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Mechanisms of mixed-lineage leukemia

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Summary

Advances in our understanding of the genetic determinants of leukemia have translated to better treatment options and improved survival of patients with acute myeloid and acute lymphoid leukemia. However, some leukemias, such as those bearing 11q23 (*MLL*) translocations, result in aggressive diseases with a relatively poor prognosis, despite improved treatments such as allogeneic hematopoietic stem cell transplantation. This article will briefly review the functions and regulation of wild-type MLL during normal hematopoiesis, while focusing on recent advances in our understanding of the molecular mechanisms governing *MLL* leukemias. The transcriptional targets, cooperating signaling pathways and molecular machinery involved in *MLL*-associated leukemias will be discussed, as well as how these may be harnessed for more personalized treatment of this disease.

MLL-associated leukemia

Since its discovery, the mixed-lineage leukemia (*MLL*) gene, located on chromosome 11q23, has garnered much attention both from a hematologic and a basic science standpoint because of its role in epigenetics and leukemia. A number of genetic aberrations including chromosomal translocations, internal tandem duplications, internal deletions and amplifications of the *MLL* gene have been observed in hematologic malignancies [1–6]. The most frequent abnormalities involving 11q23 are chromosomal translocations that constitute more than 70% of acute lymphoid leukemia in infants under the age of 1 year and between 35 and 50% of infant acute myeloid leukemia (AML) [7–9]. Greater than 70 recurrent translocations and more than 50 different partner genes have been discovered, leading to the expression of chimeric oncogenic fusion proteins [10,11]. Rearrangements of the *MLL* gene are also found in adult leukemias and therapy-related leukemias arising in patients after treatment with topoisomerase II inhibitors for other malignancies, and is seen in approximately 10% of human leukemias overall [12,13]. Generally, 11q23 abnormalities are considered a poor prognostic factor and patients are treated with high-risk protocols [14]. In a study of infant acute lymphoid leukemia, patients with germline *MLL* displayed a good response to treatment, with a 5-year event-free survival (EFS) of approximately 80% compared with an EFS of 15% for patients with rearranged *MLL* [15]. A slightly better 5-year EFS of approximately 44% is observed in AML patients with *MLL* rearrangements; however, survival rates vary largely within the 11q23 subgroup based on age, lineage, translocation partner and the presence of cooperating mutations [16]. For example, patients with a t(1;11)(q21;q23) coding for the MLL–AF1p fusion protein had a favorable outcome,

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with a 5-year EFS of 92% compared with 29% for patients with t(4;11)(q21;q23), which generates the MLL–AF4 fusion protein. These characteristics demonstrate the complexity of 11q23 leukemia, and underscore the importance of understanding the molecular mechanisms of these diseases.

Hematopoiesis & MLL

Cloning of the breakpoint region on chromosome 11q23 from leukemic cells led to the discovery of *MLL*, a gene orthologous to the *Drosophila melanogaster trithorax* [1–3]. Trithorax group proteins, including MLL, play fundamental roles in development, acting antagonistically to Polycomb group proteins through the epigenetic regulation of target genes such as the clustered *Hox* genes [17]. Hox proteins are spatio–temporally regulated transcription factors involved in a number of developmental processes including anterior–posterior patterning and hematopoietic differentiation (for review, see [18]). Deletion of *Mll* results in impaired expression of *Hox* genes, including *Hoxc8* and *Hoxa7*, and embryonic lethality at E10.5 [19]. Within both the developing fetus and adult hematopoietic systems, Mll plays a central role in the survival and/or proliferation of the hematopoietic stem cell (HSC) and progenitor compartments through the proper regulation of *Hox* genes [20–22]. Defects to hematopoietic colony-forming potential are rescued by expression of *Hox* genes, confirming the importance of the MLL–HOX axis [23]. In the absence of Mll, *Hox* gene transcription is initiated normally but not properly maintained, suggesting a role for Mll in establishing epigenetic cellular memory [24].

