Misregulation of DNA Methylation Regulators in Cancer

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Abstract Epigenetic modifications at the DNA level play a central role in establishing the chromatin state and thereby influencing biological function. Several disorders arise from aberrant epigenetic patterns on DNA, cancer being widely explored as an epigenetic disorder. In fact several cancers are associated with a hypermethylator phenotype, which essentially functions as a 'driver' of tumorigenesis. Aberrant DNA methylation patterns arise from disrupting the 'writers' or 'erasers' of the DNA methylation pathway, coordinately functioning to regulate DNA epigenetic marks. Cancer associated deregulatory mechanisms targeting functional disruption of the molecular components of the DNA methylation pathway, and their contribution to cancer initiation and progression are being increasingly appreciated. Understanding these mechanisms of deregulation is central to identifying new targets for therapeutic intervention, in both cancer prevention and treatment.

Keywords DNA methylation pathway • Hypermethylator phenotype • DNMT • TET • Epimutation • Oncogenic • Tumorigenesis

Abbreviations

AML	Acute Myeloid Leukemia
CGI	CpG island
CIMP	CpG hypermethylator phenotype
DNMT	DNA methyltransferase
EBF1	Early B-Cell Factor 1
EMT	Epithelial to mesenchymal transition
FH	Fumarate hydratase (fumarase)
G-CIMP	Glioma specific CIMP

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GSC	Glioblastoma Stem Cells
HIF	Hypoxia inducible factor
HPC	Hematopoietic presursor cells
HRE	Hypoxia response element
HSC	Hematopoietic stem cells
IDAX	Inhibition of the Dvl and Axin Complex
IDH	Isocitrate dehydrogenase
MBD	Methyl binding domain
mCG	CpG methylation
mCH	Non-CpG methylation
MDS	Myelodysplastic Syndromes
MLL	Mixed-lineage leukemia
MPN	Myeloproliferative Neoplasm
MTase	Methyltransferase
PCNA	Proliferating cell nuclear antigen
SDH	Succinate dehydrogenase
TDG	Thymine-DNA glycosylase
TET	Ten-eleven translocation
UHRF	Ubiquitin-Like with PHD and Ring Finger Domains
α-KG	Alpha-ketoglutarate

1 Introduction

Cancer is a complex diseased state arising from impaired cellular homeostasis. Cellular homeostasis is essentially defined by the underlying dynamic transcriptome, coding for the functional proteome, which is temporally modulated by intrinsic and extrinsic cues. However, the transcriptome is only an effector of the epigenetic changes occurring at the different components of chromatin – DNA, histones, and nucleosomes, which essentially control the progression of central biological processes. At the DNA level, epigenetic information is carried in the form of cyclic modifications at the C5 position of cytosine, frequently but not exclusively within 'CpG' dinucleotides (cytosine preceding guanine). The primary modification at C5 is methylation, resulting in 5-methylcytosine (5mC), and is catalyzed by a family of enzymes, the **DNA M**ethyl**t**ransferases (DNMTs). 5mC can be further oxidized by the **T**en-**e**lven **t**ranslocation (TET) family of dioxygenases, sequentially, to 5-hydroxymethyl cytosine (5hmC), 5-formyl cytosine (5fC), and lastly 5-carboxyl cytosine (5caC), which can be viewed as secondary, tertiary, and quaternary modifications at C5. All four DNA marks carry distinct epigenetic information and functional implications, and are central to driving development, differentiation, and maintaining cellular homeostasis. In fact, aberrant patterns of these C5 modifications are associated with the initiation and progression of several cancers. Misregulation of the molecular components of the DNA methylation pathway forms

the basis of most, if not all cancers. This chapter will provide an overview on how the molecular components of the DNA methylation pathway are deregulated to facilitate cancer initiation and progression, and their mechanistic contribution to achieving the hallmarks of cancerous cells.

1.1 Molecular Components of the DNA Epigenetic Pathway: Establishment, Interpretation, and Turnover of DNA Modifications

As in all epigenetic pathways, the DNA methylation pathway has three major components – writers, readers, and erasers. The DNMTs, which include three catalytically active enzymes, DNMT1, 3A, and 3B, and a catalytically inactive DNMT3L (3-Like), function as the 'writers' of the DNA epigenetic pathway. The structurally homologous de novo DNMT3A/3B, along with the accessory DNMT3L, establish 5mC patterns during early embryonic development and are implicated in development [1], differentiation, and lineage commitment [2–4] while the maintenance DNMT1 faithfully maintains and propagates established 5mC patterns during DNA replication in somatic cells [5]. Both DNMTs 3A and 3B possess a variable N-terminal domain with a proline-tryptophan rich – PWWP domain, followed by the ATRX–DNMT3–DNMT3L (ADD) domain, which contains a CXXC zinc finger and an atypical plant homeodomain (PHD) finger domain, and lastly a catalytic methyltransferase (MTase) domain at their C-terminal end [6]. DNMTs 3A and 3B are highly expressed during early embryonic development and in differentiating cells, but are generally lowly expressed in terminally differentiated somatic cells. However, somatic cells do express catalytically inactive isoforms of the DNMT3 enzymes [7], suggesting their pivotal role in non-epigenetic mechanisms. DNMT1 has similar structural organization as the DNMT3s, with a C-terminal catalytic and N-terminal regulatory domain, but shares very little homology with the DNMT3s. DNMT1 has a 30- to 40-fold higher preference for hemi methylated DNA [8], and is recruited at the replication fork via its interactions with PCNA [9, 10] and UHRF1 [11, 12], where it functions to copy and maintain DNA methylation patterns during replication.

DNA methylation patterns are interpreted by 'readers', which thereby affect local chromatin structure by recruiting histone modifying enzymes and chromatin remodeling complexes at these sites. The Methyl Binding Domain (MBD) family of proteins and the zinc-finger proteins ZBTB4, 33, and 38 are currently known to function as readers of DNA methylation [13–15]. The mammalian MBD-family has five members which recognize 5mC using a similar mechanism but with differing specificities. MeCP2, the first of the members to be identified, is highly expressed in neuronal tissue [16] and recognizes 5mC at specific genomic loci [17], whereas the other members are expressed more ubiquitously and exhibit a more general recognition pattern. All MBD family members act as transcriptional repressors by

recruiting repressive epigenetic complexes at the 5mC sites recognized by them. The ZBTB readers belong to the BTB-POZ family of zinc-finger proteins, currently composed of 49 structurally homologous members, most of which are implicated in driving B-cell and T-cell function, primarily through transcriptional repression. All ZBTB family members possess an N-terminal BTB domain, which mediates interactions with other transcriptional co-regulators, (e.g. N-CoR, SMRT, HDACs, SIN3), C-terminal C2H2-type zinc fingers (2-14 in number) which enable sequence-specific DNA binding, and in some cases an AT-hook domain that mediates binding by non-specific interactions with the minor groove in A-T rich DNA sequence binding, which include centromeric satellite repeats [18]. Of the entire family, just three members, ZBTB4, 33, and 38 are known to function as 5mC readers. Of these, Kaiso (ZBTB33) has been widely studied and is capable of recognizing both unmethylated and methylated Cs, at different recognition motifs [19, 20], however, its ability to recognize 5mC in vivo has been questioned [21].

Finally, the TET family of O_2, Fe^{2+}, and α-ketoglutarate dependent demethylases, TETs 1, 2, and 3, erase 5mC through step-wise oxidation to 5hmC, 5fC, and 5caC which are diluted during replication passively diminishing epigenetic marks on DNA, or are actively replaced by the base-excision repair pathway, specifically by thymine DNA glycosylase (TDG), to cytosine [22, 23]. The TET genes are believed to have arisen from segmental duplication of a single gene. The three family members share a great degree of homology at their carboxyl terminal catalytic domain, which consists of a cysteine-rich region and a His-Xaa-Asp/Glu signature motif in the double stranded B-helix (DSHB) fold. However, the TETs differ at their amino-terminal ends, with TET1 and TET3 possessing the CXXC motif, which is absent in the TET2 enzyme [22, 23]. The TET2 associated CXXC domain is carried on a separate gene, owing to an evolutionary gene inversion event, the IDAX (CXXC4) gene. The CXXC domains are capable of binding DNA and their function in the TETs is speculatively to direct the enzymes to their target sites [23, 24], though the catalytic domain is capable of non-specific DNA binding by itself [23].

