Chapter 1

A Short Introduction to DNA Methylation

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CONTENTS

1.1 INTRODUCTION

Almost all cells on an organism share the same genetic material encoded in the DNA sequence, but display a broad range of morphological and functional diversity. Epigenetics can be defined as the study of changes of a phenotype such as the gene expression patterns of a specific cell type not caused by underlying changes in the primary DNA sequence. These changes are mitotically and maybe in some cases meiotically heritable. Epigenetic regulation mediates genomic adaption to an environment thereby ultimately contributing toward the phenotype and "brings the phenotype into being" (1).

Epigenetics consists of a variety of molecular mechanisms including post-transcriptional histone modifications, histone variants, ATP-dependent chromatin remodeling complexes, polycomb/trithorax protein complexes, small and other non-coding RNAs including siRNA and miRNAs, and DNA methylation. These diverse molecular mechanisms have all been found to be closely intertwined and stabilize each other to ensure the faithful propagation

of an epigenetic state over time and especially through cell division. Nonetheless epigenetic states are not static, but change with age in a stochastic manner as well as in response to environmental stimuli. This review gives a brief introduction to the multiple biological facets of DNA methylation, probably the best-studied epigenetic modification, and its potential use in clinical applications.

1.2 PATTERNS OF DNA METHYLATION

DNA methylation is a post-replication modification almost exclusively found on the 5 position of the pyrimidine ring of cytosines, (Figure 1.1), in the context of the dinucleotide sequence CpG, of which around 29 million are found in the human (haploid) genome (2). The additional methyl group is located at the major groove edge in a DNA double helix

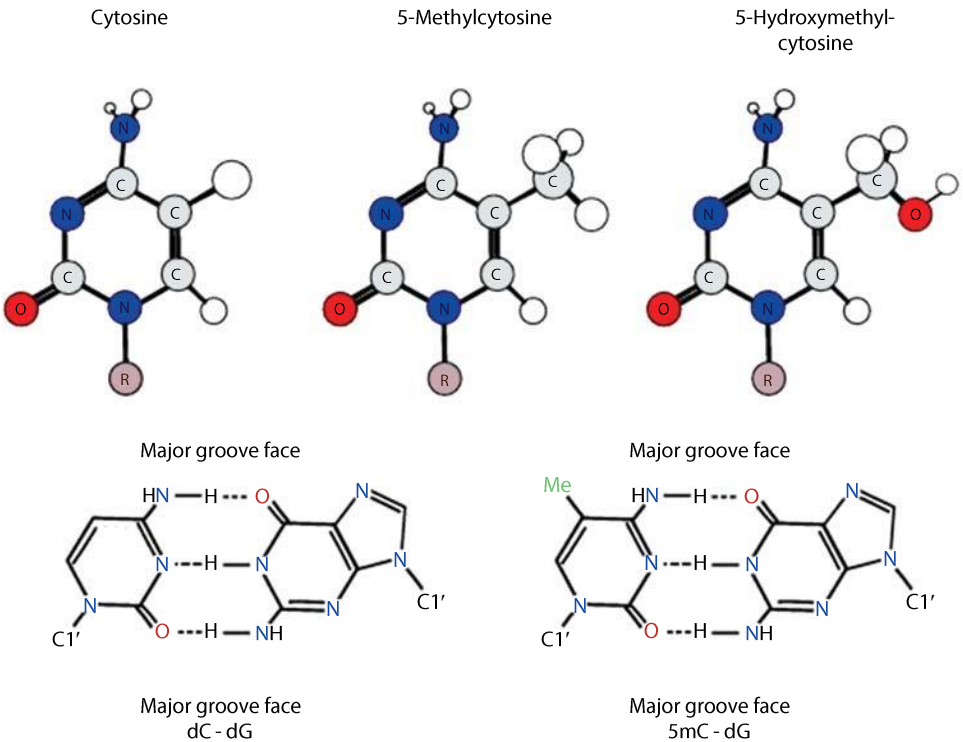

FIGURE 1.1 Chemical structure of cytosine, 5-methylcytosine, and 5-hydroxymethylcytosine. R denotes the sugar moiety, which has been omitted for simplification. Cytosine is incorporated into the DNA using deoxycytidinetriphosphate as a building block and methylated after its incorporation by DNA methyltransferases. 5-hydroxymethylcytosine is created by oxidation of methylcytosine by the TET enzymes. DNA methylation standing out in the major grove of the DNA double helix shows identical Watson-Crick base pairing compared to cytosine.

