



# A SALL4/MLL/HOXA9 pathway in murine and human myeloid leukemogenesis

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**The embryonic self-renewal factor SALL4 has been implicated in the development of human acute myeloid leukemia (AML). Transgenic mice expressing the human *SALL4B* allele develop AML, which indicates that this molecule contributes to leukemia development and maintenance. However, the underlying mechanism of SALL4-dependent AML progression is unknown. Using *SALL4B* transgenic mice, we observed that *HoxA9* was significantly upregulated in SALL4B leukemic cells compared with wild-type controls. Downregulation of *HoxA9* in SALL4B leukemic cells led to decreased replating capacity in vitro and delayed AML development in recipient mice. In primary human AML cells, downregulation of *SALL4* led to decreased *HOXA9* expression and enhanced apoptosis. We found that *SALL4* bound a specific region of the *HOXA9* promoter in leukemic cells. *SALL4* overexpression led to enhanced binding of histone activation markers at the *HOXA9* promoter region, as well as increased *HOXA9* expression in these cells. Furthermore, we observed that *SALL4* interacted with mixed-lineage leukemia (*MLL*) and co-occupied the *HOXA9* promoter region with *MLL* in AML leukemic cells, which suggests that a SALL4/MLL pathway may control *HOXA9* expression. In summary, our findings revealed a molecular mechanism for SALL4 function in leukemogenesis and suggest that targeting of the SALL4/MLL/HOXA9 pathway would be an innovative approach in treating AML.**

## Introduction

*SALL4*, a member of the zinc finger transcription factor SALL gene family, is the human homolog of the *Drosophila* homeotic gene *spalt*. The role of *SALL4* as a transcription factor has been very well established in early embryonic development, as *Sall4*-null mice die shortly after implantation (1). Our group and others have also shown that in mice, murine *Sall4* plays an essential role in regulating the pluripotency and self-renewal properties of ES cells through directly regulating the expression of Oct4 and coordinating with Nanog to control ES cell differentiation (2). *Sall4*-deficient ES cells differentiate spontaneously and form the trophoblast in mouse blastocysts, indicating its vital role in maintaining pluripotency (3).

The protein is present in humans in 2 isoforms, SALL4A and SALL4B, as a result of alternative splicing (4). Human *SALL4* mutations are associated with Duane-radial ray syndrome (DRRS; also known as Okhiro syndrome) (5–8). This condition is an autosomal-dominant disorder involving radial-sided hand anomalies in association with Duane syndrome (DS), a congenital disorder of eye movement characterized by strabismus.

SALL4 is also involved in normal hematopoiesis and leukemogenesis. During normal hematopoiesis, SALL4 is preferentially expressed in human CD34<sup>+</sup> hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) and downregulated in CD34<sup>+</sup> cells during hematopoietic differentiation (4). Depletion of SALL4 in CD34<sup>+</sup> cells impaired their proliferation and self-renewal ability (9). Recently, SALL4 was reported to be a robust stimulator of HSC expansion (10). In addition to its role

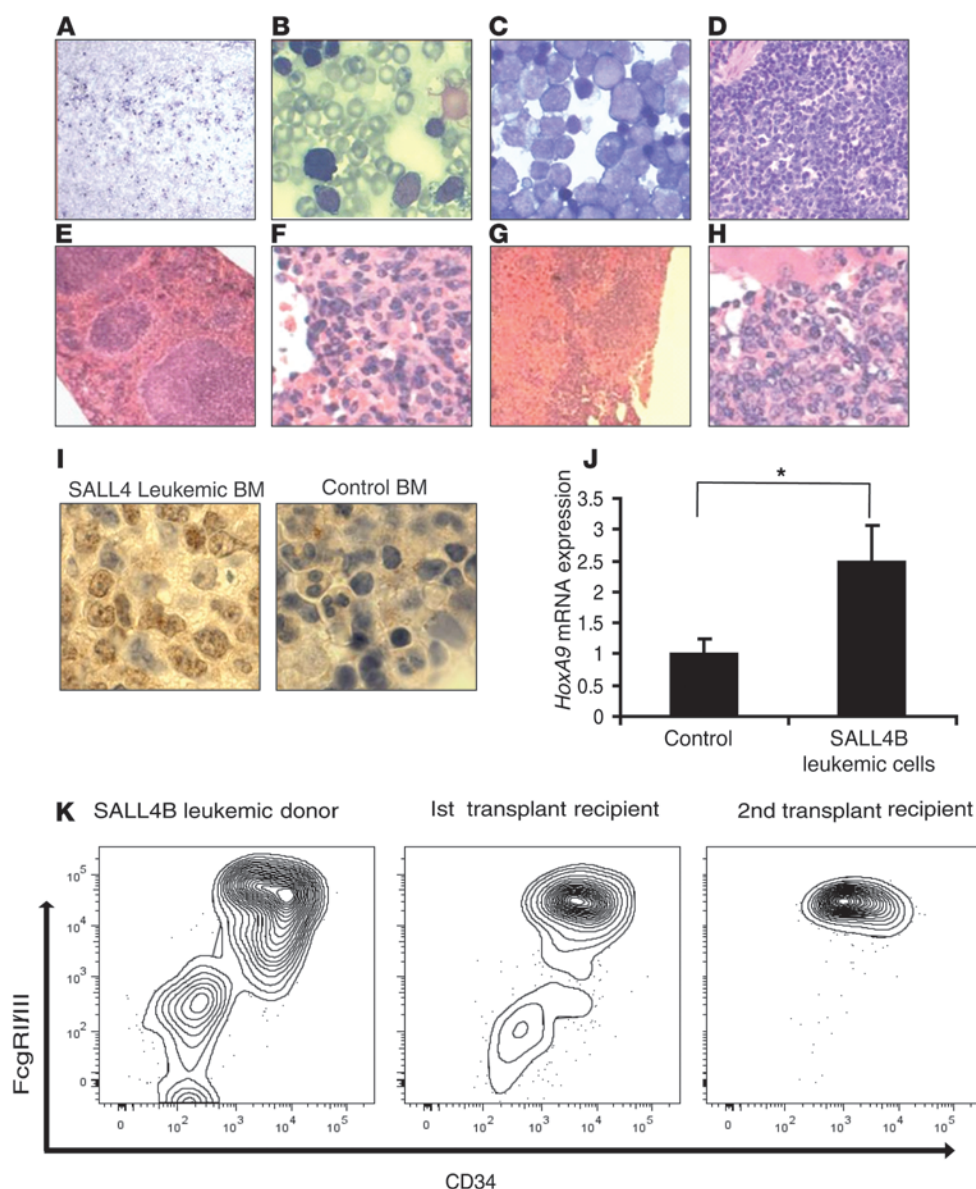
in normal hematopoiesis, we previously found that SALL4 is aberrantly expressed in myeloid leukemia cell lines and primary acute myeloid leukemia (AML) samples by using immunohistochemical staining and quantitative real-time RT-PCR (qRT-PCR) analysis (4). Moreover, SALL4 expression correlates with disease progression in human chronic myeloid leukemia (11). Its expression in AML patients correlates with treatment status (12). Further exploration revealed the role of SALL4 in drug resistance, in which SALL4 was involved in the maintenance of side population (SP) cells by regulating ATP-binding cassette drug transport genes (12). Therefore, SALL4 may be used as a marker for diagnosis and prognosis in AML.

Previously, we also demonstrated that constitutive expression of SALL4 contributes to leukemogenesis in adult mice (4). Mice transgenic for *SALL4B*, one of the SALL4 isoforms we identified, developed preleukemic myelodysplastic syndrome-like (MDS-like) features and subsequent AML, which suggests that SALL4 contributes to the initiation of leukemia (4). In addition, loss-of-function studies have demonstrated that SALL4 is a key regulator in leukemic cell survival, and downregulation of *SALL4* leads to significant apoptosis of leukemic cells (13), which suggests that SALL4 is essential for the maintenance of leukemia cells.

HOXA constitutes 1 of the 4 families of HOX genes, which are transcription factors characterized by a homeobox domain. Mutations of the HOX genes have been linked to defects of limb and genital development (14, 15). In addition, *HoxA9* is critical to murine granulopoiesis, and dysregulated *HOXA9* expression is implicated in more than 70% of human AML. Its expression is enriched in human CD34<sup>+</sup>CD38<sup>+</sup> stem cells compared with normal CD34<sup>+</sup> cells (16). Consistent with its role in leukemogenesis,

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**Figure 1**

Upregulation of *HoxA9* in SALL4B leukemic cells. (A–H) Leukemia development in recipient mice. Images are from a representative recipient of a primary transplant. Leukocytosis was observed in the blood smears (A). Blasts were present in the peripheral blood (B), BM smear (C), lymph node (D), spleen (E and F), and liver (G and H). Original magnification,  $\times 40$  (A, E, and G),  $\times 200$  (D, F, and H),  $\times 600$  (B and C). (I) SALL4 expression was determined by IHC staining in SALL4B leukemic BM cells. Original magnification,  $\times 600$ . (J) Validation of *HOXA9* upregulation in SALL4B leukemic GMP population. qRT-PCR analysis of gene expression in SALL4B leukemic GMPs compared with control normal GMPs. Measurements were from 3 individual secondary transplanted recipients ( $n = 3$ ), each performed in triplicate.  $P < 0.05$ . (K) Flow analysis of donor cell subtype after secondary transplantation.

