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Drugging the human methylome: an emerging modality for reversible control of aberrant gene transcription

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Protein and DNA methylation have emerged as critical mechanisms for the control of regulated gene transcription. In humans, the addition, recognition and removal of methyl groups are orchestrated by at least 344 proteins that we collectively refer to as the 'methylome'. The large size of the methylome likely reflects the importance of precise control over this small covalent modification. An increasing number of reports implicating the misregulation of methylation in disease make the proteins governing this modification attractive target for small molecule drug discovery. In light of the emerging opportunities for the development of therapeutics that modulate methylation-dependent pathways, this review examines the protein families that constitute the methylome, with emphasis on the methylation of arginine and lysine residues of proteins. Genetic aberrations that give rise to disease are highlighted, in addition to recent proof-of-concept successes in the development of small molecule modulators of methylome constituents.

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Introduction

Covalent modification of histones and chromosomal DNA plays a critical role in controlling gene transcription. These chromatin modifications form the basis of an epigenetic code that influences both global and local gene expressions, and are dynamically 'written' and 'erased' by specific enzymes, and 'read' by effector proteins containing protein binding modules that recognize them. Among these modifications, methylation plays a paramount role in gene regulation; the readers, writers and erasers involved in methylation are collectively referred to as the 'methylome'.

At least 344 proteins and protein domains constitute the methylome, adding, removing and recognizing protein and DNA methylation (Figure 1). Many of these proteins play critical roles in embryonic development and cellular differentiation, and are misregulated in hyperproliferative diseases such as cancer. About 96% of methylome biomass is dedicated to writing, reading and erasing lysine and arginine methylation on proteins (Figure 1). A smaller repertoire of proteins are involved in DNA methylation. Here we focus on the incipient significance of the protein methylome and prospects for targeting genetically defined diseases caused by aberrant protein methylation.

