Leukemogenic MLL Fusion Proteins Bind across a Broad Region of the *Hox a9* Locus, Promoting Transcription and Multiple Histone Modifications

Thomas A. Milne,[1] Mary Ellen Martin,[1] Hugh W. Brock,[2] Robert K. Slany,[3] and Jay L. Hess[1]

Departments of [1]Pathology and Laboratory Medicine and Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; [2]Department of Zoology, University of British Columbia, Vancouver, British Columbia, Canada; and [3]Department of Genetics, University of Erlangen, Erlangen, Germany

Abstract

Chromosome translocations involving the mixed lineage leukemia gene *MLL* are associated with aggressive acute leukemias in both children and adults. Leukemogenic MLL fusion proteins delete the MLL SET domain Lys[4] methyltransferase activity and fuse MLL to 1 of >40 different translocation partners. Some MLL fusion proteins involve nuclear proteins that are transcriptional activators, whereas others have transcriptional activating activity but instead dimerize the truncated MLL molecule. Both types of MLL fusion proteins enforce persistent expression of *Hox a9* and *Meis1*, which is pivotal for leukemogenesis through mechanisms that remain obscure. Here, we show that nuclear and dimerizable forms of MLL bind with a similar pattern to the *Hox a9* locus that overlaps the distribution of wild-type MLL and deregulate transcription of three isoforms of *Hox a9*. Induction of MLL fusion protein activity is associated with increased levels of histone acetylation and Lys[4] methylation at *Hox* target genes. In addition, the MLL-ENL-ER protein, but not dimerized MLL, also induces dimethylation of histone H3 at Lys[79], suggesting alternative mechanisms for transcriptional activation. (Cancer Res 2005; 65(24): 11367-74)

Introduction

Elevated *Hox A9* expression occurs in many human myeloid leukemias (1), and it is likely that this is important for leukemogenesis. *Hox a9* expression promotes stem cell expansion (2). Furthermore, overexpression of *Hox a7* or *Hox a9* along with the Hox cofactor *Meis1* cofactor is common in leukemias in BXH2 mice (3–5). By itself, Hox a9 is only weakly oncogenic in transplantation models; however, cotransduction of bone with *Meis1* dramatically shortens the latency period and results in leukemia in 100% of mice (6).

Three major *Hox a9* transcripts have been identified, including a long transcript *a9a* with an upstream start site (7) and two alternatively spliced downstream transcripts *a9b* and *a9T* (8). Both the *Hox a9a* and *Hox a9T* isoforms seem important because they are conserved between human, rat, mouse, chicken, and *Xenopus* (9). The *a9T* transcript is of particular interest because it produces a truncated protein that lacks the *Hox a9* homeodomain (8, 9). Because the truncated protein is unlikely to bind DNA, one possibility is that it regulates the activity of full-length Hox a9 protein as a dominant-negative inhibitor. To date, the relative levels of expression of all three of these transcripts and, particularly, their significance in leukemia have not been examined.

High level *Hox A9* expression is consistently seen in leukemias with rearrangements of the mixed lineage leukemia gene *MLL* located on chromosome 11q23 (10–12). The most common *MLL* rearrangements are balanced translocations that fuse the amino portion of *MLL* in frame with up to 50 different potential fusion partners, creating novel fusion proteins (13). MLL is homologous to the *Drosophila* protein Trithorax in a central zinc finger–containing domain as well as in a COOH-terminal SET domain that is required for the activation of *Hox* genes during both hematopoiesis and embryogenesis (14–17). Regulation of *Hox* genes by wild-type MLL involves both SET domain–mediated histone H3 Lys[4] methylation (17, 18) as well as recruitment of histone acetyltransferases, such as CBP (19) and MOF (20). The most common translocation partners (e.g., MLL-ENL, MLL-AF9, and MLL-AF4) are nuclear proteins that function as transcriptional activators (13). A second class of translocation partners, many of which are cytoplasmic proteins, dimerizes the truncated MLL (21, 22). Both classes of fusion proteins lack both the MLL SET methyltransferase as well as the CBP and MOF interaction domain yet up-regulate *Hox a9* and *Meis1* expression, leaving their mechanism of transcriptional activation obscure.