*HoxA9*-knockout mice demonstrate the most severe phenotype of all the *Hox* knockout models, with multilineage hematopoietic differentiation defects (17, 18), as well as defects in HSC repopulation and proliferation (19). Additionally, the ABD *HOXA* genes, *HoxA7*–*HoxA10*, are overexpressed in mice with expression of a partial tandem duplication of the mixed-lineage leukemia (*MLL*) allele (*MLL* PTD), which has been described in AML (20–22). This overexpression is also correlated with increased histone acetylation in *HOX* gene promoters, suggesting a unique role of epigenetic

modification in the regulation of *HOX* genes (20). Downregulation of *HOXA9* has been shown by Armstrong's group to be critical for survival in human leukemia with *MLL* rearrangement (23).

The *HOXA* and *SALL* gene families have been linked during development through protein-protein interactions. Both have been implicated in segmentation across various species. In fact, the *SALL* family modulates *HOX* expression in murine limb development (24). We recently showed that *SALL4* can regulate *HOXA9* during normal human myelopoiesis (9). While both the homeo-



tic gene *SALL4* and the homeobox gene *HOXA9* play important roles in myeloid leukemogenesis, the connection between them during leukemia development has not yet been investigated. Here, through gene expression profiles of cells from *SALL4B*-induced leukemic mice (referred to herein as *SALL4B* leukemic cells), we identified *HoxA9* among the upregulated genes. Functional studies showed that downregulation of *HoxA9* in *SALL4B* leukemic cells led to decreased replating capacity in vitro and delayed AML development in recipient mice. Using human AML leukemic cells, we further confirmed that *HOXA9* expression was regulated by *SALL4*, through its interaction and co-occupation of the *HOXA9* promoter region with MLL. In summary, we demonstrated a unique *SALL4*/MLL/*HOXA9*-mediated process in murine and human AML, a novel pathway to be targeted in AML.

## Results

**Upregulation of *HoxA9* in *SALL4B* leukemic cells.** To investigate the functional role of *SALL4* in leukemogenesis, we generated a transgenic *SALL4B* mouse model that develops AML and is transplantable (4). To explore the molecular mechanism of *SALL4B* in the development of leukemia, we used serial transplantations to identify the leukemic initiating cells (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI62891DS1). First, we sorted the Lin<sup>-</sup>Sca-1<sup>+</sup>c-kit<sup>+</sup> (LSK) and HPC subpopulations, such as common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), and megakaryocyte/erythroid progenitors (MEPs), from primary leukemic *SALL4B* transgenic donor mice according to previously described methods (25). The sorted *SALL4B* leukemic cells were then transplanted into immunodeficient NOD-SCID mice, and leukemia development was monitored in the recipients. Aggressive fatal AML with onset ranging 1–3 months developed in the recipient mice after primary transplantation of *SALL4B* leukemic cells. The transplanted disease was characterized by immature blasts (c-kit<sup>+</sup>) in the peripheral blood, BM, and tissues such as the liver, lymph nodes, and spleen (Figure 1, A–H). These were the same phenotypes as reported previously for *SALL4B*-induced AML donor mice, and *SALL4* expression was maintained in the transplanted leukemic cells as well (Figure 1I).

The HPC subpopulations from primary transplanted leukemic recipient mice were sorted again and used for secondary transplantations. All secondary recipients from leukemic GMP donors developed AML within 30 days after transplantation. The GMPs continued to expand in the leukemic recipients, becoming the dominant HPC population after secondary transplantation (Figure 1K). We then isolated the enriched *SALL4B* leukemic GMP cells from 3 independently transplanted NOD-SCID recipients and compared their gene expression profiles with their counterpart GMP populations from recipient control NOD-SCID as well as *SALL4B* wild-type littermates. Analysis using dChip revealed an expression signature for *SALL4B* leukemic cells, consisting of upregulated (83 probe sets) and downregulated (394 probe sets) genes (Supplemental Table 1). *HoxA9* was among the top upregulated genes, as determined by the level of change in expression in *SALL4B* leukemic cells compared with those from controls, and this was verified by qRT-PCR (Figure 1J).

**Downregulation of *HoxA9* in *SALL4B* leukemic cells leads to decreased leukemogenic potential.** *HoxA9* has been reported to be important for myeloid leukemogenesis and is a key survival factor for human leukemia with MLL rearrangement (23). We tried to reduce *HoxA9*

expression in *SALL4B* leukemic cells to test whether this can, at least in part, decrease *SALL4B*-mediated leukemogenesis. *HoxA9* lentiviral shRNA viruses were generated as previously reported by Armstrong's group (23).

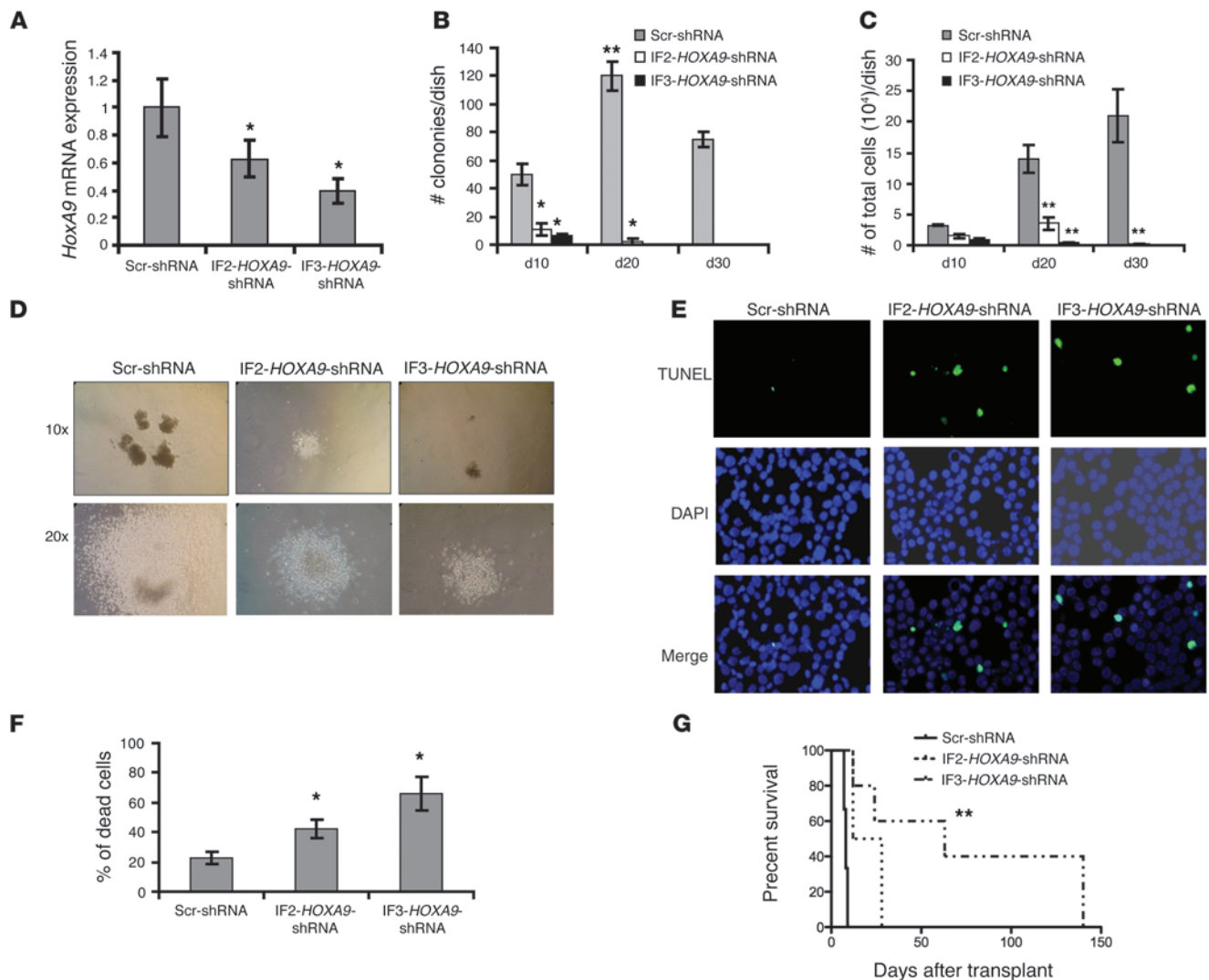
During our serial transplant experiments, we noticed that the leukemic development in the recipient mice became more aggressive, with a shortened leukemia-free period. In addition, fewer unsorted donor leukemic cells were needed for the induction of leukemic phenotype in the recipients. After the second transplant, BM cells from leukemic recipients were isolated by Ficoll-Paque PLUS and labeled with GFP to trace donor cells. Limited numbers of donor cells (10 or 100 cells) were transplanted into recipient mice to test their leukemogenic ability. We found that as few as 10 of these GFP<sup>+</sup> cells could give rise to leukemic phenotype in the recipients (Supplemental Figure 2). We then used secondary transplants as an enrichment method for leukemic initiation cells. In the subsequent mouse experiments, Ficoll-Paque-isolated leukemic cells after 2 rounds of transplants from recipients were used. Knockdown of *HoxA9* was performed using IF2-*HOXA9* shRNA and IF3-*HOXA9* shRNA, which have been demonstrated by Armstrong's group to specifically downregulate *HOXA9* expression in both murine and human hematopoietic cells (23). Using qRT-PCR, we found that the 2 pairs of *HOXA9* shRNAs had some differences in their ability to downregulate murine *HoxA9* expression in *SALL4B* leukemic cells (Figure 2A). To test whether *HoxA9* downregulation in *SALL4B* leukemic cells has any functional effects, we used a previously described replating assay (23).