and does not change the Watson-Crick base pairing (Figure 1.1). 5-methylcytosine (5mC) accounts for ~1% of all bases, varying slightly in different tissue types and the majority (60%–80%) of CpG dinucleotides throughout mammalian genomes are methylated (Figure 1.2). Other types of methylation such as methylation of cytosines in the context of CpNpG or CpA sequences have been detected in mouse embryonic stem cells, neurons, and plants, but are generally rare in somatic mammalian/human tissues. DNA methylation marks are part of the cellular identity and memory and the sequence symmetry of CpG dinucleotides allows for the transmission of DNA methylation marks through cell division. CpGs are underrepresented in the genome, as a result of their increased mutation potential with mutation rates at CpG sites to be about 10–50 times higher than other transitional

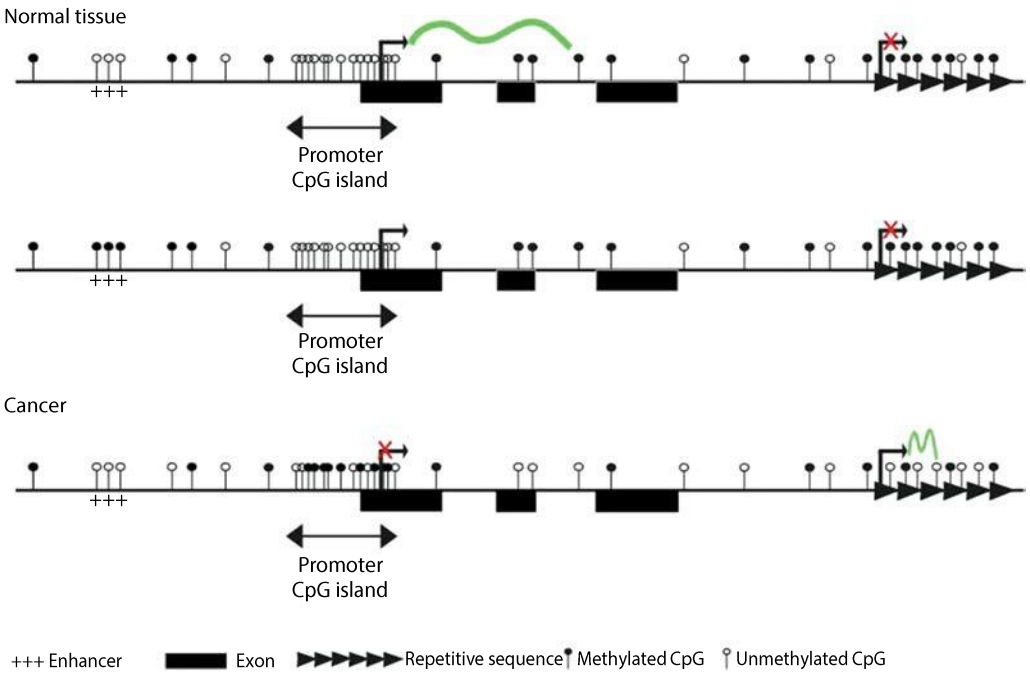

FIGURE 1.2 Distribution of DNA methylation in normal tissue and cancer. In the normal tissue, most promoter CpG islands are free of DNA methylation (indicated by white circles) even if the gene is not expressed. Repetitive elements as well as interspersed CpG dinucleotides are mostly methylated (indicated by black circles). Methylation changes at intergenic gene regulatory regions, such as enhancers, which can change the expression status of the associated gene while the methylation status of the gene does not change. In tumors, a global loss of DNA methylation (hypomethylation of the cancer genome) is observed while some promoter CpG islands become methylated in a tumor-type specific manner. Methylation patterns are dynamic and also change to a lesser extent compared to cancer with age and in response to environmental factors.

