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# The Role of Chromatin-Associated Proteins in Cancer

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## Abstract

The organization of the chromatin structure is essential for maintaining cell-type-specific gene expression and therefore for cell identity. This structure is highly dynamic and is regulated by a large number of chromatin-associated proteins that are required for normal development and differentiation. Recurrent somatic mutations have been found with high frequency in genes coding for chromatin-associated proteins in cancer, and several of these are required for cancer maintenance. In this review, we discuss recent advances in understanding the role of chromatin-associated proteins in transcription, development, and cancer. Specifically, we focus on selected examples of proteins belonging to the histone methyltransferase, histone demethylase, or bromodomain families, for which specific small molecule inhibitors have been developed and are in either preclinical or clinical trials.

## 1. INTRODUCTION

One of the most exciting and unexpected findings from the systematic sequencing of primary tumor material has been the high frequency of recurrent somatic mutations found in genes that code for chromatin-associated proteins (Watson et al. 2013). These proteins could have potential roles in epigenetic regulation, and although the involvement of DNA methylation in cancer has been appreciated for several decades, epigenetic therapies based on inhibiting DNA methyltransferases and histone deacetylases have so far only been approved for a limited number of blood cancers (Issa & Kantarjian 2009, Khan & La Thangue 2012, Wagner et al. 2010, X. Yang et al. 2010). In this review, we discuss some novel developments in our understanding of the role of chromatin-associated proteins in cancer and how these insights can be used to develop targeted drugs.

Eukaryotic genomes are packaged in chromatin, which consists of DNA, histones, and associated proteins. Covalent modifications, such as acetylation, methylation, and ubiquitylation, of the histone subunits on specific amino acid residues are one mechanism by which chromatin is organized. These post-translational modifications are deposited ("written") by several classes of enzymes, including histone methyltransferases and histone acetyltransferases, and can be removed ("erased") by other classes of enzymes, including histone demethylases and histone deacetylates. The modifications can be interpreted ("read") and transmitted by specific chromatin-associated proteins, containing domains that have an affinity for post-translational modifications, such as bromodomains, chromodomains, and PHD domains. Although current results suggest that the modifications do not determine whether genes are turned on or off, they facilitate or introduce barriers for gene expression regulated by signaling events and transcription factors (see, e.g., Laugesen & Helin 2014). Thus, epigenetic control provides a mechanism for guiding cell-fate choices. Given that cancer is a disease of cell-fate choice and proliferation, it is perhaps not unexpected that the genes coding for chromatin-associated proteins are mutated with such a high frequency in primary human tumors.

We focus on recent advances in understanding the role of chromatin-associated proteins in regulating transcription, development, and cancer. Given space limitations, we provide selected examples of how they work and emphasize those for which specific inhibitors are now in preclinical or clinical trials.

## 2. HISTONE METHYLTRANSFERASES

Histone methylation occurs primarily on arginine and lysine residues and is catalyzed by the approximately 100 members of two distinct protein families. These proteins all use *S*-adenosyl methionine (SAM) as a cofactor and donor for the methyl transfer reaction, which can lead to the mono-, di-, or trimethylation of lysine, or the monomethylation, disymmetric, or di-asymmetric methylation of arginine (reviewed in Copeland et al. 2013). Post-translational modifications of histones by these enzymes are required to regulate gene expression during development and homeostasis, and many of the enzymes are therefore essential for normal differentiation and development. A vast amount of data has implicated histone methyltransferases in various types of diseases, including cancer.

### 2.1. EZH2 and H3K27 Methylation

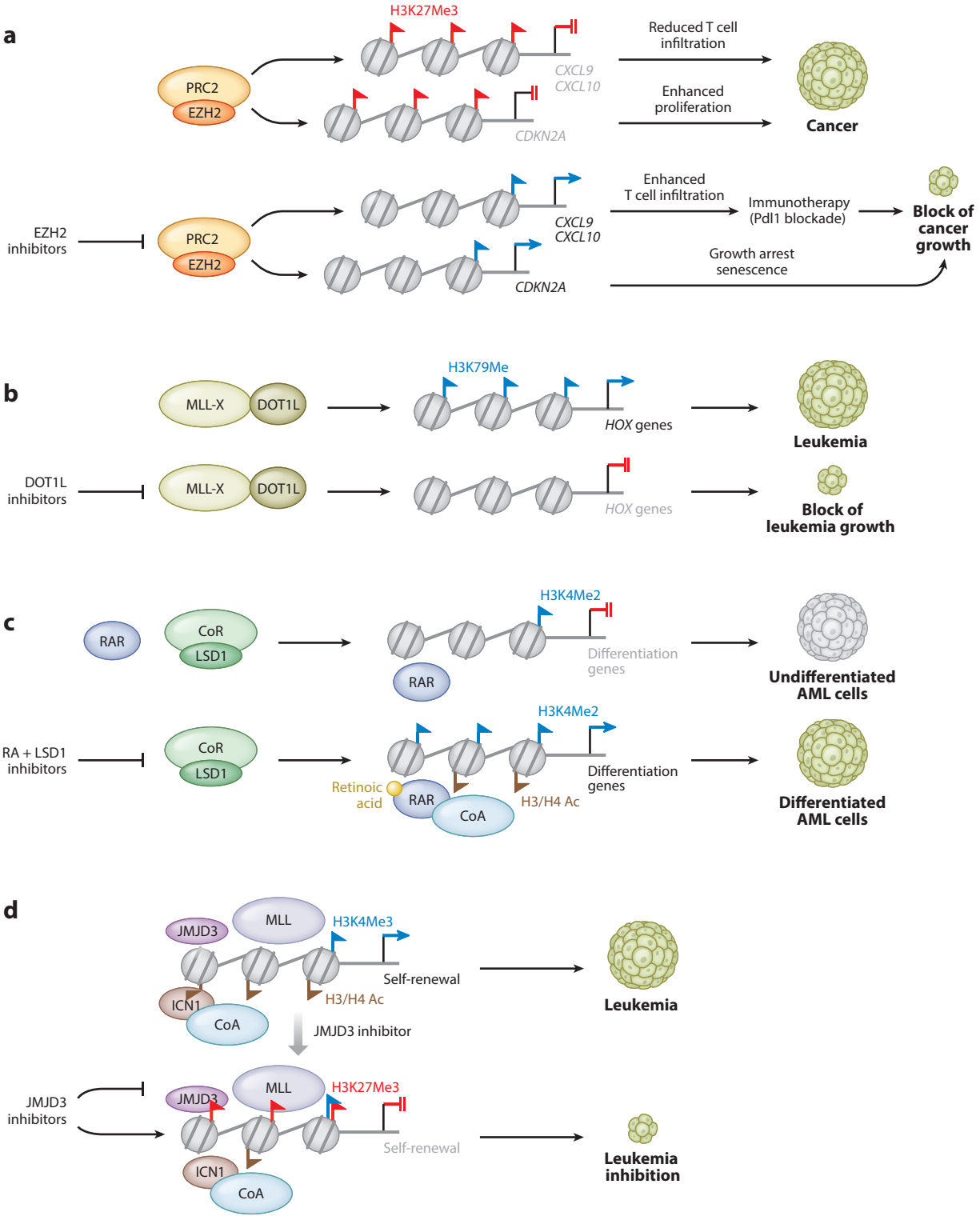
The Polycomb group (PcG) protein EZH2 is the catalytic subunit of the Polycomb repressive complex 2 (PRC2), which in addition to EZH2 also contains two other PcG proteins, EED and SUZ12 (reviewed in Laugesen et al. 2016). The three proteins form the core complex that catalyzes the mono-, di-, and trimethylation of H3 lysine 27 (H3K27). Additional proteins are also found to

