Targeting histone methylation for colorectal cancer

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Abstract: As a leading cause of cancer deaths worldwide, colorectal cancer (CRC) results from accumulation of both genetic and epigenetic alterations. Disruption of epigenetic regulation in CRC, particularly aberrant histone methylation mediated by histone methyltransferases (HMTs) and demethylases (HDMs), have drawn increasing interest in recent years. In this paper, we aim to review the roles of histone methylation and associated enzymes in the pathogenesis of CRC, and the development of small-molecule modulators to regulate histone methylation for treating CRC. Multiple levels of evidence suggest that aberrant histone methylations play important roles in CRC. More than 20 histone-methylation enzymes are found to be clinically relevant to CRC, including 17 oncoproteins and 8 tumor suppressors. Inhibitors of EZH2 and DOT1L have demonstrated promising therapeutic effects in preclinical CRC treatment. Potent and selective chemical probes of histone-methylation enzymes are required for validation of their functional roles in carcinogenesis and clinical translations as CRC therapies. With EZH2 inhibitor EPZ-6438 entering into phase I/II trials for advanced solid tumors, histone methylation is emerging as a promising target for CRC.

Keywords: colorectal cancer, drug targets, epigenetic regulation, histone demethylase, histone methyltransferase

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Introduction

Over 1.3 million new cases of colorectal cancer (CRC) are recorded each year, with more than 0.6 million deaths worldwide [Torre *et al.* 2015].

Current management for CRC includes surgery, radiofrequency ablation, radiation therapy, chemotherapies, and targeted therapies. For patients in cancer stage III or IV, chemotherapy or targeted therapies are normally used. Based on biomarker analysis, targeted therapies such as epidermal growth factor receptor (EGFR) monoclonal antibodies, cetuximab and panitumumab, can significantly improve therapeutic effects in patients [Pritchard and Grady, 2011]. However, due to molecular heterogeneity and drug resistance, new therapies are required for patients who do not respond to current treatment approaches.

In-depth understanding of pathogenesis will lead to novel therapies for CRC. It has been widely accepted that CRC results from the sequential accumulation of both genetic [Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996] and epigenetic changes [Grady and Carethers, 2008; Wong *et al.* 2007] that induce the transformation of normal glandular epithelium into invasive adenocarcinomas. Both genetic and epigenetic alterations contribute to the tumor formation by activating oncogenes or inactivating tumor suppressors that regulate CRC-associated signaling pathways. These pathways include wingless-type MMTV integration site family (WNT)-, tumor protein 53 (TP53)-, transforming growth factor (TGF)/bone morphogenetic protein(BMP)/SMAD-, receptor tyrosine kinase (RTK)-, NOTCH-, and phosphoinositide 3 kinase (PI3K)-signaling pathways, which affect functions like proliferation, migration, differentiation, adhesion and cell death [Van Engeland *et al.* 2011]. They also include microsatellite instability (MSI)-, chromosomal instability (CIN)-, and CpG island methylator phenotype

(CIMP)-pathways, which regulate the genomic stability [Al-Sohaily *et al.* 2012].

In recent years, the importance of epigenetic alterations in CRC has been rapidly realized. Epigenetic alterations affect many components of epigenetic regulation, including DNA methylation, histone modifications, nucleosomal occupancy and remodeling, chromatin looping and noncoding RNAs, and contribute to the development of CRC by affecting cancer-associated pathways [Van Engeland *et al.* 2011]. DNA methylation is one of the mostly well characterized epigenetic alterations in cancer. By searching 'DNA methylation and cancer' in PubMed on 28 March 2016, the author got 17,270 publications. However, taking a close look at the number of publications by year between 2001 and 2015, this topic was found to reach a peak in 2014, and flatten in 2015 (Figure S1a, available online). The same tendency has also been observed in the area of 'DNA methylation and CRC'.

Like DNA methylation, histone modifications have been frequently linked with CRC. Histone modifications are important epigenetic markers that regulate transcription, repair, replication and recombination of genes by affecting the chromatin structure, recruiting remodeling enzymes or transcription-complex proteins [Bannister and Kouzarides, 2011]. Many modifications have been found within histones, with reference to acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation [Bannister and Kouzarides, 2011]. Among them, acetylation and methylation are mostly investigated since the pioneering studies by Allfrey and colleagues in the early 1960s [Allfrey *et al.* 1964]. By searching 'histone acetylation or methylation and cancer' in PubMed, the number of relevant publications was 1392 and 513, respectively. Unlike DNA methylation, the topics of 'histone acetylation or methylation and cancer' have made much faster progress in the past 15 years (Figure S1a, available online). A similar pattern also exists in the area of 'histone acetylation or methylation and CRC' (Figure S1b, available online).

In line with these observations, the importance of DNA methylation and histone acetylation in CRC were highlighted by a series of reviews [Bardhan and Liu, 2013; Khare and Verma, 2012; Mottamal *et al.* 2015; Vaiopoulos *et al.* 2014; West and Johnstone, 2014]. Several DNA methyltransferase inhibitors (DNMTi) and histone deacetylase inhibitors (HDACi), such as azacitidine, decitabine, vorinostat and romidepsin, have been approved by the US Food and Drug Administration for cancers, including chronic leukemia, and more recently, panabinostat for myeloma.

However, less attention has been paid to histone methylation in CRC, although in recent years, we have witnessed rapid progress in this area, which grows even faster than histone acetylation (Figure S1b, available online). Histone-methylation modulators have entered into phase I/II trials for advanced solid tumors, giving hope to the idea that regulating histone methylation can be developed as a novel therapy for CRC. This review will focus on histone methylation, associated enzymes, and potential modulators' development for treatment of CRC.

