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Epigenetics

Everybody's welcome: the big tent approach to epigenetic drug discovery

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The rapid expansion of epigenetics research is fueled by the increasing understanding that epigenetic processes are critical to regulating cellular development and dysfunction of epigenetic programs is responsible for a diverse set of human pathologies, including cancer, autoimmune, and neurodegenerative diseases. The expansive set of components contributing to epigenetic disease mechanisms and the often reversible nature of epigenetic lesions provide prime opportunities for the development of novel therapeutic strategies. Here, we provide an overview of epigenetics and its relationship to disease, discuss current epigenetics-based therapies and suggest new avenues for the identification of therapies targeting deregulated epigenetic programs in disease.

An epigenetics primer

The definition of epigenetics, originally coined by C.H. Waddington to describe the changes in gene activity during development, has evolved over time. Waddington defined epigenetics as the 'causal interactions between genes and their products, which bring the phenotype into being' [1]. This definition has been updated over time and the version generally accepted today emphasizes the molecular components of epigenetic inheritance and states that epigenetics is 'the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence' [2].

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Central to the modern understanding of epigenetics is chromatin, the packaging of DNA with proteins that serves as the physiological substrate for all DNA-templated processes, including replication, transcription, DNA repair and chromosome segregation. The basic repeating unit of chromatin is the nucleosome, composed of an octamer of the core histones H3, H4, H2A and H2B, around which 147 basepairs of DNA are wrapped. These 'beads on a string' are folded and packaged into higher order structures, forming the chromatin fiber. The components of this fiber are key players of epigenetic inheritance: heritable changes to gene function that are independent of DNA sequence are due to frequent changes in chromatin structure [3].

Based on its underlying structure, chromatin can be roughly divided into two states. 'Euchromatin' is broadly defined as open and accessible chromatin that is permissive to transcriptional activation of genes. By contrast, 'heterochromatin' is defined by its closed and compact nature, and genes within heterochromatic regions are generally transcriptionally repressed. These states are established by complex patterns that integrate multiple types of molecular signals, including DNA methylation, covalent post-translational modification (PTM) of histones, incorporation of noncanonical histone variants into nucleosomes, nucleosome positioning and spacing, and noncoding RNAs [4]. Although all of these mechanisms are important for chromatin function, the scope