Using a semisolid medium, we could replate *SALL4B* leukemic cells treated with control scrambled shRNA for more than 3 rounds (30 days). However, *SALL4B* leukemic cells with moderate *HoxA9* reduction (i.e., treated with IF2-*HOXA9* shRNA) could not be replated for more than 2 rounds (20 days), while cells treated with the more potent IF3-*HOXA9* shRNA could only be replated once (Figure 2B). In addition, upon *HoxA9* knockdown, the total colony number and total cell number in each plate was significantly decreased compared with the scrambled shRNA-treated group (Figure 2, B and C). Furthermore, the *SALL4B* leukemic colonies treated with scrambled shRNA viruses were larger than those treated with *HOXA9* shRNA viruses (Figure 2D).

The decreased replating capacity of *SALL4B* leukemic cells after downregulation of *HoxA9* could be due to cell death and apoptosis, since *HoxA9* has been implicated in maintaining leukemic cell survival (23). To test this possibility, *SALL4B* leukemic cells were treated with scrambled or *HOXA9* shRNA for 3 days with puromycin selection on semisolid medium. The cells were then harvested and cytospun upon coverslips, and apoptotic cells were measured using TUNEL assay. As shown in Figure 2E, there was an increase in cell apoptosis in *HOXA9* shRNA-treated groups compared with the scrambled control, which suggests that *HoxA9* suppression can decrease *SALL4B* leukemic cell replating capacity by promoting cell apoptosis. This was further supported by the increased cell death in the *HOXA9* shRNA-treated group, as measured by Trypan blue staining (Figure 2F).

To evaluate the effect(s) of *HoxA9* downregulation in vivo,  $1 \times 10^3$  *SALL4B* leukemic cells after second transplants treated with scrambled or *HOXA9* shRNAs were transplanted into NOD-SCID mice. Scrambled shRNA-treated recipient mice developed fatal leukemia within 2 weeks, with a median survival of 8.5 days ( $n = 11$ ). In contrast, recipient mice treated with the potent IF3-*HOXA9* shRNA showed a statistically significant ( $P = 0.004$ ) delay



**Figure 2**

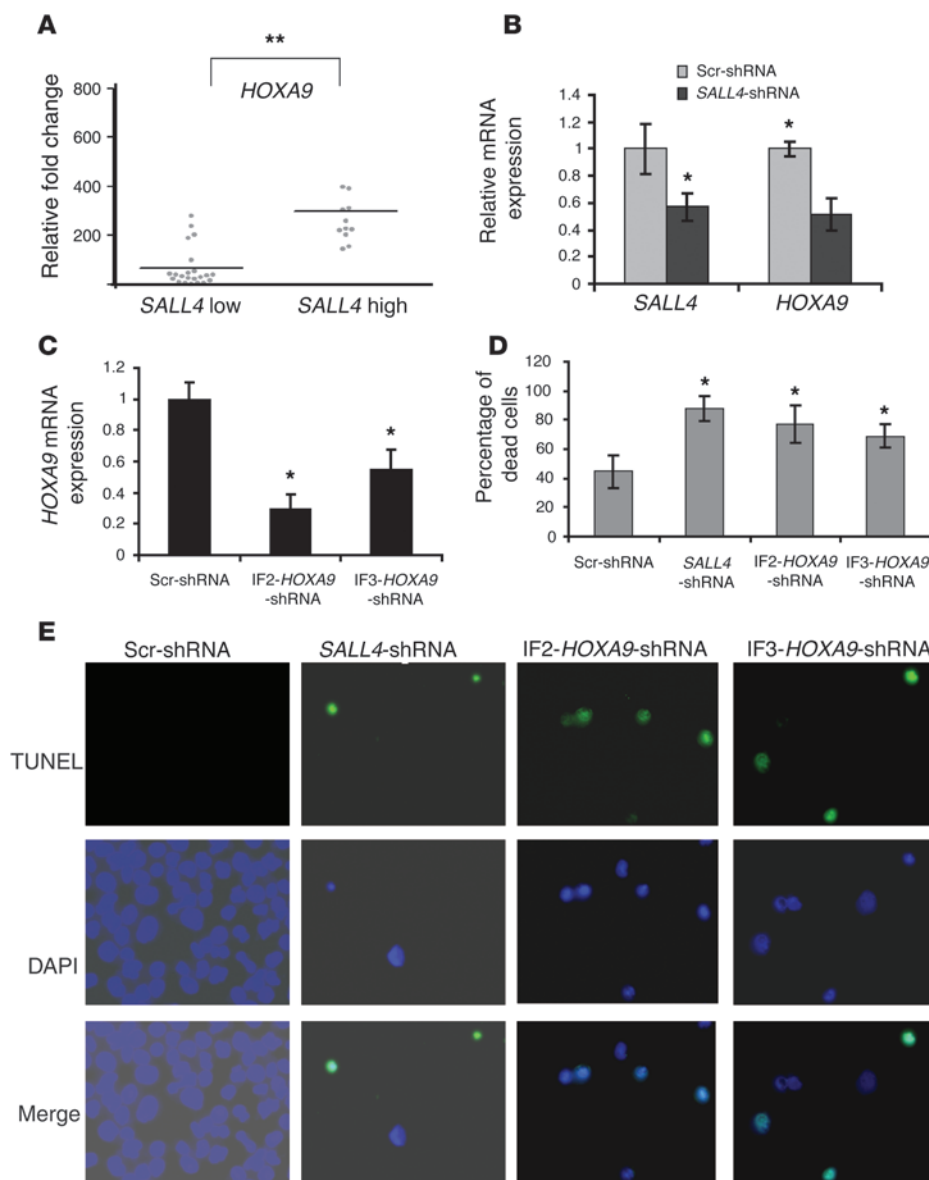
*HoxA9* is critical in *SALL4*-mediated AML. (A) qRT-PCR analysis of *HoxA9* expression in *SALL4B* leukemic cells after *HOXA9* shRNA or control scrambled shRNA (Scr-shRNA) virus infection following puromycin (1  $\mu$ g/ml) selection for 72 hours on semisolid medium. (B) *SALL4B* leukemic cell colony formation and replating capacity after *HoxA9* suppression. 1,000 *HOXA9* shRNA- or scrambled shRNA-treated *SALL4B* leukemic cells were seeded on semisolid medium with puromycin (1  $\mu$ g/ml) and replated every 10 days. The number of colonies per dish was recorded after each round of replating. (C) Total number of cells per dish after each round of replating. (D) Representative colony images 10 days after the first round of plating. Original magnification,  $\times 10$  (top);  $\times 20$  (bottom). (E and F) Increased cell death in *HOXA9*-knockdown cells was observed by TUNEL assay (E) and Trypan blue stain (F) 3 days after the first round of plating. Original magnification,  $\times 20$  (E). (G) Log-rank (Mantel-Cox) survival curve of mice transplanted with *SALL4B* leukemic cells treated with IF2-*HOXA9* shRNA ( $n = 8$ ), IF3-*HOXA9* shRNA ( $n = 7$ ), or scrambled shRNA ( $n = 11$ ). Data are mean  $\pm$  SD from 3 independent experiments. \* $P < 0.05$ , \*\* $P < 0.001$ .

in leukemic development, with a median survival of 63 days ( $n = 7$ ). Treatment of the *SALL4B* leukemic cells with the less-effective IF2-*HOXA9* shRNA also prolonged the survival of its recipient mice, with a median survival of 20 days ( $P = 0.06$ ,  $n = 8$ ; Figure 2G and Supplemental Table 2). Once the *HOXA9* shRNA-treated recipient mice developed leukemia, expression of *HoxA9* in the leukemic BM samples was back to its baseline level, as tested by qRT-PCR, which suggests that the leukemic cells could come from the non-*HoxA9*-knockdown cells.

Taken together, the upregulation of *HoxA9* observed in *SALL4B* leukemic cells and the loss-of-function study of *HoxA9* in these

cells provide direct evidence that *HoxA9* is an important downstream target of *SALL4* in the *SALL4B* leukemic murine model.

*SALL4* is essential for proper *HOXA9* expression and leukemic cell viability in primary human AML samples. To test whether the *SALL4*/*HOXA9* pathway is also important in human primary AML, we first evaluated the correlation of expression between *SALL4* and *HOXA9* in primary human AML samples. Using publicly available data sets, we performed analysis on *SALL4* and *HOXA* cluster gene expression correlation, derived from cDNA microarray analysis of 385 previously described AML specimens (see Methods and ref. 26). There was positive correlation of expression between *SALL4* and

**Figure 3**

Downregulation of *SALL4* decreases *HOXA9* expression in human primary AML and leads to apoptosis. (A) Expression of *SALL4* and *HOXA9* was correlated in human primary AML samples. qRT-PCR was performed on 34 primary samples with primers for *SALL4* and *HOXA9* mRNA. Mean value of *SALL4* was used to stratify the samples to *SALL4*<sup>hi</sup> or *SALL4*<sup>lo</sup> groups. Each dot represents a patient. Data are mean  $\pm$  SD from 3 independent experiments.  $^{**}P < 0.001$ . (B and C) Downregulation of *SALL4* or *HOXA9* expression in primary AML. Primary AML cells were transduced at a density of  $1 \times 10^6$  cells/ml with lentiviruses expressing scrambled, *SALL4*, or *HOXA9* shRNA. After 2 days of culturing in media containing cytokines (10 ng/ml IL-3, 25 ng/ml SCF, and 10 ng/ml IL-6), transduced cells were selected with 1  $\mu$ g/ml puromycin for 3 days on semi-solid medium. Expression of *SALL4* (B) and *HOXA9* (C) in primary AML after *SALL4*, *HOXA9*, or scrambled shRNA treatment was measured by qRT-PCR. (D and E) Increased apoptosis after downregulation of *SALL4* or *HOXA9* using the approach described above in primary AML cells was further evaluated by TUNEL assay (D) and Trypan blue staining (E). Original magnification,  $\times 20$  (E). Data are mean  $\pm$  SD from 3 independent experiments.  $^{*}P < 0.05$ .