DNA methylation signatures are cell type and cell stage specific, and are established by regulated cross-talk between the writers, readers, and erasers of the DNA methylation pathway, while integrating instructions from epigenetic pathways acting on different components of chromatin. The functional implication of DNA methylation is positional and context dependent, i.e. it shows variations in interpretation across the genome.

1.2 Genome-Wide Variation in DNA Modifications, How They Are Achieved, and Their Functional Interpretation

Initially, DNA methylation was largely studied at CpG sites (mCG) in the context of promoters of repressed genes, and it was largely perceived as a repressive epigenetic mark. However, recent advances have led to a transformation of this view on

DNA methylation. Firstly, methylation occurs at cytosines outside of CpG sites. This non-CpG methylation, termed 'CH' methylation (H = Adenosine/Thymine/Cytosine), is abundant in oocytes [25], embryonic stem cells, and a subset of neurons [26], and present in small amounts in other human cell types and tissues [27]. Secondly, mCG sites are not abundant in promoters alone, but show 'mosaicism' with respect to genome-wide distribution. The occurrence of 5mC, and thereby its functional impact, is dependent on the density of CpGs at a locus, the underlying genomic sequence, and the surrounding chromatin environment [28].

Across the genome, CpG sites are unequally distributed. Sites of high CpG density (at least 200 bps long with a GC content greater than 50 percent) are termed CpG islands (CGIs), and are largely exempt from DNA methylation. CGIs are by and large abundant in promoter elements. Surrounding the CGIs, 2 kb on either side of the island, are CGI shores, regions with relatively lower CG frequency. 2 kb on either side of the CGI shores are termed CGI shelves [29]. Very recently, the term CG canyons (or valleys) was coined to describe large regions of low methylation, distinct from CGIs, and frequently associated with transcription factor binding sites [30]. Additionally, isolated CG sites are seen across the genome, and these are mostly methylated. It is a general observation that CGIs in active promoters are devoid of 5mC, while CGIs are heavily methylated in repressed promoters. Isolated CG sites across the genome show a cell type specific methylation pattern, thus defining the associated active transcriptome, and the CpG sites within repeat elements, including centromeric and telomeric repeats, are largely methylated to maintain them in a constitutively repressed state thereby preventing spurious expansion of these elements [28]. A consequence of demethylation of repeat elements is genome instability [31], frequently observed in cancer, as is aberrant methylation patterns across the genome. Since irregularities in 5mC patterns across genomic features can have profound deleterious effects on cellular function, understanding the molecular pathways involved in establishing and regulating these patterns is of utmost importance.

Members of both families, DNMT and TET, show some degree of non-redundant function in regulating DNA methylation patterns, as observed by various selective knockdown and over-expression studies [32–34]. One mechanism by which distinct, cell-type specific, 5mC patterns are established, is by selectively targeting the DNMTs at particular genomic loci. How selective targeting is achieved across the genome, has been a deeply investigated question in the field of DNA methylation. Interaction with sequence-specific DNA-binding proteins mediating locus-specific recruitment of the DNMTs has been one school of thought [35]. These DNA-binding proteins can be selectively expressed, or post-translationally regulated, in a cell-type and cell-stage specific manner to establish differential methylation patterns. Several studies have isolated unique and common interactors of each of the DNMTs. For instance, DNMT3B is recruited by the transcription factor E2F6 to mediate silencing of the germ-line genes, Slc25a31, Syce1, Tex11, and Ddx41. DNMTs also cooperate with histone marks and chromatin complexes to achieve locus-specific targeting [36]. Interactions with the H3K9 methylating enzymes, Suv39h1, Setdb1 and G9a, target DNMTs to heterochromatin to establish 5mC [37]. Specifically, DNMT3B is recruited to centromeric and pericentromeric

repeat regions via interactions with CENP-C, an essential core component of the centromere, where it establishes DNA methylation and coordinates with other epigenetic components to mediate constitutive heterochromatization of the centromeric region [38].

2 Cancer: The Result of a Deregulated DNA Methylation Pathway

As DNA methylation pathways are fundamental to normal progression of several biological processes, dysregulation in its molecular pathway inevitably results in deleterious effects on cellular function. One of the most widely studied consequences of altered methylation patterns is cancer. It has been known for sometime that cancers are associated with globally hypomethylated genomes, accompanied by local hypermethylation events [39]. The local hypermethylation events generally accumulate at promoters of tumor suppressor genes (TSGs), enabling transcriptional silencing of these genes, consequently promoting tumor initiation and facilitating tumor progression. Aberrant DNA methylation patterns, in addition to distorting the normal transcriptome, also promote genomic instability, a significant contributor to tumor progression. Several studies have reported altered DNA methylation patterns to be the effector interface between a driver genetic mutation in a non-epigenetic gene and the resulting cancer associated transcriptome. However, cancers driven purely by epimutations with no associated genetic alterations are being identified. Ependymomas, a recurrent pediatric brain tumor, are driven by epigenetic events and are associated with very low recurring frequencies of somatic mutations and chromosomal aberrations [40].

Epimutations refer to non-genetic, heritable, aberrant lesions in the expression of a gene, arising from DNA modifications or other epigenetic modifications on local chromatin. Epimutations are typically not associated with base changes in DNA, either in -cis or -trans, but show transgenerational inheritance by mitotic transmission of the epigenetic mark. Epimutations can be classified into ‘somatic’ or ‘constitutional’ depending on whether it originated in differentiated cells and is thus contained in a specific tissue type (somatic), or it originated in germ-line cells and is thus present in all of the organisms’ cells (constitutional) [41]. Evidence in several tumor types suggests the association of a particular DNA methylation signature with tumor progression, signifying its aggressiveness and having a diagnostic and prognostic value. Such specific 5mC signatures are more widely seen in, but not restricted to, promoter elements, and are termed CpG Island Methylator Phenotype (CIMP) [42].

Tumorigenic epigenetic events driven by CIMP can be attributed to deregulation of individual molecular components of the DNA methylation pathway by various mechanisms;

(i) Inactivating or hyper activating mutations in the DNMTs or TETs.
(ii) Mutations in co-factors of DNMTs/TETs altering their catalytic activity

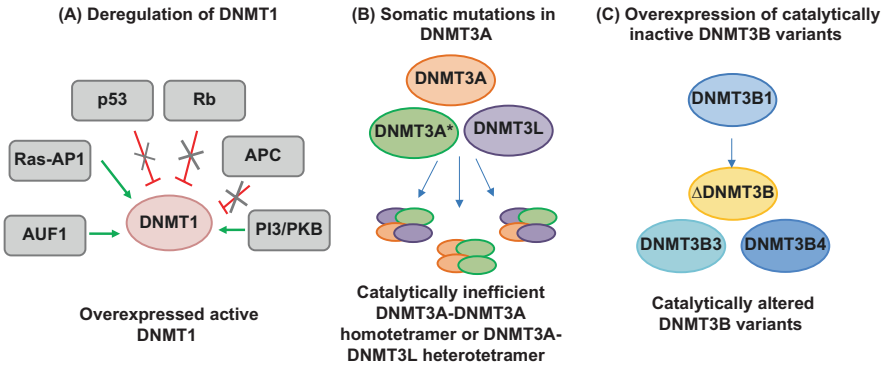

Fig. 1 Mechanisms deregulating the DNMT family to promote cancer. (**a**) DNMT1 is deregulated by hyperactivated signaling pathways. (**b**) DNMT3A is mainly inactivated by somatic mutations. (**c**) DNMT3B function is compromised by overexpression of catalytically inactive, truncated variants (DNMTΔ3B), or alternative splice variants (DNMT3B3/4). (* = mutant)

(iii) Mutations in mediators of signaling pathways regulating the DNMTs/TETs transcriptionally, post-transcriptionally, and post-translationally.
(iv) Mutational disruption of the DNMT/TET functional interactome resulting in their inappropriate targeting across the genome

Each of the above mentioned strategies can be employed by cancer cells to generate aberrant DNA methylation patterns subsequently facilitating tumorigenesis.

