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STATE-OF-THE-ART REVIEW

Molecular regulators of HOXA9 in acute myeloid leukemia

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Keywords
acute myeloid leukemia; epigenetic regulators; gene regulation; HOXA9; pharmacological inhibitors; transcription factors

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(Received 19 May 2021, revised 30 September 2021, accepted 5 November 2021)

doi:10.1111/febs.16268

Dysregulation of the oncogenic transcription factor HOXA9 is a prominent feature for most aggressive acute myeloid leukemia cases and a strong indicator of poor prognosis in patients. Leukemia subtypes with hallmark overexpression of *HOXA9* include those carrying *MLL* gene rearrangements, *NPM1c* mutations, and other genetic alternations. A growing body of evidence indicates that *HOXA9* dysregulation is both sufficient and necessary for leukemic transformation. The HOXA9 mRNA and protein regulation includes multilayered controls by transcription factors (such as CDX2/4 and USF2/1), epigenetic factors (such as MLL-menin-LEDGF, DOT1L, ENL, HBO1, NPM1c-XPO1, and polycomb proteins), microRNAs (such as miR-126 and miR-196b), long noncoding RNAs (such as HOTTIP), three-dimensional chromatin interactions, and post-translational protein modifications. Recently, insights into the dynamic regulation of HOXA9 have led to an advanced understanding of the HOXA9 regulome and provided new cancer therapeutic opportunities, including developing inhibitors targeting DOT1L, menin, and ENL proteins. This review summarizes recent advances in understanding the molecular mechanisms controlling HOXA9 regulation and the pharmacological approaches that target HOXA9 regulators to treat HOXA9-driven acute myeloid leukemia.

Abbreviations
AFF1/4, AF4/FMR2 family member 1/4; ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; ASXL1, additional sex combs like transcriptional regulator 1; BAP1, BRCA1-associated protein 1; BCOR, BCL6 corepressor; BCORL1, BCL6 corepressor-like 1; CALM, calmodulin; CDX2/4, caudal-type homeobox 2/4; CRISPR, clustered regularly interspaced short palindromic repeats; CTCF, CCCTC-binding factor; DNA, deoxyribonucleic acid; DNMT3A, DNA (cytosine-5)-methyltransferase 3a; DNMT3B, DNA (cytosine-5)-methyltransferase 3b; DOT1L, disruptor of telomeric silencing 1-like; DOTCom, DOT1L containing complex; EAF1/2, ELL-associated factor-1; EED, embryonic ectoderm development; ELL1/2/3, elongation factor for RNA polymerase II; ENL, eleven-nineteen-leukemia protein; EZH2, enhancer-of-zeste homolog 2; Flt3, Fms-related receptor tyrosine kinase 3; HAT, histone acetyltransferases; HBO1, human acetylase binding to ORC1; HOX, homeobox; HSC, hematopoietic stem cell; HSPC, hematopoietic stem and progenitor cell; KMT2A, histone-lysine N-methyltransferase 2A; LEDGF, lens epithelium-derived growth factor; lncRNA, long noncoding RNA; MEIS1, Meis homeobox 1; miRNA, microRNA; MLL, mixed-lineage leukemia; Myb, myeloblastosis proto-oncogene; MYC, MYC proto-oncogene; NF-Y, nuclear factor Y; NPM1c, nucleophosmin 1c; NUP214, nucleoporin 214; NUP98, nuclear pore complex protein 98; PRC1, polycomb repressive complexes 1; PRC2, polycomb repressive complexes 2; PRMT5, protein arginine methyltransferase 5; PSIP1, PC4 and SFRS1 interacting protein 1; P-TEFb, positive transcription elongation factor; RNA, ribonucleic acid; SEC, super elongation complex; SET, su(var)3-9, enhancer-of-zeste and trithorax; SHP2, src homology 2 (SH2) domain-containing phosphatase 2; SUZ12, suppressor of zeste 12 protein; TAD, topologically associating domain; TF, transcription factor; USF2/1, upstream stimulation factor-2/1; XPO1, exportin 1; YEATS, Yaf9-ENL-AF9-Taf14-Sas5.

Introduction

HOX gene family

The homeobox gene family was first discovered through genetic characterization of functional genes responsible for Drosophila development [1,2]. *HOX* gene family is the central homeobox gene family of transcription factors (TFs), and members of this family are highly conserved, carrying a 61 amino acid helix-turn-helix DNA binding homeodomain [3–6]. The *HOX* gene family played a fundamental role in controlling gene expression in early development, including body specification, pattern formation, and cell fate determination during metazoan development [7–9]. The TFs coded by the *HOX* gene cluster are evolutionarily conserved. A total of 39 *HOX* genes in mammals have been classified into four clusters, including *HOXA* on chromosome 7, *HOXB* on chromosome 17, *HOXC* chromosome 12, and *HOXD* on chromosome 2. Within each cluster, there are 13 paralog genes marked by sequence similarity and position. There are two exons and one intron in each *HOX* gene, and a 120-nucleotide sequence in exon 2 encodes a conserved homeobox domain [10]. During normal development, the expression of *HOX* genes within each cluster corresponds to their positions following the direction from the 3′ side (anterior) to the 5′ (posterior) along the anterior–posterior axis. In general, the *HOX* genes expressed earlier at 3′ than those at the 5′ in the cluster during development [11,12]. *HOX* genes' strict temporal and spatial control is critical to establish patterning and morphogenesis in the vertebrate embryos [13,14].

Role of HOXA9 in normal hematopoiesis

During normal hematopoiesis, most expressed *HOX* genes belong to the *HOXA*, *HOXB*, and *HOXC* clusters [15]. In general, *HOX* genes are highly expressed in hematopoietic stem cells (HSCs) and immature progenitor cells, while they are downregulated in more lineage-committed and terminally differentiated cell populations [16,17]. Different *HOX* clusters are expressed in specific lineage-restricted patterns. For instance, *HOXA* cluster genes are frequently expressed in myeloid cells, *HOXB* cluster genes in erythroid cells, and *HOXC* cluster genes in lymphoid cells [18]. *HOXA* 5-10 genes, including *HOXA9*, are highly expressed in hematopoietic stem and progenitor cells (HSPCs) and are crucial for maintaining HSPCs [16]. As HSPCs differentiate and become fully mature, the *HOXA* 5-10 genes are downregulated and epigenetically silenced [16]. This coordinated regulation of *HOXA* gene expression is mediated by various epigenetic factors modulating histone methylation, acetylation, and DNA methylation (Fig. 1). In general, two master regulators of HOXA9 expression, the mixed-lineage leukemia proteins and the polycomb group histone methyltransferases, activate and repress *HOXA9* transcription, respectively. Mixed-lineage leukemia (MLL) methyltransferase MLL1 (KMT2A) positively regulates *HOXA9* expression through trimethylation of histone 3 lysine 4 (H3K4me3) at its promoter [19]. In contrast, the *HOXA9* transcription is repressed by the sequential activity of polycomb repressive complexes PRC1 and PRC2, responsible for trimethylating histone 3 lysine 27 (H3K27me3) [20]. In addition, *HOXA9* expression is highly correlated with H3K79me2 methylation status, and Dot1L, an H3K79 methyltransferase required for sustaining *Hoxa9* expression in HSCs [21]. HSC differentiation also leads to the accumulation of DNA methylation at the *HOXA* 5-10 cluster, mediated by *de novo* methyltransferases DNMT3A and DNMT3B, further ensuing gene silencing and protecting aberrant *HOX* gene activation in more mature hematopoietic cells [22,23].