mutations. As the deamination of methylated CpGs to TpGs yields a naturally occurring DNA base, it is less well corrected. Despite this general trend, relatively CpG rich clusters, so-called CpG islands, are found in the promoter region and first exons of ~65% genes containing about 7% of all CpGs (Figure 1.2) (3). Depending on the employed set of parameters, a CpG island is defined as having a C + G content of more than 50% (55%), an observed versus expected ratio for the occurrence of CpGs of more than 0.6 (0.65) and a minimum size of 200 (500) base pairs (4). They are mostly non-methylated in all tissues and throughout all developmental stages corresponding to an open chromatin structure and a potentially active state of transcription (Figure 1.2). There are around 30,000 CpG islands in the human genome. As CpG islands are mainly unmethylated in the germline, they are less susceptible to deamination and have therefore retained the expected frequency of CpGs. Binding of transcription factors, exclusion of nucleosomes, and the presence of H3K4 methylation and the associated histone methyltransferases protect most CpG islands from DNA methylation. It should be noted that a number of CpG islands have been identified that are methylated in a tissue-specific manner in normal tissues, but concern mainly intragenic CpG islands (5,6). CpGs islands associated to genes not expressed in a specific cell type acquire the repressive histone modification H3K27Me$_3$, but rarely DNA methylation. In contrast, regions located up- and downstream of CpG islands, termed CpG island shores, show variable tissue-specific DNA methylation patterns and these are often altered in tumorigenesis (7). In contrast to CpG islands, gene bodies are commonly highly methylated, where DNA methylation has been associated with enhanced gene expression maybe by facilitating transcriptional elongation and preventing initiation of spurious transcription events (8). Intragenic methylation has in addition been associated with the repression/use of alternative promoters or different splice variants (6,9).

1.3 DNA METHYLTRANSFERASES

Both local and global epigenetic patterns are dictated by the composition of the genome depending on CpG spacing as well as sequence motifs and DNA structure (10), while in turn DNA methylation will have a major influence on DNA shape (11). The transfer of a methyl-group from the universal methyl donor S-adenosyl-l-methionine (SAMe) is carried out by DNA methyltransferases. During the methylation reaction a methyl group is transferred from SAMe to the cytosine, thereby leaving S-adenosylhomocysteine, which at high concentrations inhibits the action of DNA methyltransferases.

Four DNA methyltransferases have been identified (*DNMT1, DNMT3A, DNMT3B,* and *DNMT3L*) (Figure 1.3) (12). DNMT3L, however, lacks a catalytic domain, but is in complex with DNMT3A important for maternal genomic imprinting and male spermatogenesis.

Simplified, DNMT1 acts as a maintenance methyltransferase as it prefers hemi-methylated templates. It is located at the replication fork during the S phase of the cell

