Menin as a hub controlling mixed lineage leukemia

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Mixed lineage leukemia (MLL) fusion protein (FP)-induced acute leukemia is highly aggressive and often refractory to therapy. Recent progress in the field has unraveled novel mechanisms and targets to combat this disease. Menin, a nuclear protein, interacts with wild-type (WT) MLL, MLL-FPs, and other partners such as the chromatin-associated protein LEDGF and the transcription factor C-Myb to promote leukemogenesis. The newly solved co-crystal structure illustrating the menin–MLL interaction, coupled with the role of menin in recruiting both WT MLL and MLL-FPs to target genes, highlights menin as a scaffold protein and a central hub controlling this type of leukemia. The menin/WT MLL/MLL-FP hub may also cooperate with several signaling pathways, including Wnt, GSK3, and bromodomain-containing Brd4-related pathways to sustain MLL-FP-induced leukemogenesis, revealing new therapeutic targets to improve the treatment of MLL-FP leukemias.

Keywords:
chromatin; leukemia; menin; MLL; therapy

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Abbreviations:
BM, bone marrow; **HSC,** hematopoietic stem cell; **LSC,** leukemia stem cell; **MLL-FP,** MLL fusion protein.

Introduction

Chromosomal translocations involving the mixed lineage leukemia (*MLL*) gene occur with one of multiple partner genes, leading to the formation and expression of MLL fusion proteins (MLL-FPs) and the development of acute leukemias [1]. These acute leukemias can be lymphoid (ALL), myeloid (AML), or biphenotypic in nature [2]. *MLL* translocations are found in ~10% of all leukemias and the majority of infant leukemia cases, and patients harboring this genetic abnormality have a particularly poor prognosis [3, 4].

The wild-type (WT) MLL protein is post-translationally cleaved by the protease taspase-1 into N-terminal (MLL-N) and C-terminal (MLL-C) fragments, which then re-associate to form the MLL complex [5]. WT MLL is recruited via MLL-N to target genes through interactions with various proteins, including menin, LEDGF, and C-Myb, as well as its CxxC domain, leading to transcriptional activation of these targets (Fig. 1A) [6–9]. Target gene activation is mediated, at least in part, through histone H3 lysine 4 trimethylation (H3K4m3) catalyzed by the MLL C-terminal SET domain, in concert with the cofactors Wdr5, Ash2L, and Rbbp5 (Fig. 1A) [10–12]. Direct WT MLL targets include *HOX* genes, which are activated in a controlled manner during normal hematopoiesis, with high expression in progenitor cells, but decreased expression during cell differentiation [13, 14].

MLL-FPs retain a portion of MLL-N, including domains important for its recruitment to MLL target genes, but lack a large C-terminal portion of the protein, including the H3K4-methylating SET domain (Fig. 1B,C). Although MLL-C is lacking in MLL-FPs, MLL-FP expression still leads to the upregulation of *HOX* genes [15, 16], which restrain progenitor cells from differentiation and cause leukemia when overexpressed in mouse bone marrow (BM) [17]. These findings appear to suggest that leukemogenesis does not require the C-terminal portion of MLL (Fig. 1B,C).

However, one allele of WT *MLL* remains intact in MLL-FP-expressing leukemia cells. Recent work has demonstrated a critical role for the remaining WT allele of *MLL* in MLL-AF9-mediated leukemogenesis [8, 18]. The biochemical mechanisms by which MLL-FPs affect chromatin modification and transcriptional elongation have been extensively reviewed [19–23]. This review focuses on the roles of, and interplay between, menin, WT MLL, and MLL-FPs in gene transcription and leukemic

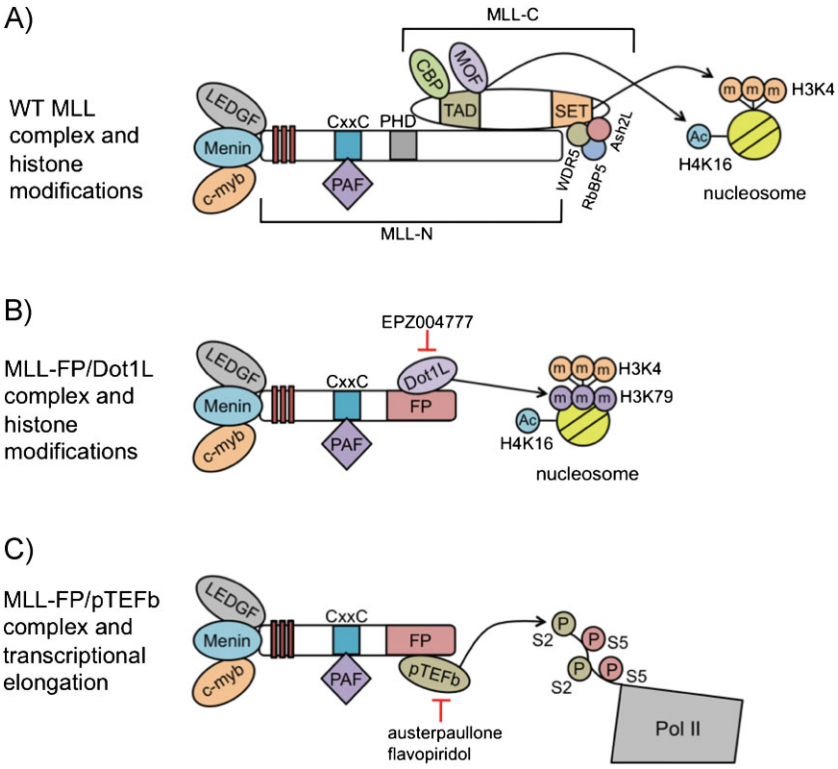

Figure 1. Menin/WT MLL and menin/MLL-FP complexes, and chromatin modifications catalyzed by these complexes. **A**: The MLL-C SET domain catalyzes H3K4m3. The MLL-C TAD interacts with the histone acetyltransferases CBP and MOF. **B**: Many MLL-FPs interact with and recruit the H3K79 methyltransferase Dot1L to MLL target genes. Dot1L can be inhibited by EPZ004777. **C**: Many MLL-FPs also recruit the pTEFb (cyclin T/CDK9) kinase complex to MLL target genes, leading to dysregulated transcriptional elongation. pTEFb can be inhibited with the drugs austerpaullone and flavopiridol. The indirect interaction of menin with other partners such as Dot1L or pTEFb is hypothetical based on deduction. The size of proteins is not drawn to scale.

transformation in MLL-FP-mediated leukemia, as well as their potential interplay with other proteins and signaling pathways. Recent findings highlight menin as a central component regulating MLL-FP-mediated leukemia, and a potential target for the pharmacological inhibition of MLL-FP leukemias.

