Chapter 26
DNA Methylation and Cancer

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Abstract Cancer has been considered a genetic disease with a wide array of well-characterized gene mutations and chromosomal abnormalities. Of late, aberrant epigenetic modifications have been elucidated in cancer, and together with genetic alterations, they have been helpful in understanding the complex traits observed in neoplasia. "Cancer Epigenetics" therefore has contributed substantially towards understanding the complexity and diversity of various cancers. However, the positioning of epigenetic events during cancer progression is still not clear, though there are some reports implicating aberrant epigenetic modifications in very early stages of cancer. Amongst the most studied aberrant epigenetic modifications are the DNA methylation differences at the promoter regions of genes affecting their expression. Hypomethylation mediated increased expression of oncogenes and hypermethylation mediated silencing of tumor suppressor genes are well known examples. This chapter also explores the correlation of DNA methylation and demethylation enzymes with cancer.

26.1 Introduction

Cancer is a complex and heterogeneous disease characterized by uncontrolled growth and cellular machinery that has gone haywire. In order to account for the diverse molecular changes that occur in different cancer types it has been conceptualized that cancer encompasses many diseases. Pathologists view cancer as acquiring properties of cells belonging to different developmental stages but appearing inappropriately in the tumors (Pitot 1986). The two-hit hypothesis proposed by Knudson (1971) remains the basis of correlating the genetic events with cancer

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initiation even today. For instance, in retinoblastoma, two genetic events are required in the retinal cell, which would result in the inactivation of both copies of the tumor suppressor – retinoblastoma gene (*RB*). In the familial set up where all cells of a person inherit a mutated allele of *RB*, a single mutation in the normal functional allele can give rise to cancer whereas in non-hereditary cancer, both mutations need to take place in the same cell. This genetic model of cancer has been supported by characterization of a wide array of molecular changes that occur among cancer types including the mutations which in small numbers are seen in benign, non-invasive tumors and the large-scale genetic changes and genetic instability found in invasive and metastatic tumors (Feinberg et al. 2006). However, there are a few shortcomings to the Knudson's two-hit model. Firstly, the model does not take into account the heterogeneity and complexity observed in tumors and secondly, tumors that show aneuploidy, polyploidy, and complex karyotypes are often incompatible with the two-hit hypothesis (Dutrillaux et al. 1991). Moreover, and of late it is becoming amply clear that genetic mechanisms alone cannot account for all the diverse molecular changes seen in tumors. Epigenetic mechanisms which provide an additional layer of control for gene expression have been proposed to be an important factor in cancer development and postulated to provide for one of the hits required for carcinogenesis.

26.2 Cancer as an Epigenetic Disease

The term "epigenetics" defines the study of all meiotically and mitotically heritable changes in gene expression patterns that are not coded in the DNA sequence itself (Egger et al. 2004). Epigenetic modifications like DNA methylation and histone modifications can bring about changes in gene function or dosage similar to those obtained in case of mutations, chromosomal rearrangements and gene duplications. Studies in tumor tissues have revealed that a given gene can exhibit multiple epigenetic changes just as the numerous possible genetic alterations (Feinberg et al. 2006). Thus, integrating the data on epigenetic regulation of gene expression with their genetic alterations is of utmost importance for understanding the mechanisms underlying cancer development. In 1983, through the pioneering efforts of Andrew Feinberg and Bert Vogelstein, loss of methylation at CpG dinucleotides in cancer cells was identified, and can be considered as the first report on the correlation of epigenetics with cancer. They found that a substantial proportion of CpG dinucleotides in the human genome had lost DNA methylation in cancer cells (Feinberg and Vogelstein 1983). Around the same time, Ehrlich's group also found that the global levels of 5-methylcytosine were reduced in cancer cells (Gama-Sosa et al. 1983). Though there was initial skepticism about this correlation, today the role of DNA methylation has become well established in multiple cancers (Jones and Baylin 2002; Feinberg and Tycko 2004). This loss of methylation has been reported in every tumor type studied; both benign and malignant; and interestingly even pre-malignant adenomas exhibit altered DNA methylation (Goelz et al. 1985; Feinberg et al. 1988).

Although hypomethylation of CpG islands was the first epigenetic change identified in cancers, it has not been given as much importance as has been to hypermethylation of CpG islands. Baylin and colleagues in 1986 identified site-specific hypermethylation of *CALCITONIN* gene in small-cell lung cancer (Baylin et al. 1986). The first correlation of CpG island hypermethylation with the inactivation of a tumor-suppressor gene was established for the Retinoblastoma gene (*RB*) when *RB* promoter was found to be methylated in a significant subset of sporadic and hereditary retinoblastomas (Greger et al. 1989). Promoter hypermethylation has now been shown for several other loci to be the cause of gene inactivation in cancer. Hypermethylation is observed within the CpG islands of specific genes and represents a change in 5-methylcytosine distribution at specific loci in the genome rather than an overall increase in the total amount of methylation. In many cases, this *de novo* methylation of CpG islands occurs early in the process of carcinogenesis and surprisingly can even be detected in the apparently normal epithelium of patients-a process that is associated with aging (Issa et al. 1994; Ahuja et al. 1998) and inflammation (Coussens and Werb 2002; Nelson et al. 2004; Lu et al. 2006). In fact, aging is considered the most important risk factor for the development of most malignancies of adulthood as DNA methylation patterns in aging cells have a tendency to be aberrant, similar to what is seen in transformed cells (Neumeister et al. 2002).

Another important link that has broadened the role of epigenetics in cancer is the correlation between chromatin organization and DNA methylation. The earliest of experiments probing this link were done in the Cedar and Graessmann laboratories. They found that pre-methylated, naked DNA templates when transfected or microinjected into cells became transcriptionally silent after getting packaged into a repressive form of chromatin (Keshet et al. 1986; Buschhausen et al. 1987). Foremost amongst the chromatin modifications are the covalent modifications of histones that can change the chromatin conformation and control gene activity. Cytosine methylation can attract methylated DNA-binding proteins and histone deacetylases to the methylated CpG island, leading to chromatin compaction and gene silencing (Jones et al. 1998; Nan et al. 1998). This has further strengthened the link between the two major epigenetic components of gene regulation. In addition, correlation has also been made between covalent histone modifications and nucleosomal remodeling (Esteller 2006). It is now well established that the three processes of DNA cytosine methylation, histone modification and nucleosomal remodeling are intimately linked and that alterations in these processes result in the epigenetic changes which would lead to events like the permanent silencing of cancer-relevant genes and genomic instability. Working on mouse models, Jaenisch and colleagues demonstrated that *Dnmt1* hypomorphic mutation reduces the frequency of intestinal neoplasia when crossed to Apc^{Min} mice (Gaudet et al. 2003) whereas, a high frequency of lymphomas were observed in mice with hypomorphic *Dnmt1* allele (Eads et al. 2002). Together, these data indicated that cancer risk is associated with a disruption in the balance of methylation rather than hypomethylation and hypermethylation *per se*.