The writers, erasers and readers of the protein methylome

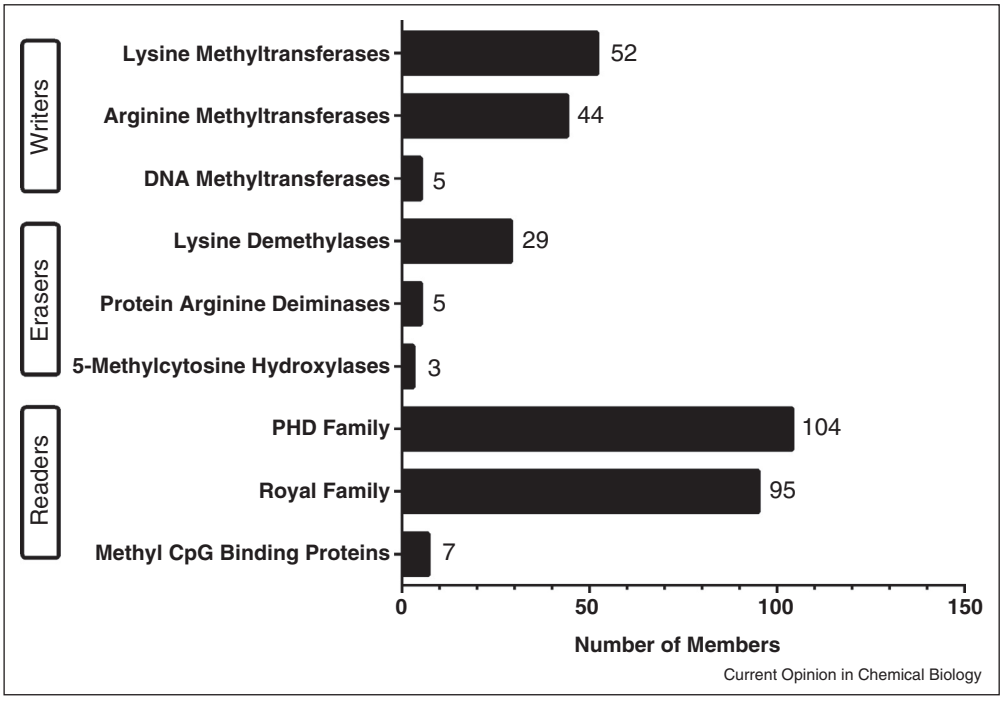
Writers

Protein methyltransferases (PMTs) transfer methyl groups from *S*-adenosylmethionine (SAM) to side chain nitrogen atoms of lysine and arginine residues [1] on both nuclear and cytosolic proteins [2,3]. From the perspective of transcriptional regulation, the histone proteins of chromatin are clearly of greatest importance; the location of known histone methylation sites is illustrated in Figure 2a. The PMT class divides into two distinct families of enzymes: protein lysine-methyltransferases (PKMTs) and protein arginine-methyltransferases (PRMTs) [4••], with the single exception of DOT1L, a lysine methyltransferase that aligns more closely with the PRMTs. Methyl groups can be added to the target lysine or arginine multiple times by the same enzyme or by a combination of enzymes. Thus, lysine can be monomethylated, dimethylated and trimethylated while arginine can be monomethylated and symmetrically dimethylated or asymmetrically dimethylated. These eight different states of lysine and arginine methylation are shown in Figure 2b and lead to distinct transcriptional effects on specific genes.

Erasers

Lysine demethylases (KDMs) remove methyl groups via oxidative mechanisms that release the methyl group as formaldehyde. The KDM class clusters into two major families: the lysine-specific demethylase (LSD) family and the Jumonji domain-containing family (JmjC). Only two enzymes comprise the LSD family, LSD1 and LSD2 [5•]. Both are flavin adenine dinucleotide (FAD)-dependent enzymes that utilize FAD^+ to oxidize monomethylated and dimethylated lysine to an imine intermediate that then undergoes hydrolysis. [6]. JmjC is a larger family of 27 enzymes [5•] that use iron and 2-oxoglutarate to oxygenate the methyl group [7]. Like PMTs, the KDMs act primarily on histones, but can also demethylate

Figure 1



The writers, erasers and readers of the methylome tabulated by protein family within the human genome.

non-histone substrates [3]. While there have been no reports of a direct arginine demethylase, the protein arginine deiminases (PADs) can lead to demethylation of arginine via conversion of arginine to citrulline [8,9].