2.1 Compromised Writers and Their Contribution to Cancer

DNMTs are frequently deregulated in cancer to achieve an altered DNA methylome. Of the three, DNMT1 and DNMT3B function as oncogenes, and are frequently activated in cancer, whereas DNMT3A functions as a tumor suppressor gene, and is functionally inactivated to promote cancer. Mechanisms targeting each of the DNMTs and their role in tumorigenesis are briefly described in this section (Fig. 1).

2.1.1 DNMT1

Single base mutations disrupting the entire DNMT1 catalytic domain were first defined in a small population of colorectal cancers (7%) [43]. Typically, DNMT1 has high affinity for hemimethylated DNA, and acts as a maintenance methyltransferase with little or no de novo methyltransferase activity. This preference for hemimethylated DNA is explained by an auto-inhibition model of the DNMT1 enzyme. Structural studies on DNMT1 have shown that the mammalian enzyme is a multimodular protein, composed of a replication foci-targeting sequence domain (RFTS domain), a DNA-binding CXXC domain, a pair of bromo-adjacent

homology (BAH) domains, and a C-terminal catalytic domain. A stretch of acidic amino acid residues, termed the autoinhibitory linker region, lies between the CXXC and BAH domains and functions to prevent methylation at unmethylated CpGs, thus conserving the preference of the MTase domain for hemimethylated CpGs. Unmethylated CpG is recognized and bound by the CXXC domain, which prevents the catalytic domain from binding to it [44]. Additionally the RFTD mediates autoinhibitory effects by occulding unmethylated CpG binding at the catalytic domain [45]. Mutations in the autoinhibitory linker region as well as the RFTD evidently affect the catalytic efficiency of DNMT1 at unmethylated CpGs in vitro [44, 46]. Although both gain of function and loss of function mutations are proto-oncogenic in vitro, genetic mutations in DNMT1 are not a frequent event in tumorigenesis. However, deregulation of DNMT1 activity is central to tumor progression, suggesting alternative mechanisms targeting DNMT1 to be the underlying oncogenic phenomenon. DNMT1 levels are elevated in lung, hepatocellular, acute and chronic myelogenous leukemia, colorectal, gastric, and breast cancers [47–52], suggesting that DNMT1 is a transcriptional target of several oncogenic signaling pathways.

One of the pathways positively regulating DNMT1 expression is the Ras-AP1 pathway. AP1 (activating-protein 1), is a collective term for a group of basic leucine-zipper (bZIP) transcription factors, and constitutes proteins belonging to the Jun, Fos, Maf and ATF sub-families. AP-1 functions as a dimeric transcription factor recognizing either 12-O-tetradecanoylphorbol-13-acetate response elements (TPA, 5′-TGAG/CTCA-3′) or cAMP response elements (CRE, 5′-TGACGTCA-3′). Functionally, AP1 regulates cellular proliferation, survival, death, and differentiation, mostly by promoting gene expression, although cases of gene repression have also been reported [53]. Evidence suggests that neoplastic transformation relying on AP1-mediated mechanisms exert their effects in part through increased DNMT1 levels, which thereby methylate and represses expression of negative regulators of cell growth, conferring an advantage on cell growth. The 5′ regulatory region in the DNMT1 gene has three AP1 response elements, which are heavily methylated in early embryonic stem cells and normal somatic cells (preventing AP1 binding), but lose methylation in transformed cells allowing binding and induction by AP1 [54]. Cellular transformation by continuous c-Fos expression, which heterodimerizes with c-Jun to form a functional AP1 bZIP, subsequently induces DNMT1 expression to drive and maintain neoplastic transformation. cFos-mediated transformation can be reverted by direct abrogation of cFos, or through DNMT1 depletion, suggesting cFos relies significantly on DNMT1 to functionally disrupt cell growth regulation [55].

The APC/β-catenin/TCF pathway, which is critical to maintaining homeostasis in the gastrointestinal system, also regulates DNMT1 transcriptionally. APC, which functions as a tumor suppressor gene, is frequently mutated in cancers of the GI tract. Mutational inactivation of APC leads to upregulation of the Wnt/β-catenin/TCF pathway, conferring growth advantage on cancer cells and facilitating metastasis by promoting epithelial to mesenchymal transition (EMT). DNMT1 is

transcriptionally inhibited by APC, and inactivating mutations in APC lead to overexpression of DNMT1, facilitating tumor initiation [56]. DNMT1 is also the transcriptional target of the p53 and Rb pathways. Pathways involving both p53 and Rb negatively regulate DNMT1 expression levels, and are frequently deregulated in several cancers, allowing overexpression of DNMT1 and hypermethylation of tumor suppressor genes. In some tumors, p53 acquires mutations in its DNA-binding domain, disrupting its ability to bind to the p53 consensus sequence in the promoters of its target genes. Over-expression of Wild-type p53, but not mutant p53 (Mut R248L or Mut R273H), could bind at the DNMT1 promoter and repress it transcriptionally in lung cancer cell lines – A549 and H1299 [57]. The p53 binding site within the DNMT1 promoter was mapped to the exon 1 region (−19 to +317), which contains putative Sp1, p53, and E2F binding sites. p53 binding at the DNMT1 promoter is Sp1 dependent. Sp1, p53, HDAC1, and HDAC6 form a complex at the DNMT1 promoter, and p53 cannot suppress DNMT1 in the absence of Sp1 [57]. However, Sp1 can function as a transcriptional activator of DNMT1, and the stoichiometric ratio of p53 and Sp1 determines the effect of Sp1 on DNMT1 transcription. Sp1 levels regulate p53 nuclear-cytoplasmic distribution thereby modulating MDM2 mediated ubiquitination and degradation of p53. At high levels of Sp1, p53 is degraded in the cytoplasm and its inhibitory effect on DNMT1 is released, resulting in DNMT1 transcriptional activation and hypermethylation of tumor suppressor genes – p16INK4a, RARβ, FHIT, RASSF1A, and hRAB37, which are frequently hypermethylated in lung cancer [57]. Rb plays a crucial role in regulating cell cycle progression, especially passage through the restriction point, and is inactivated by several mechanisms to promote tumor progression. Increased DNMT1 is invariably associated with Rb inactivation. The increase in DNMT1 is attributable to enhanced E2F1 activity in the absence of Rb, which directly binds at the DNMT1 promoter activating it to bring about methylation-mediated silencing of tumor suppressor genes [58].

In addition to modulating DNMT1 transcript levels, post-transcriptional and post-translational modifications also serve as mechanistic modulators of DNMT1. AUF1, the RNA binding protein, regulates DNMT1 mRNA stability in a cell cycle specific manner and functions to regulate the epigenetic integrity of the cell during cell division [59]. DNMT1 protein stability is regulated by the PI3K/PKB (phosphatidylinositol 3-kinase/protein kinaseB) pathway, mainly responsible for cell growth, viability, and metabolism. PI3K/PKB pathway inhibits Gsk3β (glycogen synthase kinase 3β) mediated DNMT1 proteasomal degradation contributing to elevated DNMT1 protein levels [60]. DNMT1 levels are also regulated by its replication fork-targeting factor, UHRF1, an E3 ubiquitin ligase, which ubiquitinates and directs DNMT1 degradation [61]. In vitro mutational analysis suggests that UHRF1 also stimulates DNMT1 catalytic activity [62], suggesting a dual role for UHRF1 in DNMT1 regulation. Mutations disrupting the DNMT1/UHRF1/PCNA complex result in loss of DNMT1 recruitment to the replication fork and global DNA hypomethylation, thereby promoting initiation of tumorigenesis [63, 64].

