Leukemogenesis via aberrant self-renewal by the MLL/AEP-mediated transcriptional activation system

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Abstract
Homeostasis of the hematopoietic system is achieved in a hierarchy, with hematopoietic stem cells at the pinnacle. Because only hematopoietic stem cells (HSCs) can self-renew, the size of the hematopoietic system is strictly controlled. In hematopoietic reconstitution experiments, 1 HSC can reconstitute the entire hematopoietic system, whereas 50 multipotent progenitors cannot. This indicates that only HSCs self-renew, whereas non-HSC hematopoietic progenitors are programmed to differentiate or senesce. Oncogenic mutations of the mixed lineage leukemia gene (MLL) overcome this "programmed differentiation" by conferring the self-renewing ability to non-HSC hematopoietic progenitors. In leukemia, mutated MLL proteins constitutively activate a broad range of previously transcribed CpG-rich promoters by an MLL-mediated transcriptional activation system. This system promotes self-renewal by replicating an expression profile similar to that of the mother cell in its daughter cells. In this transcriptional activation system, MLL binds to unmethylated CpG-rich promoters and recruits RNA polymerase II. MLL recruits p300/CBP through its transcriptional activation domain, which acetylates histone H3 at lysines 9, 18, and 27. The AF4 family/ENL family/P-TEFb complex (AEP) binds to acetylated H3K9/18/27 to activate transcription. Gene rearrangements of MLL with AEP- or CBP/p300-complex components generate constitutively active transcriptional machinery of this transcriptional activation system, which causes aberrant self-renewal of leukemia stem cells. Inhibitors of the components of this system effectively decrease their leukemogenic potential.

KEYWORDS
leukemia, molecular therapy, self-renewal, transcriptional machinery

Abbreviations: AEP, AF4 family/ENL family/P-TEFb complex; ALL, acute lymphoid leukemia; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; HAT, histone acetyltransferase; HMT, histone methyltransferase; HSC, hematopoietic stem cell; LEDGF, lens epithelium-derived growth factor; MBM, menin-binding motif; MLL, mixed lineage leukemia; MLL-r, MLL-rearranged; MPP, multipotent progenitor; P-TEFb, positive transcription elongation factor b; RNAP2, RNA polymerase II; SL1, selectivity factor 1.
1 | INTRODUCTION

In a multicellular organism, different types of cells form a society in which they influence one another. In the hematopoietic system, homeostasis is achieved in a hierarchy, with HSCs at the pinnacle. Because only HSCs can self-renew, the size of the hematopoietic system is strictly controlled. In hematopoietic reconstitution experiments in which hematopoietic cells are transplanted into lethally irradiated mice, 1 HSC can reconstitute the entire hematopoietic system in the recipient mice, whereas 50 multipotent progenitors cannot (Figure 1), indicating that only HSCs self-renew, whereas non-HSC hematopoietic progenitors are programmed to differentiate or senesce. Non-HSC hematopoietic progenitors cannot self-renew, presumably, because they lose a portion of their identity in every cell division. However, oncogenic mutations overcome this “programmed differentiation” by conferring the self-renewing ability to non-HSC hematopoietic progenitors. Consequently, immature progenitors expand indefinitely, occupy the bone marrow, overflow to the bloodstream, and infiltrate other organs. A transcriptional activation system specialized to reactivate previously transcribed gene promoters promotes self-renewal by replicating an expression profile similar to that of the mother cell in the daughter cells. Constitutive activation of this transcriptional activation system due to mutations leads to leukemia. This review describes the mechanism of leukemogenesis as a consequence of aberrant self-renewal of non-HSC hematopoietic progenitors and introduces several promising molecularly targeted therapies.

2 | MOLECULAR MECHANISMS OF MLL-REARRANGED LEUKEMIA

The MLL gene (also known as KMT2A) is altered by chromosomal translocations frequently observed in leukemia patients. MLL-rearrangements generate the fusion genes encoding the MLL fusion protein, which is constituted by the N-terminal half derived from MLL and the C-terminal half derived from its fusion partner (Figure 2A). To date, more than 80 MLL fusion partners have been reported. MLL gene rearrangements are responsible for 6%-7% of both acute myeloid leukemia and acute lymphoid leukemia (ALL) cases.

