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Review

5-Hydroxymethylcytosine: An epigenetic mark frequently deregulated in cancer

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ABSTRACT

The epigenetic mark 5-hydroxymethylcytosine (5hmC) has gained interest since 2009, when it was discovered that Ten-Eleven-Translocation (TET) proteins catalyze the conversion of 5-methylcytosine (5mC) into 5hmC. This conversion appears to be an intermediate step in the active DNA demethylation pathway. Factors that regulate DNA hydroxymethylation are frequently affected in cancer, leading to deregulated 5hmC levels. In this review, we will discuss the regulation of DNA hydroxymethylation, defects in this pathway in cancer, and novel therapies that may correct deregulated (hydroxy)methylation of DNA.

Contents

1. Introduction

DNA methylation is essential for normal development and plays an important role in many processes, including X-chromosome inactivation, imprinting and transcriptional regulation. Methylation of promoter regions is associated with transcriptional repression, while actively transcribed genes may contain high levels of gene body methylation (from the transcription start site to the end of the transcript) [1]. DNA methylation is mediated by DNA methyltransferases (DNMTs) and predominantly occurs on cytosine residues present in CpG context. DNMT3A and DNMT3B are responsible for *de novo* methylation, while DNMT1 is mainly involved in maintaining methylcytosine marks during DNA replication (Fig. 1) [2].

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DNA demethylation may take place as a passive process due to lack of maintenance methylation during DNA replication. In addition, recent studies presented evidence for an active DNA demethylation pathway initiated by the Ten-Eleven Translocation (TET) protein family. The TET proteins are responsible for the conversion of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) through three consecutive oxidation reactions [3,4]. 5fC and 5caC marks are recognized by thymine DNA glycosylase (TDG). TDG activates the base excision repair pathway (BER), which replaces the modified cytosine with an unmodified cytosine (Fig. 1) [5].

Many proteins and cofactors control the balance between methylation and demethylation. In cancer, this balance is frequently deregulated, leading to altered methylation patterns. These changes in methylation can lead to repression of tumor suppressor genes or activation of oncogenes. Recently, it has been shown that hydroxymethylation levels are also altered in various types of cancer [6–9]. Key events that contributed to the elucidation of the link between deregulated 5hmC levels and cancer are summarized in Fig. 2. Several mechanisms have been described that may be involved in these changes in 5hmC, which will be the focus of this review. Insights into these mechanisms may open new possibilities for treatment.

2. DNA hydroxymethylation and its regulation

5hmC was initially found in the DNA of bacteriophages [10], and was first reported to be present in mammalian DNA in 1972 [11]. After 1972, the interest for this cytosine modification was lost until 2009, when it was shown that TET proteins catalyze the conversion of 5mC to 5hmC [3]. Currently, 5hmC is well-accepted as an intermediate in demethylation. In addition, several studies suggest that it has additional functions as well. First of all, various tissues accumulate substantial levels of 5hmC, which is unexpected if 5hmC would only be a transient intermediate [12]. The levels differ considerably between tissues, with the highest levels observed in neuronal and adult liver cells [13,14]. Secondly, proteins have been described that specifically bind to 5hmC [15]. Our knowledge about these proteins is still limited, but they may function as so-called 5hmC 'readers'.

The regulation of DNA hydroxymethylation is mediated by several factors. As mentioned, the TET protein family, consisting of TET1, TET2 and TET3, is responsible for the formation of 5hmC marks. TET proteins belong to the family of α-ketoglutarate- (αKG) and Fe^{2+}- dependent dioxygenases, and require αKG as a co-substrate and Fe^{2+} and ascorbate as co-factors [3,16]. The availability of these 3 factors may influence the activity of the TET proteins. The co-substrate αKG is produced by the isocitrate dehydrogenase (IDH) protein family, consisting of IDH1, IDH2 and IDH3. IDH proteins catalyze the oxidative decarboxylation of isocitrate to αKG, which is an intermediate step in the tricarboxylic acid (TCA) cycle (Fig. 3).

TET1 and TET3 contain a CXXC domain which is implicated in DNA binding. Due to a chromosomal inversion event during evolution, the ancestral CXXC domain of TET2 is located upstream of the *TET2* gene and transcribed in the opposite direction. This CXXC domain currently functions as a separate gene, called *CXXC4* (or *IDAX*) [17]. Recently Ko et al. [18] showed that CXXC4, and another CXXC protein called CXXC5 (or RINF), are able to downregulate TET2 protein levels in a dose-dependent manner, with a concomitant decrease in 5hmC levels. They showed that CXXC4 binds to unmethylated DNA and recruits TET2 to the genome to exert its function. At the same time, DNA-bound CXXC4 triggers degradation of TET2 in a caspase-dependent manner. A similar autoregulatory function was observed for the CXXC domain present in the TET3 protein.

5hmC levels are also partly regulated by microRNAs (miRNAs). Recently, several miRNAs were found to target the TET proteins and other components of the demethylation pathway (Table 1). So overall, the 5hmC mark is regulated on many levels.