Readers

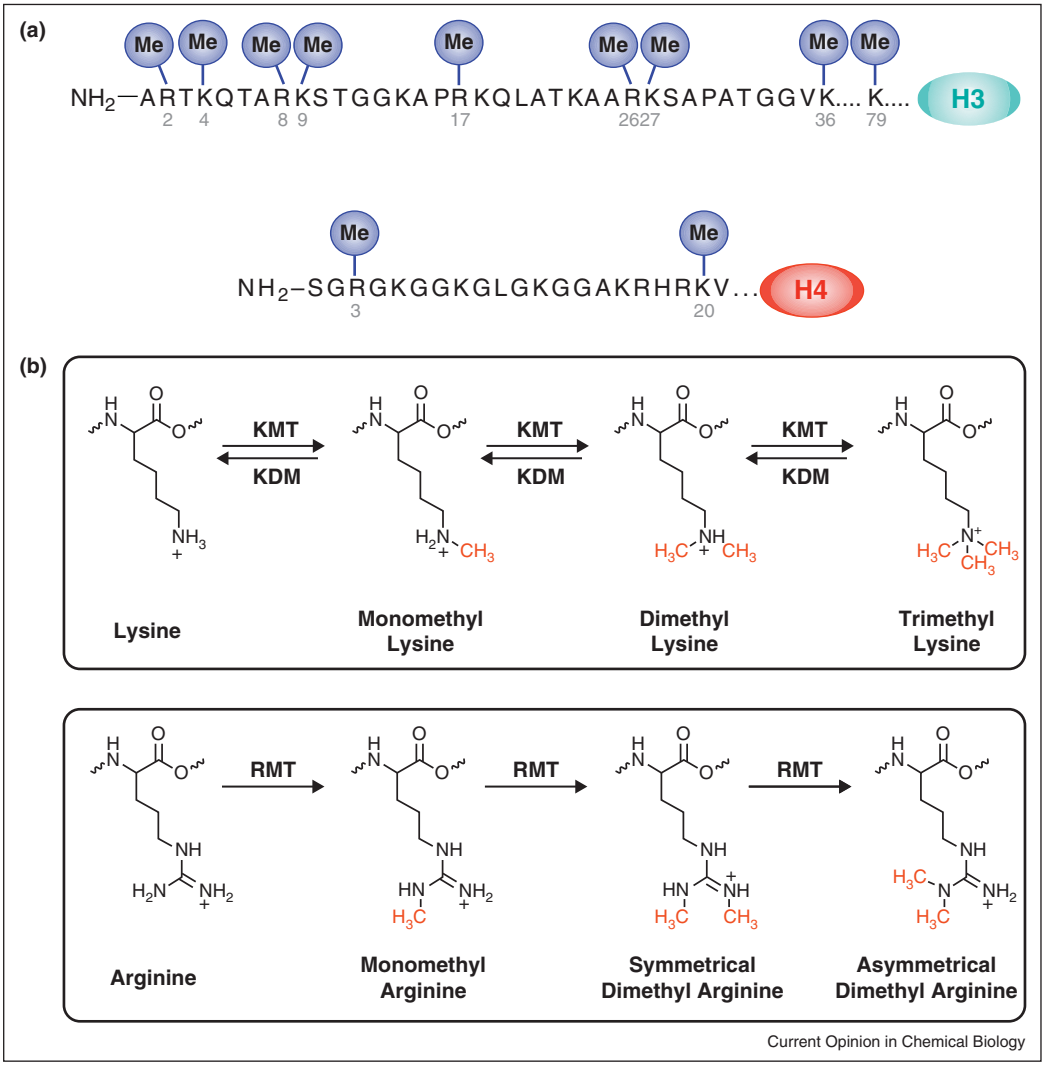
A wide array of enzymes, transcription factors and auxiliary proteins contain reader modules that specifically recognize unmodified or modified amino acids on histones. Many contain multiple reader domains that simultaneously bind to multiple marks in a sequence-dependent and modification-dependent fashion. Methyl-lysine is an abundant histone modification, and not surprisingly a diversity of methyl-lysine reader domains exist [5•]. The methyl-lysine readers (KMe readers) are divided into two families: PHD zinc finger domains, and the 'Royal Family', comprising Tudor, MBT, chromodomain, and PWWP domains [10–12]. KMe reader domains usually comprise less than 100 residues and fold to form pockets that contain an electron rich cage of 2–4 aromatic residues that interact with the lysine through π-cation interactions. The methylation state of the lysine is recognized by the presence of 0–2 acidic functionalities at the base of the pocket that interact with the nitrogen of the lysine side-chain [11]. While the bulk of methylation recognition appears to be focused on lysine, there are reports of proteins that act as arginine readers on non-histone substrates [13].

Genetic alterations of the protein methylome in cancer

Sequencing of tumors has revealed a broad spectrum of genetic alterations in the methylome, making specific methylome members attractive targets for drug discovery. A survey of the literature, summarized in Table 1, identifies 13 methylome proteins that have genetic alterations leading to enhanced methylation or mistargeting of methylation in cancer. These genetic alterations may cause either diminished expression of tumor suppressor genes or increased expression of oncogenes. In some cases the genetic alteration results in a unique dependency of the cancer cell on the altered methylome protein for proliferation. Small molecule inhibitors of these proteins would be expected to therefore impede proliferation in altered cancer cells. It thus follows that patients bearing these genetic alterations are most likely to benefit from treatment with drugs targeted to the altered methylome protein. This concept has been termed 'personalized cancer therapy' [14•] and has already succeeded in producing drugs against kinase targets for oncology indications. Parallel examples exist within the methylome; two examples of this are provided by the PKMTs EZH2 and DOT1L, described below.