2.1.2 DNMT3A

DNMT3A is frequently mutated in several hematological malignancies. Mutations are generally heterozygous and span various domains of the enzymes, but the most frequently mutated site is R882 [65]. This mutation results in formation of a hypomorphic enzyme which impedes catalytic activity by functioning in a dominant negative fashion [66]. DNMT3A functions by forming homotertramers with itself and heterotetramers with DNMT3L [67, 68], and the R882 mutant competes with WT DNMT3A encoded by the non-mutant allele, to form a dysfunctional homo- or hetero-tetramer, which exhibits reduced DNA binding and catalytic activity [66]. The R882 mutation is highly prevalent in de novo Acute Myeloid Leukemia (AML) patients (frequency of 22%) as compared to its occurrence in other hematological malignancies, including myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPNs) and chronic myelomonocytic leukemia (CMML). Other catalytically inactivating mutations, and truncating mutations (missense, non-sense, frame-shift, and splicing mutations), have also been mapped across functional domains of DNMT3A [65]. Most of these mutations have been biochemically characterized in vitro, and their effect on disrupting de novo methylation by DNMT3A can be explained by reduced catalytic activity (e.g. R664), reduced DNA binding (e.g. R831), loss of co-factor – S-adenosyl-L-methionine (AdoMet) binding (e.g. C710) [69], and loss of interactions with locus-specific recruiting histone marks (e.g. Q308) [70, 71]. How do these mutations affect cellular biology to facilitate tumorigenesis? In hematopoietic malignancies, loss of DNMT3A function alters the differentiation potential of hematopoietic stem cells while preserving their self-renewal and expansion properties, thus presumably creating a pool of stem cells predisposed to tumorigenesis upon acquisition of additional mutagenic insults. This presumption is partially substantiated by studies in murine models. Conditional genetic ablation of Dnmt3a in HSCs hampers their differentiation potential while favoring stem cell renewal. Paradoxically, loss of Dnmt3a results in hypermethylation of CGIs associated with the Basp1, Pdxdc1, and Wbscr17 genes, presumably through aberrant activity of DNMT1 and/or DNMT3b [3]. HSC fingerprint genes, which are repressed in differentiated cells, become overexpressed upon Dnmt3a ablation while differentiation specific genes are silenced, conferring the cells with a stem cell phenotype while blocking their differentiation. However, no leukemia was observed, suggesting functional disruption of Dnmt3a is a pre-leukemic event and is not sufficient to initiate tumorigenesis by itself [3].

Indeed, DNMT3A mutated HSCs acquire secondary mutations that induce tumorigenesis resulting in several blood malignancies. Mutations in the nucleophosmin gene (NPM1) and tandem duplication of the receptor tyrosine kinase FLT3 gene (FLT3ITD) [65], are the two mutations most frequently co-occurring with DNMT3A. The interactive contribution of these co-occurring mutations to leukemia is not completely understood and warrants further investigation. Mutations in DNMT3A show a strong association with mutations in the spliceosome factor SF3B1 (splicing factor 3b, subunit 1), in MDS patients. Positive association with mutations in the spliceosome factor U2AF1 (U2 small nuclear RNA auxiliary

factor 1), and negative association with mutations in serine/arginine-rich splicing factor 2 (SRSF2) have been reported [71]. Interestingly, DNMT3A mutations co-occur with IDH1/2 mutations in AML derived from MDS, suggesting a possible interactive mechanism in progression of AML from MDS [72].

DNMT3A is also inactivated by mechanisms other than mutations. UHRF1, known to regulate DNMT1 mediated methylation, along with UHRF2, negatively regulates DNMT3A by functioning as E3 ligases to promote DNMT3A degradation [73]. Both UHRF1 and UHRF2 are overexpressed in cancer. UHRF1 is exclusively involved in regulation of maintenance methylation, by directly controlling DNMT1 levels and its catalytic activity. UHRF2, however, is not associated with maintenance methylation, but is evidently involved in degrading DNMT3A, thus providing an explanation for the global hypomethylation associated with UHRF2 overexpressing tumors. DNMT3A is also subject to regulation by the MDM2/Rb pathway. In lung cancers, it was reported by Tang et al. that, depleted Rb levels, owing to overexpression of the Rb regulating E3 ubiquitin ligase MDM2, resulted in transcriptional activation of DNMT3A thereby resulting in downstream silencing of tumor suppressor genes by promoter methylation, thus promoting lung cancer [74]. The DNMT3A promoter possesses E2F binding sites, and is transcriptionally silenced by a repressive Rb-E2F complex formed at these sites. MDM2 attenuates DNMT3A repression by degrading Rb, allowing de novo methylation and silencing of multiple TSGs [74]. In this scenario, DNMT3A functions like an oncogene as opposed to its tumor suppressor role in myeloid malignancies. However, a mouse model of lung cancer contradicts the oncogenic role DNMT3A, since genetic ablation of DNMT3A promoted lung cancer progression, pointing again toward a tumor suppressor role for DNMT3A [75].

2.1.3 DNMT3B

Mutations in DNMT3B have not been observed in cancers. However, polymorphisms in the DNMT3B promoter are associated with cancer risk. The C to T polymorphism (C46359 > T) -149 bps upstream to the DNMT3B start site enhances promoter activity resulting in increased DNMT3B levels [76], potentially contributing to CIMP events occurring at tumor suppressor genes. This polymorphism, also represented as –149 C > T, is associated with an increased risk for lung cancer [77] and carcinoma of the head and neck [78]. The C46359T SNP positively correlates with age dependent Hereditary Nonpolyposis Colorectal Cancer [79], but shows no co-relation in breast cancers [80]. In fact, two DNMT3B polymorphisms, –283 T > C and –579 G > T, are associated with reduced cancer risk [81], although these results have been disputed [82].

Though mutations in DNMT3B are not associated with tumors, deregulation of DNMT3B expression levels, catalytic activity, and targeting across the genome are essential epigenomic driver events in tumorigenesis. To achieve DNMT3B mediated aberrant methylation patterns, tumors may rely on several mechanisms. One mechanism is expressing truncated DNMT3B variants generated by aberrant

splicing, to bring about redistribution of methylation patterns. A family of truncated variants, termed ΔDNMT3B, are overexpressed in non-small cell lung carcinoma (NSCLC) [83] and contribute to lung tumorigenesis by modulating DNA methylation at the promoters of tumor suppressor genes, p16INK4a and RASSF1A [84]. The ΔDNMT3B family is produced by non-conventional pre-mRNA splicing and consists of seven members, ΔDNMT3B1-4 lacking the N-terminal domain while preserving the PWWP and catalytic domains, and ΔDNMT3B5-7, which lack enzymatic activity. In NSCLC, the ΔDNMT3B1, ΔDNMT3B2, and ΔDNMT3B4 variants are highly expressed. ΔDNMT3B2/4 regulate promoter methylation of RASSF1A but not p16INK4a, suggesting a non-redundant function in regulating de novo methylation by the different truncated DNMT3B isoforms [84].