HOXA9 plays a crucial role in hematopoiesis [24]. Overexpression of *HOXA9* in mice enhances the proliferation of hematopoietic stem and myeloid progenitor cells, leading to leukemogenesis in the long run [18]. Conversely, knockout of *HOXA9* in mice diminishes the number of myeloid progenitors, inducing cell differentiation into the erythroid lineage with maturation [25]. Similarly, *HOXA9*-deficient mice show marked deficiencies in myeloid progenitors, granulocyte/monocyte precursors, and lymphoid precursors [26,27]. Taken together, *HOXA9* functions as a critical regulator of hematopoiesis, essential for the maintenance of HSC and their differentiation into myeloid lineages.

HOXA9 deregulation in leukemia

Overexpression of *HOXA9* is found in about 70% of acute myeloid leukemia (AML) cases and a subset of acute lymphoid leukemia (ALL) cases (Table 1, Fig. 2). *HOXA9* deregulation often coincides with genetic alterations, including *MLL* rearrangements (MLL-r), nucleophosmin 1 in cytoplasmic mutations (*NPM1*c), *NUP98*- fusions, and caudal-type homeobox 2 (*CDX2*) overexpression [28–31]. Other genetic alterations, such as *EZH2* loss-of-function mutation [32], *BCOR*/*BCORL1* [33], *ASXL1* [34], and *DNMT3A* [35], have also been linked to *HOXA9* overexpression. *HOXA9* overexpression is also found in ~ 10% of ALL cases, mostly associated with MLL translocations [36]. The MILE cohort patient's data confirmed that

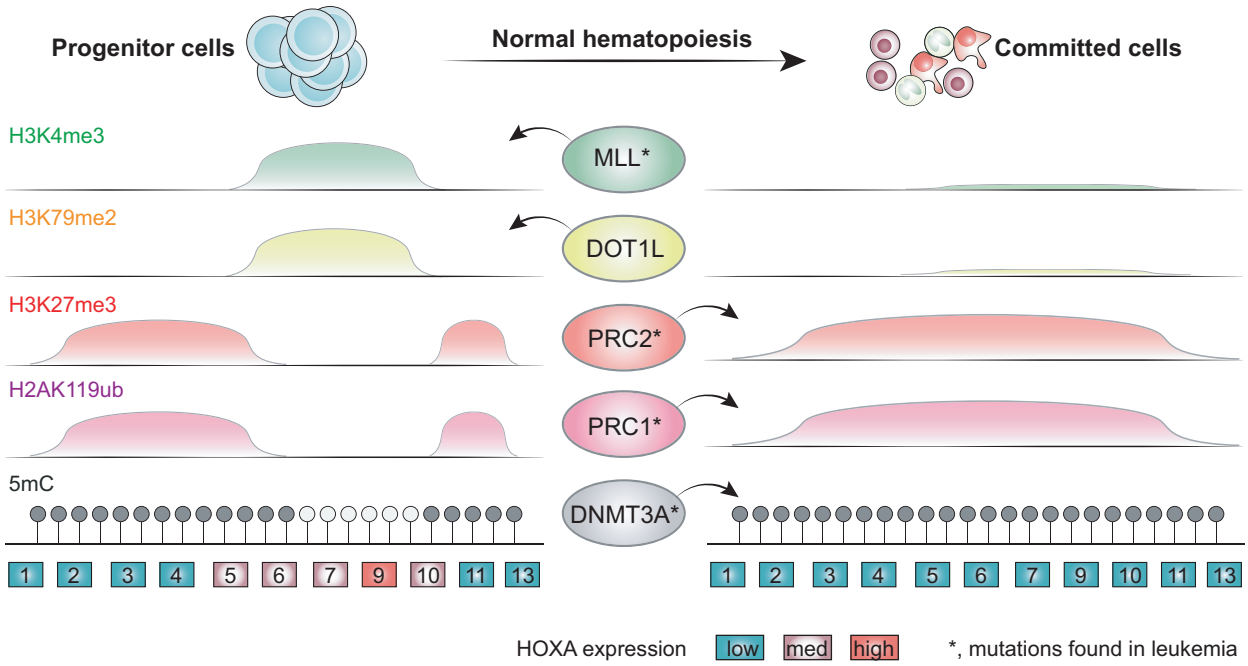

Fig. 1. Schematic diagram of epigenetic landscape and chromatin regulators of HOXA9 during normal hematopoiesis. *HOX* genes are expressed with lineage and differentiation stage-specific patterns. *HOXA5-10*, including *HOXA9* genes, are highly expressed in uncommitted hematopoietic stem and progenitor cells (HSPCs) and are epigenetically repressed during differentiation and maturation. *HOXA5-10* expression in progenitor cells is associated with MLL complex-mediated methylation of histone H3 at lysine 5, and DOT1L methyltransferase-mediated methylation of histone H3 at lysine 79. During differentiation, PRC1 and PRC2 polycomb group proteins repress *HOXA5-10* expression through catalyzing H2AK119 ubiquitination and H3K27 trimethylation, respectively. *De novo* methyltransferase DNMT3A further induces DNA hypermethylation at HOXA5-10 to ensure transcription silencing in more committed cells. Mutations of genes involved in this orchestrated epigenetic regulation are usually found in leukemias.

HOXA9 is extremely highly upregulated in MLL-r AML and B-ALL patients than other leukemia subtypes [37]. More specifically, in AML and ALL, *HOXA9* expressed at a high level at 100% of AML with t(11q23)/MLL group, 83% of AML complex aberrant karyotype group, 75% of AML with normal karyotype group, and 81% of Pro-B-ALL with t (11q23)/MLL group than all other patient's case groups compared with healthy bone marrow groups. Only 21% of AML with inv(16)/t(16;16), 6% of ALL with t(1;19), 4% of c-ALL/Pre-B-ALL without t(9;22), and 1% of c-ALL/Pre-B-ALL with t(9;22) patients showed overexpression of HOXA9. On the contrary, there are rare cases of AML with t(8;21), AML with t (15;17), ALL with hyperdiploid karyotype, ALL with t (12;21), and mature B-ALL with t(8;14), overexpressing *HOXA9* compared with healthy bone marrow patient's cases [37] (Fig. 2). In addition, T-ALL patients bearing inv(7), *CALM-AF10*, or *SET-NUP214* fusions also exhibit *HOXA9* activation [38,39]. These diverse oncogenic pathways that lead to *HOXA9* overexpression imply that *HOXA9* plays an important role in promoting leukemogenesis.