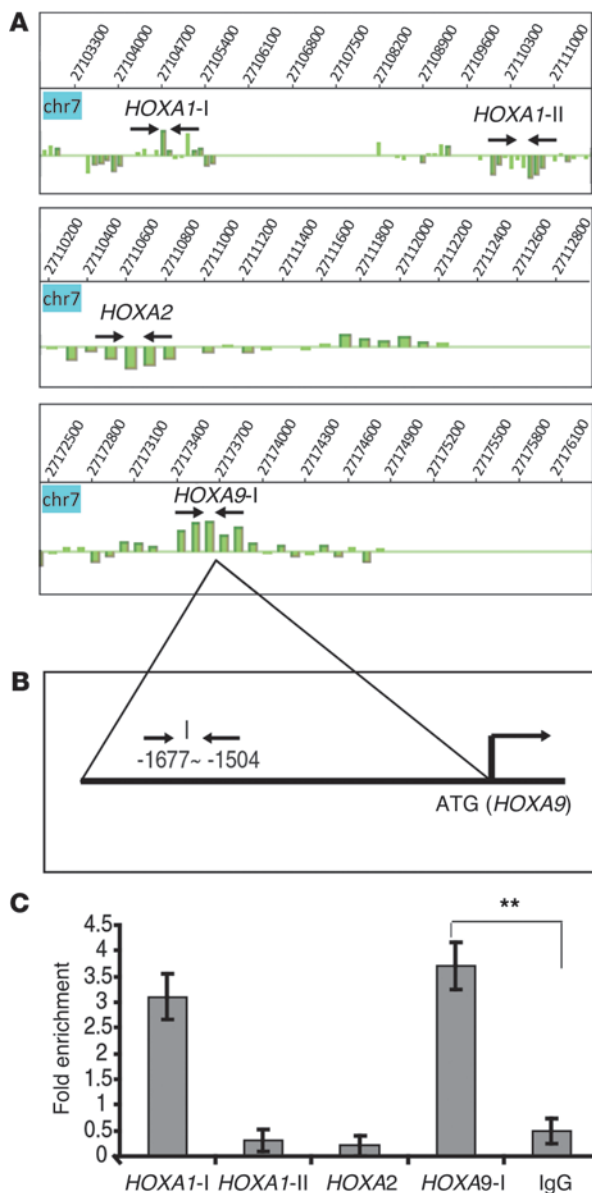
*HOXA* genes, particularly in AML M4 subgroups (Supplemental Table 3). We then verified the expression correlation of *SALL4* and *HOXA9* by qRT-PCR. 34 primary AML samples were subjected to qRT-PCR (Supplemental Table 4) and stratified into *SALL4*<sup>hi</sup> and *SALL4*<sup>lo</sup> groups using the mean intensity of *SALL4* as a threshold. Expression of *HOXA9* in the primary samples in these 2 groups was then examined. *HOXA9* showed positive correlation with *SALL4*; its expression was significantly higher in the *SALL4*<sup>hi</sup> versus the *SALL4*<sup>lo</sup> group ( $P = 0.0001$ ; Figure 3A). Next, we performed *SALL4* knockdown experiments using a lentiviral mediated shRNA approach. Treatment of 3 independent human primary AML samples expressing both *SALL4* and *HOXA9* with this *SALL4* shRNA significantly decreased *SALL4* expression (Figure 3B).

We then tested whether knocking down *SALL4* affected the expression of *HOXA9* in primary human AML cells. Using qRT-PCR, we found that *HOXA9* expression was reduced 50% compared with that of scrambled shRNA-treated cells (Figure 3B). We next tested whether

downregulation of *SALL4* and/or *HOXA9* could lead to increased apoptosis/cell death in primary AML samples. Transduction of human primary AML cells with IF2-*HOXA9* shRNA and IF3-*HOXA9* shRNA reduced *HOXA9* expression by 70% and 50%, respectively (Figure 3C). Downregulation of *SALL4* or *HOXA9* in these AML samples resulted in increased apoptosis, as determined by Trypan blue stain and TUNEL assay (Figure 3, D and E).

In summary, the correlation of expression and the loss-of-function studies of *SALL4* and *HOXA9* in primary human AML and murine leukemic model indicate that the *SALL4*/*HOXA9* pathway is maintained in human and murine leukemia and is critical for leukemic cell survival.

*SALL4 binds to a specific region of HOXA9 promoter.* As part of the search for the transcriptional network responsible for the function of *SALL4*, we conducted a parallel genome-wide analysis of *SALL4* target genes in primary CD34<sup>+</sup> cells and murine ES cells using ChIP followed by a promoter microarray (ChIP-chip; refs. 9, 27).

**Figure 4**

SALL4 binds to the promoter region of *HOXA9* in the AML cell line. (A) Analysis of ChIP-chip data showing SALL4 binding sites at the promoter regions of HOXA genes. Arrows indicate location of primers for validation by ChIP-qPCR. (B) Human *HOXA9*-I promoter region. (C) SALL4 bound to the promoter region of *HOXA9* in KG1a cells, as evaluated by ChIP-qPCR. All values represent the average of at least 2 separate pulldowns (biological duplicates) and qPCR assays. Standard error bars were calculated from the SD of separate trials (biological repeats). Promoter regions from *HOXA1*-II and *HOXA2* were used as internal negative controls, the region for *HOXA1*-I was used as a positive control, and rabbit IgG was used as a negative control for antibody specificity. \*\* $P < 0.001$ .

This analysis demonstrated that SALL4 bound to the promoter regions of several HOXA genes, including *HOXA9* (Figure 4A). Comparison of SALL4 leukemic GMP expression signature with global ChIP-chip data sets also revealed that *HOXA9* was among

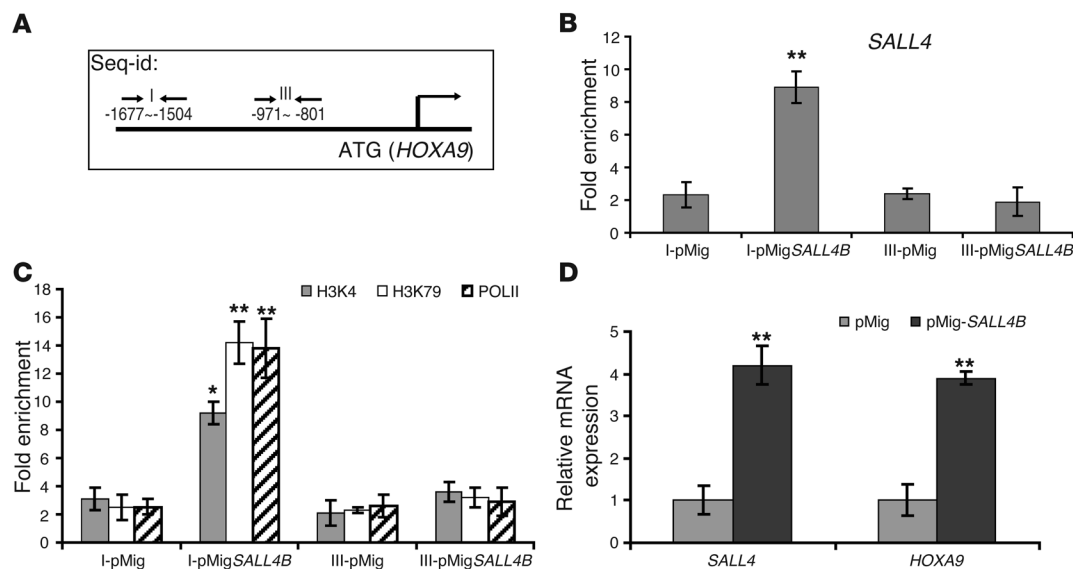
the overlapping genes (Supplemental Figure 3). In order to validate SALL4 binding to the *HOXA9* promoter, we used ChIP coupled with qPCR assay (ChIP-qPCR). Leukemic CD34<sup>+</sup> KG1a cells endogenously expressing both SALL4 and *HOXA9* were used for this experiment. Enrichment of DNA fragments pulled down from these cells using our previously validated anti-SALL4 antibody (refs. 9, 27, and Supplemental Figure 4) was compared with input control. Primers were designed around positive binding peaks according to the ChIP-chip data. Additional regions from *HOXA1*-I, *HOXA1*-II, and *HOXA2* locus served as positive and negative controls (see Supplemental Table 5 for primers). As shown in Figure 4, B and C, SALL4 bound specifically to the *HOXA9* promoter region (–1,677 to –1,504 upstream of the ATG site) compared with the negative region control and IgG control.