Histone methylation in colorectal cancer

Histone methylation occurs on the side chains of lysine and arginine (Figure 1). Two enzyme families mediate the addition and removal of methyl groups: histone methyltransferases (HMTs) and histone demethylases (HDMs). Distinguished by substrates, HMTs are further divided into protein lysine methyltransferases (PKMTs) and protein arginine methyltransferases (PRMTs). PKMTs catalyze transferring of the methyl group from the cofactor S-adenosylmethionine (SAM) to the ε-amino group of the lysine side chain, which can be mono-, di-, and trimethylated [Luo, 2012]. Similarly, PRMTs catalyze the methyl group transferring to the ω-guanidino group of arginine with the same methyl donor, SAM. The arginine side chain can be mono-, and symmetrically or asymmetrically di-methylated. Compared with histone acetyltransferases, HMTs are more substrate-specific, in terms of methylation sites and states [Luo, 2012].

Unlike histone acetylation, histone methylation does not change electrostatic charge of histones or affect the chromatin structure. Instead, it creates docking sites that can be recognized by structural motifs like Tudor-, malignant brain tumor (MBT)-, PWWP-domains, and chromodomains [Bonasio *et al.* 2010; Holdermann *et al.* 2012; Pek *et al.* 2012; Qin and Min, 2014]. These structural domains normally exist in proteins comprising transcriptional complexes or other molecular machines. Histone lysine methylation is associated with both transcriptional activation and
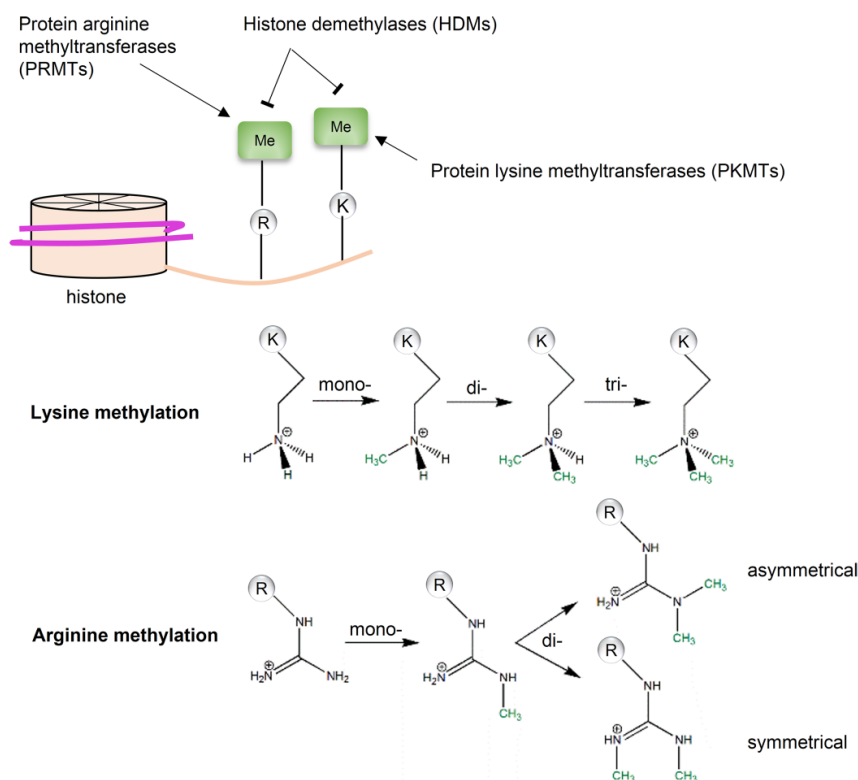
Figure 1. Histone methylation. Histone methylation is regulated by two families of enzymes: histone methyltransferases (HMTs) and histone demethylases (HDMs). The methylation occurs at side chains of both lysine and arginine, which are catalyzed by protein lysine methyltransferases (PKMTs) and protein arginine methyltransferases (PRMTs), respectively. The methylation states are substrate specific: for lysine, there are mono-, di-, and trimethylation; for arginine, there are mono-methylation and symmetrical/asymmetrical dimethylation.

repression. For example, trimethylation of histone 3 lysine 4 (H3K4me3) is a conserved marker for transcription activation, while trimethylation of histone 3 lysine 9 (H3K9me3) and histone 3 lysine 27 (H3K27me3) are signals for gene silencing [Bannister and Kouzarides, 2011; Kouzarides, 2007]. Histone arginine methylation is also involved in transcriptional regulatory mechanism [Di Lorenzo and Bedford, 2011]. For instance, asymmetrical dimethylation of histone 4 arginine 3 (H4R3me2a) is a transcriptional activating marker, while the symmetrical dimethylation of histone 4 arginine 3 (H4R3me2s) is associated with transcriptional repression [Bedford and Clarke, 2009]. Beside gene transcription, histone-methylation markers also recruit proteins associated with DNA repairing and other functions. For instance, trimethylation of histone 3 lysine 36 (H3K36me3) recruits hMutSα, the mismatch recognition protein, *via* direct interactions between H3K36me3 and the PWWP domain of human mutS homolog 6 (hMSH6) [Li *et al.* 2013a].

Methylated lysine can be restored by the flavin-dependent enzymes of lysine-specific histone demethylase-1, 2 (LSD-1, 2) [Fang *et al.* 2010; Shi *et al.* 2004], or the Jumonji family of 2-oxoglutarate-dependent demethylases [Tsukada *et al.* 2006]. Initially, converting arginine to citrulline *via* a deamination reaction was considered an indirect approach to reversal of arginine methylation [Cuthbert *et al.* 2004]. Recently, Jumonji domain-containing 6 (JMJD6) was reported to directly demethylate histone 3 arginine 2 (H3R2) and histone 4 arginine 3 (H4R3) [Chang *et al.* 2007].

Histone methylation not only regulates many biological functions, including gene transcription, nucleosomal positioning, DNA replication and repair, but also influences the carcinogenesis of cancers by affecting various cancer pathways [Esteller, 2007; Jones and Baylin, 2007]. Indeed, aberrant histone methylation has been frequently found in CRC tumor samples and cell lines (Table 1).

Table 1. Aberrant histone-methylation markers in colorectal cancer.