The MLL protein is a 3969-amino-acid histone H3 lysine 4 (H3K4) methyl transferase transcription factor that is proteolytically cleaved by TASPASE1 into 320 kDa N-terminal (MLL^N) and 180 kDa C-terminal (MLL^C) fragments that self-associate in the nucleus [25]. MLL contains several highly conserved structural domains, including three AT-hooks and a CxxC domain that, in the latter case, binds specifically to nonmethylated DNA and protects from *de novo* methylation, and a C-terminal Su[var]3–9, enhancer of zeste, trithorax (SET) domain with intrinsic H3K4 methyltransferase activity [26–31]. A set of four plant homeo domain (PHD) fingers with an embedded atypical bromodomain located downstream of the CxxC domain play an important role in the localization and post-translation regulation of wild-type MLL [32]. The third PHD finger binds trimethylated H3K4 and contributes to MLL recruitment [33,34]. Recently, the second PHD finger of MLL and MLL's closest homolog, MLL4, were shown to possess intrinsic E3 ubiquitin ligase activity, leading to auto-degradation [35]. The bromodomain and PHD4 of MLL associate with the ECS^{ASB2} E3 ubiquitin ligase complex, and together these activities play a role in auto-ubiquitination and proteasome-mediated degradation during hematopoietic differentiation [36]. MLL stability is also regulated through ATR-mediated phosphorylation events that control ubiquitination during cell-cycle progression by the SCF^{Skp2} and APC^{Cdc20} E3 ligase complexes during S and M phase, respectively [37,38].

The MLL protein functions within a large protein complex that makes several direct associations with MLL. A core complex of proteins including ASH2l, WDR5 and RbBP5 assemble around the SET domain of MLL and are required for full methyltransferase activity [39–41]. Several histone acetyltransferases, including the H4K16 acetyltransferase MOF and CBP/p300, also associate with MLL and contribute to full transcriptional activation of target genes such as *Hoxa9* [42,43]. In addition to the functions listed above, PHD3 of MLL mediates binding of the nuclear peptidyl-prolyl isomerase Cyp33 that regulates the binding of a number of co-repressor proteins including CtBP, HPC2, BMI-1 and HDAC1 [32,44]. Importantly, the sequences mediating these interactions are deleted in MLL leukemia and do not contribute to MLL fusion protein function. However, a number of

interactions are preserved in MLL fusion proteins and relate to the mechanisms of MLL leukemia.

MLL mechanisms of disease

Molecular machinery of MLL leukemia

With the identification of more than 70 different MLL translocation partners, developing a unifying model for MLL-mediated transformation is challenging. Still, some recurring themes have emerged. In the event of an *MLL* rearrangement, amino terminal sequences of MLL up to and including the CxxC domain are retained, while the PHD fingers and beyond are deleted, resulting in the loss of both recruitment and transcriptional activation activities (Figure 1). Despite truncation of MLL and loss of H3K4 methyltransferase activity, the resultant chimeric MLL fusion protein leads to deregulated activation of leukemic target genes such as *HOXA9* and *MEIS1* that is observed in the vast majority of MLL-associated leukemia [45,46].

DNA-binding domains, such as the AT-hooks and CxxC domain, are retained in the MLL fusion protein and, in the case of the CxxC domain, are essential for MLL fusion protein transformation [47]. In the case of MLL partial tandem duplications, the N-terminal region of MLL containing these domains is duplicated at the breakpoint cluster region, leading to increased self-renewal and transformation of hematopoietic stem and progenitor cells (Figure 1) [48,49]. Other than preferential binding to nonmethylated CpG islands by the CxxC domain [26], these domains lack sequence specificity, necessitating other mechanisms for the recruitment of MLL fusion proteins to target loci. One mechanism that has attracted interest from a therapeutic standpoint occurs between the extreme N-terminus of MLL and the tumor suppressor protein Menin [50,51]. Menin is coded by the *MEN1* gene and commonly mutated in multiple endocrine neoplasia type 1. Two Menin-binding motifs reside within the N-terminus of MLL and mediate the formation of a trimolecular complex consisting of MLL, Menin and the chromatin-associated LEDGF protein [52,53]. The proper formation of this complex is critical to the targeting of MLL fusion proteins to target loci, such as *Hoxa9*, and necessary for MLL fusion protein-mediated leukemogenesis. It has been demonstrated that MLL fusion proteins also rely on an interaction with the PAF complex (PAFc) for proper targeting to gene loci. The PAFc interaction domain of MLL flanks the CxxC domain of MLL and is invariably retained in all MLL fusion proteins [34,54]. The PAFc is a transcriptional activation complex that associates directly with RNA pol II and promotes elongation by facilitating the deposition of several epigenetic marks associated with active transcription, including H2BK120 ubiquitination, H3K4, H3K36 and H3K79 methylation [55,56]. Like the Menin–LEDGF interaction, MLL fusion proteins are dependent on the PAFc interaction for leukemogenesis.