*Overexpression of SALL4 results in enrichment of binding of SALL4 and epigenetic activation markers at the HOXA9 promoter region.* We then tested whether increased SALL4 expression can have an effect on its binding at the *HOXA9* promoter region. In addition to the *HOXA9*-I binding site (–1,677 to –1,504; Figure 4B), we also included another region of *HOXA9* promoter, designated *HOXA9*-III (–971 to –801), for the subsequent ChIP-qPCR studies (Figure 5A). After overexpression of SALL4B in KG1a cells, we evaluated the binding enrichment at these 2 regions of the *HOXA9* promoter. Increased SALL4 binding (4-fold) was only observed using primers spanning *HOXA9*-I (Figure 5B). We next investigated the mechanism by which SALL4 activated *HOXA9* expression. We observed that epigenetic activation markers, including H3K4 (methylation of lysine 4 of histone 3) and H3K79 (methylation of lysine 79 of histone 3), were enriched at least 6-fold in the same *HOXA9*-I region bound by SALL4. After overexpressing SALL4B, we also observed that RNA polymerase II (POLII), a marker for gene transcription, bound to this specific region of *HOXA9* promoter (Figure 5C). Along with binding of SALL4 and induction of epigenetic activation markers, increased expression of SALL4B led to increased *HOXA9* RNA expression (Figure 5D), consistent with our previous observation of upregulated *HoxA9* expression in SALL4B leukemic cells (Figure 1J).

In summary, our data indicate that SALL4 can promote *HOXA9* expression by binding to the *HOXA9* promoter region, and this process is associated with enriched binding of epigenetic activation markers.

*SALL4 binds to a specific site in the HOXA9 promoter.* In order to further confirm that SALL4 binds to the *HOXA9* promoter region, we designed 3 pairs of oligonucleotide probes and performed EMSAs (Figure 6A). As shown in Figure 6, B and C, probe 3 (–1,575 to –1,535) was markedly bound by SALL4 overexpressed in 293T cells as well as by endogenous SALL4 in THP1 cells. We further demonstrated that this SALL4/probe complex could be abolished by incubation with an anti-SALL4 antibody, but not by control IgG (Figure 6D). To further define the SALL4 binding site in the *HOXA9* promoter region, we made a series of 5 mutants of probe 3. Among these, only mutant 2 could not compete with the binding between SALL4 and wild-type probe (Figure 6E), which indicates that the mutated residues were required for DNA sequence-specific binding of SALL4 to the *HOXA9* promoter.

*Interaction between SALL4 and MLL.* Overexpression of SALL4 induced POLII binding on the *HOXA9* promoter. In addition, the MLL complex has been reported to recruit POLII to activate *HOXA9* expression (28). One possibility is that there is a direct



**Figure 5**

Overexpression of SALL4 induces enrichment of SALL4 binding and epigenetic activation markers at the *HOXA9* promoter region and increases *HOXA9* expression. **(A)** Human *HOXA9* promoter region. The locations of the primers used in ChIP-qPCR (arrows) are relative to the translation start ATG. **(B)** ChIP-qPCR demonstrated that *SALL4* binding at *HOXA9*-I was enriched by overexpression of *SALL4B* in KG1a cells compared with cells treated with control vector (pMig). In contrast, *HOXA9*-III showed no enrichment of *SALL4* binding. ChIP was performed using *SALL4* antibody with KG1a cells transduced with control pMig-GFP or pMig-GFP-*SALL4B* vectors. GFP<sup>+</sup> cells were used in this experiment. All values are averages calculated from 3 separate *SALL4* pulldowns. **(C)** Enrichment of epigenetic markers in the *HOXA9* promoter region induced by *SALL4B* overexpression. ChIP was performed using H3K4, H3K79, or POLII antibody on KG1a cells treated as in **B**. **(D)** qRT-PCR analysis of *SALL4B* and *HOXA9* expression in pMig-*SALL4B*- and control vector-transduced GFP<sup>+</sup> KG1a cells. Data in **B–D** are mean  $\pm$  SD from 3 independent experiments. \* $P < 0.05$ , \*\* $P < 0.001$ .

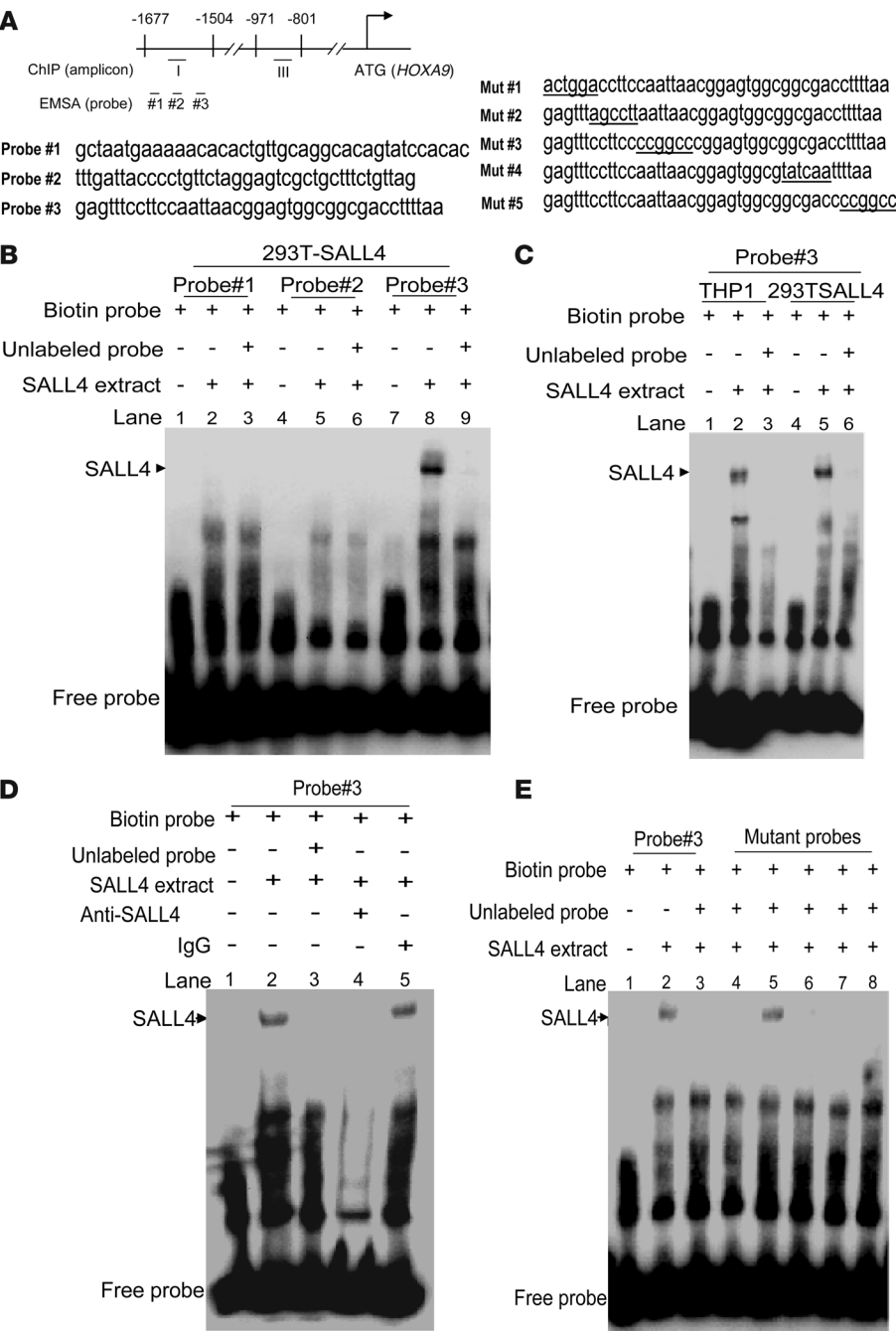
connection between SALL4 and MLL in regulation of *HOXA9* expression. To test this hypothesis, we first carried out co-IP experiments. Extracts from 293T cells overexpressing SALL4 were subjected to IP with the SALL4-specific antibody, followed by Western blotting with antibodies against various components of the MLL complex. Members of the MLL complex, including RbBp5 and Menin, were found in the SALL4 pulldown (Figure 7A). In addition, the N terminus of MLL is known to interact with other proteins (29, 30), raising the possibility that MLL might interact with SALL4 through its N-terminal domain. To test this possibility, 293T cells coexpressing SALL4 with either a longer N-terminal Flag-tagged MLL construct (MLL-BP; 1–1,393 aa), including the protein interacting domain R1/R2, or a shorter N-terminal Flag-tagged MLL construct (MLL-N; 1–1,052 aa) lacking this region (Figure 7B), were subjected to IP with an anti-Flag antibody, followed by Western blot analysis with the SALL4 antibody. Interestingly, only MLL-BP, not MLL-N, could interact with SALL4 (Figure 7C). Notably, the MLL-BP domain was preserved in both wild-type MLL and MLL fusion proteins, such as MLL-AF9. We extended our experiments mapping the interaction between SALL4 and MLL by making several SALL4-truncated proteins. When tested for their abilities to interact with MLL-BP, in addition to full-length SALL4A and SALL4B, the N terminus of SALL4 (N-1581) could interact with MLL-BP, whereas the C terminus of SALL4 (C-1581) failed to show any binding. Additional mapping focused on the N terminus of SALL4 demonstrated that the N-523 fragment (1–523 bp) retained its ability to interact with MLL-BP (Figure 7, D and E).