HOXA9 protein shows a significant correlation with poor prognosis in AML patients. It has been demonstrated that out of almost 7000 genes, *HOXA9* was the single most highly overexpressed gene in patients with treatment failure and the strongest predictor of poor prognosis [40]. A study comprising 258 patients has shown that patients with higher *HOXA9* expression levels had a reduced complete remission rate and low survivals in AML [41]. A similar study with AML patient samples reported that *HOXA9* levels were significantly inversely correlated with survival [42]. In addition, the complete remission rate in AML patients with higher *HOXA9* mRNA levels is substantially reduced compared with those AML patients with lower *HOXA9* expression following the chemotherapy [43]. These independent studies underscore that *HOXA9* is one of the most predictive factors for poor prognosis outcomes in AML.

Role of *HOXA9* in leukemia

HOXA9 dysregulation is both sufficient and necessary for leukemic transformation [28]. Forced expression of

Table 1. Leukemia-associated genetic alterations linked to HOXA9 overexpression.

Alterations	Cancer type	Percentage	Subtype	References
MLL Fusion	Leukemia	33	Therapy-related leukemia	[144]
		10	*De novo* Leukemia	[144]
	AML	10	Therapy-related AML	[145]
		3	*De novo* AML	[145]
NPM1	AML	35	All of AML	[18]
		45–55	Normal karyotype AML	[18,146]
		8–10	Pediatric AML	[18,147]
DNMT3A	AML	22	All of AML	[148,149]
		87	Adult AML	[150]
		27	Cytogenetically normal AML	[150]
EZH2	AML	2–13	All of AML	[151]
ASXL1 mutation	AML	6.50	*De novo* AML	[152]
		30	Secondary AML	[152]
BCOR/BCORL1	AML	4.5–7.4	Adult AML	[153,154]
NUP98 fusion	AML	3.80	Pediatric AML	[155]
		35	AML with 11p15 abnormality	[156]

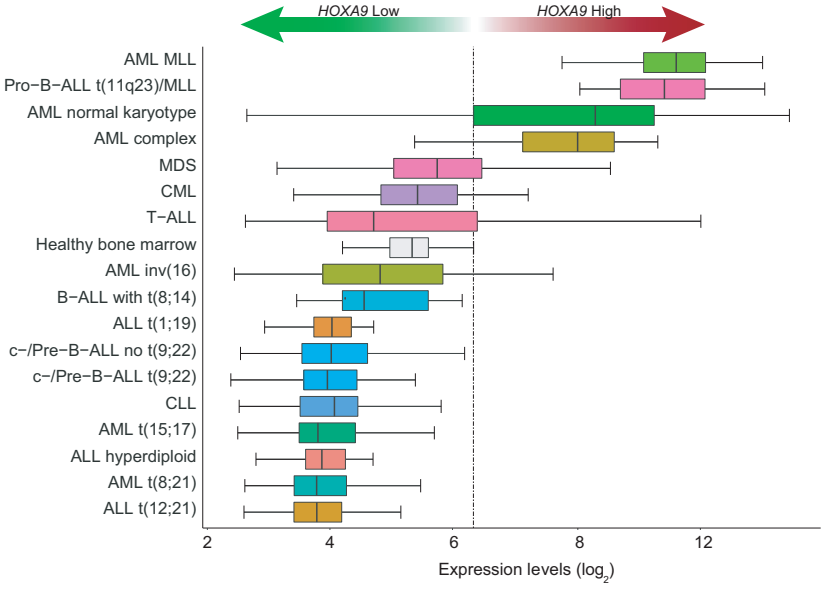

Fig. 2. HOXA9 expression in the MILE leukemia study cohort (Bloodspot). Box plot showing relative expression of *HOXA9* in leukemia subtypes. Overexpression of *HOXA9* is defined as a more than a twofold increase in *HOXA9* expression when compared to the median *HOXA9* expression in healthy bone marrow (vertical dashed line). Box, interquartile range, 25–75 percentiles.

HOXA9 enforces aberrant self-renewal, impairs myeloid differentiation of murine bone marrow progenitors, and ultimately leads to the late onset of leukemia transformation, which can be accelerated by coexpression of the partner *MEIS1* [44,45]. Conversely, knocking down of *HOXA9* in MLL-r AML cells results in leukemic cell differentiation, apoptosis, and reduced disease progression [46].

The mechanistic understanding of *HOXA9*-mediated leukemogenesis has been improved by high-throughput chromatin immunoprecipitation sequencing (ChIP-seq) technologies. HOXA9 protein is predominantly located at gene enhancers and transcriptionally activates a list of proto-oncogenes involved in leukemia development, such as *Erg*, *Flt3*, and *Myb* [47,48]. Moreover, *HOXA9* is reported as a pioneer transcription factor that creates leukemia-specific enhancers by recruiting the MLL3/MLL4 complex, which is essential for controlling gene expression and leukemia development [49].

Given the frequent overexpression and critical role of *HOXA9* in leukemia, *HOXA9* becomes an attractive molecular target. However, due to a lack of druggable domains, *HOXA9* is not quite ideal for therapeutic interventions. Therefore, uncovering the molecular mechanisms governing *HOXA9* expression holds a grand promise for developing practical approaches that inhibit *HOXA9* expression or activity in HOXA9-driven leukemia. This review will summarize the

transcriptional and post-transcriptional regulators of *HOXA9* (Table 2) in leukemia and describe the recent development of small-molecule inhibitors (Table 3) that target *HOXA9*'s regulators for antileukemia therapies.

Transcriptional regulation of HOXA9

Transcription factors

CDX2/4

Caudal genes CDX2 and CDX4 are homeobox transcription factors. When first considering the role of CDX2 in leukemia, it was discovered that the *CDX2* gene locus could be translocated to form a fusion with ETV6; however, this is a rare event that occurs in only a small subset of AML cases with t(15;17) and t(8:21) [31]. The fusion protein ETV6-CDX2 leads to an increased expression of CDX2 protein and subsequent upregulation of *HOXA9* gene expression [50]. Later, *Cdx2* overexpression in a murine model accelerated leukemic development at a similar latency compared with *EVT6-CDX2* [51]. Although this information identified that CDX2 alone could promote leukemia development, the study also found that the ectopic *Cdx2* overexpression group did not upregulate

Table 2. Transcriptional regulators of HOXA9 expression.

