Rearrangements of the Mixed-Lineage Leukemia (MLL) gene are found in > 70% of infant leukemia, ~ 10% of adult acute myelogenous leukemia (AML), and many cases of secondary acute leukemias. The presence of an MLL rearrangement generally confers a poor prognosis. There are more than 60 known fusion partners of MLL having some correlation with disease phenotype and prognosis. The most common fusion proteins induce the inappropriate expression of homoeotic (Hox) genes, which, during normal hematopoiesis, are maintained by wild-type MLL. MLL-rearranged leukemias display remarkable genomic stability, with very few gains or losses of chromosomal regions. This may be explained by recent studies suggesting that MLL-rearranged leukemias are largely driven by epigenetic dysregulation. Several epigenetic regulators that modify DNA or histones have been implicated in MLL-fusion driven leukemogenesis, including DNA methylation, histone acetylation, and histone methylation. The histone methyltransferase DOT1L has emerged as an important mediator of MLL-fusion-mediated leukemic transformation. The clinical development of targeted inhibitors of these epigenetic regulators may therefore hold promise for the treatment of MLL-rearranged leukemia.

**Introduction: MLL-rearranged leukemia**

Leukemias bearing translocations involving chromosome 11q23 are of particular interest due to unique clinical and biological characteristics. 11q23 rearrangements are found in > 70% of leukemias in infants < 1 year of age whether the immunophenotype is more consistent with acute lymphoblastic leukemia (ALL) or acute myelogenous leukemia (AML). Infants diagnosed with ALL harboring an 11q23 translocation have a particularly poor prognosis, with an overall survival of ~ 50%, whereas children with ALL that does not harbor a MLL translocation have an overall survival of > 80%. In contrast to other poor-prognosis ALL, the prognosis of most infant MLL-rearranged leukemia is not improved by the use of allogeneic stem cell transplantation. A subgroup of infants with additional high-risk features may benefit from hematopoietic stem cell transplantation (HSCT) during the first complete remission, but the 5-year event-free and overall survival rates in this group remain in the 50% range. Leukemias that develop as a result of treatment with topoisomerase II inhibitors frequently harbor 11q23 rearrangements and also have a poor prognosis. Some infant leukemias express cell-surface antigens characteristic of both lymphoblasts and monoblasts, and are often designated mixed phenotype acute leukemia (MPAL). The association of 11q23 rearrangements with ALL, AML, or MPAL is unique in that most other chromosomal rearrangements are not improved by the use of allogeneic stem cell transplantation. The fusion occurs in the 50% range. Leukemias that develop as a result of treatment with topoisomerase II inhibitors frequently harbor 11q23 rearrangements and also have a poor prognosis. Some infant leukemias express cell-surface antigens characteristic of both lymphoblasts and monoblasts, and are often designated mixed phenotype acute leukemia (MPAL). The association of 11q23 rearrangements with ALL, AML, or MPAL is unique in that most other chromosomal rearrangements tend to be associated with leukemias of a particular hematopoietic lineage. These observations led to the name Mixed-Lineage Leukemia (MLL) for the gene that resides on 11q23.

The most common MLL translocation found in lymphoblastic leukemias is t(4;11)(q21;q23). Multiple investigators cloned the MLL-AF4 gene formed as a result of this translocation in the early 1990s. MLL-AF4 encodes a protein of 2304 amino acids, with the NH2-terminal 1439 amino acids derived from MLL on chromosome 11 and the COOH-terminal 865 amino acids from the AF4 gene on chromosome 4. Subsequently, > 60 different translocations have been identified, all of which appear to produce a fusion protein possessing the NH2-terminus of MLL fused in-frame to the COOH-terminus of the fusion partner. Whereas MLL translocations can be found in either ALL or AML, particular translocations show lineage specificity, with the t(4;11) found most often in ALL, the t(9;11)(p21;q23) found most often in AML, and the t(11;16) found most often in myelodysplastic syndrome and secondary leukemia. A recent retrospective analysis demonstrated that the prognosis of MLL-rearranged leukemias may also be influenced by the fusion partner. Survival associated with the rare t(1;11)(q21;q23) translocation was good, in contrast to very poor outcomes in with the more frequent t(4;11)(q21;q23), t(10;11)(p12;q23) (MLL-AF10), t(10;11)(p1.2;q23) (MLL-ABI-1), and t(6;11)(q27;q23) translocations in this series. This points to the translocation partner as having a role in the disease phenotype and functional heterogeneity of MLL fusions, but the molecular details of these associations are unclear.

