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*MINIREVIEW*

# Writing and Rewriting the Epigenetic Code of Cancer Cells: From Engineered Proteins to Small Molecules

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**ABSTRACT**

The epigenomic era has revealed a well-connected network of molecular processes that shape the chromatin landscape. These processes comprise abnormal methylomes, transcriptosomes, genome-wide histone post-transcriptional modifications patterns, histone variants, and noncoding RNAs. The mapping of these processes in large scale by chromatin immunoprecipitation sequencing and other methodologies in both cancer and normal cells reveals novel therapeutic opportunities for anticancer intervention. The goal of this minireview is to summarize pharmacological strategies to modify the epigenetic landscape of cancer cells. These approaches include the use of novel small molecule inhibitors of epigenetic processes specifically deregulated in cancer cells and the design of engineered proteins able to stably reprogram the epigenetic code in cancer cells in a way that is similar to normal cells.

## Introduction

The concept of "epigenetics" was pioneered by Conrad H. Waddington in 1942 as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being" (Goldberg et al., 2007; Waddington, 2012). "Epi" comes from the Greek word "over," and thereby epigenetics is the study of the molecular, cellular, and environmental aspects of heredity, which are not explained by simple changes in the underlying DNA sequence. Epigenetic processes influence the *chromatin structure*, not the gene sequence per se, but the tridimensional folding and package of the DNA and associated proteins, referred to as chromatin (Strahl and Allis, 2000; Kouzarides, 2007). Heterochromatin, comprising pericentromeric regions, satellite DNA, and retroviral long terminal repeats are condensed and silenced areas of the genome (Zakrzewski et al., 2011). In contrast, euchromatin comprises the loci being actively transcribed (Gilbert et al., 2004). There are two important characteristics of the epigenetic state of cells. First, unlike somatic mutations and other genetic alterations, which irreversibly alter the genes, epigenetic processes are reversible and thus provide environmental plasticity and cell adaptation to new microenvironments. Second, the chromatin structure is inherited, and in absence of perturbations, the chromatin state has an intrinsic memory and it is transmitted over cell generations (Lange and Schneider, 2010; Margueron and Reinberg, 2010).

In the last decade it has become clear that epigenetic disruptions play a critical role in tumor initiation and progression (Berdasco and Esteller, 2010). Recent research in cancer has focused on the prognosis value of epigenetic signatures in cancer, with the advent of genome-wide large-scale profiling of tumor specimens. The emerging view of cancer epigenetics is that epimutations might affect early events of cancer, which could prime subsequent events in the progression of the disease (Carmona and Esteller, 2011; Rodriguez-Paredes and Esteller, 2011; Sandoval and Esteller, 2012). Epigenetic aberrations in cancer include both abnormal silencing (for example, silencing of tumor/metastasis suppressors) and reactivation of silenced regions (which leads to oncogenic activation). In many cases, epigenetic aberrations can collaborate with genetic changes, as for example in the case of silencing of a second allele of a tumor suppressor

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**ABBREVIATIONS:** AID, activation-induced cytidine deaminase; ATF, artificial transcription factor; CpG, cytosine-phosphate-guanine; DNAme, DNA methylation; DNMT, DNA methyltransferase; ES, embryonic stem cells; GLP, G9a-like protein; HP1, heterochromatin protein 1; MBT, malignant brain tumor domains; PHDs, plant homeodomains; PKMTs, protein lysine methyltransferases; PRMTs, protein arginine methyltransferases; SETD7, SET domain containing (lysine methyltransferase) 7; SETDB1, SET domain, bifurcated 1; SUV39H1, suppressor of variegation 3-9 homolog 1 (*Drosophila*); TALEs, transcription activator-like effectors; TET proteins, ten-eleven translocation family of proteins; TF, transcription factor; ZF, zinc finger.

gene, such as BRCA (Liu et al., 2008a; Lim et al., 2009) and p16 (Arima et al., 2012). Numerous examples involve mutations in chromatin modifiers themselves, which have a profound impact in neoplastic progression. In addition to epigenetic modifiers and chromatin remodelers, multiple molecular constituents impact chromatin structure, such as transcription factors (TFs), signal transduction proteins, histone variants, and noncoding RNA, outlining the complexity and molecular crosstalk that sculpts the epigenome (Baylin and Jones, 2011).

In light of the recognition of the importance of epigenetic disruptions in cancer cells, strategies to block or revert these changes are of growing interest in cancer therapeutics. This review will focus on novel pharmacological approaches to reprogram and remodel the epigenome of cancer cells into a pattern that is similar to normal somatic cells. Ideally, this reprogramming would entail hereditary changes in chromatin resulting in long-lasting phenotypic alterations of tumor cells, namely phenotypic memory. In the first part of this minireview we will summarize the language of chemical modifications in chromatin and the main chromatin modifiers ("writers") whose expression/and or activity is disrupted in cancer cells relative to normal tissue. Next, we will discuss novel state-of-the art approaches to revert or "rewrite" those aberrant epigenetic processes in cancer cells using both small molecule inhibitors and targeted engineered proteins.

## The "Language" of Chromatin Modifications

The genome-wide epigenetic status ("epigenetic landscape") of cells is dictated by comprehensive sets of chemical modifications that occur at both the DNA and in the histone tails. Methylation of the DNA (DNAme) occurs in position 5 in cytosine residues (Fig. 1). In mammals, the vast majority (98%) of DNAme occurs in CpG (cytosine-phosphate-guanine) dinucleotides in somatic cells. In embryonic stem (ES) cells, however, about one-quarter of all DNAme occurs in non-CpG context (Lister et al., 2009). In contrast to DNAme, histones are subjected to several chemical modifications in their N-terminal tails (Strahl and Allis, 2000), which are rather unstructured and largely exposed in the context of the three-dimensional structure of the nucleosomes (Dutnall and Ramakrishnan, 1997; Luger et al., 1997) (Fig. 2). There is large chemical diversity and a great number of histone modifications, including methylation, acetylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, deamination, and protein isomerization. Some histone modifications, such as H3K9me, H3K27me, and H4K20me, are typically associated with repressive chromatin. In contrast, other modifications, such as H3K4me, H3K9Ac, H3K36me, and H3K79 are connected with actively expressed genes (Kouzarides, 2007; Henikoff and Shilatifard, 2011; Turner, 2012).

Why is chemical language of histones so degenerate? In principle, chemical modifications can modulate chromatin structure by primarily altering the physical properties of the DNA and or/associated proteins. For example, among all the histone modifications, phosphorylation adds negative charges to the histones, whereas acetylation merely reduces the number of positive charges in chromatin. Because the DNA helix is negatively charged, chemical modifications could act to unravel the DNA from the histones, promoting chromatin relaxation. Second, chemical modifications act as binding platforms or epitopes for specific endogenous proteins ("binders" or "readers"). For example, DNAme marks are recognized by methyl-binding proteins, such as methyl-binding protein-1, histone methylation is recognized by chromo-like domains, acetylation by bromodomains, and phosphorylation by 14-3-3 proteins. "Readers" are selective proteins able to recognize specific histones and even different states of methylation, for example heterochromatin protein 1 (HP1) recognizes H3K9me3, polycomb 2 proteins recognize the H3K27me3 (Kouzarides, 2007). In the cell, endogenous readers form specific complexes with chromatin modifiers possessing catalytic activity ("modifiers" or "writers"). The complexity of epigenetic regulation is further exemplified by the discovery of multiple enzymes able to erase specific epigenetic marks. These include histone deacetylases, lysine demethylases (Klose et al., 2006; Arrowsmith et al., 2012), and the