A separate family of DNMT3B splice variants, resulting again in truncated variants exhibiting absence, or varying degrees of catalytic activity, is responsible for global DNA methylation changes associated with cancer progression [85]. One such catalytically inactive splice variant, DNMT3B7, is overexpressed in several cancer cell lines of diverse origin [86]. In breast cancers, DNMT3B7 expression leads to promoter hypermethylation and silencing of the E-cadherin gene, activating the β-catenin pathway and conferring growth advantage. DNMT3B7 expression increases between stages I and II, implying its role in facilitating tumor progression [87]. In neuroblastoma cells, however, DNMT3B7 shows an anti-tumorigenic effect. Neuroblastoma cell lines express DNMT3B7 as well as other truncated DNMT3B variants, but the more aggressive forms show depleted DNMT3B7 levels and its forced overexpression results in inhibition of growth and increased global methylation. This suggests that a finely regulated interplay between the DNMT3B variants drives tumorigenesis. In hepatocellular carcinoma (HCC), overexpression of the variant DNMT3B4, lacking conserved methyltransferase motifs IX and X is associated with demethylation of pericentromeric satellite DNA, thus contributing to heterochromatin instability and promoting tumorigenesis [88]. DNMT3B4 overexpression in chronic myeloid leukemia (CML) is associated with demethylation of LINE-1 elements. In both, HCC and CML, the catalytically inactive DNMT3B3 is also expressed, and an increased DNMT3B4 to DNMT3B3 ratio promotes tumorigenic demethylation events. Although both isoforms lack MTase activity, the two have different effects on the catalytic activity of the functional heterodimer formed with DNMT3A. DNMT3B3 enhances DNMT3A activity, while DNMT3B4 attenuates DNMT3A, thus functioning in a dominant negative fashion [85].

In contrast to DNMT3A, DNMT3B functions like an oncogene and is often overexpressed in cancer. Analysis of an array of breast cancer cell lines showed that DNMT3B overexpression is positively associated with the hypermethylator phenotype characterized by silencing of the tumor suppressor genes CDH1, CEACAM6, CST6, ESR1, LCN2, and SCNN1A [89]. Cell lines not showing a robust CIMP, did not exhibit DNMT3B overexpression. DNMT3B is also overexpressed in lung cancer and is regulated by MDM2/FOXO3. As in the case of DNMT3A, which is regulated by MDM2/Rb, DNMT3B is negatively regulated by FOXO3, typically an

activating transcription factor. The DNMT3B promoter contains two FOXO3 binding sites which when occupied by FOXO3, leads to transcriptional silencing. FOXO3 is a target for degradation by MDM2 mediated ubiqitination, and is thus repressed in MDM2 overexpressing lung cancer cell lines relieving its inhibitory control on DNMT3B [90].

2.1.4 DNMT3L

The catalytically inactive DNMT3L, though incapable of depositing 5mC by itself, plays a crucial role in establishing 5mC patterns through its influence on DNMT3A and DNMT3B activity. DNMT3L directly binds DNMT3A and enhances its catalytic activity by enhancing its binding affinity for the co-factor AdoMet, and increasing its catalytic processivity [91–93]. A similar enhancement of DNMT3B activity by interaction with DNMT3L has also been reported [94]. Additionally, DNMT3L mediates locus-specific recruitment of the de novo DNMTs, through interactions with unique sequence specific transcription factors. For instance, DNMT3L forms a complex with p65-NFkB and DNMT3B, recruiting DNMT3B to specific genomic loci to mediate their methylation [95]. DNMT3L is highly expressed in germ cells and undifferentiated pluripotent stem cells, and plays an essential role in gametogenesis. Recent evidences suggest DNMT3L may have an oncogenic role in tumors arising from early developmental stages, involving germ cells. Both seminomatous and nonseminomatous testicular germ cell tumors (TGCTs), associated with a unique 5mC profile, showed an overexpression of DNMT3L [96]. Hypomethylation of the DNMT3L promoter is observed in cervical cancer [97] and in ocular surface squamous neoplasia [98], although the biological significance of this is yet to be elucidated. DNMT3L was also reported to affect promoter methylation, and therefore expression, of the thymine DNA glycosylase (TDG) gene in an array of human gastric cancer cell lines [99]. These findings point toward a pro-oncogenic function of DNMT3L.

2.2 Dysregulated Erasers

The TET proteins are involved in actively recycling the 5mC marks, and in the process produce additional functional epigenetic marks. The three mammalian TET proteins are non-redundant, share structural homology, and function via similar mechanisms. However, they show distinct expression patterns and are associated with a unique set of interacting proteins. TET proteins play a crucial role in embryonic development, hematopoiesis, and neurogenesis, and mutations in the TETs are observed in several solid tumors as well as leukemia. Mutations in metabolic genes, especially IDH1/IDH2, are also frequent in tumors, and manifest their effects through deregulation of TET activity. In general, the TETs are oncoprotective, and

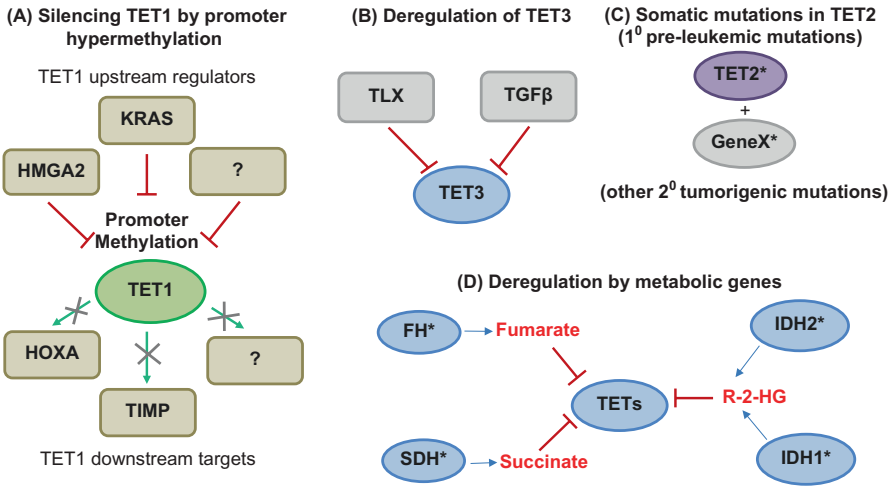

Fig. 2 Mechanisms deregulating the TET family to promote cancer. (**a**) TET1 is transcriptionally silenced by KRAS/ERK- or HMGA2-mediated promoter methylation. (**b**) TET3 levels are transcriptionally regulated by TLX and TGF β, and post-translationally regulated by IDAX. (**c**) TET2 is inactivated by somatic mutations, and upon acquisition of secondary mutations facilitates tumorigenesis. (**d**) Mutations in metabolic genes produces oncometabolites, which inhibit TET catalytic activity. (1^0 –primary, 2^0 –secondary, * = mutant, red font = oncometabolites)

TET2 is a bona fide tumor suppressor gene. Tumorigenic mutations in TET or their associated factors generally target a reduction in TET demethylation activity, thus preserving and allowing 5mC deposition and promoting the hypermethylator phenotype underlying tumorigenesis. The epigenetic basis of several cancers has been traced to deregulated activity of each of the TETs (Fig. 2).

2.2.1 TET1

The pluripotency factors, OCT4, NANOG, and SOX2, transcriptionally regulate TET1 [100], to promote high expression in embryonic stem cells where TET1 functions to maintain pluripotency by contributing to active demethylation. TET1 is also expressed in some neuronal cells and differentiated adult cell types, where it functions as a maintenance demethylase, by occupying hypomethylated CGIs via its CXXC domain, hydroxymethylating the CGI boundaries, and preventing 5mC spreading by occluding DNMTs [101]. Since TET1 was first identified as a fusion partner of MLL1 in AML, it was suspected to play an oncogenic role. However, evidence suggests otherwise. Cimmono et al., demonstrated that genetic ablation in a mouse model promotes lymphomagenesis, particularly the formation of follicular lymphoma (FL) and diffuse large B cell lymphoma (DLBCL) suggesting that the presence of TET1 is oncoprotective and loss of its function promotes B cell

lymphoma. Human FL and DLBCL samples showed no associated TET1 loss of function mutations, though a small percentage were associated with mutations in TET2. All FL and DLBCL samples unassociated with TET1 mutations, exclusively showed diminished TET1 expression as a result of promoter hypermethylation. This is indicative of transcriptional, post-transcriptional and post-translational mechanisms acting to diminish functional TET1 as an oncogenic event in the absence of loss-of-function genetic mutations in TET1 [102].

