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INVITED REVIEW

Evolving insights on histone methylome regulation in human acute myeloid leukemia pathogenesis and targeted therapy

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Acute myeloid leukemia (AML) is an aggressive, disseminated hematological malignancy associated with clonal selection of aberrant self-renewing hematopoietic stem cells and progenitors and poorly differentiated myeloid blasts. The most prevalent form of leukemia in adults, AML is predominantly an age-related disorder and accounts for more than 10,000 deaths per year in the United States alone. In comparison to solid tumors, AML has an overall low mutational burden, albeit more than 70% of AML patients harbor somatic mutations in genes encoding epigenetic modifiers and chromatin regulators. In the past decade, discoveries highlighting the role of DNA and histone modifications in determining cellular plasticity and lineage commitment have attested to the importance of epigenetic contributions to tumor cell de-differentiation and heterogeneity, tumor initiation, maintenance, and relapse. Orchestration in histone methylation levels regulates pluripotency and multicellular development. The increasing number of reversible methylation regulators being identified, including histone methylation *writer, reader,* and *eraser* enzymes, and their implications in AML pathogenesis have widened the scope of epigenetic reprogramming, with multiple drugs currently in various stages of preclinical and clinical trials. AML methylome also determines response to conventional chemotherapy, as well as AML cell interaction within a tumor-immune microenvironment ecosystem. Here we summarize the latest developments focusing on molecular derangements in histone methyltransferases (HMTs) and histone demethylases (HDMs) in AML pathogenesis. AML-associated HMTs and HDMs, through intricate crosstalk mechanisms, maintain an altered histone methylation code conducive to disease progression. We further discuss their importance in governing response to therapy, which can be used as a biomarker for treatment efficacy. Finally we deliberate on the therapeutic potential of targeting aberrant histone methylome in AML, examine available small molecule inhibitors in combination with immunomodulating therapeutic approaches and caveats, and discuss how future studies can enable posited epigenome-based targeted therapy to become a mainstay for AML treatment.

Acute myeloid leukemia (AML) is characterized by uncontrolled proliferation of clonal hematopoietic precursor cells. This interrupts normal hematopoiesis and may lead to bone marrow failure. It occurs predominantly in older adults, with the average age at diagnosis being 68 years [1]. Intensive chemotherapy combined with hematopoietic stem cell (HSC) transplantation has considerably improved outcomes

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in younger adults. However, about 80% of older adults still succumb to the disease or to the associated therapeutic toxicity [2]. Recent advancements in molecular and cytogenetic analyses have helped identify genetic abnormalities that contribute to AML initiation and maintenance [3]. Approximately 55% of AML cases harbor recurrent chromosomal translocations, which have been considered one of the most important prognostic factors for clinical outcome prediction. However, the remaining 45% cases harbor normal karyotypes, indicating that chromosomal rearrangement is not the only determinant of the disease. Compared with other cancers, AML has a low mutational load, but is highly heterogeneous in terms of genetic background and clinical presentation [4]. Though mutations are few, they frequently occur in genes encoding epigenetic regulators [5]. Some of the common examples are *DNMT3A, TET2, IDH1,* and *IDH2* (regulating DNA methylation), *CBP* and *P300* (regulating histone acetylation), and *EZH2, ASXL1,* and *KDMs* (regulating histone methylation). Additionally, mutations in the CTCF and cohesin complex have also been identified that regulate three-dimensional chromatin conformation.

Unlike genetic mutations, which are hardwired, epigenetic modifiers work by transcriptional regulation of their downstream target genes and are reversible. DNA methylation was one of the first characterized epigenetic regulations. In addition to DNA methylation, histone tail modifications, including acetylation, methylation, and ubiquitination, have been identified that significantly contribute to transcriptional output. Many of these modifications are frequently dysregulated in AML. Histone methylation, unlike other epigenetic modifications, was initially considered irreversible. This perception changed dramatically with the discovery of two families of enzymes capable of demethylating histone lysine residues [6,7]. Thereafter, histone methylation has come to be recognized to play a key role in the initiation and maintenance of several cancers including AML. In this review, we discuss the importance of histone methylation to epigenetic dysregulation in AML. We also try to understand the correlation between histone methylation and clinical outcome and discuss available epigenetic therapies and their limitations and issues, which remain unaddressed.

HMTs in AML development

Histone methyltransferases (HMTs) catalyze addition of methyl groups to specific histone residues. Depending on the position and nature of the methylated residues, histone methylation can either promote or repress transcription. In general, methylation on H3K4, H3K36, and H3K79, as well as asymmetric dimethylation of H4R3 activates gene expression, whereas methylation on H3K9, H3K27, and H4K20 and symmetric dimethylation of H4R3 are associated with transcription repression [8,9].

MLL1

MLL1, also known as KMT2A, is a member of the family of SET domain-containing enzymes. It methylates H3K4, resulting in transcription activation. Several studies have reported that Mll1 is required for definitive hematopoiesis, and regulates repopulating ability of both fetal and adult HSCs [10,11]. MLL1 is involved in translocations in approximately 5% to 10% of AML cases [12]. MLL1 translocations result in fusion proteins that lack the wild-type SET domain, hence its transformation capacity is not attributed to methyltransferase activity. This is comparable to its function in normal hematopoiesis wherein H4K16ac, through MLL1 interacting partners, is more important than its HMT activity for maintaining expression of downstream targets [13]. MLL1 fusions in leukemia occur with about six common partner genes. These usually encode super-elongation-complex nuclear proteins, such as AF4/6/9/10, ELL, and ENL, with MLL1−AF9 (MLL−AF9) being the most common and accounting for about 30% of all MLL1 fusions in AML [12]. The MLL1 fusion genes arising from these translocations have been characterized as potent oncogenes. Though MLL1 fusions themselves are devoid of HMT activity, several methylation-related enzymes, such as EZH2, LSD1, and DOT1L, have been found to be essential components of this leukemogenic program.