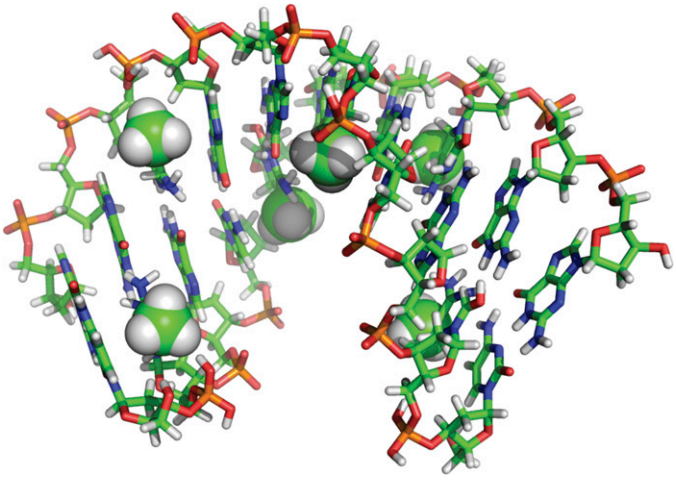

**Fig. 1.** Structure of a double-stranded DNA duplex containing meCpG (383D).

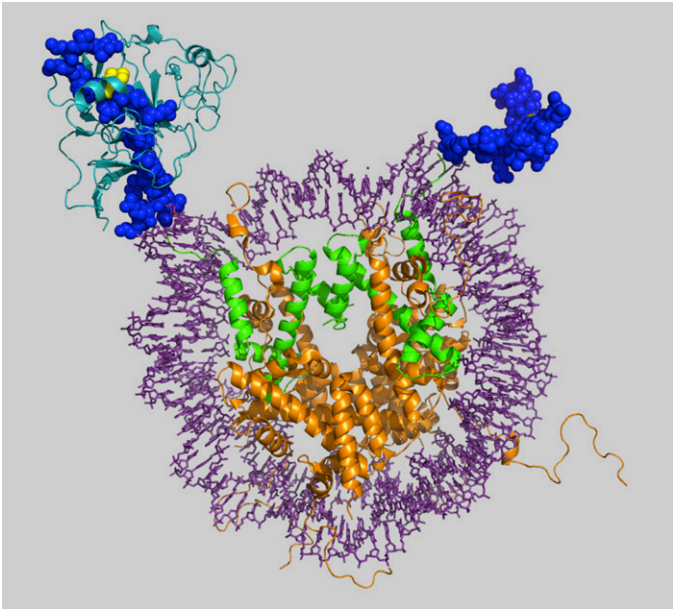

**Fig. 2.** Structure of a nucleosome showing the core histones H3 in green and the histone tail protruding out of the nucleosome outlining the H3K9me2 mark in yellow (1KX5) and superimposed with the H3K9 lysine methyltransferase (2RFI).

more recently discovered DNA-demethylases, which are involved in excision-repair DNA pathways (Rai et al., 2008; Bhutani et al., 2010, 2011) erasing DNA methylation patterns during development, differentiation, and reprogramming.

It has long been proposed that the chemical modifications in chromatin act in a combinatorial manner to define an *epigenetic code*, which coordinately shapes chromatin structure (Strahl and Allis, 2000). However, the large number of histone modifications and the possible combinations of all these primary modifications (many of them in the same histone tail) clearly add several layers of complexity and outline the plasticity of the epigenome. Among all the epigenetic modifications, DNAme and H3K9me are regarded as stable and inherited repressive marks (Margueron and Reinberg, 2010; Han and Brunet, 2012). Notably, DNAme is a key modification that has traditionally been thought to "irreversibly canalize" or "force cells" to engage into specific cell fates (Hochedlinger and Plath, 2009). In the following sections, the relationships and crosstalks between epigenetic modifications, particularly DNAme and histone modifications, will be outlined. Finally, we will briefly describe some of the key mechanisms associated with DNA demethylation in mammalian cells.

## Interconnections between DNA Methylation and Histone Methylation

The interactions between "readers" and "writers" provide regulatory opportunities for the physical transmission of epigenetic marks along the chromosome (spreading) for the transmission of epigenetic marks from a mother cell to a daughter cell (inheritance of epigenetic information) and define crosstalks of multiple epigenetic constituents (Cheng and Blumenthal, 2010; Hashimoto et al., 2010). The interactions between readers and writers can be direct or indirect. Direct interactions include physical associations between epigenetic modulators. For example, the lysine methyltransferase G9a, which deposits H3K9me2 and H3K9me1 in euchromatin (Wu et al., 2010), interacts with DNA methyltransferases through ankyrin domains (Feldman et al., 2006). In self-renewal gene promoters, such as Oct4, this recruitment promotes an irreversible epigenetic switch, which forces stem cells to differentiate (Feldman et al., 2006; Tachibana et al., 2008). During DNA replication, the protein UHRF1 recognizes hemimethylated DNA and forms a complex with several proteins, including DNA methyltransferase 1 (DNMT1) (which maintains DNAme patterns during replication) and lysine methyltransferase G9a. These physical interactions link DNAme with H3K9me in the replication fork, making sure that both DNAme and H3K9me are spatiotemporally coupled processes faithfully transmitted during replication (Bostick et al., 2007; Sharif et al., 2007; Kim et al., 2009; Hashimoto et al., 2010). Another crosstalk between DNAme and H3K9me during DNA replication is mediated by methyl-CpG-binding-proteins, such as the methyl-CpG binding protein 1 (MBD1). In heterochromatin, methylated DNA is bound by MBD1, which recruits the H3K9 methyltransferase SET domain, bifurcated 1 (SETDB1) (Schultz et al., 2002), an enzyme that deposits H3K9me3 (Ichimura et al., 2005; Uchimura et al., 2006; Cheng and Blumenthal, 2010; Hashimoto et al., 2010). The latter mark is recognized by HP1 (Bannister et al., 2001; Lachner et al., 2001), which in turn recruits histone lysine methyltransferases, such as SUV39H1/2 (Krouwels et al., 2005). In addition, HP1 directly binds to the PhD-like domain of DNMT3A in vitro, which suggests that H3K9me could in turn influence DNAme (Fuks et al., 2003). The above suggest that DNAme and H3K9me are mutually reinforcing silencing marks, which might serve to synchronize these two processes during DNA replication, ensuring the inheritance of the epigenetic information (Fig. 3). The crosstalk between DNAme and H3K9me has also been described in plants (Johnson et al., 2007).

A link between DNAme and histone deacetylation has been long demonstrated through the discovery of the methyl-CpG-binding protein 2 (Scarsdale et al., 2011). Methyl-CpG-binding protein 2 carries a transcriptional repressor domain, which recruits the corepressor histone deacetylase (Singal et al., 2002). Reciprocally, histone acetylation is a mark associated with promoter activation and gene transcription. H3/H4 lysine acetylation is recognized by bromodomain-containing proteins, often physically linked to plant homeodomain (PHD) fingers and catalytic domains of histone