Based on the subtle differences observed in the epigenetic profiles of stem cells and the cancer cells, several researchers have proposed the theory of cancer stem

cells, which suggests that stem cells are the more likely targets of epigenetic disruption leading to cancer. “Cancer stem cells” (CSCs), which constitute a small minority of neoplastic cells within a tumor, are the abnormal stem cells generated from normal stem cells upon accumulation of series of progressive genetic/epigenetic changes. CSCs are now well characterized in the hematopoietic system and colon cancers (O’Brien et al. 2007; Mani et al. 2008). The incompetence of many of the anti-cancer chemotherapeutic agents has been attributed to the presence of cancer stem cells, against which these drugs are less effective (Ren 2005).

Thus, in the past 20 years, cancer epigenetics has transformed into a full-fledged field with a focus on the different mechanisms involved in epigenetic regulation including DNA methylation, histone modifications and nucleosomal remodeling. In addition, the discovery of epigenetically regulated imprinted genes and their role in cancer has also added another dimension to this field (Jirtle 1999; Jelinic and Shaw 2007; Hirasawa and Feil 2010). While the finer mechanistic detail of the role of epigenetic modifications in cancer progression still needs to be worked out, epigenetic events are recognized as possibly one of the hits required in Knudson’s hypothesis (Yu and Shen 2002). Initially, the clonal genetic model of cancer was widely accepted, wherein cancer arises through a series of mutations in oncogenes and tumor suppressor genes. This would give rise to a monoclonal population of tumor cells and epigenetic changes were viewed as surrogate correlates of cancer. But according to the recently proposed epigenetic progenitor model, cancer begins with an epigenetic alteration of stem/progenitor cells within a tissue, which is followed by a gatekeeper mutation involving a tumor suppressor gene or an oncogene, leading to genetic and epigenetic instability (Feinberg et al. 2006).

26.3 DNA Methylation and Cancer

The epigenetic landscape is maintained by interplay between the key modifications: DNA methylation, histone tail modifications, and small RNA molecules. However, in this review we focus on the interplay between DNA methylation machinery and carcinogenesis. In the altered epigenetic setup of a cancerous cell, aberrant DNA methylation is one of the most well studied epigenetic mechanisms affecting gene expression. Collated below is data pertaining to the role of DNA methylation machinery in establishing the normal and altered epigenetic makeup of the human genome.

26.3.1 CpG Islands and CpG Methylation

Methylation of DNA is a stable modification and can be inherited through cell divisions. Methylation of DNA in mammals primarily takes place at the 5′ position of cytosine in the context of 5′-CG-3′ dinucleotide (Sinsheimer 1955; Bird 2002).

Although non-CpG methylation has been reported in mammals (Ramsahoye et al. 2000), its presence is miniscule as compared to CpG methylation. The haploid human genome contains approximately 50 million CpG dinucleotides, which represents $2^{50,000,000}$ different permutations of CpG methylation. CpG dinucleotides are unevenly distributed across the human genome with vast DNA stretches deficient in CpGs, interspersed by CpG clusters called CpG islands (Laird 2003). CpG islands were identified almost 20 years ago on the basis of the strikingly discordant patterns of digestion of genomic DNA by restriction enzyme isoschizomers that differed only by their sensitivity to cytosine methylation (Singer et al. 1979). CpG islands have been defined by different groups based on various criteria. Initially they were defined as stretches of DNA (200 bp or longer) with a (G+C) content of 0.50 or greater and an observed to expected CpG dinucleotides ratio of 0.60 or greater. Later on, Takai and Jones (2003) found that increasing the size threshold to 500 bp and the (G+C) content threshold to 0.55 biased the definition against repetitive sequences and included only unique CpG sequences. According to computational estimates, there are at least 29,000 CpG islands in the human genome (Lander et al. 2001; Venter et al. 2001). CpGs are vastly under-represented in the genome mainly because deamination of cytosine gives rise to uracil, which is recognized as foreign in the DNA and hence replaced. Moreover, deamination of methylcytosine gives rise to thymine, which is less readily recognized as foreign and therefore prone to mutation (C:G to T:A transition) and depleted in the genome (Duncan and Miller 1980). Interestingly, CpG islands are found at the promoter sites of approximately 50% of the genes in the human genome (Loshikhes and Zhang 2000), most of which are the 'housekeeping' genes, where they are kept free from methylation (Larsen et al. 1992; Ponger et al. 2001). On the other hand, CpG islands within transposable elements are heavily methylated (Yoder et al. 1997). The methyl group in CpG dinucleotides protrudes from the cytosine nucleotide into the major groove of the DNA and has two main effects: it displaces transcription factors that normally bind to the DNA; and it attracts methyl binding proteins, which in turn are associated with gene silencing and chromatin compaction (Fazzari and Greally 2004). Methylation of DNA in the common 'B' form facilitates a conformational change to the 'Z' form, increases the helical pitch of DNA and alters the kinetics of cruciform extrusion (Murchie and Lilley 1989; Zacharias et al. 1990). Promoters with a few exceptions are generally inactive when the CpG island within them are methylated (an exception, for example, is seen in the case of the H-2K gene promoter, Tanaka et al. 1983). The transcriptional machinery can respond to CpG methylation in different ways. Either methylation at specific sites within the CpG island can interfere with the efficiency of expression (as seen in the case of Herpes simplex thymidine kinase and the Epstein-Barr virus latency C promoters) or it can be the density of methylation (human α- and γ-*GLOBIN*, mouse *MyoD1* promoter) that is responsible for interfering with transcription (Zingg and Jones 1997). Moreover, since the structure of 5-methylcytosine is similar to thymine, the methylation of cytosine might lead to generation of new consensus sequences for some transcription factors. This has been seen in the case of methylation at CpG in a low affinity AP-1 binding site that converts it to high affinity site (Tulchinsky et al. 1996) or in the CRE sequence