Truncation of MLL is not sufficient for transformation, indicating the presence of a translocation partner is required for leukemia [57]. While the above interactions likely aid in MLL fusion protein recruitment, the translocation partner plays a significant role in transcriptional activation. MLL translocation partners include both nuclear proteins implicated in transcriptional activation and cytoplasmic proteins. Despite the apparent complexity of revealing a mechanism for transformation, some themes have emerged. For example, many of the most common MLL translocation partners, including *MLLT3* (*AF9*), *MLLT1* (*ENL*), *AFF4* (*AF5q31*) and *AFF1* (*AF4*), function within a common transcriptional elongation complex that includes p-TEFb (Figure 2) [58]. The p-TEFb is composed of cyclin T1 and CDK9 and functions in transcriptional elongation by phosphorylating the C-terminal domain of RNA pol II. A variation of this complex has been reported to include the elongation factors ELL2, ELL3, EAF1 and EAF2 [59]. Interestingly, the Polycomb group protein CBX8, normally associated with transcriptional repression within the PRC1

complex, associates with this complex and contributes to transcriptional activation through recruitment of the HAT protein TIP60 [60]. Notably, HAT activity also contributes to MLL leukemias by direct fusion of CBP/p300 to MLL [61]. The AF9 and ENL translocation partners also reside within a protein complex termed DotCom that includes MLLT10 (AF10) and MLLT6 (AF17), two nuclear translocation partners of MLL, and the histone H3K79 methyltransferase DOT1l leading to transcriptional activation [62]. Furthermore, DOT1L and p-TEFb have been found together in transcription activation complexes including AF4, ENL, AF9 and AF5q31 [63–65]. Recruitment of DOT1l is required for transformation by some MLL fusion proteins by upregulating *HOXA9* and *MEIS1*, as well as the WNT signaling pathway that has been shown to be essential for MLL-mediated leukemogenesis [66–69]. Indeed, MLL leukemic cells display robust H3K79 methylation patterns across the *HoxA* locus indicative of Dot1l recruitment. These studies have distilled a common mechanism for many MLL fusion proteins that implicates p-TEFb and DOT1l in the upregulation of *HOX* genes.

Cooperating events

Deep sequencing has revealed MLL leukemias show increased genetic stability compared with other leukemias [70], which may reflect the highly potent oncogenicity of MLL fusion proteins. Still, analyses of 11q23 leukemias have revealed strong correlations with activation of both the FLT3 and the Ras signaling pathway, suggesting cooperating events contribute to MLL leukemia [71]. Consistent with the two-hit leukemic model whereby activated kinase pathways cooperate with altered DNA-binding transcriptional machinery [72], MLL mutations found *in utero* require a latency period before giving rise to leukemia, likely reflecting the need for cooperating mutations [73]. FLT3 mutations are found in up to 30% of AML, leading to enhanced proliferation and survival [74]. Although activating mutations are less common in MLL leukemias [75], an independent analysis of FLT3 expression in cytogenetic AML subgroups showed the highest correlation with MLL leukemias [76]. Furthermore, recent data has demonstrated up to approximately 30% of pediatric MLL-rearranged AML also contains Ras mutations [16]. Experimentally, the disease latency in mouse models of MLL leukemia is significantly shortened with the introduction of constitutively active FLT–internal tandem duplications or k-Ras^{G12V} in support of a cooperative model of leukemogenesis [77–79]. Supporting the involvement of the Ras signaling pathway, the Ras-like proteins Rac1 and Rac2 GTPases were shown to be necessary for MLL–AF9 transformed cells through regulation of the antiapoptotic Bcl2 proteins [80]. A full characterization of the cooperating events in MLL rearranged cells will present a more comprehensive view of the mechanisms and potential therapeutic targets of these leukemias.

