

Chapter 2

DNA Hypomethylation and Hemimethylation in Cancer

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Abstract In contrast to earlier views that there was much compartmentalization of the types of sequences subject to cancer-linked changes in DNA epigenetics, it is now clear that both cancer-associated DNA hypomethylation and hypermethylation are found throughout the genome. The hypermethylation includes promoters of tumor suppressor genes whose expression becomes repressed, thereby facilitating cancer formation. How hypomethylation contributes to carcinogenesis has been less clear. Recent insights into tissue-specific intra- and intergenic methylation and into cancer methylomes suggest that some of the DNA hypomethylation associated with cancers is likely to aid in tumor formation and progression by many different pathways, including effects on transcription in *cis*. Cancer-associated loss of DNA methylation from intergenic enhancers, promoter regions, silencers, and chromatin boundary elements may alter transcription rates. In addition, cancer-associated intragenic DNA hypomethylation might modulate alternative promoter usage,

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production of intragenic noncoding RNA transcripts, cotranscriptional splicing, and transcription initiation or elongation. Initial studies of hemimethylation of DNA in cancer and many new studies of DNA demethylation in normal tissues suggest that active demethylation with spreading of hypomethylation can explain much of the cancer-associated DNA hypomethylation. The new discoveries that genomic 5-hydroxymethylcytosine is an intermediate in DNA demethylation, a base with its own functionality, and a modified base that, like 5-methylcytosine, exhibits cancer-associated losses, suggest that both decreased hydroxymethylation and decreased methylation of DNA play important roles in carcinogenesis.

2.1 Introduction

Altered methylation of DNA in human cancers was first described as overall genomic hypomethylation in various cancers vs. a wide variety of normal tissues [1] and as hypomethylation of a few gene regions in colon adenocarcinomas vs. normal colonic epithelium [2]. Almost all types of cancers exhibit both hypermethylation of some DNA sequences and hypomethylation of others relative to appropriate controls that account for the tissue specificity of DNA methylation [3]. The cancer-associated hypermethylation and hypomethylation of the genome are generally independent of each other [4, 5]. Until recently, it appeared that cancer-specific changes in DNA methylation were usually hypermethylation of unique gene regions and hypomethylation of DNA repeats, albeit with many notable exceptions [6–11]. Deep sequencing of the genome has revealed far greater size and complexity to the transcriptome than previously appreciated [12]. Similarly, recent whole-genome analysis of the cancer methylome demonstrates that there is much more cancer-linked hypomethylation of unique gene sequences and hypermethylation of repeated sequences than previously found, although there are differences in the frequency with which subsets of sequences undergo hypo- or hypermethylation [13–18].

This chapter reviews new insights into genome-wide DNA and chromatin epigenetics in normal cell populations as well as in cancers [19–29]. Recent studies are drawing attention to previously unsuspected roles of epigenetic marks in the body of genes as well as at promoters and intergenic transcription control regions. These findings are likely to be relevant to the biological impact of cancer-associated DNA hypomethylation. In addition to effects on normal gene expression, cancer-associated DNA hypomethylation probably favors oncogenesis by enhancing recombination [30–33]; occasionally activating a small number of endogenous retroviral elements [34, 35]; altering the intranuclear positioning of chromatin; and modulating the sequestration transcription factors at tandem DNA repeats, as reviewed previously [3, 6]. In addition, the little-studied area of DNA hemimethylation in cancer is discussed in this chapter in the context of our growing understanding of pathways for the conversion of genomic 5-methylcytosine (5mC) residues to C residues.

2.2 Genomic Hypomethylation Profiles in Cancer and Their Relevance at Promoters and Enhancers

Until recent high-resolution genome-wide analyses of DNA methylation, cancer-specific portions of methylomes were considered to consist predominantly of hypomethylated DNA repeats and hypermethylated gene regions [3, 7, 36]. DNA repeats are often used as a surrogate for average genomic methylation changes (usually losses of 5mC), with DNA epigenetic changes in some classes of repeats more closely associated with certain tumor types [6, 18, 35, 37–39]. In our 1983 analysis of global DNA hypomethylation in human cancers by high performance liquid chromatography analysis of enzymatic DNA digests [1], we fractionated one adenocarcinoma DNA into highly repetitive, moderately repetitive, and unique sequence classes. Because we found that each of these cancer DNA fractions had similar ratios of mol% 5mC to those from normal human tissues, we concluded that cancer-linked hypomethylation was not confined to repeated DNA. Indeed, cancer-linked DNA hypomethylation often occurs in unique sequences in and around genes, including metastasis-associated genes, as originally revealed in studies using CpG methylation-sensitive restriction endonucleases or sodium sulfite-based methods to study individual gene regions [2, 6, 40].

Recent genome-wide studies of DNA methylation in various normal and cancer cell populations indicate much tissue specificity throughout the genome in normal samples and pervasive cancer-linked DNA hypomethylation and hypermethylation [13, 15, 16, 41–45]. Regions of cancer-associated changes in DNA methylation are found in short interspersed or clustered regions as well as in long blocks [7, 42, 44, 46, 47]. There is increasing evidence for cause-and-effect relationships between normal tissue-specific DNA hypomethylation and increased transcription as well as many associations between cancer-linked hypomethylation and cancer-linked increases in gene expression [16, 17, 19, 21, 24, 48–55]. The inverse relationships between expression and DNA methylation include imprinted genes implicated in carcinogenesis [56].