Additionally, cells harboring MLL fusions may retain a copy of the wild-type MLL1. Several studies have indicated dependence of MLL fusions on the intact MLL1 allele for their functioning. Studies in mouse fibroblasts demonstrated recruitment of MLL−AF9 to the HoxA9 locus is mediated by wild-type Mll1 [14]. Similarly in MLL−AF9 murine AML cells, menin-mediated recruitment of both wild-type Mll1 and MLL−AF9 fusion is required for Hox gene activation and leukemia progression [15]. However, other studies indicate that HMT activity of wild-type MLL1 is dispensable for hematopoiesis and leukemogenesis of MLL−AF9-driven leukemia [13]. Moreover, not Mll1, but its closest orthologue Mll2, another H3K4 HMT, is required for MLL fusion-driven leukemogenesis [16]. In contrast, loss of Mll3 or Mll4 did not influence H3K4 methylation on Hox loci or their expression [17]. These contradictory findings indicate cell type specificity, redundancy, and interdependence among the MLL family of genes. The menin−MLL interaction is critical for MLL-fusion binding to target loci [18], and this dependency has been exploited to develop drugs that specifically disrupt this interaction. VTP50469, an orally bioavailable small-molecule inhibitor, displaces menin from the MLL-containing protein complex and reduces MLL1 and DOT1L binding to target loci, resulting in downregulation of key genes of the MLL-rearranged (MLL-r) transcriptional program, such as *MEIS1*, *MEF2C*, *KDM3C,* and *PBX3* [18]. VTP50469 treatment successfully eradicated disease in

patient-derived xenograft (PDX) models of MLL-r AML. Menin–MLL interaction plays a key role in both MLL-r and NPM1-mutant ($NPM1^{mut}$) AMLs, which frequently also harbor activating *FLT3* mutations. *FLT3* is a downstream target of *MEIS1*, one of the transcriptional factors downregulated on VTP50469 treatment. Combining menin–MLL inhibition with quizartinib, a potent and highly selective *FLT3* inhibitor, reduced FLT3 phosphorylation, suppressed its target genes, and synergistically enhanced apoptosis and differentiation in models of human and murine $NPM1^{mut}$ and MLL-r leukemias harboring an *FLT3* mutation [19].

Since the last decade, researchers around the globe have been trying to address initiation and evolution of AML from premalignant clones. Targeting such clones could effectively lead to preventive therapies. Npm1c/Dnmt3a mutant mice exhibit a period of extended myeloid progenitor cell proliferation and self-renewal before leukemia, presenting a premalignant model of AML development. Menin–MLL inhibition using VTP50469 abrogated self-renewal of these myeloid progenitor cells, suggesting its potential as a preventive therapy [20]. MLL-r AML is associated with increased activation of Rac GTPases, through elevated Frat expression [21]. Frat1 and Frat2 are associated with canonical and noncanonical Wnt signaling respectively. Thus, through Frat upregulation, MLL fusions promote integration of canonical and noncanonical Wnt signaling, which somewhat justifies the paradoxical requirement of both canonical Wnt signaling and GSK3 activity in MLL-r leukemia [22]. Another study has shown RUNX1 to be a direct target of MLL–AF4 and critical for its transformation potential [23].

PRC2

Polycomb repressive complex 2 (PRC2), which promotes gene repression, consists of four core subunits: EED, SUZ12, RBBP4, and either of the two catalytic subunits EZH1 and EZH2. Being the more common catalytic subunit, EZH2 regulates expression of numerous genes critical for stem cell renewal by controlling H3K27 methylation at "poised" promoters [24]. Ezh2 is essential for fetal, but not adult, HSC function [25]. Ezh2 deletion in adult bone marrow compromises specifically lymphopoiesis [26]. Unlike Ezh2, which is ubiquitously expressed, Ezh1 is highly expressed in HSCs in the bone marrow, compared with those in the fetal liver, indicating that Ezh1 complements Ezh2 in the adult bone marrow, but not in the fetal liver. *EZH2* is frequently mutated in different leukemia types. In contrast to B-cell lymphoma, where *EZH2* mutations are *gain of function* [27], the majority of *EZH2* mutations found in myeloid disorders are inactivating mutations, resulting in loss of its H3K27 methyltransferase activity [28]. *EZH2* is found in the 7q region, which is frequently deleted in myeloid neoplasms. Deletion of *Ezh2* in a mouse model induced MDS-like disease, suggesting tumor suppressor function [29]. However, a recent report indicated mutations in *EZH2* rather exert stage-dependent and opposing effects on AML. When *Ezh2* was deleted before transformation with MLL–AF9 or AML1–ETO9a oncogenes, it accelerated AML progression and shortened survival, indicating a tumor suppressor role. In contrast, when *Ezh2* was deleted in secondary recipients during the maintenance phase of AML, disease severity was attenuated and survival enhanced [30]. Other members of the PRC2 complex, including ASXL1, JARID2, and SUZ12, have also been implicated in the development of AML, with most of the mutations in these genes resulting in *loss of function* of the complex.