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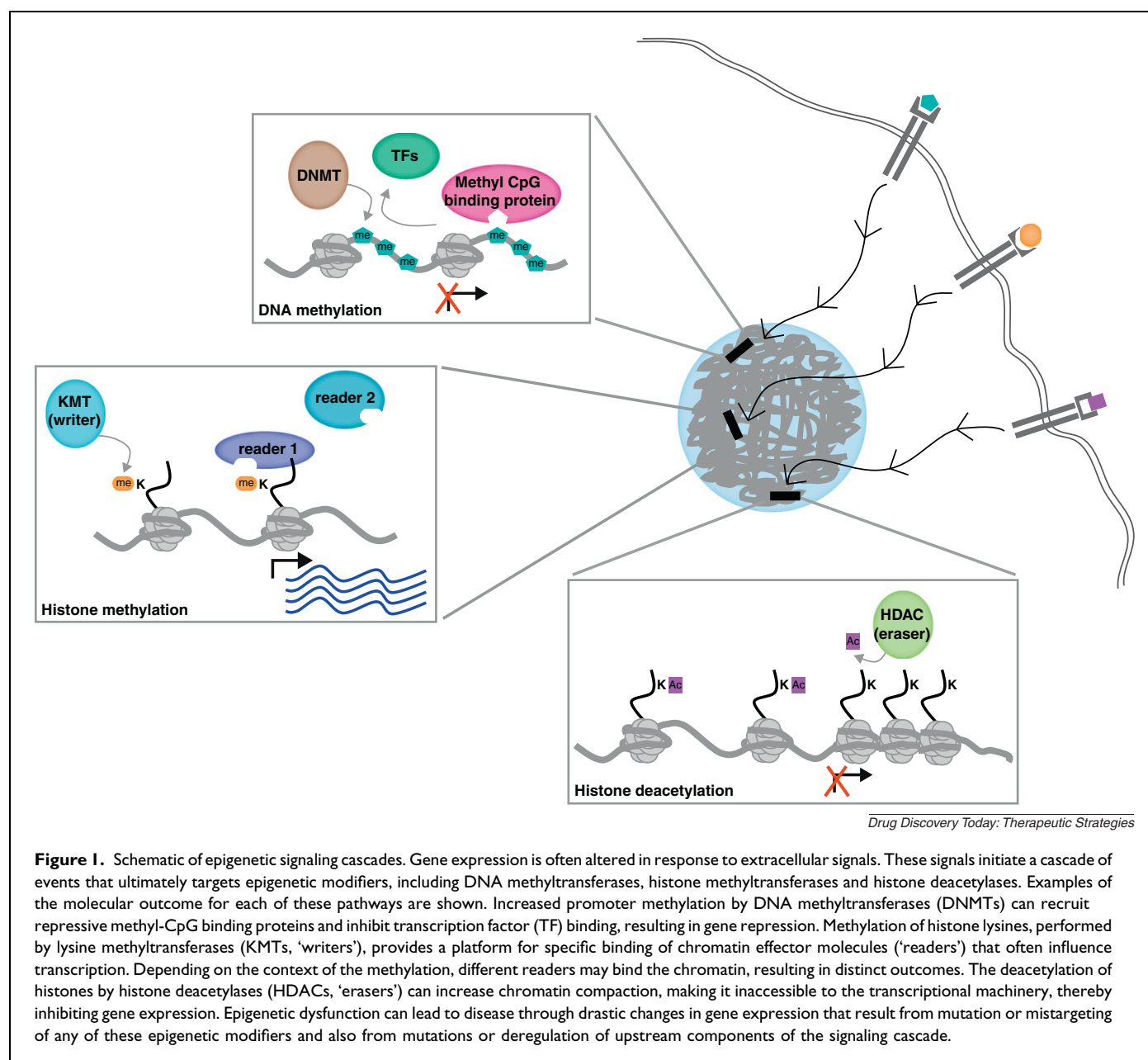
of this review will focus on DNA methylation and histone PTMs.

The establishment of chromatin states by the molecular signatures discussed above is often initiated by signals from the environment or other extracellular cues (Fig. 1). This initiating signal launches a cascade that culminates as a signal at chromatin to change gene function, for example activation of gene expression. Once the alteration to chromatin structure has been established, the signal, such as DNA methylation or a histone PTM, is maintained to ensure the heritability of the chromatin state following cell division. Therefore, the signal at chromatin persists even though the environmental cue and the initial signaling cascade may no longer be active, providing a means of epigenomic memory. Specific examples

of how both DNA methylation and histone PTMs are established at chromatin and the molecular mechanisms by which they influence gene expression are discussed below.

DNA methylation

The most common site for methylation of DNA is at position 5 of cytosine in CpG dinucleotides and is largely associated with transcriptional repression. Methylation of CpG islands in promoters acts to silence genes either through the recruitment of methyl-CpG-binding proteins that repress transcription or through the inhibition of transcription factor binding [5]. DNA methylation is performed by one of the three enzymes in mammals: DNMT1, DNMT3A or DNMT3B. DNMT3A and DNMT3B are *de novo* methyltransferases that



establish methylation patterns on DNA, whereas DNMT1 is a maintenance enzyme that re-establishes methylation patterns following DNA replication [4]. The activity of DNMT1, in particular, is essential to the propagation of methylation from one generation to the next. Through a complex interplay with histone modifications and other epigenetic signals, DNA methylation is therefore a key epigenetic regulator that establishes and maintains heritable patterns of gene expression.