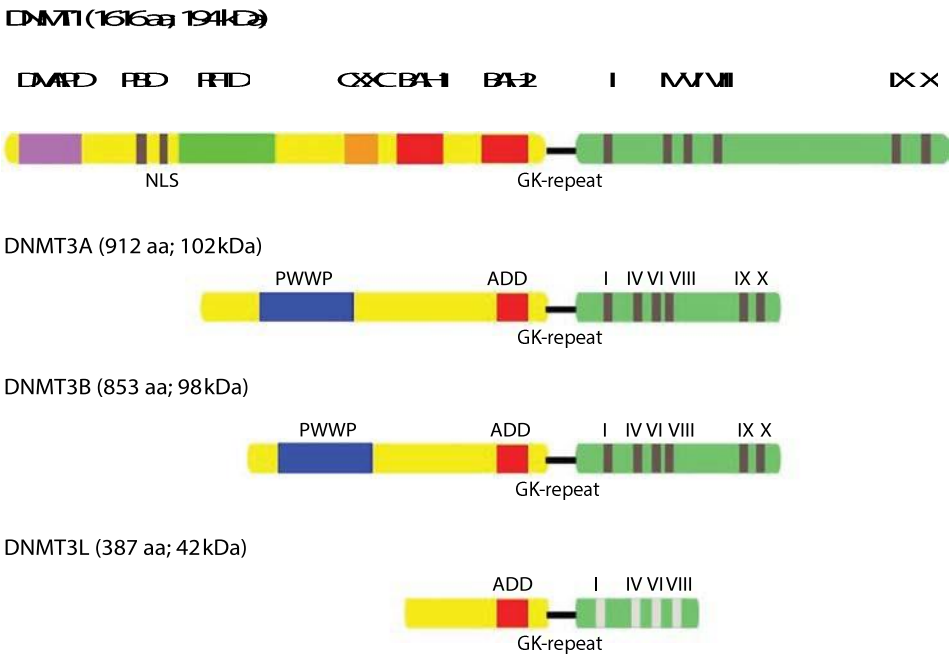

FIGURE 1.3 Schematic illustration of the domain structure of the mammalian DNMTs. All DNMTs with the exception of DNMT3L contain a catalytic domain with 10 motifs characteristic for DNA methylation activity. The DMAPD domain of DNMT1 binds DNMAP1, a factor repressing transcription through interactions with HDACs. The proliferating cell nuclear antigen (PCNA) binding domain, binds to the PBD of DNMT1, which recruits DNMT1 to replication foci at the early and middle stage of the S-phase and binds a number of factors required for replication. The RFTD domain localizes DNMT1 to the region undergoing replication by interacting with UHRF1, which recognizes hemimethylated CpGs. The CXXC domain contains two zinc atoms forming zinc fingers, which bind unmethylated CpGs. However, the exact function of this domain is currently unclear. The two BAH domains are involved in chromatin remodeling, but their exact function needs further investigation. The PWWP domain of DNMT3A and 3B is involved in protein-protein interactions, tethers the DNMTs to chromatin regions including pericentromeric heterochromatin regions marked with $H3K26Me_3$. The cysteine-rich plant homeodomain (PHD)- like ADD domain interacts with a multitude of proteins including H3K9 methylases, co-repressors, and heterochromatin protein 1. DNMT3A and 3B interact with the C-terminal domain of DNMT3L increasing *de novo* DNA methylation activity. Abbreviations used: DMAPD: DNA methyltransferase-associated protein 1 interacting domain; PBD: PCNA-binding domain; NLS: Nuclear localization signal; RFTD: Replication foci targeting domain; CXXC: CXXC domain; BAH1/2: Bromo-adjacent homology domain 1 and 2; GK-repeats: Glycine-lysine rich repeats; PWWP: PWWP domain; ADD: ATRX-DNMT3-DNMT3L domain.

cycle and methylates the newly synthesized DNA strand using the parent strand as a template with high fidelity (13). The symmetric sequence of CpGs thus allows to pass the epigenetic information to pass through cell generations. A number of proteins associated with the local chromatin structure such as LSD1 and URHF1 are required to ensure the specificity and stability of the DNA methylation reaction associated with DNA replication. However, DNMT3A and DNMT3B are also required for methylation maintenance (14). *De novo* methylation is carried out by the methyltransferases DNMT3A and DNMT3B. These enzymes have certain preferences for specific targets (e.g., DNMT3A together with DNMT3L methylates maternal imprinted genes and DNMT3B localizes at minor satellite repeats as well as the gene bodies of actively transcribed genes), but also work cooperatively to methylate the genome (12,15). Possible trigger mechanisms to initiate *de novo* methylation include preferred target DNA sequences, RNA interference, but mostly chromatin structures induced by histone modifications and other protein-protein interactions (16,17). Histone modifications such as $H3K9Me_3$ are thought to initiate heterochromatin formation and DNA methylation comes in as a secondary molecular alteration to ensure the stable silencing of the repressed sequences.

1.4 5-HYDROXYMETHYLATION AND THE DNA DEMETHYLATION PROCESSES

Mechanisms for DNA demethylation mechanism have long been searched for as active demethylation occurs at different stages of development and a global hypomethylation is associated with many cancers. DNA demethylation has been proposed to be either passive, where the 5mC is removed owing to a lack of maintaining the methylation during several cycles of replication, or as an active process, with direct removal of the methyl group independently of DNA replication (18). The active process is initiated through the enzymatic oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) (Figure 1.1) as described below in this paragraph. However, 5hmC is now considered to be not only an intermediate in oxidative DNA demethylation, but constitutes a distinct layer in the complex process of epigenetic regulation with its own distribution and regulatory functions. The reaction yielding 5hmC is catalyzed by the ten-eleven translocation (TET) methylcytosine dioxygenase family of enzymes, consisting of three mammalian subtypes, TET1-3 (Figure 1.4) (12,19). 5hmC is most abundant in human brain tissue and embryonic stem cells, but at levels approximately tenfold lower than those of 5-methylcytosine (20). TET enzymes are expressed in a tissue/cell-type and developmental stage dependent manner with 5hmC decreasing during cell differentiation. Active demethylation of gene regulatory sequences plays a key role in activating specific genes required for proper tissue-specific gene expression programs. 5hmC levels do not correlate with 5mC levels of the respective tissue and 5hmC was found enriched at specific active functional elements of the genome, in particular enhancers, promoters, and

