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Research Article **Hematology**

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Mixed-lineage leukemia (*MLL*) is a proto-oncogene frequently involved in chromosomal translocations associated with acute leukemia. These chromosomal translocations commonly result in MLL fusion proteins that dysregulate transcription. Recent data suggest that the *MYB* proto-oncogene, which is an important regulator of hematopoietic cell development, has a role in leukemogenesis driven by the MLL-ENL fusion protein, but exactly how is unclear. Here we have demonstrated that c-Myb is recruited to the MLL histone methyl transferase complex by menin, a protein important for MLL-associated leukemic transformation, and that it contributes substantially to MLL-mediated methylation of histone H3 at lysine 4 (H3K4). Silencing *MYB* in human leukemic cell lines and primary patient material evoked a global decrease in H3K4 methylation, an unexpected decrease in *HOXA9* and *MEIS1* gene expression, and decreased MLL and menin occupancy in the *HOXA9* gene locus. This decreased occupancy was associated with a diminished ability of an MLL-ENL fusion protein to transform normal mouse hematopoietic cells. Previous studies have shown that *MYB* expression is regulated by Hoxa9 and Meis1, indicating the existence of an autoregulatory feedback loop. The finding that c-Myb has the ability to direct epigenetic marks, along with its participation in an autoregulatory feedback loop with genes known to transform hematopoietic cells, lends mechanistic and translationally relevant insight into its role in MLL-associated leukemogenesis.

Introduction

The *MYB* proto-oncogene was first identified as the cellular homolog of the *v-myb* oncogene carried by the avian myeloblastosis viruses (AMVs) and E26 (1). *MYB*'s mRNA and protein (c-Myb) are highly expressed in immature hematopoietic cells and downregulated upon terminal differentiation (2–4). The hypothesis that c-Myb might be an obligate hematopoietic transcription factor was first suggested by transient gene silencing studies (5) and unequivocally demonstrated by the death in utero of $Myb^{-/-}$ mice at day 15 of embryonic life secondary to disruption of definitive hematopoiesis in fetal liver (6).

The molecular and biochemical basis for *MYB*'s importance in normal hematopoiesis has become clearer through the efforts of many laboratories. c-Myb plays a direct role in lineage fate selection, cell cycle progression, and differentiation of both myeloid and B and T lymphoid progenitor cells (5, 7–18). The mechanistic basis for c-Myb's myriad effects on blood cell development remains to be fully elucidated, but it participates, critically, in all of these activities through modulation of its effective intracellular levels (12, 19) and through effects, both activating and suppressive, on gene expression. These in turn are mediated either by direct binding to gene targets or as a result of interaction with various protein binding partners, such as p300 (20, 21), CREB-binding protein (CBP) (22), and FLASH (23). For example, c-Myb and p300 interactions have been reported to play a vital role in regulating normal thrombopoiesis (24) and more globally to control the proliferation and differentiation of hematopoietic stem and progenitor cells (21). Disruption of normal c-Myb protein interactions might also contribute to its ability to transform cells by removing control of a so-called pacifying protein (25, 26).

The *MLL* (mixed lineage leukemia) gene, a human homolog of *Drosophila melanogaster* trithorax (*trxG*), was the first *trxG* gene identified as a proto-oncogene (27–30). MLL is a very large protein (~430 kDa) with a myriad of functions. It has been known to be required for maintenance of *Hox* gene expression during embryonic life (31), an attribute that may derive, at least in part, from its intrinsic histone methyltransferase (HMT) activity (32, 33). It is also known to be cleaved by the threonine aspartase taspase 1 into 2 fragments, MLL^N and MLL^C, which have opposing effects on transcription. MLL^N silences transcription when it partners with corepressor proteins, while MLL^C is a strong activator when partnered with CBP (34). The *MLL* gene is frequently involved by chromosomal translocations in acute leukemia, and at least 50 different chimeric MLL proteins have been reported to result from these translocations (35). These chimeric proteins appear to be functional, resulting in dysregulated transcription.

Recent progress in purifying MLL-containing protein complexes from cell lines has shown that the wild-type protein has great propensity to interact with other proteins. These interactions lead to a plethora of functionally diverse roles for MLL in cell development and function as a result of the ability to also affect chromatin remodeling (36–39) and RNA processing (40). Consistent core components of these complexes are the SET1 domain–associated proteins WDR5, Ash2L, and RbBP5, which are required for the assembly and targeting of the native MLL complex (41, 42). Specifically, they are thought to orient the C-terminal SET domain adjacent to the PHD domain (43, 44) so that methylation of histone H3 at lysine 4 (H3K4) can proceed efficiently (32, 45, 46).

Conflict of interest: The authors have declared that no conflict of interest exists.

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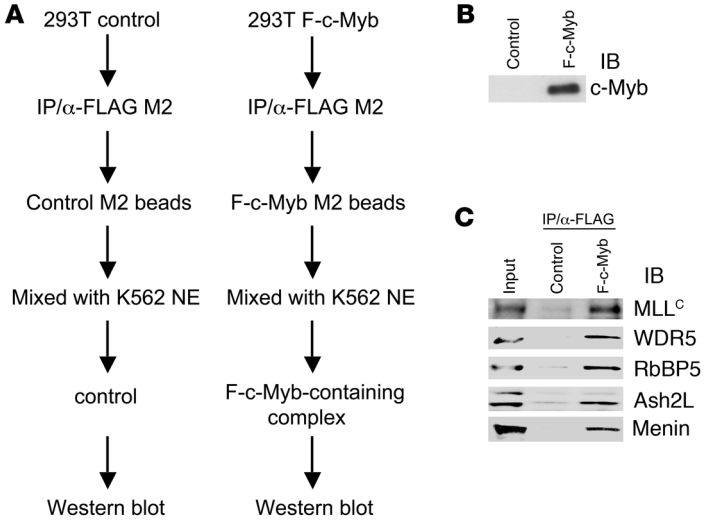

Figure 1
c-Myb coprecipitates with the menin and MLL complex. (**A**) Schematic depiction of experimental scheme. HEK 293T cells were transiently transfected with expression vector encoding FLAG-tagged c-Myb. FLAG-tagged empty vector was used as a control. Cell lysates prepared from transfectants were mixed with α-FLAG antibody affinity M2 agarose to purify FLAG–c-Myb protein. After washing with lysis buffer (1% NP-40), FLAG–c-Myb–containing M2 beads were incubated with nuclear extracts (NE) from K562 cells to isolate the c-Myb–containing complex. Beads were washed twice with nuclear extract buffer (0.05% NP-40). (**B** and **C**) Isolated samples were probed by immunoblotting with α–c-Myb (**B**), α-menin, α-MLL^C, α-WDR5, α-RbBP5, and α-Ash2L antibodies (**C**).

Menin, the product of the *MEN1* gene mutated in familial multiple endocrine neoplasia type 1, has also been found in MLL family HMT complexes (39, 47). Menin binds MLL through the consensus RXRFP sequence within the first 10 amino acids of MLL. Menin and MLL both associate with the *HOXA9* promoter, and in the absence of menin, MLL and its fusions fail to regulate *HOXA9* expression, which is believed to be critical for transformation by MLL fusion proteins (39, 48). Very recently, it has been suggested that the sole function of menin is to recruit proteins into the MLL complex, and one of these, LEDGF, has been shown to be critical for leukemic transformation (49, 50). It was speculated that other, as-yet-unidentified proteins, might also be recruited to the MLL complex and that such proteins might also be important for inducing the leukemic phenotype. *MYB* has recently been shown to be essential for MLL-ENL–mediated transformation (51), suggesting that it too might interact in some manner with MLL. Herein we provide data, in both cell lines and primary patient material, that strongly suggest that menin also recruits c-Myb to the MLL complex and that this interaction has important functional significance with respect to expression of downstream MLL gene targets on MLL HMT activity, on MLL fusion–mediated transformation, and on global methylation of H3K4.

Results

c-Myb associates with the MLL-menin complex. c-Myb has been reported to be an important downstream element of the MLL/HoxA/Meis1 leukemic transformation pathway (51). To better understand the relationship among these proteins, we first examined the possibility that c-Myb might physically interact with them. As described in Figure 1A, we began by transfecting HEK 293T cells with a FLAG-tagged c-Myb (F–c-Myb) expression vector and then purifying the protein from cell lysates by immunoprecipitation with anti-FLAG affinity M2 beads. 293T cells transfected with empty FLAG-tagged vector served as a control. We then mixed the M2 agarose beads, prepared from either F–c-Myb– or control vector–transfected cells, with nuclear extracts derived from untransfected K562 cells. The beads were washed, and bound proteins were eluted. As shown in Figure 1B, c-Myb was detected in the immunoprecipitate derived from F–c-Myb–expressing cells, but not in control vector–transfected cells. Immunoblot assays (Figure 1C) revealed that endogenous MLL as well as other core components of the MLL complex (WDR5, RbBP5, and Ash2L) were coprecipitated with purified F–c-Myb. Menin was also detected in the F–c-Myb immunoprecipitates, indicating that this protein too was associated with the MLL complex.

c-Myb interacts with the MLL complex through menin. The above experiments suggested that c-Myb could specifically interact with the MLL complex proteins. We next sought to determine how this association was constructed. We first tested the hypothesis that c-Myb might directly interact with MLL and sought to determine which domains might be involved. To this end, we generated 6 MLL fragments derived from both the MLL^N region (M1–M4), and the MLL^C region (M5, M6) by in vitro transcription/translation reactions and then utilized them for direct binding assays. Under the conditions used for these studies, none of the 6 fragments was demonstrated to interact with c-Myb (Figure 2A). Additional experiments of this type carried out with in vitro translated WDR5, RbBP5, and Ash2L proteins suggested that c-Myb does not directly bind to any of these SET domain superfamily proteins either (Figure 2B). These results strongly suggest that c-Myb does not directly interact with either MLL or the 3 core SET domain–associated proteins.