MLL fusion proteins promote *HOX* gene expression and leukemogenesis

The driving force behind MLL-FP leukemia is the fusion protein itself, as its expression causes *HOX* gene upregulation and leukemic transformation [16, 24, 25]. Leukemogenesis is achieved even though a large C-terminal portion of WT MLL, which is normally required for the activation of *HOX* gene transcription, is lost due to the chromosomal translocation. Some less frequently occurring MLL-FPs have cytosolic fusion partners, such as AF6, GAS7, EEN, and septin proteins, and MLL-GAS7 does not require *Hoxa9* for BM transformation [20, 26]. However, the most common MLL-FPs have nuclear fusion partners, and these MLL-FPs upregulate *HOX* genes to promote leukemogenesis [20]. It was initially puzzling how these MLL-FPs activated *HOX* genes to an even greater extent than WT MLL. Several important studies have since led to a greater understanding of the mechanism of MLL-FP-mediated *HOX* gene upregulation and leukemogenesis.

Dot1L interacts with MLL-FPs, and is required for MLL-FP-mediated transformation

Many MLL-FPs recruit the histone H3 lysine 79 (H3K79) methyltransferase Dot1L to target genes, promoting leukemogenesis. MLL translocation partners, including AF9, ENL, and AF10, exist in a complex with Dot1L, and MLL-FPs containing these fusion partners retain the ability to interact with Dot1L, leading to increased H3K79 methylation at target genes [27, 28]. Although it is not yet known how H3K79 methylation regulates transcription, it is frequently found at active genes [29].

Enhanced transcription of MLL targets is associated with MLL-FP recruitment of Dot1L to these genes. *Hox* gene upregulation and leukemic transformation is contingent upon the interaction between MLL-AF10 and Dot1L [30, 31]. Additionally, direct fusion of Dot1L to MLL-N transforms mouse BM, while a Dot1L catalytic mutant fused to MLL-N fails to do so [30]. These findings suggest that Dot1L catalytic activity is necessary for MLL-FP-mediated leukemogenesis. Also, MLL-FP-mediated BM transformation is suppressed by deletion or catalytic inhibition of Dot1L [32–36]. These observations support a model whereby MLL-FPs recruit Dot1L to target genes, leading to enhanced H3K79 methylation, upregulation of these genes, and leukemogenesis (Fig. 1B).

However, it is unclear how H3K79m2 might promote *HOX* gene expression, and an MLL-Dot1L fusion that retains its catalytic activity, but cannot recruit pTEFb (see below) to target genes, is unable to transform mouse BM [37], suggesting that recruitment of Dot1L catalytic activity alone is insufficient for leukemic transformation. In addition, MLL-AF4, which interacts with pTEFb but not Dot1L, causes leukemia [37], suggesting that direct Dot1L recruitment is not absolutely required for leukemogenesis mediated by at least some MLL-FPs. Even so, H3K79 methylation is still enriched at MLL targets in MLL-AF4 cells [38], and Dot1L catalytic inhibition is effective in reducing *Hox* gene expression and

viability in these cells [36]. Future work may provide a clearer understanding as to how Dot1L regulates transcription and its precise role in MLL-FP leukemic transformation.

PTEFb interacts with MLL-FPs, and promotes *HOX* gene transcription and leukemogenesis

MLL-FP recruitment of the pTEFb complex to target genes promotes transcriptional elongation, providing another mechanism for transformation by MLL-FPs. Some of the same MLL fusion partners that interact with Dot1L, such as AF9 and ENL, as well as other fusion partners including AF4, are part of a distinct complex containing pTEFb – a kinase consisting of the cyclin T and CDK9 proteins that promotes transcriptional elongation [27, 39, 40]. Transcriptional elongation is regulated at many developmentally relevant genes that have an initiated or ''poised'' RNA polymerase II (Pol II) resting at their promoters [41]. Initiated Pol II is phosphorylated by pTEFb at serine 2 of its C-terminal domain (CTD), causing Pol II to be released from the promoter and allowing transcriptional elongation [42].

The expression of MLL-FPs, including MLL-AF9, -ENL, and -AF4, leads to pTEFb recruitment to MLL target genes and enhanced transcriptional elongation (Fig. 1C) [37, 43]. MLL-FP leukemia cell lines are more sensitive to the CDK9 inhibitors flavopiridol and alsterpaullone than non-MLL-FP cell lines, suggesting that dysregulated transcriptional elongation at MLL targets is at least one mechanism for leukemic transformation by MLL-FPs (Fig. 1C) [43]. The finding that MLL-FPs recruit Dot1L and/or pTEFb to target genes, leading to their upregulation, has provided an insight into the mechanism of MLL-FP-mediated leukemogenesis.

Wild-type MLL is required for MLL-FP-mediated *HOX* gene upregulation and leukemogenesis

In addition to the MLL-FP, expression of non-translocated WT MLL is required to maintain the transformed state of MLL-FP leukemia cells, likely through C-terminal domains that are lacking in the fusion protein. While MLL-FP cells have one translocated allele, which results in the expression of MLL-FPs, the other allele of *MLL* remains intact and expresses full-length WT MLL. WT MLL is not only expressed, but binds target genes in MLL-FP cell lines [18, 44]. MLL-FPs lack a large C-terminal portion of WT MLL that is normally necessary for target gene activation. However, MLL-C function is still carried out in MLL-FP cells by expression of WT MLL from the non-translocated allele.