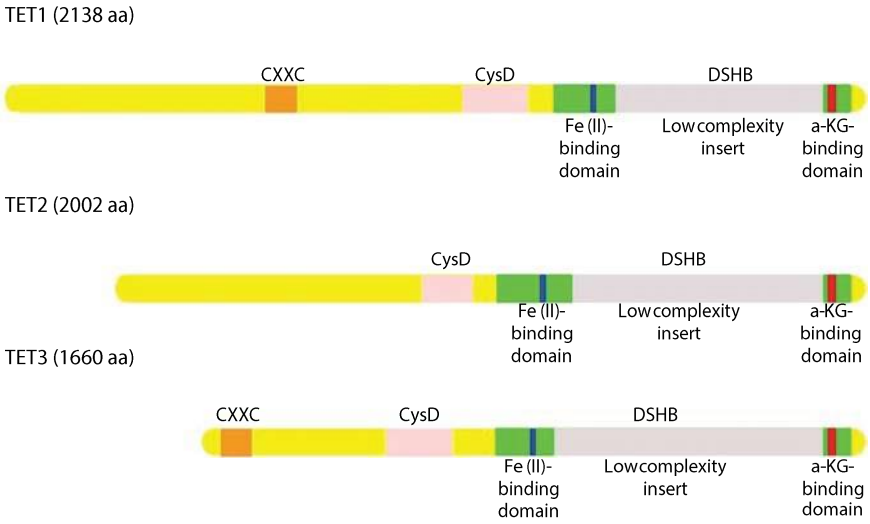

FIGURE 1.4 Domain structure of the mammalian TETs. The N-terminal region is involved in chromatin remodeling and methylation sensing and can directly bind to DNA, while the C-terminal catalytic domain consisting of a cysteine-rich domain and the double-stranded β-helix dioxygenase domain, including an Fe(II)-binding HxD motif and an α-ketoglutarate-binding domain separated by a low complexity spacer of unknown function, recognizes CpGs and oxidizes them. For TET2 the N-terminal domain is provided by a separate protein (IDAX).

gene bodies associating 5hmC with open chromatin and transcriptional activity (20,21). 5hmC levels are globally reduced in cancer and alterations of the TET enzymes have been reported for various cancers (19,22). This observation suggests that 5hmC alterations may have a distinct role in the development and progression of malignancies.

In addition to its regulatory function, 5hmC is an intermediate in the active demethylation process (23), where it is further oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) again by the TET enzymes, with the latter two modifications being present at barely detectable levels in the human genome (Figure 1.5). Both the carboxyl and the formyl groups can be removed enzymatically with or without base excision, generating an unmethylated cytosine.

1.5 TRANSCRIPTION AND GENOME STABILITY

Transcription does not occur on naked DNA but in the context of chromatin, which critically influences the accessibility of the DNA to transcription factors and the DNA polymerase complexes. DNA methylation, histone modifications and chromatin remodeling are closely linked and constitute multiple layers of epigenetic modifications to control and modulate gene expression through chromatin structure. DNMTs and histone