Post-translation modifications of histones

Covalent post-translational modification of histones is fundamental to the regulation of chromatin structure. Well-characterized histone PTMs include acetylation, methylation, phosphorylation and ubiquitylation. A highly regulated set of enzymes catalyzes the addition and removal of histone PTMs, often in response to environmental cues and diverse cellular signaling events. As an example, methylation of lysine residues on histones is performed by lysine (K) methyltransferases (KMTs; 'writers'), which can add a mono-, di- or tri-methyl mark [6]. These methylation marks can be removed by lysine demethylases (KDMs; 'erasers') [7]. The extent and sequence context of the methylation event dictate the association with chromatin of methyllysine-binding proteins ('readers'), which translate the methylation signal into a biological outcome, such as a change in gene expression [8]. In certain contexts, histone methylation also inhibits association of proteins with chromatin, for example, methylation of lysine 4 of histone H3 blocks the interaction between a binding partner, BHC80, of the demethylase LSD1 and chromatin [9]. With some variability and exceptions, similar mechanisms exist for placing, removing and interpreting other histone PTMs. However, unlike small, neutrally charged methyl marks, other PTMs can alter the local chromatin environment through additional mechanisms, such as neutralizing the positive charge on lysine residues (e.g. acetylation) and by sterically altering chromatin structure (e.g. ubiquitylation).

Epigenetics and disease: opportunities for therapeutic intervention

Aberrant expression of either individual genes or complex genetic programs underlies numerous human pathologies. The origins of pathologic gene expression patterns can be genetic, epigenetic or a combination of both. However, the critical advantage of targeting epigenetic mechanisms of disease for therapy is that unlike genetic lesions, epigenetic dysfunction has the potential to be reversible. Reversing epigenetic abnormalities using targeted therapies can be achieved in two ways. First, if gene expression is globally misregulated in the diseased cells, such as in many cancers, the cells can be reprogrammed back to a normal state by altering the epigenetic mechanisms that enforce the

pathologic genomic expression patterns. Alternatively, if the disease etiology is dependent on misregulation of individual genes, the epigenetic modifiers controlling the expression pattern of the disease-causing gene or genes can be directly targeted.

Drastically altered patterns of gene expression are the hallmarks of many human cancers, implicating chromatin-modifying enzymes in oncogenesis. Disruption of the regulation of these enzymes, such as by chromosomal deletions or translocations, results in significant changes in gene expression patterns and increased genomic instability, as discussed below. The epigenome of cancer cells is characterized by distorted patterns of epigenetic modifications, such as DNA methylation and histone acetylation and methylation [10,11]. Drug discovery efforts aimed at the enzymatic activity of writers and erasers, in particular, have yielded several therapeutic options. However, the scope of these efforts needs to be broadened to fully realize the potential of epigenetics-based therapies. Here, we briefly describe drugs targeting epigenetic mechanisms that are currently in clinical use, and then discuss developing targets that may provide new therapeutic opportunities.

Established targets: DNA methylation and histone deacetylation

The most established epigenetic therapies are those that target abnormal DNA methylation and histone acetylation patterns common to solid tumors and hematologic malignancies. Overexpression of DNMTs is observed in several cancers, including colon and acute leukemias, with high levels of expression often predictive of a poor prognosis [12]. Similarly, the 'erasers' of histone and non-histone protein acetylation, type I histone deacetylases (HDACs), have altered levels of expression in many tumor types, including breast, prostate, and gastric cancers [12]. Moreover, HDACs are often recruited to specific genetic loci by interacting with site-specific DNA-binding oncogenic fusion proteins derived from chromosomal translocations [13,14]. Although there remain many unanswered questions surrounding the mechanisms by which altered function of these chromatin-modifying enzymes contributes to neoplastic transformation, aberrant genomic localization of both of these enzyme classes is believed to repress expression of tumor suppressor genes in cancer cells [14]. In the case of DNA methylation, therapies directed at eliminating spurious methylation by incorporating nucleoside analogs into replicating DNA have shown promise in the clinic for treating myelodysplastic syndromes [14,15]. The catalytic activity of HDACs has been shown to be inhibited by a diverse set of small molecules that generally possess anticancer properties, such as slowing proliferation by inducing cell cycle arrest or apoptosis [13,15]. These molecules are in various stages of clinical development (Table 1), with numerous drugs in Phase II and Phase III trials,