**MLL function in hematopoiesis**

The MLL gene, located on the human chromosome 11q23, is the ortholog of the *Drosophila melanogaster* trithorax gene. Trithorax group (trxG) and polycomb group (PcG) genes were discovered in *Drosophila* as activators and repressors of homeobox (Hox) genes. Hox genes are a group of transcription factors involved in the development of cell identity and anteroposterior patterning in mammals, which also play a critical role in hematopoiesis. Mll is also fundamentally involved in the development of the axial skeleton and the hematopoietic system, at least in part via direct regulation of Hox gene expression. The MLL gene encodes a 3969-amino acid DNA-binding protein that possesses multiple recognizable protein motifs, including an NH2-terminal DNA-binding domain, transcriptional activation and repression domains, and a COOH-terminal SET domain that contains histone H3 lysine 4 (H3K4) methyltransferase activity. Recent biochemical studies have identified MLL as a member of a large multiprotein complex...
that contains proteins involved in chromatin modification/remodeling. The complex notably includes histone deacetylases (HDACs) and members of the Swi/Snf chromatin-remodeling complex. In addition, MLL is recruited to the promoters of select cell cycle-regulatory genes by the protein product of the MEN1 tumor-suppressor gene, suggesting a role for MLL in tumor suppression and cell-cycle control. These data support the hypothesis that the MLL protein regulates gene expression and cell-cycle control via chromatin modification.

Analysis of Mll-knockout mice suggests that Mll plays an important role in development and hematopoiesis through maintenance of appropriate homeotic (Hox) gene expression. Detailed studies assessing the specific role of Mll in hematopoietic development have shown that Mll is necessary for definitive hematopoiesis and expansion of hematopoietic progenitors and stem cells found in the aorta-gonad-mesonephros region of the developing embryo. The defect in hematopoietic progenitor expansion can be rescued by reexpression of Hox genes, confirming the importance of Mll-mediated Hox gene expression during hematopoiesis.

Multiple studies have demonstrated the ability of Hox genes to induce leukemia in mice, and the t(7;11)(p15;p15) translocation found in some human AML cases results in a fusion of the HOXA9 gene to the nucleoporin NUP98. Given the apparent importance of Hox genes in leukemogenesis, and the fact that MLL-fusion proteins generated by MLL translocations activate Hox gene expression (as described below), hox gene activation is likely a critical path of the leukemogenic program driven by MLL-fusion proteins. Further support for Hox genes as central regulators of MLL-induced leukemogenesis comes from gene-expression studies finding multiple HOXA cluster genes more highly expressed in MLL-rearranged myelogenous and lymphoblastic leukemias compared with MLL-germline leukemias.

**Gene-expression programs in MLL-rearranged acute leukemia**

Gene-expression studies of human MLL-rearranged B-precursor ALL demonstrated that hundreds of genes are differentially expressed compared with other B-precursor ALLs. Based on the magnitude of the differences in gene expression, MLL translocations appear to specify a unique lymphoblastic leukemia. Other large gene-expression studies have also shown that ALLs with distinct chromosomal rearrangements have unique gene-expression profiles, which provides support for this concept. The genes that are relatively highly expressed in MLL-rearranged B-precursor ALL are those associated with hematopoietic progenitors and developing myeloid cells, whereas the genes expressed at lower level are genes associated with lymphoid identity. Specific gene-expression signatures have also been associated with MLL translocations in primary human AML blasts. Even though there are clear differences between MLL-rearranged ALL and MLL-rearranged AML in the expression of lineage-associated genes, there appears to be a core gene-expression profile found in all MLL-rearranged human leukemias independent of the lineage markers. Presumably, MLL-fusion proteins directly regulate a subset of these genes. This is further supported by the fact that MLL-associated signatures across all studies and phenotypes contain multiple highly expressed HOX genes.