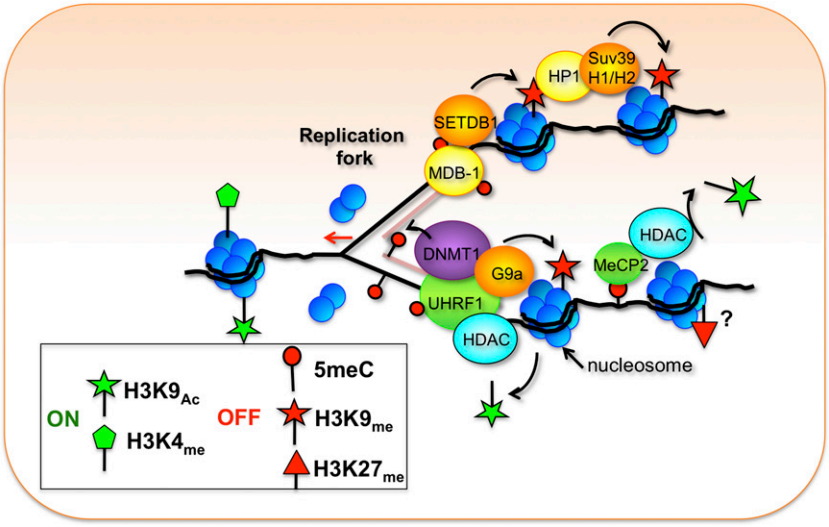


**Fig. 3.** A model outlining the crosstalk of some epigenetic modifications during replication and after the replication fork. HDAC, histone deacetylase; Suv39, suppression of variegation 39 homolog 1 or 2 (Drosophila); UHRF, Ubiquitin-like with PHD and Ring Finger domains 1.

acetyltransferases, such as pCAF (Clements et al., 1999; Nagy and Tora, 2007) and p300 (Liu et al., 2008b; Wang et al., 2008a). Bromodomains in the subunits of chromatin-remodeling enzymes, such as switch/sucrose nonfermentable family, interact with acetylated histones (Reinke and Horz, 2003; Kundu et al., 2007; Gao et al., 2008; Kizilyaprak et al., 2010), which anchor remodeling activities in the nucleosomes and serve to promote nucleosome eviction (Suganuma and Workman, 2011). In summary, direct physical interactions between readers and writers, and readers and erasers, could contribute to a quick spread of a given mark(s) along the genome, leading to effective chromosomal condensation and gene silencing or chromatin relaxation and gene activation.

The interaction or crosstalk between epigenetic processes could be also indirect. A residue of a given histone can be a substrate of multiple modifications. For example, the incorporation of H3K9me by histone lysine methyltransferases could indirectly block the acetylation of the same histone residues by histone acetyltransferases. Another indirect example of epigenetic crosstalks is the incorporation of DNAme, which might negatively affect H3K4me. Methylated DNA is particularly refractory to binding of some DNA-binding proteins, including the zinc finger protein Cfp1, which preferentially binds unmethylated DNA. Cfp1 interacts and recruits H3K4 methyltransferase SETD1, and thereby induction of DNAme might indirectly trigger a decrease on H3K9me (Xu et al., 2011), potentially explaining the dramatic reverse correlation between these two marks (Thomson et al., 2010). Reciprocally, high levels of Cfp1 in the genome could protect these regions from DNA methylation and gene silencing.

The relationship between DNAme and the silencing mark H3K27me3 is currently less well understood. Multiple groups had proposed that in cancer cells H3K27me3 could mark genes subsequently silenced by DNAme, although many developmentally regulated genes are marked H3K27me3 in the absence of DNAme (Ohm et al., 2007; Schlesinger et al., 2007; Widschwendter et al., 2007). The existence of a mutually reinforcing mechanism linking DNAme and H3K27me3 is a topic of intensive research (Hon et al., 2012).

## Context-Dependence of the Epigenetic Modifications

The advent of genome-wide approaches for the mapping of chromatin modifications, such as chromatin immunoprecipitation sequencing, has established the relationships and context dependence of epigenetic modifications, both in embryonic, induced pluripotent, and somatic cells (Chari et al., 2010; Zhou et al., 2011). For example, in promoter regions, DNAme strongly correlates with some histone modifications, specifically with high levels of H3K9me and low levels of H3K4me. In fact, DNAme is a better predictor of H3K9me and H3K4me than the underlying genome context (Mikkelsen et al., 2007; Meissner et al., 2008). Unlike promoter regions, DNAme inside the gene bodies is not correlated with silencing. Widespread low-density of methylation has been proposed to serve as a mechanism to avoid inappropriate transcription initiation from cryptic promoters (Bird, 1993; Tran et al., 2005).

Genome-wide maps of histone modifications in stem and lineage-committed progenitor cells have revealed that H3K4me3 marks the genes that are actively expressed. The "bivalent state" (H3K4me3 and H3K27me3) labels developmental genes poorly expressed that are poised for either expression or repression (Bernstein et al., 2006). In addition, the H3K27me3 mark is retained in genes that are stably repressed. H3K9me3 and H4K20me3 are characteristic of satellite, telomeric, and long-terminal repeats. Finally, H3K36me3 marks coding and noncoding transcripts (Mikkelsen et al., 2007; Meissner et al., 2008).

## Mechanisms of DNA Demethylation

The discovery of active and passive mechanisms to erase DNAme has opened the door to a new paradigm: DNAme and demethylation may not be "one-way" streets as initially thought but highly dynamic and regulated processes (Bhutani et al., 2011). The current view of DNA demethylation involves the integration of multiple interconnected pathways. DNA demethylation can be the result of passive mechanisms, for example, by dilution during replication or inactivation of DNMTs. In addition to passive demethylation, several enzymatic activities have been recently associated with active demethylation and DNA repair. Much excitement has surfaced since the discovery of 5-hydroxymethylcytosine in the mammalian genome in neuronal and ES cells (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009) and its role as an intermediate and key player in DNA demethylation during DNA repair. The ten-eleven translocation (TET) protein family members catalyze the active modification of 5meC to give 5-hydroxymethylcytosine. TET proteins are 2-oxoglutarate and Fe(II)-dependent dioxygenases that catalyze the conversion of 5-methylcytosine in 5hC. TET1 was first identified in acute myeloid leukemia as a fusion protein with the mixed lineage leukemia factor and was designated as leukemia associated protein (Ono et al., 2002). TET1 contains a CXXC zinc finger DNA-binding domain that binds preferentially CpG-rich sequences near the transcription start site of the targeted genes (Williams et al., 2011; Wu et al., 2011). TET1 and TET2 are expressed in mouse ES cells and are necessary for cell lineage specification (Ficz et al., 2011; Koh et al., 2011). TET3 mediates the conversion of 5-methylcytosine to 5hC in the paternal pronucleus after fertilization (Gu et al., 2011; Iqbal et al., 2011).

Recently, the activation-induced cytidine deaminase (AID) has been recognized to participate in DNA demethylation. AID has a pivotal role in generating antibody diversity in lymphocytes. Unlike TET proteins, AID lacks of a DNA binding activity. IAD catalyzes deamination of cytosine residues to uracil. This step is followed by an excision repair or mismatch repair pathway, which is error prone. In addition to this function to generate the antibody repertoire, the AID enzyme has been implicated in demethylation in zebrafish (Rai et al., 2008) and in mammals. Hence, mice lacking AID exhibit global DNA hypermethylation in the primordial germ cells (Popp et al., 2010). Active DNA demethylation is believed to be enzymatically coupled with the replacement of the modified cytosine via DNA repair. DNA glycosylases, such as those belonging to the base excision repair pathway have been involved in the removal of modified cytosine in plants (Gehring et al., 2009). In mammals, these enzymes could act in concert with TET and AID enzymes to promote demethylation. In this model, 5-methylcytosine and 5-hydroxymethylcytosine are first deaminated by AID to thymine and 5-hydroxymethyluracil,

respectively, followed by glycoylase-mediated thymine and 5-hydroxymethyluracil excision repair (Cortellino et al., 2011).