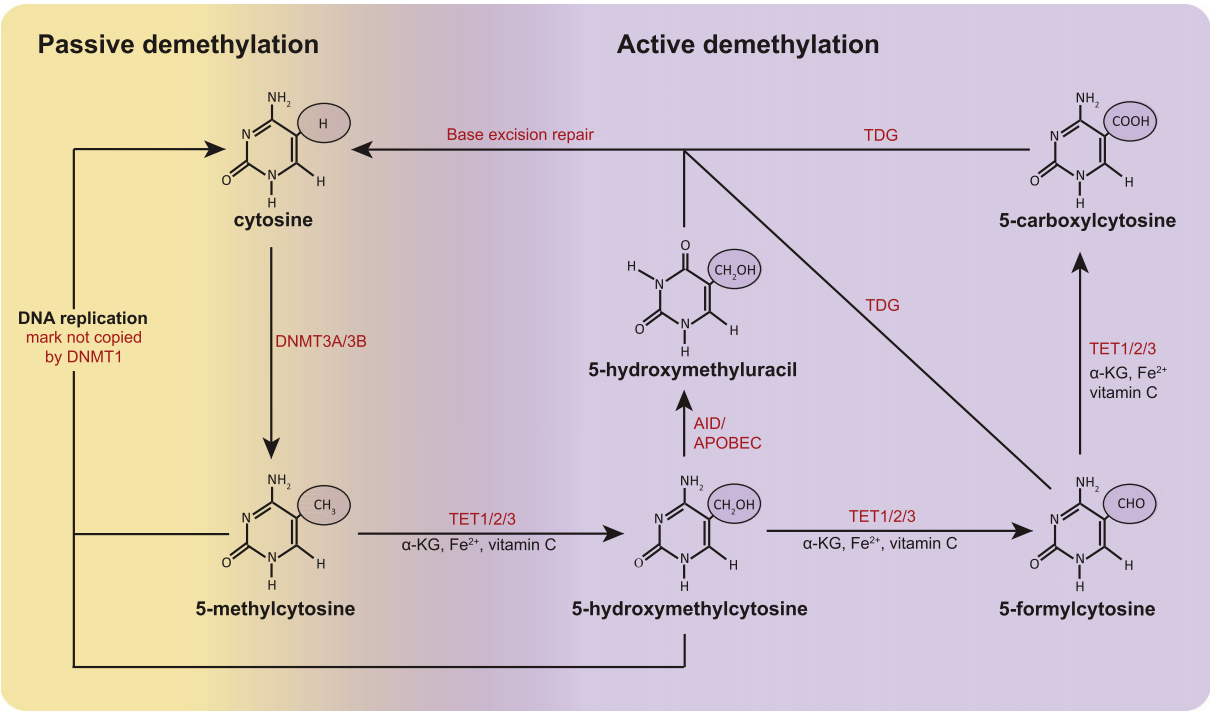

Fig. 1. DNA methylation and demethylation pathways. DNMT proteins are responsible for the methylation of cytosines in the DNA. DNMT3A/B are the most important regulators of *de novo* methylation, while DNMT1 is mainly involved in maintaining the 5mC mark during DNA replication. When DNMT1 is not able to copy the mark, this can lead to passive demethylation during cell division. Recently, two pathways of active demethylation have been described. Most evidence exists for a pathway in which TET proteins convert 5mC into 5hmC, 5fC and 5caC through three consecutive oxidation reactions. Subsequently, 5fC and 5caC are recognized by TDG proteins which activate the base excision repair pathway. In addition, evidence exists for a pathway in which AID/APOBEC proteins deaminate 5hmC to 5hmU followed by TDG-mediated base excision repair [149].

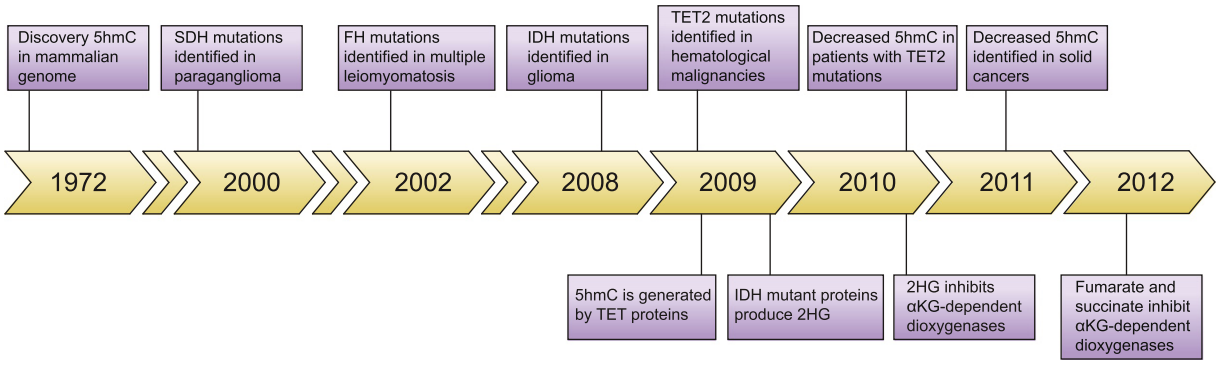

Fig. 2. Timeline illustrating the key events that contributed to elucidation of the link between deregulated 5hmC levels and cancer.

3. Deregulation of DNA hydroxymethylation in cancer

Several genes that influence hydroxymethylation are mutated in cancer. First of all one of the *TET* genes, *TET2*, is affected by mutations in different hematological malignancies. Furthermore, four genes that play a role in the Krebs cycle, namely *IDH1*, *IDH2*, *SDH* and *FH*, are mutated in hematological malignancies and various types of solid cancers. These 4 genes are able to affect the activity of the TET proteins by changing the levels of metabolites that compete with the TET co-factor αKG. In addition, changes in expression of *TET*, *IDH* and *CXXC* genes are linked to altered 5hmC levels in cancer. All these individual factors will be discussed in the next sections.

3.1. TET2 mutations

In 2009, *TET2* aberrations were discovered in different hematological malignancies (Table 2) [19,20]. The *TET2* gene can be affected by deletions as well as by splice site, missense, and nonsense mutations.