Non-translocated WT MLL is critical for maintaining the transformed state of MLL-FP leukemias, and the survival of MLL-AF9 mice is prolonged by WT MLL knockout [18]. WT MLL depletion in human MLL-AF9 cells not only leads to a decrease in *HOX* gene transcription and WT MLL-mediated H3K4m3, but also reduces MLL-AF9-induced H3K79m2, indicating that MLL-FP function requires WT MLL [18]. WT MLL is necessary for MLL-AF9 recruitment to *Hox* genes in MEFs, but there is no physical interaction between MLL-AF9 and WT MLL [8]. Therefore, WT MLL function may indirectly lead to MLL-FP recruitment through chromatin modifications mediated by C-terminal domains that are lacking in MLL-FPs, such as the trans-activation domain (TAD) and SET domain (Fig. 1A).

The potential role of WT MLL-mediated H3K4m3 in MLL-FP leukemias

MLL-C SET domain-catalyzed H3K4m3 is associated with transcriptional activation, and may be important for the activation of WT MLL/MLL-FP target genes through the recruitment of certain proteins that specifically bind this chromatin modification. Mice expressing MLL lacking the SET domain are viable, but exhibit skeletal defects and decreased Hox expression, suggesting that the SET domain and H3K4m3 are required for optimal expression of MLL target genes during development [12].

H3K4m3 is specifically recognized by various ''reader'' proteins. One of these readers is WT MLL itself, which recognizes H3K4m3 via its third PHD (PHD3) domain at target gene promoters (Fig. 2A) [45, 46]. The H3K4m3 mark is recognized by PHD3 through a hydrophobic cavity, ''reading'' the gene as active, and retaining the active chromatin state [45]. It is likely that the recognition of H3K4m3 by PHD3 allows the propagation of active histone modifications catalyzed by WT MLL and its interacting partners, maintaining the chromatin in a conformation conducive to MLL-FP binding. MLL-FPs invariably lack the WT MLL PHD domains. Interestingly, overexpression of MLL-ENL containing the PHD3 domain fails to transform mouse BM [47]. However, it is not yet understood why MLL-FPs must contain MLL-N lacking the PHD domains.

Another reader of H3K4m3 is BPTF, a subunit of the NuRF chromatin-remodeling complex. BPTF specifically binds nucleosomes that contain both H3K4m3 and acetylated histone H4 at lysine 16 (H4K16Ac) via adjacent PHD- and bromo-domains (Fig. 2B) [48–50]. BPTF has also been found to bind *Hox* loci [49]. *Hox* gene expression is altered by BPTF knockdown in *Xenopus* during development, but it is not yet known whether BPTF regulates *HOX* gene expression in MLL-FP leukemia cells [48].

In MLL-AF9 leukemia cells, the chromodomain-containing protein CHD1 also recognizes H3K4m3, and localizes to *Hox* genes, but has not yet been implicated in MLL-FP-mediated leukemogenesis (Fig. 2C) [9, 51]. Future studies will determine whether WT MLL complex-catalyzed H3K4m3 and the reading of this mark by CHD1 and BPTF are involved in restructuring chromatin to allow MLL-FP binding and *HOX* gene upregulation (Fig. 2B,C).

The potential role of the WT MLL TAD and histone acetylation in MLL-FP leukemias

In addition to the SET domain, the WT MLL TAD, which promotes histone acetylation, a feature of actively transcribed genes, may have a role in the activation of MLL target genes in MLL-FP cells. WT MLL interacts with the histone acetyltransferase (HAT) enzymes MOF and CREB binding protein (CBP; Fig. 1A) [52, 53]. CBP directly interacts with the WT MLL TAD, and CBP is required for MLL transactivation activity in normal cells [52]. An MLL-CBP fusion aberrantly recruits CBP to MLL targets, causing myeloproliferative disease, supporting a

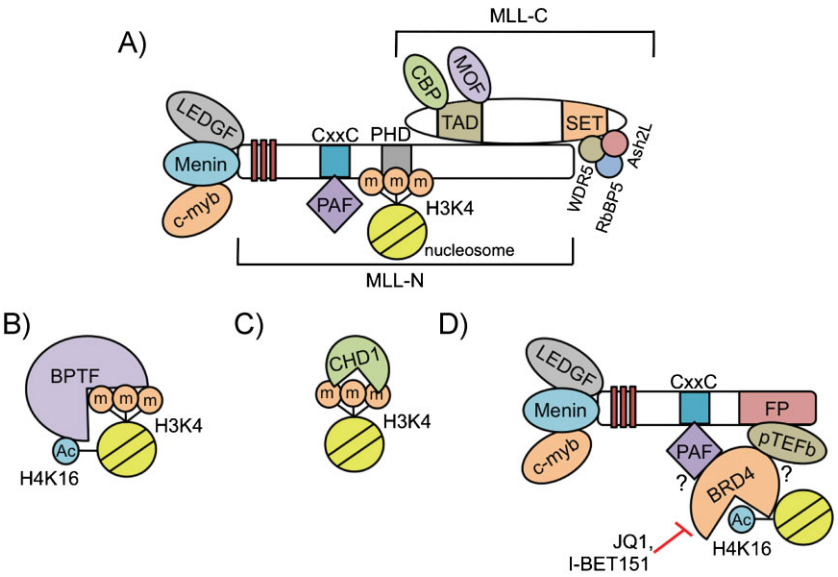

Figure 2. Readers of epigenetic modifications mediated by menin/WT MLL. **A**: The WT MLL PHD domain specifically associates with H3K4m3. **B**: BPTF (part of the NuRF complex) binds H3K4m3 and H4K16Ac via its tandem PHD- and bromo-domains. **C**: CHD1 specifically recognizes H3K4m3. **D**: Brd3/4 bromodomain-containing proteins interact with acetylated histone and may recruit MLL-FPs to target genes through PAF/pTEFb interactions. The Brd protein inhibitors JQ1 and I-BET151 cause MLL-FP leukemia cell death.

potential role for CBP in MLL-FP pathogenesis [54]. The WT MLL TAD also interacts with MOF, an H4K16 acetyltransferase [53].