A small percentage of annotated gene promoters overlap tissue-specific (T-DMR) or cancer-specific (C-DMR) differentially methylated DNA regions [49, 57]. However, most of the non-imprinted, autosomal T-DMR promoters are not the main type of vertebrate DNA promoters, which are part of CpG islands (CGIs, a class of CpG-rich regions surrounded by CpG-poor DNA). Among the genes with T-DMR promoters are some that become activated upon experimentally induced demethylation with a low dose of 5-deoxyazacytidine but not upon treatment with a histone deacetylase inhibitor, trichostatin A [49].

Enhancers too sometimes show a correlation between upregulation of expression of the associated gene and DNA demethylation in normal cells. For example, the binding of FoxA1/FOXA1 to enhancers is inhibited by site-specific DNA methylation at the corresponding binding site [58]. This differentiation-associated transcription regulatory factor can open up DNA compacted in chromatin of inactive enhancers (as a “pioneer” factor) and then recruit effector transcription factors to

make the enhancer active [59–61]. A window of DNA demethylation provided by previous binding of FoxD3, another pioneer factor, allows recruitment of FoxA1 and conversion of the enhancer to a state that is poised for activity. Moreover, in embryonal stem cells, local DNA demethylation per se, rather than any changes in histone H3K27 or H3K9 methylation, is associated with the binding of pioneer factors to certain tissue-specific non-CGI promoters [58]. Pioneer factors, including FOXA1, are implicated in various types of carcinogenesis [62]. Given the extensive hypomethylation of DNA in cancers, many known and yet more unknown enhancer regions are likely to become demethylated specifically in tumors. However, specific losses of DNA methylation from transcription regulatory regions might facilitate, but not independently cause, changes in expression [63].

Broad DNA regions enriched in hypomethylation are sometimes also associated with increases in copy number of DNA regions and can, thereby, synergistically increase expression of some of the affected genes [13, 33, 42]. Such broad regional hypomethylation (which can encompass occasional sites of persistent methylation) might reflect higher order chromatin structure. The latter is influenced, in turn, by the type, frequency, and spacing of DNA repeats; the G+C and CpG contents of subregions; the gene density; the nucleosome density; broad regions of distinct histone composition modification; and the presence of clusters of co-regulated genes. Nonetheless, a long region of cancer-linked DNA hypermethylation can be adjacent to a region of cancer-linked DNA hypomethylation with a sharp border between them, as demonstrated for a tandem repeat array (D4Z4) and its border sequences [9]. Despite evidence for functionality, DNA demethylation in cancer probably involves frequent overshooting of targeted sequences. These are referred to as *passenger* DNA methylation changes [64]. The hypomethylation in cancers of many more sites than are biologically relevant is probably due to a relaxed specificity of the demethylation apparatus during carcinogenesis and tumor progression and to the spreading of DNA demethylation patterns.

2.3 Genomic Hypomethylation in Cancer Within Gene Bodies

Recent findings implicate intragenic epigenetic marks in the regulation of normal gene expression. T-DMRs have been found inside many genes, and increased methylation in the central gene body or downstream promoter-flanking region of certain subsets of genes is associated with increased transcription [23, 65–68]. Moreover, there are nonrandom associations between positions of CpG methylation within genes and exon–intron boundaries, distance from the transcription start site, and distance from the 3' end of the gene [66, 69]. Besides first exons, T-DMRs are present in various exonic and intronic sequences, including internal CGIs, sequences adjacent to internal CGIs (“CGI shores”), insulators, intragenic ncRNA genes, and 3' terminal regions [17, 19, 28, 59, 70, 71]. They are present in both repeated and unique sequences. These findings are consistent with the many interrelationships between DNA and chromatin epigenetics and tissue-specific chromatin epigenetic marks inside genes [65, 68, 72, 73].

Differentiation-related DNA and/or chromatin epigenetic marks within genes may help determine alternative promoter usage, modulate the rate of transcription initiation or elongation, and possibly help direct the choice of alternative splice sites [19, 21, 24, 26, 27, 29, 34, 52, 74, 75]. The average DNA methylation level in the central portion of moderately expressed genes is associated with higher average transcription levels, possibly by being related to nucleosome positioning [76]. For example, immediately downstream of proximal CpG-poor promoters, it was unexpectedly found that methylation of sequences antagonizes binding of Polycomb repressor complexes [68]. Methylation of gene-body CGIs appears to be associated with repression of intragenic promoters [28]. However, for some sets of genes under certain conditions, lower expression was correlated with increases in gene-body methylation [69].

With respect to alternative splicing, evidence implicates certain histone modifications in helping to regulate the choice of splice junctions by altering rates of transcription, nucleosome positioning, or direct interactions with proteins that mark exon–intron junctions of pre-mRNA [77, 78]. Changes in physiological conditions can alter the chromatin modifications at these junctions and concomitantly modulate exon skipping [78]. DNA methylation may also be involved in regulating alternative splicing because of the many DNA methylation/chromatin epigenetic interrelationships and the finding that intron–exon junctions are enriched in sharp transitions in DNA methylation levels [66]. A recent report that malignant prostate cancer cells have enrichment of DNA hypermethylation at exon–intron junctions [45] is consistent with the cancer-linked involvement of DNA methylation levels in determining alternative splicing.

Programmed changes in DNA methylation in intra- and intergenic regions are not restricted to differentiation-related events. For example, electroconvulsive stimulation of mouse neuronal cells *in vivo* was recently demonstrated to cause rapid decreases and increases in DNA methylation in a substantial minority of CpG sites, especially at CpG-poor regions [69]. The physiologically linked DNA demethylation included rapid demethylation of exons and introns in various positions of the genes. Importantly, there was enrichment in these DNA epigenetic changes in the vicinity of brain-related genes. Thus, there is ample precedent from studies of normal cell functioning to suggest that cancer-associated DNA hypomethylation in intronic and exonic sequences can modulate the amount and type of gene products and thereby contribute to tumor formation or progression.