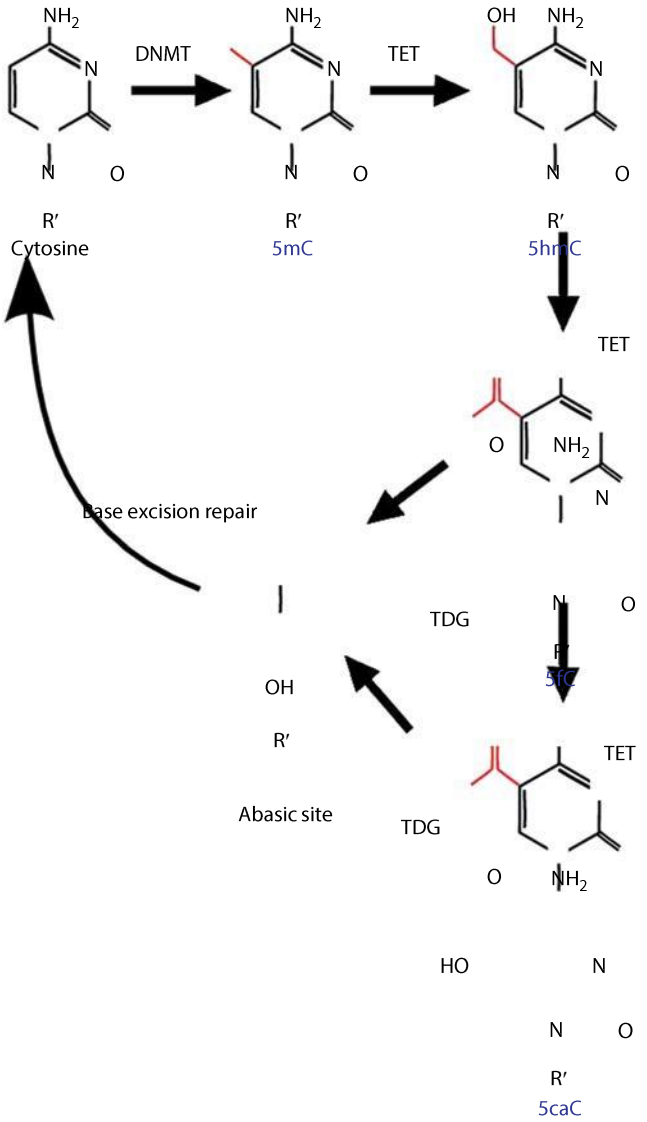

FIGURE 1.5 Demethylation pathway. TET enzymes successively oxidize 5mC to 5hmc, and 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) are dependent on α-ketoglutarate and Fe(II). 5fC and 5caC are replaced by unmodified cytosines through a thymine DNA glycosylase (TDG) initiated base excision repair (BER) mechanism, although additional mechanisms for DNA demethylation do exist.

deacetylases (HDACs) are found in the same multi-protein complexes and methyl CpG-binding domain proteins (MBDs) interact with HDACs, histone methyltransferases as well as with the chromatin remodeling complexes. Furthermore, mutations or loss of members of the SNF2 helicase/ATPase family of chromatin remodeling proteins such as ATRX or

LSH lead to genome-wide perturbations of DNA methylation patterns and inappropriate gene expression programs.

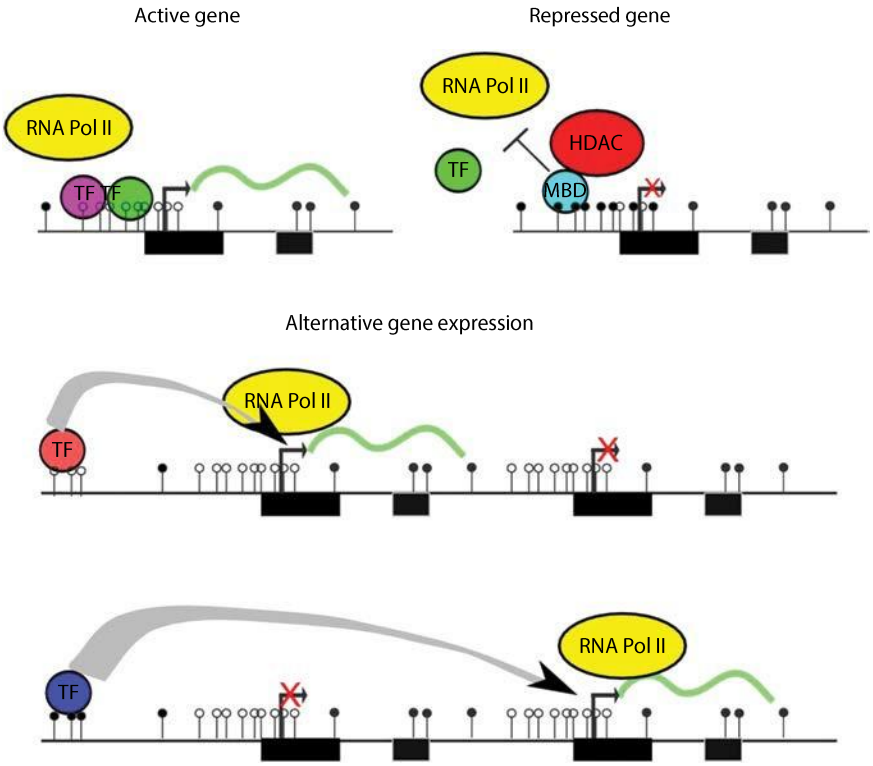

FIGURE 1.6 Methylation sensitivity of transcription factors. A simplified view how DNA methylation might influence transcription factor (TF) binding. Some TFs are only binding to unmethylated DNA and methylation will impede binding. Methylation will on the other hand attract methyl binding proteins which in complex with HDACS and other repressors will silence gene expression. Other transcription factors might also have a higher affinity for methylated compared to unmethylated DNA. Methylation changes at upstream regulatory elements such as enhancers might induce changes in the TF occupancy of the regulatory element, which can lead to activation of alternative genes or transcripts.