## Epigenetic Alterations in Cancer

Tumor cells show multidimensional aberrations affecting many levels of regulation, including genetic changes, but also profound modifications in the epigenetic landscape. The "cancer methylome" is therefore distinct from normal, non-transformed tissue. Characteristic hallmarks of cancer include a global genome-wide hypomethylation and also hypermethylation of CpG islands (which is typically associated with gene repression) (Berdasco and Esteller, 2010; Portela and Esteller, 2010; Rodriguez-Paredes and Esteller, 2011; Sandoval and Esteller, 2012). However, a substantial fraction of DNA methylation occurs in the cancer methylome away from the transcription start site (~2 kb or more), comprising microRNAs and mirtrons (Dudziec et al., 2011)—these dinucleotides are named CpG shores (Irizarry et al., 2009; Sandoval et al., 2011).

Global DNA hypomethylation and local hypermethylation has recently been confirmed by whole genome shotgun sequencing of bisulfite-treated DNA (methyl-sequencing) of a low-passage breast cancer cell HCC1954 relative to normal epithelial cells (HUMEC cell line). This study revealed that the majority of hypomethylated regions in cancer cells correlated with partially methylated domains in normal cells. Surprisingly, in the tumor cells, these regions exhibited monoallelic DNA methylation, one allele displaying DNAme and the other allele possessing the repressive histone marks H3K9me3 or H3K27me3. Thus, hypomethylated regions in cancer cells were correlated with silenced gene expression and repressed chromatin (Hon et al., 2012). In an independent study, differentially methylated regions have been mapped in colon cancer relative to normal tissue. These regions were associated with high gene expression variability. Whole genome bisulfite sequencing has also confirmed hypomethylation in discrete blocks in about half of the cancer genome. These data suggest a model of neoplastic progression involving loss of epigenetic stability and tumor heterogeneity (Hansen et al., 2011). In another recent study in colon cancers versus matched normal tissue, focal CpG island hypermethylation was also detected within long-range (>100 base pairs) hypomethylation regions. In this case, the widespread changes of DNA methylation in cancer was correlated with repressive chromatin and by the three-dimensional organization of the chromatin inside the nucleus (Berman et al., 2012)

In mammalian cells, DNA methylation outside promoter regions frequently occurs in long-terminal repeats of viral origin and other repetitive regions named satellite regions. The methylation of these sequences in healthy cells could serve to protect the genomes by preventing the expression of exogenous promoters. Hypomethylation of preferentially repetitive sequence classes including short interspersed elements, long interspersed elements (e.g., LINE-1 elements), satellite, subtelomeric, and Alu repeats, has been associated with tumor progression in several cancer models, such as colon (Rodriguez et al., 2008; Sunami et al., 2011), squamous cell carcinomas of the lung (Pfeifer and Rauch, 2009), head, neck (Szpakowski et al., 2009), and mammary tumors (Costa et al., 2006). Hypomethylation of repetitive regions could prime genomic instability and also reactivate potential oncogenes during transformation, tumor progression, and metastasis. Importantly, the use of global demethylating agents for the treatment of cancers (e.g., 5-Aza-2′dC) in in vitro models has indicated that these inhibitors could prime tumor progression by re-expression of silenced oncogenes, such as Sox2 in chondrosarcoma cells (Hamm et al., 2009) and c-Met in colon carcinoma cell lines (Weber et al., 2010).

The genome-wide mapping of DNAme changes in tumor versus normal cells could help investigators gain valuable prognosis information in cancer patients and help not only to identify single gene hits important for tumor progression, but also to define prognosis signatures. Esteller et al. (2000) demonstrated that hypermethylation of CpG islands in the promoter of the gene O-6-methylguanine-DNA methyltransferase is a predictor of good response of glioma cancer patients to alkylating agents (Esteller et al., 2000). Since then, genomic approaches have identified groups of biomarkers associated with poor prognosis and responsiveness to therapeutic drugs, such as in pancreatic (Gupta et al., 2012) and colon cancers (Esteller et al., 2001). In glioblastoma multiforme, a genome-wide study by Martinez and colleague demonstrated that genes hypermethylated in glioblastoma multiforme were highly enriched for targets of the polycomb repressive complex 2 in embryonic stem cells, and thus, these tumors exhibited a methylome with stem cell-like features (Martinez et al., 2009; Martinez and Esteller, 2010).

During the process of immortalization, tumorigenesis, and tumor progression precancerous cells might acquire a "hypermethylator phenotype" affecting several tumor suppressor gene promoters (Sato et al., 2010). Methylation of p16 is a key gateway control for cells to overcome senescence barriers, and together with overexpression of telomerase these events might be crucial for the onset of the tumorigenic process (Gallardo et al., 2004; Beltran et al., 2011a). Another example in breast and ovarian cancers is the methylation of the BRCA1 tumor suppressor (Jacinto and Esteller, 2007; Stefansson et al., 2011), a gene associated with DNA repair, thereby promoting further genomic instability (Jacinto and Esteller, 2007). Mutations of BRCA genes have been associated with expansion of stem/progenitor cells and basal-like cancer (Liu et al., 2008a; Rakha et al., 2008). For these genes, methylation and genetic alterations can contribute to inactivating both copies of the tumor suppressor. Some tumor suppressors, namely class II tumor suppressor genes, are solely inactivated by epigenetic mechanisms. One classic example is the tumor suppressor *mammary serine protease inhibitor* (*maspin*), in which the degree of promoter methylation clearly correlates with disease stage and metastatic behavior in multiple cancer models, such as breast, lung, and prostate cancer (Zou et al., 1994; Futscher et al., 2002, 2004). In the *maspin* promoter, DNAme clearly correlates with H3K9me2 (Wozniak et al., 2007), which could be explained by the tight epigenetic crosstalk established between DNA methyltransferases and the H3K9 methyltransferase G9a.

The era of cancer epigenomics has recently identified the extent of the histone modifications in the cancer genome relative to normal tissue, leading to aberrant gene activation or repression (Rodriguez-Paredes and Esteller, 2011; Sandoval and Esteller, 2012). In non-small lung cancer, high dimethyl-H3K4 and low acetyl-H3K9 has been associated with favorable prognosis (Song et al., 2012). As discussed earlier, aberrant H3K4me and H3K27me have also been detected in

prostate cancer cells (Ke et al., 2009) and human colorectal cancer (Enroth et al., 2011).

What is the mechanistic basis of aberrant epigenetic regulation in cancer cells? In many cases, mutations or disruption in gene expression of the chromatin modifiers themselves have been identified. For example, the polycomb group of methyltransferases, such as enhancer of zeste homolog 2 (drosophila), are often overexpressed in basal-like breast cancers, particularly in African-American women (Chase and Cross, 2011; Pang et al., 2012). The histone methyltransferase G9a (Chen et al., 2010) and other lysine methyltransferases have also been associated with tumorigenesis and targeted by screening and structure-based design for the identification of specific inhibitors (Unoki, 2011). Deregulation of TF gene expression might cause profound effects in the epigenetic landscape of cells. During reprogramming of somatic cells toward pluripotency, the exogenous delivery of Oct4, Sox2, Klf4, and c-Myc TFs is sufficient to change the epigenetic architecture of the genome in a way that is similar to embryonic ES cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Moreover, intensive research revealed that induced pluripotent stem cells are not identical to ES cells, retaining epigenetic features (epigenetic memory) from the cell of origin (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Kim et al., 2010; Polo et al., 2010; Lister et al., 2011; Ziller et al., 2011). The "pluripotency" TFs activate a cellular network involving many other self-renewal TFs and chromatin remodelers. An imbalance in normal gene expression due to changes in expression or activity of specific TFs could lead to dysregulation of the epigenome of cells, resulting in cancer predisposition. In the mammary gland, our laboratory has demonstrated that overexpression of the TF Oct4 leads to aberrant epithelial to mesenchymal transition and to the generation of clones possessing tumor initiation capabilities (Beltran et al., 2011a).