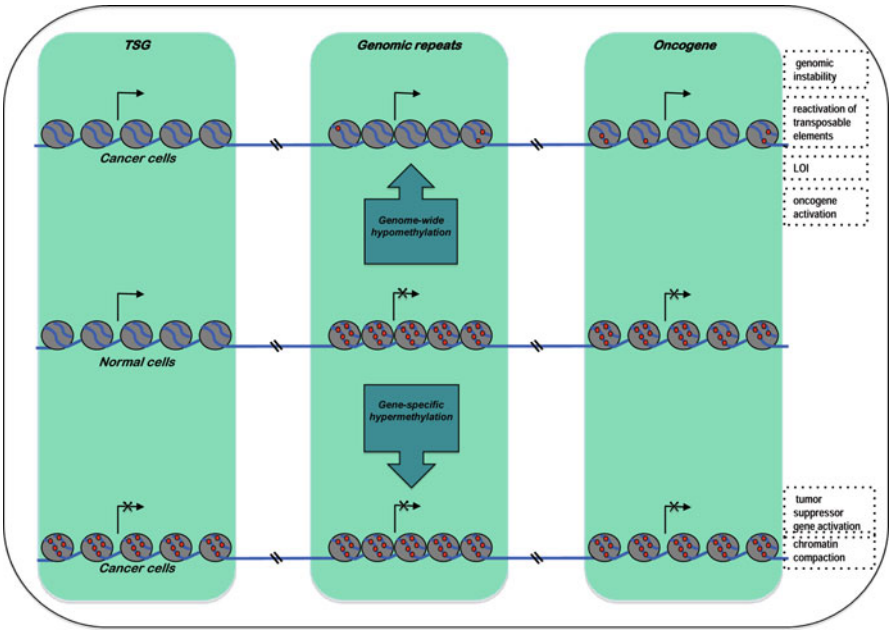

Fig. 26.1 Aberrant methylation during cancer progression. In normal cells, while promoter CpG islands of most genes lack methylation, repeat elements and promoters of oncogenes are kept inactive by DNA methylation. Genome-wide hypomethylation causes activation of oncogenes and activates aberrant transcripts from within repeat elements leading to genomic instability. On the other hand, hypermethylation of tumor suppressor genes (TSG) leads to their silencing. *LOI* loss of imprinting, *TSG* tumor suppressor gene. *Raised arrows* denote transcriptional activation; *crossed arrows* denote transcriptional repression. *Red circles*: Methyl CpG and *circles* with *curved lines* denote nucleosomal organization of chromatin

which enhances the binding of C/EBPα transcription factor (Rishi et al. 2010). Similarly, the binding of several other DNA binding proteins (AP-2, Ah receptor, CREB/ATF, E2F, EBP-80, c-Myc, NF-κB) is inhibited when the CpG within their recognition sequence is methylated (Jones and Gonzalgo 1997).

26.3.2 Aberrant DNA Methylation in Cancer

Global hypomethylation and gene-specific hypermethylation are the hallmarks of most cancers studied (Fig. 26.1 and Feinberg and Tycko 2004). Global alterations in DNA methylation have been observed not only in fully developed cancers, but also in the precancerous stage, including chronic inflammation, persistent viral infection and cigarette smoking. Furthermore, aberrant DNA methylation is significantly associated with aggressiveness of cancers and the poor outcome in cancer patients.

26.3.2.1 Genome-Wide Hypomethylation

The earliest evidence linking DNA methylation to cancer came with the discovery of global hypomethylation in tumors (Riggs and Jones 1983). Many CpG islands are normally methylated in somatic tissues (Strichman-Almashanu et al. 2002). During global hypomethylation, these methylated islands can become hypomethylated in cancers activating the nearby genes. High-throughput genomic DNA methylation studies have identified that the frequency of hypomethylated sites might be quite high in tumors and numerous genes have been identified that lose DNA methylation in different cancers (Adorjan et al. 2002; Lacobuzio-Donahue et al. 2003). The important link between DNA hypomethylation in cancer and chromosomal instability was established by Ehrlich and colleagues (Tuck-Muller et al. 2000). DNA hypomethylation was found to be particularly severe in pericentromeric satellite sequences or other DNA repeat elements, and several cancers including Wilms tumor, ovarian and breast carcinomas frequently contain unbalanced chromosomal translocations with break points in the pericentromeric DNA of chromosome 1 and 16 (Qu et al. 1999; Ehrlich 2009; Yoshida et al. 2011). Hypomethylation of satellite sequences might predispose them to breakage and recombination. Jaenisch and colleagues showed that neurofibromatosis 1 $(Nf1)^{+/-}$ $Trp53^{+/-}$ mice were 2.2 times more prone to loss of heterozygosity (LOH) when a hypomorphic *Dnmt1* allele was introduced (Eden et al. 2003). Similarly, hypomethylation of L1 retrotransposons promote chromosomal rearrangements in colorectal cancer (Suter et al. 2004; Ogino et al. 2008). LINE1 hypomethylation has been implicated in cancers of bladder, ovary, liver and colon (Kim et al. 2009; Dammann et al. 2010; Schernhammer et al. 2010; Wilhelm et al. 2010). The mechanism behind global hypomethylation in cancers largely remains unknown but several experiments point towards the involvement of SWI/SNF chromatin-remodeling complexes. Individuals with the developmental disorder ATRX (α-thalassaemia, myelodysplasia) have mutations in the *ATRX* gene, which encodes a SNF2-family helicase. In mutant ATRX cells, the ribosomal DNA repeats are hypomethylated (Gibbons et al. 2000). *Lsh*, a SNF2-family member is required for maintenance of normal DNA methylation as its knockout leads to global defect in genomic methylation and chromosomal instability (Fan et al. 2003). A common splice variant of the *de novo* DNA methyltransferase-*DNMT3B* (*DNMT3b4*) that was identified in patients with liver cancer, is also associated with hypomethylation of pericentromeric satellite sequences (Saito et al. 2002). Further, it was shown that mice carrying a hypomorphic allele for the maintenance methyltransferase *Dnmt1* (which reduces expression of Dnmt1 to 10% of wild type levels), developed tumors early and showed chromosomal instability (Gaudet et al. 2003). Hypomethylation of CpG islands has been implicated in overexpression of *CYCLIND2* and *MASPIN* in gastric carcinoma, *MN/CA9* in human renal cell carcinoma, *S100A4* metastasis-associated gene in colon cancer and human papillomavirus 16 (*HPV16*) in cervical cancer (Nakamura and Takenaga 1998; Cho et al. 2001; Badal et al. 2003; De Capoa et al. 2003; Lacobuzio-Donahue et al. 2003; Oshimo et al. 2003; Piyathilake et al. 2003; Sato et al. 2003).