be associated with PRC2, such as the nucleosome binding proteins RBBP4/7 and other proteins (AEBP2, JARID2, PCL1–3, C17ORF96, and C10ORF12) that contribute to the recruitment of PRC2 to chromatin. EZH1 is a less well-studied homolog of EZH2, which also forms a PRC2 complex with SUZ12 and EED that catalyzes H3K27 methylation. However, because EZH1's contribution to the overall H3K27 methylation activity is relatively minor, and it is dispensable for normal development, this protein has received less attention (Laugesen et al. 2016, O'Carroll et al. 2001).

The PcG proteins were first identified in *Drosophila* as being required to maintain transcriptional repression of homeotic genes during development (Lewis 1947, 1978). The PRC2 proteins are conserved in all multicellular organisms and are essential for normal development. Mouse embryos, for instance, with the targeted deletion of *Ezh2*, *Eed*, or *Suz12*, die around day e7.5 during gastrulation (O'Carroll et al. 2001, Pasini et al. 2004, Shumacher et al. 1996). In mammalian cells, PRC2 binds by default to CpG islands of nontranscribed genes to maintain their transcriptional repression (Riising et al. 2014), which in *Drosophila* has been shown to require the methylation of H3K27 (Pengelly et al. 2013). Thus, the role of PRC2 is to maintain the gene expression pattern in any given cell and therefore protect cell identity. In this way, PRC2 establishes a threshold for gene activation and cell-fate changes (Laugesen & Helin 2014), and somatic mutations in genes required for PRC2 activity are therefore predicted to interfere with normal development, which is also observed in mutant embryos.

EZH2 is highly expressed in several types of cancer, and its levels have been correlated with advanced stages and poor prognosis (Bachmann et al. 2006, Bracken et al. 2003, Kleer et al. 2003, Orzan et al. 2011, Varambally et al. 2002, Weikert et al. 2005). Given that several in vitro cell–based studies also concluded that EZH2 is required for the proliferation of tumor cell lines, multiple pharmaceutical and biotech companies started to develop small molecule inhibitors to EZH2 (Helin & Dhanak 2013). Meanwhile, somatic heterozygous mutations that specifically lead to residue changes in the catalytic domain of EZH2 (Y646, A682, and A692) were described in germinal center B cell diffuse large B cell lymphoma (GCB DLBCL) and in follicular lymphoma (FL). Subsequently, mutations leading to EZH2 Y646 mutants were found in melanoma and in sporadic parathyroid adenomas (Cromer et al. 2012, Hodis et al. 2012, Krauthammer et al. 2012). These mutations lead to the expression of hyperactive forms of EZH2 that preferentially produce H3K27me3 (Bodor et al. 2011, McCabe et al. 2012, Morin et al. 2010, Sneeringer et al. 2010). The causal role of the mutant EZH2 in cancer has been shown in mouse, in which expression of $Ezh2^{Y641}$ (corresponding to human $Ezh2^{Y646}$) in mouse B cells or melanocytes leads to high-penetrant B cell lymphoma or melanoma, respectively (Souroullas et al. 2016). Moreover, $Ezh2^{Y641}$ also cooperates with other oncogenes in inducing B cell lymphomas and melanomas in mouse (Beguelin et al. 2013, Berg et al. 2014, Souroullas et al. 2016). Taken together, these studies demonstrate that hyperactive Ezh2 can be a driving oncogene and suggest that increased levels of H3K27me3 can contribute to tumorigenesis. Although the mechanism by which this occurs is not completely understood, it appears to involve the silencing of target genes that are required for normal differentiation or are promoting a fail-safe response to hyperproliferative signals (e.g., induction of senescence or apoptosis) (**Figure 1*a***).

Unexpectedly, however, inactivating mutations of *EZH2*, *SUZ12*, and *EED* were reported for a variety of tumors, including myelodysplastic syndrome, T cell acute lymphoblastic leukemia (T-ALL), and malignant peripheral nerve sheath tumors (De Raedt et al. 2014, T. Ernst et al. 2010, Lee et al. 2014, Nikoloski et al. 2010, Ntziachristos et al. 2012, Simon et al. 2012). Additionally, experiments using mouse models have shown that these mutations can lead to tumor development (Ntziachristos et al. 2012, Sashida et al. 2014, Simon et al. 2012). EZH2 (PRC2) therefore has a context-dependent role in tumorigenesis, and caution should be taken when small molecule


a
H3K27Me3
PRC2
EZH2
CXCL9
CXCL10
CDKN2A
Reduced T cell infiltration
Enhanced proliferation
Cancer
EZH2 inhibitors
Enhanced T cell infiltration
Immunotherapy (Pdl1 blockade)
Block of cancer growth
Growth arrest senescence
b
H3K79Me
MLL-X
DOT1L
HOX genes
Leukemia
DOT1L inhibitors
Block of leukemia growth
c
RAR
CoR
LSD1
H3K4Me2
Differentiation genes
Undifferentiated AML cells
RA + LSD1 inhibitors
Retinoic acid
CoA
H3/H4 Ac
Differentiated AML cells
d
JMJD3
MLL
H3K4Me3
Self-renewal
ICN1
CoA
H3/H4 Ac
Leukemia
JMJD3 inhibitor
JMJD3 inhibitors
H3K27Me3
Leukemia inhibition

inhibitors to EZH2 are systemically administered to patients (**Figure 2**). In support of this notion, recent studies have shown that short-term depletion of Ezh2 can improve survival in a mouse model for glioblastoma (GBM), whereas prolonged depletion of Ezh2 can lead to tumor progression (de Vries et al. 2015). These mechanistic studies are important to keep in mind when assessing the currently ongoing clinical trials using EZH2 inhibitors (**Table 1**).