Transcription may be affected by DNA methylation in several ways (Figure 1.6). First, the binding of transcriptional activators such as Sp1 and Myc may be inhibited directly by the methylated DNA through sterical hindrance, while other transcription factors especially homeodomain transcription factors are attracted by methylated target recognition sequences (24–26). Methylation of CpG sites in a target sequence can thereby lead to change in transcription factor occupancy at the same sequence and activation of tissue-specific genes (27). Second, methylated DNA is bound by specific methyl-CpG binding domain (MBD1, MBD2, and MBD4) proteins or methyl-CpG binding proteins (MeCP2) as well as

proteins of the Kaiso family (12,28,29). They recruit transcriptional co-repressors such as histone deacetylating complexes, polycomb proteins and chromatin remodeling complexes, thereby establishing a repressive closed chromatin configuration (Figure 1.6). Mbd3 binds specifically hydroxymethylated cytosines.

In many cases, DNA methylation occurs subsequently to changes in the chromatin structure and is used as a molecular mechanism to permanently and thus heritably lock the gene in its inactive state. It should be underlined that an unmethylated state of a CpG island or gene regulatory element does not necessarily correlate with the transcriptional activity of the gene, but rather that the gene can be potentially activated. The simple presence of methylation does therefore not necessarily induce silencing of nearby genes. Only when a specific core region of the promoter that is often—but not necessarily—spanning the transcription start site becomes hypermethylated, the expression of the associated gene is modified (30). In CpG-poor intergenic gene regulatory regions DNA methylation is highly dynamic, CpG dinucleotides are mostly highly methylated, but methylation is reduced when the region or the methylated CpG is bound by transcription factors (31).

DNA methylation plays an important role in the maintenance of genome integrity by transcriptional silencing of repetitive DNA sequences and endogenous transposons (32). DNA methylation may prevent the potentially deleterious recombination events between non-allelic repeats caused by these mobile genetic elements. In addition, methylation increases the mutation rate leading to a faster divergence of identical sequences and disabling many retrotransposons.

The functional relevance of DNA methylation (and other epigenetic changes) can now be interrogated by epigenetic editing using mainly a nuclease deficient version of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein (Cas) 9 system, which allows recruiting chromatin modifying and remodeling complexes to specific target sequences (33). The fusion of the core catalytic domain of a DNA methyltransferase (e.g., DNMT3A) or demethylase (e.g., TET1) to a modified nuclease deficient Cas9 (dCas9) has been shown to induce specific targeted epigenetic changes either locally if a promoter is targeted or more regionally if a distant gene regulatory element such as an enhancer is targeted (33–35).

1.6 EPIGENETIC CHANGES IN HEALTH AND DISEASE

Cytosine methylation is essential for normal mammalian development including X chromosome inactivation and correct setting of genomic imprinting. Epigenetics holds the promise to explain at least a part of the influences the environment has on a phenotype as described in Chapter 5 and is an integral part of aging and cellular senescence whereby the overall content of DNA methylation in the mammalian and human genome decreases with age in all tissues especially at repetitive elements (36,37). Despite its stochastic nature,

DNA methylation levels at a number of specific loci have been shown to correlate very well with lifetime (chronological) age and several DNA methylation signatures have been developed for the accurate prediction of the age through the analysis of DNA methylation patterns (36,38). Accelerated epigenetic changing has been associated with earlier all- cause mortality in later life (39), while people with exceptional longevity show a decreased epigenetic age compared to their chronological age (40).

DNA methylation and chromatin structure are strikingly altered in many complex diseases. Mutations in genes that are part of the molecular machinery responsible for correct establishment and propagation of the epigenetic modifications through development and cell division lead to the neurodevelopmental disorders such as ICF (immune deficiency, centromeric instability, and facial abnormalities, *DNMT3B*) (41) or Rett syndrome (*MeCP2*) (42), while mutations in *TET2* have been found in multiple hematological malignancies, where they probably among the earliest genetic events of the disease (43).

Cancer is by far the most studied disease with a strong epigenetic component (44,45). In tumors, a global loss of DNA methylation (hypomethylation) of the genome is observed. This hypomethylation has been suggested to initiate and propagate oncogenesis by inducing aneuploidy, genome instability, activation of retrotransposons, and transcriptional activation of oncogenes and pro-metastatic genes (46). The overall decrease in DNA methylation is accompanied by a region- and gene-specific increase of methylation (hypermethylation) of multiple CpG islands frequently associated with transcriptional silencing of the associated gene (44,45). This hypermethylation is not random as it occurs primarily at gene promoters that are targets of the polycomb repressive complex 2 (PRC2) and marked by $H3K27Me_3$ in (embryonic) stem cells and resembles the DNA methylation changes observed during aging albeit at a much greater amplitude.