Transcriptional pathways

The clustered *HOX* genes are some of the best-understood targets of MLL and MLL fusion proteins. High-level expression of *A* cluster *HOX* genes, as well as the HOX protein cofactor, *MEIS1*, is highly characteristic of MLL rearranged leukemia. In fact, gene-expression profiling has demonstrated *HOXA9* is the most highly correlated gene for poor prognosis in AML [81]. The importance of Hox proteins in MLL leukemia is highlighted by studies showing *Hoxa9*-deficient cells are not capable of transformation by MLL fusion proteins [82]. Furthermore, overexpression of *Hoxa9* is sufficient to induce leukemia in mice; however, disease onset is accelerated substantially by co-expression of *Meis1* [83]. It should be noted that some experimental systems involving MLL fusion proteins are capable of initiating leukemia independent of Hoxa9, which is likely due to compensation by other *HoxA* cluster genes [84,85]. Indeed, overexpression of almost all *HOXA* cluster genes leads to immortalization in serial replating assays with the exception of HOXA2 and HOXA5 [86]. HOX proteins contribute to leukemogenesis by blocking differentiation and promoting

survival of transformed cells [18]. Hoxa9 was recently shown to associate with enhancer regions, in collaboration with lineage-specific transcription factors such as PU.1, controlling the expression of known proto-oncogenes, such as *Flt3*, *Lmo2*, *Runx1* and *Sox4* [87]. Interestingly, PU.1, which is required for hematopoietic differentiation, is regulated by RUNX1-mediated recruitment of MLL methyltransferase activity, suggesting a possible feedback mechanism involving HOX proteins [88]. In addition, following direct activation of the *RUNX1* locus by MLL–AF4 fusion proteins, the RUNX1 protein associates with the reciprocal chromosomal fusion product AF4–MLL [89]. This association can contribute to both activation of RUNX1 target genes and the proleukemic capabilities demonstrated for the AF4–MLL reciprocal fusion protein [90].

Several additional targets of MLL fusion proteins have been identified that also contribute to leukemogenesis. For example, upregulation of the transcription regulatory factors Myb, Hmgb3 and Cbx5 by MLL fusion proteins establish an embryonic stem cell-like gene-expression program in hematopoietic progenitor cells, leading to transformation independent of Hoxa9/Meis1 [91,92]. MLL fusion proteins are also reported to regulate *MEF2C* and *EVI-1*, whose expression contributes to leukemic stem cell homing and tumor growth [93–95]. Interestingly, the MLL fusion proteins may recognize only a small subset of wild-type MLL target genes, and the localization of the fusion protein may be dependent on the fusion partner [66,96]. Some additional target genes that aid in MLL-mediated leukemogenesis include *Myc* and *Epha7* [97–99]; however, with additional genome-wide localization studies this list is likely to expand.