Y641 and A677 point mutations altering the enzymatic activity of EZH2 have been identified in 10–24% of diffuse large B-cell (DLBCL) and follicular lymphoma

Figure 2



Overview of protein methylation. **(a)** The location of known histone H3 and H4 lysine and arginine methylation sites. **(b)** The 8 potential methylation states of lysine and arginine.

(FL) patients [15•,16,17]. The wild-type EZH2 displays decreasing catalytic activity against substrates of increasing methylation state at H3K27, such that the order of substrate turnover is as follows: H3K27 > H3K27me1 ≫ H3K27me2. The exact opposite pattern of substrate utilization is observed for all of the lymphoma-associated mutant enzymes; the mutant enzymes all show the following order of substrate turnover: H3K27me2 > H3K27-H3K27me1 ≫ H3K27 [18••,19,21]. Patients who are heterozygous for these mutations display elevated levels of the tumorigeneic H3K27me3 state, as a result of the coupled activities of wild-type and mutant EZH2.

A second example is provided by MLL-rearranged leukemia, in which chromosomal translocations at 11q23 result in fusion protein formation between MLL and a variety of AF and ENL family proteins. The MLL fusion partners (AF and ENL family proteins) share a common ability to bind to and thus recruit the PKMT DOT1L to MLL-targeted gene locations, where it catalyzes the transcriptionally activating methylation of H3K79 [22••]. This aberrant H3K79 methylation drives leukemogenesis in MLL-rearranged leukemia, accounting for >70% of infant pediatric leukemias and ~10% of adult AML [23].

Additional examples of genetic alterations in the methylome that are hypothesized to play causal roles in disease are summarized in Table 1. An important goal of future chemical biology efforts directed at the methylome should be to enable the interrogation of these testable disease hypotheses, through the discovery and

Table 1

Genetic alterations in members of the methylome in cancer

Target	Genetic alteration	Disease	Ref.
Writers			
EZH2	Heterozygous activating mutations occurring at Y641, A677 and A687 that result in hypertrimethylation of H3K27	Lymphoma	[15•,16,17,18••, 20,21,39]
	Deletion of miR-101 leads to EZH2 overexpression	Prostate cancer	
	Deletion of SNF5 leads to EZH2 dependency	Malignant rhabdoid tumors	
DOT1L	11q23 chromosomal translocations fusing MLL1 (without its catalytic SET domain) to DOT1L binding partners such as AF4, AF9, AF-10 and ENL leading to abberant H3K79 methylation. Additionally, CALM-AF10 and SET-NUP214 fusions are known to mistarget DOT1L	Leukemia	[22••]
NSD1	t(5;11)(q35;p15.5) translocation create NSD1-NUP98 fusions	Acute myeloid leukemia	[40]
WHSC1	t(4:14)(p16:q32) chromosomal translocations that places *WHSC1* gene under the control of the *IGH* promoter and results in the overexpression of WHSC1	Multiple myeloma	[41,42]
WHSC1L1	t(8;11)(p11.2;p15) chromosomal translocations fuses WHSC1L1 to NUP98	Acute myeloid leukemia	[43–45]
		Breast cancer	
	8p11–12 focal amplifications	Squamous cell lung cancer	
SETDB1	1q21 amplifications	Melanoma	[46]
SMYD2	1q32 amplifications	Esophageal squamous cell carcinoma	[47]
Erasers			
JMJD2C	9p23-24 amplifications	Esophageal squamous cell carcinoma Squamous cell lung cancer Medulloblastoma Basal breast cancer	[45,48–50]
	t(9;14)(9p24.1q32) translocations creating fusions to *IGH*		
LSD2	6p22 amplifications	Urothelial carcinomas	[51]
UTX	Inactivating mutations of UTX lead to pro-oncogenic hypertrimethylation of H3K27 and dependence on EZH2	Multiple blood and solid cancers	[52]
Readers			
JARID1A	t(11;21;12)(p15;p13;p13) translocations create fusions of JARID1A PHD domain and NUP98.	Acute myeloid leukemia	[33,53]
PHF23	t(11;17)(p15;13) translocations create fusions of PHF23 PHD domain to NUP98	Acute myeloid leukemia	[33,34]
PHF1	t(1;6)(p34;p21) translocation create MEAF6-PHF1 fusions may misdirect HAT activity towards PHF1 targets	Endometrial stromal sarcoma	[35]