*SALL4 and MLL co-occupy the same HOXA9 promoter region in AML cells.* To verify whether the interaction between SALL4 and the MLL complex could occur in a leukemic context, we repeated the co-IP experiments using SALL4-expressing AML cells (Supplemental Figure 5). As shown in Figure 8A, a MLL-specific antibody could efficiently pull down endogenous SALL4 from leukemic KG1 cells. In addition, since the MLL-AF9 fusion protein has been extensively studied for its role in leukemogenesis, we also tested whether SALL4 could interact with the MLL-AF9 fusion protein. THP1, a human acute myelomonocytic leukemia cell line with the MLL-AF9 translocation, was chosen as a cell model. Notably, in this line harboring the MLL rearrangement, SALL4 protein was still detected in the MLL pulldowns. More importantly, when tested in a primary AML patient sample, we observed the interaction between SALL4 and MLL as well (Figure 8A).

Since both SALL4 and the MLL complex can regulate *HOXA9* protein expression by binding to its promoter region, we next tested whether SALL4 and MLL could co-occupy the same region on the *HOXA9* promoter. The MLL binding region on the *HOXA9* gene has been well studied. Based on published data on the MLL binding site (31), we designed a sequential ChIP-qPCR assay, referred to herein as ChIP-Re-ChIP. As shown in Figure 8B, specific co-occupancy was observed for SALL4 and MLL in KG1, THP1, and primary AML cells using primers spanning the region of *HOXA9*-I.

The molecular mechanism of MLL and its fusion proteins in AML development occurs, at least in part, through activation of the HOX genes, including *HoxA9* (32). The upregulation of



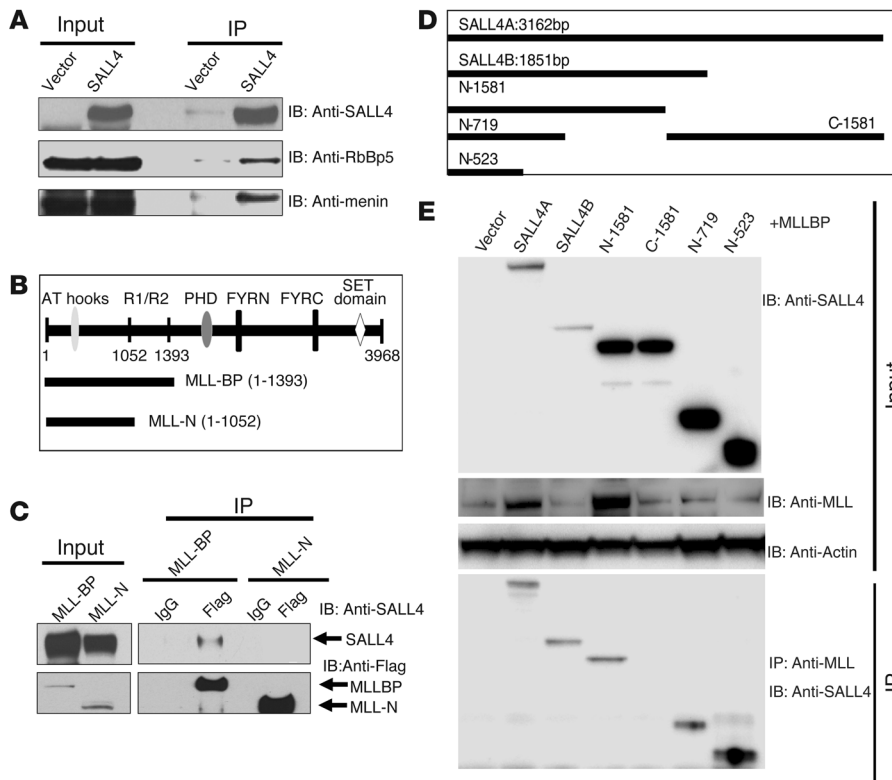


**Figure 6** Identification of a SALL4 DNA binding site in the promoter region of *HOXA9* by EMSA assays. (A) Human *HOXA9* promoter region. Corresponding locations of amplicons for qPCR and oligo probes for EMSA are indicated. Left: 3 probe sequences. Right: Mutant probe sequences of probe 3. (B) EMSAs were performed to identify the SALL4 DNA binding cells to the *HOXA9* promoter region using nuclear extracts from 293T cells transfected with a SALL4 expression construct. Of 3 pairs of oligonucleotide sequences, only probe 3 was specifically bound by SALL4 protein (lane 8); however, this shift could be prevented by competition from 200-fold excess of non-labeled probe (lane 9). (C) Endogenous SALL4 from THP1 nuclear extracts bound probe 3 specifically (lanes 1–3). Lanes 4–6 show nuclear extracts from 293T-SALL4 as a positive control. (D) SALL4 antibody abolished SALL4 and probe 3 binding (lane 4), but not IgG control antibody (lane 5). (E) Among the 5 mutant probes, only mutant probe 2 (lane 5) failed to inhibit wild-type probe 3 binding to SALL4 protein.

*HoxA9* in SALL4B leukemic cells and the interaction between SALL4 and MLL prompted us to compare our SALL4B leukemic cell gene expression profiling with that from a MLL-AF9 murine model (17). Gene set enrichment analysis (GSEA) was used for this comparison study. GSEA is an algorithm used to compare 2 sets of expression profiles to determine whether a particular expression signature is enriched or depleted (32–34). There was a statistically significant similarity of the gene expression signatures between SALL4B leukemic GMPs and MLL-AF9 leukemic GMPs (Supplemental Figure 6); the top 50 genes shared by the 2 groups, including *HoxA9*, are listed in Supplemental Table 6. In order to identify whether other MLL target genes are modu-

lated by the SALL4/MLL interaction, we overexpressed SALL4 in the KG1a AML cell line. A SALL4 mutant lacking the ability to interact with MLL was used as a control. Using qRT-PCR, we found that SALL4 overexpression significantly upregulated most of a set of well-known MLL target genes (8 of 10 tested; refs. 35–39), such as *HOXA* cluster genes and *MEIS1*. In contrast, the SALL4 mutant lacking the MLL interaction domain had no effect on the expression of these MLL target genes (Supplemental Figure 7). Taken together, our data strongly suggest that SALL4 regulates *HOXA9* expression through interacting with MLL, and this novel SALL4/MLL/*HOXA9* pathway may play a critical role in human leukemogenesis.





**Figure 7**

SALL4 interacts with MLL. (A) Co-IP with anti-SALL4 antibody was done using 293T cell lysates after SALL4 overexpression. The protein complex from Co-IP was resolved in SDS-PAGE and immunoblotted using antibodies against SALL4, RbBP5, and Menin. (B) MLL full-length and truncated proteins (MLL-BP and MLL-N). (C) 293T cells were cotransfected with SALL4 and Flag-tagged truncated MLL constructs. IP was performed with Flag antibody, followed by Western blot analysis with SALL4 antibody, which demonstrated that SALL4 could only be detected in pulldown with MLL-BP. Flag antibody could pull down both overexpressed MLL-BP and MLL-N proteins from 293T transfected cells. (D) SALL4 truncated vectors used for interaction with MLL-BP. (E) 293T cells were cotransfected with various SALL4 vectors and Flag-tagged truncated MLL-BP constructs. IP was performed with MLL antibody, followed by Western blot analysis with SALL4 antibody. Whereas the C terminus of SALL4 (C-1581) did not interact with MLL-BP, the N terminus (N-523) retained its ability to bind to MLL-BP.

## Discussion

While both *HOXA9* and *SALL4* play important role(s) in myeloid leukemogenesis, the connection between these 2 transcription factors has not been investigated. Further characterization of the underlying mechanism should allow for better understanding of leukemogenesis and provide potential therapeutic targets for AML treatment. Here, through gene expression profiling of our previously characterized *SALL4B* transgenic mouse model (which develops AML), we observed upregulated *HoxA9* expression in the *SALL4B* leukemic cell population. Downregulation of *HoxA9* in *SALL4B* leukemic cells led to apoptosis and decreased replating ability in vitro as well as decreased AML development in recipient mice in vivo. Parallel human studies demonstrated that expression of *SALL4* and *HOXA9* was correlated in primary AML patient samples. Furthermore, downregulation of *SALL4* in human primary AML cells leads to decreased

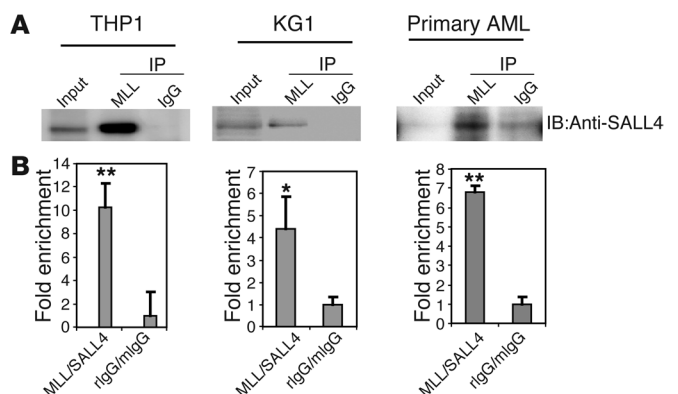
*HOXA9* expression, and loss-of-function studies of each gene in primary human AML samples showed a similar cell apoptosis/death phenotype. This prompted us to examine whether *HOXA9* is a direct target of *SALL4*, and whether *SALL4* can regulate *HOXA9* in the hematopoietic system.