Histone markers	Alterations in CRC	Effects on CRC	Affected functions	Reference
H4K20me3	Decreased in cell lines and primary tumor tissue	Poor prognosis	Hypomethylation of DNA repetitive sequences	Fraga *et al.* [2005], Benard *et al.* [2014]
H3K4me3	Elevated in tumor tissue of patients and cells	Unclear	Interacting with β-catenin and promoting WNT-signaling target genes	Salz *et al.* [2014]
H3K4me1/2/3	Decreased at MLH1 promoter under hypoxia	Unclear	Silencing MLH1 and resulting in DNA mismatch repair defect	Lu *et al.* [2014]
H3K9me3	Increased in invasive CRC tissue; increased under hypoxia	Metastasis	Promoting cell motility; repression of APAK	Yokoyama *et al.* [2013]; Olcina *et al.* [2016]
H3K27me3	Elevated in tumor tissue of patients; increased in patients with poor prognosis	Poor prognosis	Unclear	Benard *et al.* [2013, 2014];
H3K79me2	Elevated in patients with poor prognosis	Poor prognosis	Promoting IL-22 induced cancer stemness	Kryczek *et al.* [2014]

CRC, colorectal cancer; WNT, wingless-type MMTV integration site family; APAK, ATM and p53-Associated KZNF Protein; IL, interleukin.

Initially, loss of trimethylation of histone 4 lysine 20 (H4K20me3) was identified as one of the common hallmarks of human cancers [Fraga *et al.* 2005]. Consistently, high expression of H4K20me3 and H3K9me3, and low nuclear expression of H3K4me3, were associated with good prognosis in early-stage CRC patients [Benard *et al.* 2014]. As a well known gene activation marker, H3K4me3, was found to be elevated in tumor tissue of CRC patients and several cell lines, resulting in activated expression of WNT-signaling target genes through interaction between SET Domain containing 1A (SETD1A) and of β-catenin [Salz *et al.* 2014]. Interestingly, H3K4me1/2/3 were all decreased at the MutL Homolog 1 (MLH1) promoter in SW480 cells under hypoxia, leading to silence of MLH1 and DNA mismatch repair defects, a key process in the development of sporadic CRC [Lu *et al.* 2014]. Meanwhile, well known transcription repression marker, H3K9me3 was increased in invasive tumor tissue of CRC patients, resulting in enhanced cell motility [Yokoyama *et al.* 2013]. It was also found that the H3K9me3 level was elevated along ATM and p53-Associated KZNF Protein (APAK) loci under hypoxia, leading to repression of APAK and p53-dependent apoptosis [Olcina *et al.* 2016]. The transcription-repression

marker H3K27me3 was found to be increased in tumor tissue of CRC patients with poor prognosis [Benard *et al.* 2013, 2014]. Additionally, dimethylation of histone 3 lysine 79 (H3K79me2) was elevated in CRC patients with poor prognosis, enhancing IL-22-induced cancer stemness [Kryczek *et al.* 2014].

Very recently, direct mutations in histone-methylation sites have been found to contribute to abnormal histone-methylation profile, then cancer development. Histone 3 lysine 36-to-methionine (H3K36M) mutation was identified in a CRC sample [Shah *et al.* 2014]. This mutation has been proved to impair mesenchymal progenitor cell differentiation and promote undifferentiated sarcoma *in vivo* [Lu *et al.* 2016], suggesting that H3K36 methylation is an important epigenetic marker for tumor suppression.

Histone-methylation enzymes and colorectal cancer

Histone methylation in CRC is regulated by HMTs and HDMs. Targeting histone-methylation enzymes may restore normal methylation profile, therefore there is a potential to develop the therapeutic reagents. To evaluate the prospects of

Figure 2. Histone-methylation enzymes in colorectal cancer (CRC). Enzymes of histone methyltransferase (HMT) and histone demethylase (HDM) are plotted in a polygenetic tree based on sequence homology. The gene name of each enzyme is highlighted by different colors according to its role in CRC: orange, oncoproteins and blue, tumor suppressor. Gene name in bold represents a validated target. If the enzyme has potent chemical probes, it is labeled with 'C' in a circle; if it has crystal structure(s) which contains the catalytic domain, it is labeled with 'S' in a circle.
HMTs, histone methyltransferases; HDMs, histone demethylases.

histone-methylation enzymes as drug targets in CRC, evidence from preclinical studies was collected. Among the 87 histone-methylation enzymes accessed in this study, including 60 HMTs and 27 HDMs, 25 proteins were found to have links with CRC, namely 17 oncoproteins and 8 tumor suppressors (Figure 2; Table 2).

Histone-methylation enzymes as oncoproteins in colorectal cancer

H3K4 methylation-associated enzymes. KMT2B/ MLL4, KMT2D/MLL2, and SETD1A are all H3K4 methyltransferases [Denissov *et al.* 2014; Nguyen *et al.* 2008; Nightingale *et al.* 2007] that promote the development of CRC. Knockdown of KMT2B by antisense suppressed tumor growth in CRC xenograft-implanted nude mouse. Further experiments in cancer cells revealed that KMT2B regulated expression of several critical

cell-cycle regulatory genes; while knockdown of KMT2B affected cell-cycle progression and induced apoptosis [Ansari *et al.* 2012]. Further, KMT2D was found to be significantly elevated in tumor tissue compared with adjacent benign mucosa in CRC patients' samples and cell lines [Natarajan *et al.* 2010]. KMT2D could be a potential oncoprotein in CRC, but such a role remains to be validated by knockdown or pharmacological inhibition experiments. Levels of SETD1A and H3K4me3 were elevated in human CRC cells and patient samples; while depletion of SETD1A inhibited CRC cell growth and affected about 50% WNT target genes [Salz *et al.* 2014].

Interestingly, H3K4 demethylases, KDM1A/ LSD1 and KDM5B/JARID1B [Secombe and Eisenman, 2007] also promote the development of CRC, suggesting that the local H3K4 methylation profile might be better associated with CRC.

Table 2. Histone-methylation enzymes associated with colorectal cancer.