In vitro knockdown studies imply an inhibitory effect of TET1 on cell proliferation [103], explaining why it is downregulated in several cancers. Analysis of adenocarcinomas originating from lung, colon, breast, and rectum, at stages I to IV, showed that TET1, but not TET2/3, was downregulated in stage1 adenocarcinoma, suggesting repression of TET1 is an early event in tumorigenesis [104]. As in FL and DLBCL, hypermethylation of the TET1 promoter results in transcriptional silencing, and is a significant contributor to CIMP associated with colorectal cancers [105]. In primary colorectal cancer cells, TET1 inhibition facilitates tumorigenesis via activation of the Wnt signaling pathway, mainly a result of repression of negative regulators of Wnt, DKK3 and DKK4, which are TET1 transcriptional targets, and in its absence are repressed by promoter methylation [104]. In addition to controlling proliferation, TET1 also negatively regulates invasion and metastatic potential. Members of the Tissue inhibitors of metalloproteases (TIMP) family, TIMP2 and TIMP3, are directly bound by and regulated by TET1-mediated demethylation. Suppression of TET1 in invasive cancers results in repression of the TIMPs, thereby resulting in derepression of matix metalloproteinases (MMPs), an essential step towards gaining invasiveness and promoting metastasis [106]. This mechanism of MMP reactivation through TET1 inhibition was also observed in breast cancers dependent on HDAC-mediated epigenetic events [107]. Breast cancer metastasis has also been attributed to inhibition of HOXA (HOXA7 and HOXA9), which is targeted by TET1-mediated promoter demethylation to bring about transcriptional activation. Overexpression of the tumorigeneic architectural transcription factor, HMGA2, in breast cancer cells, results in transcriptional silencing of TET1 and thereby its downstream targets - HOXA7 and HOXA9. TET1, which autoregulates itself by preventing 5mC deposition at its promoter, is silenced by promoter methylation in HMGA2-overexpressing breast cancer cells, thus implying the involvement of an HMGA2/TET1/HOXA signaling pathway in promoting breast cancer metastasis [103]. Another signaling pathway dependent on TET1 repression to promote TSG repression is the KRAS-ERK signaling pathway. KRAS overexpression is oncogenic, results in cellular transformation, and is observed in numerous cancers. KRAS overexpression is concomitant with reduced 5hmC levels and increased 5mC levels, particularly at the promoters of TSGs. Of these, DAPK, MGMT, and DUOX1 are direct targets of TET1, and KRAS overexpression results in reduced TET1 occupancy at the promoters of these TSGs resulting in their silencing by hypermethylation [108]. TET1 activity is also affected by hypoxic conditions, and will be discussed in Sect. 4.

2.2.2 TET2

This family member lacking the N-terminal CXXC domain plays an essential role in myelopoiesis, and is highly expressed in normal myeloid progenitor cells, granulocytes, and erythroid cells. TET2 is an established tumor suppressor of myeloid malignancies and is frequently mutated in myelodysplastic syndromes and myeloproliferative disorders, including AML. The first reported mutations in the TET2 gene were myeloid cancer associated chromosomal aberrations-microdeletions and uniparental disomy involving the chromosomal region 4q24, where the human TET2 gene is located [109]. Thereafter, several TET2 point mutations were identified across several blood malignancies, including MDS, CMML, primary AML, blastic plasmacytoid dendritic neoplasm, myeloproliferative neoplasms (MPNs) such as polycythemia vera, primary myelofibrosis, and B- and T-cell lymphomas. These somatic mutations, encompassing insertions, deletions, missense, nonsense, and frameshift mutations, were heterozygous and mapped to the TET2 catalytic domain, potentially resulting in TET2 enzymatic deficiency [110]. However, TET2 mutations are only pre-leukemic, potentiating tumor initiation, but not causative on their own. Evidence suggests that TET2 mutations are acquired at an early stage in the onset of hematological malignancies. Analysis of HSCs from MDS patients show accumulation of monoallelic TET2 mutations with progressive accumulation of secondary mutations at the MDS stage, suggesting that TET2 inactivation creates a clonal population of HSCs poised for oncogenesis upon accumulation of secondary mutations. This notion is supported by studies in different models of Tet2 knockout mice. Disrupting Tet2 in HSCs or hematopoietic progenitor cells (HPCs) led to decreased 5hmC levels, an increase in self-renewal capacity, and expansion of the stem cell compartment with concordant blockage in myeloid differentiation. Interestingly, lymphoid differentiation remains unaffected [111]. A similar effect was seen when the Tet2 catalytic domain was selectively ablated. The HSC and HPC compartments exhibited enhanced self-renewal and expansion in serial transplantation assays, with impaired differentiation down the myeloid lineage [112]. The enhanced self-renewal capacity and antagonistic differentiation can be attributed to increased expression of self-renewal factors, Meis1 and Evi1 with concomitant decrease in differentiation/myeloid specific factors [111]. To further support the idea that acquisition of TET2 mutations is an early event in hematological malignancies, Zhao et al. specifically disrupted TET2 in different compartments of the hematopoietic system, and showed that mutations in the HSC/HPC, but not the more differentiated cell types, led to myeloid malignancies [113]. TET2 mutations have been shown to accumulate in healthy ageing individuals showing clonal hematopoiesis, predisposing them to developing hematological malignancies [114].

In order to initiate tumorigenesis, TET2 mutated hematopoietic clones acquire a second hit. Several genes are co-mutated with TET2 across different cancer types. In MDS, TET2 mutations are associated with the splicing factor, SF3B1. Additionally, mutations in SRSR2, EZH2, and ASXL1, also reportedly co-exist with TET2 mutations in MDS [110]. Mouse models with co-mutations in TET2 and

EZH2/ASXLI recapitulate the MDS phenotype [115, 116]. The gene encoding the small GTPase of the Rho family, RHOA, is exclusively co-mutated with TET2 in peripheral T-cell lymphoma (PTCL) [117], while DNMT3A co-mutations are observed in angioimmunoblastic T-cell lymphoma (AITL) [118]. Mutations in IDH2 have been reported to co-occur with TET2 mutations in MDS [119], but IDH1 and TET2 mutations are still believed to be mutually exclusive.

While inactivating mutations in TET2 seem to be the major route towards achieving oncogenic potential, alternate mechanisms affecting TET2 function have also been reported. The TET2 associated CXXC domain is carried as a separate gene, IDAX, which binds promoters and CpG islands in the genome. IDAX directly interacts with TET2, and mediates TET2 degradation in a caspase-dependent manner [120]. By negatively regulating TET2 protein levels, IDAX abrogates TET2's tumor suppressor function, to promote cancer initiation and/or progression. TET protein levels are also regulated by the calcium-dependent cysteine proteases, calpains (Calpain1 – Tet1/2, Calpain2-Tet3) [121], which are overexpressed in cancers [122], providing an additional mechanism by which TET2 can be negatively controlled to facilitate and sustain cancer.

Lastly, loss of TET2 targeting across the genome could result in the hypermethylator phenotype underlying tumor progression. Mutations in a TET2 interacting transcription factor, Wilms Tumor 1 (WT1), occur in AML and are mutually exclusive of TET2, IDH1, and IDH2 mutations. WT1 recruits TET2 to specific target sites to mediate transcriptional activation. AML associated mutations in TET2 disrupting its interaction with WT1 result in loss of transcriptional activation of WT1 target genes, signifying the dependence of WT1 on TET2 to mediate transcriptional activation [123].