The dual bromodomain-containing protein Brd4 recognizes acetylated histones [55]. However, it is not known whether Brd4 recognizes histone acetylation mediated by WT MLL at target genes. Nonetheless, Brd4 may have a role in MLL-FP recruitment to target genes. Brd4 recruits PAFc and pTEFb to gene loci [56, 57], and PAFc/pTEFb interact with MLL-FPs [37, 58], providing a link between MLL-FPs and chromatin (Fig. 2D). In support of this model, Brd4 inhibition causes a decrease in PAFc, pTEFb, and PolII CTD phospho-S2 at the promoter of *Bcl-2*, a direct MLL-FP target gene [57].

Also, BPTF recognizes H3K4m3 and H4K16Ac simultaneously, suggesting that the TAD may act in concert with the SET domain to maintain chromatin in a conformation conducive to MLL-FP binding [48, 49]. Future studies will determine whether WT MLL SET domain-catalyzed H3K4m3 and/or WT MLL TAD-mediated histone acetylation are involved in MLL-FP-mediated leukemogenesis.

WT MLL and MLL-FPs share N-terminal domains critical for their recruitment to target genes

Although the C-terminal portions of WT MLL and MLL-FPs possess quite distinct activities, the N-terminal regions of these proteins, which are responsible for recruitment to target genes, are mostly shared and function in a similar manner with only subtle differences (Fig. 1).

Menin captures the N-terminus of MLL through a deep pocket

The interaction between MLL-N and menin is pivotal to the physiological, as well as the pathological, roles of MLL [9, 59–61]. MLL-dependent transcription and MLL-FP-mediated leukemic transformation also require the LEDGF protein, which interacts with MLL-N and menin [6]. Recently, crystallographic studies of the structures of menin in its free form and in complex with MLL or an MLL-LEDGF heterodimer explain how menin acts as a central ''hub'' through its role in recruiting both WT MLL and MLL-FPs to target genes [62, 63].

Menin interacts with a bipartite motif at the extreme N-terminus of MLL consisting only of residues 6–25 ($MLL1_{MBM}$: *m*enin-*b*inding *m*otif), a region that is found in both WT MLL and MLL-FPs. The crystal structures of menin alone and in complex with the MLL_{MBM} peptide reveal that menin adopts a rectangular-shaped conformation, resembling a curved left hand, with a deep pocket formed by its thumb and palm domains (Fig. 3A,B). The thumb domain of menin is structurally similar to the transglutaminase-like fold of the transglutaminase superfamily, and the palm domain contains three TPR motifs that usually serve as protein–protein interaction modules. The MLL_{MBM} peptide adopts a highly coiled conformation and plugs into the deep pocket of menin. The interaction affinity and specificity between menin and MLL_{MBM} is mainly determined by extensive hydrophobic contacts at the bottom of the deep pocket of menin (Fig. 3C). Disruption of the interaction between menin and MLL-N diminishes WT MLL-mediated H3K4m3 and MLL-AF9 enrichment at the *Hoxa9* promoter and decreases Hoxa9 expression in transformed mouse BM, highlighting the importance of the menin–MLL-N interaction for MLL-FP-mediated leukemogenesis (Fig. 4) [60].

Menin serves as a scaffold protein to assemble a menin–MLL–LEDGF ternary complex

Menin/WT MLL and menin/MLL-FP complexes are guided to MLL target genes through additional interactions with the chromatin-associated LEDGF protein. [6, 62]. Notably, LEDGF binds to menin/MLL-N with high affinity, whereas neither menin nor MLL-N alone stably associate with

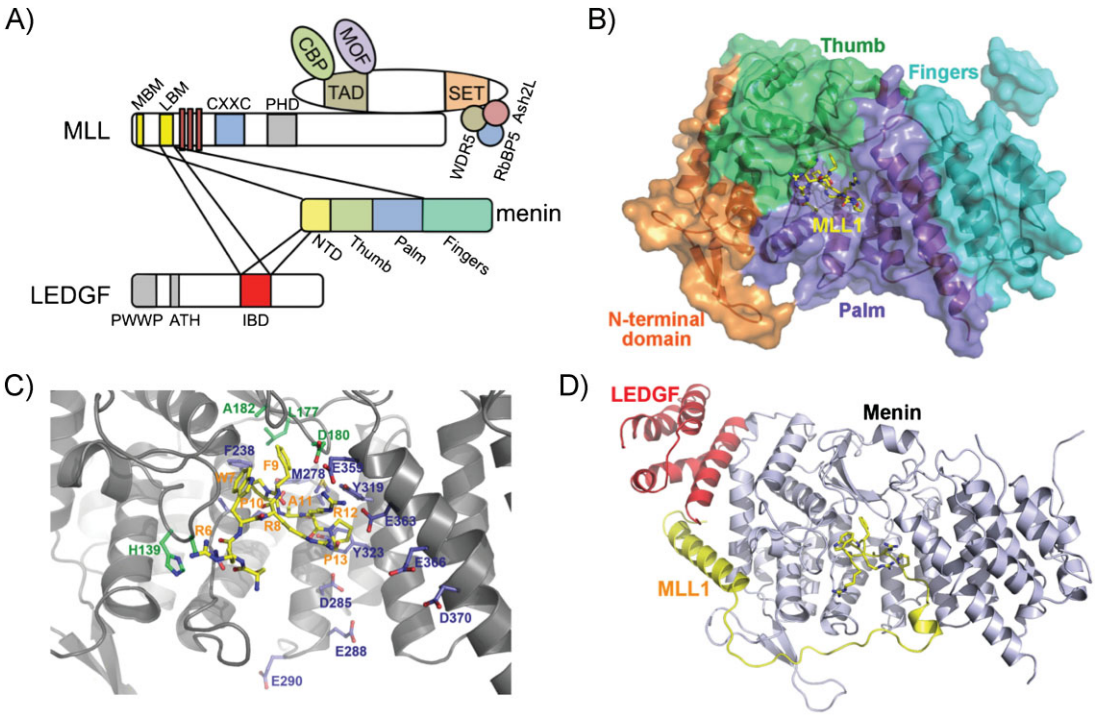

Figure 3. Structural insights into the menin/MLL interaction. **A**: Domain organization of menin, MLL, and LEDGF. Interactions among the three proteins are denoted. **B**: Overall structure of the menin-MLL_{MBM} complex. **C**: Detailed view of the menin-MLL_{MBM} interface. **D**: Ribbon diagram of the menin/MLL/LEDGF complex.