Other HMTs and reader proteins

Apart from MLL and PRC2, there are methyltransferases regulating methylation at the other common residues, H3K9, H3K36, and H3K79. SUV39H1 methylates H3K9. MECOM, a potent proto-oncogene known to be involved in stem cell self-renewal and leukemogenesis, physically interacts with SUV39H1 and has been implicated in disease progression of AML [31]. Loss of *G9a*, another H3K9 HMT, was reported to suppress leukemogenesis in a mouse model of leukemia induced by HOXA9 [32]. H3K36 trimethylation, which promotes transcription elongation, is catalyzed by SETD2. Homozygous *Setd2* deficiency during early hematopoietic development causes pancytopenia, splenomegaly, and overall bone marrow hypocellularity, along with a reduced total number of HSCs [33]. *SETD2 loss of function* mutation is common and has been reported in more than 20% of MLL-r leukemias [34]. Like EZH2, SETD2 may have a context-dependent tumor suppressor or oncogenic function. Partial *SETD2* loss enhances leukemogenesis and leads to drug resistance [35], while complete *SETD2* loss delays leukemia progression, suggesting a possible gene dosage effect [35,36].

CBX7, a chromodomain-containing member of PRC1 recognizing H3K27me3, has been reported to promote self-renewal of human normal and AML stem cells and progenitors, which involves nonhistone protein interactions with H3K9 methyltransferases SETDB1, EHMT1, and EHMT2 [37]. Contribution of plant homeodomain (PHD) motif-containing proteins, which are able to read H3K4me3 marks, to AML development has also been suggested [38]. Additionally, HMTs and HDMs are erratic for nonhistone targets as well as methylation-independent roles. These complexities warrant further investigation. DOT1L is the only lysine methyltransferase known to be responsible for H3K79 methylation in mammalian systems.

Dot1l-null mice are embryonic lethal, with disrupts erythroid development and causes severe anemia [39]. In MLL-r leukemia, aberrant recruitment of DOT1L occurs on promoters and gene bodies of MLL targets, aiding their expression [40]. DOT1L is critical to the MLL-r oncogenic transcriptional program, and DOT1L inhibition suppresses this program and leukemia development [41]. Interestingly DOT1L also presents as a therapeutic target for the treatment of *DNMT3A*-mutant AML [42]. Methylation of arginine residues within histone tails (H3 and H4), though less common than lysine methylation, is regulated by protein arginine methyltransferases (PRMTs). PRMT1, the founding member of the PRMTs, has been reported to be required for leukemia initiation by the fusion proteins MLL–GAS7 and MOZ–TIF2, and its silencing was able to block leukemia transformation [43]. PRMT4 and PRMT5 enzymes also play important roles in AML development [44,45].

HDMs in AML pathogenesis

Histone demethylases (HDMs) catalyze removal of methyl groups from specific residues on histone tails, counteracting the function of methyltransferases. A dynamic balance between these two groups of enzymes ensures efficient histone methylome regulation, which is often disrupted in most malignancies, including AML (Figure 1).

LSD family

HDMs consist of two families of enzymes, LSD and JMJC. LSD1 (also known as KDM1A), the first demethylase discovered, is an amine oxidase that demethylates di- and monomethylated H3K4 and H3K9 residues. H3K4 methylation and H3K9 methylation play antagonistic roles; thus, LSD1 may function as either a repressor or an activator of transcription depending on its interacting partners. It is usually associated with gene repression and is a critical component of transcription repressor complexes such as CoREST and NuRD. LSD1, along with CoREST, associates with the transcriptional repressors Gfi-1/1b during hematopoietic differentiation [46]. *Lsd1* loss de-represses Gfi-1/1b lineage-specific transcriptional programs, hampering terminal differentiation of erythroid, megakaryocytic, and granulocytic cells [47]. LSD1 is frequently overexpressed in AML. *LSD1* loss impairs proliferation and increases differentiation and apoptosis in MLL- and AML1-rearranged leukemias [48].

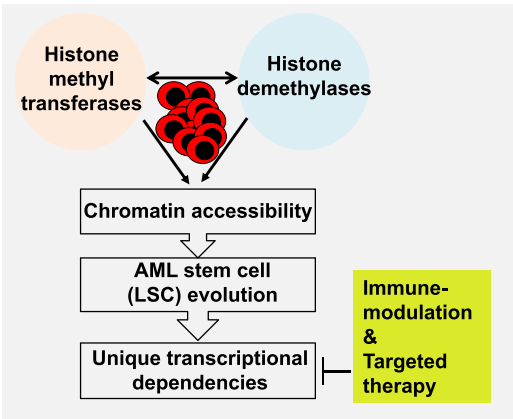

Figure 1. Schema representing molecular derangements and cross-talk between HMTs and HDMs impinging on an aberrant histone methylome and their implications in AML pathogenesis and targeted therapy.