2.2.3 TET3

TET3 is highly expressed in oocytes and has been functionally implicated in regulating methylation patterns in the male pronucleus. Like TET1, mutations in TET3 contributing to cancer development are rare. Nonetheless, a tumor suppressor function has been attributed to TET3 in a few tumor types. Inhibition of TET3 is critical to maintaining self-renewal and tumorigenic potential in glioblastoma stem cells (GSCs), and is achieved by repression by the transcription factor TLX. TLX binds to the TET3 promoter to transcriptionally silence it and promote GSC tumorigenecity, possibly through repression of the tumor suppressor genes, BTG2, TUSC1, BAK1, LATS2, FZD6 and PPP2R1B. The TLX-TET3 inverse regulatory axis, if disrupted, results in reduced oncogenic potential of GSCs [124]. The tumor suppressor role of TET3 was further elucidated in ovarian cancer cells. TET3 was targeted for repression by TGFβ1-induced EMT, and EMT was blocked upon overexpression of TET3 in ovarian cancer cell lines. TET3 mediates its oncoprotective function in ovarian cancer by regulating promoter demethylation and hence activation of miR-30d, a proven inhibitor of EMT [125].

3 Modulation of DNA Methylation Regulators by Metabolic Mechanisms

3.1 IDH1/IDH2

The genes coding for the enzyme isocitrate dehydrogenase (IDH) are frequently mutated in myeloid malignancies (AML, MDS, and MPN), neural malignancies (astrocytoma, oligodendrocytoma, and glioblastoma), and less frequently in other solid tumors (cholangiocarcinoma, chondrosarcoma, colorectal cancer, esophageal cancer, bladder cancer, melanoma, prostate carcinoma, and breast adenocarcinoma). The IDH family converts isocitrate to αKG via oxidative decarboxylation and consists of three active enzymes, IDH1 which localizes to the cytosol and peroxisomes, and IDH2 and 3, which localize to the mitochondria. IDH1 and IDH2 function as homodimers and use nicotinamide adenine dinucleotide phosphate (NADP+) as a cofactor, whereas IDH3 functions as a heterotetramer (consisting of two alpha, one beta and one gamma subunit) in the TCA cycle, utilizing nicotinamide adenine dinucleotide (NAD+) as a cofactor. However, somatic mutations in IDH1/2, but not IDH3 have been attributed to promoting tumorigenesis by altering metabolism, specifically α-ketoglutarate levels, thereby inhibiting the function of α-KG dependent dioxygenases which include the TET family, JmjC domain-containing histone demethylases, and EglN prolyl-4-hydroxylases.

Somatic mutations, mostly missense mutations, in IDH1/2, are restricted to one of three arginine residue in the catalytic pocket of the enzyme essential for isocitrate binding. In IDH1, this residue is invariably R132, while in IDH2, the R172 and R140 residues are targets for somatic mutations. As opposed to inactivating enzymatic mutations, frequently underlying mechanisms promoting cancer, IDH1/2 mutations are activating and produce a neomorphic enzyme that catalyzes the reduction of α-ketoglutarate (α-KG) to the R-enantiomer of 2-hydroxyglutarate (R-2-HG), an oncometabolite [126]. Accumulation of R-2-HG promotes proliferation while inhibiting differentiation, and competitively inhibits the dioxygenase activity of the α-KG and Fe^{2+} dependent TET enzymes [127]. The consequence of inhibiting TET activity in this manner is DNA hypermethylation, and in fact IDH mutant tumors exhibit CIMP. Expression of the mutant IDH1 in an in vitro system redefines the methylome to recapitulate the hypermethylator phenotype observed in IDH1 mutant tumors. This was independently shown in isogenic human primary astrocytes and an isogenic colorectal cancer cell line, HCT116, both genetically engineered to express IDH1 (R132H) [128].

IDH mutations have been suggested to be causative, rather than simply contributing, to CIMP. Studies involving low grade glioma (LGG) showed that the G-CIMP+, but not the G-CIMP- LGGs were associated with IDH1 mutations. G-CIMP+ tumors are associated with hypermethylation at CGIs and shores at loci enriched for PRC2 targets, and indeed showed deposition of H3K27me3, a histone mark positively correlating with/permissive to 5hmC deposition [129]. Hypermethylation in IDH mutant primary gliomas also show a loss in CTCF-binding, disrupting the

organization of topologically associated domains (TADs), resulting in aberrant expression of oncogenes. The term TAD refers to the three-dimentional subdomain arising from spatially favourable conformations of locally interacting chromatin. Boundaries between TADs ensure maintenace of the environment within them, and are maintained by the insulator binding protein - CTCF. PDGFRA, an established glioma associated oncogene, is activated by this mechanism. G-CIMP associated with IDH1 mutation results in disruption of the boundary between PDGFRA and FIP1L1 (from an adjacent TAD) leading to association of the PDGFRA promoter with a constitutive enhancer, resulting its constitutive expression and oncogenic signaling [130]. How does IDH1 mutation lead to CIMP? Inhibition of TET catalytic activity is one explanation, however, loss of TET recruitment has also been reported. Chondrosarcomas driven by IDH1 and IDH2 mutations exhibit a CIMP in regulatory regions, including promoter associated transcription start sites and CpG islands and shores at genes enriched to function in the retinoic acid pathway. These genes were co-bound by EBF1 and TET2, suggesting the hypermethylation was a result of altered TET2 targeting by EBF1 at these sites. Recruitment of both EBF1 and TET2 was altered at three of the most differentially hypermethylated loci – CCND2, FABP3 and FBRSL1, as determined by ChIP-seq. EBF1 and TET2 co-immunoprecipitate in the chondrosarcoma cell line SW1353 [131].

3.2 Hypoxia

Hypoxia is a cancer prevalent microenvironment promoting tumor growth by influencing cellular processes that confer upon cells aggressive pro-survival phenotypes of uncontrolled proliferation, invasion, evasion from apoptosis, while also facilitating angiogenesis. The adaptive response to hypoxic conditions is mediated by the oxygen tension-dependent hypoxia inducible factor-1 (HIF-1). HIF1 functions as a heterodimeric transcription factor, and regulates expression of genes containing a 5′-ACGTG-3′ hypoxia-response element (HRE) in their associated promoters or enhancers [132]. The active transcription factor consists of a hypoxia inducible HIF-1α subunit and a ubiquitously expressed HIF-1β subunit. HIF1α levels are regulated by targeted degradation by O_2, Fe^{2+}, and α-ketoglutarate-dependent prolyl hydroxylases, which have reduced activity under hypoxia, resulting in HIF1α accumulation. It is well-accepted that hypoxia is accompanied by global hypomethylation events, which have been attributed to induction of TET enzymes, direct transcriptional targets of HIF1. In N-type neuroblastoma cells, TET1 is directly bound and activated by HIF1 at the HRE within its proximal promoter, resulting in transcriptional activation and a concordant increase in 5hmC levels. Studies suggest that TET1 is specifically bound by HIF2α, and not HIF1α, and HIF1 interaction with TET1 enhances its transcriptional activity, independent of catalytic activity [133], and functions as a co-activator to regulate expression of genes involved in glucose metabolism (glucose transporter 3 (GLUT3), hexokinase 1 (HK1), phosphoglycerate kinase 2 (PGK2), pyruvate kinase M (PKM), and lactate

dehydrogenase A (LDHA)). An important mediator of the Hypoxia/HIF1/TET1 gene regulation is INSIG (insulin induced gene 1), which is activated under hypoxic conditions by promoter demethylation to regulate glucose metabolism [134]. Hypoxia induced cellular proliferation and invasion, properties responsible for achieving EMT, can be abrogated by TET1 depletion, demonstrating its central role in the process [134]. In addition to TET1, TET3 (but not TET2) was also shown to be a transcriptional target of HIF1, and is induced under hypoxic conditions along with TET1 to regulate cancer phenotypes in breast cancer cells [135]. It is surprising that TET1 functions as an oncogene and is transcriptionally activated in hypoxic conditions, but is a tumor suppressor gene that is inactivated to promote tumorigenesis in other instances (myeloid malignancies). In addition to activating TET enzymes, demethylation under hypoxia is also achieved by silencing of the DNMT family of enzymes, as shown in colorectal cancer cells, which results in demethylation of the p16INK4a gene promoter [136].