Our goal in these experiments was to provide information on MLL fusion protein function that clarifies their role in *Hox* gene deregulation. We first used isoform-specific quantitative PCR to define which of the three known *Hox a9* isoforms are regulated by MLL fusion proteins. We then examined where MLL fusion proteins bind within the *Hox a9* locus to exert their effect. We specifically determined whether this pattern varies between different types of MLL fusion proteins, MLL-ENL, an MLL fusion protein involving a nuclear translocation partner, and MLL-FKBP, a dimerized MLL fusion protein, to help assess if their mechanism of action differs. A pivotal question we address here is what the relationship is between MLL fusion proteins and wild-type MLL. Specifically, we determined whether MLL fusion proteins displace MLL so that it is not localized to target genes. A final key question we sought to clarify is what the downstream effectors are of MLL fusion protein activity. These are likely covalent modifications of histone tails implicated in both transcriptional activation and repression (23). Here, we present comprehensive data on how the "histone code" is altered by MLL fusion proteins and compare this with modifications known to be

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Jay L. Hess, Department of Pathology, University of Michigan Medical School, 1301 Catherine Road, M5240 Medical Science 1, Ann Arbor, MI 48109-0602. Phone: 734-763-6384; Fax: 734-763-4782; E-mail: jayhess@umich.edu.

doi:10.1158/0008-5472.CAN-05-1041

regulated by wild-type MLL. In aggregate, our data suggest a potential role for wild-type MLL and a range of histone modifications, including Lys^{79} methylation in MLL fusion protein–mediated *Hox* gene deregulation.

Materials and Methods

Cell lines. MLL-AF9 cell lines were established and grown in media containing interleukin-3 (IL-3) and stem cell factor as previously described (21). MLL-FKBP cells were grown in similar media either with or without 50 nmol/L AP20187 dimerizer (Ariad Pharmaceuticals, Cambridge, MA). MLL-ENL-ER cells were grown in media containing IL-3, IL-6, granulocyte macrophage colony-stimulating factor (GM-CSF), stem cell factor, and 100 nmol/L 4-OHT as previously described (24).

Chromatin immunoprecipitation. Chromatin immunoprecipitation was done as described, with one modification for mouse antibodies. Mouse antibodies were incubated overnight, incubated with 2 μg anti-mouse IgG for 7 hours, and then incubated with agarose A for 4 hours, all at 4°C. Chromatin immunoprecipitation was quantified relative to inputs using Taqman Real-time PCR (Applied Biosystems, Foster City, CA) as previously described (21). Taqman primer and probe sequence are provided upon request.

RNA expression analysis. Real-time PCR quantitation of gene expression was done as previously described (17). *GAPDH* and *β-actin* were both used as internal reference standards. Taqman probe and primer sequences for gene expression are available upon request.

Antibodies. Anti-MLL^C was a generous gift of Drs. James J-D. Hsieh (School of Medicine, Washington University, St. Louis, MO) and Stanley Korsmeyer (Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, MA). Anti–estrogen receptor (ER) antibodies Ab10 (TE111.5D11) and Ab3 (AER308) were from Lab Vision/Neomarkers (Fremont, CA). As a negative control Ab1 (AER314), which, unlike Ab10 and Ab3, recognizes endogenous ER but not the ER in the fusion protein, was used. Antibodies for histone modifications included histone H3 trimethyl Lys^4 from Abcam (Cambridge, MA), histone H3 trimethyl Lys^4, dimethyl Lys^{79}, acetyl Lys^9, acetyl Lys^{14}, acetyl Lys^{27}, histone H4 acetyl Lys^5, acetyl Lys^8, acetyl Lys^{12}, acetyl Lys^{16}, and polyacetylated histone H3 and H4, all from Upstate Biotechnology (Charlottesville, VA). The Abcam and Upstate Biotechnology antibodies for histone H3 Lys^4 methylation gave similar results; the Abcam antibodies were used for all the figures in all the different cell systems. Anti-mouse IgG was purchased from Upstate Biotechnology. Antibodies to caspase-3 and cleaved caspase-3 were from Cell Signaling Technology (Beverly, MA).

Results

MLL-ENL-ER and MLL-FKBP regulate expression of multiple Hox a9 transcripts in addition to *Hox a7* and *Meis1*. Previously, we showed that withdrawal of 4-OHT inactivates the MLL-ENL-ER fusion protein causing down-regulation of target gene expression by 72 hours and terminal differentiation by 10 days (24). Multiple genes are down-regulated in the absence of 4-OHT (24), but *Hox a9* and *Meis1* are of central importance because their cooperative expression is sufficient to maintain transformation in the absence of an active MLL-ENL-ER protein (24). Therefore, we focused our analysis on these two target genes and specifically examined whether the major *Hox a9* isoforms are differentially regulated by the MLL fusion proteins.