In addition to coding regions, many noncoding RNAs have been associated with tumor progression (Veeck and Esteller, 2010). An explosion of studies has demonstrated global changes in expression of micro-RNA signatures between normal and tumor cells in multiple tumor types, including a recent study in lung cancer (Guan et al., 2012). Interestingly, some microRNAs are epigenetically silenced and act as tumor suppressors, whereas many others act as oncogenes (Cho, 2012). The significance of the complex noncoding-RNA circuitry in cancer development is not well understood; microRNAs could fine tune the threshold levels of critical targets involved in development and perhaps in cancer, illustrating the complexity of the regulatory network controlling cancer progression.

## Strategies to Revert the Epigenetic Landscape of Cancer Cells Using Small Molecules and Engineered Proteins

The epigenetic deregulation of tumor cells comprising the aberrant silencing of tumor suppressors and the hypomethylation of potential oncogenes creates the basis of novel therapeutic strategies to revert their chromatin state. The endogenous silencing of tumor suppressors can be reverted with epigenetic inhibitors, such as DNA methyltransferase, histone methyltransferase, and histone deacetylase inhibitors. An active area of research aims at developing chemical approaches to inhibit specific "writers" or "erasers," some of which will be reviewed in the following sections. Moreover, it is believed that these agents' lack of locus selectivity may result in potential off targets and might cause adverse effects in cancer patients. Another approach is the design of artificial proteins or artificial transcription factors (ATFs) able to recognize specific sequences in the genome, directing a chromatin modification or combinations of chromatin modifications, in specific loci.

## Selective Inhibitors of Bromodomains and Methyl-Lysine Readers

The acetylation and methylation of lysine residues play a central role in chromatin function, substantially through creation of binding sites for the readers of these marks (Taverna et al., 2007; Wang et al., 2008b). Although acetylation of lysine eliminates the residue's positive charge (akin to the significant change in chemical properties and charge upon phosphorylation of serine, threonine, and tyrosine), methylation is a more subtle post-translational modification, simply shifting the chemical properties of lysine toward a more diffuse, polarizable positive charge as methylation proceeds from monomethyl (Kme1) to trimethyl (Kme3) (Zacharias and Dougherty, 2002; Hughes et al., 2007). Recently, small molecule probes of bromodomains, the readers of acetylated lysine, have been reported, and their utilization has revealed bromodomain regulation of critical gene transcription events such as the regulation of the c-myc oncogene (Filippakopoulos et al., 2010; Dawson et al., 2011; Delmore et al., 2011). Although the current repertoire of acetyl lysine readers in the human genome is limited primarily to the structurally homologous 57 bromodomains and possibly a subset of PHDs (Zeng et al., 2010), there are more than 200 methyl-lysine (Kme) reader domains described within several protein families: PHD; the so-called "royal family" made up of Tudor, Agenet, chromo, (Pro-Trp-Trp-Pro), and MBT (malignant brain tumor) domains; the WD40 repeat proteins consisting of WDR5, and EED (Adams-Cioaba and Min, 2009; Margueron et al., 2009). This large Kme reader family seems destined to expand further as research in epigenetics continues (Kuo et al., 2012; Nakamura et al., 2012). A unifying feature of all of these domains is the presence of an "aromatic cage" that comprises the Kme binding pocket (Li et al., 2007b; Santiago et al., 2011). Computational analysis suggests that this target class has significant drugability (Santiago et al., 2011), and progress toward chemical probes for Kme readers is summarized here (Frye et al., 2010; Herold et al., 2011a).

In contrast to many protein target classes, such as G protein-coupled receptors, nuclear receptors, and protein kinases, where ligand discovery sometimes proceeded determination of high-resolution protein structures by decades, Kme binding sites were relatively well explored structurally prior to any reports of potent, small molecule ligands (Ruthenburg et al., 2007; Adams-Cioaba and Min, 2009). As a consequence, approaches toward ligands have been based primarily upon computational and structural techniques (Campagna-Slater and Schapira, 2010; Campagna-Slater et al., 2010; Kireev et al., 2010). In the first report of experimentally confirmed Kme binding domain small molecule ligands (MBT), Kireev et al. (2010) used both

a two-dimensional and three-dimensional virtual screening approach to select ligands from a computationally curated and procurable set of 5.8 million small molecules. Of 51 compounds tested in a histone-peptide competitive dose response proximity assay (Wigle et al., 2010a), 19 compounds produced a measurable $IC_{50}$ in the 5–100 $\mu$M range. All active compounds from this screen contained a mono- or dialkylamine that presumably satisfies the mono- and dimethyllysine histone peptide preference of the MBT domains (Kireev et al., 2010). Subsequently, Herold et al. (2011b) described the structure- and pharmacophore-based design of UNC669 (Fig. 4) that demonstrates a 5 $\mu$M dissociation constant (Kd) versus L3MBTL1 (Herold et al., 2011b). The conformationally rigid piperidino-pyrrolidine Kme mimic in UNC669 results in a ligand with fivefold greater affinity for L3MBTL1 than the H4K20me1 peptide—a presumed endogenous binding partner. UNC669 demonstrated moderate to high selectivity as a ligand for L3MBTL1 versus a small panel of other Kme binders with the next highest affinity being for L3MBTL3 (Herold et al., 2011b). A subsequent structure-activity relationship study of the UNC669 template revealed that pyrrolidine is an optimal size and shape for binding in the aromatic cage of L3MBTL1 that recognizes Kme and that an increase in ring size to piperidine was not tolerated (Herold et al., 2012).

Although these first reports of antagonists of Kme binding domains have described ligands of modest potency, the functional conservation of the pi-cation Kme recognition motif across this large and structurally diverse domain family bodes well for improvements in affinity as a further structure-activity relationship is developed (Herold et al., 2011a).

## Selective Inhibitors of Protein Lysine Methyltransferases

Protein lysine methylation is increasingly recognized as an important type of post-translational modification. This modification has been heavily studied in the context of epigenetic regulation of gene expression through histone lysine methylation (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Martin and Zhang, 2005; Bernstein et al., 2007; Kouzarides, 2007; Gelato and Fischle, 2008), but numerous nonhistone substrates suggest that the impact of lysine methylation is not limited to chromatin biology (Huang et al., 2006, 2007, 2010; Rathert et al., 2008). Protein lysine methyltransferases (PKMTs, also known as histone methyltransferases) catalyze mono-, di-, and/or trimethylation of lysine residues of histone and nonhistone proteins (Kouzarides, 2007; Copeland et al., 2009). Since the first PKMT was characterized over a decade ago (Rea et al., 2000), more than 50 PKMTs have been identified (Kouzarides, 2007; Copeland et al., 2009; Wu et al., 2010). Growing evidence suggests that PKMTs play critical roles in various human diseases including cancer (Bedford and Richard, 2005; Martin and Zhang, 2005; Fog et al., 2007; Cole, 2008; Esteller, 2008; Copeland et al., 2009; Spannhoff et al., 2009a, 2009b; Bissinger et al., 2010; Frye et al., 2010; Yost et al., 2011; He et al., 2012 ). Selective inhibitors of PKMTs are valuable tools for dissecting biologic functions of these proteins and assessing their potential as therapeutic targets. In this part of the review, we briefly discuss the selective PKMT inhibitors discovered to date (Fig. 5).