Family	Enzyme	Synonyms	Substrates	Role in CRC	Target validation	Reference
HMT	KMT2B	MLL4	H3K4	Oncoprotein	Knockdown	Ansari *et al.* [2012]
	KMT2C	MLL3	H3K4	Tumor suppressor	Not yet	Watanabe *et al.* [2011]; Li *et al.* [2013b]; Huhn *et al.* [2014]
	KMT2D	MLL2	H3K4	Oncoprotein	Not yet	Natarajan *et al.* [2010]
	SETD1A	hSETD1A	H3K4	Oncoprotein	Knockdown	Salz *et al.* [2014]
	SUV39H1	KMT1A	H3K9	Oncoprotein	Knockdown	Kang *et al.* [2007]; Yokoyama *et al.* [2013]
	EHMT2	G9a	H3K9	Oncoprotein	Knockdown; pharmacological inhibition	Zhang *et al.* [2015b]
	PRDM2	RIZ;RIZ1	H3K9	Tumor suppressor	Not yet	Chadwick *et al.* [2000]; Emterling *et al.* [2004]
	PRDM16	MEL1	H3K9	Oncoprotein	Not yet	Burghel *et al.* [2013]
	SETDB1	KMT1E	H3K9	Tumor suppressor	Not yet	Kim *et al.* [2012a]; Olcina *et al.* [2016]
	EZH2	KMT6A	H3K27	Oncoprotein	Knockdown	Fluge *et al.* [2009]; Wang *et al.* [2010]; Takawa *et al.* [2011]; He *et al.* [2015]; Liu *et al.* [2015]
	DOT1L	KMT4	H3K79	Oncoprotein	Pharmacological inhibition	Kryczek *et al.* [2014]
	SMYD3	KMT3E	H4K5	Oncoprotein	Knockdown	Xi *et al.* [2008]; Van Aller *et al.* [2012]; Peserico *et al.* [2015]
	WHSC1	MMSET; NSD2	H4K20	Oncoprotein	Not yet	Hudlebusch *et al.* [2011]
	PRDM5	PFM2	Unknown	Tumor suppressor	Overexpression	Watanabe *et al.* [2007]; Bond *et al.* [2015]
	CARM1	PRMT4	H3R17 H3R26	Oncoprotein	Knockdown	Di Lorenzo and Bedford [2011]; Ou *et al.* [2011]
	PRMT5	SKB1	H3R8 H4R3	Oncoprotein	Knockdown; pharmacological inhibition	Zhang *et al.* [2015a]
HDM	KDM1A	LSD1	H3K4	Oncoprotein	Knockdown	Ding *et al.* [2013]; Jie *et al.* [2013]; Jin *et al.* [2013]
	KDM5B	JARID1B	H3K4	Oncoprotein	Knockdown	Ohta *et al.* [2013]
	KDM3A	JMJD1A	H3K9me2	Tumor suppressor	Not yet	Zuo *et al.* [2008]; Liu *et al.* [2013]
	KDM3B	JMJD1B	H3K9	Tumor suppressor	Not yet	Liu *et al.* [2013]
	PHF2	JHDM1E	H3K9me2	Tumor suppressor	Not yet	Lee *et al.* [2015]
	KDM4B	JMJD2B	H3K9; H3K36	Oncoprotein	Knockdown	Liu *et al.* [2013]; Berry *et al.* [2014]
	KDM4C	JMJD2C	H3K9	Oncoprotein	Knockdown; pharmacological inhibition	Kim *et al.* [2014]
	KDM6B	JMJD3	H3K27	Tumor suppressor	Knockdown	Tokunaga *et al.* [2016]
	JARID2	JMJ	Unknown	Oncoprotein	Not yet	Tange *et al.* [2014]

HMT, histone methyltransferases; HDM, histone demethylase.

Overexpression of KDM1A was found in colon cancer specimens, and associated with advanced Tumor-Node-Metastasis (TNM) stages and metastasis [Ding *et al.* 2013; Jie *et al.* 2013].

Depletion of KDM1A in human CRC cell line HCT116 resulted in reduced cell proliferation both *in vitro* and *in vivo* [Jin *et al.* 2013]. KDM5B is involved in CRC maintenance, and depletion of KDM5B led to loss of epithelial differentiation and suppression of CRC cell growth [Ohta *et al.* 2013].

H3K9 methylation-associated enzymes. SUV39H1 and PRDM16 are two H3K9 methyltransferases [Pinheiro *et al.* 2012; Rea *et al.* 2000] found to be associated with CRC. Increased level of SUV39H1 mRNA was found in 25% of 219 CRC cases [Kang *et al.* 2007]. SUV39H1-mediated H3K9me3 was specifically increased in invasive regions of CRC tissue [Yokoyama *et al.* 2013]. CRC cell migration was activated by overexpression of wild-type SUV39H1 and reduced by knockdown of SUV39H1 [Kang *et al.* 2007], indicating that SUV39H1 is an oncoprotein in CRC. PRDM16 was one of the gained focal-minimal common-region genes identified in 53 microsatellite-stable sporadic CRC cases [Burghel *et al.* 2013]. It is a potential oncoprotein, but such a role remains to be established. EHMT2/G9a is responsible for dimethylation of H3K9 (H3K9me2) [Tachibana *et al.* 2002]. Very recently, EHMT2 was found to be much higher expressed in CRC tumor tissue than peritumoral counterparts. Knockdown of EHMT2 by antisense inhibited proliferation and induced DNA damage of CRC cells [Zhang *et al.* 2015b]. These data suggest that EHMT2 is an oncoprotein in CRC.

KDM4B and KDM4C are both demethylases of H3K9 [Berry and Janknecht, 2013]. High expression of KDM4B was correlated with lymph node status, Duke's classification and tumor invasion of CRC patients [Liu *et al.* 2013]. Consistent with this finding, KDM4B was upregulated in colon and rectal adenocarcinomas, which stimulated β-catenin and colon cancer cell growth; downregulation of KDM4B by shRNA resulted in β-catenin/TCF4 target genes [Berry *et al.* 2014], indicating that KDM4B is an oncoprotein in CRC. Overexpression of KDM4C was found in colon cancer cell lines, while the downregulation of KDM4C led to reduced growth and clonogenic capacity of colon cancer cells [Kim *et al.* 2014], suggesting that KDM4C is also an oncoprotein in CRC.