FIGURE 1 Leukemogenesis by aberrant self-renewal. One hematopoietic stem cell can reconstitute the entire hematopoietic system, whereas 50 multipotent progenitors fail to reconstitute it. Leukemia stem cells, which overcome “programmed differentiation,” aberrantly self-renew to hijack the hematopoietic system. CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; HSC, hematopoietic stem cell; MPP, multipotent progenitor.
by very few additional mutations\textsuperscript{15,16} and is uniquely prevalent in malignant infant ALL\textsuperscript{17}

Immature hematopoietic progenitors proliferate briefly ex vivo when treated with myeloid cytokines, but they eventually differentiate and stop forming proliferative colonies in semisolid media.\textsuperscript{5} During this differentiation process, cells drastically change their expression profiles and morphologies. cKit-positive immature hematopoietic cells express high levels of HOXA9 and MEIS1 when freshly isolated from the bone marrow but lose their expression after ex vivo culture for 1 wk. Conversely, forced expression of HOXA9 promotes self-renewal and immortalizes cKit-positive myeloid progenitors ex vivo.\textsuperscript{18} When MLL fusion genes are exogenously expressed in myeloid progenitors, they express high levels of Hoxa9 and become immortalized.\textsuperscript{2,3,5,19} Therefore, MLL fusion proteins cause uncontrolled self-renewal of hematopoietic progenitors by maintaining the expression of genes programmed to be suppressed during differentiation.

The N-terminal MLL portion contains several evolutionarily conserved structures that bind to proteins and DNA. The MLL fusion protein associates with menin and lens epithelium-derived growth factor (LEDGF) through the menin-binding motif (MBM) and LEDGF-binding domain, respectively.\textsuperscript{20,21} Because the MLL portion has a CXXC domain that selectively associates with unmethylated CG sequences (CpG),\textsuperscript{22,23} MLL fusion proteins associate with a broad range of target promoter recognition by MLL fusion proteins. A, The structure and function of MLL fusion proteins. The CXXC domain of MLL specifically binds to unmethylated CpG, while the PWWP domain of LEDGF recognizes H3K36me2/3 marks. B, Menin-dependent promoter recognition by MLL fusion proteins. More than 80 different fusion partners have been reported\textsuperscript{13} and discussed further in Figure 4. An MLL fusion-menin complex associates with LEDGF and unmethylated CpG at the target promoter. LEDGF, lens epithelium-derived growth factor; MLL, mixed lineage leukemia.
of CpG-rich promoters. These proteins first associate with menin and subsequently bind to LEDGF and unmethylated CpGs on chromatin (Figure 2B). LEDGF binds to di-/tri-methylated histone H3 lysine 36 (H3K36me2/3) through its PWPP domain,24,25 H3K36me3 marks are introduced by SETD2 histone methyltransferase (HMT) in a manner coupled with transcription26 (Figure 3). Therefore, LEDGF preferentially binds to transcriptionally active chromatin. Consequently, the MLL fusion complex localizes to previously transcribed CpG-rich promoters.6 Recently, our group reported that MLL not only binds to unmethylated CpGs but also recruits RNA polymerase II (RNAP2) through its CXXC domain.27 Localization of RNAP2 at MLL target promoters substantially decreases in MLL-depleted cells, indicating that MLL promotes the early stages of transcription by recruiting RNAP2 to CpG-rich promoters.