**Is there a multistep pathogenesis of MLL-rearranged leukemias?**

Mouse models of leukemia predict multiple genetic events are necessary for the development of acute leukemias. “Knock-in” models of MLL-rearranged AML have been developed, in which the fusion genes generated by translocations found in human leukemias are under the control of the endogenous promoter. Mice containing an MLL-AF9 fusion gene under control of the Mll promoter spontaneously develop AML with a latency of 4 months to >1 year. This is widely interpreted as a requirement for a second genetic event during leukemogenesis. A multistep model of leukemogenesis is further supported by mouse models such as an Mll-Chp knock-in or an Aml1-Eto knock-in model, which do not spontaneously develop leukemia. In these models, either irradiation or chemical mutagenesis is necessary to induce leukemias, thus they clearly require multiple events for the development of leukemia. Several lines of evidence suggest that a multistep model of leukemogenesis also applies to human acute leukemia. Elegant epidemiologic studies suggest that childhood leukemias require at least 2, and probably more, genetic events for leukemia development. In particular, lymphoblastic leukemias with TEL-AML1 rearrangements appear to develop after a multistep process. TEL-AML1 rearrangements are often detected in blood taken at birth from a child who will develop ALL 3-5 years later. A recently described twin pair, one with TEL-AML1 ALL, and one clinically healthy, allowed the isolation of a clearly abnormal, TEL-AML1-bearing preleukemic clone that up to that point had failed to cause clinically overt leukemia in the unaffected twin. This suggests that TEL-AML1 rearrangements are the first genetic event, but that additional mutations are required for the development of ALL. Similar studies performed on newborn blood spots from children who subsequently develop MLL-rearranged ALL show that MLL-translocations clearly arise in utero and are detectable at birth, whereas the clinical disease develops some time in the first year of life.

Mutant signaling molecules are attractive candidates for cooperation with translocation-associated fusion proteins during leukemogenesis. Ever-increasing evidence suggests that activated kinases play a central role in the pathogenesis of leukemias and myeloproliferative syndrome. The most dramatic evidence for such a role is activation of the ABL tyrosine kinase by the BCR-ABL fusion produced by the t(9;22) and its inhibition by imatinib (Gleevec). Other mutant kinases frequently identified in AML are the receptor tyrosine kinases FLT3 and c-KIT. Recent mouse experiments support the hypothesis that DNA-binding fusion proteins generated by leukemia-associated translocations perform different functions than activated tyrosine kinases. DNA-binding fusions induce either a block in differentiation or activate self-renewal in developing hematopoietic progenitors, whereas activated kinases may provide a survival or proliferation signal. These data prompted the hypothesis that at least 2 different classes of mutations are necessary for leukemogenesis. The identification of FLT3 mutations and RAS mutations in MLL-rearranged ALLs suggests that a multistep process may also be necessary.

Given the widespread epigenetic dysregulation (described below), the question arises as to whether the model of multistep pathogenesis might not apply to MLL-rearranged leukemias. Rather, epigenetic changes alone may be sufficient for oncogenesis driven by MLL-fusion proteins. In this scenario, mutations such as those described in FLT3 or RAS are acquired after the development of leukemia and represent clonal outgrowth of leukemia cells that
either proliferate more rapidly or are less likely to undergo apoptosis. This interpretation is supported by careful tracking of the clonal evolution of (non-MLL-rearranged) ALL, which has revealed that bulk leukemias are comprised of several clones and subclones with different cytogenetic abnormalities that over time contribute to various extents to the clinical disease.\(^43,44\) Multiple mutations might therefore influence the disease phenotype or confer a selective advantage to the respective subclone, but not be necessary for initial leukemia development. Recent genome-wide studies have demonstrated very few gains or losses of chromosomal regions in MLL-rearranged ALLs, providing further support for the concept that MLL-rearranged leukemias may be largely a result of epigenetic dysregulation.\(^45\) If MLL-rearranged leukemias are largely driven by epigenetic changes, then therapeutics targeting chromatin structure might be particularly useful in this disease. Possible approaches that focus on targeting epigenetic modifications are described below.