There are currently five ongoing phase I/II clinical trials testing three different, although structurally similar, EZH2 inhibitors produced by Constellation, Epizyme, and GlaxoSmithKline (**Table 1**). The clinical trials focus on patients with tumors showing high levels of EZH2 expression (such as B cell lymphoma), carrying *EZH2* gain-of-function mutations (such as DLBCL and FL), or harboring mutations in genes coding for components of the SWI/SNF nucleosome-remodeling complex (**Table 1**). In particular, mutations in *SMARCB1* (also known as *INI1*, *SNF5*, or *BAF47*) of the SWI/SNF complex, which is present with very high frequency in rhabdoid tumors, lead to elevated levels of EZH2 and have been shown to confer oncogenic dependency on EZH2 (or synthetic lethality to EZH2 inhibition) (Knutson et al. 2013, Wilson et al. 2010). In agreement with a tumor-suppressive role for the SWI/SNF complex, mutations have been found in 9 of the 15 genes coding for its subunits in approximately 20% of all cancers (Kim et al. 2015), and recent data suggest that many of these tumors are dependent on EZH2 (Kim et al. 2015). The preliminary reported results from the clinical trials indicate limited adverse effects of EZH2 inhibition using tazemostat and a time-dependent objective response in 9 of 15 patients with B cell lymphoma (Ribrag et al. 2015a). Moreover, Epizyme has provided promising data in meeting reports for antitumor activity of EZH2 inhibitors in SMARCB1-negative and SMARCA4-negative tumors (Ribrag et al. 2015b).

In summary, even though the precise mechanism for how EZH2 contributes to cancer progression is yet to be determined, and the fact that EZH2 has a general role in regulating transcription and normal development, the ongoing clinical EZH2 inhibitors as a monotherapy are encouraging. Moreover, EZH2 inhibitors have a large potential in their use in combination therapy, for instance, together with immunotherapy. Studies in ovarian cancer models have shown that EZH2-mediated histone H3K27 trimethylation and DNA methylation repress the expression of T helper chemokines (CXCL9 and CXCL10) by tumor cells, and restoration of their expression by treatment with EZH2 inhibitors increases effector T cell tumor infiltration, slows down tumor progression, and improves the therapeutic efficacy of checkpoint inhibitors (Peng et al. 2015).

**Figure 1**

Histone methyltransferases and histone demethylases can have transcriptional activating or repressive functions in tumorigenesis, and their targeting by epigenetic drugs can lead to multiple antitumor effects. (*a*,*b*) Examples of altered function of histone methyltransferases and (*c*,*d*) of histone demethylases. (*a*) Following activating mutations/enhanced expression, EZH2 in the PRC2 complex trimethylates histone H3K27 at multiple genes involved in the control of cell proliferation and of the immune response, maintaining their transcriptional repression and favoring cancer growth and immune escape. EZH2 inhibitors can restore expression of senescence and growth arrest–inducing genes, thus reducing tumor growth, and lead to enhanced recruitment of immune cells that stimulates a strong antitumor immune response when combined with immunotherapy. (*b*) The H3K79 histone methyltransferase DOT1L associates with several MLL fusion proteins (MLL-r) in acute myeloid leukemia (AML) and is required to maintain expression of key genes (such as *HOX* genes) for leukemogenesis. DOT1L inhibitors block transcription of these genes and inhibit leukemia growth. (*c*) LSD1 [in complex with other corepressor (CoR) activities, such as histone deacetylases] maintains reduced levels of expression of several differentiation-associated genes in AMLs, through regulation of histone H3K4me2 levels. Several of those genes are also targets of nuclear receptors (RAR). Inhibition of LSD1 in combination with retinoic acid (*yellow sphere*) leads to enhanced H3K4me2 levels, recruitment of coactivators (CoA), such as histone acetyltransferase, which induce histone acetylation, and induction of differentiation and death of AML cells. (*d*) Intranuclear Notch (ICN1) recruits several coactivating complexes, and JMJD3-mediated demethylation of methylated histone H3K27 contributes to setting a permissive chromatin structure at genes involved in self-renewal of leukemic cells. JMJD3 inhibitors lead to enhanced levels of histone H3K27me3, reduced expression of self-renewal genes, and loss of stemness of leukemic cells, inhibiting leukemia growth.