H3K27 methylation-associated enzymes. EZH2 methylates H3K27 [Kuzmichev *et al.* 2004]. This PKMT belongs to the polycomb group genes involved in the tumor-suppressor gene silencing. Overexpression of EZH2 was found in tumor tissue compared with adjacent nonneoplastic tissue in CRC patients [Fluge *et al.* 2009; Wang *et al.* 2010], which was further validated by two independent studies [Liu *et al.* 2015; Takawa *et al.* 2011]. EZH2 was responsible for the methylation-dependent resilencing of RUNX3 after the removal of demethylating agents [Kodach *et al.* 2010]. EZH2 was regulated by the ERK and AKT pathways, which resulted in silencing integrin alpha2 and enhancing the epithelial–mesenchymal transition associated with metastasis [Ferraro *et al.* 2013, 2014]. The vitamin D receptor (VDR) has also been identified as an EZH2 target; and the downregulation of VDR contributes to the EZH2-induced CRC cell invasion [Lin *et al.* 2013]. Further study revealed that HAND1 [Tan *et al.* 2014] and CLDN23 [Maryan *et al.* 2015] are also silenced by EZH2 in CRC tissue. EZH2 knockdown by siRNA led to the inhibited proliferation and migration of SW620 cells and apoptosis [He *et al.* 2015]. These results suggested that EZH2 is deeply involved in the carcinogenesis of CRC as an oncoprotein.

Others. DOT1L is the only non-SET-domain-containing PKMT that methylates H3K79 [Steger *et al.* 2008]. High expression of DOT1L in CRC tissue is a predictor for poor prognosis, and it was found that IL-22-dependent colon cancer stemness is regulated by DOT1L *via* H3K79 methylation. When using treatment with selective DOT1L inhibitor, EPZ004777, primary colon cancer sphere formation was inhibited [Kryczek *et al.* 2014]. SMYD3, the methyltransferase of H4K5 [Hamamoto *et al.* 2004] was found to be overexpressed in the majority of colorectal carcinomas [Van Aller *et al.* 2012; Xi *et al.* 2008]. Overexpression of SMYD3 was thought to be induced by KRAS mutation [Gaedcke *et al.* 2010]. RNAi-mediated SMYD3 knockdown inhibits CRC cell proliferation [Peserico *et al.* 2015]. WHSC1/MMSET/NSD2 is responsible for the methylation of H4K20 [Pei *et al.* 2011]. The WHSC1 protein is highly expressed in carcinomas of the gastrointestinal tract, including stomach, colon, anal canal, and the expression level was correlated with tumor aggressiveness [Hudlebusch *et al.* 2011]. WHSC1 could be a potential oncoprotein in CRC, but such a role remains to be established. CARM1, also known as PRMT4, methylates H3R17 and H3R26 [Di

Lorenzo and Bedford, 2011]. CARM1 is overexpressed in human colon cancer cells and positively modulates β-catenin-mediated gene expression. Depletion of CARM1 by shRNA suppresses clonal survival and growth [Ou *et al.* 2011]. PRMT5 catalyzes symmetric dimethylation on histone 3 arginine 8 (H3R8me2s) and histone 4 arginine 3 (H4R3me2s), and induces transcriptional repression [Pal *et al.* 2004; Zhao *et al.* 2009]. It was found that PRMT5 was highly expressed in CRC tumor tissue and associated with poor patient survival. Knockdown of PRMT5 by siRNAs downregulated expression of oncogenes FGFR3 and eIF4E, led to inhibition of CRC cell proliferation and colony formation [Zhang *et al.* 2015a].

JARID2/JMJ, is found as a Polycomb-repressive complex-2-interacting component [Li *et al.* 2010]. JARID2 is involved in the TGF-β-induced epithelial–mesenchymal transition in HT29 colon cancer cells [Tange *et al.* 2014]. JARID2 could be a potential oncoprotein in CRC, but such a role remains to be established.

Histone-methylation enzymes as tumor suppressors in colorectal cancer
H3K4 methylation-associated enzymes. KMT2C/ MLL3 catalyzes the methylation of H3K4 [Herz *et al.* 2012]. Frameshift mutations of KMT2C in both CRC cells and primary tumor were confirmed more commonly in cases with MSI [Watanabe *et al.* 2011]. Insertion mutation in the KMT2C was found in a pedigree with CRC and acute myeloid leukemia (AML). This insertion caused a premature truncation at codon 827 of KMT2C [Li *et al.* 2013b]. In line with these findings, an Single Nucleotide Polymorphism (SNP) in KMT2C had the strongest association with CRC risk and survival [Huhn *et al.* 2014]. These genetic alterations in KMT2C suggest that it is a potential tumor suppressor in CRC, but such a role remains to be established.

H3K9 methylation-associated enzymes. SETDB1 and PRDM2 are two H3K9 methyltransferases [Congdon *et al.* 2014; Schultz *et al.* 2002] involved in prevention of CRC development. SETDB1 mediates suppressing the expression of WNT target genes in human CRC cells [Kim *et al.* 2012a]. Consistent with this finding, SETDB1-mediated H3K9me3 repressed APAK and enhanced the hypoxia-induced p53-dependent apoptosis in

CRC [Olcina *et al.* 2016]. SETDB1 could be a potential tumor suppressor in CRC, but such a role remains to be established. Many frameshift mutations of PRDM2 were revealed in hereditary and sporadic CRC; these mutations resulted in reduced or absent mRNA expression of PRDM2 [Chadwick *et al.* 2000]. In one study examining the MSI of Swedish patients, mutations of PRDM2 were detected in 31% of 29 MSI tumors [Emterling *et al.* 2004]. PRDM2 could be a potential tumor suppressor in CRC, but such a role remains to be established.

KDM3A, KDM3B/JMJD1B, and PHF2 are all H3K9 demethylases [Kim *et al.* 2012b; Wen *et al.* 2010; Yamane *et al.* 2006]. KDM3A is involved in the transcriptional reactivation of silenced 15-LOX-1 in CRC cells *via* demethylating H3K9me2 [Zuo *et al.* 2008]. Low expression of KDM3B was correlated with the lymph node status, Duke's classification and TNM staging of CRC patients [Liu *et al.* 2013]. PHF2 was downregulated in human colon cancer tissue. PHF2 was also required for activation of the p53 pathway in the HCT116 xenograft model treated by oxaliplatin and doxorubicin [Lee *et al.* 2015]. Taken together, KDM3A, KDM3B and PHF2 are all potential tumor suppressors in CRC, but such roles remain to be established.