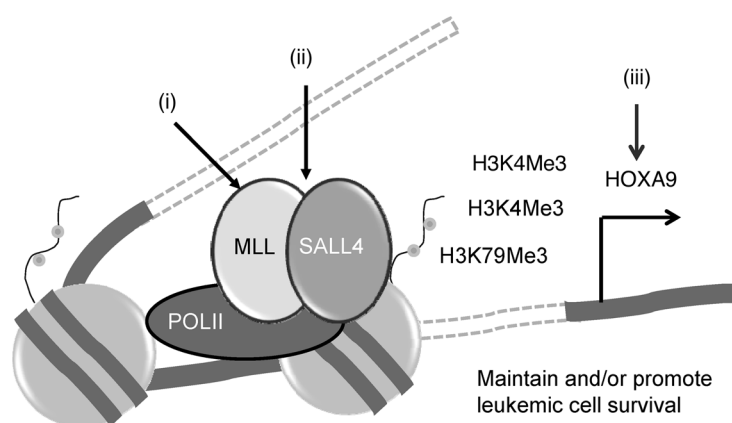
Additional ChIP-chip studies suggested that *HOXA9* is a potential *SALL4* target. We confirmed this finding by ChIP-qPCR, which showed that *SALL4* bound to the specific promoter region of *HOXA9* in leukemic cells at the endogenous level. Furthermore, overexpressing *SALL4* enhanced markers of histone activation, such as H3K4 and H3K79 methylation, as well as POLII binding in the same promoter region, which increased *HOXA9* expression in these cells.

*MLL*, which codes for a histone methyltransferase, is a global gene transcription regulator. The molecular mechanism of *MLL* and its fusion proteins in AML development occurs, at least in

**Figure 8**

Co-occupancy of the *HOXA9* promoter region by *SALL4* and *MLL* in primary AML cells and cell lines. (A) Endogenous *SALL4* protein was subjected to IP using *MLL* antibody in KG1, THP1, and primary AML cells, followed by Western blot analysis with *SALL4* antibody. (B) ChIP-Re-ChIP demonstrated co-occupancy of the *HOXA9* promoter region by *SALL4* and *MLL*. The first ChIP assay was performed with antibody against *MLL*, and the second ChIP (Re-ChIP) was done with antibody against *SALL4*. Controls for the Re-ChIP assay were performed with anti-rabbit and anti-mouse IgG (rIgG/mlgG). Data are representative of at least 2 independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .





**Figure 9**

Targeting the SALL4/MLL/HOXA9 pathway in AML. SALL4 can upregulate HOXA9 expression by interacting with MLL, which results in H3K4 and H3K79 methylation. The SALL4/MLL/HOXA9 pathway is critical for leukemic cell survival, as disruption of this pathway results in leukemic cell death. Based on this model, we propose the following novel therapeutic approaches in targeting AML: (i) inhibiting the function of MLL and its complex (MLL inhibitor); (ii) disrupting the SALL4/MLL interaction; and (iii) targeting HOXA9 (HOXA9 inhibitor).

part, through activation of HOX genes, including *HoxA9* (32). We then explored whether SALL4 regulates HOXA9 through interaction with MLL. Using both 293T cells and human leukemic cells, we observed that SALL4 not only physically interacted with MLL, but also co-occupied on the *HOXA9* promoter region with MLL. Others have shown that both MLL fusions and the normal MLL allele are important for leukemogenesis (40). Our finding that SALL4 interacted with both wild-type MLL and MLL fusion proteins prompted us to compare our SALL4B leukemic cell gene expression profile with that of a MLL-AF9 murine model (17). There was a statistically significant similarity of the gene expression signatures between SALL4B leukemic GMPs and MLL-AF9 leukemic GMPs (Supplemental Figure 6), further supporting the connections among SALL4, HOXA9, and MLL.

In conclusion, we propose that a novel SALL4/MLL/HOXA9 pathway plays an important role in myeloid leukemogenesis by promoting leukemic survival (Figure 9). Based on this model, we propose the following novel therapeutic approaches in targeting AML: (a) inhibiting the function of MLL or its complex with menin, as recently proposed by others (41); (b) disrupting the SALL4/MLL interaction, which we have recently shown to disrupt the protein interaction between SALL4 and NuRD (42); and (c) targeting HOXA9 directly. As the HOXA and SALL gene families are linked during development through protein-protein interactions (24), it is possible that SALL4 forms a complex with HOXA9 and coregulates common downstream target genes in various cellular biological processes, such as myeloid differentiation, proliferation, and cell survival. Given that SALL4 interacts with both wild-type MLL and a MLL fusion protein, further studies will determine whether modulation of the SALL4/MLL/HOXA9 pathway or interruption of the connection between SALL4 and MLL represent novel approaches in treating AML, including leukemia with MLL rearrangements. As SALL4, MLL, and HOXA9 have all been implicated in the development of leukemia, our findings provide new insights into the pathogenesis of AML.

## Methods

Further information can be found in Supplemental Methods.

**Mice and leukemic transplantation experiments.** *SALL4B* transgenic mice were generated as previously described as well as murine leukemic transplants (4). Whole BM cells, spleen cells, and sorted LSKs, CMPs, GMPs, and MEPs from *SALL4B*-induced leukemic mice were transplanted into NOD-SCID mice by tail vein injection. The transplantation cell dose range was  $1 \times$

$10^6$  for whole BM and spleen;  $3.9 \times 10^3$  (primary) or 1,334 (secondary) for LSKs; and  $8 \times 10^4$  (primary) or 2,300 (secondary) for GMPs.  $8 \times 10^3$  CMPs or  $80 \times 10^3$  MEPs were used for primary leukemic transplants; there were no leukemic CMPs or MEPs for secondary transplant, since only leukemic GMPs remained. For *HoxA9* knockdown experiments, BM cells were harvested from serial transplant leukemic mice, and blast cells were isolated with Ficoll-Paque separation. 1,000 cells were transplanted into NOD-SCID recipient mice by tail vein injection.

**Fluorescence-activated cell sorting (FACS) analysis and cell depletion.** HSCs were isolated from mouse BM cells as described previously (25). Briefly, murine lineage-positive cells were depleted with antibodies against CD3, CD4, CD8, Gr-1, CD19, and B220/CD45RA (Invitrogen) using magnetic beads (Dyna Beads, Invitrogen). After depletion, cells were stained for the following markers: Sca-1, CD34, Fcγ receptors II and III (FcγRII/III), and c-kit (BD Biosciences – Pharmingen). Finally, cells were resuspended in 1x PBS with 2% heat-inactivated FBS and 1 μg/ml PI, then analyzed or sorted using a MoFlo cell sorter (Dako). The cell surface marker Sca-1, obtained as a biotin conjugate, was visualized using a streptavidin-APC-Cy7 antibody (BD Biosciences – Pharmingen).

**PCR and qRT-PCR.** PCR and qRT-PCR were performed as previously described (4). Briefly, a PCR kit (Qiagen) was used to genotype the *SALL4B* founder mice and transmission of the transgene. Genomic DNA was purified from mouse tail using a high-quality DNA kit (Gentra Systems). *SALL4B* primer sequences were as follows: forward, 5'-AGCAGAGCTC-GTTTAGTGAACCG-3'; reverse, 5'-CTGTCATTCATGATGAGGACAGG-3'. Total RNA was isolated using a phenol-free and filter-based RNA isolation system (Qiagen) digested with DNase I to remove DNA contamination. Primer sequences for qRT-PCR, designed using Primer Express software (Applied Biosystems), were as follows: *GAPDH* forward, 5'-GAAGGTGAAGGTCGGAGTC-3'; *GAPDH* reverse, 5'-GAAGATG-GTGATGGGATTTC-3'; *SALL4B* forward, 5'-ACATCTCCGCGGTG-GATGT-3'; *SALL4B* reverse, 5'-TGCTCCGACACTTGTGCTTG-3'; *Gapdh* forward, 5'-ACTCCACTCACGGCAAATTC-3'; *Gapdh* reverse, 5'-TCTC-CATGGTGGTGAAGACA-3'; *Hoxa9* forward, 5'-ATGGCATTAACCT-GAACCG-3'; *Hoxa9* reverse, 5'-GTCTCCGCGCTCTCATTTC-3'. All reactions were performed in an ABI-7000 sequence detection system using TaqMan PCR core reagents according to the manufacturer's instructions (Applied Biosystems). For each sample, *GAPDH* expression was used to normalize the amount of investigated transcript.

**Gene expression profiling and data analysis.** Total RNA of various populations ( $2\text{--}5 \times 10^4$  cells/population) from BM of wild-type mice, NOD-SCID mice, and NOD-SCID mice with leukemic cell transplants was extracted using TRIzol reagent (Invitrogen). RNAs (50 ng) were reverse transcribed



to cDNAs, then amplified by single primer isothermal amplification (SPIA). Finally, amplified products (3.75 µg) were fragmented and biotin labeled by Ovation RNA Amplification System V2 and FL-Ovation™ cDNA Biotin Module V2 kits (NuGEN Technologies), according to the manufacturer's instructions. Labeled cDNA was hybridized to an Affymetrix Mouse Genome 430A 2.0 Array and detected by the Microarray Core Facility at the Dana-Farber Cancer Institute.

SALL4 and HOXA cluster gene expression correlation was analyzed using publicly available data sets, derived from cDNA microarray analysis of 385 AML specimens (accession no. GSE14468; ref. 26). Gene expression data were deposited in the European Bioinformatics Institute Array-Express database (accession no. E-MEXP-2072; <http://www.ebi.ac.uk/arrayexpress>). Data were analyzed by dChip (<http://biosun1.harvard.edu/complab/dchip/>) and GSEA 2.0 algorithm (<http://www.broad.mit.edu/gsea/>). With dChip, raw expression data were normalized to account for differences in chip intensities and then filtered using  $0.4 < SD/SEM < 1,000$ ; presence call percentage in the arrays used  $\geq 20\%$  and expression level of at least 20 in  $\geq 20\%$  samples. Hierarchical clustering was performed among the filtered genes. For comparisons of gene expression between the 2 groups, transcripts were considered to be up- or downregulated when their transcript levels in *SALL4B* transgenic mice changed 1.5-fold (increase or decrease) compared with control samples, with  $P < 0.05$  and lower confidence bound  $> 90\%$ . GSEA was carried out as described by Subramanian et al. (33).