Global DNA hypomethylation is associated with progression of multiple types of cancers including cervical, ovarian, multiple myeloma, chronic lymphocytic leukemia and breast cancer (Cho et al. 2010; Missaoui et al. 2010; Fabris et al. 2011; Walker et al. 2011). In case of gastric, tongue and esophageal carcinogenesis, global DNA hypomethylation was negatively correlated with invasiveness (Baba et al. 2009; Tomita et al. 2010).

26.3.2.2 Gene-Specific Hypermethylation

Aberrant transcriptional silencing of genes associated with DNA hypermethylation of their promoter region is probably the most intensely studied epigenetic abnormality in cancers. It is difficult to answer as to why certain genes become methylated during carcinogenesis, but several hypotheses have been proposed to address this issue. The Darwinian theory suggests the selective growth advantage conferred to cells upon inactivation of particular genes as the reason why they become methylated in tumors (Esteller 2005). Also, it has been suggested that genes which are under the control of polycomb proteins are more vulnerable to DNA methylation (Ohm and Baylin 2007). Like DNA hypomethylation, hypermethylation can play a seminal role in neoplastic evolution. Promoter DNA hypermethylation can silence specific genes including tumor suppressor genes in cooperation with histone modifications. For example, hypermethylation was found to be associated with deacetylation of histone H3 and H4, loss of histone H3- lysine4 methylation and gain of H3K9 methylation (Baylin and Ohm 2006).

While Retinoblastoma (*RB*) was the first tumor suppressor gene shown to be silenced by DNA hypermethylation, $p16^{ink4A}$ is one of the most common tumor suppressors exhibiting loss of function following DNA hypermethylation. It exhibits DNA hypermethylation during progression of lung cancers and even in preneoplastic lesions (Belinsky et al. 1998; Nuovo et al. 1999) and its germ line loss leads to increase in hematopoietic stem cell (HSC) life span in terms of their ability for tissue maintenance and repair (Janzen et al. 2006; Krishnamurthy et al. 2006; Molofsky et al. 2006). The silencing of $p16^{ink4A}$ gene has also been reported in preinvasive stages of breast, colon and other cancers. Furthermore, experimental loss of $p16^{ink4A}$ appears to facilitate early tumorigenesis by being permissive for subsequent emergence of genomic instability (Kiyono et al. 1998) and may directly allow for additional epigenetic silencing of other genes (Reynolds et al. 2006). Germ line mutations of many tumor suppressors cause familial forms of cancers. The same tumor suppressor genes have been found to be promoter DNA hypermethylated in subsets of non familial cancers such as *VHL* in renal, *APC* in colon and *BRCA1* in breast cancers (Ting et al. 2006a, b). Till date, a large number of tumor suppressor or candidate tumor suppressor genes have been identified to be DNA hypermethylated in multiple cancer types, including but not limiting to: *p53, RASSF1A, p14ARF, CDKN2A, p16, p21, TIMP3, ECRG4, HIC1* (Cohen et al. 2003; Amatya et al. 2005; Chanda et al. 2006; Gotze et al. 2009; Juhlin et al. 2010; Dadkalos et al. 2011; Radpour et al. 2011).

Tumor suppressors are not the only genes to exhibit DNA hypermethylation; a multitude of other genes like *hMLH1, MGMT, E-CADHERIN, CALCITONIN*, etc. are silenced in cancers (Esteller 2007; Jacinto and Esteller 2007). Apart from individual gene hypermethylation, in some cancers, groups of genes were found to exhibit increased DNA methylation levels. Two such examples identified recently in cancers are, CpG island methylator phenotype (CIMP) and Long Range Epigenetic Silencing (LRES). CIMP defines a group of cancers with a 3–5 fold elevated frequency of aberrant gene methylation especially in case of *INK4A, MLH1* and *THBS1* apart from harboring microsatellite instability (Toyota et al. 1999). The clustering pattern suggestive of CIMP has been confirmed in glioblastoma, gastric cancer, liver cancer, pancreatic cancer, esophageal cancer, ovarian cancer, acute lymphocytic leukemia and acute myelogenous leukemia apart from colorectal cancer (Issa 2004). Though the causes leading to CIMP are largely unknown, its presence is associated with a poor outcome in multiple malignancies (Issa 2003). Age-related methylation, life-style, exposure to epimutagens, chronic inflammations have all been thought of as important contributors of CIMP. Long range epigenetic silencing (LRES) is a phenomenon where large regions of chromosomes can be coordinately suppressed. Typically, LRES can span megabases of DNA and involve broad heterochromatin formation accompanied by the hypermethylation of clusters of contiguous CpG islands within the region. This process is usually associated with DNA and histone hypermethylation and can in turn lead to DNA methylation of flanking, non-methylated genes. Such hypermethylated regions spanning hundreds of kilobases involving gene family clusters have also been found in esophageal, neuroblastoma, breast and colorectal cancers (Frigola et al. 2006; Clark 2007).

26.3.3 The DNA Methyltransferases

DNA methyltransferases (DNMTs) are the enzymes, which catalyze the covalent addition of methyl groups to cytosine in the CpG dinucleotide context. Broadly they have been classified into: maintenance methyltransferases (*DNMT1*) and *de novo* methyltransferases (*DNMT3A, DNMT3B, DNMT3L*). *DNMT2*, with its weak methyltransferase activity has not been classified into either of the groups. The DNMTs are characterized by a 'C' terminal catalytic domain, which transfers methyl group from S-Adenosyl-L-methionine (SAM) to cytosine and contains five conserved amino acid motifs, namely I, IV, VI, IX and X. Motifs I and X form the AdoMet binding site.