While the contribution of somatic mutations to carcinogenesis has long been recognized, it has become evident that epigenetic changes leading to transcriptional silencing of tumor suppressor genes constitute an at least equally contributing mechanism (45,47). DNA methylation changes and genetic mutations co-occurring in the same tumors show different dynamics and evolve differently during tumor progression supporting the functional relevance of epigenetic changes on the phenotype of the tumor (48). Epigenetic changes occur at higher frequency compared to genetic changes and may be especially important in early-stage human neoplasia (49).

Examples for the use of DNA methylation-based biomarkers include therefore early detection with the commercial FDA-approved Epi proColon blood test being a prime example. This test analyzes the methylation profile of an intronic sequence of the *SEPT9* gene for the population-wide screening of colorectal cancer achieving a sensitivity of 50%–80% depending of the stage of the cancer and a very high specificity (>95%) (50,51). A number

of DNA methylation changes have been linked to prognosis of different cancers (52). In addition to the application for early detection of cancers, the analysis of the methylation status of CpG islands can be used to characterize and classify cancers as for examples recently demonstrated for subtypes of Ewing sarcoma, which are all characterized by the same recurrent genetic alteration (53). Furthermore, as the DNA methylation patterns of distant metastases will at least partly carry the tissue-specific DNA methylation signature of the primary tumor, analysis of the DNA methylation profile of the metastases has shown to permit the identification of the tissue of the primary tumor (54). Similarly, the DNA methylation profile of cell-free DNA from plasma/serum contains the information of the tissue it is released from and allows detecting the tissue-of-origin of a cancer (55). DNA methylation changes detect tumor recurrence as well as predict and monitor patients' response and the effectiveness to a given anti-cancer therapy with the prediction of the response of patients with glioblastomas to the alkylating agent temozolomide based on the DNA methylation status of the DNA repair gene *MGMT* being the prime example (56). As DNA methylation is a non-mutational and therefore—at least in principle—a reversible modification, it can be used as point of departure for anti-neoplastic treatment by chemically induced demethylation (57). Two DNA methyltransferase inhibitors (DNMTis) (azacytidine [Vidaza] and 5-aza-deoxycytidine [decitabine, Dacogen, Decitibine]) have been approved for the treatment of several hematological malignancies (58,59). At low doses azacytidine and decitabine induce their effect through demethylation of silenced genes associated with reduced apoptosis, cell differentiation and proliferation, whereas at higher doses the main cytotoxic effect is due to DNA damage after incorporation (60). Second-generation DNMTis with improved pharmacology and lower toxicity such as the prodrug SGI-110 show high potential for the use in the treatment of several different malignancies (58). Epigenetic therapy is rapidly evolving with many combination therapies now under investigation.

The field of epigenetics of other complex diseases is still relatively young, but epigenetics may provide the missing link between the genetic susceptibility and the phenotype by mediating and modulating environmental influences. Several neuropsychiatric disorders have been linked to epigenetic changes (61). Epigenetic dysregulation in cognitive disorders such as Alzheimer's disease as well as age-related memory decline has also been reported (62,63). DNA methylation patterns are also disturbed in atherosclerosis (64), diabetes (65) as well as inflammatory, autoimmune, and allergic diseases (66,67). In a number of studies the epigenetic changes are located in the same genomic region as genetic variation previously associated with the disease (68,69). It is difficult to infer causality from most of studies, but at least in some case DNA methylation seems to mediate the effects of genetic variation to yield the phenotype.

1.7 CONCLUSION

Although our knowledge on the regulation of DNA methylation has been rapidly increasing over the last years, the gained information has mainly led to the insight that the complexity of the gene regulatory network, in which DNA methylation plays a pivotal role, had been underestimated. CpG islands, which had been the focus of research for decades, might contribute little to the plasticity of the cells necessary for cell-type specific differentiation and to cope with internal and environmental cues. New functional tools allow now for the first time to assess the functional consequences of DNA methylation changes during development and disease. Biomedical applications of DNA methylation as biomarkers for cancer and other complex diseases, but also for prenatal testing, have become routine and first assays have been approved by regulatory agencies paving the way for a more widespread acceptance and use of DNA methylation-based tests.

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