Table 1. A subset of epigenetic drugs in clinical development [12–15]

Drug	Epigenetic target	Diseases	Status
Vidaza (5-azacytidine)	DNA methylation	Leukemia, myelodysplastic syndromes, solid tumors	FDA approved, Phase II
Decitabine (5-aza-2' deoxycytidine)	DNA methylation	Leukemia, myelodysplastic syndromes	FDA approved, Phase II
Zebularine	DNA methylation	Liver cancer, urinary bladder cancer	Preclinical
Procaine	DNA methylation	Breast cancer, liver cancer	Preclinical
DNMT1 antisense oligo (MG98)	DNA methylation	Renal cell carcinoma, solid tumors	Phase I/II
Vorinostat (SAHA)	HDACs	Cutaneous T-cell lymphoma, leukemias	FDA approved, Phase II
Romidepsin (depsipeptide)	HDACs	Cutaneous T-cell lymphoma, leukemias	FDA approved, Phase II
Valproic acid	HDACs	Epilepsy, bipolar disorder, leukemias, breast and ovarian cancer	FDA approved, Phase II
Mocetinostat (MGCD0103)	HDACs	Hodgkin's lymphoma, AML	Phase II
Belinostat	HDACs	Peripheral T-cell lymphoma, AML, multiple myeloma	Phase II
Trichostatin A	HDACs	Breast and ovarian cancer	Preclinical
Apicidin	HDACs	Leukemias	Preclinical

and full FDA approval obtained for vorinostat (SAHA) and romidepsin (depsipeptide) in the treatment of cutaneous T-cell lymphoma [12,15]. Furthermore, given the crosstalk between DNA methylation and histone deacetylation, combined therapy of their respective inhibitors provides an opportunity to assault multiple complementary, inter-connected pathways within the cell, and has proven therapeutically effective [16–18]. The development of anticancer therapeutics targeting DNA methylation and histone deacetylation has been extensively reviewed elsewhere [13–15]. Although in-depth study of these therapeutics and their targets is still necessary, the lessons learned and successes realized to date can be applied to exploring the untapped potential in other epigenetic regulators, which will not only present opportunities to treat more diseases, but will also allow for greater exploration of combination epigenetic therapies.

Developing targets: histone lysine methyltransferases (KMTs)

Altered levels and disrupted dynamics of lysine methylation on histones are associated with cellular transformation, and

many histone KMTs are deregulated in cancer [19]. The genetic lesions that disrupt KMT function to promote oncogenic transformation are diverse and include many of the following events, singly or in combination: chromosomal translocations that generate neomorphic fusion proteins or drastically alter expression levels of KMTs, gene amplification, silencing or deletion, and abnormal recruitment of KMTs to genetic loci, resulting in aberrant expression patterns of target genes (Table 2, [19]). Numerous KMTs have both oncogenic and non-oncogenic roles, such as the H3 K27 methyltransferase Ezh2, which not only is a potential therapeutic target for breast and other cancers [20], but also functions in B-cell development [21] and tissue differentiation [22]. G9A and GLP, two enzymes that mono- and di-methylate at H3K9 have been shown to have diverse roles, including in cognitive behavior and inflammation [23,24]. H3K9 methylation is also known to be critical for modulating expression of highly regulated inflammatory genes [25] and recent work has shown that a signaling cascade initiated by methylation of the nuclear factor (NF)- κ B subunit RelA by the KMT SETD6 stabilizes the H3 K9 methyltransferase GLP at chromatin to repress inflammatory gene expression programs [24]. Moreover, c-Rel-targeted H3K9

Table 2. Common sites of histone methylation in humans and a subset of KMTs published to utilize these sites as substrates.