Dnmt1 was the first methyltransferase to be identified. Homologs of *Dnmt1* have been found in nearly all eukaryotes that have DNA bearing 5-methylcytosine, but not in species that lack 5-methylcytosine. It is a large protein of 1,620 amino acids, having an 'N' terminal regulatory region containing an NLS, a region that targets *Dnmt1* to replication foci, PHD like domain, proliferating cell nuclear antigen (PCNA)-binding domain apart from the 'C' terminal catalytic domain. An 'N' terminal truncated but enzymatically active, oocyte-specific isoform of *Dnmt1*

(*Dnmt1o*) has also been identified, which accumulates to high levels in the cytoplasm of embryos (Doherty et al. 2002). Being a maintenance methyltransferase, Dnmt1 is involved in the bulk of DNA methylation and has also been implicated in non-CpG methylation (Grandjean et al. 2007). The DNMT3 family comprises *de novo* methyltransferases: *DNMT3A, DNMT3B* and an enzymatically inactive paralogue *DNMT3L*. Both DNMT3A and DNMT3B have a regulatory 'N' terminal domain containing an ATRX-like Cys-rich domain (PHD domain) and a PWWP domain, which are involved in interaction of these enzymes with other proteins and in targeting them to heterochromatin (Gowher et al. 2005). DNMT3L on the other hand doesn't possess any catalytic activity owing to mutations within all the conserved motifs that contain the catalytic residues of DNA-(cytosine-C5)-methyltransferase. It interacts with and regulates the *de novo* methyltransferases DNMT3A and 3B, stimulating their activity (Chedin et al. 2002; Suetake et al. 2004; Kareta et al. 2006). Recently, it has also been shown that DNMT3L interacts with Histone 3 Lysine 4 (H3K4) when it is unmethylated, providing a link between *de novo* DNA methylation and histone modifications (Ooi et al. 2007).

Homozygous deletion of Dnmts: *Dnmt1, Dnmt3a, Dnmt3b* are lethal in mice (Li et al. 1992; Lei et al. 1996; Okano et al. 1999). The deletion of *Dnmt3a* and *Dnmt3b* abolishes *de novo* methylation, while *Dnmt1* depletion leads to bulk DNA demethylation (Okano et al. 1999). Embryos of *Dnmt1*$^{-/-}$ mice are stunted, show delayed development, and do not survive past mid-gestation (Li et al. 1992). Though *Dnmt3a*$^{-/-}$ homozygous mutant mice developed to term and appeared normal at birth, they became runted and died at about 4 weeks of age (Okano et al. 1999). On the other hand, *Dnmt3b*$^{-/-}$ homozygous mutant mice were not viable, though they appeared to develop normally till E 9.5. These embryos displayed multiple developmental defects including growth impairment and rostral neural tube defects (Okano et al. 1999). Similarly, *Dnmt3a*$^{-/-}$ and *Dnmt3b*$^{-/-}$ double homozygous embryos were smaller in size and showed abnormal morphology at E 8.5, E 9.5 and died before E 11.5. A closer analysis of the embryos revealed that their growth and morphogenesis were arrested shortly after gastrulation (Okano et al. 1999).

Conditional deletion of *Dnmt3b* was also shown to result in DNA hypomethylation leading to chromosomal instability and spontaneous immortalization in mouse embryonic fibroblasts (Dodge et al. 2005). The DNMT3 family also plays an important role in genomic imprinting. Pericentromeric satellites are one of the specific targets of *DNMT3B*. Furthermore, inactivation of *DNMT3B* in humans leads to ICF syndrome, which is associated with low methylation in pericentromeric satellite regions. *Dnmt3a* conditional mutant females died *in utero* and lacked methylation and allele-specific expression at many maternally imprinted loci (DMRs of *Snrpn, Igf2r, Peg1*), whereas *Dnmt3a* conditional mutant males showed impaired spermatogenesis and lacked methylation at a few paternally imprinted loci examined in spermatogonia (*H19* DMR, *Dlk1-Gtl2* IG-DMR) (Kaneda et al. 2004). DNMT3A2, a germ-cell-specific isoform of *DNMT3A* is also required for genomic imprinting.

Though enzymatically inactive, DNMT3L is required for DNA methylation in ES cells where it is expressed in high amounts compared to differentiating somatic

cells (Ooi et al. 2010). Mice obtained after disruption of *Dnmt3l* by targeted mutation were viable and of normal phenotype in both heterozygous and homozygous conditions (Bourc'his et al. 2001). The male progeny however were found to be sterile and displayed azoospermia as their spermatocytes failed to complete meiosis, apart from exhibiting decrease in methylation at the differentially methylated regions of *H19* and *Rasgrf1* imprinted genes (Webster et al. 2005). On the other hand, the heterozygous progeny of homozygous females died before mid gestation (at 9.5 days post coitum) and these embryos were found to lack methylation imprints at *Snrpn* and *Peg1* imprinted genes apart from other pre-natal growth defects (Bourc'his et al. 2001). The role of Dnmt3l in establishing patterns of DNA methylation for several imprinted genes during gametogenesis has also been established by other groups (Webster et al. 2005; Arima et al. 2006).

26.3.4 The DNA Demethylase Enzymes

In contrast to the well characterized DNA methyltransferases, the enzymes that catalyze the removal of methyl moiety from methylcytosine have remained enigmatic. It has been easy to envisage passive mode of demethylation, wherein cytosine methylation is lost upon replication without being followed by maintenance methylation of hemimethylated DNA. However, active demethylation observed in the zygote (specifically for the paternal genome) and during germ cell formation has been hard to explain (Mayer et al. 2000; Ooi and Bestor 2008; Wu and Zhang 2010). Only very recently, a few proteins have been identified that modify methylcytosine either to thymine or hydroxymethylcytosine, which can be directly or indirectly converted to cytosine by DNA repair enzymes (Morgan et al. 2004; Rai et al. 2008; Kriaucionis and Heintz 2009; Tahiliani et al. 2009; Ito et al. 2010).

Conversion of methylcyotsine to thymine in mammals is catalyzed by Apolipoprotein B RNA-editing catalytic component-1 (Apobec-1) and Activation Induced Deaminase (AID, also known as Apobec2), members of the cytidine deaminase family (Morgan et al. 2004; Rai et al. 2008). The role of these enzymes in active demethylation of methylcytosine has been confirmed by various experiments directly or indirectly. In 2004, it was demonstrated that AID had 5-methylcytosine deaminase activity *in vitro* (Morgan et al. 2004). Moreover, it was found that AID is expressed in primordial germ cells (PGCs), oocytes and cells from early mouse embryos, cells which are known for their active demethylation activity (Morgan et al. 2004; Wu and Zhang 2010). That AID is indeed involved in DNA demethylation during PGC specification was confirmed by Popp et al. (2010) when they showed that erasure of DNA methylation mark during PGC formation is hindered in AID deficient mice. The role of AID in DNA demethylation was further strengthened by the observation that AID deficiency prevents the demethylation of pluripotency specific genes (*OCT4, NANOG*) during the process of converting fibroblasts into pluripotent iPS cells (Bhutani et al. 2010).