### Targeting DNA methylation in MLL-rearranged leukemia

Recent studies have focused on the role of DNA methylation at CpG dinucleotides in normal hematopoiesis, leukemia development, and therapeutic response. The majority of DNA methylation found in cells is controlled by the de novo methyltransferases DNMT3A and DNMT3B and by the maintenance methyltransferase DNMT1, whereas a recently described form of DNA methylation, 5-hydroxymethylation, is generated by the proteins TET1-TET3.\(^46\) These DNA modifications likely play multiple roles in the control of gene expression and/or genome integrity via modulation of chromatin state and access of transcriptional complexes to their target genes. However, the most well-described function of DNA methylation is repression of gene expression as a result of methylation at CpG islands located near genes. Recent studies in mice have demonstrated a critical role for DNA methylation in the development and maintenance of normal hematopoietic stem cells and murine leukemias through genetic inactivation of Dnmt1.\(^47,48\) Inactivation of Dnmt1 led to inappropriate expression of myeloid cell developmental programs in hematopoietic stem and progenitor cells, leading to inappropriate cell fate decisions and stem cell loss. Inactivation of Dnmt1 also delayed the onset of MLL-AF9–mediated AML and suppressed the development of MYC-induced leukemia/lymphomas.\(^48\) These initial studies demonstrate that DNA methylation is indeed critical for normal hematopoiesis and leukemia development, providing support for therapeutic approaches that target DNA methylation in leukemia.

Recent clinical studies have, with some success, assessed the therapeutic activity of hypomethylating agents in patients with myelodysplastic syndrome.\(^49,50\) Similar studies are ongoing in patients with various leukemias and solid tumors.\(^51,52\) Given that MLL-fusion proteins transform cells via aberrant epigenetic programs, as described below, it is possible that modifying the DNA-methylation state in MLL-rearranged leukemia cells could lead to relevant changes in gene expression and thus have therapeutic efficacy. Indeed, studies in cell lines have shown that treatment of MLL-rearranged ALL cells with hypomethylating agents leads to the reactivation of tumor suppressor gene expression and the inhibition of cell growth and proliferation.\(^53\) Furthermore, DNA-methylation profiling of MLL-rearranged ALLs showed unique profiles for these leukemias compared with other ALLs and also an association between elevated DNA methylation and therapeutic outcome.\(^54,55\) Whereas there are little data on the use of hypomethylating agents against MLL-rearranged leukemias in vivo, MLL-rearranged leukemia cell lines do appear to be more sensitive to hypomethylating agents in vitro than some other subtypes of leukemia.\(^55\) In addition, TET1, the founding member of the TET family of proteins that are responsible for 5-hydroxymethylation of DNA, was initially identified in a leukemia possessing a t(10;11)(q22; q23) translocation that generates an MLL-TET1 fusion protein. This points to hydroxymethylation as potentially relevant in some subsets of leukemias with MLL translocations.\(^56\) Whereas further study is clearly required to fully understand the relevance of DNA methylation in MLL-rearranged leukemias, targeting DNA methylation is an approach worthy of further evaluation.

### Targeting histone acetylation in MLL-rearranged leukemia

An initial hint that histone acetylation might play a role in MLL-rearranged leukemia stems from the binding of HDACs to the MLL wild-type protein.\(^57\) A recent study demonstrated that the recruitment of HDACs to MLL-bound promoters is mediated by the MLL-PHD3-Bromo cassette stabilized by Cyp33, resulting in silencing of the respective genes. This may represent an important mechanism for the physiological repression of MLL-regulated gene-expression programs as cells differentiate.\(^58\) In MLL-rearranged leukemias, the PHD3-Bromo cassette is replaced by the fusion partner, and loss of the MLL-PHD domain has been confirmed to be required for the transforming activity.\(^59,60\) The loss of HDAC-mediated silencing of MLL-target genes may contribute to MLL-fusion–mediated leukemogenesis.