Regulators	Regulator role on *HOX* expression	Translocation states	Context	References
Transcriptional regulators				
CDX2/4	Upregulatory	t(8;12); t(12;13)(p13;q12) in MLL-r, t (15;17) and t(8;21) in AML	AML preclinical mouse models	[51,55]
USF2/1	Upregulatory	MLL-r: t(4;11), t(9;11)	MLL-rearranged leukemia (MLL-r), human hematopoietic stem cells	[37,56]
Epigenetic regulators				
MLL1-menin-LEDGF	Upregulatory	t(9;11), t(3;5)(q25;q34), t(9;9)(q34;q34), t(3;21)(q26;q22), t(8;16)(p11;p13), t (10;11)(p13;q14-21)	MLL-r and NPM1c mutant leukemia, MLL-AF9 fusion, Chronic myeloid leukemia	[61,66–69]
DOT1L	Upregulatory	MLL-r: t(9;11)(p22;q23), t(4;11)	MLL-r, MLL fusion, NPM1c mutants, DNMT3A mutants, MLL-AF9 fusion	[19,71,111,130,157]
ENL	Upregulatory	t(11;19)/MLL-ENL	MLL fusion, AML cell line models	[77–79,158]
EZH2	Downregulatory	MLL-r: t(9;11)	MLL-AF9 fusion, AML mouse models	[94,159]
DNMT3A	Upregulatory	Rare fusion transcripts like AML1/ETO, PML/RARA, MLL/AF9 and CBF/βMYH11 Almost always associated with translocations t(15;17), inv(16) and t (8;21) in a mutually exclusive manner	AML patient samples	[35,160,161]
BCOR/BCORL1	Downregulatory		Myeloid murine cells, ML patient samples, Mouse models with hematologic malignancies	[33,102]
Others				
Long non coding RNAs (lncRNAs)	Both Downregulatory [117], Upregulatory [118]	Tumor suppressor role in AML with t (8;21)	AML cell lines	[111,112,162]
CTFC chromatin organization	Both	MLL-r AML: t(9;11), t(11;19), RUNX1-RUNX1T1: t(8;21)	AML cell lines	[113,115–117]
microRNA	Downregulatory	Bcr-Abl fusion: t(9;22)(q34;q11), MLL-r: t(9;11), CA-AML: t(8;21)	AML cell lines	[118–121]
Post-translational modifications	Both		AML cell lines	[122–127]

Table 3. Pharmaceutical inhibitors that target HOXA9 upregulation.

Targets	Regulatory role	Representative compounds	Effect in HOXA9	Clinical development	References
DOT1L	H3K79 methyltransferase	EPZ004777	Statistic significant downregulation in MLL-r AML	Preclinical	[132]
		EPZ-5676		Phase I/II	[132]
		SGC0946		Preclinical	[134]
MENIN	Chromatin associated protein	VTP50469	Statistic significant downregulation in MLL-r or NPM1c AML	Preclinical	[65]
		MI-3545		Preclinical	[136]
		KO-539		Phase I	[137]
		SNDX-5613		Phase I/II	[138]
		MI-2-2		Preclinical	[163]
		MI-463		Preclinical	[164,165]
		MI-503		Preclinical	[165]
ENL	Histone acetylation reader	SR-1114	Statistic significant downregulation in MLL-r AML	Preclinical	[140]
		SR-0813		Preclinical	[140]
XPO1	Nuclear-cytoplasmic transport protein	KPT-8602	Some downregulation in AML	Preclinical	[141]
		KPT-330		Phase I/II	[142]
		KPT-185		Preclinical	[89]
		KPT-276		Preclinical	[89]
HBO1	Histone acetyltransferase	WM-3835	Significant downregulation in AML	Preclinical	[80,81]

HOXA9 gene expression compared with control animals [51]. A later study from the same group found that the N-terminal domain of the *Cdx2* gene, the transactivation domain provides the binding sites for coregulators and transcription factors. The deletion of the N-terminal domain rendered a truncated CDX2 protein that demonstrated a reduction in *HOXA* gene expression compared with control [52]. In addition to ETV6-CDX2 fusion gene expression, ectopic CDX2 expression was observed in t(12;13)(p13;q12) positive AML and is a transforming event in the mouse model of t(12;13) AML (p13;q12) AML [51]. Moreover, another caudal-related gene, CDX4, has been shown as an important regulator for maintaining HOXA9 expression during embryonic hematopoiesis in zebrafish [53,54] and can regulate both HOXA9 and HOXA10 expression [55].

USF2/1

Two transcriptional factors, upstream stimulation factor-1 (USF-1) and upstream stimulation factor-2 (USF-2), have been linked to *HOXB* gene expression during normal hematopoiesis [56,57]. Recently, by utilizing an unbiased CRISPR screen targeting 1,639 human transcription factors in a *HOXA9*-mCherry reporter MLL-r leukemia cell line, we have identified USF2 as a novel positive regulator of HOXA9. USF2 directly binds to a conserved motif at *HOXA9* promoter, and USF2 depletion downregulates *HOXA9* expression in MLL-r leukemia cells and impairs cell growth, which can be rescued by ectopic expression of *HOXA9* [37].

Epigenetic modulators

MLL-menin-LEDGF complex

The mixed-lineage leukemia gene MLL (MLL1, KMT2A) encodes a histone methyltransferase that contains a C terminus SET domain for catalyzing the methylation of lysine 4 of histone 3 (H3K4) [58,59]. Leukemia-associated MLL gene rearrangements, which affect only one allele of the endogenous MLL gene, would produce a fusion oncoprotein that directly binds and constitutively activates *HOXA9* and the HOX cofactor *MEIS1* [45,60]. The MLL gene fusion results in a loss of the C terminus SET methyltransferase domain. Still, it retains the N-terminal domain involved in interaction with chromatin cofactors such as menin [61]. Wild-type MLL is required to maintain *HOX* gene expression during normal hematopoiesis [62]. In MLL-r AML, the remaining wild-type MLL allele was initially shown to be essential for *HOX* gene expression and leukemogenesis [63]; however, a more recent study reported that MLL2, but not MLL, is required for the cell growth of MLL-r leukemia [64]. In non-MLL-r leukemia subtypes such as NPM1c AML, the wild-type MLL remains critical for leukemia development [65].