Since c-Myb and MLL did not appear to interact directly, we next explored the possibility that c-Myb might interact with the complex through menin. This seemed a tenable alterative, since the MLL-menin interaction is known to be important for the induction of leukemia (48), and menin is reported to interact with a myriad of protein partners (52). A similar strategy was employed. HEK 293T cells were cotransfected with vectors expressing c-Myb and FLAG-tagged menin (F-menin). Analysis of anti-FLAG affinity M2 immunoprecipitates from transfected cell lysates revealed the presence of c-Myb along with F-menin when the 2 proteins were coexpressed, but not when c-Myb was expressed along with an empty control vector (Figure 2C). Immunoprecipitation of K562 cell nuclear extracts with anti-menin antibody also showed the presence of endogenous c-Myb protein in the menin immunoprecipitate (Figure 2D). Together, these results provide strong evidence of an interaction between c-Myb and menin in living hematopoietic cells.

As the association between c-Myb and menin was demonstrated in cell lysates, it was not certain whether this interaction was direct

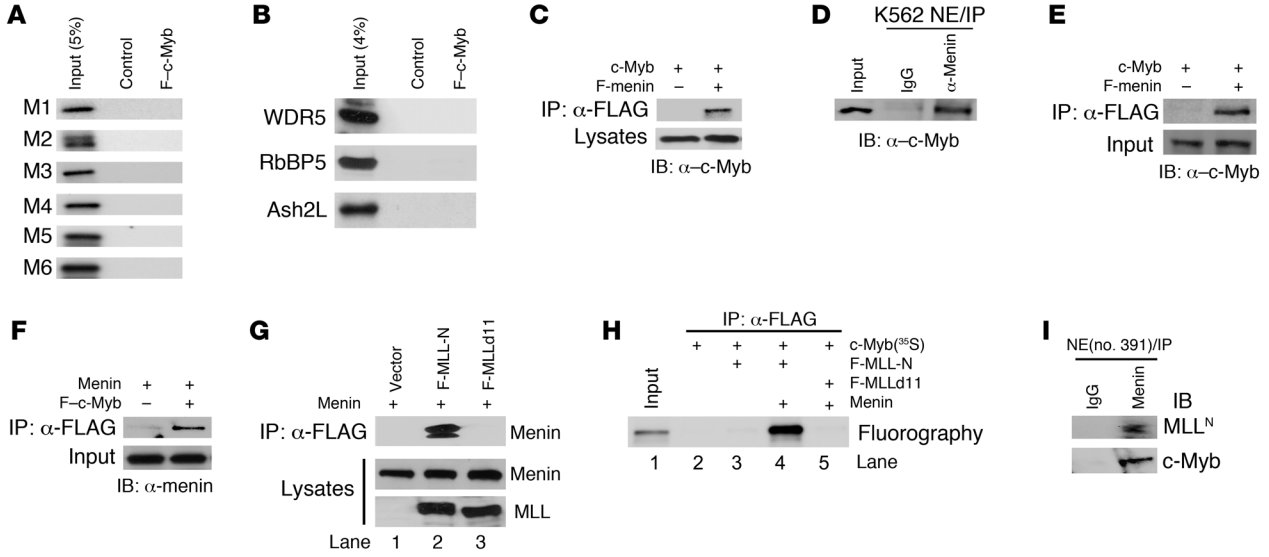

Figure 2

c-Myb associates with MLL through menin. (**A**) In vitro translated, ^{35}S-labeled MLL fragments (indicated on the left) and F–c-Myb (indicated at the top) were mixed, followed by IP with anti-FLAG M2 agarose. Samples were resolved on SDS-PAGE, then amplified, dried, and fluorographed. (**B**) In vitro protein-binding assay of WDR5, RbBP5, and Ash2L with F–c-Myb as described in **A**. (**C**) Cell lysates prepared from 293T cells cotransfected with c-Myb and FLAG-menin were subjected to IP with anti-FLAG M2 agarose, followed by immunoblotting with α–c-Myb antibody. (**D**) Nuclear extracts prepared from K562 cells were subjected to IP with α-menin antibody or control IgG. Immunoprecipitates were analyzed by immunoblot with α–c-Myb antibody. (**E** and **F**) c-Myb and menin proteins were in vitro translated with or without a FLAG tag separately. The various proteins were mixed as indicated in the blots of **E** and **F** and then subjected to IP with anti-FLAG M2 agarose. Immunoprecipitates were analyzed by immunoblotting with α–c-Myb (**E**) or α-menin (**F**) antibody. (**G**) FLAG-tagged MLL deletion mutants (F-MLL-N: aa 1–400; F-MLLd11: aa 12–400) and menin were transiently expressed in 293T cells. Cell lysates were subjected to IP with α–FLAG M2 agarose. Immunoprecipitates were analyzed by immunoblotting with α-menin antibody. (**H**) In vitro translated proteins were mixed as indicated on the right, followed by IP with α-FLAG M2 agarose. Samples were resolved on SDS-PAGE, then amplified, dried, and fluorographed. (**I**) Nuclear extracts from human leukemia patient (UPN no. 391) were immunoprecipitated by α-menin antibody or general IgG as a control. Samples were resolved on 5% SDS-PAGE, followed by immunoblotting with α-MLL^N or α–c-Myb antibody.

or required an adapter protein. We addressed this question by expressing both proteins, with or without a FLAG tag, using an in vitro transcription/translation system. The expressed proteins were then employed in in vitro binding assay. We found that c-Myb was coprecipitated when it was mixed with F-menin, but not when mixed with sample generated from empty vector (Figure 2E). Similar reciprocal binding assays showed that menin was only detected when it was mixed with F–c-Myb (Figure 2F), indicating clearly that c-Myb directly interacts with menin.

To further define the various protein interactions within the c-Myb–MLL complex, 2 FLAG-tagged fragments of MLL (MLL-N: aa 1–400; MLLd11: aa 12–400) were transiently expressed in 293T cells that were also engineered to express menin. The ability of these MLL fragments to interact with menin was assessed by immunoprecipitation and Western blotting. As shown in Figure 2G, lane 2, menin was easily detected in the anti-FLAG immunoprecipitate prepared from cells expressing F–MLL-N. In distinct contrast, menin was not detected in the immunoprecipitate prepared from cells expressing F–MLLd11, a mutant that lacks aa 1–11 at the N terminus (Figure 2G, lane 3). This site has previously been defined as a high-affinity menin binding site (48). The previously described in vitro translated protein binding assay also demonstrated that MLL-N directly interacts with menin (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI38030DS1). A further binding assay using in vitro translated proteins indicated that c-Myb only coprecipitates with MLL-N in the presence of menin (Figure 2H, lane 4). In the absence of menin, c-Myb failed to immunoprecipitate with MLL-N (Figure 2H, lane3). As expected, even in the presence of menin, c-Myb did not immunoprecipitate with MLLd11 (Figure 2H, lane 5). These results demonstrate that the N-terminal menin binding site is required for the association of MLL with c-Myb, and in aggregate with the rest of these studies, they strongly support the suggestion that c-Myb associates with MLL complex via a direct interaction with menin.

Finally, to ascertain the potential physiologic relevance of this observation, we investigated whether a c-Myb–menin–MLL complex could be detected in human leukemia cells isolated from a patient with a t(6;11) chromosomal translocation of *MLL* gene. To this end, we carried out an immunoprecipitation with anti-menin antibody on nuclear extract prepared from this patient's cells and clearly detected both c-Myb and MLL, which strongly suggested the existence of such a complex in vivo (Figure 2I). Furthermore, the c-Myb–menin–MLL complex was also identified in human primary T lymphocytes stimulated in culture with phytohemagglutinin (PHA) and IL-2 (Supplemental Figure 1C), as well as in SEM-K2 leukemia cells, which express the MLL-AF4 fusion as a consequence of t(4;11) chromosomal translocation (Supplemental Figure 1B). Taken together, these results indicate that c-Myb forms a multiprotein complex with MLL through menin both in vitro and in vivo, in human normal primary cells and human leukemia cell lines with or without the MLL chromosomal translocation.

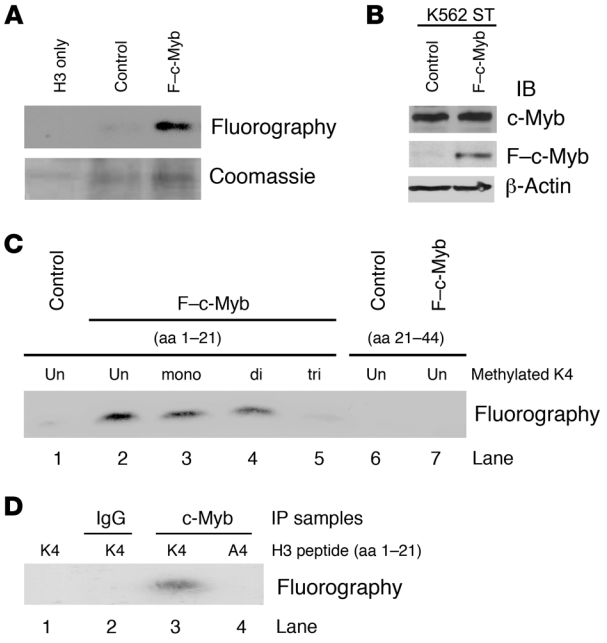

Figure 3
The c-Myb–containing complex methylates histone H3 on lysine 4. (**A**) F–c-Myb or control immunoprecipitate (described in Figure 1A) was incubated with recombinant human histone H3 and the methyl donor [^{3}H]SAM. Samples were resolved on 15% SDS-PAGE, stained with Coomassie blue (bottom panel), amplified, dried, and flourographed (top panel). (**B**) Cell lysates (top panel), α-FLAG immunoprecipitates (middle panel) prepared from F–c-Myb stably transfected K562 cells, or empty vector control K562 stable cells were immunoblotted with α–c-Myb and α-FLAG antibodies. (**C**) Methylation of histone H3 peptides with different levels of K4 premethylation (aa 1–21) or without methylation (aa 21–44) by F–c-Myb–containing complexes prepared from K562 stable cells. (**D**) Mutation of H3 peptide from K4 to A4 abrogated the methylation activity of the c-Myb complex.

c-Myb–containing MLL complexes have HMTase activity specific to histone H3K4. We next explored the functional significance of c-Myb's association with the MLL by determining c-Myb's effect on the HMT activity of the MLL complex (Figure 3). To do this, we first performed the immunoprecipitations using anti-FLAG affinity M2 beads from vector control or F–c-Myb–transfected 293T cell lysates and then mixed the beads with nuclear extracts of K562 cells as described in Figure 1A. Immunoprecipitates were incubated with recombinant human histone H3, together with the radioactive methyl donor *S*-adenosyl-L-[methyl-^{3}H] methionine ([^{3}H]SAM). We observed that the immunoprecipitate derived from lysates made from F–c-Myb–transfected cells methylated histone H3 robustly, while the immunoprecipitate derived from control vector–transfected cells was completely inactive (Figure 3A).