LEDGF. The crystal structure of the menin/MLL-N/LEDGF complex also indicates that the integrase-binding domain of LEDGF ($LEDGF_{IBD}$) binds to a V-shaped groove formed by both menin and MLL-N (Fig. 3D). Menin acting as a scaffold protein to mediate the MLL-N/LEDGF interaction underscores the central role of menin in MLL-FP leukemias (Fig. 4).

C-Myb interacts with menin and is critical for MLL-FP-mediated transformation

In MLL-FP leukemia cells, menin also interacts with the C-Myb transcription factor, which is essential for WT MLL, and presumably, MLL-FP recruitment to target genes (Fig. 1) [7]. C-Myb is required for optimal *HOX* gene expression in human MLL-FP leukemia cell lines, and MLL-ENL-mediated transformation is suppressed by expression of a C-Myb fragment that binds DNA, but lacks the menin-interaction domain [7]. The role of the menin/C-Myb interaction in MLL-FP leukemias again highlights menin as a central player in this disease (Fig. 4).

The CxxC domain is critical for WT MLL and MLL-FP recruitment to target genes

Another common feature of WT MLL and MLL-FPs is the CxxC domain, which interacts with the PAFc complex and is required for WT MLL and MLL-FP binding to *Hox* genes (Fig. 1) [8, 58]. The CxxC domain also interacts with unmethylated CpG DNA sites [64–66]. Interestingly, disruption of the CxxC-DNA interaction with a point mutation (K1176A) abrogates MLL-AF9, but not WT MLL binding to the *Hoxa9* promoter [64]. This discrepancy could be due to the fact that MLL-FPs lack PHD3, which may stabilize WT MLL at target genes, further suggesting that an ''open'' chromatin conformation is critical for MLL-FP binding and stabilization at such target genes.

However, the necessity of the CxxC-DNA interaction for MLL-AF9 recruitment is still unclear, as another point mutation abrogating CxxC DNA binding (C1188D) has no effect on MLL-AF9 binding in MEFs, but is still required for MLL-AF9-mediated leukemogenesis, potentially by affecting methylation of CpG islands at the *Hoxa9* promoter [66]. As WT MLL and MLL-FP recruitment are controlled by the same interacting proteins, which are largely coordinated by menin, menin may be particularly important for regulating two distinct functions controlling *HOX* gene upregulation and leukemogenesis (Fig. 4).

Direct transcriptional targets of menin/WT MLL/MLL-FPs

MLL-FP recruitment to *HOX* genes and other recently identified target genes causes enhanced transcriptional elongation and subsequent overexpression of these genes. These direct MLL-FP targets play various roles in promoting leukemic disease, highlighting the importance of investigating genes that are upregulated by MLL-FPs. Understanding MLL-FP downstream effectors has yielded further insight into the mechanism by which expression of these fusion proteins causes leukemia.

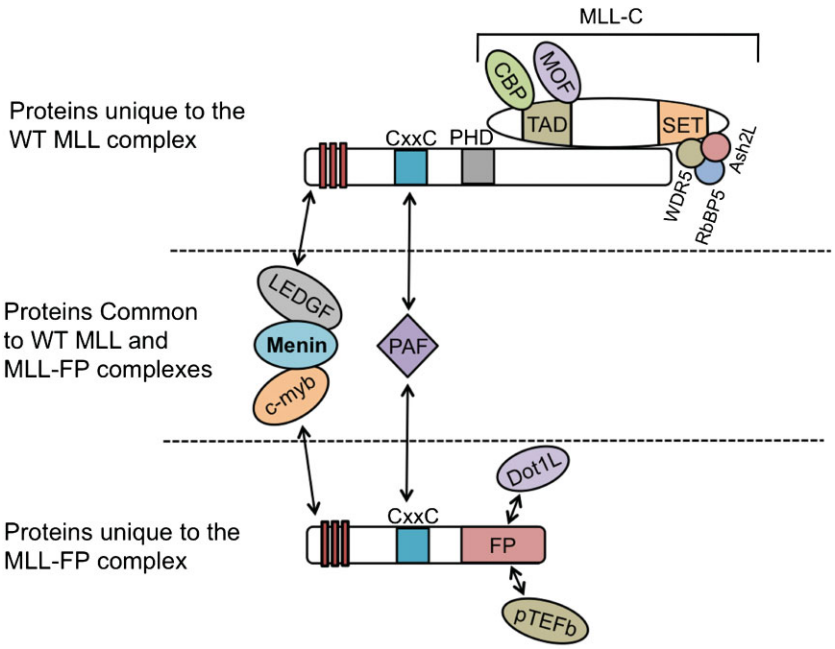

Figure 4. Menin as a central hub regulating WT MLL and MLL-FPs. Menin binds the N-terminus of either WT MLL or MLL-FPs, together with C-Myb and LEDGF (middle). The interaction between menin and LEDGF requires MLL-N. The PAF complex interacts with the CxxC domain of both WT MLL and MLL-FPs. The proteins interacting with the C-terminal part of WT MLL or MLL-FPs are distinct (top and bottom). WT MLL and MLL-FPs are recruited to many of the same target genes.