Menin is a chromatin-associated nuclear protein essential for the transcriptional regulation of MLL target genes and maintenance of *HOX* gene expression by MLL fusion proteins [66]. A 5-amino acid RWRFP sequence near the N terminus of MLL is essential for interaction with menin [66]. The resulting menin-MLL interaction plays a critical role in the pathogenesis of

MLL leukemia [61,66]. Menin might act as an adapter protein because it lacks known protein motifs but interacts with MLL complex components such as LEDGF [67]. Moreover, it has been reported that menin, along with Cdx4, co-activated the *HOXA* genes by binding to the same regulatory region at the *HOXA9* locus [68]. In addition, inactivation of menin leads to differentiation arrest and reduced oncogenic potential of MLL fusion [66]. Menin can interact with wild-type MLL and MLL fusion proteins to facilitate MLL-mediated gene expression regulation [69].

LEDGF/PSIP1, a transcriptional coactivator recruited to the MLL complex by menin, is also critical for MLL-r AML [70]. Blocking the interaction between LEDGF and MLL/menin can downregulate MLL's target HOXA9 by impairing cell cycle progression and growth of MLL fusion-transformed human and mouse HSCs [70] (Fig. 3A).

DOT1L and super elongation complex (SEC)

The disruptor of telomere silencing 1-like (DOT1L) is a histone-lysine methyltransferase that methylates lysine 79 residues of histone H3 [71]. DOT1L is associated with several members of the super elongation complex (SEC), which consists of RNA polymerase II elongation activators or coactivators, including P-TEFb, ELL1/2/3, AFF1/4, ENL, AF9, and EAF1/2 [72]. Because the fusion partners of MLL are often components of the SEC, the MLL fusion protein can recruit DOT1L to its target genes for aberrant H3K79 methylation modification [19,71] (Fig. 3A). This H3K79 methylation then facilitates constitutive activation of *HOX* genes and other oncogenes [19]. In addition, the aberrant methyltransferase activity of DOT1L is required for the leukemogenesis of several non-MLL-r leukemias, including leukemias with *NPM1* mutation [67] and *DNMT3A* mutation [73,74].

ENL

ENL belongs to the chromatin histone acetylation reader protein family with a distinct amino-terminal named YEATS domain and a disordered carboxy-terminal protein–protein interaction interface [75]. Recently, the ENL YEATS domain has been implicated in interaction with acetylated histone H3 [76]. Studies have shown that ENL is crucial for *HOXA9*/10, *MEIS1*, and *MYC* gene expression and aids in blocking cell differentiation in MLL-rearranged leukemia [77]. Similarly, Wan *et al.* [78] have also shown that ENL is required for AML maintenance. Wan's finding is accomplished through the binding of ENL to the acetylated histone H3 and colocalization with H3K27ac and H3K9ac on the promoters of genes essential for leukemia and inducing active transcription [78]. Generally, in MLL-rearranged leukemia, MLL fusion proteins interact with the super elongation complex (SEC) or the DOT1L containing complex (DOTCom) and modulate gene expression in both cases. The protein ENL can associate with both of these complexes and, interestingly, can interact with both the fused (MLL fusion/SEC/DOTCom) and nonfused complexes (wild-type MLL) to drive leukemia [79]. Taken together, ENL serving as a histone acetylation reader regulates oncogenic transcriptional programs in acute myeloid leukemia.

HBO1

HBO1 histone acetyltransferase (HAT), also known as KAT7, is a member of the MYST HAT family and is responsible for histone H3 lysine 14 acetylation [80,81]. HBO1 maintains leukemia stem cells by maintaining higher expression of *HOXA9* and *HOXA10* through H3K14 acetylation followed by RNA pol II activation in leukemia [80] and is a potential therapeutic target in AML [81]. Recently, it has also been identified that HBO1-MLL interaction is a crucial step in promoting AF4/ENL/P-TEFb-mediated leukemogenesis [80,82].

NPM1c-XPO1

Nucleophosmin (NPM1) is a ubiquitously abundant nucleolar protein that maintains genome integrity, DNA repair, and ribosome biogenesis [83]. Under the normal physiologic condition, NPM1 protein is localized in nucleoli. However, the AML-related NPM1 mutations at its C terminus (i.e., NPM1c) lead to abnormal cytoplasmic dislocation of the protein [84]. *NPM1c* AML is associated with aberrant activation of *HOXA* and *HOXB* cluster genes, including *HOXA9* [85,86]. The relocalization of NPM1c from the cytoplasm to the nucleus or targeted NPM1c degradation results in disruption of the oncogenic program through downregulation of HOX genes followed by induction of myeloid cell differentiation [87]. Xportin 1 (XPO1), also known as chromosomal region maintenance 1 (CRM1), a major nuclear-cytoplasmic transport protein, interacts with NPM1c and transports NPM1c to the cytoplasm [88,89]. Genetic or pharmaceutical inhibition of XPO1 blocks NPM1c transport and subsequently results in AML growth arrest and differentiation [90] (Fig. 3B, Model I). Intriguingly, a much recent study identified a second possible