To delineate which lysine residues in histone H3 are specific substrates for the c-Myb–MLL complex, we employed K562 stable cell lines (K562ST) transfected with F–c-Myb or an empty vector that served as a control. Immunoblots indicated that F–c-Myb was only present in the F–c-Myb stable cells and that the total amount of c-Myb protein in F–c-Myb stable cells (F–c-Myb plus endogenous c-Myb) was approximately the same as in the K562 vector control cells (Figure 3B). This result alleviated the potential concern that gross overexpression of F–c-Myb in the K562 lysate might result in activity artifacts. Accordingly, we then incubated anti-FLAG immunoprecipitates prepared from nuclear extracts of control or F–c-Myb K562 stable cells with histone H3 peptides (aa 1–21) that were either non-, mono-, di-, or trimethylated on K4 (Figure 3C, lanes 1–5) or nonmethylated H3 peptide (aa 21–44) (Figure 3C, lanes 6 and 7). The F–c-Myb immunoprecipitates showed

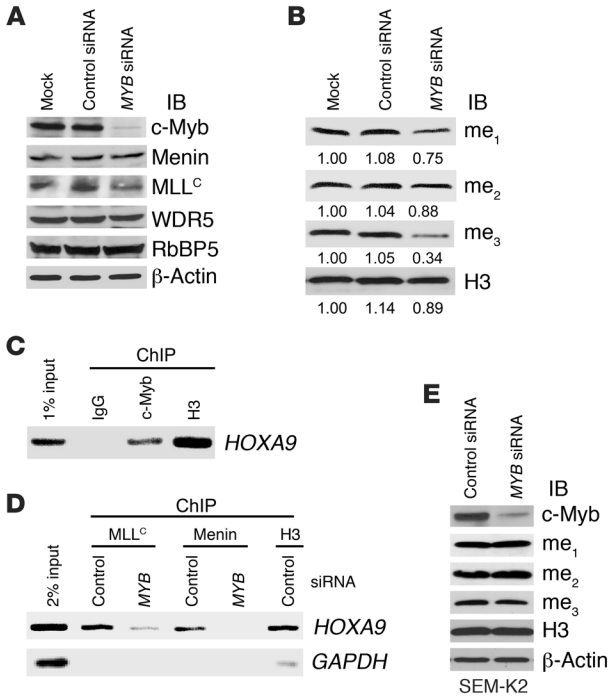

Figure 4
Loss of c-Myb affects global H3K4 methylation level in K562 cells but not in human SEM-K2 leukemic cells. (**A** and **B**) K562 cells were nucleofected with control siRNA or *MYB* siRNA. Whole-cell extracts were prepared 3 days after the initial nucleofection according to the cell number counts and probed by immunoblotting with α–c-Myb, α-menin, α-WDR5, α-MLL^C, α-RbBP5, α–β-actin (**A**), α-H3K4(Me)$_{1-3}$, and α-H3 (**B**) antibodies. Mock indicates K562 cells nucleofected with the same amount of nuclease-free water without siRNA. (**C**) ChIP assay was performed to assess the occupancy of c-Myb on the *HOXA9* gene locus of HL-60 cells. Chromatin was immunoprecipitated with α–c-Myb antibody. The presence of the *HOXA9* locus DNA in the chromatin precipitates was assessed by standard PCR. Negative and positive controls consisted of IgG and α–histone H3 antibody, respectively. (**D**) ChIP analysis of HL-60 cells nucleofected with control siRNA or *MYB* siRNA was performed using antibodies specific for MLL^C and menin. The presence of the *HOXA9* locus DNA in the chromatin precipitates was assessed by standard PCR. ChIP using α–histone H3 antibody served as a positive control. (**E**) SEM-K2 cells were nucleofected with control siRNA or *MYB* siRNA. Whole-cell extracts were prepared 3 days after the initial nucleofection according to the cell number counts and probed by immunoblotting with α–c-Myb, α-H3K4(Me)$_{1-3}$, α-H3, and α–β-actin antibodies.

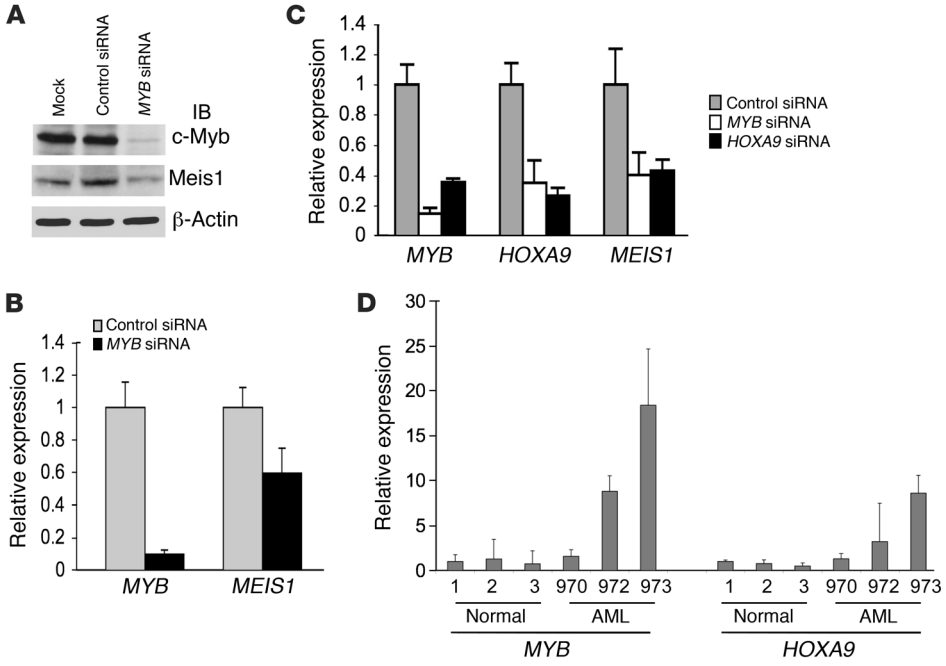

Figure 5

Loss of c-Myb results in a decrease in *HOXA9* and *MEIS1* gene expression. (**A**) K562 cells were nucleofected with control siRNA or *MYB* siRNA. Whole-cell extracts were prepared 3 days after the initial nucleofection. Proteins were subjected to immunoblot analysis with α–c-Myb, α-Meis1, and α–β-actin antibodies. Mock indicates K562 cells nucleofected with the same amount of nuclease-free water without siRNA. (**B**) K562 cells were nucleofected with control siRNA or *MYB* siRNA. RNA samples, prepared 2 days after the initial nucleofection, were reverse transcribed and used for qRT-PCR analysis for *MYB* and *MEIS1* expression, determined in triplicate. Relative expression of *MYB* and *MEIS1* to *GAPDH* is shown, with error bars indicating standard deviations. (**C**) Human ML-2 leukemia cells were nucleofected with control, *MYB*, or *HOXA9* siRNA. RNA samples were prepared 2 days after initial nucleofection and subjected to qRT-PCR using *GAPDH* as an internal standard. Error bars represent standard deviations based on assays performed in triplicate. (**D**) RNA samples prepared from normal bone marrow donors (nos. 1, 2, and 3) and AML patients (UPN nos. 970, 972, and 973) were subjected to qRT-PCR for *MYB* and *HOXA9* mRNA expression levels as described in **B**. Error bars represent the standard deviation of assays performed in triplicate.

unambiguous HMT activities on non-, mono-, and dimethylated H3 peptides (aa 1–21) (Figure 3C, lanes 2–4). In contrast, control cell immunoprecipitates showed no activity on these 2 nonmethylated peptides (Figure 3C, lanes 1 and 6). The inability of the trimethylated H3K4 peptide and nonmethylated H3 peptide (aa 21–44) to be further methylated by the F–c-Myb–containing immunoprecipitates (Figure 3C, lanes 5 and 7), despite the fact that these peptides have unmethylated K9, K27, and K36 residues, demonstrates convincingly that the c-Myb–containing MLL complex has a preference for methylating K4. Finally, there was no detectable H3 methylation when a mutated (K4A) H3 peptide (1–21 aa) (Figure 3D, lane 4) as employed in the same assay. Hence, the c-Myb–containing HMT activity is specific for H3 lysine (K4).

Deletion of c-Myb decreases global H3K4 methylation levels in K562 cells but not in SEM-K2 cells. To provide additional proof of c-Myb's role in facilitating MLL complex–mediated methylation of H3K4, we nucleofected K562 cells with *MYB*-targeted siRNA. Mock-nucleofected or control siRNA–nucleofected K562 cells served as controls for nonspecific effects of the transfection procedure itself or the siRNA, respectively. Whole-cell lysates were prepared for immunoblotting from all of these cells 72 hours after nucleofection.

The *MYB* siRNA resulted in an efficient knockdown of c-Myb protein, with an at least 80% reduction in protein expression level relative to that observed with mock- or control siRNA–treated cells (Figure 4A, top panel). The expression levels of menin and other components of MLL complex (MLL, WDR5, and RbBP5) were unaffected by either the *MYB*-targeted or control siRNA (Figure 4A). Remarkably, in K562 cells in which c-Myb levels were downregulated with siRNA, levels of H3K4 methylation were also reduced. The changes in H3K4 methylation and histone H3 protein were quantitated (Figure 4B). Trimethylation of H3K4 decreased by 30%–50%, while a more modest effect on monomethylation was observed (~15%). There was no apparent effect on the dimethylated form of H3K4 (Figure 4B). A similar pattern of effects on H3K4 methylation was reported after knockdown of WDR5 (38), suggesting that c-Myb might interact with WDR5 in some as-yet-unspecified manner. The expression level of histone H3 was unchanged.