Whereas locus-specific loss of HDAC activity may contribute to leukemogenesis, more global modulation of histone acetylation might reorganize the chromatin state such that critical epigenetic programs are lost. Several HDAC inhibitors have entered clinical trials for hematologic malignancies and have shown responses in selected patients.\(^52,61\) In preclinical studies, pharmacologic inhibition of HDACs with various pan-HDAC inhibitors was reported to exhibit in vitro antiproliferative and pro-apoptotic effects on a panel of B-ALL cell lines, including several bearing an MLL rearrangement, and in primary MLL-rearranged ALL samples.\(^52,62\) Analysis of HDAC isoform–specific inhibitors revealed that compounds selectively targeting class I HDACs also inhibited proliferation and induced apoptosis in MLL-rearranged B-ALL cell lines in a dose range that is consistent with specific target inhibition. Compounds with even further specificity for HDAC1 and HDAC2 also exhibited toxicity toward the B-ALL cell line panel, including those with MLL rearrangements. A phase 1A/2 study of the pan-deacetylase inhibitor panobinostat (LBH589) showed 5 responses in 26 response-evaluable patients with AML. One of the 2 observed complete remissions was achieved in a patient with an MLL-CBP fusion (t(11;16)(q23;p13)).\(^64\) CBP (also called CREB binding protein or CEBBP) is a histone acetyltransferase and transcriptional coactivator. Recurrent mutations of CBP have also been described recently in samples of relapsed ALL (18.3%, including one case with an MLL rearrangement).\(^65\) Functional in vitro studies suggest that CBP mutations may negatively affect glucocorticoid responsiveness. Glucocorticoid response has been validated in multiple clinical studies as an independent prognostic marker in MLL-rearranged infant ALL. These findings suggest that HDAC inhibitors may be interesting therapeutic approaches for MLL-rearranged leukemias, possibly those with MLL-CBP or MLL-p300 rearrangements or perhaps glucocorticoid-resistant MLL-rearranged infant ALL in particular.
Targeting histone methylation in MLL-rearranged leukemia

Biochemical studies have shown that MLL-fusion oncoproteins copurify with protein complexes normally involved in transcriptional elongation. This suggests that MLL-fusion proteins induce leukemia via unregulated transcriptional regulation at MLL-fusion target genes. At least 3 related complexes, PAFc, DOT1L, and pTEFb (also designated AEP or SEC), have been shown to bind to MLL-fusion proteins. The polymerase-associated factor complex (PAFc) associates with the NH3-terminal portion of wild-type MLL and MLL-fusion proteins and regulates RNA polymerase II. The pTEFb complex (CDK9/cyclinT) releases stalled RNA polymerase II and thus stimulates transcriptional elongation. pTEFb interacts with MLL-fusion partners such as ENL, ELL (as well as ELL2, ELL3), AF4, and AF5, and copurifies with the respective MLL fusions. The third, the DOT1L complex, consists of DOT1L, a histone methyltransferase that modifies histone H3 on lysine 79 (H3K79), and multiple MLL-fusion partners such as AF9, ENL, AF17, and AF10. Based on biochemical interactions, it appears that MLL fusions regulate transcription via recruitment of one or several of these protein complexes. ENL has been identified as a core member of both the pTEFb and Dot1I complexes. However, whether and how these complexes interact, and the extent to which each contributes to MLL-fusion-mediated leukemia development is just beginning to be understood. Another example of the recruitment of a histone-modifying enzyme by an MLL-fusion protein is the MLL-ENL fusion, which requires an arginine methyltransferase for its transforming activity. Because studies have linked histone methylation and particularly methylation of H3K79 to positive transcriptional regulation, the aberrant recruitment of H3K79 methyltransferase activity or arginine methyltransferase activity might directly influence gene expression. Therefore, at least for these fusion proteins, association with a unique histone methyltransferase may be important for leukemogenic transformation.

The association of MLL-fusion proteins with the histone methyltransferase DOT1L has prompted assessment of H3K79 methylation in MLL-rearranged patient cells and leukemia models. Early evidence that MLL fusions may regulate gene expression via histone modification came from studies assessing the MLL-ENL fusion. Upon expression of MLL-ENL in immortalized murine hematopoietic progenitors, H3K79 levels associated with the HoxA9 and Meis1 promoters increased. Further studies demonstrated enhanced H3K79 methylation associated with genes overexpressed in MLL-rearranged leukemia cell lines and murine leukemia models on a genome-wide level. These observations hold true for primary human leukemia samples. Similar to the mouse model, enhanced H3K79 methylation is associated with the HOXA cluster in human leukemia cells. Genome-wide analysis of primary MLL-rearranged leukemia samples has revealed a distinct H3K79 methylation profile and a unique transcription profile that can distinguish MLL-rearranged leukemias from leukemias with germline MLL.