**ChIP followed by qPCR and ChIP-Re-ChIP.** We previously described this assay when it was used to identify SALL4 targets in ChIP experiments in leukemic NB4 and murine ES cells (13, 27, 43). Briefly, cells were cross-linked with 1% formaldehyde at room temperature for 10 minutes and washed once with ice-cold PBS containing protease inhibitor cocktail (Roche Diagnostics). Sonication conditions were optimized for Omni Sonic Ruptor 400 for 7 sonications. In addition to sonication, the DNA was also sheared via an enzyme shearing kit (Active Motif) according to the manufacturer's instructions. These ChIP samples were incubated overnight with ChIP-qualified antibodies (anti-H3K4me3, catalog no. MC315; anti-H3K79me2, catalog no. NL59; anti-RNA POLII, catalog no. 17-620; all from Millipore; and our own SALL4 antibody) (13, 27, 43). Samples were subjected to reverse crosslinking with NaCl (final concentration, 200 mM) at 65°C followed by RNase A and proteinase K treatment and phenol-chloroform extraction. Nucleotide sequences were obtained from NimbleGen ChIP-chip design files for positive probes, and real-time PCR primers were designed for the promoter regions to include these positive probes in their product fragments (average size, 160 bp). qPCR was performed on the immunoprecipitated DNA fragments with the BioRad SYBR Green real-time PCR kit. To detect enrichment of SALL4 binding, expression of SALL4 pulldown DNA was compared with that of input (no IP) and IgG controls using Gene Expression Macro software (provided by BioRad) designed to analyze qPCR and qRT-PCR data.

For ChIP-Re-ChIP experiments, protein-DNA complexes after the first ChIP with anti-MLL-N antibodies (catalog no. A300-086A, Bethyl Laboratories) were washed with washing buffer and Tris-EDTA buffer as described above. Complexes were eluted by incubation for 30 minutes at 37°C in 50 µl elution buffer (1× TE containing 2% SDS, 15 mM DTT, and protease inhibitors). After centrifugation, the supernatant was diluted 30 times with dilution buffer supplemented with protease inhibitors. Samples were then subjected to the ChIP procedure with SALL4 antibody (27) and IgG control.

**EMSA.** EMSA was carried out as previously described (12). Briefly, nuclear extracts from SALL4-transfected 293T and THP1 cells was prepared with nuclear extract kit (catalog no. 78833, Pierce) according to the manufacturer's instructions. Synthetic complementary of oligonucleotides

were labeled using the biotin 3'-end DNA labeling kit (catalog no. 89818, Pierce). Binding reactions were carried out for 20 minutes at room temperature in the presence of 50 ng/µl poly (dI-dC), 0.05% Nonidet P-40, 10 mM EDTA, and 2.5% glycerol in 1× binding buffer (LightShift chemiluminescent EMSA kit, catalog no. 20148, Pierce), using 20 fmol biotin-end-labeled target DNA and 2 µg nuclear extract. Unlabeled target DNA (4 pmol), 1 µl anti-SALL4 antibody, or normal rabbit IgG (Cell Signaling) was added per 20 µl of binding reaction, as indicated.

**Construction of pMIG-SALL4 plasmid, production of retroviral particles, and transduction of KG1a cells.** The human *SALL4B* coding sequence was inserted into the pMIG retroviral plasmid. The amphotropic packaging cell line Phoenix was transfected using calcium phosphate/chloroquine. Retroviral supernatants were harvested and used for infection of KG1a cells. After addition of 1 ml viral particles (viral titer adjusted to  $1 \times 10^6$  transducing units/ml) to  $1 \times 10^6$  cells and supplementation with 8 µg/ml polybrene, spinoculation was performed at 1,044 g for 90 minutes at 37°C. 2 infection cycles were performed, after which cells were placed back in original complete growth medium. After 48 hours, GFP<sup>+</sup> cells were sorted and used for ChIP-qPCR or RT-PCR analysis.

**Co-IP.** Subconfluent 293T cells were cotransfected with pCXN2-FLAG-MLL-BP (1–1,393 aa) or pCXN2-FLAG-MLL-N (1–1,052 aa) and pCDNA3-SALL4 using Fugene 6 (Roche). Various SALL4 vectors were gifts from Y. Ma (Nevada Cancer Institute, Las Vegas, Nevada, USA). At 48 hours after transfection, cells were lysed in 50 mM Tris (pH 8.0), 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 10% glycerol, 1% NP-40, 10 mM NaF, and protease inhibitor. Flag antibody-agarose (Sigma-Aldrich) or mouse IgG-protein bead as a control was added into lysate and incubated overnight at 4°C. Next, protein complexes were washed 5 times with 1 ml lysis buffer and resolved in 3%–8% SDS-PAGE, after which Western blots were performed with SALL4 antibody (27). For detecting MLL components, cells were transfected with the SALL4 construct, subjected to IP with anti-FLAG-agarose, and immunoblotted with the antibodies anti-RbBp5 (catalog no. A300-109A, Bethyl Laboratories), anti-Menin (catalog no. A300-105A, Bethyl Laboratories), anti-MLL1 (catalog no. A300-087A, Bethyl Laboratories), and anti-Flag (catalog no. F1804, Sigma-Aldrich). Endogenous MLL and SALL4 co-IP was performed in KG1, THP1, and primary AML cells using the regular co-IP protocol with MLL antibody (catalog no. A300-086A, Bethyl Laboratories). Clean-Blot Detection kit (catalog no. 21230, Thermo Scientific) was used to reduce the background after co-IP.

**Lentiviral virus production and transduction.** The *SALL4* shRNA construct (Puri-7412 and Puri-7410) and scrambled control vectors were verified by our previous studies (13, 44). IF2-*HOXA9* and IF3-*HOXA9* shRNA constructs were purchased from Invitrogen; sequences were as follows: *SALL4* shRNA, 5'-GCCTTGAAACAAGCCAAGCTA-3'; IF2-*HOXA9* shRNA, 5'-CACGCTTGACACTCAGCTTT-3'; IF3-*HOXA9* shRNA, 5'-GTG-GTTCTCCTCCAGTTGATA-3'; scrambled shRNA, 5'-CCTAAGGTTA-AGTCGCCCTCG-3'.

Lentiviral supernatants were obtained in 293T cells by cotransfection of the shRNA plasmids and packaging plasmids containing VSV-G and pH8.9. For lentiviral infection of primary murine SALL4B or human AML patient samples,  $1 \times 10^5$  cells were seeded in 12-well plates (200 µl/well) in the appropriate culture media. Polybrene (hexadimethrine bromide, Sigma-Aldrich) was added at a final concentration of 8 µg/ml. After addition of 1 ml lentiviral particles (titer of each lentiviral shRNA construct adjusted to  $1 \times 10^6$  transducing unit/ml, to achieve MOI of 10 transducing units/cell), spinoculation was performed at 669 g for 90 minutes at 37°C. Next, cells were brought back to 500 µl in volume using the appropriate fresh culture media and incubated at 37°C, 5% CO<sub>2</sub>, until being used for subsequent applications.

**Primary AML samples.** For gene expression studies, 34 AML samples were





collected from patients. In addition, 3 primary AML leukemic patient samples were obtained from Brigham and Women's Hospital. Culture conditions were adapted from a previously published protocol (23) that supports 40%–50% viability at 72 hours of post-thaw culturing, based on our experience. Only live cells were used for subsequent experiments.

**Serial replating assays.** 1,000 leukemic cells were plated in methycellulose (StemCell Technologies) containing a full complement of cytokines (GM-CSF, IL-3, IL-6, stem cell factor, and erythropoietin) in the presence of 1 µg/ml puromycin. Cells were cultured for 10 days, collected, and counted, and 1,000 cells were then placed into methylcell medium for the next round of culture.

**Analysis of cell death and apoptosis.** Virus-transduced human primary AML cells or murine leukemic cells were seeded on semisolid medium as described above with 0.5–1 µg/ml puromycin selection for 72 hours. Next, cells were cytocentrifuged onto microscope slides, and apoptotic cells were measured using a TUNEL apoptosis kit (catalog no. G3250, Promega). Additional cells were stained with Trypan blue (Invitrogen).

**Statistics.** Results are expressed as mean ± SD from at least 3 independent experiments. Statistical significance between 2 groups was determined by unpaired 2-tailed Student's *t* test (GraphPad Prism). The Kaplan-Meier method was used to estimate survival curves for murine leukemic transplant data, and log-rank test was used to evaluate statistical differences. A *P* value less than 0.05 was considered significant.

**Study approval.** All animal work was conducted according to relevant national and international guidelines and in accordance with the recommendations of the Weatherall report under protocol 10-10-1832 at Chil-

dren's Hospital Boston animal facility. The 34 primary AML samples were collected from patients with informed consent under protocol 2007/00173 from the Domain Specific Review Board of National Healthcare Group of Singapore (NHG DSRB); use of these samples was also approved by NHG DSRB under protocol 2009/00495. The 3 primary AML leukemic patient samples obtained from Brigham and Women's Hospital were approved under IRB protocol 2011-P-000096/1.

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