Cancer-linked DNA hypomethylation in the gene body is illustrated in Fig. 2.1 for three genes whose expression has been reported to be altered in certain cancers [79–81]. *TGFB2* has an intronic Alu repeat that was hypomethylated in some cancer cell lines relative to a wide variety of normal tissues (Fig. 2.1a) and untransformed cell cultures. The only exceptions to this intronic region being highly methylated in normal tissues and cell strains were found in skeletal muscle (Fig. 2.1a), myoblasts, and myotubes (data not shown). Their hypomethylation at this site might be related to the significant upregulation of *TGFB2* in myoblasts and myotubes vs. 19 types of non-muscle cell cultures [82] and is an example of the frequent relationship between targets for cancer-associated hypo- or hypermethylation and targets for differentiation-associated epigenetic changes [17, 83]. Like *TGFB2*, *PRDM16* (Fig. 2.1b) exhibited gene-body hypomethylation in

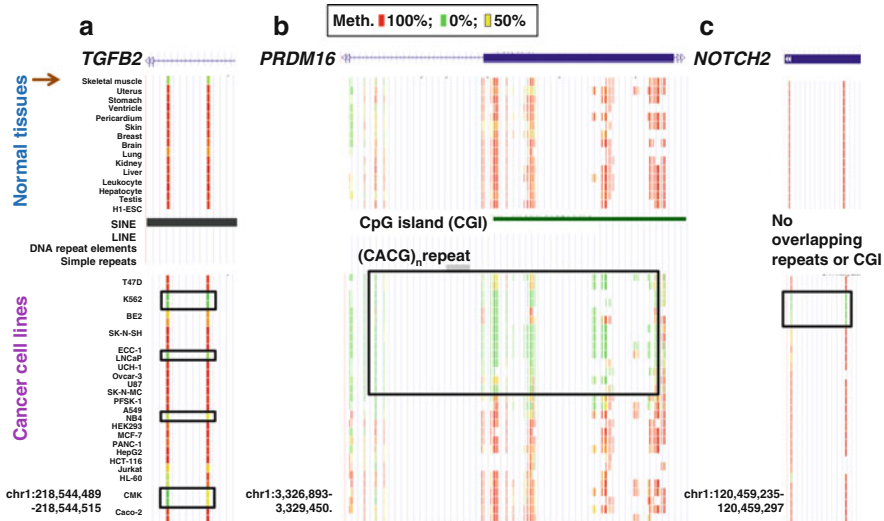


Fig. 2.1 Examples of cancer cell-associated hypomethylation (*boxed*) within gene bodies and overlapping a DNA repeat (**a**), a CGI (**b**), or neither (**c**) as determined by whole-genome analysis using reduced representation bisulfite sequencing (RRBS). (**a**), *TGFB2*, intron 1; the cancer hypomethylation overlaps an Alu repeat that is also hypomethylated in skeletal muscle (see *arrow*). (**b**), *PRDM16*, exon 9 and intron 8; the cancer hypomethylation overlaps a CGI and CGI shore. (**c**), *NOTCH2*, exon 34; no overlapping repeats or CGI. In contrast to the cancer-derived cell lines, non-immortalized cell strains (not shown) showed the same hypermethylation seen in normal tissues with the exception of myoblasts and myotubes for *TGFB2*. Myoblasts and myotubes overexpress *TGFB2* relative to 19 other types of cultured cell populations. All analyses were done in duplicate, and representative duplicates are shown

some of the cancer cell lines; however, this hypomethylation was in a region largely overlapping a CGI in an exon. *NOTCH2* (Fig. 2.1c) also showed gene-body hypomethylation in several cancer cell lines, but this hypomethylation was neither in a subregion with a CGI nor a DNA repeat. We note that some of the cancer cell lines with *TGFB2* or *PRDM16* gene hypomethylation also displayed cancer cell-linked promoter hypermethylation (data not shown).

Recently, the presence of 5-hydroxymethylcytosine (5hmC) as the sixth naturally programmed base in vertebrate DNA has been established [84]. It is generated from 5mC by hydroxylation via the enzymes TET1, TET2, or TET3 and is even more highly tissue specific in its relative levels in DNA than is 5mC [84–86]. It is implicated in stem cell renewal and distinct types of differentiation [87–89], as described further in an accompanying chapter by Pradhan and Kinney. Like 5mC, 5hmC is enriched in certain intragenic regions and exhibits major decreases in its genomic levels in cancer [84–86]. However, unlike 5mC, exons, intragenic CGIs, and enhancers have significantly elevated 5hmC levels relative to other portions of the genome [87, 90, 91]. These findings further highlight the need for studies of the functional significance of decreases in intragenic DNA epigenetic marks in cancer. In addition, they introduce a complication into almost all studies to date of 5mC that use either bisulfite or conventional CpG methylation-sensitive

restriction analysis to distinguish 5mC from unmethylated C, as these methods cannot resolve 5hmC and 5mC [69, 92, 93]. Therefore, a caveat to conclusions about 5mC distribution is that 5hmC might have been monitored instead, especially in exonic or enhancer regions in more 5hmC-rich tissues like brain [84, 85]. However, in some other cell types, like breast, heart, cell lines, and cancers, 5hmC is very much lower [84–86, 93], and 5hmC levels are also low in intronic and intergenic regions [90, 94].