It was recently discovered that TET family of proteins including TET1, TET2 and TET3 can convert methylcytosine into hydroxymethylcytosine (Tahiliani et al. 2009; Ito et al. 2010). JBP1 and JBP2 proteins catalyze conversion of methyl-thymine into β-D-glucosyl-hydroxymethyluracil in Trypanosomes. TET proteins were identified to be their homologs in mammals based on iterative sequence profile computational search (Tahiliani et al. 2009). This study also demonstrated that cytosine does exist in the hydroxmethylcytosine form in the mammalian genomes (also shown by Kriaucionis and Heintz 2009) and TET1 does convert methylcytosine to hydroxymethylcytosine *in vitro* (Tahiliani et al. 2009). Like Activation induced deaminase, TET proteins mediated conversion of methylcytosine to hydroxymethylcytosine has also become established as a part of the DNA demethylation process. TET1 has been shown to be involved in maintaining ES cell pluripotency and in the specification of inner cell mass during embryogenesis (Ito et al. 2010). Just after fertilization and before the first cell division, the male pronucleus has been shown to get actively demethylated (Mayer et al. 2000). A very recent study showed that unlike the previous established notion, methylcytosines in male pronucleus gets converted into hydroxymethylcytosine and not cytosine (Iqbal et al. 2011). Moreover, conversion back to cytosine is not an active process as hydroxymethylcytosine stays in the genome even after several cell divisions (Iqbal et al. 2011). The same paper also indicated on the basis of transcriptional profiling that TET3 rather than TET1 was probably responsible for the conversion to hydroxymethylcytosine (Iqbal et al. 2011).

26.3.5 DNMTs and Cancer

Being the effectors of DNA methylation, DNMTs have an important role to play in the aberrant DNA methylation observed in cancer cells (Fig. 26.2). DNMT protein levels and activities were found to be elevated in various cancer types, including gastric, bladder, brain, leukemia, colon and lung (Issa et al. 1993; Belinsky et al. 1996; Melki et al. 1998; De Marzo et al. 1999; Robertson et al. 1999; Ramsahoye et al. 2000; Girault et al. 2003; Li et al. 2003; Xiong et al. 2005; Amara et al. 2010; Qu et al. 2010). Moreover, multiple DNMTs have been found to co-localize to promoters of hypermethylated genes and also have been defined as components of the transcriptional repression complexes (Di Croce et al. 2002; Kim et al. 2002; Datta et al. 2003). However, it has been difficult to correlate the increased expression of DNMTs with CpG hypermethylation in several cancers (Miremadi et al. 2007). While the maintenance methylation activity of DNMT1 is primarily observed in somatic cells, the *de novo* methylation activities are seen in germ cells and at embryonic stages. This paradigm of separation of methylation activity has been challenged in the cancer setting.

Though aberrant DNA methylation patterns observed in cancer is an undisputed fact, it is still not clear as to which of the DNMTs are primarily responsible. The maintenance methyltransferase *DNMT1* has been implicated because its expression

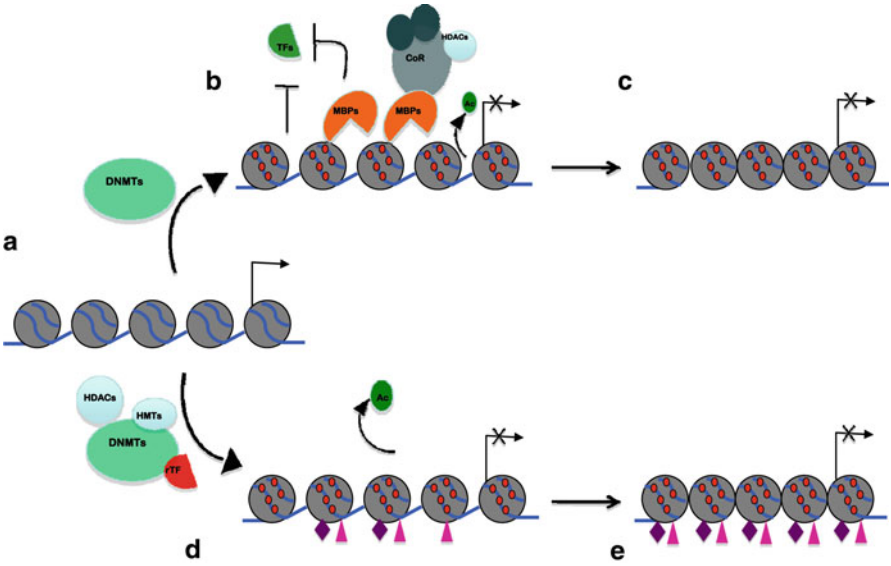

Fig. 26.2 Dual role of DNMTs in silencing of gene expression. *DNMTs* can be targeted directly or with the help of transcriptional repressors (*rTF*) to CpG dinucleotides. (**a**) Nascent chromatin strand (**b** & **c**) DNMTs can get directly targeted to a genomic locus due to a stimulus like hemi-methylated DNA and lead to CpG methylation. This could cause inhibition of transcription factor (*TF*) binding either directly or due to the binding of *MBPs* (Methyl binding proteins). The MBPs can recruit co-repressor complexes (*CoR*) and *HDACs* leading to nucleosomal remodeling and chromatin compaction. (**d** & **e**) The DNMTs are also found in complex with histone deacetylase and histone methyltransferases. In this scenario DNA methylation is coupled to setting up of repressive histone marks including H3K9me3 and H3K27me3 eventually leading to condensed and tightly packed chromatin. *Raised arrows* denote transcriptional activation; *crossed arrows* denote transcriptional repression. *Red circles*: Methyl CpG; *Green circles*: H3K9 acetylation; *Circles* with *curved lines* denote nucleosomal organization of chromatin; *Diamonds* represent H3K9me3; *Triangles* represent H3K27me3

levels are higher in cancers and it also exhibits low levels of *de novo* methylation activity against unmethylated substrates (Jair et al. 2006). DNMT1 overexpression resulted in detectable *de novo* methylation of CpG island in human fibroblasts (Vertino et al. 1996) and induced transformation in NIH3T3 cells (Wu et al. 1993). On the other hand, severe depletion of DNMT1 produced only minor decrease in overall methylation, minimal loss of promoter hypermethylation and undetectable re-expression of silenced tumor suppressor genes in colorectal cancer cells (Rhee et al. 2000, 2002; Ting et al. 2004, 2006a, b). However, a double knock out of *DNMT1* and *DNMT3b* (*DNMT1*$^{-/-}$ *DNMT3b*$^{-/-}$) in HCT116 cells resulted in >95% loss in genomic methylcytosine content and complete promoter demethylation apart from re-expression of aberrantly silenced genes (Rhee et al. 2002; Akiyama et al. 2003; Suzuki et al. 2004). *DNMT1* is thus one candidate that is capable of initiating aberrant CpG island hypermethylation in cancer cells. Indeed, DNMT1 has been