development of potent, selective and cell permeable tool compounds directed against the altered methylome component. Indeed, the causal role of genetic alterations affecting EZH2 in DLBCL and FL, and DOT1L in MLL-rearranged leukemia have recently been addressed with small molecule inhibitors, providing preclinical proof-of-concept for these targets (*vide infra*).

Chemical probes and *in vivo* proof-of-concept for protein methylome inhibitors

Biochemical, chemical biological and structural biological studies have recently led to the identification and characterization of a significant number of methylome-targeted ligands. Inspection of the published methylome ligands presented in Table 2 leads to several observations that are discussed further in this section: Firstly there is a spectrum of inhibition modalities for PMT inhibitors, including SAM-competitive, substrate-competitive and allosteric mechanisms. Among the demethylases, LSD-family inhibitors are mechanism-based inactivators and reported JmjC-family inhibitors are all metal chelators. All the KMe reader inhibitors bind in the lysine binding pocket and compete with the methyl-lysine residue. Secondly the PMT class has the most potent and selective inhibitors; demethylase inhibitors are less specific and generally exploit common features shared among all enzymes in the family, while the KMe reader inhibitors are of modest affinity and specificity. And finally comparing Tables 1 and 2, the PMT class stands out as having the greatest number of high quality inhibitors against therapeutically relevant targets today.

The availability of highly potent and selective inhibitors provides a strong foundation for the unambiguous validation of methylome targets, based on phenotypic effects seen upon cell treatment with such compounds. The

Table 2

Inhibitors of the human methylome

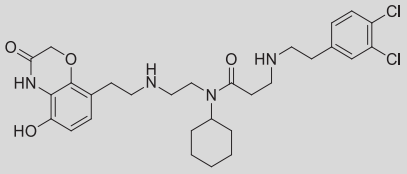
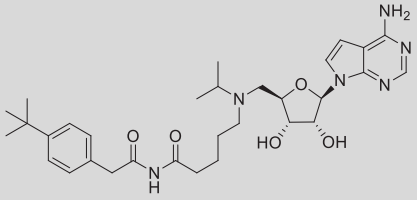
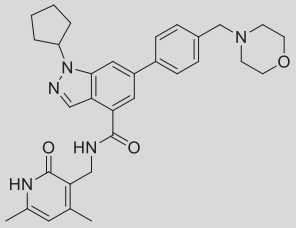
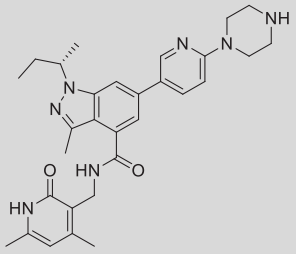
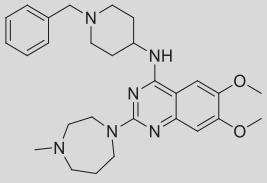
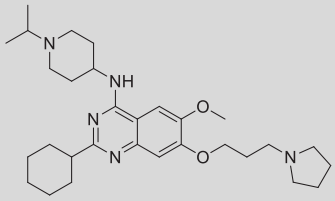
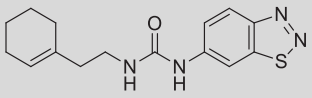
Compound	Structure	Activity	Ref.
Writers			
AZ-505		SMYD2; IC_{50} = 120 nM Substrate-competitive	[54]
EPZ004777		DOT1L; K_i = 0.3 nM SAM-competitive	[25••,26]
EPZ005687		EZH2; K_i = 24 nM SAM-competitive	[27]
GSK126		EZH2; K_i = 0.5M nM SAM-competitive	[28••]
BIX-01294		G9a; IC_{50} = 2.7 μM Substrate-competitive	[55]
UNC0638		G9a; K_i = 3 nM GLP; IC_{50} = 19 nM Substrate-competitive	[24•]
Compound 1		PRMT3; IC_{50} = 2500 nM Allosteric	[56]