JMJC family

The JMJC family encompasses most of the known demethylases. They have a catalytic C-terminal Jumonji (JmJC) domain and, unlike the LSD family, can demethylate mono-, di-, and trimethylated residues. The JMJC demethylases consist of many subfamilies with multiple members implicated in normal and malignant hematopoiesis. KDM2B, which demethylates H3K36, is overexpressed in leukemia stem cells (LSCs) and is required for their neoplastic transformation [49]. *Kdm2b*-deleted mice exhibit a reduced number of long-term HSCs as well as defective lymphopoiesis, with a resultant upregulation of myeloid differentiation [50]. KDM2B also functions as the DNA binding subunit of the noncanonical PRC1.1 complex. Downregulation of KDM2B suppressed in vitro AML cell growth, as well as abrogated leukemogenesis in vivo, in PDX models, revealing the role of the PRC1.1 complex in regulating leukemia progression [51]. KDM3A, specific for H3K9, has been reported to maintain myeloma cell survival. The 5q31 genomic region, which contains a portion of the *KDM3B* gene, is frequently deleted in myelodysplastic syndromes (MDS) and AML. Therefore, KDM3B is thought to play a role in tumor suppression. In contrast, another study reported that KDM3B is involved in transcriptional activation of the LMO2 oncogene in leukemia [52]. Though KDM3C is dispensable for healthy adult hematopoiesis as well as for JAK2V617F-driven MPN disease initiation, it has recently implicated in the progression of AML1–ETO- and HOXA9-mediated leukemias [53]. KDM3C was identified as a co-activator in AETFC, a complex formed by AML1–ETO, where KDM3C maintained low level of H3K9me2, hence enhancing gene expression of AML1–ETO targets.

KDM4, which targets both H3K9 and H3K36, has been characterized primarily as proto-oncogenic. KDM4A, KDM4B, and KDM4C in conjunction have been reported to mediate survival of leukemia cells by enhancing the expression of interleukin (IL)-3 receptor-α, a key initiator in the JAK–STAT pathway, in MLL–AF9-translocated AML [54]. Additionally, conditional *Kdm4a/Kdm4b/Kdm4c* triple-knockout mice exhibit high H3K9me3 at transcription start sites and

concomitant repression of several genes involved in HSC maintenance [55]. KDM6 enzymes are exclusively H3K27 demethylases. KDM6A (UTX) has been found to direct migration of HSCs in response to SDF-1/CXCR4 signaling [56]. Though KDM6A is mutated in many cancers and classified as a tumor suppressor, we had observed that it is also overexpressed in some cases of AML [57]. We found that KDM6A interacts and cooperates with an MBD3-deficient nucleosome remodeler histone deacetylase (NuRD) complex to promote CBP recruitment and H3K27 acetylation at *DOCK5/8* loci, thereby inducing Rac GTPase activation and AML cell migration [58]. KDM6A, being encoded by the X chromosome, has sex-specific effects; homozygous female *Kdm6a*-null mice exhibit MDS and suppressed erythro-megakaryocytopoiesis, whereas males have normal hematopoietic development [59]. KDM6B (JMJD3) regulates transcriptional elongation, and overexpression of KDM6B is reported in MDS hematopoietic stem and progenitor cells (HSPCs) [60]. In addition, recent reports have also identified it as playing an oncogenic role in AML.

Arginine demethylases

Arginine demethylation is a relatively unexplored avenue. Although many of the lysine demethylases also exhibit in vitro arginine demethylation capability, to date, only two bona fide arginine demethylases (PAD4 and JMJD6) have been discovered. PAD4 catalyzes conversion of monomethylated arginine to citrulline on H3R17 and H4R3, while JMJD6 directly converts methylarginine to arginine by removing the methyl group. PAD4, along with LEF1 and HDAC1, represses c-myc expression and regulates proliferation of lineage$^-$Sca-1$^+$c-Kit$^+$ (LSK) mouse bone marrow multipotent progenitor cells [61]. Furthermore, PAD4, acting as a co-activator by influencing H3R2me2a, facilitates expression of the Tal1 target IL6ST [62]. This provides control over IL6ST expression during lineage differentiation of HSPCs. Recent studies have revealed involvement of PAD4 in ATRA-mediated differentiation of AML cells. On ATRA exposure, PAD4 translocates into the nucleus and downregulates SOX4 expression, which in turn relieves transcriptional repression of PU.1 [63]. Thus, PAD4 controls ATRA-mediated differentiation in a SOX4-dependent manner. JMJD6 removes dimethyl groups from H3R2 and H4R3. Though JMJD6 has not yet been implicated in AML pathogenesis, reports have indicated that it can influence AML cell sensitivity to BET protein inhibitors.

Histone mutations in AML

Apart from alterations in histone-modifying enzymes, mutations in the histone gene itself can alter methylation dynamics. A key example is diffuse intrinsic pontine glioma (DIPG), wherein heterozygous mutations (*K27M* and *G34R/V*) in *H3F3A*, the gene encoding the histone variant H3.3, are identified [64]. Apart from DIPG, it also occurs in up to 60% of pediatric glioblastoma multiforme patients, in whom the presence of *K27M−H3.3* predicts poor survival [65]. Primary human samples, as well as cell lines containing the *K27M−H3.3* mutation, exhibit reduced levels of H3K27me2/3 and DNA hypomethylation at many loci. Similarly, H3K27 methylation also plays a critical role in AML [66]. Loss of the H3K27me3 mark is associated with the multidrug resistance phenotype in AML [67]. Like glioblastoma, sequencing of the 16 histone H3 genes in AML identified 7 recurrent mutations, with H3K27 mutations having the highest frequency. These mutations occur with a higher frequency in secondary AML and have been found to exist in preleukemic HSCs and major leukemic clones [68]. A previous study reported reduced H3K27me2/3 in patients with H3K27M/I mutations and accelerated disease progression in an AML1-ETO mouse model [66]. In vivo functional assays using mutant human HSCs (CD34$^+$CD38$^-$) revealed that H3.1K27M/I mutations increased stem cell-enriched population and engraftment potential in secondary recipients, along with a blockage in erythroid differentiation [68]. Whether H3K27 mutations or methylation levels provide a clonal advantage, whether this is associated with drug resistance/relapse, and whether this alteration can be exploited to specifically target LSCs are important questions that need to be addressed.