Many of the 80-plus MLL fusion partners have functional domains that activate transcription. A select few fusion partners with transcriptional activation functions account for the majority of the MLL-r leukemia cases. The most frequent fusion partners are AF4 family proteins, including AF4 (also known as AFF1) and AF5q31 (also known as AFF4), and ENL family proteins, including ENL (also known as MLLT1) and AF9 (also known as MLLT3). These 2 families account for two-thirds of the MLL-r leukemia cases. AF10 (also known as MLLT10), ELL, and AF6 (also known as MLLT4 or AFDN) are considered major fusion partners. The AF4 family and ENL family proteins form a biochemically stable complex with the positive elongation factor b (P-TEFb), a complex of CDK9 and cyclin T1/T2.7,28-30

We named this complex AEP (AF4 family/ENL family/P-TEFb complex).7 Similar complexes have been characterized as transcription elongation factors by others and are referred to as the super elongation complex.28-30 MLL has a transactivation domain that associates with CBP/p300 histone acetyltransferases (HATs).31 MLL recruits CBP/p300 HATs cooperatively with sequence-specific transcriptional factors (TFs) such as MYB and CREB31,32 (Figure 3), to generate acetylation marks of histone H3 lysine 9/18/27 (H3K9/18/27ac)23,34. Because AEP binds to H3K9/18/27ac through the YEATS domain of ENL family proteins,35,36 AEP is recruited to the promoter regions where MLL resides.27 AEP further recruits the selectivity factor 1 (SL1) complex37 which is known as a core transcription factor in RNA polymerase I-dependent transcription.38 AEP activates RNAP2-dependent transcription initiation presumably by loading the TATA-binding protein to the promoter through SL137 and promotes transcription elongation by phosphorylating RNAP2 and other transcriptional regulators, such as DSIF and NELF, through the P-TEFb complex.7,28 (Figure 3). MLL fusion proteins bind to CpG-rich promoters that have been transcribed6 and recruit RNAP2 through the CXXC domain,27 setting up transcriptional activation. MLL-ENL and MLL-AF4 constitutively recruit AEP components via direct interaction to activate transcription initiation and elongation7,37 (Figure 4A). Genetic analyses indicate that this MLL/AEP-mediated transcriptional activation system is active in normal HSCs and required for the maintenance of the HSC pool.39,40 It is presumed that the recruitment of CBP/p300 HATs and AEP is tightly regulated and progressively suppressed during differentiation in normal hematopoiesis, because non-HSC hematopoietic progenitors cannot maintain the expression of MLL target genes. However, the oncogenic mutants of MLL constitutively recruit either AEP or p300/CBP HATs to reactivate the genes previously expressed in the mother cell to produce daughter cells nearly identical to the mother cell. Consequently, MLL fusion proteins continuously activate the genes expressed in HSCs.27

MLL also fuses with AF10 family genes, including AF10 and AF17 (also known as MLLT6). AF10 family proteins form a complex with the DOT1L HMT and ENL family proteins.7,41,42 DOT1L is responsible for the mono-/di-/tri-methylation of lysine 79 of histone H3K79 (H3K79me1/2/3).43 This methylation maintains a transcriptionally active state of chromatin by counteracting transcriptional repressors such as SIRT1.44 Genetic inactivation of DOT1L in MLL fusion-immortalized cells induces differentiation.45-48 An artificial fusion construct of MLL and DOT1L transforms myeloid progenitors ex vivo.8 This MLL-DOT1L fusion loses its transforming property when its ENL-binding domains are deleted, indicating that it transforms hematopoietic progenitors by recruiting ENL to the target chromatin.8 Therefore, MLL-AF10 family fusion function as ENL recruiters that load ENL onto promoter-proximal chromatin to establish AEP (Figure 4B).

AFX (also known as FOXO4) forms an MLL-AFX fusion in leukemia patients. It transforms hematopoietic progenitors through the transactivation domain of AFX.49 This domain binds to CBP/p300 HATs, thereby depositing H3K9/18/27ac on the target chromatin, which subsequently recruits AEP.27 MLL also fuses with CBP/p300 HATs and transforms hematopoietic progenitors through its HAT activity.50-52 These MLL fusions function as acetyl mark providers and constitutively recruit AEP (Figure 4C).