In addition to being associated with actively transcribed genes, H3K79 methylation appears to be associated with direct MLL-fusion target genes. A recent study determined the genome-wide promoter occupancy of the MLL-AF4 protein and showed that H3K79 methylation was associated with MLL-AF4-target genes in a human MLL-rearranged cell line. Similarly, aberrant H3K79 methylation has been demonstrated on direct MLL-AF9 targets in a murine model of MLL-AF9 fusion-driven leukemia. In this study, detailed analysis of multiple epigenetic modifications and correlation with expression levels revealed abnormally high peaks and broader spread of H3K79 methylation, specifically on fusion target genes. This was specific to MLL-AF9 leukemia cells, because H3K79 methylation profiles were normal when the same loci were expressed under physiologic regulation in normal hematopoietic progenitors.

Functional studies that suppress or inactivate Dot1L expression also support a critical role for H3K79 methylation in MLL-fusion-mediated transformation and leukemogenesis. shRNA-mediated suppression of DOT1L in human leukemia cell lines has been shown to suppress MLL-fusion-mediated target gene expression, and this suppression leads to growth arrest and apoptosis in MLL-AF4 ALL and MLL-AF9 AML cell lines. Several groups of investigators have recently developed conditional loss-of-function mouse models and have shown that inactivation of DOT1L in MLL-fusion-transformed cells leads to differentiation and apoptosis in vitro.3,4 Furthermore, inactivation of Dot1L in mouse MLL-AF9-mediated AML inhibits leukemia development.3,4 On a molecular level, loss of Dot1L and H3K79 methylation led to the collapse of a leukemia-associated gene-expression signature that contained many of the MLL-AF9 target genes. In contrast, global gene expression levels did not change despite association of H3K79 methylation with as many as 11% of all genes. The notion that the loss of widespread chromatin marks may not affect the majority of genes associated with this modification, but rather may induce very specific gene-expression changes on a small subset of genes, is supported by recent studies on epigenetic regulation in yeast. It is therefore possible that the recruitment of Dot1L and the institution of abnormal histone methylation patterns at key loci of leukemic transcriptional programs is a crucial step in MLL-fusion-mediated transformation and in the maintenance of MLL-fusion protein-mediated gene expression. Initial studies on hematopoiesis in conditional Dot1l-knockout mice show that hematopoietic cells can develop in neonatal mice in the absence of Dot1L, albeit less efficiently than in wild-type animals and that inactivation of Dot1L in adult mice leads to pancytopenia, within 1-2 months. Furthermore, hematopoietic cells transformed by other leukemogenic oncogenes such as HoxA9 and Meis1 do not appear to be affected in their growth. These studies support the hypothesis that DOT1L is a potential therapeutic target in this disease and that continued development of DOT1L inhibitors is warranted.

Conclusions

The recent development of a specific small-molecule inhibitor of DOT1L will be instrumental in addressing some fundamental questions about the therapeutic potential for inhibition of H3K79 methylation, in addition to providing proof of principle for the clinical development of DOT1L inhibitors. EPZ004777 is a competitive inhibitor of the methyl donor S-adenosyl-methionine. Analysis of this compound on a panel of human leukemia cell lines revealed antiproliferative activity in the nanomolar or low micromolar range that was remarkably selective for cell lines bearing MLL rearrangements. On a molecular level, treatment with the inhibitor causes a decrease in mRNA expression of known MLL-fusion
target genes, including HOX9 and MEIS1. In addition, inhibitor-treated, MLL-rearranged cell lines exhibited cell-cycle arrest in G0/G1, an increase in expression of differentiation markers in MLL-rearranged AML cells, and death by apoptosis. These data provide a very compelling rationale for the clinical development of DOT1L inhibitors as targeted therapeutics for MLL-rearranged leukemias.

Disclosures
Conflict-of-interest disclosure: S.A.A. has consulted for Epizyme. K.M.B. declares no competing financial interests. Off-label drug use: None disclosed.

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