Crosstalk between HMTs and HDMs in AML

Although histone-modifying enzymes appear to individually regulate their targets, epigenetic regulation is rather a concerted effort and extensive functional crosstalk exists between HMTs and HDMs to regulate locus-specific gene expression, global chromatin architecture, and cellular states. Emerging studies, using *state-of-the-art* genetic models, single-cell analyses, and modern technologies in chromatin biology, are helping us to further explore this crosstalk and appreciate the intricate molecular regulation of histone methylation in AML. Recently, it was reported that DOT1L antagonizes recruitment of SIRT1 and SUV39H1 to their targets [41]. On DOT1L inhibition, SIRT1 and SUV39H1 bound to MLL targets and repressed their expression. Thus MLL-r AML sensitivity to DOT1L inhibitors depends on SIRT1 and SUV39H1 levels, indicating the importance of the functional crosstalk between these enzymes. Similarly, in MLL-AF9 AML, KDM4C together with PRMT1 co-regulates transcription of MLL downstream targets [43]. Another interesting example is that KDM4A inhibition restores H3K36me3 and sensitizes SETD2-mutant AML to cytarabine treatment [35]. Though not yet demonstrated in AML, KDM6A mutation sensitizes multiple myeloma cells to

EZH2 inhibition [69]. Studies have also found that MLL fusion oncoproteins promote EZH2 transcription, indicating a role for PRC2 in MLL-r leukemia. PRC2, in turn, represses genes that are critical to the myeloid differentiation program [70]. In MLL-r AML, both EZH2 and EZH1 are expressed and compensate each other to promote leukemogenesis; thus, simultaneous disruption of both enzymes is required to inhibit growth of leukemia carrying MLL–AF9.

Chromatin accessibility and AML stem cell function

Chromatin architecture plays a key role in determining the accessibility of transcription factors to gene regulatory regions. This controls cell type- as well as development stage-specific gene expression. Chromatin markers define stem cell function, and orchestrated changes in chromatin accessibility ensure efficient progression of HSCs through maturational stages to form the differentiated blood cells [71–73]. Aberrant chromatin structure is often associated with disruption of this regulation, leading to malignancies [5,74,75]. Profiling of genomewide histone methylation marks has revealed that LSCs in MLL-r leukemia are characterized by high H3K4me3 and low H3K79me2. KDM5B, the H3K4-specific demethylase, negatively regulates LSC potential, demonstrating importance of the H3K4 methylome in determining LSC fate [76]. H3K4me3 ensures an open chromatin structure and accessibility of leukemogenic MLL to its downstream targets *Hoxa9* and *Meis1*. In addition to MLL-r AML, the HoxA cluster is upregulated in AML1–ETO-induced AML as well. HMGN1, a chromatin modulator, is frequently amplified in AML and is associated with high H3K27ac and increased accessibility and expression of HoxA cluster genes [77]. HMGN1 overexpression decreases quiescence and induces HSC proliferation. It functions through cooperation with AML1–ETO oncoprotein, blocking myeloid differentiation and enhancing LSC activity. Inhibition of the H3K27 histone acetyltransferases CBP/p300, with concomitant reduction in HMGN1-associated H3K27ac, relieves differentiation block [77]. Thus, balance between H3K27 methylation and acetylation states regulates HMGN1-mediated LSC potential. H3K9me3, another repressive mark, also differentiates gene expression between normal HSCs and LSCs.

ALKBH5 is an m^6A demethylase required for LSC function and is regulated by H3K9me3 levels at its promoter [78]. KDM4C, which is upregulated in AML, removes the repressive H3K9me3 at the ALKBH5 promoter, increasing chromatin accessibility. This facilitates recruitment of MYB and Pol II, increasing ALKBH5 expression. It has also been observed that chromatin architecture differs for AMLs with different underlying genetic signatures, which in turn affect their stemness. Patients harboring NPM1 mutations or MLL-fusion genes have greater chromatin accessibility of HOX-family genes and have high self-renewal capacity and stemness and a poorer prognosis [79,80]. In contrast, patients with RUNX1 or spliceosome mutations depend mainly on IRF family regulators for the downstream transcriptional program [80]. Thus, histone methylation states play an indispensable role in regulating chromatin architecture, transcriptional accessibility, and AML stem cell function.