2.4 Hypomethylation of DNA Repeats in Cancer

Global losses of DNA methylation with less numerous increases in methylation in other portions of the genome are typical of cancer [5, 6] although there are exceptions [18]. A major contributor toward the overall DNA hypomethylation is hypomethylation of tandem and interspersed DNA repeats, which is observed in most examined cancers [6, 95–97]. Most hypomethylation of DNA repeats in cancers is apparently the result of demethylation and not preexisting hypomethylation in a cancer stem cell [3], with the exception of seminomas as discussed below. Besides the effects on transcription and possible effects on alternative splicing described in the previous section, hypomethylation of a minor portion of interspersed DNA repeats may occasionally cause induction of retroviral element transcription [35]. In addition, hypomethylation of certain promoter-containing interspersed DNA repeats may affect chromatin boundaries resulting in effects on transcription of nearby genes [98, 99].

In a study of mononuclear cells from a few patients with chronic lymphocytic leukemia vs. the analogous cells from controls, Dante et al. described hypomethylation of LINE-1, a highly repeated interspersed repeat [100]. Hypomethylation of LINE-1 and Alu repeats was subsequently observed in many other types of cancers [38, 101–104]. Similarly, we found that tandem repeats in centromeric and juxta-centromeric satellite DNA are frequently hypomethylated in breast adenocarcinomas, ovarian epithelial cancers, and Wilms tumors [30, 105, 106], as confirmed for many other types of cancers [3, 107]. Additional classes of tandem repeats (including macrosatellite DNAs) and segmental duplications are also susceptible to DNA hypomethylation in malignancies [9, 18, 39, 43, 83, 108–110], although different subclasses of DNA repeat families can vary in their susceptibility to loss of DNA methylation in cancer [38, 39, 102, 111–113]. In some cancers, satellite DNA repeats showed the strongest DNA hypomethylation of all types of sequences analyzed [18, 33].

The frequency of cancer-associated hypomethylation of DNA repeats depends on the grade, the stage, and the individual tumor specimen [46, 114]. This hypomethylation is seen sometimes in non-tumor tissue adjacent to the cancer and in benign neoplasms and tissue lesions such as breast fibroadenomas and ovarian cystadenomas, although often to a lesser extent than in cancers [13, 51, 95, 105, 106, 112, 115]. In a mouse model of prostate tumor progression, repeat DNA hypomethylation was observed at the stage of prostatic intraepithelial neoplasia and prior to promoter hypermethylation [116]. However, depending on the tumor type

or specimen, repeat DNA hypomethylation may increase with tumor progression, a relationship inferred since the 1980s [1, 117]. In many types of cancer, repeat DNA hypomethylation is a highly informative prognostic marker and/or predictor of survival [46, 107, 118–122].

2.5 DNA Hypomethylation and Germ Cells: Comparison to Cancer Hypomethylation

Differential methylation of testes-specific genes has some similarities to cancer-associated DNA hypomethylation. Most genes that are specifically expressed in testis (like the cancer-testis genes) have little or no methylation in their promoter regions in testis and sperm although they are highly methylated, and transcriptionally repressed, in somatic tissues [123]. In sperm, as well as in many cancers, tandem DNA repeats and certain subclasses of interspersed DNA repeats display low methylation levels compared with normal postnatal somatic tissues [38, 112, 124–126]. Reminiscent of the tendency (with many exceptions, as described above) towards DNA repeats and unique sequences having opposite methylation changes in cancer, single-copy genes become demethylated but tandem and interspersed repeats retain their methylation in murine primordial germ cells at 12.5–13.5 dpc [123].

Another interface between the germ line epigenome and cancer is seen in the exceptionally strong global DNA hypomethylation in seminomatous testicular germ cell tumors. In our 1982 study of 62 tumors representing 23 different types, we found that a testicular seminoma had only 1.4% of its genomic C present as 5mC, while the next lowest 5mC level for a cancer was 2.4% [1]. The range of genomic 5mC levels among the normal tissues that we studied was 3.5–4.1% of C residues methylated. Smiraglia et al. confirmed the extraordinary depletion of 5mC in the genomes of many seminomas [127]. This finding has been ascribed to the origin of seminomas from primordial germ cells that had undergone massive demethylation before oncogenic transformation without subsequent de novo methylation thereafter [127, 128]. Importantly, seminomas generally show none of the CGI hypermethylation so prevalent in other types of cancer, but rather display extreme overall DNA hypomethylation [127]. Therefore, cancers can develop without gene region hypermethylation but with extreme overall genomic hypomethylation.

2.6 Opposite Cancer-Linked Changes in DNA Methylation in DNA Repeats: Hypo- and Hypermethylation

Opposite types of cancer-linked DNA methylation changes can occur in the same DNA sequence, as we found in a Southern blot study of methylation of NBL2, a 1.4-kb sequence repeated in tandem mostly near the centromeres of acrocentric chromosomes [39]. NBL2 was hypomethylated at HhaI sites (5'-CGCG-3' sites) in