It has been reported that MLL binding in the *HOXA9* locus is associated with the ability to methylate H3K4, and in particular to trimethylate the dimethylated form of H3K4 (36, 38). One might then anticipate that if c-Myb was responsible for targeting the MLL complex to this locus, then a *MYB* knockdown ought to be associated with a decrease in MLL and menin at this site. To test this hypothesis, we carried out a series of ChIP assays in HL-60 cells using DNA primers specific for the first exon of *HOXA9*, a region previously reported by Dou et al. (36) and Wysocka et al. (38) to be occupied by MLL. We first carried out a ChIP assay to confirm the presence of c-Myb in the *HOXA9* locus in untreated HL-60 cells (Figure 4C). We then investigated whether *MYB* silencing would affect the occupancy of MLL and menin on the *HOXA9* locus. For this experiment, we nucleofected HL-60 cells with control or *MYB*-targeted siRNA. ChIP assays were performed 72 hours after nucleofection using anti-MLL^C or anti-menin antibodies. The occupancy of MLL and menin on the *HOXA9* locus was substantially decreased or undetectable, depending on which primary antibody was employed for the immunoprecipitation (Figure 4D). To demonstrate that these results were not dependent on the use of a particular cell line, we carried out identical silencing experiments and ChIP assays using KCL-22 cells (human chronic myeloid leukemia). As showed in Supplemental Figure 2, B–E, the results obtained with these cells were entirely consistent with those obtained with HL-60 cells. Of note, there are several canonical c-Myb–binding sequences in 2 *HOXA9* exons (2 in exon 1 and 6 in

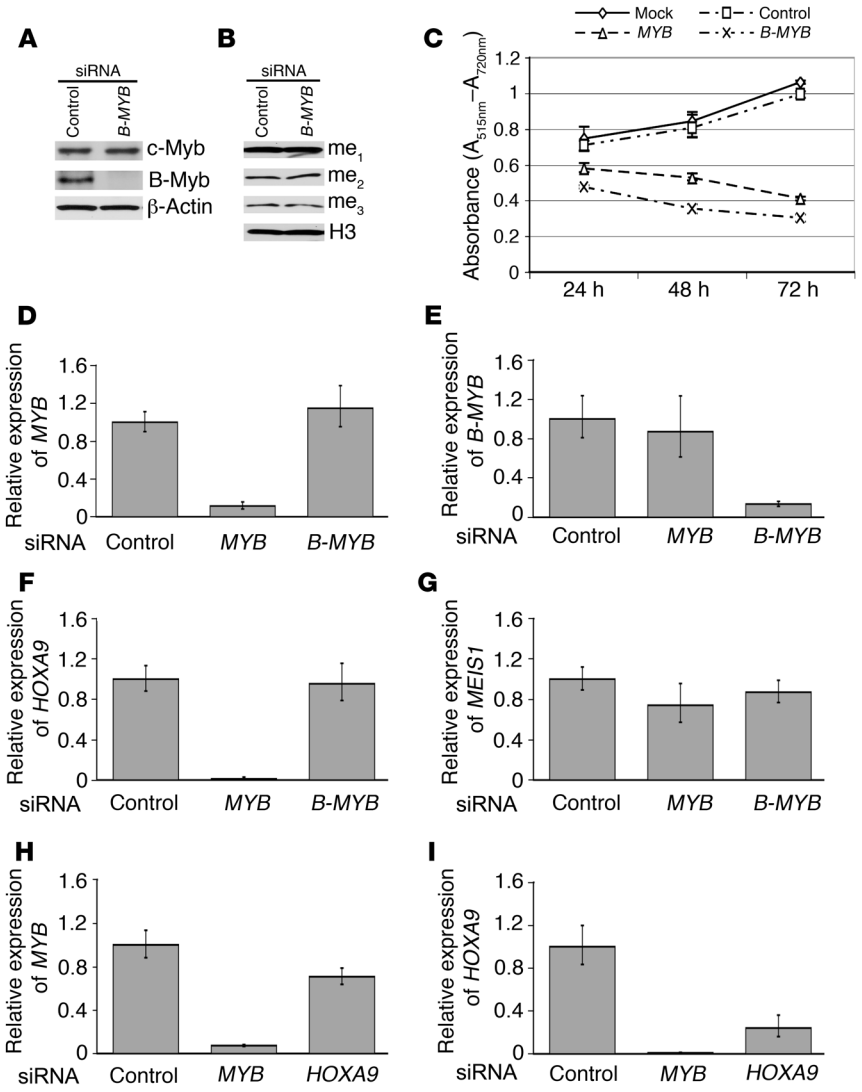

Figure 6
Decrease in trimethylation of H3K4 and *HOXA9* gene expression is specific for *MYB* silencing but not secondary to the antiproliferation effect of *MYB* silencing. (**A** and **B**) KCL-22 cells were nucleofected with control siRNA or *B-MYB* siRNA. Whole-cell extracts were prepared 3 days after the initial nucleofection according to the cell number counts and probed by immunoblotting with α–c-Myb, α–B-Myb, and α–β-actin (**A**), α-H3K4(Me)$_{1\text{-}3}$, and α-H3 (**B**) antibodies. (**C**) Cell proliferation assays were carried out with KCL-22 cells nucleofected with mock, control, *MYB*, and *B-MYB* siRNAs at indicated time points. Silencing *MYB* or *B-MYB* inhibited cell proliferation. (**D**–**G**) KCL-22 cells were nucleofected with control, *MYB*, or *B-MYB* siRNA. RNA samples, prepared 2 days after the initial nucleofection, were reverse transcribed and used for qRT-PCR analysis for *MYB* (**D**), *B-MYB* (**E**), *HOXA9* (**F**), and *MEIS1* (**G**) gene expression determined in triplicate as described in Figure 5B. (**H** and **I**) KCL-22 cells were nucleofected with control, *MYB*, or *HOXA9* siRNA. RNA samples, prepared 2 days after the initial nucleofection, were reverse transcribed and used for qRT-PCR analysis for *MYB* (**H**) and *HOXA9* (**I**) gene expression determined in triplicate as described in Figure 5B. Error bars represent the standard deviation of assays performed in triplicate (**C**–**I**).

exon 2) (Supplemental Figure 3A). A ChIP analysis conducted on KCL-22 cells with primer sets specific for the c-Myb–binding sites revealed that c-Myb, menin, and MLL are localized to these exons (Supplemental Figure 3, B–E). These results strongly suggest that c-Myb recruits MLL and menin to the *HOXA9* locus and that it does so by binding to its canonical recognition sites. In support of this statement, we also found that ChIP experiments conducted in cells expressing a c-Myb construct with deletion of the DNA-binding domain (c-Myb 194–636) led to the decrease in binding of MLL and menin on the *HOXA9* locus (see below).

Finally, to further investigate c-Myb's role in regulating overall H3K4 methylation, and specifically whether c-Myb might augment the activity of other cellular HMTs, we determined the effect of a *MYB* knockdown on H3K4 methylation in human SEM-K2 leukemia cells. SEM-K2 cells were derived from a child with ALL, and though they retain a copy of the wild-type *MLL* gene, they express an MLL-AF4 fusion protein as a consequence of a t(4;11) chromosomal translocation (53). Accordingly, we postulated that a decrease in H3K4 methylation might result from the loss of non-MLL c-Myb–dependent HMT activity. The SEM-K2 cells were nucleofected with identical control or *MYB*-targeted siRNA. Whole-cell lysates were prepared 72 hours after nucleofection. Immunoblotting with anti–c-Myb antibody showed that c-Myb protein expression levels in the *MYB* siRNA–treated SEM-K2 cells was approximately the same as in K562 cells (~20%) relative to that observed with control siRNA–treated cells (Figure 4E). However, in contrast to the results obtained with the myeloid K562 cells, no detectable change in the levels of mono- or dimethylated H3K4 was observed, while the trimethylated form was reduced only approximately 6% (Figure 4E). Again, the total protein level of histone H3 was unchanged. These results suggest that c-Myb has limited ability to promote non–MLL-dependent HMT activity, at least in this particular lymphoid leukemia cell line.

MYB silencing is associated with decreased HOXA9 and MEIS1 gene expression. The results described above suggested that c-Myb might well influence the biological activity of the MLL complex. To investigate this possibility, we investigated the effect of *MYB* silencing on the ability of the MLL complex to regulate *HOXA9* and *MEIS1* expression in different types of leukemia cells. In K562 cells, c-Myb protein and mRNA expression were effectively eliminated using siRNA (Figure 5, A and B), and specificity was suggested by lack of effect on β-actin (Figure 5A). The *MYB* knockdown resulted in