Recent work has uncovered unique miRNA-expression patterns associated with leukemias bearing *MLL* rearrangements. miR-196b, which resides within the *HOXA* cluster locus, is directly regulated by MLL and MLL fusion proteins. Hoxa9/Meis1 and Fas are targeted by miR-196b, which likely plays a role in primitive hematopoietic cells [100]. The importance of miR-196b expression has been demonstrated in MLL leukemia (and others with high-level *HOX* expression), where mi-196b expression is functionally linked with upregulation of *HOXA* genes, thus contributing to MLL-mediated transformation [101,102]. The overexpression and inhibition of miR-196b can delay MLL leukemia, which may reflect a delicate balance of targets such as *HOX* and *FAS* in leukemic cells [100]. Likewise, the miRNA cluster (miR-17–92) is frequently overexpressed in various hematologic and solid tumors, including MLL rearranged leukemias, and is directly bound by MLL fusion proteins. This cluster appears to regulate p21 expression, leading to inhibition of both cell differentiation and apoptosis of the MLL transformed cells [103,104]. Important MLL collaborating proteins, such as FLT3 and MYB, are targeted by miR-150, which is negatively regulated by MLL fusion proteins through a MYC/LIN28 axis. miR-150, along with miR-495, both appear to be down-reulgated in MLL leukemias and contribute to disease progression [105].

Targeting MLL in the leukemic stem cell

Inhibition of MLL fusion protein complexes

Evolution of leukemic stem cells poses a serious challenge to effectively targeting *MLL* rearranged leukemia. This model suggests the self-renewing leukemic stem cell (LSC) gives rise to the leukemic cell burden in a patient that can further evolve both genetically and epigenetically, leading to various clonal subpopulations of different frequency. Selective pressures, such as chemotherapy, redefine the leukemic population in favor of previously existing subclones that can vary significantly from the disease characterized at the time of diagnosis [106,107]. However, since LSCs will likely remain dependent on initiating driving genetic events, MLL fusion proteins represent an excellent therapeutic target.

Several recent studies have reported either enzymatic inhibition or disruption of protein interactions within the MLL fusion protein complex with small-molecule inhibitors. The identification of a common biochemical complex shared by many of the most common MLL fusion proteins (discussed above) (Figure 2) raises the prospect of targeting MLL fusion protein complexes. One attractive target of this complex is the transcriptional elongation complex, p-TEFb. The flavonoid flavopiridol shows activity against this kinase and may be effective in treating MLL leukemia, but clinical trials have raised concerns about efficacy and toxicity [108]. The H3K79 methyltransferase DOT1l also associates with the AEP complex and has recently been targeted with the small molecule EPZ004777 (Figure 2). This compound binds as a SAM structural analog and inhibits DOT1l function and selectively slows the growth of human MLL rearranged leukemic cells in xenograft transplantation assays [109]. Another target, which is shared among all MLL fusion proteins, is the MLL–Menin interaction. Small molecules developed to bind Menin and inhibit the MLL–Menin interaction have shown remarkable specificity for inducing both the apoptosis and differentiation of MLL leukemic cells (Figure 2) [110]. The PAFc is predicted to associate with all MLL fusion proteins and poses another potential therapeutic target. A physical association between BRD4 and the PAFc, as well as p-TEFb, has demonstrated the usefulness of targeting the histone-acetyl-binding pocket of BRD4 [111]. Exposure of MLL leukemia cells to small molecules (I-BET151 and JQ1) designed to bind the acetyl-binding bromodomain of BRD4 led to cell cycle arrest and monocytic differentiation (Figure 2) [97,98]. These data confirm the importance of DOT1l, Menin and BRD4 in MLL leukemias as potential therapeutic targets, and suggests critical interacting proteins, such as CBX8 and the PAFc, may show similar promise.