Others. PRDM5/PFM2 is another tumor suppressor in CRC. Methylation of PRDM5 promoter was more frequently seen in BRAF mutant- than BRAF wild-type CRC [Bond *et al.* 2015]. Consistently, PRDM5 was found to be silenced in CRC and gastric cancer cell lines by DNA methylation; overexpression of PRDM5 suppressed cancer cell growth [Watanabe *et al.* 2007]. KDM6B/JMJD3 is responsible for dimethylation of H3K27 [Agger *et al.* 2007]. Decreased KDM6B was found to be an independent predictor for poor prognosis in 151 CRC patients. Knockdown of KDM6B in CRC cell lines resulted in increased proliferation, *via* apoptosis suppression and cell-cycle progression [Tokunaga *et al.* 2016]. These data suggested that KDM6B is a tumor suppressor in CRC.

Drugging histone-methylation enzymes for colorectal cancer
Given the fact that many histone-methylation enzymes play important roles in development of CRC, targeting histone-methylation enzymes by
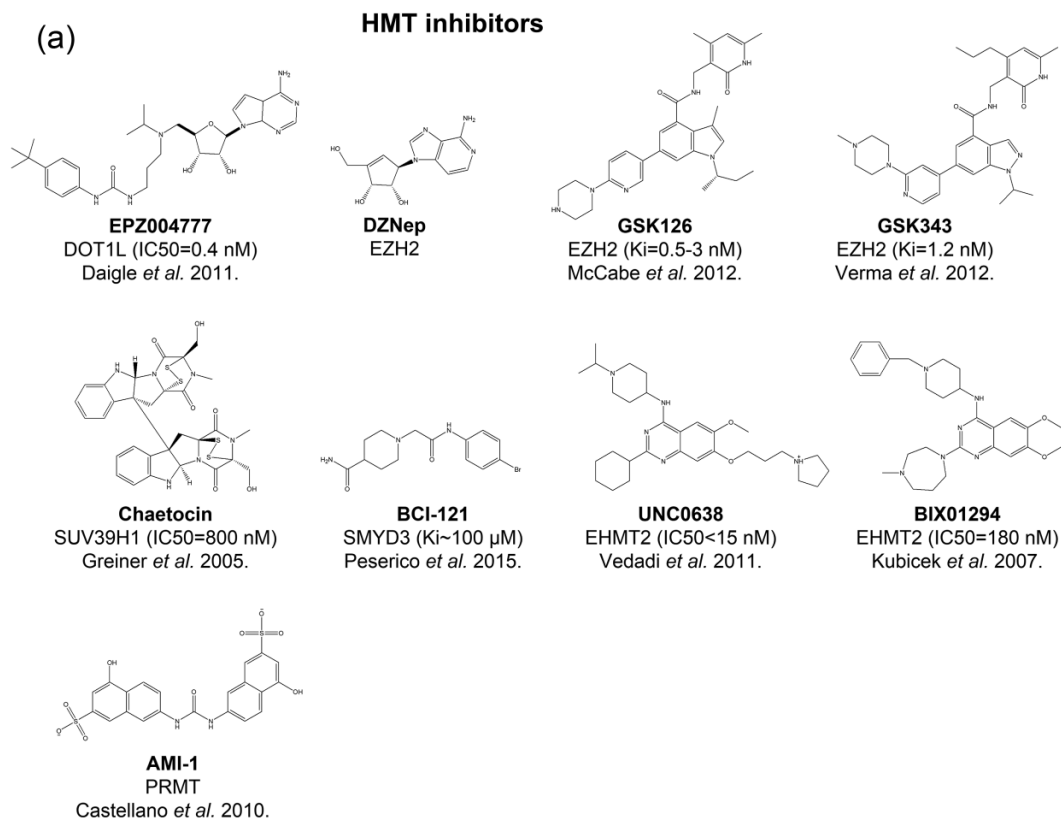
Figure 3. Histone methyltransferase and histone demethylase inhibitors in preclinical studies of colorectal cancer. The structure, name, target(s), potency, and the discoverers of inhibitors are shown.
HDM, histone demethylase; HMT, histone methyltransferase.

small-molecule modulators could be effective therapy for CRC. Currently, there are a number of small molecules targeting histone-methylation enzymes that have been used for CRC in preclinical studies (Figure 3 and Table 3).

EPZ00477 is a potent inhibitor of DOT1L with IC_{50} of 0.4 nmol [Daigle *et al.* 2011]. Treatment with EPZ004777 resulted in inhibited sphere formation in primary colon cancer and suppressed DLD-1 cell line *in vitro* at 10 μmol [Kryczek *et al.* 2014]. BCI-121, which was identified as SMYD3 inhibitor by virtual screening, suppressed the growth of CRC cells [Peserico *et al.* 2015]. Chaetocin is a fungal metabolite that potently inhibits SUV39H1 with IC_{50} of 800 nmol [Greiner *et al.* 2005, 2013]. Chaetocin inhibited the activity of SUV39H1 and the migration of CRC cells [Yokoyama *et al.* 2013]. BIX01294 and UNC0638 are two potent and selective EHMT2 inhibitors competing with substrates rather than

cofactors [Kubicek *et al.* 2007; Vedadi *et al.* 2011]. BIX01294 and UNC0638 inhibited proliferation of CRC cell lines with IC_{50} ranging from 1 to 20 μmol [Zhang *et al.* 2015b]. EZH2 is the most promising PKMT target in experimental CRC, as validated by pharmacological inhibition. DZNep is an indirect EZH2 inhibitor [Tan *et al.* 2007], which increased apoptosis in CRC cell lines and colon cancer stem cells [Benoit *et al.* 2013a, 2013b]. EZH2 inhibitor GSK346 [Verma *et al.* 2012] reduced migration of CRC cells [Ferraro *et al.* 2014]. GSK126 is a highly specific inhibitor of EZH2 with subnanomolar potency [McCabe *et al.* 2012]. Treating Colo205 and HT-29 cell lines with GSK126 resulted in reduced level of H3K27me3 and increased CLDN23 mRNA and protein level [Maryan *et al.* 2015]. AMI-1 was initially reported as type I PRMT inhibitor [Castellano *et al.* 2010], which also demonstrated inhibition activity in PRMT5 [Zhang *et al.* 2015a]. AMI-1 inhibited

Table 3. Inhibitors of histone-methylation enzymes in treating experimental colorectal cancer.