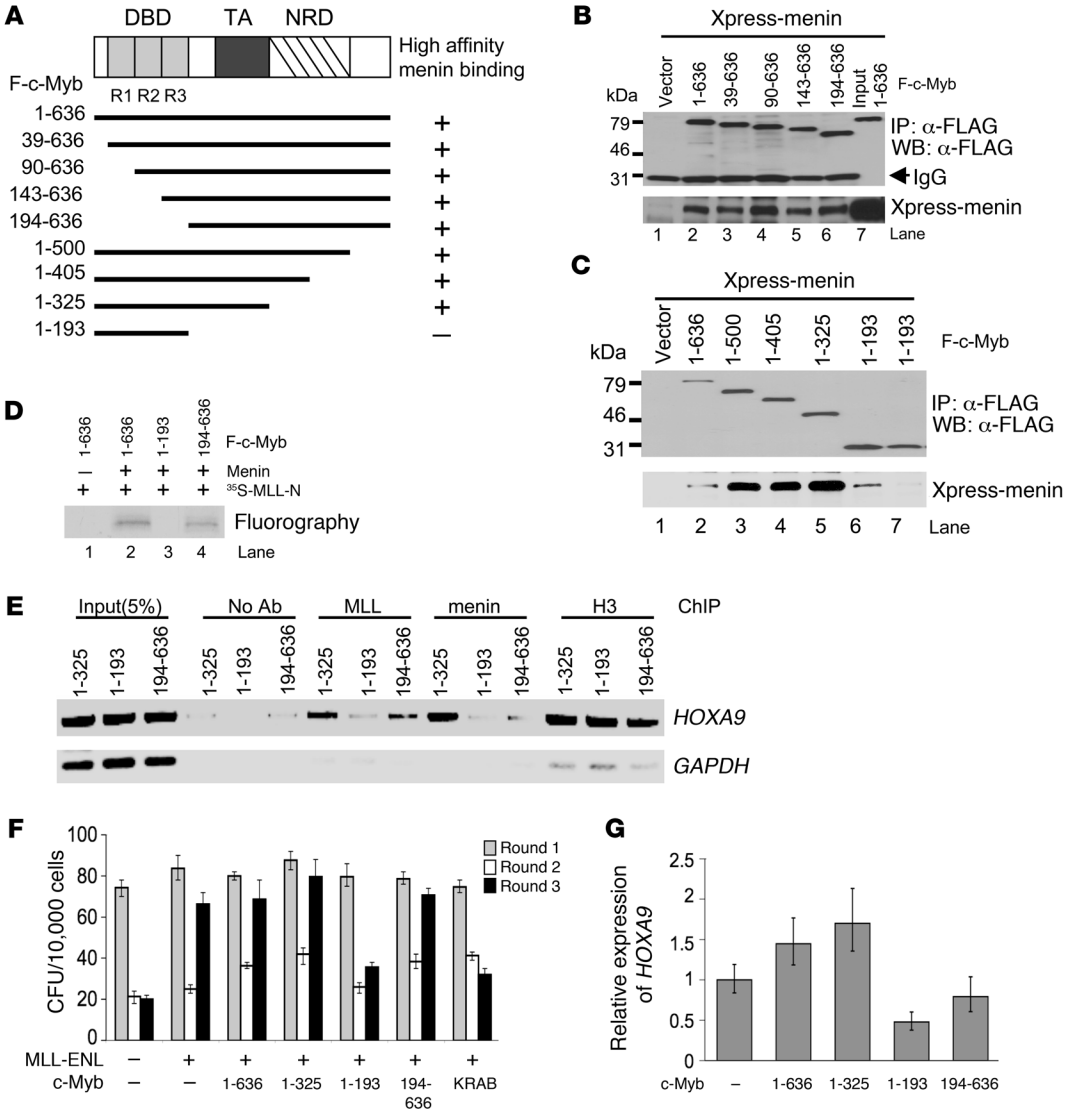

Figure 7

The interaction between c-Myb and the menin-MLL complex is required for localization of MLL and menin on the *HOXA9* gene, transformation of myeloid progenitors, and *HOXA9* gene expression. (**A**) Schematic representation of the constructions used. DBD, DNA binding domain; TA, transactivation domain; NRD, negative regulation domain. Various c-Myb mutants containing FLAG tag at their N termini and Xpress-tagged menin were transiently transfected in 293T cells. Cell lysates prepared from transfectants were subjected to IP with anti-FLAG M2 agarose. High-affinity menin-binding capacities are indicated on the right. (**B** and **C**) Immunoprecipitated proteins were separated by 10% SDS-PAGE and immunoblotted with anti-FLAG antibody (upper panel) or anti-Xpress antibody (bottom panel) antibody. (**D**) F–c-Myb mutants, menin, and ^{35}S-labeled F–MLL-N were generated by in vitro transcription/translation. c-Myb was incubated with protein reaction mixtures as indicated on the right in the presence of α-FLAG M2 agarose. Samples were resolved on 10% SDS-PAGE, amplified, dried, and fluorographed. (**E**) ChIP assay of HL-60 cells transfected with c-Myb mutants was performed using the antibodies indicated at the top. Amplicons upstream of the *HOXA9* and *GAPDH* genes were analyzed. (**F**) CFU per 10^4 plated cells are shown for each round of plating. Error bars represent standard deviations of 3 independent experiments. (**G**) Relative expression levels of *HOXA9* gene are shown in SEM-K2 cells transfected with c-Myb or its mutants. Error bars represent the standard deviation of assays performed in triplicate.

a considerable reduction in Meis1 protein relative to the decrease observed with mock- or control siRNA–treated cells (Figure 5A). Quantitative real-time RT-PCR (qRT-PCR) analysis also showed that knockdown of *MYB* expression resulted in a moderate downregulation (~40% decrease) in *MEIS1* mRNA level relative to that of *GAPDH* (Figure 5B). The effect of a *MYB* knockdown on *HOXA9* mRNA could not be determined in these experiments because *HOXA9* was undetectable in control (untreated) K562 cells. Therefore, to further establish whether c-Myb is required for expression of *HOXA9* as well as *MEIS1*, we also performed a *MYB*-directed siRNA silencing experiment in human ML-2 leukemia cells (Figure 5C). These cells were of particular interest because they bear a t(6;11) chromosomal translocation and thus express an MLL-AF6 fusion protein exclusively. After silencing *MYB*, qRT-PCR analysis showed that *HOXA9* expression was moderately downregulated (50% reduction) in the *MYB*-knockdown cells compared with mock- or control

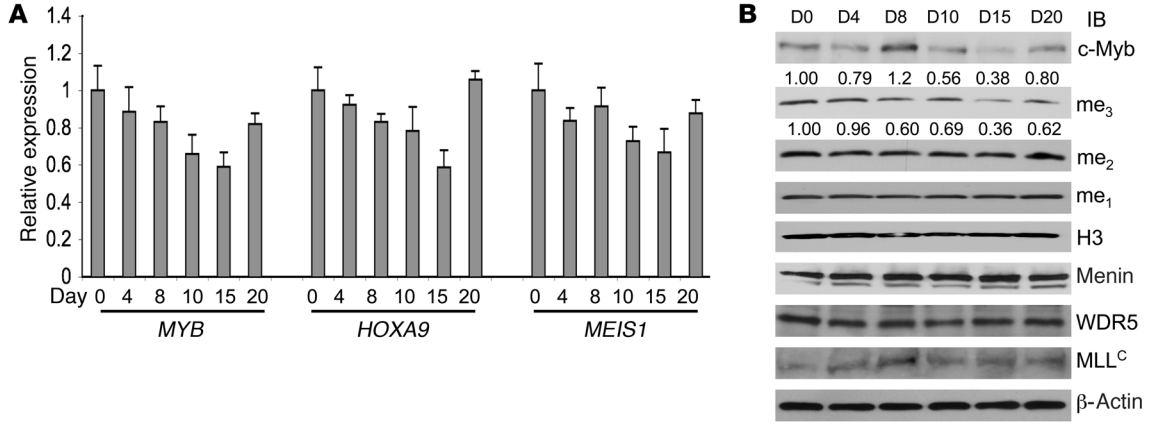

Figure 8

Treatment of AML patient no. 866 with *MYB* antisense oligonucleotides resulted in a reduction in *HOXA9* and *MEIS1* gene expression, as well as a decrease in trimethylation of H3K4. (**A**) RNA samples were prepared from cells isolated from patient no. 866 at the indicated days and subjected to qRT-PCR for *MYB*, *HOXA9*, and *MEIS1* mRNA expression levels as described in Figure 5D. Error bars represent the standard deviation of assays performed in triplicate. (**B**) Whole-cell lysates prepared from the same patient at indicated days were analyzed by immunoblotting with a variety of antibodies indicated on the right. Numbers between the top rows are densitometry measurements of scanned bands relative to the day 0 value, which was arbitrarily set at 1.00.

siRNA–treated cells (Figure 5C). Interestingly, the converse was also found to be true. That is, siRNA-mediated knockdown of *HOXA9* caused a large downregulation of *MYB* expression (Figure 5C), a result consistent with the recent observation of the Hess group (51). Unexpectedly however, silencing of *MYB* was also associated with decreased expression of *MEIS1*, as was a siRNA-mediated knockdown of *HOXA9*. In accordance with these results, in AML patient samples (nos. 70, 972, 973; Supplemental Table 1) in which *MYB* expression was found to be elevated compared with that in normal bone marrow cells (sample nos. 1, 2, and 3), qRT-PCR analysis revealed that *HOXA9* mRNA expression was elevated as well, and in proportion to the elevation of *MYB* (Figure 5D).

It has been reported that c-Myb plays a direct role in cell proliferation (8–12). Therefore, one could question whether the observations reported above were the specific result of *MYB* silencing or merely secondary phenomena resulting from decreased cell proliferation. To address this issue, we examined the effects of silencing *B-MYB*, a Myb family member also involved in regulating cell proliferation (54, 55), on H3K4 methylation and expression of *HOXA9* and *MEIS1*. For these experiments, all assays were carried out with *MYB* and *B-MYB* siRNAs in KCL-22 human leukemia cells. Silencing *B-MYB* had no effect on c-Myb protein levels (Figure 6A) or on H3K4 methylation (Figure 6B). As expected, however, silencing *MYB* or *B-MYB* inhibited cell proliferation to a similar extent (Figure 6C). Specificity of silencing was confirmed by qRT-PCR (Figure 6, D and E). Consistent with results obtained in other cell lines, silencing *MYB* in KCL-22 cells decreased the trimethylation of H3K4 (Supplemental Figure 2C). Silencing *MYB* also led to the very large decrease in *HOXA9* gene expression (≥90%) in KCL-22 cells and a more modest reduction in *MEIS1* gene expression (~25%). Silencing *B-MYB* did not affect the expression of either gene (Figure 6, F and G). Taken together, these results indicate that the observed decrease in trimethylation of H3K4 and *HOXA9* gene expression is not a secondary phenomenon related to decreased cell proliferation but is in fact specific to the loss of c-Myb. Finally, we noted that as expected, silencing *HOXA9* reduced *MYB* gene expression (Figure 6H), but quite unexpectedly, silencing *MYB* led to a dramatic decrease in *HOXA9* gene expression (Figure 6I). These results suggest the existence of an autoregulatory feedback loop between *MYB* and *HOXA9* that has not previously been described and whose exact control remains to be defined.