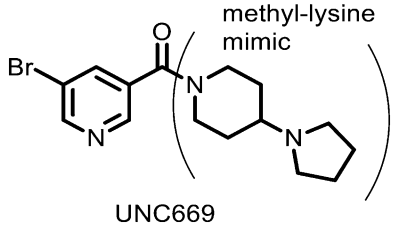


**Fig. 4.** Structure of UNC669. See PDB 3P8H for structure of UNC669 bound to L3MBTL1.

The discovery of BIX01294, the first selective PKMT inhibitor by Jenuwein and colleagues (Kubicek et al., 2007) was a major milestone in the PKMT inhibitor field. BIX01294 has good in vitro potency against G9a and G9a-like protein (GLP) and is selective for G9a and GLP over SUV39H1 and SETDB1 and SETD7 (Kubicek et al., 2007; Chang et al., 2009). BIX01294 is also active in cells. However, it has a poor separation of functional potency and cell toxicity (Chang et al., 2010; Vedadi et al., 2011; Yuan et al., 2012). Optimization of this quinazoline scaffold via structure-based design and synthesis by Jin and coworkers led to the discovery of UNC0638 (Vedadi et al., 2011), a chemical probe of G9a and GLP and several other potent and selective inhibitors of G9a and GLP (Liu et al., 2009, 2010, 2011). UNC0638 has balanced high in vitro potency versus G9a/GLP and desirable physicochemical properties and is >100-fold selective for G9a and GLP over a broad range of epigenetic and nonepigenetic targets (Vedadi et al., 2011). Importantly, UNC0638 has robust on-target activities in cells and an excellent separation between functional potency and cell toxicity (Vedadi et al., 2011). Cheng et al. (2010) also discovered a potent G9a and GLP inhibitor E72 based on the quinazoline template via structure-based design and synthesis. E72 has high in vitro potency versus G9a and GLP and is inactive against SUV39H2 (Chang et al., 2010). E72 is also active in cells and shows reduced cell toxicity compared with BIX01294 (Chang et al., 2010). X-ray cocrystal structures of these G9a/GLP inhibitors in complex with GLP or G9a reveal that these quinazoline-based inhibitors occupy the substrate binding groove and do not occupy the cofactor binding site (Chang et al., 2009, 2010; Liu et al., 2009; Vedadi et al., 2011). These findings were confirmed by mechanism of action studies, which reveal that UNC0638 and its analog are competitive with the peptide substrate and noncompetitive with the cofactor (Wigle et al., 2010b; Vedadi et al., 2011). More recently, Schreiber and coworkers discovered a cofactor-competitive G9a inhibitor BRD9539 (Yuan et al., 2012), which is structurally distinct from quinazoline-based G9a/GLP inhibitors. BRD9539 has moderate in vitro potency versus G9a and EZH2 and is selective over SUV39H1, NSD2, DNMT1, 16 other chromatin-modifying enzymes, and 100 kinases (Yuan et al., 2012). BRD4770, the methyl ester of BRD9539, is active in cells with a cellular potency similar to the in vitro potency of BRD9539 (Yuan et al., 2012).

The discovery of EPZ004777, the first highly selective, cofactor-competitive PKMT inhibitor by Pollock and coworkers (Daigle et al., 2011) was also a significant advancement in the PKMT inhibitor discovery field. EPZ004777, a nucleoside-based inhibitor, is highly potent versus DOT1L ($K_i$ = 0.3 nM) and >1,000-fold selective for DOT1L over other PKMTs and PRMTs (protein arginine methyltransferases) tested (Daigle et al., 2011). In addition, EPZ004777 selectively kills

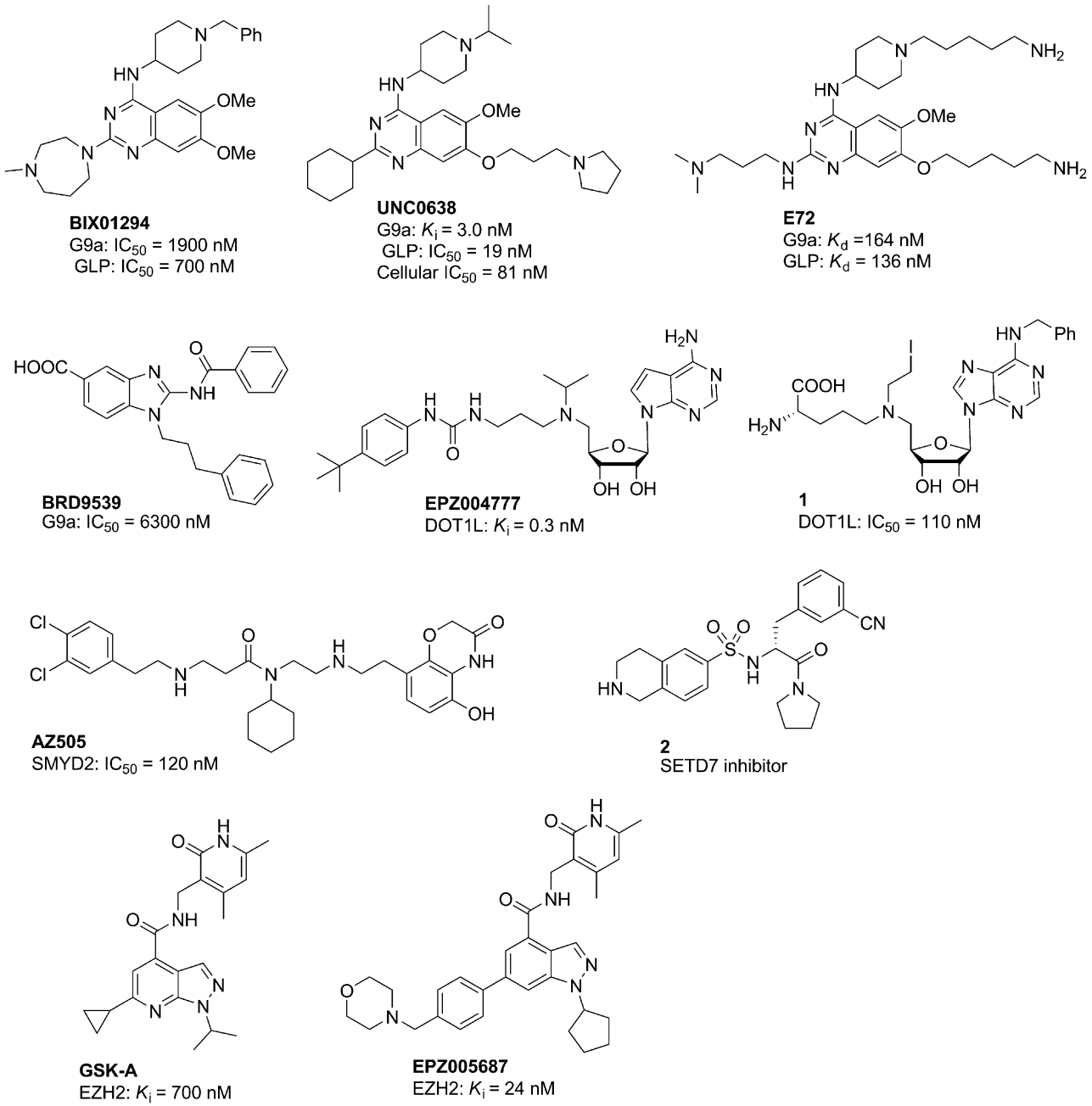


**Fig. 5.** Selective Inhibitors of PKMTs. DOT1L, DOT1-like; SMYD2, SET and MYND domain containing 2.

off cells bearing mixed lineage leukemia translocation and continuous infusion of EPZ004777 via a mini-pump increases animal survival in a mouse mixed lineage leukemia xenograft model (Daigle et al., 2011). Song and coworkers also discovered potent, selective, cofactor-competitive, nucleoside-based inhibitors of DOT1L and obtained an X-ray cocrystal structure of DOT1L in complex with one of their inhibitors (Yao et al., 2011). Compound 1, the most potent and selective inhibitor in their series, was proposed to be capable of covalently modifying the enzyme (Yao et al., 2011).