MLL also fuses with ELL, which directly binds to AF4 family proteins.52 Therefore, MLL-ELL presumably functions as a direct AEP recruiter, similar to MLL-AF4. However, the ELL portion associates with the EAF family of proteins, and an artificial fusion of MLL and EAF1 transforms hematopoietic progenitors,53 indicating that EAF1 recruitment is also important. The precise molecular mechanism of MLL-ELL-mediated leukemogenesis remains to be elucidated (Figure 4D).

MLL fusion proteins with a dimerization domain are also able to transform hematopoietic progenitors. MLL-AF6 and MLL-GAS7 have been shown to employ this dimerization-mediated mechanism.54,55 Although it is unclear why a dimerization domain confers oncogenic ability, MLL-AF6 colocalizes with AEP and DOT1L at target promoters, suggesting that dimer type fusions also recruit AEP by unknown mechanisms7 (Figure 4D). These findings indicate that MLL fusion proteins bind to CpG-rich promoters with H3K36me2/3 marks through the N-terminal MLL portion. They further recruit RNAP2 through the CXXC domain and activate transcription by constitutively recruiting AEP through the C-terminal fusion partner. AEP activates both transcriptional initiation and elongation. The DOT1L complex plays an important role in the MLL/AEP-mediated transcriptional activation system, as
FIGURE 3  MLL-/AEP-mediated transcriptional activation system. After promoter entry, the MLL recruits RNAP2 and MOZ. In cooperation with sequence-specific TFs such as MYB and CREB. MLL recruits CBP/p300 HAT and generates H3K9/18/27ac marks. The DOT1L complex loads ENL onto acetylated nucleosomes to recruit the AEP components. AEP initiates transcription through SL1 and subsequently promotes elongation through P-TEFb. The DOT1L complex methylates histone H3K79 to repel SIRT1 transcriptional repressors. SETD2 associated with elongating RNAP2 generates H3K36me3 marks. Menin-MLL interaction inhibitors perturb the promoter binding of MLL, whereas DOT1L HMT inhibitors induce SIRT1-mediated transcriptional repression. AEP, AF4 family/ENL family/P-TEFb complex; HAT, histone acetyltransferase; HMT, histone methyltransferase; P-TEFb: positive transcription elongation factor b; RNAP2, RNA polymerase II; SL1, selectivity factor 1
it provides ENL to establish AEP and methylates histone H3K79 to counteract transcriptional repressors. Consequently, the CpG-rich promoters that are actively transcribed in the mother cell are also activated in the daughter cells after cell division, so that the daughter cells would express the same set of genes (Figure 5). This is likely to be the mechanism by which the MLL fusion protein promotes self-renewal to transform non-HSC hematopoietic progenitors into leukemia cells.

However, it should be noted that this "oncogenic self-renewal" does not occur by the exact same mechanism of self-renewal as that of normal HSCs. Only a fraction of the HSC-specific genes is maintained by MLL fusion. Indeed, the immune phenotype of the leukemia stem cells (LSCs) is Mac1/Gr1-positive in AML. Therefore, hyperactivation of the MLL/AEP-mediated transcriptional activation system does not ensure complete replication of the expression profile of the mother cell. It replicates its expression profile imperfectly, which is sufficient to maintain the expression of key proliferative genes such as Myc and Hoxa9, leading to unlimited proliferation without complete differentiation. Consequently, LSCs continue to produce their clones to induce full-blown leukemia.

3 | MOLECULAR TARGETED THERAPIES FOR MLL-R LEUKEMIAS

3.1 | Menin-MLL interaction inhibitors

MLL fusion proteins bind to menin to form a stable complex with LEDGF on the target chromatin. Therefore, inhibition of the interaction between menin and the MLL fusion protein would specifically attenuate the oncogenic property of the MLL fusion protein. Grembecka et al screened for compounds that interfere with the menin-MLL interaction and developed specific menin-MLL interaction inhibitors. Recently, compounds with improved pharmacokinetics have been developed and their clinical studies are underway (NCT04065399, NCT04067336, and NCT04811560).