Delineation of the c-Myb–menin interaction domain. To develop a more mechanistic understanding of how c-Myb and menin interact, we sought to localize more precisely the c-Myb–menin interaction domains. This was accomplished by construction of a series of c-Myb FLAG-tagged deletion mutants (Figure 7A) in pcDNA3 expression vectors. These were cotransfected with Xpress-tagged menin (Xpress-menin) in 293T cells (Figure 7, B and C). Immunoprecipitation of the various FLAG-tagged c-Myb constructs from transiently transfected 293T cells revealed that deletion of the c-Myb DNA–binding repeats R1, R2, and R3 had no effect on the ability of c-Myb to bind to menin (Figure 7B). Further, the c-Myb DNA–binding domain (aa 39–193), which is contained within the aa 1–193 expression fragment, was not only dispensable for a stable c-Myb–menin interaction; by itself, it had no ability to bind menin at all, at least under these assay conditions (Figure 7C, lanes 6 and 7). In contrast, c-Myb fragment 1–325 strongly interacted with menin (Figure 7C, lane 5). Reciprocal experiments conducted in vitro with recombinantly expressed proteins indicated that full-length c-Myb and c-Myb 194–636 were both able to form a complex with MLL in the presence of menin (Figure 7D, lanes 2 and 4, respectively). As expected from the experiments carried out with 293T lysates, c-Myb DNA–binding domain fragment 1–193 was unable to form a complex with MLL even in the presence of menin (Figure 7D, lane 3). Thus, the c-Myb–menin interaction domain was localized to a region within c-Myb spanning residues 194–325.

c-Myb binding to its specific DNA recognition site is required for localization of MLL and menin to the HOXA9 promoter locus, for HOXA9 gene expression, and for transformation of normal myeloid progenitor cells. To ascertain whether the association of c-Myb with menin-MLL is required for localization of the complex to the *HOXA9* gene promoter region in vivo, we transfected HL-60 cells with 3 of the

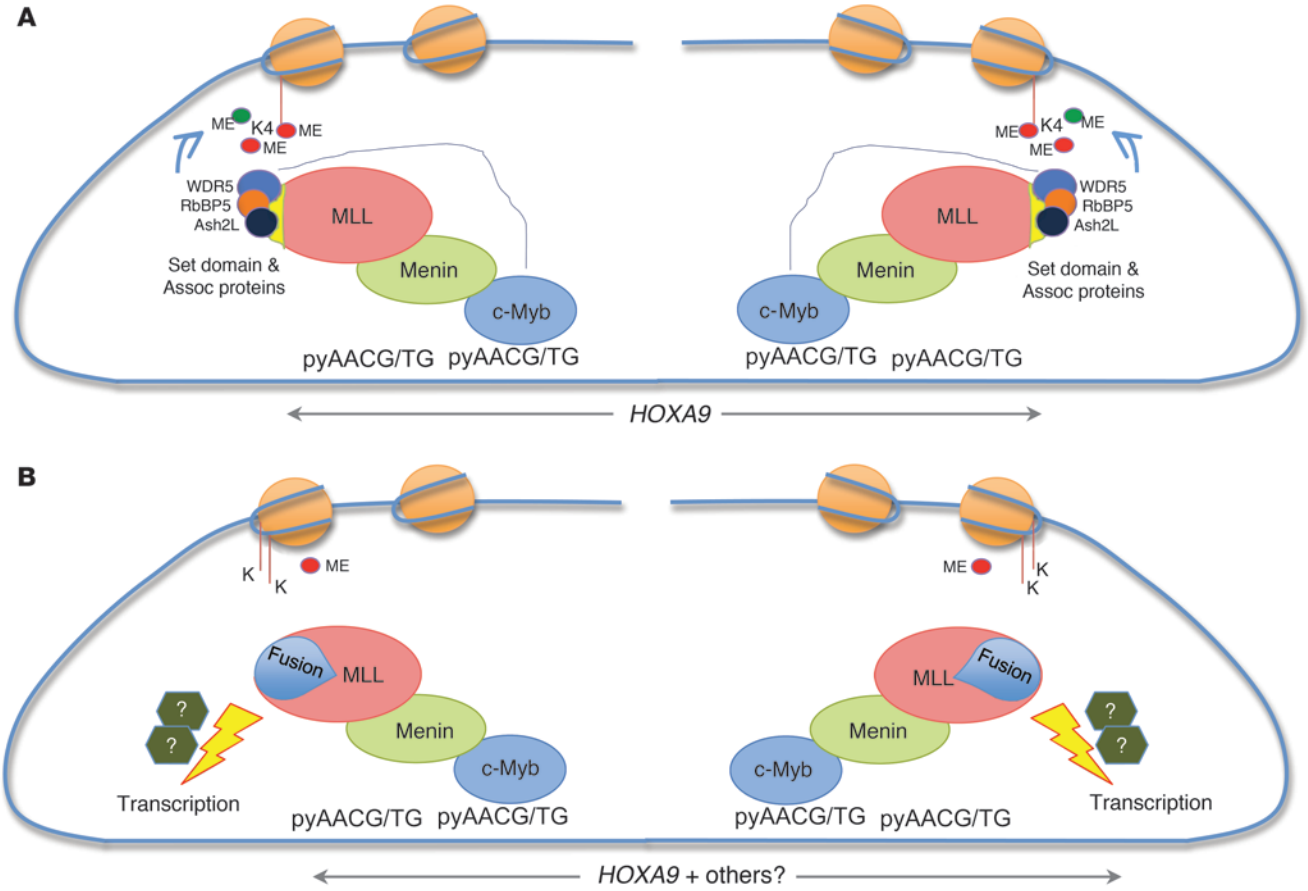

Figure 9

c-Myb binds to menin-MLL and directs the complex to canonical c-Myb–binding sites in the *HOXA9* promoter locus. (**A**) Proposed interaction of c-Myb with wild-type menin-MLL in the *HOXA9* promoter locus. c-Myb binds to its canonical recognition sites and holds the complex in place, leading to maintenance of *HOXA9* expression. Changes in surrounding chromatin, specifically H3K4 methylation in neighboring nucleosomes (orange circles), is brought about by the MLL^{c} SET domain–associating proteins, most likely WDR5, whose function may be enhanced by an as-yet-uncharacterized interaction with c-Myb, as indicated by the thin blue arrow. (**B**) c-Myb brings the menin-MLL fusion protein complex to the *HOXA9* locus, where, as in **A**, it serves to anchor the complex and sustain *HOXA9* expression. Sustained fusion protein expression leads to other, as-yet-uncharacterized transcriptional changes that likely play a role in leukemic transformation. As indicated, other unidentified cofactors are postulated to assist in transformation.

c-Myb mutants (1–325, 1–193, and 194–636, respectively) described immediately above. We then carried out ChIP assays employing antibodies specific for either menin or MLL protein and the previously described cDNA primers spanning the c-Myb–binding sites in the *HOXA9* promoter locus. We found that transfection of HL-60 cells with c-Myb 1–193 decreased the localization of both MLL and menin on the *HOXA9* promoter as compared with that observed with c-Myb 1–325, which contains the menin-binding domain (Figure 7E). These results strongly suggest that c-Myb's ability to bind to its DNA recognition region is necessary for recruitment of the menin-MLL complex to the *HOXA9* promoter. In support of this conclusion, transfection of HL-60 cells with c-Myb fragment 194–636, which is competent to interact with menin-MLL but is unable to bind DNA, recruits considerably less menin-MLL to the *HOXA9* locus (Figure 7E). We speculate that the menin-MLL protein detected in the *HOXA9* promoter in the presence of 194–636 is bound there by either endogenous c-Myb or perhaps LEDGF, which has previously been reported to recruit menin-MLL to this locus.

Finally, to investigate the potential role of c-Myb in MLL-mediated leukemogenesis, we carried out in vitro transformation assays using normal mouse bone marrow cells obtained from 5-fluorouracil–treated (5-FU–treated) donors transfected with an MLL-ENL fusion construct and c-Myb deletion mutant 1–325, 1–193, or 194–636. We then assessed the cells for growth characteristics consistent with transformation (51, 56) in serial methylcellulose replating assays (Figure 7F). A dominant-negative c-Myb expression construct consisting of a C-terminal fusion of the c-Myb DNA–binding domain (1–200 aa) to the KRAB repressor domain (1–74 aa) (DNMyb-KRAB) was used as a negative control for these experiments. After the first round of plating, cells transfected with the various constructs yielded a similar number of colonies. When the colonies present in the dishes were disaggregated and replated in a second round, colony formation was, as expected, diminished under all conditions. However, in the third replating round, cells expressing the full-length c-Myb expression fragment (aa 1–636) or the c-Myb deletion mutants capable of interacting with menin (aa 1–325 and 194–636, respectively) recovered their original clon-

ing efficiency, a hallmark of transformation (56), in contrast to those expressing the 1–193 mutant, which is incapable of binding menin, or the DNMyb-KRAB. We believe that the transformation observed in cells transfected with MLL-ENL alone occurs because of the presence of endogenous c-Myb protein in the murine marrow cells. This hypothesis is supported by the work of Hess et al. (51) and is explained mechanistically by results shown in Figure 7G. Here we explored the effect of c-Myb mutations on *HOXA9* gene expression in SEM-K2 cells. When cells were transfected with c-Myb 1–636 (full-length c-Myb) or c-Myb 1–325, *HOXA9* expression levels increased 50% and 70%, respectively, compared with cells transduced with an empty vector control. In contrast, *HOXA9* expression fell by 50% compared with control in cells transduced with the c-Myb 1–193 construct. Therefore, if c-Myb cannot bind to its DNA recognition sequence, menin-MLL is not effectively recruited to the *HOXA9* locus, *HOXA9* expression falls, and transformation diminishes accordingly. The functional relationship between these proteins holds regardless of whether MLL is wild-type or part of a fusion protein as long as the menin interaction domains persist. A molecular basis for c-Myb's role in oncogenic MLL-fusion protein transformation is therefore provided by these studies.

c-Myb inhibition is associated with decreased HOXA9 and MEIS1 mRNA expression in AML patients being treated with a MYB–targeted antisense oligodeoxynucleotide. To further explore the relationship among *MYB*, *HOXA9*, and *MEIS1* in primary leukemia cells in vivo, we examined the expression of all 3 genes in patients participating in a phase I clinical trial evaluating the toxicity of a *MYB*-targeted phosphorothioate-modified antisense oligodeoxynucleotide (ASODN; http://clinicaltrials.gov/ct2/show/NCT00780052?term=UPCC+04701&rank=1). The ASODN was delivered by continuous intravenous infusion for 7 days at a dose of 3 mg/kg/d. Peripheral blood mononuclear cells (95%–100% blasts) were isolated from 3 patients (nos. 866, 995, 1207; Supplemental Table 1) on days 0, 4, 8, 10, 15, and 20 after the start of the infusion and analyzed by qRT-PCR for *MYB* and *HOXA9* expression (Figure 8A, Supplemental Figure 4). In the case of patient no. 866, additional analysis for *MEIS1* mRNA expression as well as Western blotting for c-Myb, menin, WDR5, and MLL protein expression and H3K4 methylation was also carried out (Figure 8B). In patient 866, *MYB* mRNA levels were reduced 40% from day 0 to day 15 and returned to baseline by day 20 (Figure 8A). *HOXA9* and *MEIS1* mRNA levels in this patient's samples paralleled those of *MYB*. Also in agreement with our in vitro data, trimethylation of H3K4 correlated directly with relative c-Myb expression, whereas mono- and di-K4 methylation, also as expected, was unchanged (Figure 8B). Of note, protein expression levels of menin, WDR5, MLL^C, and histone H3 were not effected by the c-myb–targeted ASODN, suggesting specificity of the effect (Figure 8B).