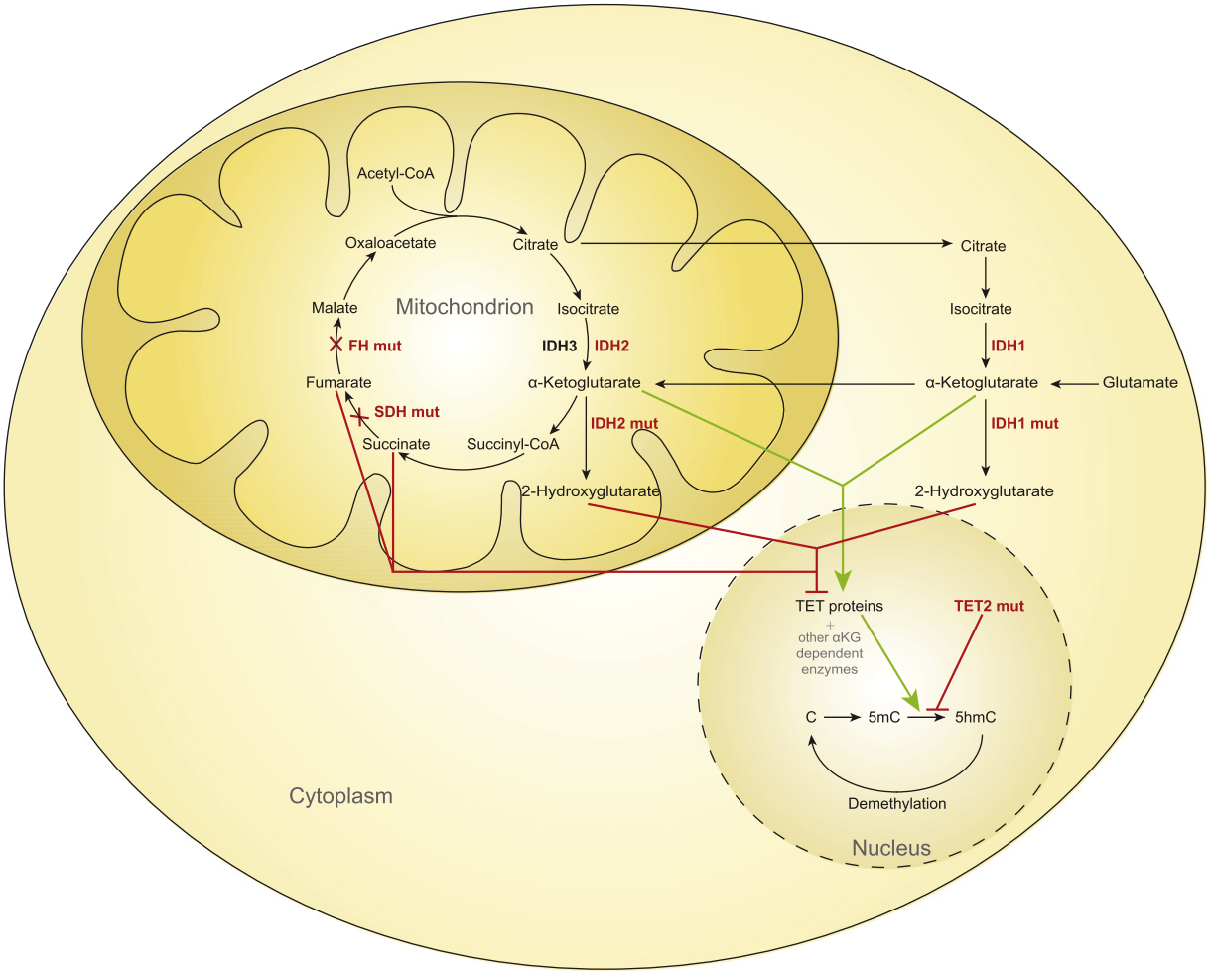

Fig. 3. Effect of TET2, IDH1, IDH2, SDH and FH mutations on the generation of 5hmC. Schematic overview of a cell, with the Krebs cycle and DNA (de)methylation pathway indicated. In red, five genes are highlighted that are frequently mutated in cancer. *TET2* mutations disturb the catalytic activity of the *TET2* gene, resulting in decreased generation of 5hmC. Mutations in IDH, SDH and FH proteins result in accumulation of the metabolites 2-hydroxyglutarate, succinate and fumarate respectively. All three metabolites are able to inhibit αKG-dependent enzymes, including the TET protein family, which leads to a decreased formation of 5hmC.

Table 1
Human TET- and TDG- targeting miRNAs.

miRNA[a,b]	Target(s)[c]	References
miR-7	TET2	[78]
miR-22	TET1,2,3	[79,80]
miR-26	TET1,2,3 and TDG	[78,110]
miR-29	TET1,2,3 and TDG	[78,81,111]
miR-30	TET2	[78]
miR-33	TET2	[78]
miR-101	TET2	[78]
miR-125	TET2	[78]
miR-202	TET2	[78]
miR-335	TET2	[78]
miR-339	TET2	[78]
miR-520d	TET2	[78]
miR-550-2	TET2	[78]
miR-767	TET2	[78]

[a] Most miRNA family members have a similar effect.
[b] From Cheng et al. [78] only miRNAs included with more than 25% repressive effect.
[c] Not the same targets tested for each miRNA.

The majority of the missense mutations target the C-terminal catalytic domain of the protein. The frequency of *TET2* mutations depends on the type of disease (Table 2), with the highest frequencies observed in chronic myelomonocytic leukemia (CMML) and a subtype of T-cell lymphomas called angio-immunoblastic T-cell lymphoma (AITL) [21,22]. In most hematological malignancies, *TET2* mutations did not significantly affect prognosis, but several studies reported an association with poor prognosis in acute myeloid leukemia (AML) [23–25]. *In vitro* studies have shown that *TET2* mutations result in loss-of-function. Cells transfected with wild type *TET2* produced high levels of 5hmC, while 5hmC could hardly be detected in cells transfected with a *TET2* mutant [7,25]. Recently, several studies reported decreased 5hmC levels in *TET2* knock-out (KO) mice [26] and patients with *TET2* mutations [7,9,27,28]. Importantly, *TET2* KO mice showed an increase in hematopoietic progenitor cells and a bias towards myeloid differentiation, and ultimately developed a CMML-like disease [29,30]. The discovery of loss-of-function *TET2* mutations in hematological malignancies provided the first link between deregulated 5hmC levels and cancer (Figs. 2, 3). Interestingly, *TET2* mutations have not been reported, or are very rare, in other malignancies where 5hmC levels are affected, including brain tumors and melanoma [31–33].

3.2. IDH mutations and elevated 2HG

Two genes of the IDH family, *IDH1* and *IDH2*, are frequently mutated in hematological malignancies as well as in certain solid tumors [34] (Table 2). *IDH* mutations are always heterozygous and occur at hotspot regions; mutations in IDH1 target arginine 132, and mutations in IDH2 target the arginines at position 140 and 172 of the protein [35]. *In vitro* studies have shown that mutant IDH proteins gain the function to convert the TET co-substrate αKG into R-2-hydroxyglutarate (R-2HG) [36]. Importantly, 2HG can act as a competitive inhibitor of αKG-dependent enzymes, including the TET protein family [37,38].