Table 2 (*Continued*)

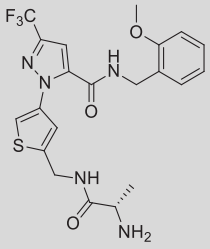
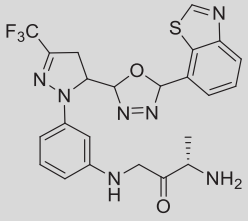
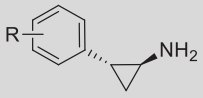
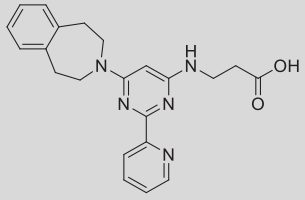
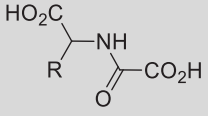
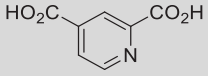
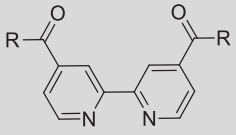
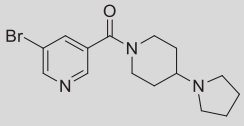
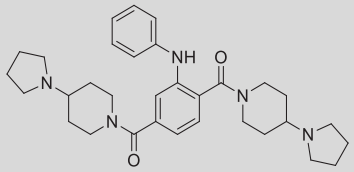
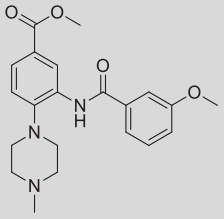
Compound	Structure	Activity	Ref.
Methylgene compound 7a		CARM1 IC_{50} = 60 nM	[57]
Bristol Myers Squibb compound 7f		CARM1 IC_{50} = 40 nM SAH-uncompetitive Substrate-competitive	[58]
Erasers			
Cyclopropyl-amines; multiple examples		LSD1 $\geq$ 600 nM Mechanism-based inactivator	[59–61]
GSK-J1		JMJD3 = 60 nM Chelator	[30]
N-oxalylamino acids; multiple examples		Multiple JmJC demethylases $\geq$ 5400 nM; chelator	[62,63]
2,4-PDCA		Multiple JmJC demethylases $\geq$ 1400 nM; chelator	[62]
Bipyridyl compounds; multiple examples		JMJD2E $\geq$ 180 nM Chelator	[62,64]
Readers			
UNC669		L3MBTL1; K_d = 5000 nM Substrate-competitive	[36]
UNC1215		L3MBTL3; K_d = 120 nM Substrate-competitive	Ingerman *et al.* [38]

Table 2 (*Continued*)

Compound	Structure	Activity	Ref.
WDR5-0103		WDR5; K_d = 450 nM Substrate-competitive	[37]

PMTs in particular, appear to be quite amenable to chemical biology, based on the multiplicity of reversible, potent and selective inhibition modalities discovered against these enzymes. Thus, selective PMT inhibitors have led to chemical probes for G9a/GLP [24•], DOT1L [25••,26] and EZH2 [27,28••] with demonstrated ability to reduce intracellular levels of the respective target-related histone methylation mark in concentration-dependent fashions. In the case of EZH2, two independent groups reported nanomolar, highly selective inhibitors of EZH2 that specifically kill lymphoma cells bearing the heterozygous Y641 or A677 mutations. In one report, these observations were extended to show that the inhibitor GSK126 effected tumor regression and a survival benefit in mouse xenograft models of DLBCL [28••].