In 2011, Ferguson et al. (2011) reported the discovery of a potent SMYD2 inhibitor AZ505 ($IC_{50}$ = 120 nM), which is selective for SMYD2 over a panel of PKMTs including SMYD3, DOT1L, EZH2, GLP, G9a, and SETD7. The X-ray cocrystal structure of AZ505 in complex with SMYD2 reveals that AZ505 occupies the peptide binding groove, which was confirmed by the findings from mechanism of action studies: AZ505 is competitive with the peptide substrate and uncompetitive with the cofactor (Ferguson et al., 2011). Most recently, an X-ray cocrystal structure of compound 2 in complex with SETD7 has been reported in the protein data bank (4E47). Although the in vitro potency of this SETD7 inhibitor has not been reported, the X-ray cocrystal structure reveals that this inhibitor occupies the substrate-binding groove and does not occupy the cofactor binding site.

GSK-A was recently reported as the first selective, cofactor-competitive inhibitor of EZH2 by Diaz et al. (2012). GSK-A ($K_i$ = 700 nM), which was identified via high throughput

screening, is selective for EZH2 over a panel of PKMTs, PRMTs, and DNMTs. It is competitive with the cofactor and noncompetitive with either the peptide or nucleosome substrate. GSK-A is cell permeable and reduces cellular levels of H3K27me3 with an $IC_{50}$ of 8 $\mu$M. Most recently, Knutson et al. (2012) discovered EPZ005687, a highly potent, selective, and cofactor-competitive inhibitor of EZH2, via high throughput screening and subsequent optimization. EPZ005687 ($K_i$ = 24 nM) is 50-fold selective for EZH2 over EZH1, >500-fold selective over 15 other PKMTs and PRMTs, and at least 60-fold selective over a broad panel of G protein-coupled receptors and ion channels. Similar to GSK-A, EPZ005687 is competitive with the cofactor and noncompetitive with the nucleosome substrate. It inhibits wild-type EZH2 and 6 Y641 or A677 EZH2 mutants with similar in vitro potencies and reduces H3K27 methylation in various lymphoma cells with high cellular potencies. Importantly, EPZ005687 selectively induces apoptotic cell killing in heterozygous Y641F or A677G mutant cells and has little effect on the proliferation of wild-type lymphoma cells.

## Artificial Transcription Factors as Locus-specific Epigenetic Modulators

Locus-specific targeting of epigenetic domains in chromatin requires minimally two modules: a DNA-recognition domain, which has sequence selectivity, and an effector domain, which is engineered to modify chromatin (Beltran and Blancafort, 2010). Most engineered proteins were constructed using zinc finger (ZF) scaffolds, and more recently, transcription activator-like effectors (TALEs), which are bacterial proteins of the genus *Xanthomonas* that are injected into infected plants (Mussolino and Cathomen, 2012). The DNA-binding specificity of a TALE is a tandem array of ~15.5–19.5 single repeats, each repeat containing ~34 conserved residues. The binding specificity is dictated by positions 12 and 13 of the repeats, known as repeat variable residues. Thereby, the TALE code is a simple 1:1 interaction, one repeat unit to one nucleotide (Moscou and Bogdanove, 2009; Deng et al., 2012;). Recently, several laboratories have designed artificial TALEs for gene regulation and genome editing using nuclease technology (Bogdanove and Voytas, 2011; Bultmann et al., 2012). The on-targets and potential off-targets of these emerging technologies are still under investigation.

Sequence specific Cys2-His2 ZFs have been engineered by several approaches, including "helix grafting" (directed mutations in the $\alpha$-helical recognition sequence), and by in vitro as well as in vivo selection experiments in both bacteria and yeast (Beltran et al., 2006)). These selection experiments have overcome the context dependence of the assembly of the ZF units, generating proteins with potentially improved specificity for the target DNA. Each ZF module recognizes primarily three base pairs of DNA, and most studies have engineered polydactyl proteins consisting of three or more ZF proteins. Thereby, 6ZF proteins will recognize an 18-base pair recognition site in the DNA with potentially unique specificity in the genome (Tan et al., 2003), although potential off-targets might also occur in the cell due to the binding of the engineered protein to closely related binding sites. ZF proteins have been linked to several activator or repressor domains, promoting specific activation or repression of the targeted gene by recruiting specific coactivator or corepressors (Sera, 2009; Stolzenburg et al., 2010). However, such a first generation of effector domains does not possess intrinsic catalytic activity to modify either the histones or the DNA. Consequently, these effectors are expected to have a transient effect in the target cell and are not capable of inducing epigenetic memory (transmission of epigenetic modifications over the cell progeny). In contrast, the linkage of ZFs with epigenetic modifiers possessing enzymatic activity is expected to directly modify the histones or the DNA. Thus, some of these effectors are potentially able to promote hereditable changes in chromatin (Rivenbark et al., 2012).

Among all epigenetic marks, DNAme and H3K9me are recognized as being hereditable marks, or transmitted over the cell progeny. Several studies have demonstrated that linkage of a catalytic domain of bacterial methyltransferases with designer ZFs leads to targeted DNAme in plasmid-based assays or integrated reporters (Carvin et al., 2003; Minczuk et al., 2006; Li et al., 2007a; Smith and Ford, 2007; Smith et al., 2008). To avoid untargeted methylation, programmable DNA methyltransferases have been constructed, in which the expression of two independent ZF proteins can complement or restore the activity of a functional DNA methyltransferase enzyme (Nomura and Barbas, 2007; Meister et al., 2010). Interestingly, triple-helix forming oligonucleotides have been coupled with a DNA methyltransferase M.SssI to promote targeted methylation of the EpCAM oncogenic promoter (van der Gun et al., 2010). Our laboratory recently reported the first endogenous targeted DNA methyltransferase activity in a cancer genome, by tethering 6ZF domains with the catalytic domain of human Dnmt3a, an enzyme that catalyzes the de novo DNAme (Fig. 6). When linked with a 6ZF promoting homing to a tumor suppressor gene promoter, such as *Maspin*, the ZFs-Dnmt3a fusions promoted targeted DNAme, which was accompanied by an increased tumorigenic ability of the breast cancer cells in vitro. In contrast, in the context of an oncogene, such as *Sox2*, 6ZF linked to Dnmt3a promoted cancer cell growth arrest. Importantly, these studies suggested that the silencing initiated by the ZFs-Dnmt3a fusions was maintained over more than 50 cell generations. In fact, DNAme patterns were faithfully transmitted over cell generations even when after the ZF protein was no longer expressed in the tumor cells (Rivenbark et al., 2012). Overall, these studies suggested that ZFs-Dnmt3a constructs were able to stably reprogram the epigenetic state of the targeted promoters. In addition to DNA methyltransferases, ZF proteins have been linked to histone methyltransferase domains, such as SUV39H1 and G9a, targeting H3K9me marks in the context of the vascular endothelial growth factor A gene. These chimeras were sufficient to target silencing of the endogenous gene (Snowden et al., 2002).