17% of ovarian carcinomas and hypermethylated in >70% of ovarian carcinomas and Wilms tumors at the same sites [39]. Various normal postnatal somatic tissues exhibited partial methylation at HhaI sites in NBL2 and were similar to each other in their methylation patterns at this tandem repeat. Using NotI (5'-GCGGCCGC-3') for Southern blotting, only the cancer-linked hypomethylation of NBL2 was previously observed [108, 110] because NotI cleaves control somatic DNA too infrequently to reveal hypermethylation in cancers. This is an example of the importance of considering the technique used in evaluating results on DNA methylation [92] as well as the appropriate control DNA for comparison to the cancer. A few cancer DNAs digested with HhaI displayed two distinct fractions of NBL2 sequences, one with overall hypermethylation and the other with overall hypomethylation relative to all the somatic controls, which suggests that the repeats at one chromosomal location underwent de novo methylation and at another underwent demethylation during carcinogenesis. Hairpin genomic sequencing [129] (see below) at two ~0.3-kb subregions of the 1.4-kb *NBL2* ([8] and Nishiyama and Ehrlich, unpublished data) confirmed that hypomethylation at NBL2 predominated in some cancers and hypermethylation in others in comparison to normal somatic tissues, which displayed much site specificity in the methylation status of individual CpG sites. Therefore, a small region of DNA can be made unstable epigenetically during carcinogenesis so that CpG sites that are very near to each other undergo opposite changes in DNA methylation. The plasticity of the directionality of methylation changes at DNA repeats in cancers has also been seen in recent genome-wide studies [15, 18].

D4Z4, a heterologous tandem array (macrosatellite) located at subtelomeric 4q and 10q, also exhibited strong hypomethylation in the bulk of the array in some cancers and hypermethylation in others of the same type [9]. Several of the cancers had extremely high levels of methylation in more than three consecutive 3.3-kb repeat units of D4Z4, indicative of the spreading of de novo methylation. This methylation spreading seems to have limits to its processivity and to be prone to stop at certain subregions of the repeat unit.

2.7 Tagging Classes of DNA Sequences for Demethylation

Because NBL2 and D4Z4 tandem repeats displayed overall hypomethylation in some cancers and hypermethylation in others, it was highly informative to compare their methylation changes in a given cancer. Among 17 ovarian carcinomas and 44 Wilms tumors, there was a significant correlation ($p < 0.001$) between the direction (either hypo- or hypermethylation) and degree of methylation change (strong, moderate, or weak) at D4Z4 and the dissimilar NBL2 [9]. This suggests that diverse sequences on different chromosomes may be similarly tagged for demethylation or de novo methylation (methylation of symmetrically unmethylated CpG dyads) during carcinogenesis. However, many cancers with extensive hypermethylation of D4Z4 and NBL2 repeats displayed hypomethylation of another, heterologous tandem repeat, juxtaacentromeric satellite 2 on chromosome 1 (Sat2) [39].

NBL2 (mostly in the short arm of the acrocentric chromosomes) and D4Z4 (in the subtelomeric region of chromosomes 4 and 10) are both rich in G+C and look like very long CGIs. However, they differ appreciably in their G+C composition (61% and 73%, respectively) and their CpG content (5.7% and 9.9%, respectively). Analysis of histone modification and DNaseI sensitivity has been done for D4Z4 and indicates that its chromatin has properties midway between constitutive heterochromatin and unexpressed euchromatin [130, 131]. In contrast, Sat2, which is in the pericentromeric region, is constitutively heterochromatic and highly condensed in interphase. It has only 38% G+C but, nonetheless, it has 5.1% CpG. Therefore, the CpG suppression seen in the overall genome is not evident in Sat2. Sometimes even Sat2, with its rather CpG-rich character, becomes hypermethylated in cancers at a CpG dyad that exhibits a low methylation level in normal somatic tissues [132].

That the G+C content and chromatin structure is important for recruiting machinery for either demethylation or de novo methylation is consistent with our findings on the HpaII site immediately proximal to the D4Z4 array. It is located in a 0.2-kb D4Z4-proximal subregion that has 43% G+C, while D4Z4 has 73% G+C in all of its essentially identical, tandem 3.3-kb repeats. This 0.2-kb sequence immediately adjacent to the array is prone to tumor-linked hypomethylation even in cancers displaying strong hypermethylation within the array [9]. Surprisingly, even the adjacent D4Z4 repeat unit at the proximal end of the array became hypomethylated in cancers with hypermethylation of the bulk of the array. Probably, the array-adjacent sequence with its much lower G+C content helps confer a different chromatin structure on the neighboring D4Z4 repeat unit, which, in turn, affects the directionality of cancer-linked methylation change. Interestingly, a study of tandem transgenic repeats in mice revealed that, in some animals, all of the (G+C)-rich transgene units became methylated except for one copy adjacent to cellular DNA [133]. Despite the regional properties of DNA and chromatin that may recruit cancer-associated DNA methylation or demethylation apparatus, there are, as mentioned above, very local sequence-specific effects which allow individual CpG dyads to circumvent regional demethylation or de novo methylation [8, 9].

DNA demethylation both influences and is strongly influenced by histone modifications. For example, histone H3 trimethylation at lysine 4 (H3K4me3) correlates best with the lack of DNA methylation around the transcription start site [66]. This was found for both CGI promoters [134] and promoters that do not contain a CGI, and for CpG methylation as well as the appreciable amount of CpA methylation in embryonal stem cells [66]. A histone H3 unmethylated at lysine 4 has been implicated as necessary for de novo methylation by DNMT3A in conjunction with its interacting partner DNMT3L [135]. Increased activity of the histone lysine demethylase LSD1 (KDM1A), which, depending on its interacting partners, demethylates K4- or K9-methylated histone H3, has been found to correlate with an adverse outcome and a less differentiated phenotype in neuroblastomas [136]. Conversely, mutation of the *Lsd1* gene blocks murine gastrulation [137] and results in global DNA hypomethylation. This may be partially due to the need for Lsd1/LSD1 to demethylate the DNMT1 enzyme itself and thereby increase its stability

[137] but also could reflect the role of this enzyme in the demethylation of H3K9me3. There are many other players that could influence DNA methylation during carcinogenesis by their effects on chromatin structure, e.g., poly(ADP-ribosyl)ation, other types of histone modifications, histone variants, nonhistone chromatin proteins, specific interactions with DNMT proteins, and modulation of the set of DNA methyltransferase isoforms produced at the RNA or protein levels [138–143]. Nonetheless, multi-functionality of LSD1 in its ability to demethylate proteins and both activating and repressive histone methylation marks may serve as a paradigm for how, paradoxically, there can be both increases and decreases in DNA methylation in a given cancer cell.