Histone methylome in AML therapy and response

As the role of the histone methylome in AML pathophysiology becomes more evident, new therapeutic strategies to target these aberrations are being increasingly explored (Table 1). Among the HMTs, DOT1L and EZH2 have emerged as promising targets. DOT1L inhibitors have extensively been used to reduce leukemia burden in a variety of MLL-r AML models. Similarly, DZNep, an EZH2 inhibitor, caused accumulation of reactive oxygen species (ROS) and induced apoptosis in MLL-r AML cells, reducing the frequency of leukemia-initiating cells (LICs) [81]. However, in many cases, simultaneous inhibition of both EZH1 and EZH2 is required [70,82]. UNC1999, an orally bioavailable dual EZH1 and EZH2 inhibitor, has emerged as a promising candidate in MLL-r leukemia [83]. Combining DOT1L and EZH2 deletion demonstrated synergy in vivo in an MLL–AF9 leukemia model [84]. However, the same combination had either a synergistic effect or an antagonistic effect when investigated in a panel of human AML cell lines. DOT1L inhibition suppressed ribosomal biogenesis and protein translation. Consequently, combination with homoharringtonine, a protein translation inhibitor, revealed an additive effect in MLL-r leukemias [84]. Additionally, PRMT1 inhibition using AMI-408 has also proved effective in MLL–GAS7 and MOZ–TIF2 fusion-carrying mouse models [43]. The dosage effect of SETD2 in AML, and its associated tumor vulnerability, also provides an excellent therapeutic opportunity to be explored.

Among HDMs, LSD1 inhibition has shown promise in MLL-r leukemia [85]. The LSD1 inhibitor tranylcypromine (TCP), either alone or in combination with ATRA, disrupted the MLL oncogenic program and induced expression of myeloid differentiation genes in MLL-r AML cells [86]. Overall, this has resulted in a proof-of-concept phase I/II pilot trial with relapsed/refractory (r/r) AML patients ineligible for intensive therapy and another phase I/II trial for MDS patients. Among 18 AML patients, the overall response rate was a meager 20%, which included 2 complete remissions and 1 partial response [87]. However, there was no hematological recovery, with the median overall survival being 3.3 months. This pilot trial indicates that

Table 1. Histone methylation-modifying enzymes implicated in AML pathogenesis

Gene	Target	Alteration	Function	Inhibitors	Clinical trial identifier
MLL1	H3K4→H3K4me1/2/3	Fusion of N-terminal DNA binding domain with C-terminal of transcription elongation factors	Oncogene	MM-401, MM-102, KO-539 (menin-MLL inhibitor), SNDX-5613 (menin-MLL inhibitor)	NCT04067336 NCT04065399
EZH1	H3K27→H3K27me1/2/3	Upregulated	Oncogene	CPI-360, UNC1999, DS-3201	
EZH2	H3K27→H3K27me1/2/3	*Loss of function* mutation, upregulated	Oncogene, tumor suppressor	El1, GSK2816126, EPZ-6438, CPI-1205, GSK343	
SUV39H1	H3K9me1→H3K9me3	None	Oncogene	Chaetocin	
G9A	H3K9me→H3K9me1/2	None	Oncogene	UNC0638, UNC0642	
SETD2	H3K36me2→H3K36me3	*Loss of function* mutation	Tumor suppressor	EPZ-040414	
DOT1L	H3K79→H3K79me1/2/3	None	Oncogene	EPZ004777, EPZ-5676, SGC0946	NCT01684150 NCT02141828
PRMT1	H4R3→H4R3me1, H4R3me2a	Upregulated	Oncogene	AMI-408, C7280948	
PRMT5	H3R8→H3R8me2s H4R3→H4R3me2s	Upregulated	Oncogene	GSK3326595	NCT03614728
LSD1	H3K4me1/2→H3K4 H3K9me1/2→H3K9	Upregulated	Oncogene	TCP, GSK-LSD1, ORY1001, IMG7289, GSK2879552	NCT02177812 NCT02273102 NCT02261779 NCT02842827
KDM2B	H3K4me3→H3K4me2 H3K36me2→H3K36me1	Upregulated	Oncogene	None	
KDM3A	H3K9me1/2→H3K9	None	Oncogene, tumor suppressor	None	
KDM3C	H3K9me1/2→H3K9	None	Oncogene	None	
KDM4A	H3K9me2/3→H3K9me1 H3K36me3→H3K36me2	Upregulated	Oncogene	CP2	
KDM4B	H3K9me2/3→H3K9me1 H3K36me3→H3K36me2	Upregulated	Oncogene	NSC636819	
KDM4C	H3K9me2/3→H3K9me1 H3K36me3→H3K36me2	Upregulated	Oncogene	SD-70	
KDM6A	H327me2/3→H3K27me1	*Loss of function* mutation, upregulated	Oncogene, tumor suppressor	GSK-J4	
KDM6B	H327me2/3→H3K27me1	Upregulated	Oncogene	GSK-J4	
PADI4	H3R17me→H3R17ci H4R3me→H4R3ci	Downregulated	Tumor suppressor	GSK199, GSK484	
JMJD6	H3R2me1/2/2a→H3R2 H4R3me1/2/2a→H4R3	None	Tumor suppressor	SKLB325	

the TCP/ATRA combination does induce differentiation and response in r/r AML, but only slightly so. The trial on MDS patients was subsequently terminated as risk–benefit analysis did not favor continuation of the study. Therefore, effects observed in vitro may not always translate into suitable drugs in vivo, advocating for improving efficacy and establishing better preclinical models. A recent study further strengthened this notion, wherein irreversible LSD1 inhibition was sufficient to induce cytotoxicity in vitro in a model of CEBPA/CSF3R mutant AML, but failed to do so in vivo. However, when combined with ruxolitinib, an inhibitor of JAK/STAT signaling, LSD1 inhibition synergized to normalize peripheral blood WBC counts and double median survival in vivo [88]. Similarly, in MLL-r leukemias, apart from ATRA, combination with mTORC1 inhibition has also been found to enhance the differentiation potential of LSD1 inhibition [89]. Leukemias derived from HSCs exhibit resistance to LSD1 treatment compared with those initiated from myeloid progenitor cells [90]. Elevated Evi1 expression in HSCs attenuates p53 apoptotic response, which can be overcome by combining LSD1 inhibition with BCL2 inhibition. Among the JMJC demethylases, the KDM4C inhibitor SD70 has so far had an excellent therapeutic effect on AML expressing MOZ–TIF2 and MLL fusions [43]. We and others have found that KDM6 inhibition using GSKJ4 also has antileukemic potential both in vitro and in vivo [57,91].