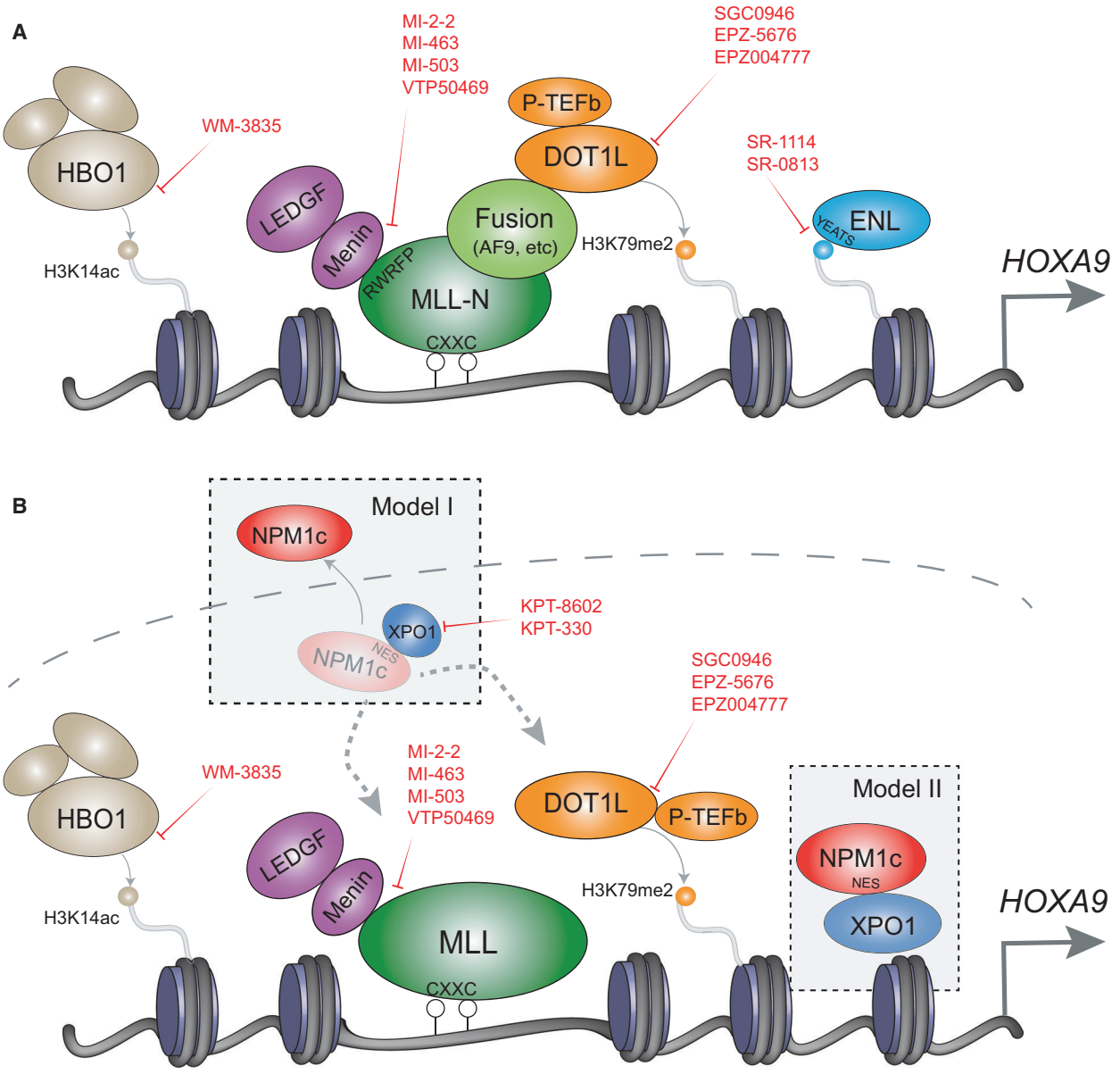

Fig. 3. HOXA9 regulation and pharmacological inhibition of HOXA9. (A) In MLL-r AML, HOXA9 is maintained by the multiple epigenetic modulators, such as the MLL-menin complex, DOT1L super elongation complex, HBO1 complex, and AF9 protein. Several small-molecule inhibitors have been developed to target these epigenetic proteins, including menin inhibitors (MI-2-2, MI-463, MI-503, VTP50469), which block menin-MLL interaction; DOT1L inhibitors (SGC0946, EPZ-5676, EPZ004777), which inhibit DOT1L methyltransferase activity; HBO1 inhibitor (WM-3835), which binds directly to the acetyl-CoA binding site of HBO1 to inhibit its acetyltransferase activity; and inhibitors of ENL (SR-1114 and SR-0813), which disrupts ENL's interaction with histone acetylation. (B) In NPM1 mutant AML, the HBO1, MLL-menin, and DOT1L complexes are also implicated in HOXA9 regulation. In addition, several hypotheses have been proposed for the mechanism of NPM1c-HOXA9 gene regulation. For example, the AML-associated *NPM1c* mutant is exported to the cytoplasm by nuclear protein export receptor XPO1. Pharmacological suppression of XPO1 in *NPM1c* AML relocalizes NPM1c to the nucleus and inhibits HOXA9 gene expression (Model I). Recently, NPM1c has also been suggested to bind to chromatin with XPO1 at HOXA genes including HOXA9 (Model II).

mechanism by which NPM1c aberrantly regulates *HOX* gene expression. This study is accomplished by the nuclear relocalization of NPM1c in an XPO1-dependent manner and their direct binding to *HOX* cluster regions to activate *HOX* genes [91] (Fig. 3B, Model II). Because the wild-type MLL is required for NPM1 mutant AML, NPM1c AML also depends on MLL-menin interaction. Small-molecule inhibition of

MLL-menin interaction reduces *HOX* gene expression and promotes leukemic cell differentiation [65,92].

EZH2

The enhancer-of-zeste homolog 2 (EZH2) belongs to polycomb group complex 2 (PRC2) that typically functions as a histone methyltransferase to add a tri- or di-methylation mark on lysine 27 of histone 3 (H3K27me3/2), causing transcriptional repression of the marked gene [93]. While gain-of-function mutations of *EZH2* are found in lymphoid malignancies [94], loss-of-function mutations of *EZH2* have been found in myeloid malignancies such as MDS, MPN, and AML [32,95–97]. Reduced *EZH2* expression is significantly associated with poor prognosis and chemoresistance in AML [98]. Interestingly, overexpression of *HOXA9* is found in myeloid malignancies with decreased EZH2 expression [98,99]. Knockdown of *EZH2* in AML cells results in elevated *HOXA9* levels [98], supporting a negative regulatory role of *EZH2* on *HOXA9* expression in MLL-r AML. The studies on the mouse models also confirmed that *Hoxa9* was depressed by *EZH2* loss at the myelodysplastic syndrome stage [100].

BCOR

BCL6 corepressor (BCOR), a crucial component of a polycomb repressive complex 1 (PRC1) variant, has an essential role in regulating cell fate transition and myeloid differentiation during normal hematopoiesis [33,101]. In one study, researchers have identified that *BCOR* acts as a repressor of *HOXA* cluster gene members (*HoxA5*, *HoxA7*, and *HoxA9*) to promote leukemia [33]. A recent study supports this notion showing that *BCOR* inactivation in hematopoietic stem cells (HSCs) results in aggressive acute leukemia [102]. Through gene expression analysis and chromatin immunoprecipitation sequencing, they have revealed differential regulation of *HOXA7* and *HOXA9* upon *BCOR* inactivation [102]. It has also been suggested that BCOR homolog (BCORL1) mutations have been characterized in many AML subtypes, and the mutation spectrum of *BCORL1* is very similar to BCOR mutations [103]. Whether *BCORL1* plays a similar role in repressing HOXA9 expression remains to be investigated.

ASXL1

Additional sex comb-like 1 (ASXL1), which encodes a regulator of gene expression, is frequently mutated in myeloid malignancies, including ~ 10–20% of AML [104,105]. ASXL1 is involved in the regulation of H2AK119ub by interacting with chromatin deubiquitinase BAP1 [106]. Loss of *ASXL1* results in loss of H3K27me3 and increased expression of *HOXA9* in leukemia cells [34]. Mechanistically, ASXL1 forms a complex with PRC2 members EZH2, SUZ12, and EED and is required for PRC2 recruitment at the *HOXA* locus for gene repression [34]. Other studies suggest that *ASXL1* mutations may lead to a hyperactive ASXL1-BAP1 complex that removes H2AK119 mono-ubiquitylation and induces *HOXA9* upregulation [107].