3.3 Fumarate Hydratase (FH) and Succinate Dehydrogenase (SDH)

Two enzymes acting at consecutive steps in the TCA cycle, succinate dehydrogenase (SDH), and fumarate hydratase (FH), are mutated in familial paraganglioma (PGL), pheochromocytoma (PCC), uterine and skin leiomyoma, and papillary renal cell carcinoma [137]. FH and SDH are tumor suppressors, and inactivating mutations in these enzymes result in an accumulation of their substrates, fumarate and succinate, which act as oncometabolites [138]. Since fumarate and succinate are structurally similar to α-KG, they effectively inhibit α-KG dependent enzymes, including the TET family, through competitive inhibition [139]. Studies have reported a global loss of 5hmC, resulting from inhibition of TET enzymatic activity in tumors carrying FH and SDH mutations [137]. Loss of genomic 5hmC can facilitate hypermethylation events contributing to CIMP. This has been reported in paraganglioma driven by SDH mutations [140]. Additionally, fumarate and succinate function as oncometabolites by inhibiting the α-KG dependent HIF prolyl hydroxylases, which leads to increased HIF1α stabilization, creating a ‘pseudohypoxic’ condition, augmenting angiogenesis and anaerobic respiration [141].

4 Summary, Conclusions, and Perspectives

The epigenomic contribution to promoting cancerous events is being increasingly appreciated, and in this chapter we have covered mechanisms by which aberrant epigenetic information on DNA may occur, and its role in tumorigenesis. Two major components of the DNA methylation pathway, the writers – DNMTs and the erasers -TETs, are frequently deregulated by multiple mechanisms during tumorigenesis. A recurring theme in cancer is global genomic hypomethylation accompanied by

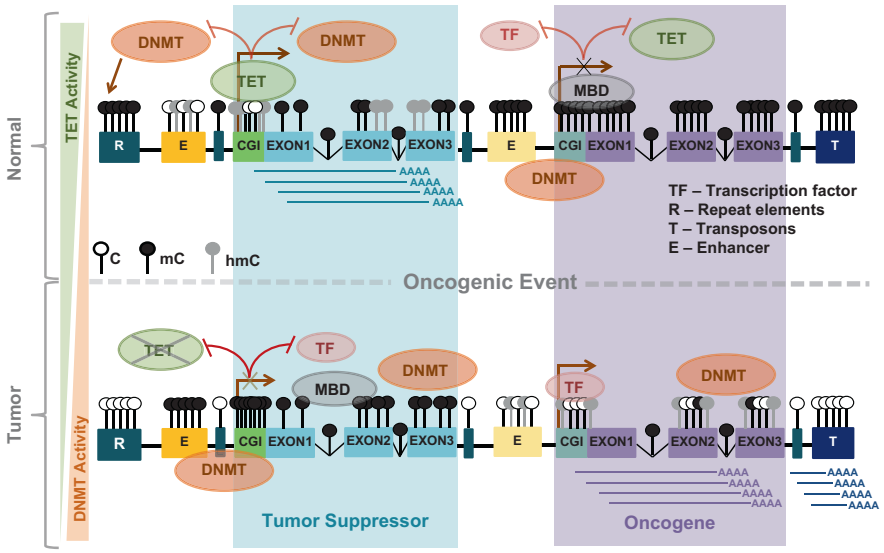

Fig. 3 Overview of how a CpG island hypermethylator phenotype is inactivates tumor suppressor genes. DNMTs in general act as oncogenes and are overexpressed/activated in tumors, whereas the TETs act as tumor suppressors and are functionally silenced or inactivated. The net outcome of these deregulation events result is CIMP, most prominently observed at promoters of TSGs. Tumors are also associated with global hypomethylation that results in demethylation of regulatory features associated with oncogenes, leading to their activation. Additionally, demethylation of repeat elements is frequently observed in cancers, resullting in spurious transcription from these elements which contibutes to genome instability

local hypermethylation events, giving rise to a 'hypermethylator phenotype' termed CIMP (Fig. 3). Since these two components are functionally antagonistic, the DNMTs are hyperactivated, while the TETs are functionally inactivated to promote CIMP in tumors. To establish tumorigenic aberrant DNA methylation patterns, the molecular components of the DNA methylation pathway are functionally intervened with somatic mutations, transcriptional regulation, mRNA stability, and protein turnover. Figure 3 gives an overview of how different genomic features undergo a switch in their DNA epigenetic marks to promote oncogenesis. This chapter addresses the pro-cancer modifications of the components directly affecting deposition (DNMTs) and erasure (TETs) of DNA methylation patterns. However, DNA methylation patterns exert their functional effects through a cascade involving other components of the chromatin-histones, nucleosomes, and larger chromosomal domains-which feedback onto DNA. This intricate cross-talk is particularly evident at the centromeric repeats, which form a part of the constitutive heterochromatin compartment silenced by DNA methylation, repressive histone modifications, specifically H3K9me3, and macromolecular repressive factors like the HP1 family. DNMT3A/3B localize to, and methylate pericentromeric repeats, and closely interact with H3K9me3 and HP1 to form a reinforcing feedback loop to ensure complete heterochromatization and structural maintenance of the centromere which is essential for preventing chromosomal aberrations like aneuploidies arising from

incomplete chromosome segregation. In addition to cooperation among epigenetic mechanisms, cooperation also exists between cellular processes. Metabolic changes influence epigenetic modifications, as exemplified by tumors with mutations in IDH/FH/SDH, and local epigenetic changes influence cellular properties including proliferation, adhesion, migratory, and invasive potential, disturbing cellular homeostasis, and promoting metastasis. Although our current knowledge of the intricate DNA methylation process, its cross-talk with other epigenetic processes, and the molecular impact on biological processes seems substantial, many lose ends remain. (i) How are writers and erasers preferentially recruited to, or excluded from particular genomic loci? (ii) What functional epigenetic boundaries exist at the DNA level and how these are established and maintained? (iii) How are epimutational hotspots generated, and how can these be exploited for therapeutic intervention or early cancer detection? Addressing these questions will provide additional insights into how the DNA methylation pathway is deregulated to facilitate cancer, and may lead to identification of new molecules for targeted therapy.

Glossary

CpG site Linear sequence of DNA where a cytosine is followed by guanine in the 5' to 3' direction.

CpG methylation (mCG) Cytosine within a CpG site, methylated by the DNMTs at the C5 position, represented as mCG.

Non CpG methylation (mCH) Methylation occurring outside of CpG sites, where H could be adenine, cytosine or thymine.

CpG Islands (CGI) Short interspersed sequence of DNA, around 200 base-pairs long, with high CpG fequency, and GC content greater than 50%.

CpG shores and shelves 2 kb on either side of CpG islands are termed CpG shores, and 2 kb on either side of the CGI shores are termed CGI shelves.

CpG Canyons Regions of low methylation, distinct from CGIs, and frequently associated with transcription factor binding sites.

Epimutations Non-genetic, heritable, aberrant lesions in the expression of a gene, arising from epigenetic DNA modifications or other epigenetic modifications on local chromatin.

CpG hypermethylator phenotype (CIMP) Hypermethylated CpG islands forming a diagnostic/prognostic tumor specific DNA methylation signature.

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