IDH1(R132H) knock-in mice had several hematological defects. They showed increased numbers of early hematopoietic progenitors, and developed splenomegaly and anemia with extramedullary hematopoiesis [39]. In *IDH*-mutated AML patients, elevated 2HG levels could be measured in myeloblasts, bone marrow aspirates, urine and serum [40]. Several reports showed the potential of using serum 2HG (often 50–100

Table 2
Mutated genes that influence 5hmC levels.

Disease/cancer type	Frequency	Prognostic impact	Reference
TET2 mutations			
Chronic myelomonocytic leukemia	44–58%	Intermediate/poor	[21,112,113]
Myelodysplastic syndrome	21–33%	Intermediate	[19,114,115]
Myeloproliferative neoplasms	12–15%	Intermediate	[20,116,117]
T-cell lymphoma	12%	ND	[29]
Angioimmunoblastic T-cell lymphoma	*47–76%*	*Intermediate*	[22,29,118]
Acute myeloid leukemia	7–13%	Intermediate/poor	[9,23,24,119]
B-cell lymphoma	2%	ND	[29]
IDH1/2 mutations			
Secondary glioblastoma	73–95%	Favorable	[120–122]
Glioma (grade II and III)	68–85%	Favorable	[120,123,124]
Angioimmunoblastic T-cell lymphoma	20–45%	Intermediate	[118,125]
Acute myeloid leukemia	15–23%	IDH1 R132 Intermediate/poor IDH2 R172 Intermediate/poor	[9,35,119,126,127]
		IDH2 R140 favorable	
Myelodysplastic syndrome	4–12%	Intermediate/poor	[114,115,128,129]
Chronic myelomonocytic leukemia	3–6%	Intermediate	[112,130,131]
Ollier disease and Maffucci syndrome	77–93%	ND	[50,51]
Chondrosarcoma and enchondroma	43–56%	Intermediate	[49,51,132,133]
Intrahepatic cholangiocarcinoma	10–28%	Favorable	[52,53,134]
Melanoma	10%	ND	[56]
Thyroid cancer[a]	8–16%	ND	[55,135]
Prostate cancer	3%	ND	[54]
SDH mutations			
Paraganglioma/pheochromocytoma	12–15% (SDHA,B,C,D)	Poor	[64,136,137]
Gastrointestinal stromal tumors	3–8% (SDHA,B,C,D)	Favorable	[59,138–140]
Renal cell carcinoma	1–4% (SDHB)	ND	[141,142]
FH mutations			
Multiple cutaneous and uterine leiomyomata	100%	ND	[143,144]
Hereditary leiomyomatosis and renal cell cancer	71–93%	ND	[145–147]
Papillary renal cell cancer	17%	ND	[145]
Leydig cell tumors	7%	ND	[148]
Paraganglioma/pheochromocytoma	<1%	ND	[61,64]

ND = Not determined.
[a] *IDH1* mutations outside hotspot region; no 2HG production.

fold elevated) as a biomarker to detect *IDH*-mutated AML [40,41]. 2HG-mediated inhibition of TET proteins resulted in decreased 5hmC levels in these AML patients [9,27]. As both *TET2* and *IDH* mutations affect 5hmC levels in AML, this may explain why mutations in these genes are mutually exclusive. However, given that αKG is a co-substrate for more than 70 αKG-dependent dioxygenases, the effect of *IDH* mutations may be broader than only inhibiting TET proteins.

The first *IDH* mutations were discovered in 2008 in gliomas [42]. Additional studies in various central nervous system tumors revealed *IDH1* and *IDH2* mutations in 75% of grade II to III gliomas and secondary glioblastomas (Table 2). As in hematological malignancies, *IDH* mutations in glioma are heterozygous and restricted to specific positions: arginine 132 in IDH1 (90% of the cases) and arginine 172 in IDH2 (10% of the cases). In contrast to AML, IDH2 R140 mutations are absent. The reason why IDH2 R140 mutations are found in hematological malignancies but not in brain tumors is currently unknown. As expected, IDH mutant glioma cells had elevated 2HG levels (~100 fold) [36]. In line with 2HG-mediated inhibition of TET proteins, *IDH*-mutated gliomas showed a hypermethylation phenotype; an increased prevalence of CpG island hypermethylation in promoter regions [43]. 5hmC levels were strongly decreased in brain tumors when compared to healthy brain tissue, however, the decreased 5hmC did not always correlate with *IDH* mutations. Several studies reported decreased 5hmC levels in *IDH* mutant tumors compared to *IDH* wild type tumors [8,37,44], while other studies could not confirm this difference [45–47]. This discrepancy might be caused by the area where biopsies were taken from, the specific types of brain tumors that were investigated or the techniques that were used to measure 5hmC. 5hmC levels in brain tumors negatively correlated with tumor grade, and low 5hmC correlated with a poor prognosis [8,47]. In contrast, *IDH* mutations in brain tumors are associated with a relatively favorable prognosis [42,48]. Additional studies should be performed to clarify this inconsistency.

IDH1 R132 and IDH2 R172 mutations have also been identified in cartilaginous tumors, which range from benign enchondromas to highly malignant chondrosarcomas [49]. *IDH* mutations are found in 56–70% of central chondrosarcomas and enchondromas [50]. In addition, somatic mosaic *IDH1* and *IDH2* mutations have been detected in 77–91% of patients with Ollier disease and Maffucci syndrome, two syndromes characterized by multiple benign enchondromas. Elevated 2HG levels and hypermethylation were detected in these *IDH* mutated tumor tissues [50,51].

IDH1 R132 and IDH2 R172 mutations also occur in 10–23% of intrahepatic cholangiocarcinomas, tumors composed of epithelial cells from the bile duct. These *IDH*-mutated tumors are characterized by elevated 2HG levels, decreased 5hmC levels, increased 5mC levels and a hypermethylation signature [52,53]. In this disease, *IDH1/2* mutations are an independent favorable prognostic factor [52].

In addition, *IDH* mutations have been detected at lower frequencies in prostate cancer (3%), thyroid cancer (8–16%) and melanoma (10%) (Table 2) [54–56]. Of note, most *IDH* mutations in thyroid cancer are located outside the hotspot regions and do not produce 2HG, and therefore 5hmC levels will presumably not be affected [57]. Future research is required to determine how relevant these mutations are for the pathogenesis of thyroid cancer.