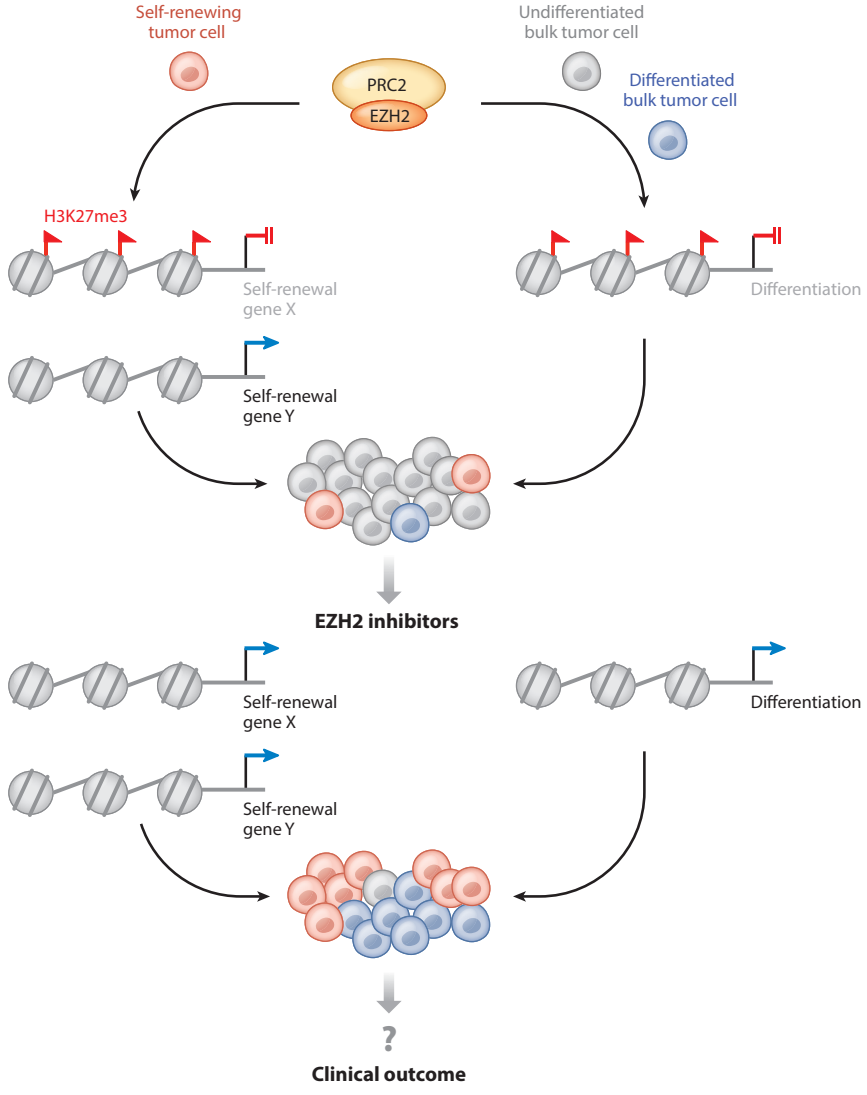



**Figure 2**

Context-dependent activity of epigenetic targets may lead to difficult-to-predict clinical outcomes. Chromatin-associated targets may have different functions in different tumor cell subcompartments. Here, the hypothetical case of EZH2 is shown. The tumor cell mass can be distinguished into self-renewing tumor cells, which sustain continuous growth of the tumor, and non-self-renewing bulk tumor cells, at different stages of differentiation and with limited growth potential. In self-renewing cells, EZH2/PRC2 may contribute to repressing the expression of genes associated with self-renewal, but other EZH2-independent self-renewal genes/pathways are on, and tumor cells show a low to intermediate degree of self-renewal. In bulk tumor cells, EZH2 keeps differentiation genes repressed, and these cells show an undifferentiated phenotype. Upon treatment with EZH2 inhibitors, most bulk tumor cells re-express differentiation-associated genes and assume a more differentiated phenotype that may lead to reduced growth in the short term. Self-renewing cells, however, show enhanced self-renewal, which in the long term may lead to a redistribution of the cell subpopulations within the tumor, with self-renewing cells becoming the most abundant ones and the tumor ultimately acquiring a more aggressive phenotype. These contrasting effects due to EZH2 inhibition may lead to an initial clinical response, followed by tumor regrowth: This can be erroneously interpreted as the emergence of resistance, but in fact it is the consequence of the context-dependent functions of the target.

**Table 1 Small molecule inhibitors in ongoing clinical trials**

| Target | Compound | Conditions | Phase | Sponsor | Reference |
|---|---|---|---|---|---|
| EZH2 | EPZ-6438, tazemostat | B cell lymphoma, rhabdoid tumors, synovial sarcoma, solid tumors | I/II | Epizyme | NCT01897571 |
| | GSK2816126 | Relapsed/refractory DLBCL, transformed FL | I | GlaxoSmithKline | NCT02082977 |
| EZH2 | CPI-1205 | B cell lymphoma | I | Constellation | NCT02395601 |
| DOT1L | EPZ-5676, pinometostat | AML, ALL, MDS, pediatric AML | I | Epizyme/Celgene | NCT01684150, NCT02141828 |
| LSD1 | GSK2879552 | AML, SCLC, midline carcinoma, other solid tumors | I/II | GlaxoSmithKline | NCT02034123, NCT02177812, NCT01943851, NCT01587703 |
| | ORY-1001 (RG6016) | Relapsed/refractory acute leukemia | I | Roche/Oryzon | EudraCT Number-2013-002447-29 |
| | 4SC-202 | AML, ALL, MM, MDS, CLL | I | 4SC | NCT01344707 |
| | Tranylcypromine | AML, MDS | I/II | None | NCT02717884 NCT02261779 NCT02273102 |
| BRD | OTX015/MK-8628 | Midline carcinoma, AML, ALL, DLBCL, MM, advanced solid tumors | I/compassionate | Oncoethix | Stathis et al. 2016; NCT01713582, NCT02259114, NCT02698189, NCT02698176 |
| | GSK525762 | Midline carcinoma, other solid tumors | I/II | GlaxoSmithKline | NCT01587703 |
| | GSK2820151 | Advanced or recurrent solid tumors | I | GlaxoSmithKline | NCT02630251 |
| | INCB054329 | Advanced cancer | I/II | Incyte | NCT02431260 |
| | BMS-986158 | Advanced solid tumors | I/IIa | Bristol-Myers Squibb | NCT02419417 |
| | CPI-0610 | Progressive lymphoma, pretreated multiple myeloma, AML, MDS, myelofibrosis | I | Constellation | NCT01949883, NCT02157636, NCT02158858 |
| | FT-1101 | Relapsed/refractory acute leukemia, high-risk MDS | I | Forma Therapeutics | NCT02543879 |
| | ZEN003694 | Metastatic prostate cancer | I | Zenith Epigenetics | NCT02705469 |
| | TEN-010 | AML, MDS | I | Tensha Therapeutics | NCT02308761 |

National Clinic Trial (NCT) numbers are available at **http://www.clinicaltrials.gov**, and the Eudract number is available at **http://www.clinicaltrialsregister.eu**. Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; MDS, myelodysplastic syndrome; MM, multiple myeloma; SCLC, small-cell lung carcinoma.

## 2.2. The H3K79 Methyltransferase DOTL1

In contrast to most other studied methylated residues on histones, methylation of H3 at lysine 79 occurs on the globular core of the nucleosome. This methylation, which can occur in the mono-, di-, or trimethylated form, is catalyzed by a unique enzyme, DOT1L. Disruptor of telomeric silencing 1 (DOT1) was first identified through a screen for proteins whose overexpression would lead to impaired telomere silencing in *Saccharomyces cerevisiae* (reviewed in Chen & Armstrong 2015, Vlaming & van Leeuwen 2016). DOT1L is unique because, unlike other lysine methyl transferases, it does not contain a conserved catalytic SET domain, but instead has a protein fold that is more similar to protein arginine methyltransferases (PRMTs) (Copeland et al. 2013). Knockout studies have shown that DOT1L is the major (if not only) H3K79 methyltransferase, and that DOT1L is required for normal embryogenesis (see, e.g., Feng et al. 2010, Jones et al. 2008).

H3K79 methylation is generally associated with actively transcribed regions of the genome (Kuntimaddi et al. 2015, Steger et al. 2008). However, despite DOT1L and H3K79 methylation being associated with the majority of active genes, the deletion of *DOT1L* leads only to relatively few transcriptional changes (Bernt et al. 2011, Steger et al. 2008). H3K79 methylation is therefore not generally required to activate gene expression, but is most likely present on actively transcribed genes to prevent silencing. This would be in agreement with how DOT1 was first identified, and with recent studies of leukemia demonstrating that DOT1L inhibits chromatin localization of a SIRT1 histone deacetylase repressive complex (Chen et al. 2015).