HOX genes are critical downstream effectors of MLL-FP-mediated leukemogenesis

In MLL-FP leukemias, *HOX* genes have been implicated in promoting cell survival and blocking differentiation to promote leukemogenesis. In mammalian cells, there are 39 *Hox* genes residing in four distinct clusters on four respective chromosomes [67]. In particular, *HOXA9* and its dimerization partner *MEIS1* are the most well characterized direct targets of the MLL-FP pathway. MLL-AF9 leukemic disease can be mimicked by overexpression of HOXA9 and MEIS1 [16, 17]. Also, Hoxa9/Meis1 overexpression is able to rescue BM colony formation defects caused by menin or WT MLL depletion, demonstrating that *Hox* genes are critical downstream mediators of MLL-FP-mediated leukemia [9, 18]. In addition, shRNA-mediated depletion of HOXA9 in human MLL-AF9 cells causes growth arrest, increased expression of differentiation-associated genes, and apoptosis, indicating various roles for *HOX* genes in leukemogenesis [68]. As *HOX* genes themselves are transcription factors, it is likely that MLL-FP-mediated *HOX* gene upregulation leads to increased transcription of multiple HOX target genes involved in leukemogenesis. Further investigation into the direct downstream targets of HOXA9/MEIS1 will yield more insight into the yet uncharacterized mechanisms by which these genes function to regulate critical processes in MLL-FP leukemia cells.

MLL-FPs directly activate additional genes that have a role in leukemogenesis

Recently, global approaches have led to the discovery of previously unrecognized direct MLL-FP target genes that promote MLL-FP-mediated transformation. A comparison of MLL-FP-expressing cells with cells containing only WT MLL has demonstrated that MLL-FPs regulate a subset (~15%) of WT MLL target genes [44]. Two of these genes, *Six1* and *Eya1*, are able to transform mouse BM, demonstrating a role for these proteins in leukemogenesis [44]. Additionally, *Evi1*, a gene that was discovered as a common retroviral integration site in murine leukemias, was found to be a direct MLL-AF9 target in mouse hematopoietic stem cells (HSCs) [69]. Future experiments may lead to the discovery of more direct MLL-FP targets involved in leukemogenesis.

Signaling pathways regulating MLL-FP leukemias

In addition to direct downstream targets of MLL-FPs, which are critical for leukemic transformation, signaling pathways have been found that cooperate with MLL-FP function to regulate leukemogenesis. These pathways include GSK-3 signaling, which modulates Hoxa9/Meis1 function, Wnt signaling, and the Akt/Foxo pathway. Understanding pathways that function in concert with, or in parallel to, MLL-FPs has led to a broader understanding of the cellular context in which MLL-FPs function.

GSK-3 regulates HOX function downstream of MLL-FPs

The GSK-3 serine/threonine kinase promotes MLL-FP-mediated leukemogenesis by increasing the recruitment of HOXA9 and its cofactor MEIS1 to target genes. GSK3 has a role in multiple cellular pathways, such as Wnt signaling. Wnt signaling is repressed by GSK-3, which induces β-catenin phosphorylation and degradation [70, 71]. Interestingly, independent of its role in Wnt signaling, GSK-3 has an opposing pro-oncogenic role in MLL-FP leukemia, as its inhibition leads to G1 arrest [72]. Further investigation into how the cell cycle is regulated by GSK-3 demonstrated its ability to directly phos-