A recent study described elevated 2HG levels in a subset of breast cancer tumors with predominantly basal-like and mesenchymal origin [58]. Although the 2HG levels were comparable to those found in *IDH*-mutated leukemia or glioma samples, no *IDH* mutations were present in these breast tumors. The exact mechanism leading to this elevated 2HG is not yet known; however *MYC* pathway activation and increased glutamine metabolism were linked to elevated 2HG levels in these patients. These tumors were associated with a hypermethylation phenotype and poor clinical outcome. Although 5hmC levels were not measured in this study, an increase in 2HG will most likely lead to decreased generation of 5hmC by TET proteins.

3.3. SDH and FH mutations

Fumarate hydratase (FH) and succinate dehydrogenase (SDH) are mutated in a subset of human cancers (Table 2). Both enzymes play an important role in the Krebs cycle (Fig. 3). FH catalyzes the conversion of fumarate to malate, and SDH, which is composed of 5 subunits (SDHA, SDHB, SDHC, SDHD and SDHAF2), catalyzes the oxidation of succinate to fumarate. Mutations targeting the *FH* gene are found in multiple cutaneous and uterine leiomyomata (MCUL, ~100%), hereditary leiomyomatosis and renal cell cancer (HLRCC, 71–93%), papillary renal cell cancer (17%), Leydig cell tumors (7%), and paraganglioma and pheochromocytoma (<1%). *SDH* genes are mutated in renal cell carcinoma (1–4%), gastrointestinal stromal tumors (3–8%), and paraganglioma and pheochromocytoma (12–15%) [59–61]. In most cases this are germline mutations, but also some somatic mutations have been described [62]. Mutations in *FH* and *SDH* lead to a reduction of enzymatic activity, which results in an accumulation of fumarate and succinate, respectively [63]. Structurally, both fumarate and succinate are similar to αKG and 2HG. *In vitro* and *in vivo* studies have shown that fumarate and succinate are able to inhibit multiple αKG-dependent dioxygenases, including the TET family. Indeed, knockdown of *Fh* or *Sdha* in mouse liver cells led to a decrease in 5hmC, which could be rescued by transfecting with wild type but not with mutant FH or SDHA constructs [62]. Genome-wide methylation studies revealed a hypermethylation phenotype in pheochromocytoma and paraganglioma samples with *SDH* or *FH* mutations. In addition, 5hmC levels were decreased in these patients, providing *in vivo* evidence for the effect of *SDH* and *FH* mutations on 5hmC levels. *SDH* and *FH* mutations were associated with poor prognosis in pheochromocytoma and paraganglioma [61,64].

3.4. Other TET abnormalities that may influence 5hmC levels

TET1 was first identified as a gene fused to *MLL* in an AML patient with a ten–eleven translocation [65]. This translocation does not occur very frequently with only 13 cases reported in the literature to date: 11 AML and 2 ALL patients [66]. 5hmC measurements have not yet been reported in these patients.

In glioma, *TET2* mutations have not been described; however *TET2* promoter methylation has been detected in 14% of low-grade glioma patients without *IDH* mutation [32]. Since promoter methylation is associated with transcriptional repression, this suggests a decreased *TET2* expression in these patients, possibly leading to decreased levels of 5hmC. More research is required to determine the consequences and importance of this observation. In addition, Müller et al. [46] showed a strong correlation between nuclear exclusion of TET1 and decreased 5hmC levels in glioma. In their study, 61% of the tumor samples had decreased 5hmC levels; however there was no correlation between 5hmC and *IDH* mutations. Of the 5hmC negative tumors, 70% showed nuclear exclusion of TET1, or no detectable TET1 protein, thereby demonstrating an additional mechanism that may lead to decreased 5hmC in glioma.

3.5. Altered TET/IDH/TDG expression

In several types of cancer, deregulated 5hmC was accompanied by changes in *TET* or *IDH* expression. Diminished expression of *IDH2* and *TET1/2/3* was reported in melanoma, and therefore may represent one of the molecular mechanisms underlying global 5hmC loss in these tumors [31]. 5hmC levels were decreased in melanoma but not in benign nevi, showing the potential of 5hmC levels to be used as a biomarker to discriminate between benign and malignant lesions [67]. 5mC levels did not differ significantly between melanoma and benign nevi [68]. Loss of 5hmC correlated with melanoma progression, increasing nuclear diameter and decreased tumor-free survival [31,69]. Rebuilding the 5hmC landscape in melanoma by reintroducing active TET2 or IDH2,

suppressed melanoma growth and increased tumor-free survival in animal models [31].

Also in breast and liver cancer the expression of all three TET proteins was reduced when compared to matched healthy tissues [70]. In gastric cancer all three TET proteins, IDH2, and TDG were downregulated; however the loss of 5hmC was mainly correlated with the downregulation of TET1 [71]. In colorectal cancer *TET1* expression was found to be reduced [72], while in oral squamous cell carcinoma reduced 5hmC levels were accompanied by decreased *TET2* expression levels [73]. In gastric cancer and hepatocellular carcinoma, 5hmC levels negatively correlated with tumor size and tumor stage, and reduced 5hmC predicted a poor overall survival [74,75].

In addition, several genes involved in regulating the levels of 5hmC were differentially expressed between glioblastoma subtypes. mRNA expression of all three TET proteins was decreased in the aggressive mesenchymal subtype of glioblastoma. Moreover, the expression of several genes involved in the active demethylation pathway, including *TDG*, was increased in this glioblastoma subtype. Reduced *TET1* or *TET3* expression was associated with inferior overall survival. The expression levels of *TDG* and *TET* genes were not directly correlated with 5hmC levels; however decreased *TET* expression and increased expression of genes involved in demethylation (e.g. *TDG*), both suggest a reduction in 5hmC levels [47].

Huang *et al.* observed increased *TET1* expression in leukemia patients with *MLL*-rearrangements. They described an oncogenic role for TET1 in *MLL*-rearranged leukemia. Knockdown of TET1 inhibited MLL-AF9-induced leukemogenesis in mice, and led to a significant downregulation of *HOXA9*, *MEIS1* and *PBX3*, three genes frequently overexpressed in *MLL*-rearranged leukemia [76]. In accordance with high *TET1* expression, we found that most AML patients with *MLL* rearrangements had high 5hmC levels [9].