Family	Enzyme	No. of potent ligands*	Inhibitors used in treating CRC	Structure of catalytic domain available	Reference
HMT	KMT2B	N/A	N/A	No	N/A
	KMT2C	N/A	N/A	Yes	N/A
	KMT2D	N/A	N/A	No	N/A
	SETD1A	N/A	N/A	No	N/A
	SUV39H1	1	Chaetocin	Yes	Yokoyama *et al.* [2013]
	EHMT2	36	UNC0638; BIX01294	Yes	Zhang *et al.* [2015b]
	PRDM2	N/A	N/A	Yes	N/A
	PRDM16	N/A	N/A	No	N/A
	SETDB1	N/A	N/A	Yes	N/A
	EZH2	20	GSK126;GSK343; DZNep	Yes	Benoit *et al.* [2013a, 2013b]; Ferraro *et al.* [2014]; Maryan *et al.* [2015]
	DOT1L	28	EPZ004777	Yes	Kryczek *et al.* [2014]
	SMYD3	N/A	BCI-121	Yes	Peserico *et al.* [2015]
	WHSC1	N/A	N/A	Yes	N/A
	PRDM5	N/A	N/A	No	N/A
	CARM1	17	N/A	Yes	N/A
	PRMT5	N/A	AMI-1	No	Zhang *et al.* [2015a]
HDM	KDM1A	56	Tranylcypromine	Yes	Ding *et al.* [2013]
	KDM5B	N/A	N/A	Yes	N/A
	KDM3A	5	N/A	No	N/A
	KDM3B	N/A	N/A	Yes	N/A
	PHF2	N/A	N/A	Yes	N/A
	KDM4B	N/A	N/A	Yes	N/A
	KDM4C	10	FLLL-32	Yes	Lin *et al.* [2010]
	KDM6B	1	N/A	Yes	N/A
	JARID2	N/A	N/A	No	N/A

CRC, colorectal cancer; N/A, not applicable; HMT, histone methyltransferases; HDM, histone demethylase.
*Data acquired from CHEMBL database (version 20).

proliferation of CRC cells and xenograft mouse models [Zhang *et al.* 2015a].

Tranylcypromine, previously used as an antidepressant drug, was discovered as a potent KDM1A inhibitor [Lee *et al.* 2006; Yang *et al.* 2007a, 2007b]. Treated with tranylcypromine at 2.5 mmol in SW620 cells, invasion and growth were significantly suppressed [Ding *et al.* 2013]. FLLL-32, one of the curcuminoids, inhibits KDM4C *in vitro* [Kim *et al.* 2014]. FLLL-32 inhibits STAT3 phosphorylation, resulting in the inhibition of cell proliferation of CRC cell lines [Lin *et al.* 2010].

We also noticed that among the 25 CRC-associated histone-methylation enzymes, only a few of them (nine) have potent chemical probes available (Table 3). Moreover, specific chemical probes, which can accurately modulate the enzymatic activity *in vitro* and *in vivo*, are also lacking. Both may hamper testing their roles in CRC development, or hinder utilizing their therapeutic value in CRC treatment.

Crystal structures of histone-modifying enzymes could provide opportunities to meet the requirements of developing selective and potent small-molecule modulators as novel epigenetic therapies for CRC. We searched all the available structures of CRC-associated histone-methylation enzymes in the PDB Data Bank. In total, 134 catalytic-domain-containing structures of 10 enzymes (including 5 HMTs and 5 HDMs) were found. These structures, either in *apo* form or in complex with substrates/cofactors/inhibitors/activators,
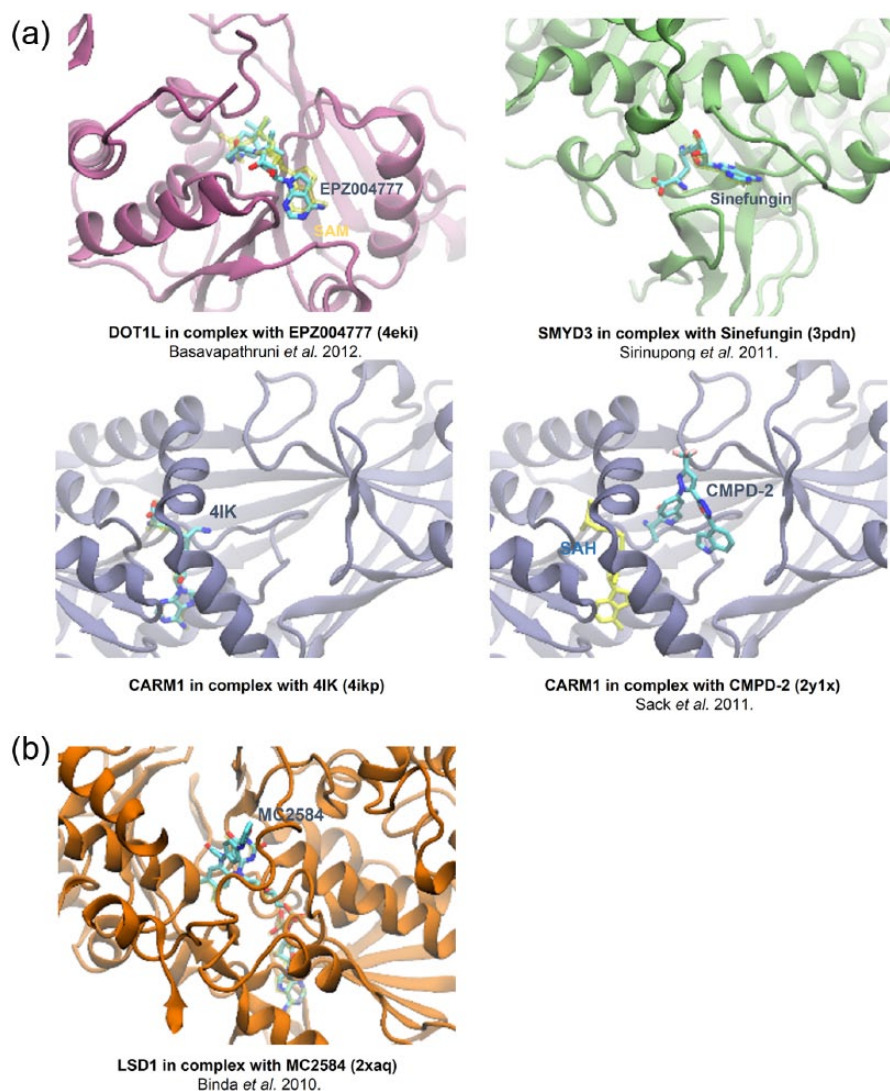
Figure 4. Crystal structures of histone methyltransferase and histone demethylase in complex with different inhibitors. (a) DOT1L binding to EPZ004777 at the cofactor site (top left); SMYD3 binding the Sinefungin at the cofactor site (top right); CARM1 binding to 4IK at the cofactor site (bottom left); CARM1 binding to CMPD-2 at the substrate site (bottom right). (b) LSD1 binding to MC2584 at the cofactor site.
SAM, S-adenosylmethionine cofactor; SAH, S-adenosylhomocysteine cofactor.