reported to repress transcription through its interactions with methyl CpG binding proteins, histone deacetylases and histone methyltransferases (Bogdanovi and Veenstra 2009). On the other hand, global methylation levels were affected indirectly through the regulation of DNMT1 by BRCA1 and miRNA29b (Garzon et al. 2009; Shukla et al. 2010). In the Apc^{Min} mice model, a reduction of *Dnmt1* activity, due to heterozygosity of *Dnmt1* gene, in conjugation with treatment using DNMT inhibitor like 5-Aza-cytidine reduced the average number of intestinal adenomas (Laird et al. 1995). Similar observations in genetically engineered mice involving *Dnmt1* clearly demonstrate a causal relationship between alteration of DNA methylation and cancer. But the requirement of the maintenance methyltransferase *DNMT1* in maintaining promoter hypermethylation and gene silencing in cancer cells is still debatable. Mutational inactivation of *DNMT1* has not been observed in most of the cancers though colorectal cancers infrequently harbored these mutations (Kanai 2008). Among the other DNMTs, germ line single nucleotide polymorphisms (SNPs) in *DNMT3B* have been associated with risk of breast cancer, gastric cancer, hepatocellular carcinoma, lung adenocarcinoma and lung cancer (Cebrian et al. 2006; Ezzikouri et al. 2009; Hu et al. 2010).

Recently data from our own laboratory showed loss of methylation at *DNMT3L* promoter in cervical cancer and few other cancer types (Gokul et al. 2007; Manderwad et al. 2010 and unpublished results). This decrease in methylation was associated with an increased expression of DNMT3L. Moreover, overexpression of DNMT3L in HeLa cells lead to increased cell proliferation and anchorage-independent growth in a time and passage dependent manner (Gokul et al. 2009). Microarray analysis revealed that the expression patterns of genes important in nuclear reprogramming, development and cell cycle were misregulated. Interestingly, among this misregulated gene set, many imprinted genes were found to be downregulated; consistent with the role of DNMT3L in imprinting. DNMT3L was also identified to be essential for the growth of human testicular germ cell tumors (Minami et al. 2010).

26.3.6 DNA Demethylase Enzymes and Cancer

Though the role of AID in DNA demethylation has been identified only recently, its role in creating somatic hypermutations and in class-switch recombination of human immunoglobulin genes has been known for some time now (Revy et al. 2000; Marusawa and Chiba 2010). Several studies have reported unregulated expression of AID in cancer (Matsumoto et al. 2007; Klemm et al. 2009). An interesting correlation that has been investigated is the link between *Helicobacter pylori* infection, aberrant expression of Activation Induced Deaminase and gastric cancer (Matsumoto et al. 2010; Touati 2010; Endo et al. 2011). A few studies have also highlighted the role of aberrant AID expression in causing widespread genomic instability (Klemm et al. 2009; Robbiani et al. 2009).

TET1 is an abbreviation for Ten-Eleven Translocation-1 and was named so because of its involvement in a t(10;11)-associated leukemia (Lorsbach et al. 2003).

It was simultaneously identified by Ono et al. (2002) who found *TET1* to be fused to *MLL* gene in Acute Myeloid Leukemia. Later studies have also reported fusion of *TET1* (also known as *LCX*) with *MLL* suggesting a role for *TET1* in carcinogenesis (Shih et al. 2006; Burmeister et al. 2009, reviewed in Dahl et al. 2011). The fusion of *TET1* and *MLL* is also interesting from an epigenetic perspective as MLL itself is H3K4 histone methyltransferase (Krivtsov and Armstrong 2007). Deletions and mutations have also been reported for *TET2* in several myeloid cancers (Figueroa et al. 2010; Ko et al. 2010; Langemeijer et al. 2011). Along with the mutations in TET proteins, a recent report showed that the levels of hydromethylcytosine were also altered in myeloid cancer (Ko et al. 2010) indicating that the process of DNA demethylation itself might be key to the process of carcinogenesis.

26.3.7 DNA Methylation Binding Proteins and Cancer

The transcriptional repressive activities of DNA hypermethylation are primarily interpreted and mediated by a family of proteins which harbor the methyl-CpG-binding domain (MBD) – the protein motif responsible for binding methylated CpG dinucleotides (Bird and Wolffe 1999; Bird 2002; Burgers et al. 2002). These include MECPs (Methyl CpG binding protein-MECP2), MBDs (Methylcytosine-binding protein MBD1, 2, 3, 4) and the novel protein Kaiso (Prokhortchouk et al. 2001). Except for MBD3, the other methyl binding proteins specifically recognize methyl-CpG (Klose and Bird 2006). These proteins can mediate silencing of gene expression by recruiting other members of the epigenetic machinery, primarily the chromatin remodeling co-repressor complexes (Jones et al. 1998; Ng et al. 1999; Wade et al. 1999; Zhang et al. 1999; Sarraf and Stancheva 2004). The MBDs have a high specificity towards gene promoters and they have been found at hypermethylated and aberrantly silenced cancer gene loci (Ballestar and Esteller 2005; Ting et al. 2006a, b). For example, MBD2 binds to aberrantly methylated promoter of tumor suppressor genes: *p14/ARF* and *p16/ink4A* in colon cancer cell lines (Magdinier and Wolffe 2001; Martin et al. 2008) and suppress their expression. Importantly, MBDs are found to be associated with complexes that contain HDACs; for example the methyl binding proteins, MECP2, MBD1 and MBD2 have been found to associate with transcriptional co-repressors, such as SIN3A, which are known to bind HDACs directly (Jones et al. 1998; Ng et al. 1999). This binding results in compaction of chromatin and stable repression of the target gene. MBD1 interacts with histone H3 methyltransferase (SETDB1) linking epigenetic marks on DNA to histone modifications, whereas MBD4 is thought to act as a thymine DNA glycosylase, repairing G:T or G:U mismatches at CpG sites (Kanai 2008). The newly identified methyl binding protein- Kaiso, associates with the histone deacetylase containing-N-CoR co-repressor complex bringing about repression of methylated genes (Yoon et al. 2003). Interestingly, mice lacking Kaiso have been shown to exhibit resistance to intestinal tumorigenesis (Prokhortchouk et al. 2006). Polymorphisms (SNPs) in many of these MBD proteins (MBD1, MBD2 and MBD4) were found to be associated