DNMT3A

DNMT3A, a *de novo* DNA methyltransferase, establishes DNA methylation at CpG sites during development and disease [108]. Frequent alterations of *DNMT3A* have been recently noticed in a wide variety of hematologic malignancies, which seems to confer negative predictive values in AML patients [35]. DNA hypomethylation at *HOXA* genes is observed in AML patient samples with *DNMT3A* mutations and mouse models with *DNMT3A* knockout or hot spot mutations [23,74,86]. Several studies have shown that *DNMT3A* mutants in hematopoietic progenitor cells facilitate *HOXA9* gene upregulation in mouse models [74,109,110].

Long noncoding RNAs

HOX gene expression has also been shown to be regulated by long noncoding RNAs (lncRNAs). Though direct regulation of *HOXA9* by lncRNA has not yet been established, researchers have demonstrated both the repressive and expressive regulatory mechanisms underlying lncRNA regulation of other *HOX* gene members. A seminal study by Rinn *et al.* identified a trans-regulatory system termed HOTAIR. This noncoding RNA is expressed initially from the *HOXC* locus and regulates *HOX* gene expression in the *HOXD* cluster. They reported that HOTAIR could recruit PRC2 member SUZ12 and increase H3K27 trimethylation to repress *HOXD* gene clusters (but not *HOXC* and *HOXB* gene members) [111]. In contrast, Wang and colleagues have reported that HOTTIP functions through a cis-regulatory manner, in which this long noncoding RNA is transcribed from the *HOXA* cluster and binds and activates *HOXA* genes. HOTTIP appears to accomplish this through chromosomal looping, bringing HOTTIP near the target genes, and increasing H3K4 trimethylation to activate gene transcription [112].

CTCF and three-dimensional chromatin organization

Recently, a few studies focused on the molecular mechanisms underlying the role of three-dimensional chromatin architecture associated and aberrant *HOXA9* expression by tackling CTCF-binding sites [113–115]. While global CTCF deletion in the genome by CRISPR knockout or shRNA induced a significant survival crisis and led to biased phenotypes [116], CRISPR-mediated genomic editing of minimal CTCF-binding consensus sequences holds the promise to reveal CTCF's function in regulating *HOXA* expression. Deletion of CTCF-binding sites (CBS) within *Hox* clusters disrupted topological boundaries and caused the spreading of active transcription into previously repressed domains in mouse embryonic stem (ES) cells [117]. In MLL-rearranged AML cell line MOLM-13, disruption of a CTCF boundary between *HOXA7* and *HOXA9* genes perturbs chromatin structure was reported to reduce *HOXA* gene transcription and represses AML engraftment in mouse models [115]. The role of CTCF in regulating gene expression can be highly context-dependent and cell-type-dependent. In another independent study, targeted deletions of CBS in the NPM1 mutant OCI-AML3 AML cell line eliminated CTCF binding occupancy but had minimal influence on HOXA expression [113]. Therefore, the precise regulation of *HOXA9* by CTCF-dependent chromosomal architectures warrants further investigations in genetically defined leukemia models.

Post-transcriptional regulation of HOXA9

In addition to regulating HOXA9 at transcriptional levels, many post-transcriptional cascades, including mRNA processing and post-transcriptional modifications, regulate HOXA9 protein levels and function (Table 2). Although relatively less studied than transcriptional regulation, these processes represent additional layers of control of HOXA9 and could provide new therapeutic opportunities for targeting *HOXA9*.

Regulation by microRNAs

MicroRNAs (miRNA) are small, noncoding RNAs that play essential roles in post-transcriptional gene regulation [118]. Several miRNAs, such as miR-126 [119], miR-196b [120], and miR-181 [121], have been identified as regulators of *HOXA9* expression. miR-126 is the first experimental validated microRNA regulator of *HOXA9* [119]. miR-126 binds to *HOXA9* homeobox and inhibits *HOXA9* protein levels in MLL-ENL cells [119]. miR-196b is a miRNA located adjacent to *HOXA9* at the *HOXA9* cluster and is co-expressed with *HOXA9* in human AMLs [120]. Interestingly, miR-196b directly targets *HOXA9* and its partner, *MEIS1*, for gene repression, suggesting a negative feedback loop of *HOXA9* regulation in AML [120]. miR-181a and miR-181b are associated with favorable outcomes in cytogenetically abnormal AML [121]. Ectopic expression of miR-181b leads to decreased *PBX3* and *HOXA* cluster gene expression levels and delayed leukemogenesis [121].

Regulation by post-translational modifications

In various biological contexts, post-translational modifications such as ubiquitination [122], methylation [123,124], and phosphorylation [125] have been identified as regulators of HOXA9 protein. Zhang and colleagues have identified that CUL4A, a member of the cullin protein family of ubiquitin-protein ligases, promotes HOXA9 ubiquitination and subsequent proteasome-dependent degradation in myeloid progenitor cells [122]. During cardiomyocyte hypertrophy or inflammation in endothelial cells, protein arginine methyltransferase 5 (PRMT5) binds to HOXA9 and induces arginine methylation on HOXA9 to modulate *HOXA9* expression or activity [123,124]. A consensus sequence in the N-terminal region of the HOXA9 homeodomain has been found to be phosphorylated by protein kinase C (PKC) and casein kinase II, which alters the affinity of *HOXA9* for DNA binding [126]. Moreover, additional and distinctive phosphorylation sites of *HOXA9* have been suspected in various contexts [127].

Pharmacological targeting HOXA9 expression by small-molecule inhibitors

DOT1L inhibitors

DOT1L inhibitors (Table 3) have shown to have some promising efficacy for MLL-rearranged leukemia and are currently under investigation in clinical trials to investigate the therapeutic benefits of targeting DOT1L. In an induced homozygous deletion of the Dot1L mouse model, the mice's death was due to severe anemia, hypocellularity in the bone marrow, and depletion of hematopoietic stem cells [128]. Another study, using a hematopoietic cell knockout model, confirmed that not all developed cells via hematopoiesis are dependent on DOT1L. [129]. Following knockout/down studies, Dot1l contributes to MLL-AF9-mediated