provide fruitful insights into the structural basis of regulation of enzymatic activity.

Generally speaking, current structure-based drug-design efforts towards histone-modifying enzymes are primarily focused on the cofactor and substrate binding sites. PKMT and PRMT use the common cofactor SAM to catalyze the methylation of lysine and arginine. Except DOT1L, the catalytic domains of all PKMTs contain a conserved SET domain. The catalytic domain of PKMT is composed of several subdomains, including N-SET, I-SET, C-SET and post-SET. Along with the N-, C-SET domain, the I-SET and post-SET domains form the substrate and cofactor binding sites, where the substrate lysine and cofactor methyl meet at the catalytic channel. A potent and selective inhibitor of DOT1L, EPZ004777, occupies the SAM binding site [Figure 4(a), top left] [Basavapathruni *et al.* 2012]. A similar binding mode is adopted by sinefungin in SMYD3 [Figure 4(a), top right] [Sirinupong *et al.* 2011] or 4IK in CARM1 [Figure 4(a), bottom left]. The inhibitor can also occupy the substrate site of HMT. For example, CMPD-2 with IC_{50} of 27 nmol, binds CARM1 at

the arginine cavity [Sack *et al.* 2011]. HDMs are classified into two subfamilies, the flavin-dependent LSD1 and LSD2, and the iron-dependent Jumonji C-domain-containing demethylases. The tranylcypromine derivative, MC2584, binds to LSD1 at the cofactor site [Figure 4(b)] [Binda *et al.* 2010].

Development of selective and potent small-molecule modulators of histone-modifying enzymes should be emphasized in the near future. Firstly, the cofactor site is structurally conserved among family members. It is an ideal binding site for small-molecule inhibitors, like cofactor analogs, but the poor specificity is an increasing issue. To improve selectivity, bisubstrate inhibitor, which occupies both cofactor and substrate sites, might be a promising direction. Meanwhile, many crystal structures of these enzymes exhibit distinct conformers in crystal structures, like inactive or active states. It is possible to capture distinct intermediate states in transition pathways between inactive and active states by small molecules. The intermediate states are supposed to be specific for individual enzymes, which may raise hope in developing highly selective intermediate-bound inhibitors. Secondly, the allosteric site is also promising for specific inhibitors or activators. It requires thorough understanding of the regulatory domains in enzymes that are usually absent in the crystal structures. For histone-methylation enzymes as tumor suppressors in CRC, using an activator to targ*et al*losteric sites is an attractive way to confer tumors. Given the successful example in SIRT1 [Dai *et al.* 2015], it is possible to find small-molecule activators for these tumor suppressors. Thirdly, many histone-methylation enzymes are within multiprotein complexes in cells. Protein interfaces between proteins are also druggable sites for small molecules. New protein–protein interaction inhibitors for histone-modifying enzymes may be developed in the future, such as ICG-001, a good example of an inhibitor that disrupts the interaction between CBP and β-catenin [Emami *et al.* 2004].

Conclusion
Aberrant histone methylation, as well as associated enzymes have been widely linked with CRC. It is worth noting that some CRC-associated histone-methylation enzymes have not been validated as drug targets, including JARID2, KDM3A, KDM3B, KMT2C, KMT2D, PRDM2, PRDM16, SETDB1, and WHSC1. Modulating these proteins in CRC cells or animal models by overexpression, knockdown or pharmacological inhibition may shed light on their therapeutic values in CRC.

More attention should be paid on the mechanisms of histone-methylation enzymes in the development of CRC. We know that by regulating the histone-methylation profile, onco- or tumor-suppressor genes can be turned on or off. Nevertheless, current data suggest such regulation might be specific, and we should figure out exactly which genes are affected by deregulated histone-methylation enzymes. Moreover, histone-methylation enzymes can also modify nonhistone proteins and affect their functions in post-transcriptional level. Once these pathogenesis mechanisms can be elucidated, more precise treatment therapies can be expected.

The author noticed that current data of histone methylation in CRC is mainly preclinical. Intriguingly, EZH2 inhibitor, EPZ-6438 has entered into phase I/II trials for advanced solid tumor or B-cell lymphomas [ClinicalTrials.gov identifier: NCT01897571]. In this active field, we expect more histone-methylation therapies for CRC in clinical trials, identification of new histone-methylation enzymes as CRC drug targets, and discovery of new specific chemical probes of histone-methylation enzymes in coming years.

Conflict of interest
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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