Altered 5hmC levels have also been described in some benign tumors. In uterine leiomyoma, the most common benign tumor of the female reproductive tract, 5hmC levels were found to be increased, which was associated with an elevated protein expression of TET1 and TET3 [77].

3.6. MicroRNA expression

Several *TET*-targeting microRNAs have recently been described (Table 1). Changes in the expression of these miRNAs may account for the altered *TET* expression observed in several types of cancer. Cheng *et al.* [78] discovered a complete network of more than 30 miRNAs that inhibited *TET2* expression. The top 10 most effective human *TET2*-targeting miRNAs were miR-26a-1, -26a-2, -29b-1, -520d, -26b, -7-3, -125b-1, -7-1, -125a, and -30d. Overexpression of these miRNAs in cell lines resulted in decreased TET2 mRNA and protein expression, and decreased levels of 5hmC. Some TET2-targeting miRNAs, including miR-26 and 29 family members, also regulated *TET1* and *TET3* expression levels. Transplantation of bone marrow cells transduced with TET2-targeting miRNAs into lethally irradiated mice resulted in a disturbed hematopoiesis with hematopoietic expansion and/or a myeloid differentiation bias, whereas co-expression of TET2 corrected these phenotypes. Importantly, several of these TET2-targeting miRNAs, including miR-125b, miR-29b, miR-29c, miR-101 and miR-7, were predominantly overexpressed in *TET2* wild type AML, which suggests that TET2 activity can be affected by either *TET2* mutations or deregulated *TET* expression in AML patients.

In a study by Song *et al.*, *TET2* was predicted to be a target of miR-22 [79]. Transgenic mice overexpressing miR-22 in the hematopoietic compartment developed a myelodysplastic syndrome (MDS)-like disease which, in 70% of the mice, resulted in myeloid leukemia. The phenotype developed in these mice closely resembled the inactivation of TET2 in the hematopoietic compartment. TET2 mRNA and protein levels were reduced in peripheral blood and bone marrow of miR-22 overexpressing mice, as well as the expression of TET2 target genes. 5hmC levels were reduced more than 2-fold in bone marrow with a concomitant increase in 5mC levels. Micro-array data showed upregulated miR-22 expression in patients with MDS and AML. Around 60% of the MDS patients had decreased *TET2* expression which anti-correlated with miR-22 expression levels. These findings show that changes in miR-22 may affect 5hmC levels in hematological malignancies.

miR-22 also represses *TET1*, *TET2* and *TET3* in breast cancer [80]. Overexpression of miR-22 in the mammary glands of mice resulted in decreased 5hmC levels. Targeting of TET proteins by miR-22 inhibited the demethylation of the miR-200 promoter, leading to silencing of anti-metastatic miR-200 family members. miR-200 family members are known to be important regulators of epithelial-to-mesenchymal transition and mammary stem cell function. miR-22 was highly expressed in non-triple-negative breast cancer samples when compared to normal breast tissue, and high expression correlated with silencing of the TET-miR-200 axis and poor clinical outcome. This study shows that a reduced expression of *TET* enzymes, caused by miR-22 overexpression, may promote breast cancer metastasis.

Analysis of miRNA-targets across 10 cancer types (see Table 3, last row) led to the discovery of miR-29 as an important regulator of *TET1* and *TDG* [81]. *TET1* expression was strongly negatively correlated with expression of miR-29 family members in all 10 tested cancer types and of *TDG* in all but one cancer type. miR-29a was generally downregulated and *TET1* and *TDG* were upregulated as compared with representative normal samples. In line with this, the majority of TDG-associated promoters were hypomethylated in 9/10 cancer types. These results are opposite to the decreased *TET1* expression reported in melanoma, hepatocellular carcinoma, gastric cancer and the mesenchymal subtype of glioblastoma. It would be of interest to determine 5hmC levels in the analyzed tumor samples, since upregulated *TET1* suggests elevated 5hmC levels, while most papers describe decreased 5hmC levels in cancer.

3.7. CXXC4/5 expression

As explained above, CXXC4/5 proteins are able to influence 5hmC levels by modulating TET protein levels. In line with this, Ko *et al.* showed that CXXC overexpression may play a role in hematological malignancies, since CXXC4 overexpression mimicked TET2 deficiency by skewing differentiation of murine bone marrow hematopoietic stem/progenitor cells towards the myeloid lineage [18]. Importantly, deletions of chromosome 5 or the long arm of chromosome 5 (−5/del(5q)), including CXXC5, are frequently observed in leukemia. In addition, decreased *CXXC5* expression was recently reported in a subset of leukemia patients, particularly cases with−5/del(5q), *MLL* rearrangements and *AML1-ETO* translocations [82,83]. In line with this, we found that most patients with *AML1-ETO* and *MLL* rearrangements have high 5hmC levels [9].

Aberrant *CXXC4* or *CXXC5* expression has also been observed in several other types of cancer. *CXXC5* is overexpressed in a number of solid cancers (breast cancer, melanoma, thyroid cancer) and correlated with poor prognosis in breast cancer [84]. Furthermore, *CXXC4* overexpression has been described in adenomas of the colon [85], whereas *CXXC4* downregulation has been described in gastric carcinoma, where it associated with poor prognosis [86]. Future studies should examine whether overexpression or downregulation of CXXC4/5 proteins influences 5hmC levels in these types of cancer.

3.8. Potential 5hmC regulating factors

TET proteins belong to the family of αKG- and Fe^{2+}- dependent dioxygenases, and therefore require αKG as a co-substrate and iron (Fe^{2+}) as a co-factor for their activity [3]. Recently, *in vitro* studies revealed an additional co-factor, ascorbate, which enhances TET-mediated generation of 5hmC [16]. Ascorbate is required to maintain iron in the active state by reducing inactive Fe^{3+} to active Fe^{2+}. A disbalance in co-substrates and co-factors might affect the activity of

Table 3
Mechanisms deregulating 5hmC in each cancer type.