Methyl mark	KMTs	Diseases	Inhibitors
H3K4	hSET1/MLL family, SET7/SET9	Leukemia (AML, ALL, MLL), hepatocellular, colorectal and breast carcinoma	None
H3K9	SUV39H1/2, G9a, Eu-HMT1, SETDB1, PRDM2/RIZ1	B-cell lymphoma, breast, colorectal, hepatocellular carcinoma, neuroblastoma, melanoma, Huntington's disease	Chaetocin (Dm Su-(var)3–9) [27] BIX-01294 (G9a) [28]
H3K27	EZH2, EZH1, G9a	Breast, bladder, colorectal and prostate cancer, lymphoma, melanoma	DZnep (EZH2) [20]
H3K36	SETD2/HYPB, NSD1, NSD2, NSD3	Acute myeloid leukemia, multiple myeloma, Wolf–Hirschhorn and Sotos syndromes, glioblastoma multiform, breast, lung and prostate cancer	None
H3K79	DOT1L	Leukemia (AML, ALL), T-cell acute lymphoblastic leukemia	EPZ00477 [47]
H4K20	Pr-SET7/8, SUV4-20H	Lung cancer, Hutchinson–Gilford progeria syndrome	None

demethylation by the KDM AOF/LSD2 leads to derepression of NF- κ B regulated genes [26]. The potential significant roles for KMTs in diseases of inflammation, immune responses, and other conditions highlight how elucidation and therapeutic targeting of KMT functions will have broad clinical application and importance.

The strong correlation between aberrant histone methylation and disease, the pathogenic deregulation of KMTs and the reversible nature of lysine methylation make KMTs an attractive therapeutic target. Despite the broad potential of targeting these enzymes to slow or revert disease processes, the first small molecule inhibitor of a KMT was only identified relatively recently [27]. Since this initial discovery, inhibitors of a few other enzymes have been characterized (Table 2; [20,28]) although a cache of inhibitors similar to what is available for other epigenetic modifiers has not yet materialized. Studies of the G9a inhibitor, BIX-01294, have attempted to optimize its potency through the addition of a moiety that mimics lysine to the molecule [28,29], a successful approach that also significantly reduced the toxicity of the compound *in vivo*. This provides an opportunity for further development of the known inhibitors, but the identification of additional lead compounds is still necessary. Additionally, in all cases, the therapeutic potential of the available inhibitors remains unknown at this time. Below, we discuss two examples of KMTs for which targeted therapies would probably prove beneficial.

The NSD family

The NSD (nuclear receptor-binding SET domain) family of proteins, composed of NSD1, NSD2 and NSD3, are KMTs implicated in developmental disorders, several cancers and overgrowth syndromes [30–32]. Chromosomal translocations fusing both NSD1 and NSD3 (also known as WHSC1L) to NUP98 occur in acute myeloid leukemia (AML), and result in deregulation of these enzymes and activation of proto-oncogenes [33–35]. NSD2 (also known as MMSET and WHSC1) is overexpressed in up to 20% of patients with multiple myeloma, the second most common hematologic malignancy, because of the t(4;14) translocation [36]. There is much debate in the literature regarding the substrate specificity of the NSD family of enzymes [31,37–39], although evidence is coalescing around H3 K36 di-methylation as the physiologic chromatin substrate for all three enzymes [37,40]. Indeed, the structural similarities among members of this family and the shared disease processes in which they participate provide a unique opportunity for the iterative development of inhibitors that will likely lead to productive clinical treatments.

DOT1L

The gene encoding the H3 lysine 4 (H3K4) tri-methyltransferase mixed lineage leukemia (MLL) is translocated in greater

than 70% of infants with acute leukemias and approximately 10% of adults with AML, often predicting a poor prognosis. The oncogenic fusion proteins that result from these translocations lack the catalytic SET domain of MLL but force interactions between other proteins, such as the H3K79 methyltransferase DOT1L and chromatin [41–43]. In leukemias with MLL rearrangements, DOT1L is directly recruited to MLL target genes by physical interactions with MLL fusion proteins [44,45] and is specifically required for the upregulation of these target genes [46]. A small molecule inhibitor of DOT1L has been generated based on structural data of DOT1L. This inhibitor – EPZ004777 – was designed to mimic S-adenosylmethionine (SAM), the methyl donor for methylation reactions, and acts as a competitive inhibitor by binding to the DOT1L SAM-binding pocket. This molecule was shown to be highly specific to DOT1L, to reduce levels of H3K79 methylation at MLL target genes and repress expression of these leukemogenic genes. Strikingly, it also selectively eliminates MLL-translocated cells while not adversely affecting non-translocated cells, and extends lifespan in an MLL xenograft mouse model [47]. Although the poor pharmacokinetics of EPZ00477 preclude it from being an efficacious therapeutic agent, studies of structurally similar compounds might prove productive. This recent work provides proof of principle that specific inhibition of KMTs has potential as a therapeutic strategy tailored to cancers with known epigenetic perturbations, and thus warrants further exploration and clinical development.