Studies of mixed-lineage leukemia carrying MLL-r (rearrangement of the mixed-lineage leukemia) have shown that DOT1L associates with MLL fusion proteins and is required for leukemogenesis (Chen & Armstrong 2015, Vlaming & van Leeuwen 2016). MLL1 is fused to more than 60 different protein partners, with the most common ones being AF4, AF9, AF10, ELL, and ENL (Chen & Armstrong 2015, Vlaming & van Leeuwen 2016). Interestingly, DOT1L is found in a chromatin complex regulating transcriptional elongation with several of these fusion partners (AF9, AF10, and ENL), and in agreement with this, DOT1L is involved in maintaining gene expression (Chen & Armstrong 2015, Vlaming & van Leeuwen 2016). MLL1 is required for the maintenance of *HOX* gene expression (Yu et al. 1995), and DOT1L is recruited to the genes that are normally controlled by MLL1. By recruiting DOT1L, MLL fusion proteins induce leukemia by maintaining high levels of *HOXA9*, *HOXA10*, *MEIS1*, and other genes normally regulated by MLL1 (**Figure 1*b***). Several studies using conditional knockout mice and human cell lines have shown that DOT1L and its enzymatic activity are required in MLL-r leukemia (Bernt et al. 2011, Daigle et al. 2011, Nguyen et al. 2011). Moreover, the development and testing of highly selective small molecule inhibitors of DOT1L have provided further proof of concept for targeting DOT1L in MLL-r leukemia (Daigle et al. 2011, 2013; Yu et al. 2012). One of these inhibitors, EPZ-5676, is currently in phase 1 clinical trials in adult and pediatric MLL-r leukemia patients. Information regarding the clinical trials in adult MLL-r patients has been reported (Stein & Tallman 2015, Stein et al. 2015), and although the response rates have been relatively modest, the trials are providing clinical proof of concept and demonstrating that the drug is well tolerated. Moreover, because the maximal tolerable dose was not achieved, there is a possibility of increasing the dose, and perhaps more importantly of testing EPZ-5676 in combination therapy with other drugs, such as standard-of-care drugs (Klaus et al. 2014), BCL2 inhibitors (Benito et al. 2015), or SIRT1 activators (Chen et al. 2015).

## 2.3. The Arginine Methyltransferase PRMT5

Several other methyltransferases, including PRMT5, are known to contribute to oncogenic development and are known to be required for tumor maintenance. PRMT5 is a type II arginine

methyltransferase that requires association with a WD40-containing protein, MEP50, to be enzymatically active (reviewed in Stopa et al. 2015, Yang & Bedford 2013). PRMT5 can symmetrically methylate both H3R8 and H4R3, which have been associated with transcriptional repression. Consistent with this, PRMT5 has been found to be associated with several transcriptional repressor complexes, including N-CoR and NURD (Stopa et al. 2015, Yang & Bedford 2013). PRMT5 has also been shown to methylate many nonhistone substrates, and the protein has been linked to a number of different cellular processes, such as RNA processing, cell cycle regulation, and different cellular signaling pathways (Chari et al. 2008, Koh et al. 2015, Stopa et al. 2015, Yang & Bedford 2013, Zhao et al. 2016). Genetic studies have shown that PRMT5 is required for maintaining embryonic and adult stem cells and therefore that it is required for normal development and differentiation of many different tissues (Z. Li et al. 2015, Liu et al. 2015, Tee et al. 2010, Zhang et al. 2015).

PRMT5 expression is high in several primary tumors and has so far been shown to be required for the proliferation of lymphoma, acute myeloid leukemia (AML), and GBM (Alinari et al. 2015, Y. Li et al. 2015, Tarighat et al. 2016, Yan et al. 2014). Moreover, it can cooperate with cyclin D1 in inducing B cell lymphomas (Y. Li et al. 2015). Interestingly, cancer cells showing loss of the enzyme methylthioadenosine phosphorylase (MTAP), which decreases the cellular concentrations of MTA, are strongly dependent on PRMT5 (Kryukov et al. 2016, Mavrakis et al. 2016). Mechanistically, the increased levels of MTA will compete with SAM as a cofactor for PRMT5, and the MTAP negative cells therefore have reduced levels of PRMT5 activity and become more sensitive to PRMT5 inhibition. Because *MTAP* is frequently deleted due to its proximity to the tumor suppressor gene *CDKN2A*, many human tumors can therefore potentially be treated with PRMT5 inhibitors.

As PRMT5 is an attractive therapeutic target in several different types of cancer, inhibitors have been developed (Alinari et al. 2015, Chan-Penebre et al. 2015, Smil et al. 2015). One of these, EPZ015666, is a highly selective and potent inhibitor that decreases the cellular proliferation of mantle cell lymphoma cells in vitro and in xenografts in vivo (Chan-Penebre et al. 2015). A major concern in targeting PRMT5 is that the protein is involved in many cellular processes, and it is not clear how it contributes to tumorigenesis. PRMT5 inhibition may therefore be highly toxic; however, the development of a highly selective and potent PRMT5 inhibitor could help define the mechanism by which PRMT5 contributes to cancer and help in defining a future therapeutic strategy.

## 3. HISTONE DEMETHYLASES

Several histone demethylases have been identified (Højfeldt et al. 2013). These enzymes demethylate methylated histone lysine residues as well as methylated arginines via amine oxidation, hydroxylation, or deimination. Here we focus on the involvement in cancer of (*a*) LSD1, which demethylates the histone substrate through a flavin adenine dinucleotide–dependent amine oxidase reaction but is unable to catalyze the demethylation of trimethylated lysine residues, and (*b*) selected members of the Jumonji (JmjC) domain–containing demethylases, which catalyze a reaction compatible with the demethylation of mono-, di-, and trimethylated lysines.

### 3.1. LSD1/KDM1A

Histone H3K4me1/me2 is the best-characterized substrate of LSD1/KDM1A. Following association with hormone receptors, specificity of the enzyme has been reported to switch toward histone H3K9 (Metzger et al. 2005). Interestingly, a neuronal-specific LSD1 isoform with a short

insertion in the catalytic domain shows different specificity (histone H4K20 or H3K9) (Laurent et al. 2015, Wang et al. 2015, Zibetti et al. 2010). Different activity may arise from the association of LSD1 with different cofactors found in LSD1-containing macromolecular complexes: In hematopoietic cells, Rcor1 and Rcor2 associate with LSD1 to trigger H3K4 demethylation, whereas Rcor3 inhibits the enzymatic activity (Upadhyay et al. 2014).

LSD1 can act therefore either as a transcriptional corepressor or coactivator, depending on the substrate (H3K4 versus H3K9). It is found at promoters and at distal regulatory elements: The H3K4 demethylase activity of LSD1 associated with NURD has been involved in "enhancer decommissioning" in embryonic stem cells, where it contributes to shutting off enhancers that become inactive during differentiation (Mendenhall et al. 2013, Whyte et al. 2012). In addition to its role in transcription, LSD1 is also involved in the DNA damage response and in the regulation of telomere structure (Mosammaparast et al. 2013, Porro et al. 2014).

The role of LSD1 in cancer has been explored by genetic and pharmacological tools, based on the early availability of small molecule inhibitors, mainly derivatives of the monoamino oxidase inhibitor tranylcypromine, which weakly inhibits LSD1 (Lee et al. 2006). Impairment of LSD1 expression or activity showed an antiproliferative response in some cancer subtypes (AMLs and small-cell lung carcinomas) (McGrath et al. 2016, Mohammad et al. 2015). A set of 45 differentially methylated probes has been correlated with LSD1 sensitivity and resistance in small-cell lung carcinoma (Mohammad et al. 2015). Another factor modulating response to LSD1 inhibition is the expression of Myc family members (Mohammad et al. 2015).