Type of cancer	Defects possibly influencing 5hmC levels	Effect on 5hmC level
Brain tumors	IDH1/2 mutations	Decreased/No effect
	Nuclear exclusion of TET1	Decreased
	TET2 promoter methylation	ND
	Possibly: Aberrant expression of genes involved in demethylation	ND
Breast cancer	Increased miR22 expression	Decreased
	Decreased TET1/2/3 expression	Decreased
	Increased 2HG production	ND
	CXXC5 overexpression	ND
Colon cancer	Decreased TET1 expression	Decreased
	CXXC4 overexpression	ND
Gastric cancer	Decreased IDH2, TDG, TET1/2/3 expression	Decreased (TET1)
	Decreased CXXC4 expression	ND
Gastrointestinal stromal tumors (GIST)	SDH mutations	ND
Hematological malignancies	TET2 mutations	Decreased
	IDH1/2 mutations	Decreased
	Increased expression of TET–targeting miRNAs	Decreased
	TET1 overexpression	Increased
	Decreased CXXC5 expression	ND
	Possibly: MLL–TET1 translocation	ND
Liver cancer	Decreased TET1/2/3 expression	Decreased
Melanoma	IDH1/2 mutations	ND
	Decreased IDH2, TET1/2/3 expression	Decreased
	CXXC5 overexpression	ND
Oral squamous cell carcinoma	Decreased TET2 expression	Decreased
Paraganglioma/pheochromocytoma	SDH mutations	Decreased
	FH mutations	Decreased
Renal cell carcinoma	SDH mutations	ND
	FH mutations	ND
Thyroid cancer	CXXC5 overexpression	ND
Uterine leiomyoma	TET1 and TET3 overexpression	Increased
Cartilaginous tumors	IDH1/2 mutations	ND
Intrahepatic cholangiocarcinoma		Decreased
Prostate cancer		ND
Hereditary leiomyomatosis and renal cell cancer (HLRCC)	FH mutations	ND
Leydig cell tumors		
Multiple cutaneous and uterine leiomyomata (MCUL)		
Bladder urothelial carcinoma	Decreased miR–29 expression	ND
Breast invasive carcinoma		
Colon and rectum adenocarcinoma		
Glioblastoma multiforme		
Head and neck squamous–cell carcinoma		
Kidney renal clear–cell carcinoma		
Lung adenocarcinoma		
Lung squamous–cell carcinoma		
Ovarian serous cystadenocarcinoma		
Uterine corpus endometrioid carcinoma		

ND = not determined.

the TET proteins and consequently the levels of 5hmC. Therefore, it will be interesting to explore whether the levels of αKG, iron and ascorbate are altered in cancer.

TET proteins utilize molecular oxygen to oxidize 5mC, which suggests that altered oxygen levels in the tumor microenvironment may influence TET activity. In the past few years, several studies

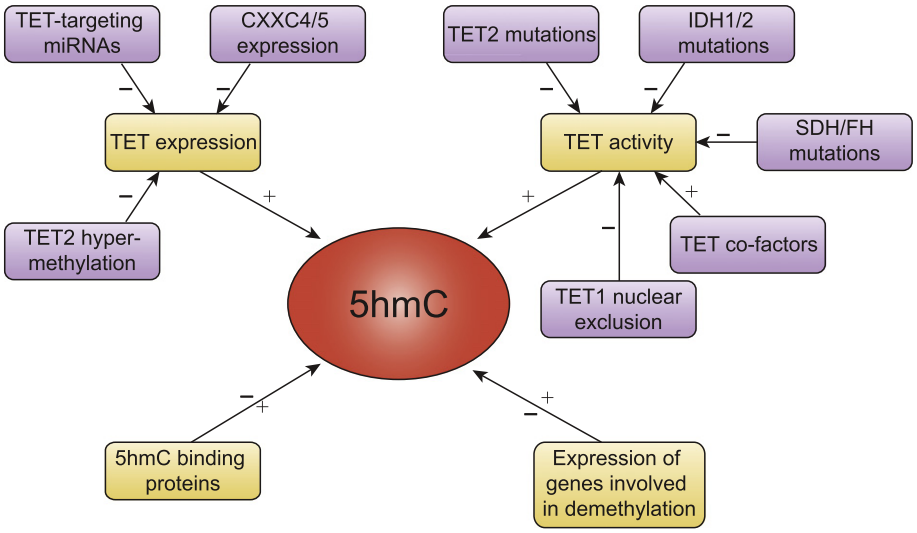

Fig. 4. Factors (potentially) influencing 5hmC levels in cancer. In cancer, 5hmC levels are mainly influenced by changes in TET expression or TET activity. In addition, changes in the expression of genes that bind 5hmC or genes that are implicated in demethylation may affect the levels of 5hmC (indicated in yellow). Several factors have been described that are able to influence the expression or activity of TET proteins (indicated in purple). + indicates positive effect, and − negative effect.

reported an effect of altered oxygen levels on TET expression or activity. It has been shown that hypoxia leads to an increased IDH-dependent carboxylation of glutamine-derived αKG, which is associated with an increased synthesis of 2HG in cells with wild type IDH1 and IDH2 proteins [87]. Elevated 2HG levels could lead to enhanced inhibition of TET activity and decreased 5hmC levels in these cells. On the contrary, it has been shown that hypoxia induces an HIF1-dependent increase in *TET1* expression in neuroblastoma cell lines, followed by an increase in 5hmC [88]. Finally, it has been reported that reactive oxygen species (ROS), induced by the drug 5-fluorouracil, upregulated TET1 expression and function in colorectal cancer, whereas antioxidants had the opposite effect [89]. More research should therefore be performed to clarify the effects of altered oxygen levels on 5hmC levels in cancer.

Several proteins have been discovered that bind to 5hmC, like MECP2, WDR76, UHRF1 and UHRF2 [15]. It may be possible that these 5hmC readers block further oxidation of 5hmC into 5fC and 5caC or, in contrast, promote active demethylation. In line with the latter, Spruijt *et al.* showed that overexpression of UHRF2 *in vitro* increased the levels of 5hmC, 5fC and 5caC, which suggests that UHRF2 stimulates TET-mediated oxidation [15].

4. Possibilities for treatment

Although the causes and consequences of deregulated 5hmC levels are still under investigation, several opportunities for targeting the underlying mechanisms have already been explored.

