availability directly modulates acetylation rate for example. This extensive interconnectedness and interdependency may be the cause of the slow degradation of epigenetic regulation of transcription, as dysfunction in one part of the system will affect the functionality of others. Two of the most important characteristics of an ageing cell are therefore the loss of transcriptional accuracy due to the degradation of modification patterns and writer/eraser balance, and changes in transcript splicing.

The most important finding in this thesis however, is that there is still a significant gap in our knowledge regarding the exact interaction(s) between the epigenetic regulatory mechanisms, cellular metabolism and the signalling pathways related to the cellular microenvironment. This blind spot currently prevents us from revealing the secret behind cellular differentiation and the exact mechanisms of epigenetic dysfunction during ageing. Despite lacking an understanding of the processes involved, multiple attempts at rejuvenating human cells and tissues have been made already. In this thesis the results of one of the more promising recent studies have been discussed. In the (*in-vivo*) experiments, murine retinal ganglion nerve was surgically damaged, preceded and followed by intravenous injection of a viral vector containing OSK genes (which induce pluripotency when expressed) controlled by an inducible promoter. The results have demonstrated that it is possible to selectively return cells to a pluripotent state and then allow them to differentiate back into healthy cells with the epigenetic modification patterns of young cells. Such cellular rejuvenation was not accompanied by total tissue regeneration however. It simply restored the treated cells to a pre-senescent state. To reverse the damage done by larger scale senescence, such as is seen in ageing organisms, this method of rejuvenation must be accompanied by other therapies.

The need for a combination of therapies to alleviate senescence-related tissue damage ties in to the other hallmarks of ageing, specifically to stem-cell depletion as discussed in Chapter 1. In young organisms senescent cells are removed by the immune system and replaced by stem cells, while the decline of stem-cell replication leads to incomplete regeneration of ageing tissues in older organisms. By introducing stem cells intravenously, a patient undergoing a cellular rejuvenation therapy might

therefore enjoy fully restored tissue function. The latter example of combining cellular rejuvenation through epigenetic programming and stem cell therapy is not fully restorative however, as it does not allow the patient's own stem-cells to divide again. In this way different hallmarks of ageing may still lead to the various age-related pathologies even if the functionality of epigenetic mechanisms is restored. Further research is therefore necessary to elucidate the exact mechanisms of all hallmarks of ageing to develop rejuvenation therapies capable of fully restoring youth in an ageing individual. This means that for now, the dream of immortality and eternal youth remains just that, a dream.

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Glossary:

1) Senescence: The condition or process of functional deterioration with age.

2) Nuclear Lamina: A fibrillar network on the inside of the nuclear membrane composed of filaments and proteins that participate in replication, cell division and chromatin organization (Gruenbaum et al., 2003)

3) Proteostasis: The balance between the formation and removal of proteins within a cell. To maintain the functionality of a cell a certain number of proteins are needed while damaged proteins are disposed of. Malfunctions in protein expression of one or more genes or of the clean-up of damaged proteins may lead to dysfunction within the cell.

4) Aggregation (of proteins): The clumping together of misfolded proteins, caused by either pre-transcriptional damage to DNA or post-transcriptional damage of the proteins themselves.

5) Haematopoiesis: The formation of all types of blood cells in the bone marrow, for example through differentiation of multipotent stem cells into platelets or haemocyte (red blood cell).

6) CpG island: A collection of CpG sequences close to one another. CpG sequences are: 5'-CG-3'. This linear sequence can be methylated by DNA methyltransferases to regulate gene expression.
Autoinhibitory domains: Domains within a protein that negatively regulate other domains or proteins. Often involved in substrate-selection within enzymes.

7) Heterotetramer: A protein complex composed of two pairs of twin proteins. For example, 2x protein A and 2x protein B.

8) Heterochromatin: Densely packed chromatin.

9) DNA methylation array: A method by which the methylation of a region of DNA can be determined using a chip containing wells for many reactions.

10) Euchromatin: Loosely packed chromatin.

11) Histone variants: Histone monomers within a nucleosome can be replaced with different types of non-standard monomers by ATP-dependent chromatin remodelling complexes. These histone variants may offer greater accessibility of DNA to transcription mechanisms, activating genes.

12) Transposable elements: Small regions of DNA capable of relocating to other locations within the genome. They can often be transcribed and serve various functions within cells, like the formation of interference RNAs that bind to mRNA to prevent translation.

13) Exon skipping: A form of alternative splicing which involves the exclusion of an exon from the formation of mRNA from pre-mRNA.

14) Yamanaka factor genes: Genes known to be able to return differentiated cells to pluripotent cells through various mechanisms.