2.8 Active Versus Passive DNA Demethylation

There are two broad classes of mechanisms by which 5mC residues can be replaced by C residues (DNA demethylation). During replicative or repair DNA synthesis there may be a failure to methylate the newly synthesized DNA strand at a symmetrically methylated CpG dyad (passive demethylation), which will initially result in a hemimethylated dyad (Fig. 2.2). If this failure occurs again at the same CpG dyad in the next round of replication, then a symmetrically unmethylated CpG dyad will be the result. Active demethylation involves 5mC residues being physically replaced with C residues (at the base or mononucleotide level) or, less likely, the methyl group being removed enzymatically. Accumulating evidence favors mainly active demethylation contributing to the naturally occurring DNA demethylation by the replacement of C residues [144, 145]. Active demethylation is consistent with

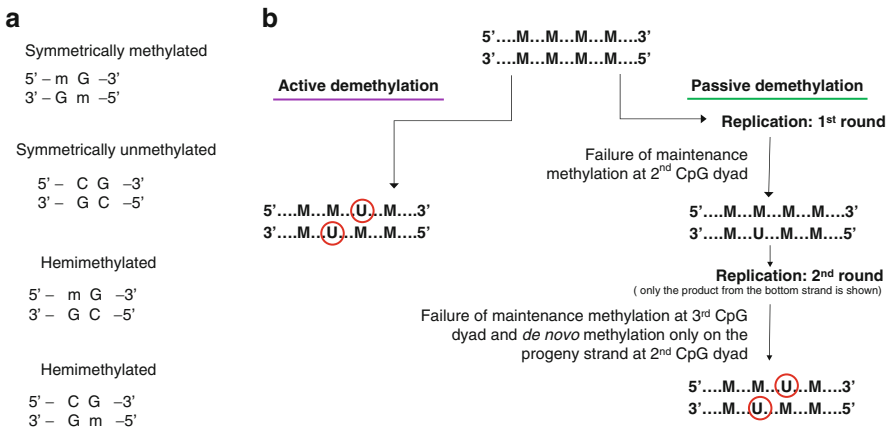


Fig. 2.2 Findings of consecutive hemimethylated dyads of opposite orientation in normal and cancer cells are best explained by active demethylation. (a) m, 5mC; C, unmethylated cytosine. (b) M, 5'-5mCpG-3'; U, 5'-CpG-3'. The generation of hemimethylated dyads of opposite orientation by passive demethylation would involve improbable changes in the second round of replication

the rapid and distributive loss of 5mC and the replication independence that has been demonstrated for many examples of naturally programmed demethylation of mammalian genomes [146, 147]. However, passive demethylation or a combination of active and passive demethylation due to inadequate maintenance methylation [148] is likely to also play a role in normal and pathological decreases in DNA methylation. Hemimethylated dyads (Fig. 2.2) can be intermediates in both active and passive demethylation of DNA as well as being intermediates in maintenance methylation.

2.9 Maintenance of DNA Methylation Patterns Through Hemimethylated Intermediates

The processes by which DNA methylation patterns are maintained are highly relevant to understanding how DNA demethylation occurs. Over 30 years ago, mechanisms for the inheritance of DNA methylation were initially proposed [149, 150]. In the traditional view, methylation at each site is assumed to be governed by the processes of de novo methylation and maintenance methylation, and these processes are independent of one another. The maintenance of methylation patterns has been attributed to the methyltransferase Dnmt1. As summarized in a 2009 review by Jones and Liang, “The basis of this model is that DNA methylation patterns are established in germ cells and in developing embryos by the activity of the de novo DNA methyltransferases Dnmt3a and Dnmt3B. Subsequently, methylation patterns are inherited after DNA replication primarily owing to the activity of Dnmt1, which has a preference for hemimethylated sites that are generated through DNA synthesis” [151]. The premise of independently acting mechanisms for de novo and maintenance methylation has led to the construction of stochastic models for methylation inheritance [152–157].

2.10 Alternative Mechanisms for Maintenance Methylation

The accepted dogma of de novo methylation catalyzed by DNMT3A/Dnmt3a, DNMT3B/Dnmt3b, and maintenance methylation through obligatory hemimethylated intermediates via DNMT1/Dnmt1 has recently been called into question. According to the original model for maintenance methylation, hemimethylated CpG dyads (Fig. 2.2) should be short-lived and difficult to detect. However, as early as 1986, demethylation with long-lived hemimethylated CpG dyads was observed at individual CpG sites in the avian vitellogenin II gene following treatment with estradiol, which suggested an active pathway through excision repair and/or enzymatic demethylation [158]. A later study of the rat alpha-actin gene promoter provided evidence for hemimethylated intermediates persisting more than 48 hours prior to becoming fully demethylated and suggested active demethylation involving