Discussion

c-Myb's ability to transactivate genes required for lineage commitment, cell proliferation, and differentiation underlie its importance for normal hematopoietic cell development (5, 6). It is intuitive that c-Myb's participation in such critical functions might also suggest mechanisms whereby c-Myb could contribute to hematopoietic cell transformation (57). Simple overexpression is one such mechanism, but overexpression of *MYB* is not found in the majority of leukemia patients (58). Indeed, direct evidence implicating *MYB* overexpression in the pathogenesis of leukemia in primary patient material has only recently been provided in a small subset of acute lymphoblastic leukemia patients (<10%) in whom a translocation that juxtaposed the *TCRB* and *MYB* loci [i.e., t(6;7)(q23;q34)] or a short, somatic gene duplication increased *MYB* expression (59, 60). Therefore, how *MYB* might facilitate leukemic transformation remains incompletely understood. What is known is that failure to downregulate *MYB* expression is associated with a block in differentiation (61, 62) and a failure of proliferation arrest (57). Yet transgenic mice that overexpress c-Myb protein do not develop leukemia (63), suggesting that c-Myb protein requires additional cell-intrinsic factors in order to manifest its transformation ability. Cells capable of supplying such factors have been hypothesized to be both rare and immature (64), and the identity of factors supplied remains uncertain. Cooperation of oncogenes is one possibility (65). A recent example of this has been described in chronic myeloid leukemia, where c-Myb cooperates with Bcr/Abl (66) to drive leukemic transformation, perhaps by blocking differentiation as a result of c-Myb–mediated downregulation of C/EBPα (67). Aberrant gene activation by alternately spliced but transcriptionally active c-Myb proteins, translated from alternately spliced *MYB* mRNAs, is another way that c-Myb may contribute to leukemogenesis (68). We now extend these findings, presenting a series of observations that not only provide mechanistic insight into how c-Myb may facilitate cellular transformation, even in the absence of overexpression, but may also explain how c-Myb exerts such a myriad of effects on cell proliferation and differentiation.

c-Myb forms a complex with MLL through menin both in vitro and in vivo. The results presented here clearly show that c-Myb associates with the MLL complex in human hematopoietic cells and that this association occurs through menin, a protein first identified as the tumor suppressor of the *MEN1* gene. The existence of a c-Myb–menin–MLL complex was confirmed in vitro, in vivo, and in a variety of normal and neoplastic cell types using a host of complementary methods including: (a) coimmunoprecipitation of F–c-Myb with menin, MLL, and its core components (including WDR5, RbBP5, and Ash2L) from K562 nuclear extracts using purified F–c-Myb protein; (b) coimmunoprecipitation of MLL and c-Myb with menin in K562 cells, KCL-22 cells, SEM-K2 cells, human leukemia cells from a patient with *MLL* gene translocation, and normal human primary T lymphocytes using anti-menin antibody; (c) studying direct in vitro interactions among c-Myb, menin, and MLL using purified proteins; and (c) demonstrating clearly that menin is required in order for c-Myb and MLL to form a complex. Copurification of these proteins was also obtained by 2-step ion exchange chromatography (data not shown). The possibility that c-Myb might be directly associated with WDR5, Ash2L, and RbBP5 was also ruled out by the failure of F–c-Myb to coprecipitate ^{35}S-labeled forms of these proteins in direct binding assays. These results strongly suggest that c-Myb interacts with the MLL complex exclusively through menin. Nonetheless, once incorporated into the complex, other indirect effects on protein members of the complex are possible, as suggested by the loss of H3K4 trimethylation, a WDR5 function (38), when c-Myb is silenced with siRNA (Figure 4B and Figure 8). Therefore, by associating with the menin-MLL complex, c-Myb may direct the complex to its cognate binding sites, where effects on expression of c-Myb target genes may be induced.

c-Myb impacts chromatin modification and Hox gene expression. Cellular gene expression programs are regulated by both transcriptional factors and chromatin modification. The nucleosome is the fundamental unit of chromatin, and it is composed of an octamer of the

4 core histones (H3, H4, H2A, H2B) around which 147 base pairs of DNA are wrapped. Nucleosomal histones, and their unstructured N-terminal tails, are the target of a variety of covalent modifications. It is now well established that posttranslational acetylation, methylation, phosphorylation, and ubiquitination of these histones play an intrinsic role in transcriptional regulation. It has also been suggested that histone modification may be involved in the propagation of the transcriptional state through cell division (69, 70).

Methylation of histone H3 on lysine 4 was first identified in trout testes (71). It has been one of the characteristic of active chromatin in various organisms (70, 72). The existence of distinct patterns of mono-, di-, and trimethylation at H3K4 suggests that the transition between methylation states is a regulated event and that different methylation states are associated with distinct regulatory outcomes. MLL family proteins have already been reported to display H3K4 methyltransferase activity (32, 73, 74).

The c-Myb–menin–MLL complex appears to be functionally similar to the previously described yeast Set1 complex, which promotes transcriptional activation by methylating H3K4. Like the Set1 complex, the isolated c-Myb–MLL complex shows robust methyltransferase activity on both recombinant human histone H3 and synthetic histone H3 peptides. More importantly, the c-Myb–MLL complex methylates H3K4 non-, mono-, and dimethylated peptides efficiently and specifically (Figure 3, C and D). Subunits of the c-Myb–associated HMTase complex are homologous to members of the yeast Set1 assembly. These results are consistent with the reported ability of the MLL complex, and specifically the MLL SET domain, to effect H3K4 methylation. In fact, the observed reduction of global H3K4 trimethylation in *MYB*-knockdown human leukemia cells suggests that the specificity of MLL methyltransferase might be influenced by c-Myb, as proteins that associate with the enzyme may affect its selection of residues to modify (75), or the degree of methylation at a specific site (42). In this specific regard, our data appear to indicate that c-Myb may perturb WDR5 function, though with respect to effects on global methylation, effects on other HMTs are not ruled out. Regardless, since methylation of H3K4 is associated with open chromatin and active transcription (32, 45, 46), this work strongly suggests that in addition to its direct effects on transcription, c-Myb may regulate hematopoietic cell gene expression by leading complexes such as menin-MLL to c-Myb–bindings sites throughout the genome where the MLL set domain may exert effects on H3K4 methylation.

MLL and menin both associate with the *HOXA9* promoter. In the absence of menin, MLL and its fusions fail to maintain *HOXA9* gene expression, an event that has been reported to be critical for MLL fusion protein–mediated transformation (39, 48). Recently, lens epithelial-derived growth factor (LEDGF) has been shown to recruit the menin-MLL complex to the *Hox* locus and, because of this activity, to play a critical role in leukemic transformation by MLL fusion proteins (49). c-Myb has also been shown to be essential for MLL-ENL–mediated transformation (51) and a critical component of the *MLL* myeloid leukemia stem cell maintenance signature (76), suggesting that it too might interact in some manner with MLL. Our observations that c-Myb recruits MLL to the *HOXA9* gene locus by binding to its canonical DNA recognition sites, that this recruitment is critically dependent on c-Myb's interaction with menin, and finally that *HOXA9* gene expression falls in the absence of c-Myb, in concert with its ability to transform normal myeloid progenitor cells, all provide mechanistic insight into c-Myb's potential role in leukemic transformation in those leukemias associated with MLL fusion proteins. Whether c-Myb plays a similar role in leukemic transformation in cells expressing wild-type MLL remains to be determined. Potential interactions between c-Myb and LEDGF and whether either makes the other dispensable with respect to transformation in vivo also remain to be determined.

MYB, HOXA9, and MEIS1 participate in an autoregulatory feedback loop. As part of our evaluation of the biological implications of c-Myb's incorporation into the MLL complex, we evaluated the expression levels of *HOXA*9 and its cofactor *MEIS1*, 2 well-established MLL target genes, in human leukemia cell lines nucleofected with *MYB*-targeted siRNA and, perhaps of greater relevance, in cells isolated from AML patients undergoing treatment with a *MYB*-targeted ASODN. We noted with interest that in cells with (ML-2) or without (K562) an *MLL* translocation expression of both *HOXA9* and *MEIS1* was specifically downregulated by the *MYB*-targeted siRNA. These results suggest that binding of c-Myb in the *HOXA9* locus, independent of effects on H3K4 methylation, is sufficient for upregulation of *HOXA9* expression. Importantly, expression of *HOXA9* and *MEIS1* paralleled *MYB* expression levels in leukemia patients undergoing treatment with a *MYB*-targeted ASODN. These results support the physiologic relevance of the observations in cell lines just mentioned. Previous work demonstrating a connection between *MYB* expression and *HOX* cluster genes placed c-Myb downstream of Hoxa9 and Meis1 (51, 77). The results reported in this study (Figures 5–8) clearly indicate that c-Myb plays a role in regulating expression of *HOXA9* and *MEIS1*. In aggregate, these studies strongly suggest that c-Myb and these Hox cluster proteins are all in linked in an autoregulatory loop, the regulation and functional significance of which is presently under investigation in our laboratory.