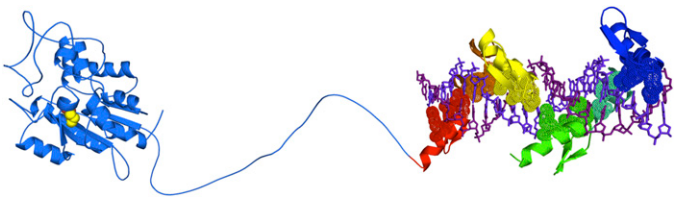

**Fig. 6.** Schematic structure of the catalytic domain of Dnmt3a (2QRV) linked to a 6ZF-DNA complex.

Reactivation of epigenetically silenced genes has also been achieved with ZF proteins. Gene activation, however, appears to be fundamentally more challenging than targeted silencing due to the decreased accessibility of the targeted region. Reactivation of silenced and proapoptotic genes has been reported for *maspin* (Beltran and Blancafort, 2011), Bax (Falke et al., 2003), and p53-dependent pathways using ZF library screenings (Blancafort et al., 2008). Highly methylated promoters, for example tumor suppressors, are associated with condensed chromatin, which could impede TFs to find their targets in chromatin. In the case of the *maspin* tumor suppressor, for example, the degree or extent of transactivation by ZF proteins is highly dependent on the targeted region of the promoter and in the DNA methylation level of the cell line examined (Beltran et al., 2011b). We have characterized an activator ATF able to reactivate silenced *maspin* in breast and lung cancer lines having high degree of promoter methylation. When delivered in these cell lines, the ATF suppressed breast cancer growth and totally abolished metastatic colonization (Lund et al., 2004; Beltran, 2011; Beltran et al., 2011b). Moreover, the effect of the ATF in reactivating endogenous *maspin* was highly synergistic with small molecule inhibitors of DNA methyltransferases and histone deacetylases (Beltran et al., 2008). Similarly, in the context of an epigenetically silenced oncogene, such as Oct4, TALE activators designed against the promoter were able to reactivate Oct4 only in presence of epigenetic inhibitors, such as 5-Aza-dC and trichostatin A (Bultmann et al., 2012). These experiments indicated that compact chromatin represented a partial blockade for ZFs and TALE technologies and generally TFs to reach high levels of regulation of silenced genes. Future engineering of both ZFs and TALEs might benefit from linkage of the DNA binding domains with chromatin remodelers, which might help to unravel the silenced chromatin. The discovery of novel DNA demethylases able to erase the 5meC of DNA represents a powerful new engineering avenue.

## ATFs Versus Small Molecule Inhibitors

The "pros" and "cons" for the use of ATFs and small molecules for anticancer treatment are summarized in Table 1. The design and optimization of DNA-binding domains in ATF backbones opens the possibility to regulate single genes in the cancer genome. Nevertheless, the spectrum of targets regulated by the ATF highly depends on the choice and primary selectivity of the DNA-binding domains used for the construction of the proteins. Investigators can now take advantage of genome-wide approaches to map the ATF-binding events in the cell and to study the specificity of regulation by chromatin immunoprecipitation sequencing and RNA-sequencing (or gene expression microarrays), respectively. Nevertheless, work done in our laboratories and others demonstrate that the genomic context of the host cell type, for example, target site selectivity and promoter context, are key factors defining the specificity of action of ZF proteins (Beltran and Blancafort, 2010; Beltran, 2011). As is often observed with siRNA technology, several ATF proteins need to be constructed to effectively regulate the desired gene (Beltran et al., 2007).

Genomic and functional studies have defined critical target genes deregulated in cancer cells relative to normal tissue, providing investigators with ad hoc information regarding which genes are the most important to correct and in which subtypes of cancers. For example, investigators can selectively downregulate specific oncogenes overexpressed in cancer tissues without altering the behavior of normal cells. In contrast, the inhibition of endogenous epigenetic enzymes using small molecules potentially yields toxicity or undesired pharmacology due to the lack of locus selectivity of these chemical-based approaches. Because both the biologic consequence of chromatin regulator inhibition and the potential off-target profile of small molecules are not predictable, experimental data, not debate, will reveal the magnitude of this challenge. By analogy to the selectivity issues often debated for protein kinase inhibitors, we anticipate clinically useful levels of efficacy and selectivity to be obtainable in the epigenetics field with progress driven by thoughtful experimentation. The potency of transcriptional regulation using ATFs is highly enhanced by using transactivator or repressor domains. In our hands, the degree of upregulation of tumor suppressors genes is considerably stronger using ATFs compared with small molecule inhibitors. This result could be explained by the effective recruitment of RNA polymerase II complex and associated factors by ATFs, which results in enhanced transactivation and strong anticancer effects (Lara et al., 2012). A linkage of multiple epigenetic effectors (e.g., methyltransferase and histone deacetylase functions) in the same ATF could improve the effect of the engineered protein. Similarly, small molecules targeting different epigenetic

TABLE 1
Comparison between artificial transcription factors and small molecule epigenetic inhibitors for anticancer intervention

| Features | Artificial Transcription Factors | Epigenetic Molecules |
|---|---|---|
| *Genomic/functional (e.g., cell type) specificity* | Design of sequence-specific DNA-binding domains can provide high selectivity for the target | Broad spectrum of cellular targets, some specificity depending on the compound |
| | Selective targeting for cancer cells by regulation of genes differentially expressed in cancer cells | Potentially high toxicity due to undesirable targeting of normal cells |
| *Transcriptional/regulatory potency* | Coupling the DNA-binding domain with transactivators/repressors can result in potent regulation | Can result in transcriptional regulation of targeted promoters |
| | Combinatorial delivery of effector functions, diversity of epigenetic domains | Combinatorial delivery of small molecules results in potent synergisms |
| *Delivery in cells/tumors/tissues* | Viral/nonviral (targeted nanoparticles) | Intravenous delivery, targeted nanoparticle delivery |
| *Probability of acquired resistance mechanisms* | Potentially diminished by using epigenetic effector domains (e.g., methyltransferase enzymes) | Drug resistance/disease recurrence is often observed during treatment |
| *Cost of synthesis/delivery* | Potentially very high cost | Large spectrum of small molecules available for treatment or in clinical trials |

processes have been shown to be highly synergistic in upregulating endogenous genes.

Certainly the strongest known limitation of the ATF technology is effective delivery into the target tissue. Targeted delivery for gene therapy has been historically addressed with the use of tissue-specific viral vectors (e.g., adeno- and adeno-associated vectors). In addition, targeted nanoparticles encapsulating a more stable and modified ATF mRNA have been used in animal models of metastatic ovarian cancer (Lara et al., 2012). These nanoparticles were able to accumulate with high doses in the tumor site and were able to inhibit tumor growth when administered intravenously. In addition to delivering the ATF in the tumor compartment, another fundamental problem of the ATFs is the high cost associated with the bulk production of ATF-containing nanoparticles required for treatment. Moreover, the ultimate hope of these ATF approaches is the generation of more stable epigenetic treatments for aggressive cancers, which might increase the longevity of the therapeutic approach, while providing a window of opportunity to combat incurable and highly drug-resistant tumors. Chimeric nanoparticles containing multiple ATFs and other anticancer agents, could be envisioned in the future to enhance therapeutic outcome.

## Summary

In conclusion, the epigenomics era has provided a multilayer view of fundamental processes disrupted in cancer cells, which include changes in DNA and histone-methylation in many different loci. The development of small molecule inhibitors for specific methyltransferases and methyl-readers (bromodomains) has provided novel strategies to target the epigenetic processes that are disrupted in malignant cells. Sequence-specific, engineered proteins attached to chromatin remodeling enzymes have the pivotal advantage to target specific locus associated with malignant progression. Combinatorial treatments of cancer cells with several distinct pharmacological approaches, for example small molecules and artificial chromatin remodeling enzymes, represent attractive approaches to stably reprogram the epigenetic landscape of cancer cells.

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#### Authorship Contributions

*Wrote or contributed to the writing of the manuscript:* Blancafort, Jin, Frye.

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