cis-acting DNA elements [159]. Subsequently, Liang et al. [160] developed an assay that allowed determination of hemimethylation at *HpaII* sites (CCGG). In mouse embryonic stem cells, levels of hemimethylation in some repetitive sequence regions were significantly higher than the traditional model of maintenance methylation by Dnmt1 would predict. By looking at gene knockouts for *Dnmt1* and *Dnmt3a* and *Dnmt3b*, they deduced that ongoing de novo methylation by Dnmt3a or Dnmt3b in a highly cooperative manner with Dnmt1 in embryonal stem cells compensated for inefficient maintenance methylation by Dnmt1 in these regions. These results suggest a constant, rather than sporadic or only differentiation-associated, role for de novo methylation in vivo. They concluded that sequences would gradually become demethylated without this constant role for de novo methylation to compensate for inefficient replication-coupled maintenance methylation. Furthermore, in a study by Chen et al. [161], loss of Dnmt1 gave only a 10% decrease in methylation overall following one cell cycle of replication in human colorectal carcinoma cells. This conditional knockout resulted in hemimethylation of 18% of sites analyzed by hairpin genomic sequencing in the CGI of an L1 transposable element. The overall level of methylation at CpG dyads in these sequences in cells with normal Dnmt1 was around 85% with no detectable hemimethylation.

In the alternative model for maintaining DNA methylation patterns that was proposed by Jones and Liang [151], DNMT1, the most abundant DNA methyltransferase is still considered to be primarily a maintenance methylase and is responsible for most of the replication-associated DNA methylation. However, they propose that DNMT3A and DNMT3B enzymes remain bound to nucleosomes that contain high levels of DNA methylation. Following replication, CpG dyads whose methylation fails to be correctly maintained by DNMT1 would then be “corrected” by DNMT3A and DNMT3B, so that these enzymes would preserve highly methylated regions without strictly “reading” the patterns on the parental strand. In this way, the methylation state of a region is maintained rather than a site-specific methylation pattern. In addition, DNMT1 might participate in some of this correction of lingering hemimethylated sites that have left the vicinity of the replication fork, perhaps recruited by proteins such as UHRF1 which recognizes hemimethylated sites (see below). This concept of repair methylation is consistent with findings that methylation patterns in highly methylated regions tend to vary among molecules and higher rates of de novo methylation are observed in highly methylated sequences [129]. Moreover, non-CpG methylation at asymmetrical sites, which is found mostly in embryonal stem cells [70], should rely on de novo methyltransferase activity for perpetuating the DNA methylation patterns, as described below.

In cancers, the frequent presence of long blocks of hypomethylated DNA [7, 16, 42, 47, 105] and the usual predominance of overall decreases rather than increases in 5mC content of the genome suggest that passive demethylation contributes to cancer-associated genomic hypomethylation. Passive demethylation might involve either a lack of methylation of hemimethylated sites by DNMT1 or a failure of DNMT3A or DNMT3B to retain dense methylation of a normally highly methylated region. However, the current, more layered view of the maintenance of DNA methylation patterns suggests that while some of the demethyla-

tion of DNA in cancer occurs by a failure of maintenance methylation, most is due to an active mechanism. Recent studies of normal differentiation- or physiology-associated DNA demethylation support an active type of DNA methylation involving enzymatically catalyzed modification of 5mC residues to 5hmC residues (and subsequent oxidation products) or thymine residues followed by DNA repair [162–164]. Three-step processes for active DNA demethylation have been proposed in which 5mC is first enzymatically modified; then demethylated on one strand, most likely by excision repair; and later fully demethylated by a mechanism that avoids inducing double-strand breaks during removal of both 5mCs of a 5mCpG dyad [165]. The last step could involve a repair mechanism that preferentially acts on hemimethylated substrates [165] or passive demethylation of a hemimethylated or hemihydroxymethylated dyad. The latter could be due to the 5hmC residues on one strand of a hemihydroxymethylated dyad not being recognized for maintenance methylation [148].

UHRF1 (also known as NP95) is a cofactor that interacts specifically with hemimethylated DNA and may participate in demethylation as well as de novo methylation of cancer epigenomes. UHRF1 also interacts with DNMT1, and even more strongly with DNMT3A and DNMT3B [166], and thereby, may be involved in the recruitment of DNMT3A/3B to unmethylated regions during tumorigenesis leading to de novo methylation [167]. However, recent work on gliomas has identified the disruption of DNMT1, PCNA, and UHRF1 interactions as a crucial oncogenic event promoting DNA hypomethylation-induced tumorigenesis in the absence of DNMT1 deficiencies [168]. Thus, while upregulation of UHRF1 may contribute to the silencing of tumor suppressors through de novo methylation, the disruption of DNMT1/PCNA/UHRF1 interactions might result in cancer-associated DNA hypomethylation affecting transcription.

2.11 Insights into Cancer-Associated DNA Demethylation from Studies of DNA Hemimethylation

The introduction of hairpin-bisulfite PCR (hairpin genomic sequencing) by Laird et al. in 2004 [129] has enabled the observation of the methylation status on both strands of individual DNA molecules on a site-by-site basis. In bisulfite-based genomic sequencing, bisulfite causes deamination of unmethylated C residues, but not methylated C residues [169]. Hairpin genomic sequencing allows analysis of methylation at every CG dinucleotide pair in a given region on covalently linked DNA strands of a restriction fragment. A caveat about these studies of DNA hemimethylation is that bisulfite-based DNA methylation analysis cannot distinguish between 5hmC and 5mC, as described above, and 5hmC on one strand at a CpG dyad is not recognized for maintenance methylation [170]. Therefore, it is possible that the detected hemimethylation is actually a CpG dyad with one unmethylated C residue and one 5hmC residue. However, in the studies of tandem DNA repeats in cancers described below, this is unlikely because 5hmC is predominantly in gene

regions and all studied cancers and cancer cell lines have extremely low levels of 5hmC [84–86].