Conclusions. Despite the advances that have been made in uncovering potential roles for c-Myb in the pathogenesis of human leukemia, the mechanistic basis for c-Myb's ability to transform hematopoietic cells remains incompletely understood. The work presented here is of considerable interest then, because it elucidates, mechanistic functions for c-Myb in the pathogenesis of MLL-associated leukemias and suggests other important ways in which c-Myb may mechanistically affect the development of normal and malignant blood cells (Figure 9). Specifically, by demonstrating that c-Myb, via menin, forms a stable complex with wild-type and translocated MLL in cell lines, normal hematopoietic cells, and primary leukemia patient material, c-Myb's role in normal and malignant hematopoiesis comes into sharper focus. The Hox genes, in particular *HOXA9*, have been shown to play an important role in leukemogenesis. c-Myb's role in bringing and anchoring the menin-MLL complex to the *HOXA9* promoter more clearly explains how expression of this gene is maintained and confirms its importance in the pathogenesis of MLL fusion protein–induced leukemias. That c-Myb plays a role in regulating Meis1, a cofactor implicated in *HOXA9* expression, is also informative with respect to leukemia pathogenesis. We provide data as well to show that c-Myb plays an important supporting role in H3K4 trimethylation, at the *HOXA9* locus, and postulate a similar effect at other c-Myb targets, perhaps through an indirect effect on WDR5. These c-Myb–supported changes in chromatin structure no doubt affect the expression of other genes that have yet to be defined but may well play a role in normal cell development and leukemia. A deeper understanding of these networks will have great translational implications and may provide new therapeutic targets for the treatment of acute leukemia.

Methods

Cell sources. 293T, K562, and HL-60 cells were originally obtained from the ATCC. ML-2, KCL-22, and SEM-K2 cells were the gift of M. Cleary (Stanford University Medical Center, Stanford, California, USA), H.P. Koeffler (UCLA, Los Angeles, California, USA), and C. Felix (Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA), respectively. Primary patient material was obtained from the Stem Cell and Xenograft Core Facility of the Abramson Cancer Center, University of Pennsylvania School of Medicine. This facility is compliant with the Health Insurance Portability and Accountability Act of 1996 and has Institutional Review Board approval for anonymous collection, storage, and distribution of materials obtained from consenting patients treated at the Abramson Cancer Center of the University of Pennsylvania School of Medicine.

Cell culture and transfection. 293T cells were cultured in DMEM supplemented with 10% FCS. K562, ML-2, HL-60, KCL-22, and SEM-K2 cells were cultured in RPMI 1640 medium supplemented with 10% FCS. 293T cells were transfected using FuGENE 6 (Roche) and collected 48 hours after transfection. K562, ML-2, HL-60, KCL-22, and SEM-K2 cells were nucleofected using Cell Line Nucleofector Kit V (Amaxa Biosystems) according to the manufacturer's instructions. Stably transfected cells were selected by G418 (750 μg/ml) for 3 weeks and confirmed by Western blotting.

Plasmids and expression vectors. FLAG-tagged menin in pcDNA3 was as described previously (78) (gift of X. Hua, University of Pennsylvania, Philadelphia, Pennsylvania, USA). pCMV-SPORT-menin was a gift from S.K. Agarwal (NIH, Bethesda, Maryland, USA). FLAG-tagged c-Myb and its mutants were constructed by inserting the cDNA amplified from human pCI-neo-c-Myb-HA (gift of O.S. Gabrielsen, University of Oslo, Oslo, Norway) into the expression vector pcDNA3-FLAG. FLAG-tagged MLL-N and MLLd11 were constructed by inserting the cDNAs amplified from pLNCX-MLL-N 1/2666 (HA) (gift from M.L. Cleary, Stanford University Medical Center, Stanford, California, USA) into the expression vector pcDNA3-FLAG. For in vitro translation assays, cDNAs encodin, menin, WDR5, RbBP5, and Ash2L were inserted into the expressing vector pcDNA4/HisMax (Invitrogen). Expressing vectors encoding M1–M6 fragments of MLL were gifts from R.G. Roeder (Rockefeller University, New York, New York, USA) (36). pMSCV-MLL-ENL and pMSCV-c-Myb-KRAB (a dominant-negative version of c-Myb) were described previously (51) (gifts from R.K. Slany, University Erlangen, Erlangen, Germany). p-MX-puro-c-Myb and its mutants were constructed by inserting the PCR products into the retrovirus vector.

Cell lysis, nuclear extract, and coimmunoprecipitation. Cells were lysed in lysis buffer (50 mM HEPES, pH 7.5, 0.15 N NaCl, 1.0% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM glycerophosphate, 0.5 mM vanadate, and 10% glycerol) supplemented with 1 mM phenylmethylsulfonyl fluoride and the recommended concentration of complete protease inhibitors (Roche). Nuclear extracts were obtained from cells by a modified Dignam procedure (79). Cell lysates or nuclear extracts containing the same amounts of total protein were incubated with antibody and protein A/G plus agarose (Santa Cruz Biotechnology Inc.) or anti-FLAG affinity M2 agarose for 4 hours (or overnight) at 4°C. Beads were collected by centrifugation and washed with the same buffer 2 times. Proteins were eluted by boiling in 1× SDS loading buffer and subjected to immunoblotting.

Antibodies. Antibodies against the following were purchased commercially: c-Myb, histone H3 trimethyl K4, MLL^N, and Meis1 (Upstate Biotechnology); H3, H3 monomethyl K4 and dimethyl K4, and WDR5 (Abcam); anti-Xpress antibody (Invitrogen); MLL^C, menin, RbBP5, and Ash2L (Bethyl Laboratories, Inc); anti-FLAG antibody and anti-FLAG M2 agarose beads (Sigma-Aldrich). Anti-WDR5 serum was provided by W. Herr (University of Lausanne, Lausanne, Switzerland).

Cell proliferation assay. Cell proliferation assays were carried out with Cell Proliferation Kit II (Roche) according to the manufacturer's recommendation. Briefly, KCL-22 cells (2×10^6) were nucleofected with mock, control, *MYB*, and *B-MYB* siRNA (2 μg) and cultured at 37°C. Cells (2×10^4) were seeded in 100 μl culture medium and then mixed with 50 μl XTT labeling mixture at 18, 42, and 66 hours after nucleofection and incubated for 6 hours at 37°C. Spectrophotometrical absorbance of the sample was measured at 515 nm, with the reference wavelength at 720 nm using a microplate (ELISA) reader (model EL800, Bio-TEK Instruments Inc).

ChIP and standard PCR detection. ChIPs were performed with the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology) using the protocol recommended by the manufacturer. qChIP DNA was detected using standard PCR and staining with ethidium bromide after electrophoresis in 2% agarose gels. Primers employed for the *HOXA9* locus studies were as follows: forward, 5′-GGTGCGCTCTCCTTCGC-3′; reverse, 5′-GCATAGTCAGTCAGGGACAAAGTGT-3′. Primers employed for human *MEIS1* and *GAPDH* were described previously (49).

In vitro translation and protein-binding experiment. Manufacturer's protocols for the TNT Quick Coupled Transcription/Translation System (Promega) were used for in vitro translation. For protein-binding assays, FLAG-tagged protein was incubated with TNT translation reaction in the cell lysis buffer with 0.1% NP-40 for 1 hour and then immunoprecipitated by anti-FLAG affinity M2 beads overnight at 4°C. Beads were washed 3 times with the same buffer. Samples were subjected to immunoblotting or fluorography.

Histone modification assays. The histone methyltransferase assay was carried out as described previously (47). Briefly, control and c-Myb–containing immunoprecipitations were carried out for 4 hours at 4°C by anti-FLAG affinity M2 agarose from cells transfected with vector or FLAG–c-Myb. After washing with the lysis buffer (1% NP-40) 3 times, agarose beads were incubated with nuclear extracts of K562 cells overnight at 4°C. Agarose beads were washed twice with nuclear extract buffer (0.05% NP-40) and once with HMTase buffer (50 mM Tris-HCl, pH 8.0, 5% glycerol, and 0.1 mM EDTA). For each HMT assay, 2 μg histone H3 or 5 μg peptide (Upstate Biotechnology) were used. Reactions were carried out at 30°C for 1 hour in the presence of 1 μM [^{3}H]SAM (GE Healthcare). Samples were applied to SDS-PAGE (histone H3) or Tris-Tricine gel (peptides). The gels were then amplified for 30 minutes (Amplify, GE Healthcare), dried (only for SDS-PAGE), and exposed to film.

RNA interference. K562 cells (1×10^6), ML-2 cells (2×10^6), HL-60 cells (1.5×10^6), KCL-22 cells, and SEM-K2 cells (2×10^6) were nucleofected with 2 μg siRNA using Cell Line Nucleofector Kit V according to the manufacturer's protocols. Cells were harvested 48 or 72 hours after nucleofection. The sequence for human *MYB* siRNA used in the assay was 5′-UAUCAGUUCGUCCAGGCAG-3′. Human *HOXA9* siRNA (M-006337-01-0005), *B-MYB* siRNA (D-010444-01), and control siRNA duplexes (D-001210-01) were purchased from Dharmacon.

qRT-PCR. RNA was isolated from cells using TRIzol (Invitrogen), and the RNA was subsequently treated with RNase-free DNase I and quantified by absorbance at 260 nm, and cDNA was synthesized from 1 μg of total RNA using oligo (dT). qRT-PCR was carried out in an ABI 7900 HT Sequence Detection System using TaqMan Master Mix according to the protocol of the manufacturer (Applied Biosystems). All data were normalized using the endogenous *GAPDH* control. All primer and probe sequences are listed in Supplemental Table 2. To quantify gene expression, a relative standard method was used. The quantities of targets and of the endogenous *GADPH* were determined from the appropriate standard curves. The target amount was then divided by the *GAPDH* amount to obtain a normalized value.

Retroviral transformation assays. pMSCV and pMX-puro retrovirus were produced by FuGENE 6 transfection of Plat-E packaging cells. Virus was harvested 48–72 hours after transfection, centrifuged, and stored at –80°C. Serial replating assays were carried out as described by Slany et al. (56). Briefly, primary bone marrow cells were isolated from 5-FU–treated (150 mg/kg)

donor mice, activated overnight in IMDM containing 15% FBS, IL-3, IL-6, and SCF. After retroviral transduction by spinoculation on 2 consecutive days, the cells were cultured in triplicate (10^4 cells/well) and replated every 7–10 days in MethoCult (M3234) methylcellulose medium (Stem Cell Technologies) containing IL-3, IL-6, and GM-CSF, all at 10 ng/ml, and SCF at 100 ng/ml under G418 and puromycin selections (only for the first round of replating). Colony morphology and number were assessed during the course of 3 rounds of serial replating of methylcellulose cultures.

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