MOZ is a HAT responsible for histone H3K14 acetylation. MOZ fuses with CBP/p300 and TIF2, which specifically binds to CBP/p300. MOZ associates with MLL and RNAP2 at CpG-rich promoters. Therefore, MOZ-TIF2 activates HOXA9 and other MLL target genes to transform non-HSC hematopoietic progenitors. The menin-MLL interaction is required for the function of wild-type

FIGURE 4 Mechanisms of AEP recruitment by various MLL fusions. Various mechanisms of AEP recruitment by MLL fusion are described. A. Fusions with ENL and AF4 family members directly recruit AEP components (direct AEP recruiter type). B. Fusions with AF10 family members load ENL through DOT1L (ENL provider type). C. Fusions with CBP/p300 or its binders induce H3K9/18/27ac marks to recruit AEP (acetyl mark provider type). D. MLL-ELL and MLL fusions with a dimerization domain recruit AEP through unknown mechanisms.
MLL as genetic ablation of menin results in decreased HOX gene expression in non-MLL-r cells.\textsuperscript{70,71} Because MOZ-TIF2 targets the chromatin in a wild-type MLL-dependent manner, menin-MLL interaction inhibitors attenuate the oncogenic property of MOZ-TIF2 in a manner similar to their inhibition of MLL fusion proteins.\textsuperscript{27} Leukemia cells with NPM1 mutations also express \textit{HOXA9} at high levels in a wild-type MLL-dependent manner. Menin-MLL interaction inhibitors have been shown to eradicate leukemia and preleukemia cells with NPM1 mutations.\textsuperscript{61,72,73} These results indicate that menin-MLL interaction inhibitors would be effective for not only MLL-r leukemia, but also for other types of leukemia that promote self-renewal in a wild-type MLL-dependent manner.
3.2 | DOT1L HMT inhibitors

Histone methylation by DOT1L inhibits transcriptional repressors, such as SIRT1, to maintain the self-renewing ability of LSCs.\textsuperscript{44} Daigle et al developed a DOT1L HMT inhibitor, EPZ-5676,\textsuperscript{74,75} which effectively inhibited the continuous proliferation of MLL- \(r\) leukemia cells in preclinical models. A clinical trial demonstrated that it induced complete remission in some patients but did not provide adequate therapeutic effects overall.\textsuperscript{76} Therefore, combination therapies were tested with chemotherapeutic agents (eg, cytarabine and daunorubicin) and DNA methylation inhibitors (eg, azacytidine and decitabine) and showed synergistic anti-tumorigenic effects in preclinical models.\textsuperscript{77} Because MLL fusion proteins constitutively recruit AEP, which cooperatively activates transcription with DOT1L,\textsuperscript{8} a combination of DOT1L HMT inhibitors and menin-MLL interaction inhibitors demonstrated higher anti-tumorigenic effects than either of the agents when used alone.\textsuperscript{8,78} In the future, DOT1L inhibitors are expected to contribute to the development of superior therapeutic strategies when used as part of combination therapies.

4 | CONCLUSION

Various biological capabilities are acquired during cancer development and are cataloged as hallmarks of cancer.\textsuperscript{79} Mutations that cause sustained proliferative signaling or resistance to cell death are prime examples. However, certain mutations found in leukemia are not regarded as the known hallmarks of cancer. Structure/function analysis revealed that gene rearrangements of MLL generate constitutively active transcriptional machinery that promotes self-renewal.\textsuperscript{6,27} Consequently, non-HSC hematopoietic progenitors acquire the ability to self-renew and develop leukemia.\textsuperscript{2,4} Therefore, promoting self-renewal is likely to be another hallmark of cancer, featured especially in hematopoietic malignancies. Whether this hallmark of cancer is acquired in non-hematopoietic malignancies remains to be elucidated. There are several molecularly targeted drugs currently in clinical trials that inhibit the components of this transcriptional activation system. They may provide therapeutic benefits to patients in the near future and be applied to other cancers that use similar mechanisms to promote aberrant self-renewal.

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