leukemogenesis by upregulation of oncogene *Hoxa9* and *Meis1*. Investigators are creating compounds to target and inhibit DOT1L for AML treatment. These compounds can inhibit DOT1L enzymatic activity by competing with the cofactor *S*-adenosyl methionine (SAM) [130]. There are three compounds under investigation: EPZ004777, EPZ-5676, and SGC0946. In a preclinical study, the researchers discovered that treating MLL-leukemic cells with EPZ004777 inhibits H3K79 methylation and, as a result, reduces the expression of downstream targets HOXA9 and MEIS1 by around 80% in MLL-AF9 transformed cells but not in normal myeloid progenitors [131]. Additionally, the DOT1L inhibitor reduced the proliferation of MLL-leukemic cells and induced apoptosis [131]. A few years later, the same group demonstrated similar results with the treatment of EPZ-5676 [132]. Similarly, SGC0946 was synthesized to be a more potent DOT1L inhibitor than EPZ004777. [133]. In leukemia patients, a phase I clinical trial of EPZ-5675, also known as pinometostat, found that patients responded well to various therapeutic dosages. Only two of the 51 participants experienced complete remission at the end of the trial; however, one of those participants experienced an aggressive relapse following the study's conclusion. This suggests that continued DOT1L targeting treatment is required [134]. Although high EPZ-5676 blood concentrations mirrored anti-tumor effects in preclinical studies, there was no significant evidence that the treatment effectively suppressed cancer. Based on this, the authors hypothesized that DOT1L should be considered in combination with other therapies in leukemia patients [134]. A phase Ib/II clinical investigation is now ongoing in MLL leukemia patients to assess the safety and efficacy of pinometostat with chemotherapy.

MENIN inhibitors

Menin is another therapeutic target being studied in MLL-rearrangement leukemias. Direct examination of patient models with *HOXA* gene overexpression reveals that AML subtypes including mutations *KMT2Ar* (11q23 rearrangements), *NpM1-MLF1* (t(3;5)(q25;q34)), *NUP98r* (11p15 rearrangements), *SET-NUP214* (t(9;9)(q34;q34)), *RUNX1-EV11* (t(3;21)(q26;q22)), *MYST3-CREBBP* (t(8;16)(p11;p13)), *CALM-AF10* (t(10;11)(p13;q14-21)), *EZH2,* and *ASXL1* have better response with menin inhibitors [135]. In a preclinical study, the compound VTP50469 was found to inhibit the binding of menin with target proteins to form leukemogenic protein complexes. The treatment of leukemic cells with this compound inhibited proliferation, induced differentiation, and promoted apoptosis. Additionally, in patient-derived xenograft models, treatment with VTP50469 was able to eradicate leukemia. Menin-MLL interaction is also required in *NPM1* mutant leukemia cells to maintain the aberrant expression of *HOXA* genes [65]. Another preclinical study found that using the menin inhibitor MI-3545 can induce remission in MLL-rearranged or *NPM1* mutant leukemias [136]. Due to the positive effects of targeting menin in preclinical studies, several phases I clinical trials are currently underway to measure the safety and efficacy of menin inhibitors in AML patients. KO-539, an oral menin inhibitor, is being evaluated in the ongoing first-in-human KOMET-001 trial in patients with relapsed or refractory AML. A preliminary report indicates that KO-539 is well tolerated in participants and demonstrates efficacy in treating leukemia depending on the mutations [137]. Additionally, another menin inhibitor SNDX-5613 is under investigation in a phase I/II clinical trial to investigate the safety and efficacy in MLL-rearranged and NPM1-mutated leukemias [138].

ENL inhibitors

An ENL YEATS domain selective inhibitor XL-13m has been reported to induce downregulation of MLL-r regulated oncogenes such as *HOXA9* by repressing ENL recruitment on chromatin [139]. A recent study by Wortzel *et al.* [140] has developed an ENL degrader, SR-1114, and an ENL YEATS domain inhibitor, SR-0813, antileukemia therapies. SR-1114 and SR-0813 selectively inhibit the growth of ENL-dependent leukemia cell lines and downregulate ENL target genes such as HOXA9/10 [140].

XPO1 inhibitors

Exportin 1 (XPO1) belongs to nuclear-cytoplasmic transport protein families and has recently emerged as a therapeutic target in leukemia [141,142]. A second-generation XPO1 inhibitor, KPT-8602, also called Eltanexor, has been reported to have potent activity against ALL in preclinical models [141]. KPT-330 (selinexor), another XPO1 inhibitor, is already under phase I/II clinical trials for CLL and AML [142]. Preclinical studies of KPT-185 and KPT-276, two orally bioavailable selective inhibitors of XPO1, have also been reported to confer promising antileukemic effects both *in vitro* and *in vivo* models AML models [89]. Furthermore, XPO1 inhibitor treatment in AML cell lines and patient samples leads to intranuclear accumulation of NPM1 followed by restoration of normal cellular homeostasis, suggesting XPO1 as an attractive

target during AML with some downregulation of *HOXA9* expression and consistent with other studies [89,143] (Fig. 3B, Model I).

HBO1 inhibitors

Studies have shown that HBO1 maintains higher *HOXA9/10* expression in leukemia through the activation of RNA pol II [80] and is a potential therapeutic target in AML [81]. Recently, a cell-permeable small HBO1 inhibitor molecule WM-3835 has been reported to show an antileukemic effect in human AML cell lines with significant downregulation *HOXA9* expression [80,81].

Concluding remarks and future directions

HOXA9 is a promising target for leukemia therapy as it is highly expressed in leukemia subtypes driven by diverse genetic mutations. Many *HOXA9* regulators, including transcription factors, epigenetic modulators, lncRNAs, microRNAs, 3D chromatin organizations, and post-transcriptional modifications, play critical roles in regulating *HOXA9* expression and function. Despite that *HOXA9* itself is a difficult drug target, pharmacological intervention with small molecules inhibiting *HOXA9* expression or function holds great promise for leukemia therapy. As the regulation of *HOXA9* is complex, therapeutic response to these small-molecule inhibitors may be highly context and subtype-dependent. It is also crucial to dissect the crosstalk of various transcriptional and epigenetic *HOXA9*-regulating pathways that influence drug response. To systematically discover regulators of *HOXA9*, our laboratory has successfully generated endogenous $HOXA9^{P2A\text{-}mCherry}$ reporter MLL-r AML and ALL cell lines that could monitor *HOXA9*'s expression in real time without affecting endogenous transcription of other adjacent *HOXA* genes [37]. We have performed CRISPR/Cas9 screening in the transcription factor library with this reporter and identified a novel positive regulator USF2 of *HOXA9* [37]. The advance of CRISPR/Cas9 genetic screen technologies and such newly developed *HOXA9* reporter cell lines would provide a robust and unbiased platform for discovering novel regulators controlling *HOXA9* expression and identifying new alternative strategies to overcome the resistance to *HOXA9*-inhibiting agents by combined genetic or drug screenings.

Acknowledgements

RL is an American Society of Hematology Scholar in Basic Science and is supported by Concern Foundation, Leukemia Research Foundation, and Institutional Research Grant the American Cancer Society. CL is supported by ALSAC and V Foundation for Cancer Research. We apologize to researchers whose work could not be cited owing to space limitations.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

SA, YZ, SW, CL, and RL wrote the manuscript. All authors confirmed the authorship before submission.

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