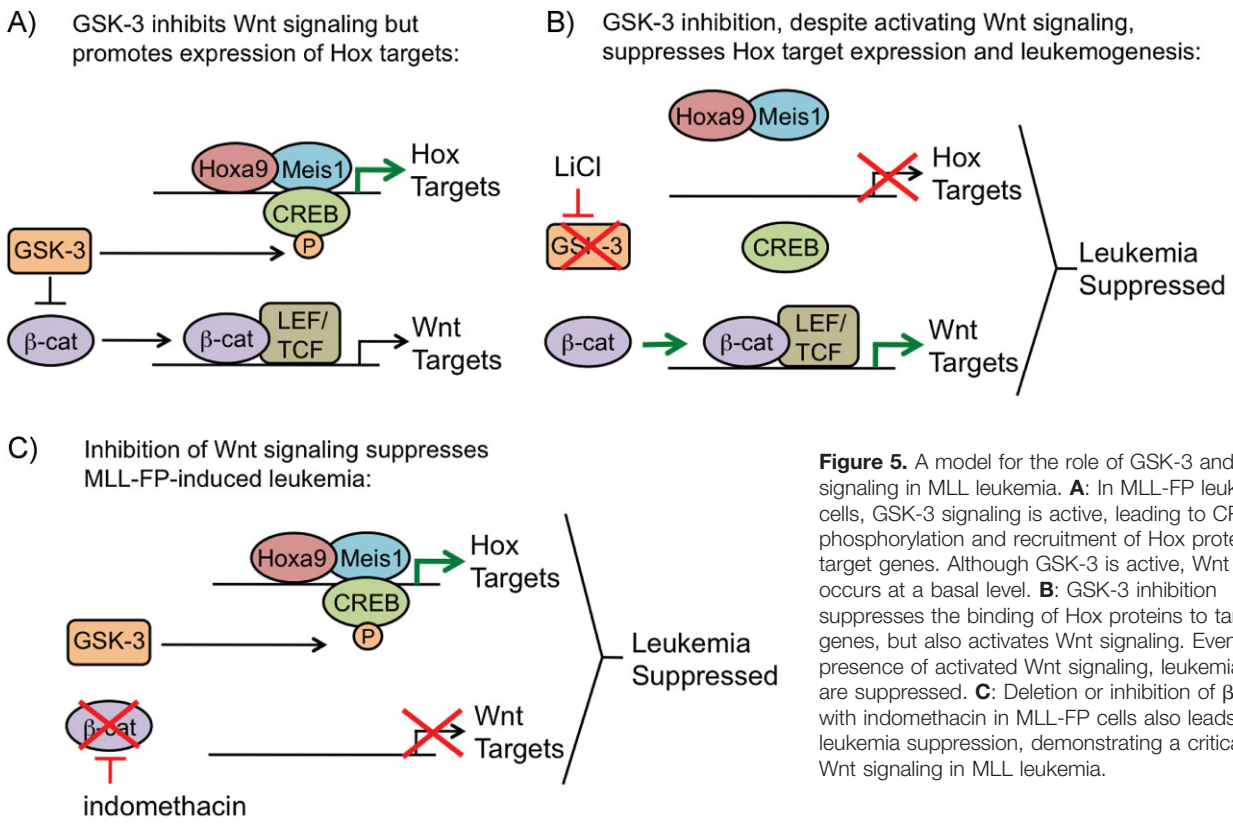

Figure 5. A model for the role of GSK-3 and Wnt signaling in MLL leukemia. **A**: In MLL-FP leukemia cells, GSK-3 signaling is active, leading to CREB phosphorylation and recruitment of Hox proteins to target genes. Although GSK-3 is active, Wnt signaling occurs at a basal level. **B**: GSK-3 inhibition suppresses the binding of Hox proteins to target genes, but also activates Wnt signaling. Even in the presence of activated Wnt signaling, leukemia cells are suppressed. **C**: Deletion or inhibition of β-catenin with indomethacin in MLL-FP cells also leads to leukemia suppression, demonstrating a critical role for Wnt signaling in MLL leukemia.

phorylate CREB, leading to the formation of a complex including CREB, MEIS1, HOXA9, and CBP that activates HOX/MEIS1 downstream targets (Fig. 5A) [73]. These results identify GSK-3-mediated HOX protein recruitment as a critical pathway for MLL-FP-mediated leukemogenesis.

Wnt signaling is required for MLL-FP-mediated leukemogenesis

The Wnt pathway is also crucial for MLL-FP-mediated leukemogenesis, as β-catenin knockout impairs the ability of MLL-AF9-expressing cells to cause leukemia (Fig. 5C) [74, 75]. Although Wnt signaling is essential for MLL-FP leukemia, inhibition of GSK-3, which activates Wnt signaling, leads to MLL-FP cell death by inhibiting binding of HOXA9/MEIS1 to target genes (Fig. 5B) [72, 73]. These results suggest that in the absence of HOXA9/MEIS1 function, Wnt signaling is unable to sustain MLL-FP-mediated leukemogenesis.

MLL-FPs may independently regulate Wnt signaling through their interaction with Dot1L. The native Dot1L complex, in addition to MLL fusion partners, also contains the Wnt pathway components TRRAP, Skp1, and β-catenin in 293T cells [28]. This finding suggests that MLL-FPs regulate Wnt signaling at least in part through their interaction with Dot1L in MLL-FP leukemia cells, possibly through stabilizing or aiding in the recruitment of the Dot1L complex to Wnt targets. Although it is unclear whether Dot1L regulates Wnt signaling in MLL-FP cells, Wnt signaling is upregulated by MLL-FPs and is essential for MLL-FP leukemia.

Low Akt signaling, resulting in Foxo activation, is required for MLL-FP leukemia stem cells

Akt activity is low in MLL-AF9 leukemia stem cells (LSCs), and overexpression of a constitutively active form of Akt leads to MLL-AF9 cell differentiation [76]. In MLL-FP leukemias, low Akt signaling leads to nuclear translocation and activation of Foxo proteins. Depletion of Foxo1/3/4 increases the survival rate of mice transplanted with MLL-AF9-transduced primary BM cells, demonstrating a pro-oncogenic role for *Foxo* genes in MLL-AF9 leukemia, in part through activation of the JNK/Jun pathway [76]. In addition to GSK-3, Wnt, and Akt/Foxo, it is likely that yet unidentified signaling pathways are involved in MLL-FP-mediated leukemogenesis.

Potential therapeutic targets of the menin-WT MLL-MLL-FP pathway

Substantial progress has been made in understanding the mechanism of MLL-FP-mediated leukemogenesis and the critical signaling pathways in these cells. Pharmacological inhibition of these targets may be effective in treating patients with this disease.

Inhibition of Wnt signaling as a potential therapy for MLL-FP leukemias

Inhibition of Wnt signaling by β-catenin knockout depletes MLL-FP LSCs and increases the survival of MLL-FP leukemic

mice, highlighting the Wnt pathway as a potential target for treating this disease [74, 75]. Importantly, Wnt signaling does not affect adult HSCs in mice [77], decreasing the likelihood of toxicity due to Wnt inhibition. The Wnt pathway inhibitor indomethacin, a commonly used anti-inflammatory drug, inhibits Cox1/Cox2 and causes β-catenin degradation in MLL-FP cells [75, 78]. Treatment of MLL-AF9 leukemic mice with indomethacin leads to a modest yet significant increase in survival time, suggesting that MLL-FP leukemia can be suppressed by pharmacological inhibition of the Wnt pathway (Fig. 5C) [75].

Since Wnt pathway components are found in a complex with Dot1L [28], it is possible that Dot1L inhibition could decrease not only *HOX* gene expression, but also Wnt signaling. It should be noted, however, that inhibition of Wnt signaling is less effective in later stages of the disease [75], suggesting that a Wnt inhibitor may only be useful in combination with other therapies to effectively treat this disease.

Inhibition of GSK-3 as a potential therapy for MLL-FP leukemias

GSK-3, through phosphorylation of CREB, leads to MEIS1 recruitment to target genes [73]. Since HOXA9/MEIS1 are critical mediators of MLL-FP-mediated leukemogenesis, inhibition of GSK-3 could be a viable treatment option for this type of leukemia. In support of this theory, GSK-3 inhibition with lithium chloride, a common treatment for bipolar disorder, prolongs the survival of MLL-AF4 leukemic mice (Fig. 5B) [73].

Although GSK-3 inhibition is initially effective, mice eventually become resistant to therapy and succumb to disease [73]. One possible mechanism for resistance is that, in addition to inhibiting HOXA9/MEIS1 targets with GSK-3 inhibition, inhibiting GSK-3 also activates the Wnt pathway, which is essential for MLL-FP leukemia (Fig. 5B). MLL-FP cells are re-sensitized to GSK-3 inhibition by Wnt pathway disruption through depletion of β-catenin, suggesting that a combination of GSK-3 and Wnt inhibition may increase treatment efficacy [74].