In leukemia, LSD1 is highly expressed in putative leukemic stem cells (LSCs), and its overexpression suppresses differentiation of normal hematopoietic progenitors (Harris et al. 2012, Wada et al. 2015). In murine AMLs driven by MLL fusion proteins, knockdown or inhibition of LSD1 leads to impaired self-renewal of AML cells and downregulation of a transcriptional LSC signature (Harris et al. 2012). In AML-sensitive cell lines, LSD1 is required for expression of a transcriptional program that depends on HOXA9, proposed to control the stemness of AML cells (McGrath et al. 2016). Consistently, in T-ALL cells, LSD1 interacts with the Notch proteolytic fragment ICN1, which acts as a transcription factor and modulates Notch signaling (Yatim et al. 2012). LSD1 is involved in the self-renewal of other cancer subtypes: In GBM, LSD1 knockdown or inhibition has almost no effect on the bulk of tumor cells, whereas it dramatically affects the GBM–stem cell compartment (Suvà et al. 2014).

Most existing studies show that LSD1 does not play a major role in controlling global levels of its histone substrates, but rather fine-tunes histone methylation, to control expression (Harris et al. 2012, McGrath et al. 2016, Schenk et al. 2012). It is unclear if this exclusively results from its enzymatic activity: Experiments with inhibitors are not conclusive, as LSD1 inhibition leads to its eviction from several target sites (though most sites remain LSD1 bound) (McGrath et al. 2016). Another point to be further understood is the relative contribution of histone/nonhistone substrates to the observed LSD1-dependent phenotypes (Hamamoto et al. 2015).

Taken together, the currently available results make LSD1 an attractive target for cancer therapy: Irreversible inhibitors derivative of tranylcypromine have entered the clinical stage recently (**Table 1**), and reversible inhibitors are being developed (Stazi et al. 2016). Combination with other epigenetic drugs such as histone deacetylase inhibitors or DNA demethylating agents shows great potential, and other drugs are likely to be highly effective (e.g., the strong cooperative effect observed by treatment with retinoic acid in AMLs) (Binda et al. 2010, Fiskus et al. 2014, Schenk et al. 2012, Wouters & Delwel 2016) (**Figure 1*c***).

LSD1 has one homolog, LSD2, which has been proposed to have tumor-suppressive functions. Data also suggest that it has additional E3 ubiquitin ligase activity against O-GlcNAc transferase, which is required for cancer cell growth (Yang et al. 2015).

## 3.2. JmjC-Domain Containing Histone Demethylases

Here we focus on selected members of the JMJ protein family, with a clear involvement in cancer.

**3.2.1. JMJD3/UTX (KDM6B/KDM6A).** The enzymes JMJD3/UTX (KDM6B/KDM6A) demethylate methylated histone H3K27, counteracting the repressive PRC2 complex (Arcipowski et al. 2016). UTX is found in complexes containing histone methyltransferase activity (different MLL3/4-containing complexes at promoters versus enhancers), so that upon demethylation concomitant methylation at H3K4 and acetylation at the demethylated H3K27 residue switch the chromatin structure to an active state (Agger et al. 2007, Di Croce & Helin 2013, Lee et al. 2007, Tie et al. 2012).

JMJD3 is frequently downregulated or lost in cancer. It contributes to the regulation of oncogene-induced senescence: JMJD3 is induced by expression of oncogenes, and by histone H3K27, demethylation leads to activation of the *ARF* locus, encoding key regulators of cellular senescence (Agger et al. 2009, Barradas et al. 2009). JMJD3 has been reported to contribute to the formation of senescence-associated heterochromatin foci, genomic domains leading to the permanent inactivation of growth-promoting genes, through demethylation of the retinoblastoma protein (Zhao et al. 2015). Consistent with a tumor-suppressive role, JMJD3 is recruited by p53 to p53 DNA binding sites in response to genotoxic stress, and its transcription is induced by several differentiation stimuli (Arcipowski et al. 2016, Bosselut 2016, Williams et al. 2014).

In contrast to these findings, however, an oncogenic role for JMJD3 has been described in different cancer types. JMJD3 controls the inflammation process and the production of several oncogenic cytokines (De Santa et al. 2007). In melanomas, JMJD3 contributes to the activation of NF-kB and BMP oncogenic transcriptional programs and affects the tumor microenvironment (Park et al. 2016). In pediatric gliomas carrying mutations of the histone H3K27 (K27M), there is a global reduction in histone H3K27 methylation, and some studies suggest that inhibition or knockdown of JMJD3 impairs tumor growth (Andor et al. 2014).

Interestingly, although they demethylate the same histone modification, JMJD3 and UTX clearly play nonoverlapping roles. This could be ascribed to differences in both catalytic and noncatalytic functions of the proteins: In *Caenorhabditis elegans*, the essential role of UTX-1 in development is dependent not on its enzymatic activity, but rather on its scaffolding role for a complex containing MLL (Vandamme et al. 2012). In NOTCH-induced models of T-ALL, where PRC2 plays a tumor-suppressive role, NF-kB induces expression of JMJD3, which associates with both NOTCH and MLL complexes (Ntziachristos et al. 2014) (**Figure 1*d***). Impairment of JMJD3 has an antitumor effect, whereas UTX has a tumor-suppressive role, and in its absence, leukemia development is accelerated with enhanced histone H3K27 trimethylation and repression of tumor suppressors such as RBBP6 and FBXW7 (Ntziachristos et al. 2014). This yin/yang contribution of enzymes with the same specificity to tumorigenesis may not represent a general phenomenon: In a subset of T-ALL dependent on the transcription factor TAL1, UTX (working as a coactivator for TAL1) is pro-oncogenic rather than tumor suppressive, and its inhibition has antitumor effects (Benyoucef et al. 2016).

Small molecule inhibitors against JMJD3/UTX have been identified and used as probes to study the role of these two proteins. Because there are no small molecules available that can selectively tackle one or the other protein, the results obtained with these probes are not necessarily straightforward to interpret, given the opposite roles frequently played by JMJD3 and UTX. As an additional note of caution in the use of these inhibitors, they may act also against other KDMs: The most widely used JMJD3/UTX inhibitor, GSK-J4, also shows clear inhibitory activity against KDM5 enzymes (Heinemann et al. 2014, Kruidenier et al. 2012).

**3.2.2. The JMJD2/KDM4 family.** The four members of the JMJD2/KDM4 family catalyze the demethylation of di- and trimethylated H3K9 and H3K36. Because these modifications are generally believed to be involved in transcriptional repression, the KDM4 demethylases are considered transcriptional coactivating enzymes (Berry & Janknecht 2013).