Signaling pathways necessary for MLL leukemias

Therapeutic targeting of cooperating signaling pathways may work synergistically with pharmaco logic inhibition of the MLL fusion protein complex to help eradicate MLL LSCs. MLL fusion proteins are capable of transforming both HSCs and committed granulocyte/monocyte progenitors into leukemic stem cells, in part, through the reactivation of the WNT/β-catenin pathway [112–114]. The targeting of Wnt/β-catenin has attracted attention due to the fact that β-catenin is dispensible for normal HSC self-renewal, but required for MLL-transformed LSCs [114,115]. Indeed, pharmacologic inhibition of β-catenin by modulating prostaglandin signaling with the cyclooxygenase inhibitor indomethacin is effective in impairing MLL–AF9 LSCs, as well as BCR–ABL-positive myeloid leukemia stem cells [114,116]. Paradoxically, GSK-3, which functions in complex with Axin and APC to degrade β-catenin, is required for the survival of MLL leukemias by promoting CREB binding to the HOX co-factor protein MEIS1. As such, GSK-3 inhibition with SB216763 displayed a highly selective growth inhibition of leukemic cells dependent on high HOX expression (Figure 2) [117,118]. Recent work has identified a requirement by MLL leukemic cells for the H3K4 demethylase KDM1a (LSD1). LSD1 inhibition with tranylcypromine slowed the growth of MLL–AF9 cells *in vivo*, but spares the clonogenic potential for normal hematopoietic progenitor cells [119]. Other cooperating pathways, such as the FLT3 signaling pathway described above, have been targeted with the inhibitor molecules PKC412 and CEP-701 and shown effectiveness in differentially killing MLL transformed cells especially following chemotherapy treatment [78,120,121].

Conclusion & future perspective

Rapid advances in our understanding of the biochemical and biological processes involved in MLL leukemia have revealed molecular contacts necessary for transformation, and ushered in new prospective therapeutic options. Exploiting these molecular points of susceptibility with drugs designed to specifically inhibit these functions shows promise for

more individualized treatment options. Future studies are likely to focus on the changes to the epigenome and epigenetic regulators that distinguish leukemic stem cells from their normal counterparts. The integration of both genetic and epigenetic models of leukemogenesis and the interplay between the two is likely to provide a more unified understanding of cancer progression, while also unlocking new therapies that may help to reverse the aggressiveness of *MLL* leukemia.

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Practice Points

- Mixed-lineage leukemia (MLL) is a histone H3K4 methyltransferase critical for normal hematopoietic development.
- Rearrangements of the *MLL* locus (11q23) are common in acute myeloid and lymphoid leukemias, and are associated with a poor prognosis.
- Leukemias harboring MLL fusion proteins display altered epigenetic profiles that contribute to disease.
- MLL fusion protein-mediated activation of proleukemic target genes, such as *HOXA9*, *MEIS1* and miRNAs, is critical for leukemogenesis.
- MLL fusion proteins associate with a transcriptional elongation complex, containing DOT1L and p-TEFb, leading to deregulated transcription.
- Targeted chemical disruption of the MLL fusion protein supercomplex effectively inhibits the growth of MLL-associated leukemic cells.