In addition to their being therapeutic targets, HMTs and HDMs often predict treatment response in AML. As previously mentioned, H3K27me3 loss promotes multidrug resistance in AML [67]. Low H3K27me3 levels predict a poor prognosis with significantly decreased overall (median 11.06 vs. 38.6 months) and disease-free survival (median 10.0 vs. 31.2 months) compared with patients with high H3K27me3. Loss of *EZH2*, the enzyme responsible for H3K27me3 levels, also leads to acquired drug resistance to tyrosine kinase inhibitors (TKIs) and cytotoxic drugs in AML [67]. Surprisingly, though KDM6A plays an antagonistic role to EZH2, its *loss of function* has also been found to result in a similar phenomenon. In 45.7% of CN-AML patients, relapse-specific loss of *KDM6A* was observed [92]. *KDM6A* loss caused reduced H3K27ac at the nucleoside membrane transporter *ENT1* locus, repressing its expression, which led to cytarabine resistance. Similarly, LSD1 expression predicts response to ATRA-mediated differentiation in MLL-r leukemia, and LSD1 inhibition appears synergistic with ATRA therapy [85]. KDM7B, an H3K9 demethylase, is also implicated to define response to ATRA-mediated differentiation of RARα fusion-driven acute promyelocytic leukemia [93].

Immunomodulation and combination therapy

In patients unresponsive to conventional chemotherapy or in elderly individuals in whom induction therapy is not an option, immunomodulatory strategies, including immune checkpoint inhibition (ICI) therapy, are being increasingly utilized. Though immunotherapy has shown promise in selected solid tumors, its effectiveness in AML remains limited. Remission achieved through allogenic HSC transplantation in AML patients was a kind of first successful "adoptive immunotherapy" cancer treatment, suggesting that AML was amenable to immunomodulation [94,95]. However, there are several challenges that make AML a difficult target for immunotherapy.

First, AML is a disseminated and systemic malignancy, which itself arises within the core of hematopoietic hierarchy that generates a spectrum of immune cells. Second, as AML is present predominantly in individuals with age-related clonal hematopoiesis with an expansion of the myeloid compartment, an adaptive immune system is intrinsically prone to immunosenescence. Third, AML has a relatively lower mutational burden, thus reducing neo-antigens for elimination by cytotoxic T lymphocytes (CTLs) [96,97]. Fourth, the immune-evasive strategies of AML blasts include inefficient cross-priming by antigen presenting cells, downregulation of antigen presentation by MHC class I and II molecules, expansion of immunosuppressive regulatory T lymphocytes (Tregs), and upregulation of checkpoint inhibitors such as PD-L1, CTLA-4, TIM-3, and LAG-3, among others [98–101]. Overall, these immune suppressive circuits make AML blasts resistant to immunotherapy, which may be improved by rationally combining targeted, monoclonal, or immune-activating approaches with epigenetic or cytotoxic therapies.

Several studies have revealed epigenetic regulators to have immunomodulatory function, thus making them ideal targets for combination therapy. Immunomodulation can be attained by the action of the drugs either on the target cells itself, on the immune cells, or on the tumor microenvironment. Control of CIITA expression, the master regulator of MHC-II, upon interferon (IFN)-γ treatment by EZH2 was first demonstrated in cervical cancer cells. EZH2 suppresses CIITA, reducing surface MHC-II, thus restricting antigen presentation to $CD4^+$ T cells, which can be reversed by EZH2 inhibition [102]. Furthermore, combining EZH2 and DNMT1 inhibition in ovarian cancer derepresses Th-1-type chemokines CXCL9 and CXCL10, increasing $CD8^+$ T-cell infiltration; enhances PD-L1 checkpoint inhibition; and reduces tumor burden [103]. Similarly, KDM1A inhibition synergizes with HDAC1 inhibition, increasing pro-inflammatory cytokine expression, effectively rewiring the tumor from an immunologically "cold" to a "hot" microenvironment [104]. Histone methylome also plays a pivotal part in determining the plasticity of multipotent memory progenitor (MP) cells and their differentiation into $CD8^+$ terminal effector (TE) cells. MP cells exhibit low H3K27me3 at pro-memory and pro-survival genes, indicative of

permissive chromatin [105]. EZH2-mediated deposition of H3K27me3 at pro-memory genes occurs during differentiation of MP cells into $CD8^+$ TE cells. Additionally, Th-cell differentiation is regulated by methylation status at H3K9 and H3K27 residues [106]. The SUV39H1–H3K9me3–HP1α pathway is essential for silencing antitumorigenic Th1-specific gene loci, favoring the protumorigenic Th2 subtype $CD4^+$ T cells [107], whereas EZH2 loss results in enhanced Th1 and Th2 cell polarization [108]. Among HDMs, KDM6 plays an integral function in inducing pro-inflammatory cytokines and has been reported to be important for Th1 lineage commitment as well as M2 macrophage activation [109].