Genetic evidence linking some members to cancer is available: KDM4C/GASC1 has been found to be amplified in several cancers, and there is a partial correlation among gene amplification, expression levels, and dependence on KDM4C (Cloos et al. 2006, Liu et al. 2009). Lack of a stronger correlation may be due to redundancy: In fact, members of the cluster can work both distinctly and combinatorially, and on most active genes, more members of the cluster, targeted to histone H3K4me3 by their double Tudor domain, are found dynamically replacing each other and independently maintain transcriptional competence of their target genes (Das et al. 2014, Pedersen et al. 2016). Although most studies focus on the role of their catalytic activity, the KDM4 proteins appear to regulate the *ATG* genes, involved in autophagy, by a demethylase-independent mechanism (Bernard et al. 2015).

KDM4B/KDM4C are transcriptional targets of HIF1 and are involved in the response of tumor cells to a hypoxic environment, stimulating angiogenesis and metastasis (Beyer et al. 2008, Luo et al. 2012, J. Yang et al. 2010). Inactivation of one or more cluster members by genetic tools or small molecules has shown antitumor activity (Berry & Janknecht 2013). In AMLs, KDM4C has been reported to associate with oncogenic fusion proteins (MOZ-TIF2, MLL fusion proteins), and cooperate with the Arg-histone methyltransferase PRMT1 to derepress HOX genes (Cheung et al. 2016). Although these studies are based on short hairpin RNA (shRNA) inactivation strategies, more recent results using knockout mice show that KDM4A/B/C are redundantly required for both establishment and maintenance of AMLs driven by MLL fusion proteins, by maintaining the expression of critical target genes required for AML cell growth and survival, such as the IL3R $\alpha$-subunit (Agger et al. 2016).

Finally, an oncogenic role has been proposed for KDM4A in promoting site-specific gene amplification, without global genomic instability (Black et al. 2013).

Taken together, these results provide important experimental support for the validation of KDM4 proteins as cancer targets and offer a rationale for the therapeutic opportunity to target the KDM4 cluster as a whole, rather than focusing on individual members. Unfortunately, the identification of selective and potent inhibitors to the KDM4 family is still in its infancy: In fact, most if not all of the inhibitors identified to date are also strong inhibitors of other KDMs, and therefore not optimally suited as probes or as potential starting points for drug development (Chin & Han 2015).

**3.2.3. The JARID1/KDM5 family.** The four members of the KDM5 cluster demethylate the di- and trimethylated histone H3K4 mark and associate with other chromatin modifiers involved in transcriptional repression (histone deacetylases, histone methyltransferases/PcG proteins) (Højfeldt et al. 2013).

KDM5A has been found to be involved in translocations in AMLs, leading to a fusion protein (NUP98-KDM5A) that through the PHD finger of KDM5A associates with H3K4me2/me3, and to silence target genes associated with an LSC phenotype (Wang et al. 2009). Enhanced expression of KDM5A has been found to be strongly associated with breast cancer metastasis and to an in vivo reduction of lung metastasis, and its knockdown causes downregulation of organotropic metastasis gene signatures in cell lines (Cao et al. 2014).

KDM5B downregulates genes involved in the control of cell proliferation and senescence, thus counteracting the tumor-suppressive role of pRB (Lin et al. 2011). KDM5B has also been described to work as an oncogene in other cancer types. In breast cancer, it is frequently overexpressed, in

part as a consequence of gene amplification, and appears to be critical for the determination of the luminal subtype of breast cancer, being involved in repression of genes expressed in the basal subtype of breast cancer and maintenance of luminal cell–specific expression programs (Yamamoto et al. 2014). In contrast with this oncogenic role are recent findings in MLL-rearranged AML cells, showing that KDM5B is an oncosuppressor involved in suppression of the LSC expression program, through regulation of the global histone H3K4 methylome (Wong et al. 2015).

Finally, KDM5A and KDM5B have been proposed to be responsible for the properties—within the tumor mass—of a cellular subpopulation that is tolerant to treatment with antitumor drugs. These cells have self-renewing properties and are required for tumor maintenance (Sharma et al. 2010). In melanoma, KDM5B-expressing cells have been characterized and shown to possess several properties commonly ascribed to cancer stem cells. However, KDM5B-expressing cells dynamically interconvert into nonexpressing cells, and therefore do not follow a canonical cancer stem cell model based on a defined cellular hierarchy (Roesch et al. 2010, 2013).

In view of these studies, many groups and companies have started the synthesis of small molecule inhibitors of KDM5. Inhibitors directed against the entire cluster have shown antitumor activity in preclinical xenograft models and are progressing to the clinical stage (Rasmussen & Staller 2014).

## 4. THE BROMODOMAIN/BET FAMILY

The bromodomain is a recognition module for acetylated histones present in several chromatin-associated proteins. The BET family of bromodomain-containing proteins, which include BRD2, -3, and -4 and BRDT, has been studied extensively. BRD4 is involved in the global control of transcription, regulating pTEF-b, and associates with the mediator and chromatin remodeling complexes. Additional, it plays a regulatory role at enhancers and superenhancer elements, where it is dynamically recruited as a function of variations of histone H3K27 acetylation, leading to transcriptional activation (Shi & Vakoc 2014).

BRD4 is also expressed as a BRD4-NUT fusion protein in midline carcinomas (French et al. 2003). Knockdown of BRD4-NUT leads to terminal squamous differentiation and arrested proliferation of midline carcinoma cells, indicating that it plays a critical role in proliferation through a blockade of differentiation (Grayson et al. 2014, Wang et al. 2014). The fusion protein occupies large regions (up to 1.4 Mb) of acetylated chromatin, forms nuclear foci, and, through NUT-mediated recruitment of p300, squelches p300 from normal binding sites, thus blocking differentiation (Alekseyenko et al. 2015).

In other cancers, BRD4 has been involved in the control of the expression of several oncogenes, including c-*MYC* and *BCL2*, thus regulating cell proliferation and survival. This could result from the preferential binding of BRD4 to superenhancers that control the expression of factors playing a critical role for tumor cells (Chapuy et al. 2013, Knoechel et al. 2014, Wyce et al. 2013).

Several small molecule BET inhibitors have been synthesized, and their effects have been extensively studied in cancer and other diseases (Shi & Vakoc 2014). They compete with acetylated peptides for binding the bromodomain and can displace BET proteins from chromatin (Dawson et al. 2011). Several inhibitors are advanced at the early stages of clinical development, and the first clinical results are promising, with complete responses observed (**Table 1**) (Stathis et al. 2016). Because downregulation of c-MYC expression has been among the earliest effects observed upon inhibition, BET inhibitors have been perhaps too simplistically considered as MYC inhibitors, playing a main role in cell proliferation through regulation of this oncogene. Several other targets beyond c-MYC have now been identified. In fact, BET inhibitors act by displacing BRD4 from superenhancer regions responsible for the control of key oncogenic pathways in tumor cells (Chapuy et al. 2013, Wyce et al. 2013).