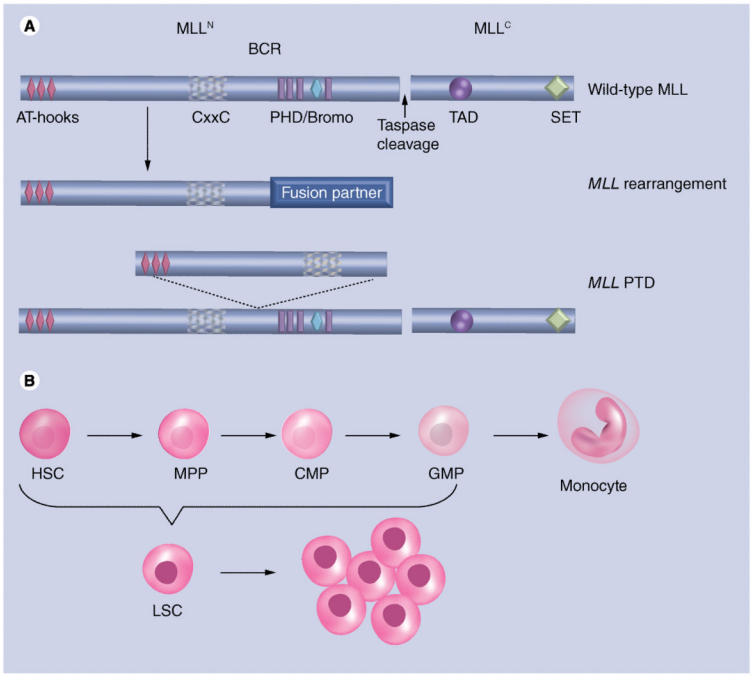

Figure 1. Normal and mutated forms of the mixed-lineage leukemia protein

(A) The wild-type MLL protein is shown schematically with conserved domains indicated and labeled. MLL is cleaved into two fragments, MLL^N and MLL^C. The site of taspase-mediated protein cleavage is indicated. AT-hooks and the CxxC domain of MLL^N bind to DNA, while the plant homeodomain/bromo region aids in localization and protein regulation. A SET and TAD domain contributes to histone methylation and recruitment of histone acetyltransferases. BCR is the site of fusion in the event of chromosomal translocations. MLL fusion proteins contain a sequence of a fusion partner protein fused in-frame to the MLL^N fragment at the BCR. MLL-PTDs duplicate the MLL sequence from the AT-hooks through the CxxC domain that is inserted at the BCR. Proteins are not drawn to scale. (B) HSC differentiation to a monocyte is shown, including intermediate progenitor cells: MPPs, CMPs and GMPs. MLL fusion proteins are capable of transforming HSCs through GMPs into LSCs that gives rise to leukemia.

BCR: Breakpoint cluster region; CMP: Common myeloid progenitor; GMP: Granulocyte/monocyte progenitor; HSC: Hematopoietic stem cell; LSC: Leukemic stem cells; MLL: Mixed-lineage leukemia; MLL-PTD: MLL partial tandem duplications; MPP: Multipotent progenitor.

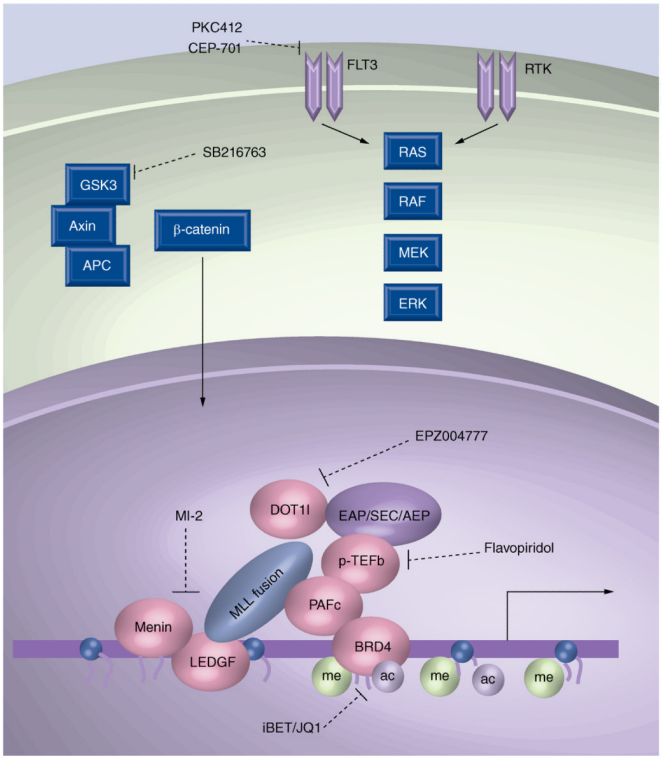

Figure 2. Targeting mixed-lineage leukemia fusion protein complexes and cooperating pathways

A representative MLL fusion protein complex is shown that recruits a transcriptional activation complex. Varieties of this complex have been termed EAP [122], SEC [59] and AEP [58]. Included in the recruitment are p-TEFb and the histone H3K79 methyltransferase DOT1l. Menin and PAFc bind directly to MLL fusion proteins, aiding in target recognition. BRD4 associates with PAFc and is required for MLL fusion protein function. GSK3 is shown associated with Axin and APC, which are involved in several signaling pathways including the WNT/β-catenin pathway. FLT3 activates several signaling pathways, including the Ras pathway that cooperates with MLL fusion proteins leading to leukemia. Compounds known to inhibit some of these processes and protein interactions are shown. ac: Acetylation; me: Methylation; MLL: Mixed-lineage leukemia; PAFc: PAF complex.