Developing targets: chromatin-binding proteins

Targeting the interaction between epigenetic ‘readers’ and chromatin is also likely to open new therapeutic avenues. The methyl-CpG-binding protein MeCP2, known to promote gene silencing and transcriptional repression, is mutated in Rett syndrome, an X-linked neurodevelopmental disorder. Importantly, phenotypic reversal of neurological symptoms has been demonstrated in a mouse model of Rett syndrome by restoration of expression of MeCP2 [48], providing evidence that neurological defects might be reversed via re-establishment of the normal epigenetic mechanisms and that targeting the chromatin-binding proteins has significant potential for reversing disease phenotypes.

The bromodomain and extra-terminal domain (BET) family of proteins bind acetylated histones via their bromodomain and stimulate gene activation, and have been implicated in pro-inflammatory gene expression and tumorigenesis. Strikingly, a small molecule inhibitor, named I-BET, binds to the bromodomain by mimicking acetylated histones and blocks BET-dependent activation of inflammatory gene expression in macrophages with high specificity [49]. Remarkably, I-BET treatment rescued mice from acute septic shock [49]. Thus, this study demonstrated a new strategy for treating diseases of hyperinflammation. In a parallel study,

treatment of NUT midline carcinoma cells, which posses an oncogenic translocation of the BET family member BRD4, with the small molecule inhibitor JQ1 reduced their proliferative capacity and promoted differentiation [50]. Together, these studies provide proof of principle that targeting the interaction between ‘readers’ and chromatin will provide new possibilities for treating epigenetics-based diseases.

Concluding remarks and future perspectives

Pursuing therapeutic strategies aimed at the underlying epigenetic mechanisms of disease holds great promise. To harness the potential in these strategies and push compounds into clinical development, we must combine both basic research and translational studies. For example, unequivocal identification of the enzymatic activity and substrate specificity of disease-associated enzymes is necessary for accurate assay development. Detailed structural studies will also provide great insight into the directed design of compounds. It is also essential to continue the search for yet unknown epigenetic modifiers, and to clearly distinguish those enzymes that are causative agents in pathogenesis from those whose abnormal regulation is a corollary to the disease process.

Here, we review the status of the more established epigenetic targets, DNA methylation and histone acetylation, and present histone methyltransferases and chromatin-binding proteins as promising candidate therapeutic targets. However, additional enzyme classes such as histone demethylases and histone arginine methyltransferases have similarly been implicated in disease mechanisms and may also provide unique opportunities for therapeutic intervention [51].

One of the challenges of studying chromatin-modifying enzymes is that many of these enzymes are known to have non-histone targets. Therefore, the effect of any directed therapies on functions unrelated to chromatin will have to be considered. Moreover, although many of these enzymes are dysfunctional in disease cells, they perform critical functions in normal cells, so the toxicity of any of these drugs, especially over the long term, will need to be evaluated. Finally, it will also be important to determine which modifications to chromatin are truly epigenetic, as targeting heritable marks is more likely to produce a stable outcome. Although the mechanism of inheritance of DNA methylation patterns is relatively well established [4], recent work has just begun to elucidate the mechanism by which histone modifications are propagated from one generation to the next [52,53]. Changes to transcription induced by specific histone modifications may be transient, and although directing therapeutic agents at these modifications may have some, probably shorter-term, benefits, targeting heritable histone modification events may provide the opportunity to reverse the disease process and revert the cell back to its normal state.

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