By sodium bisulfite-based whole-methylome analysis using next-generation sequencing, Lister et al. analyzed more than 90% of the cytosines in human H1 embryonic stem cells (H1 ES) and IMR90 fetal lung fibroblasts [70]. While nearly all of the methylcytosines detected in the IMR90 fibroblasts were in the CG dinucleotide context, considerable methylation in non-CG contexts (mCHG and mCHH, where H = A, C or T) was observed in the H1 stem cells, comprising almost 25% of the total methylation, in agreement with a recent study by Laurent et al. [66]. Methylation at mCHG sites in H1 ES was also highly asymmetrical, with 98% of such sites observed to be methylated on only one strand. Non-CpG methylation was also found to be significantly higher on the antisense strand of gene bodies, suggesting a nonrandom bias in the observed asymmetry. Non-CpG methylation disappeared upon differentiation of the H1 stem cells, but was restored in differentiated cells induced to form pluripotent stem cells. These findings suggest that asymmetrical methylation at non-CG dinucleotide sites may contribute to maintenance of the pluripotent state. They are reminiscent of the less frequent, hemimethylated CG dinucleotide sites that we and Laird et al. have seen in various DNA repeats [8, 132, 171] or single-copy sequences [129] in normal or cancer tissues.

2.12 Hemimethylated CpG Dyads in Cancer

Although reports of DNA hemimethylation in cancer are few, our studies of hemimethylated DNA in cancers support the involvement of active demethylation in generating cancer-linked genomic hypomethylation. We analyzed DNA methylation changes in depth at the above-mentioned tandem repeats NBL2 and at Sat2 in ovarian epithelial tumors and Wilms tumors by hairpin genomic sequencing [8, 132]. In a study of 13 CpGs in a 0.2-kb subregion of Sat2 in ovarian carcinomas and somatic control tissues, hairpin genomic sequencing not only revealed significantly greater clonal variability in methylation patterns in the cancers than in diverse control tissues but also provided statistically significant evidence of clustering among both hemimethylated and fully demethylated sites [132]. Runs of hemimethylated sites with identical orientation were seen at higher than expected rates in the cancers. Similarly, an analysis of 14 CpGs in the NBL2 repeat unit identified both hypomethylation and hypermethylation in ovarian carcinomas and Wilms tumors, again with a high degree of clonal variation in methylation patterns within each sample [8].

Diverse control and cancer samples contained some DNA clones derived from unusual, consecutive hemimethylated CpG dyads of opposite polarity. Figure 2.2b illustrates how an M/U (5'-5mCpG-3'/3'-GpC-5') dyad near a U/M dyad (5'-CpG-3'/3'-Gp5mC-5') could be generated by active vs. passive demethylation. Passive demethylation would require inhibition of maintenance methylation (by DNMT1 alone or in conjunction with DNMT3A and DNMT3B, as discussed above) at a single CpG dyad in one round of replicative DNA synthesis. The next round of replica-

tion would then have to involve both asymmetrical de novo methylation of only the opposite strand of this dyad and inhibition, once again, of maintenance methylation at a neighboring CpG dyad. In contrast to this highly unlikely sequence of events, active demethylation can easily explain the generation of various patterns of hemimethylation in contiguous CpG dyads with either identical or opposite orientation.

In a simulation study jointly analyzing the Sat2 and NBL2 regions, we found that the observed methylation patterns in the carcinomas were best explained by a mechanism that accounted for site-to-site correlation [157]. Prior studies have produced evidence of spreading of methylation in cancer [172–176]. Our analysis suggests that demethylation may progress by spreading as well.

We propose that during carcinogenesis a highly methylated DNA sequence becomes partially demethylated by active demethylation. The sequence may then attain a density of 5mC residues in an atypical intermediate range. This intermediate level of methylation might confer less stability during successive cell divisions for maintenance of the methylation pattern or methylation density. The stability of a given partially methylated sequence could be determined, in part, by the efficiency with which DNMT3A and DNMT3B recognize unmethylated CpG sites in the sequence for repair methylation. Abnormally low methylation levels may favor the generation of yet lower levels, with some site-specific effects superimposed on the regional ones. Thus, active demethylation might start cancer-associated demethylation and a failure of maintenance methylation (including repair methylation) might continue it. The result could explain the observation that tumor progression is frequently linked to a progressive decrease in methylation.

2.13 Conclusions

Recently, there has been a burst of studies increasing our understanding of the importance of changes in DNA methylation in intragenic, promoter, and intergenic regions during differentiation and in response to some types of physiological change. These findings suggest that much more of the cancer-associated DNA hypomethylation contributes to tumor formation and progression than previously recognized. Similarly, high-resolution analysis of cancer methylomes in comparison to appropriate controls indicates that the extent of cancer-linked hypomethylation is larger than previously appreciated and affects a greater variety of DNA sequences. We propose that the pathways for normal DNA demethylation that operate during differentiation or induction of certain physiological changes become hijacked during carcinogenesis and tumor progression, leading to the initiation of cancer-associated DNA demethylation. This demethylation then may spread in *cis* by both additional rounds of active demethylation and by passive demethylation involving failures in classical maintenance methylation and replication-associated repair methylation. The net result of some of this cancer-associated DNA demethylation could be abnormal modulation of transcription and even some aberrant posttranscriptional processing of transcripts as well as increases in DNA recombination, thereby contributing to tumor formation and progression.

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