AMG-330 is a bispecific anti-CD33/CD3 T cell-engaging construct reported to be effective in favorable-risk AML patients [110]. It is currently in a phase I clinical trial (NCT02520427). AMG-330, though effective, is susceptible to resistance mechanisms such PD-L1 upregulation and Treg expansion [111], highlighting the need to combine it with ICI therapy [112]. DNA hypomethylation has been found to upregulate expression of immune checkpoint inhibitors. Treatment with hypomethylating agents (HMAs) such as azacitidine in AML often induces PD-L1 expression, which blocks CTL activity and has been associated with azacitidine resistance. This has led to clinical trials combining PD-1/PD-L1 inhibitors with azacitidine in AML and MDS [113]. In a phase II clinical trial combining nivolumab and 5-azacitidine, 11 of the 53 patients treated (21%) achieved CR/CRi, and 7 (14%) had hematologic improvement [114]. Apart from DNA methylation, the repressive histone marks H3K9me3 and H3K27me3 also play a crucial role in determining expression of immune checkpoint inhibitors. In breast cancer PD-1, expression of CTLA-4, TIM-3, and LAG-3 is elevated on removal of the repressive histone modifications [115]. Unlike HMAs, HMT and KDM inhibitors appear to be more specific, and combining histone methylome drugs with checkpoint inhibitors presents a promising therapeutic avenue. Apart from directly modulating checkpoint molecules, histone methylation has been described to play a pivotal role in determining expression of key cytokines, thus regulating the cytokine milieu in the tumor microenvironment, which in turn affects immune cell infiltration. Thus, epigenetic reversal of aberrant histone methylation can provide for enhanced recruitment of cytotoxic immune cells, efficient targeting of AML blasts, and improved patient survival.

Discussion and future perspective

Characterization of mutational landscape in AML, owing to the advent of genome sequencing studies over the past several years, has significantly increased our knowledge of alterations involving epigenetic modifiers and the importance of epigenetic contribution in AML pathogenesis. Despite significant progress in understanding the molecular basis of AML, treatment has remained stagnant for the past four decades. Epigenetic plasticity and transcriptional dysregulation are key hallmarks in leukemogenesis, which can contribute immensely to treatment responses. In this effort we have highlighted critically emerging regulators of histone methylation and the alterations they undergo during AML transformation, as well as their potential to serve as therapeutic targets. Epigenetic therapy is an emerging proposition with an immense potential. However, there are multiple aspects and caveats that need to be considered before it can synergize with, or even substitute for, conventional chemotherapy as the mainstay of AML treatment. Specific clinical patterns of sensitivity to epigenetic therapies are associated with molecularly defined subtypes and genetic signatures. Therefore, a major challenge in determining appropriate epigenetic drugs will be based on individual genetic barcoding of the cohort. Indeed, this would demand robust sensitivity studies involving in silico and in vitro small molecule inhibitor and genetic screening, combined with an in-depth characterization, to identify specific "epigenetic lesions" and their respective drivers.

Epigenetic inhibitors often do not destroy the malignant clone, but rather promote differentiation of leukemia cells. To achieve complete remission, they need to be combined with other small molecule inhibitors, immunomodulatory drugs, or conventional chemotherapy. Thus, design of newer trials should involve an understanding of the synergism and antagonism of epigenetic drugs with current therapeutic modalities. As witnessed by recent clinical trial failures, new models to test epigenetic therapies must be developed, possibly employing patient samples and patient-derived xenografts, as well as genetically engineered mouse models that recapitulate the entire spectrum of AML mutations. Another significant challenge to AML therapy is therapy resistance and subsequent relapse. With the recent technological advancements, combining epi/genomic, transcriptomic, proteomic, and mass cytometry analysis, even at the single-cell level, with an unsupervised systems learning approach matched with in vivo clonal repopulation assays, it is now plausible to trace the evolution of individual clones to better understand AML pathophysiology and identify molecular vulnerabilities.

As AML is a heterogeneous disease, individual subclones are phenotypically and functionally different. Proteomics-based assays have identified 50 AML-enriched plasma membrane proteins, permitting isolation of individual clones from an oligoclonal patient [116]. This is integral to tracing the individual clones in a heterogeneous setting, and can be used for diagnosis, treatment, and survival prediction. Tracing of

individual clones is a robust evaluation of minimal residual disease. A clone-specific strategy employing next-generation sequencing and fluorescent in situ hybridization in 69 AMLs with known clonal architecture revealed that the presence of two or more lesions in more than 0.4% of remission cells correlates with lower overall and disease-free survival [117]. This contrasts with recent studies indicating that residual mutations in less than 5% of cells in complete remission correlates with a better survival. Lineage tracing has also enhanced our understanding of mechanisms of chemoresistance and how it can be overcome. A recent study using lentivirus-mediated DNA barcoding in human AML cells identified DNMT inhibition as preventing outgrowth of chemoresistant clones with enhanced stemness [118]. Currently, a limited number of selective inhibitors against epigenetic targets are available, and efforts to develop a broader arsenal are underway. Future studies focusing on discerning the molecular regulation of pathologically relevant histone methyl *writers, readers,* and *erasers,* their potential crosstalk, and the pivotal role of critical histone residues will be instrumental for the development of next-generation AML-targeted therapy.

Conflict of interest disclosure

The authors declare no conflicts of interest.

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