with an increased risk of cancer (lung and breast). Overexpression of MeCP2 has been observed in breast cancer (Muller et al. 2003) and it is implicated in the silencing of *IL-6* in pancreatic adenocarcinoma cell lines (Dandrea et al. 2009). MeCP2 also plays important roles in gastric and colorectal carcinogenesis (Pancione et al. 2010; Wada et al. 2010). Progeny of a cross between Mbd2-deficient mice and $Apc^{Min/+}$ mice were found to be resistant to the development of intestinal tumors and this resistance was dependent on the dosage of Mbd2 (Sansom et al. 2003). The role of MBD2 has also been positively implicated in the silencing of Nrf2 expression in a mouse prostate adenocarcinoma model (Yu et al. 2010). The hypermethylated region of human telomerase reverse transcriptase (*hTERT*) was also shown to be specifically associated with MBD2 bringing about its transcriptional repression in many cancer cell lines (Chatagnon et al. 2009).

26.3.8 DNA Methylation Inhibitors and Epigenetic Therapy

In spite of being robust, epigenetic modifications like DNA methylation and histone modifications are reversible in nature. This characteristic of epigenetic modifications makes them a useful target for cancer therapy and the enzymes that mediate and maintain DNA methylation (DNMTs) and various histone modifications (HATs, HDACs and HKMTs) are the prime drug targets in the epigenetic therapy proposed for cancer.

Various small molecules have been identified with inhibitory effects on DNA methylation, histone methylation or acetylation and it is possible to reactivate a hypermethylated tumor suppressor gene by the use of DNA demethylating agents or DNA methyltransferase inhibitors (DNMTi). Most of these molecules are nucleoside analogs, which are incorporated into replicating DNA in place of cytosine. Once incorporated, they can sequester DNMTs by the formation of a covalent bond between them, eventually depleting the cell of enzymatic activity resulting in heritable demethylated DNA (Juttermann et al. 1994; Santi et al. 1984). Due to the high replicative potential of cancer cells they are particularly prone to demethylation underscoring the efficacy of DNMTi as antineoplastic drugs. Among the best studied inhibitors are 5-aza-cytidine and 5-aza-2′-deoxycytidine which were found to bring about DNA demethylation (Sorm et al. 1964, reviewed in Momparler and Bovenzi 2000; Gal-Yam et al. 2008; Kwa et al. 2011). Both inhibitors were shown to reactivate genes silenced by aberrant methylation in various human tumor cell lines (Momparler and Bovenzi 2000) apart from inducing in vitro differentiation of human leukemic cells (Pinto et al. 1984; Momparler et al. 1985). They also exhibited potent antineoplastic activity in hematological malignancies and lung cancer (Rivard et al. 1981; Pinto and Zagonel 1993; Momparler et al. 1986; Momparler and Ayoub 2001). Presently, of the four epigenetic-based drugs that have been approved by FDA for cancer therapy, two are inhibitors of DNMTs: 5-azacytidine (Vidaza) and 5-aza-2′-deoxycytidine (Decitabine) (Kwa et al. 2011). Both drugs have been approved by the FDA for the treatment of myelodysplastic syndrome, a preleukemic disease (Pinto

and Zagonel 1993). There are few other inhibitors like Epigallocate, Procaine etc. some of which are less toxic compared to 5-aza-cytidine, but their dosage, stability and efficacy have impeded their advancement to clinical trials (Kwa et al. 2011). Zebularine is another promising DNMTi which has the advantage of higher stability and longer half-life together with the convenience of oral administration but is effective only in high doses (Champion et al. 2010; Cheng et al. 2004; Kwa et al. 2011). Recently, derivatives and variants of 5-azacytidine like S110 and 2′-deoxy-5,6-dihydro-5-azacytidine have been characterized and found to be less toxic and more stable demethylating agents (Chuang et al. 2010; Matousova et al. 2011). In spite of concerns relating to non-specific side effects, toxicity, drug resistance and inefficacy against solid tumors, DNMTis remain an attractive choice as therapeutic agent in hematological malignancies. Additionally because of their ability to cross the blood brain barrier they can be used to treat brain malignancies (Diede et al. 2010).

The interplay between histone modifications and DNA methylation in cancer cells has prompted the use of histone deacetylase inhibitors (HDACi) with DNMTi. Such combinatorial therapy was shown to increase the expression of silenced genes to higher levels than with either of the inhibitors alone (Issa 2007; Kwa et al. 2011). Cameron et al. (1999) were the first to show the synergistic transcriptional activation of the *p16CDKN2A* tumor suppressor gene upon treatment of tumor cells with 5-azacytidine and Trichostatin A, a HDACi. We in our lab also have been able to show similar 5-azacytidine transcriptional activation for the *DNMT3L* gene in cervical cancer cells upon treatment with 5-azacytidine and Trichostatin A (Gokul et al. 2009). It was also shown that the treatment of tumor cells with 5-azacytidine decreased levels of repressive histone marks (H3K9me3) while increasing active histone marks (H3K4me3) at promoters of genes that are aberrantly silenced by DNA methylation (Nguyen et al. 2002). These facts not only underscore the importance of such combinatorial therapy but also warrant the need to investigate the nature of synergy between different inhibitors to bring about an optimal response in tumors.

26.4 Concluding Remarks

The abnormalities in cancer change the “epigenetic landscape” of the cell and involve multiple aberrations in virtually every component of the epigenome. Since epigenetic silencing processes are mitotically heritable, they play the same roles and undergo the same selective processes as genetic alterations in the development of cancer. Similar to genetic mutations, epigenetic events follow the Darwin’s hypotheses for the evolution of species by which alterations in gene expression induced by epigenetic events which confer cellular growth advantage are selected for in the host, resulting in the progressive, uncontrolled growth of the tumor. Can epigenetic mechanisms initiate tumorigenesis by their own? As described in this chapter there are numerous examples of aberrant epigenetic changes occurring in various cancers as early events. For example, DNA hypomethylation in cancers has been shown to be an early event, but it has not been conclusively shown nor are there examples that

show epigenetic processes initiating cancer on their own. However, the evidence accumulated over the last two decades reiterates the complexity of the mechanisms involved in cancer development and emphasizes the fact that there is strong interplay between epigenetic and genetic events in cancer and that understanding the epigenetic basis of carcinogenesis is critical in solving the puzzle that cancer is.

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