Similarly, the DOT1L inhibitor EPZ004777 (K_i = 0.3 nM) was shown to selectively kill cell lines containing the MLL-AF4, MLL-AF9 and MLL-ENL rearrangements that are known to cause mistargeting of DOT1L activity in MLL-rearranged leukemia. Continuous infusion on EPZ004777 also led to a survival benefit in mice bearing MV4-11 xenograft tumors containing the MLL-AF4 translocation [25••]. These chemical probes have enabled the validation of both EZH2 and DOT1L as drug targets in patient populations that are identifiable through companion diagnostic tests [14•]. Continued compound optimization within the EPZ004777 pharmacophore series resulted in the DOT1L inhibitor EPZ-5676, which is currently in phase I clinical trials (http://www.clinicaltrials.gov/ct2/show/NCT01684150).

In contrast to the PMTs, known inhibitors of the KDMs are all active-site directed and display modest affinities for their targets. The LSD-family inhibitors are irreversible, mechanism-based inhibitors that react with the FAD^+ cofactor, while the JmjC inhibitors are chelators of the catalytic iron. Progress has been made in achieving greater selectivity and potency among tranylcypromine analogs [29] for the inhibition of LSD1 and metal chelators for the inhibition of JMJD3 and UTX [30]. In both cases, these compounds were used to interrogate the inhibition of the demethylases in cells, and the improved LSD1 inhibitors were dosed in one MLL-AF9-bearing mouse xenograft model, conferring a survival benefit and reducing the progression of leukemia. However, these results were confounded by drug-induced anemia [31]. These examples suggest that the existing mechanisms of inhibition may prove pharmacologically useful, although reversible inhibitors of these targets would still be desirable.

KMe readers regulate protein–protein interactions, which are often difficult drug targets. However, there is precedence for the effectiveness of targeting epigenetic readers as seen with inhibitors of the bromodomain BRD4, which binds to acetyl-lysine [32]. BRD4 inhibitors have recently entered the clinic (http://clinicaltrials.gov/ct2/show/NCT01587703) for the treatment of BRD4-NUT translocations associated with NUT midline carcinoma [32]. Similarly, there are several examples of translocated KMe reader PHD fingers that cause misreading of chromatin [33–35]. To date, however, no inhibitors of these targets have yet been reported. Examples of small molecule inhibitors of other KMe reader domains have emerged in the past 2 years. Inhibitors of L3MBTL1 [36], WDR5 [37] and L3MBTL3 [38] have recently been reported, with the latter demonstrated to be a specific and cell-active inhibitor of methyl-lysine recognition. In all cases cocrystal structures and biophysical methods were used to confirm inhibition, and guide the optimization of ligand affinity. Structure-guided design is expected to be a powerful tool in future development of KMe reader ligands as at least 126 crystal structures have been deposited into the public domain for this target class.

Conclusions

Our understanding of the pathobiology and chemical biology of histone modification has grown significantly over the past decade. As briefly reviewed here, the methylome constitutes an attractive and diverse collection of targets for the development of personalized cancer therapeutics. Genetic alterations exist among writers, erasers and readers of protein methylation that can be exploited with small molecules that block enzymatic activity or chromatin recognition. The successful examples of the inhibition of EZH2 and DOT1L in cell lines and xenografts harboring genetic alterations lend validation to the overall approach. We are on the verge of

learning whether therapies based on EZH2 and DOT1L inhibition will prove to be clinically useful as personalized cancer therapeutics. It remains to be seen whether the KDMs and KMe readers will also be tractable target classes, but current research should soon address this question.

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