A number of specific inhibitors for *IDH1* and *IDH2* mutations have been developed and tested in *in vitro* and *in vivo* settings. Inhibitors for IDH1 R132 (AGI-5198 [90], HMS-101 [91], AG1-14100 [92], ML309 [93]) were shown to specifically inhibit the ability of the mutant enzyme to produce 2HG in leukemia and glioma xenograft models. In addition, treatment with these inhibitors resulted in growth inhibition and induction of cellular differentiation [91,92]. Recently, the IDH1 inhibitor AGI-14100 was tested in combination with Ara-C in a xenograft model, which resulted in a stronger decrease of bone marrow tumor burden than either treatment alone [92]. Specific inhibitors for IDH2 R140 have also been developed; AGI-6780 [94] and AG-221 [95]. Both compounds reduce 2HG production and induce cellular differentiation. AG-221 was also shown to reverse histone and DNA hypermethylation *in vitro*. The AG-221 inhibitor is currently being tested *in vivo* in a phase 1 dose-escalation study.

In addition, changes in DNA methylation are interesting targets for therapy, because epigenetic modifications are reversible. Several clinical trials investigated the treatment of MDS and AML patients with hypomethylating agents (azacitidine and decitabine), which may result in reactivation of silenced tumor suppressor genes. These trials showed promising results, including improved complete remission, event-free survival and overall survival rates [96,97]. Azacitidine and decitabine have therefore been approved by the Food and Drug Administration (FDA) for treatment of MDS patients, and AML patients with 20–30% blasts (previously classified as RAEB-T by the French–American–British MDS classification). In addition, decitabine has been approved by the European Medicines Agency (EMA) for treatment of AML patients aged 65 years and older who are not eligible for initial standard induction chemotherapy. Because *TET2* and *IDH*-mutated AML patients are characterized by a hypermethylation phenotype, hypomethylating agents were hypothesized to be particularly beneficial for these patients. Studies by Itzykson *et al.* [98], Traina *et al.* [99] and Bejar *et al.* [100] reported an increased response rate to azacitidine for patients with a *TET2* mutation; however other recent reports did not show a preferential benefit for *TET2*- or *IDH*-mutated patients [101–103]. Future large prospective studies are required to unravel the biomarkers that predict response to hypomethylating agents in hematological malignancies. The hypomethylating agent decitabine was also tested in an *IDH1*-mutant glioma xenograft model. Decitabine treatment resulted in decreased cell growth and induction of differentiation. In addition, genome-wide DNA demethylation and upregulation of several differentiation-associated genes were observed [104]. More research is necessary to determine the effectiveness of hypomethylating agents in *IDH*-mutated glioma patients. Despite the successes achieved by these nucleoside analogs, there is still room for improvement since the specificity of these compounds is quite low and cellular toxicity has been observed. In an attempt to overcome these problems several non-nucleoside DNMT inhibitors have been explored, including hydralazine, RG108, SGI-1027, procaine, procainamide, and MG98 [105]. To date, none of these compounds has yet been approved for use as DNMT inhibitor in the clinic. In addition to the reported beneficial effects of hypomethylating agents, these drugs might also reactivate oncogenes and, in solid tumors like breast cancer,

prometastatic genes, indicating that caution is needed when using these drugs [106,107].

Based on recent discoveries, several new treatment options might be investigated. In patients where 5hmC is decreased due to elevated miRNA or CXXC expression, TET proteins are still functional, which suggests that increasing TET expression or TET activity may be beneficial for these patients. *In vitro* studies have already demonstrated that TET overexpression is able to rescue the phenotype caused by overexpression of TET-targeting miRNAs [78,80]. In addition, modulating the activity of the TET proteins by changing the availability of co-substrates and co-factors (αKG, iron and ascorbate) warrants investigation.

5. Concluding remarks and perspectives

Deregulated 5hmC levels have been observed in many types of cancer, both in solid cancers as well as in hematological malignancies. Several mechanisms can be responsible for the altered 5hmC levels, ranging from mutations to deregulated miRNA expression (Table 3, Fig. 4). The fact that altered 5hmC levels are frequently observed in cancer suggests that the (de)methylation pathway might be an important target for cancer therapy, however several unanswered questions still need to be addressed.

First of all, it is still unclear whether deregulated 5hmC levels are a cause or a consequence of cancer. In hematological malignancies, *TET2* mutations seem to play an important role in transformation, since *TET2* KO mice develop a CMML-like disease, and *TET2* mutations are often early events [29,30]. Also *IDH* mutations are often early events [108], but a sole *IDH* mutation in mice is insufficient to cause glioma or leukemia, suggesting that additional hits are required for full transformation [39]. On the other hand, in some solid cancers 5hmC levels seem to correlate with the stage of the disease [31,74,75], which suggests that 5hmC levels either change as a consequence of cancer progression, or promote cancer progression. This suggests that the role of deregulated 5hmC in pathogenesis may differ per cancer type, with mutations more likely to be involved in the initiation of cancer, while other ways of deregulating 5hmC (e.g. changes in *TET* expression) may be associated with progression of the disease. More research should be performed to clarify the involvement of 5hmC changes in the pathogenesis of different cancer types.

Secondly, it is important to unravel how altered 5hmC levels contribute to pathogenesis. A decrease or increase in global 5hmC suggests a defect in the balance of methylation and demethylation, which may lead to silencing of tumor suppressor genes or activation of oncogenes. Genome-wide (hydroxy)methylation studies should be performed to determine which genes are differentially (hydroxy)methylated between healthy and cancer tissues. This will give insights into the genes that might play a role in the pathogenesis or progression of the disease, and thereby point towards new targets for therapy. At the moment, several techniques are available to determine genome-wide 5mC distribution; however not all techniques are able to discriminate between 5mC and 5hmC marks. Techniques suitable for specific detection of 5mC are for example methylated DNA immunoprecipitation (MeDIP) and methyl binding domain (MBD)-based techniques. Determining the genome-wide distribution of 5hmC is still challenging due to the low levels; however with the rapidly improving techniques this should be possible in the future.

Finally, it will be very interesting to determine whether the levels of the other demethylation intermediates, 5-formyl- and 5-carboxylcytosine, are also altered in cancer. Genome-wide techniques to determine 5fC and 5caC have only recently been developed and might be used in the future to answer this question [109].

Transparency document

The Transparency document associated with this article can be found, in the online version.

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