Recently, cross-talk with other signaling pathways has been described: In mouse models of mammary tumors driven by c-MYC and activated PI3K, tumors are resistant to both BET and PI3K inhibitors. Upon exposure to PI3K inhibitors, AKT is inhibited only transiently, but cotreatment of PI3K inhibitors with BET inhibitors shuts PI3K signaling off permanently, through c-MYC downregulation and dissociation of BRD4 from regulatory regions of multiple receptor tyrosine kinases, thus leading to their decreased expression (Stratikopoulos et al. 2015). BET inhibitors may also sensitize T-ALL cells to treatment with Notch inhibitors, due to downregulation of c-MYC and BCL2 (and of other key T-ALL genes) (Knoechel et al. 2014).

Mechanisms of resistance to BET inhibitors have already been identified. In MLL-driven AMLs, continuous exposure to BET inhibitors leads to selection of a subpopulation of LSCs, which show maintained transcription of key BRD4 target genes (including c-MYC) even if BRD4 is displaced from chromatin upon treatment with the inhibitor (Fong et al. 2015, Rathert et al. 2015). Characterization of the resistant cells showed an increase in the activity of the TGFβ and Wnt/β-catenin signaling pathways, which appeared to be crucial, as inhibition of TGFβ increases differentiation and restores sensitivity of AML cells to BET inhibitors. Mechanistically, β-catenin is found to be associated with BRD4 target genes in resistant cells (Fong et al. 2015, Rathert et al. 2015).

Other studies of triple-negative breast cancer cells resistant to bromodomain inhibition show that BRD4 maintains its association with chromatin, as decreased PP2A activity leads to hyperphosphorylated BRD4, which binds more strongly to MED1, facilitating a bromodomain-independent chromatin recruitment mechanism. Thus, multiple mechanisms of potential resistance are emerging, which could be monitored in patients undergoing treatment with BET inhibitors (Shu et al. 2016).

Overall, BET inhibitors are advancing at an astonishing speed toward clinical validation. Many questions will be probably answered soon, when the phase 1 clinical studies are published: Particularly relevant is the issue of the potential toxicity of these drugs, given the wide range of fundamental functions played by BET proteins in normal cells. If positive, these clinical studies will also pave the way for more complex clinical studies, in which BET inhibitors can reveal an even stronger potential in combination with other drugs (see below).

## 5. CONCLUSIONS AND PERSPECTIVES

Although showing clinical efficacy, the first epigenetic drugs (histone deacetylase inhibitors and DNA demethylating agents) have so far performed below expectations. The main reason behind this partial failure lies in the lack of defined biomarkers to stratify patients, and their use in a frequently blind approach. The expectation is that the novel epigenetic drugs will do better than the approved drugs, and we are now at a stage where preclinical and clinical investigations have led to several promising results for the use of these novel inhibitors:

1. Patients can be stratified based on alleles with activating mutations, chromosomal translocations, and genetic dependencies and potentially be treated with inhibitors to EZH2, DOT1L (MLL-r), BRD4, and KDM5.
2. Synthetic lethal interactions have been identified that can be exploited for patient stratification and treatment (e.g., SWI/SNF defects with EZH2 inhibitors and *MTAP* deletions with PRMT5 inhibitors).
3. Relapsed patients showing chemoresistance can potentially be treated with epigenetic inhibitors (e.g., KDM5A/KDM5B and BET proteins).
4. Epigenetic therapies can be combined with standard of care and immunotherapy (epigenetic priming).

The size of the task of a complete dissection of the cancer(s) epigenome(s), however, remains immense. We need to define more precise genetic, epigenetic, and transcriptional signatures that can help to efficiently stratify patients and direct them to the most appropriate treatment with (epi-)drugs. The finding that several tumors harbor genetic mutations in chromatin-associated factors will likely lead to the identification of novel associations of genetic lesions with specific epigenetic alterations, and with defined spectra of sensitivity or resistance to drugs. For now, however, these studies remain relatively few (discussed above) and will need to be validated clinically. This paucity could be consequences of the youth of the technologies to map the epigenome and of the need to further develop and optimize these technologies to adapt them to the small amounts and storage conditions of patient samples (biopsies, formalin-fixed and paraffin-embedded samples). Recently, new methodologies are emerging for both proteomic and next-generation sequencing–based profiling of the epigenome from patient samples (Cejas et al. 2016, Fanelli et al. 2011, Noberini et al. 2016).

Unfortunately, studies aimed at identifying biomarkers will not be straightforward, as they have to take into account the fundamental issue of tumor heterogeneity: In fact, the same chromatin factor can play different roles in different subcompartments or cellular subpopulations of the tumor, being oncogenic versus tumor suppressive in the stem cells versus the bulk cells (**Figure 2**). Therefore, we need at a minimum to explore the association of genetic versus epigenetic versus transcriptional signatures in different cancer subcompartments, which is further complicated by the fact that those compartments are not rigidly defined. Lacking definitive markers, single-cell approaches that are now also becoming available for epigenome profiling will help to explore the epigenetic diversity of tumor cells (Buenrostro et al. 2015, Jin et al. 2015).

For the reasons sketched above, a strategy based uniquely on a "search-for-association" approach to the identification of stratification biomarkers will not suffice to unravel the complexity of the cancer epigenome. We have to ramp up the preclinical studies in which we use more sophisticated methods to understand tumor vulnerabilities that need to be transformed into robust diagnostic assays to direct treatment. Among those novel approaches, we mention here the possibility to use (*a*) tumor organoids for a systematic screen of epigenetic drugs/combination treatments, unthinkable in the in vivo setting (e.g., Van De Wetering et al. 2015), and (*b*) in vivo screening of barcoded shRNA or CRSPR/Cas9 libraries of epigenetic targets using patient-derived xenograft models well characterized at the genetic/epigenetic/transcriptional levels to identify novel synthetic interactions (e.g., Bossi et al. 2016).

Of unique relevance will be the study of combination therapies, exploiting epigenetic drugs to re-establish or maintain drug sensitivity. Largely unexplored remains the possibility of combining epigenetic approaches with novel immunotherapeutic drugs, which need to be studied more systematically and need better preclinical models (e.g., humanized mice to study immune responses) (Maio et al. 2015, Peng et al. 2015).

The complexity of the epigenome may be simplified by grouping epigenetic modifications in chromatin states (J. Ernst et al. 2010). Many of the factors described above as playing a critical role in determining those states in both normal and tumor cells, and therefore their pharmacological modulation, will likely contribute to drive tumor cells toward epigenetic landscapes compatible with an optimal clinical response.

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