Directly targeting MLL-FP function to treat MLL-FP leukemias

As MLL-FPs are the driving force behind leukemogenesis, it seems logical to directly target fusion protein function through inhibition of Dot1L or pTEFb. Along these lines, mice xenografted with MLL-AF4 leukemia cells have a modest but significant increase in survival when treated with EPZ004777, a Dot1L catalytic inhibitor, underscoring the potential for targeting Dot1L to treat MLL-FP leukemia (Fig. 1B) [36].

Also, pTEFb inhibition with the CDK9 inhibitors flavopiridol or alsterpaullone shows some selectivity towards MLL-FP cell lines (Fig. 1C) [43], and clinical trials involving flavopiridol and other CDK9 inhibitors are ongoing for the treatment of chronic lymphocytic leukemia and acute leukemias, presenting a possible avenue for MLL-FP leukemia therapy in the future [79, 80].

The use of BET bromodomain inhibitors to treat MLL-FP-induced leukemia

The potential for treating MLL-FP leukemia patients by blocking Brd4 binding to acetylated histone through chemical inhibition is promising. Two specific inhibitors that bind the first bromodomain of Brd4 with strong affinity, JQ1 and I-BET151, have been developed (Fig. 2D) [57, 81, 82]. These drugs have shown efficacy in cell culture and animal models, leading to differentiation, cell cycle arrest, and apoptosis of MLL-FP leukemia cells [57, 81]. Although the life span of leukemic mice is extended by treatment with Brd4 inhibitors, the animals do succumb to disease, suggesting that the inhibitor needs to be improved or used in combination with other therapies to treat MLL-FP leukemias.

The potential for targeting WT MLL to treat MLL-FP leukemias

WT MLL is required for MLL-FP-mediated leukemogenesis [18]. Thus, blocking WT MLL function could hold therapeutic value for patients with this disease. The development of specific inhibitors of the WT MLL SET domain may inhibit WT MLL functioning in MLL-FP-mediated leukemias. Additionally, the TAD or PHD3 could potentially be targeted. However, the exact mechanism by which WT MLL promotes MLL-FP-mediated leukemogenesis is unknown. Also, while pharmacological inhibition rarely completely inhibits the function of a protein, complete ablation of WT MLL can cause defects in adult hematopoiesis, suggesting that inhibition of WT MLL function could lead to modest or even severe suppression of normal BM [83–85].

Targeting menin, a central hub regulating WT MLL and MLL-FPs to treat MLL-FP leukemias

Menin directly interacts with WT MLL and MLL-FPs, and is necessary for directing these two critical components for leukemogenesis to *HOX* genes, establishing menin as a hub in this disease and a potential therapeutic target (Fig. 4) [9, 18, 60–63]. Further, menin directly interacts with LEDGF and C-Myb [6, 7], strengthening the view that menin acts as a central "hub" for these various complexes and is suitable for therapeutic targeting (Fig. 4). Therefore, inhibiting menin may treat MLL-FP leukemias more effectively. In addition, inhibition of menin may be less toxic, as menin depletion causes a functional defect in LT-HSCs, but does not affect steady-state hematopoiesis [14].

Crystal structures of menin complexes have revealed promising drug targets for the therapy of MLL-FP-mediated leukemia through menin inhibition (Fig. 3). The hydrophobic menin pocket that binds MLL_{MBM} is specifically shaped for the phenyl ring of phenylalanine. Substitution of the phenyl ring with an imidazole ring of histidine or a hydroxyphenyl ring of tyrosine abolishes menin–MLL interaction [62]. Thus, inhibition of the menin–MLL interaction could be achieved with small aromatic compounds.

In addition, two phenol rings of MLL_{LBM} bind the hydrophobic pocket of the $LEDGF_{IBD}$ (Fig. 3D). This interaction is another potential target for blocking the formation of the

menin–MLL–LEDGF complex. A potent inhibitor of the menin/MLL-N interaction has recently been developed. This thienopyromidine class compound, termed MI-2, inhibits the interaction between MLL-FPs and menin, and blocks MLL-FP induced transformation [60], providing a proof of principle for targeting menin in MLL-FP leukemias.

Conclusions and outlook

Recently, significant progress has been made in understanding the mechanisms by which the menin/WT MLL/MLL-FP hub upregulates a subset of MLL target genes and drives leukemogenesis. In addition to MLL target gene upregulation, MLL-FPs work in concert with various signaling pathways to promote leukemia. Menin acts as a central hub through its role in recruiting WT MLL and MLL-FPs to target genes. Menin also links C-Myb/LEDGF to the MLL N-terminus, further highlighting menin's central role in this disease. A more detailed understanding of the molecular mechanism of menin/WT MLL/MLL-FP-mediated upregulation of target genes and potential interactions with crucial signaling pathways may provide additional therapeutic targets for this disease.

Increasing knowledge about the mechanism of MLL-FP-mediated leukemogenesis has led to the possibility of treating this disease with targeted therapeutics. MLL-FP function can be suppressed by inhibition of Dot1L catalytic activity [36], and MLL-FP cells are also sensitive to the BET bromodomain inhibitors JQ1 and I-BET151 [57, 81]. JQ1 and I-BET151 are the first in a new class of small molecules inhibiting readers of epigenetic marks. The development of additional drugs in this class could be used to inhibit other reader proteins that may be important for MLL-FP-mediated leukemogenesis (Fig. 2). Inhibition of the menin/MLL-N interaction with the small molecule MI-2 is effective in blocking MLL-FP-induced transformation [60], providing proof of principle for targeting this interaction to treat patients with this disease. Inhibiting the menin/MLL/MLL-FP hub in combination with targeted therapies based on pathways interacting with the menin hub or conventional chemotherapeutic agents will likely lead to a more favorable prognosis for MLL-FP leukemia patients in the future.

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