We used Taqman probes and Real-time PCR to quantify the expression of these specific *Hox a9* transcripts (see Fig. 1*B*) as well as *Meis1* and *Hox a7* in MLL-ENL-ER–transformed cells after 4-OHT withdrawal. At 12 and 24 hours after 4-OHT withdrawal, there is no change in growth rate of the cells (Fig. 1*A*) and only a slight reduction in expression of *Meis1, Hox a7*, and *Hox a9* transcripts (Fig. 1*C*). By 60 hours after 4-OHT withdrawal, there was a noticeable reduction of cell number among −4-OHT cells (Fig. 1*A*), and the expression of *Meis1, Hox a7*, and all three *Hox a9* (*Hox a9a, Hox a9b*, and *Hox a9T*) transcripts were reduced to between 20% and 60% of the expression seen in +4-OHT cells (Fig. 1*C, gray* versus *black columns*). Expression of *Hox a1*, which is not normally a regulatory target of wild-type MLL (17), is only slightly reduced (Fig. 1*C*). Interestingly, although *Hox c8* is a target of MLL regulation in mouse embryonic fibroblast cells (17), it is not expressed in MLL-ENL-ER–transformed cells (Fig. 1*C*). *Hox c8* is also not expressed in any myeloid lineages examined, including hematopoietic stem cells (data not shown), suggesting that MLL-ENL-ER acts by enhancing expression of genes that are already transcriptionally active and cannot activate silent loci.

Dimerization of the MLL-FKBP protein was similarly required for *Hox* gene regulation in this time period. When cultured in the absence of dimerizer for 72 hours, cell growth was slowed (ref. 21; data not shown) and expression of *Meis1, Hox a7*, and the *Hox a9b* and *Hox a9T* transcripts was reduced by levels similar to that seen in MLL-ENL-ER cells (Fig. 1*D*; data not shown). Expression of *Hox a1* is only slightly reduced, whereas *Hox c8* is not expressed in these cells (Fig. 1*C* and *D*). Taken together, these data show MLL-ENL-ER and MLL-FKBP proteins regulate multiple *Hox a9* isoforms to a similar degree in addition to other targets important for leukemogenesis.

MLL fusion proteins involving nuclear and dimerizing translocation partners have similar binding patterns as MLL at the *Hox a9* and *Meis1* loci. Chromatin immunoprecipitation with quantitative PCR detection was done to determine where MLL fusion proteins bound across the *Hox a9* locus. Chromatin immunoprecipitations were quantified using Taqman probes and Real-time PCR. Signal was normalized relative to total input chromatin for each sample, as previously described (25), permitting quantitative comparisons of binding intensity with a given antibody at different locations and in different cell types. For all Taqman probe and primer sets used, no antibody chromatin immunoprecipitation controls were quantified to determine what constituted background signal. For every primer/probe set, the signal from no antibody chromatin immunoprecipitation control experiments consistently fell in the range of 0 to 0.1 relative units, with the signal usually below 0.05 (Fig. 2*B*; data not shown). Therefore, signals below 0.1 are considered within the range of background and thus insignificant.

To determine the MLL-ENL-ER binding pattern at target genes, we did chromatin immunoprecipitations with an antibody (Ab10, see Materials and Methods) that recognizes the mutant ER receptor fused to the MLL-ENL-ER protein. The resulting chromatin immunoprecipitation experiments were quantified using probes 1 to 11 across the *Hox a9* locus (see Fig. 1*B*) or probes specific for the *Meis1, Hox a1*, or *Hox c8* genes (Fig. 2*A*). Another antibody (Ab1, see Materials and Methods), which recognizes the endogenous ER but not the mutant ER in the MLL-ENL-ER protein, was used in parallel chromatin immunoprecipitation experiments as a control. Chromatin immunoprecipitation with Ab10 showed that in the presence of 4-OHT, MLL-ENL-ER bound across the entire *Hox a9* locus (Fig. 2*C, black line*) with two peaks of increased binding in the upstream noncoding (between 1 and 2 kb) and downstream (between 5 and 7 kb) coding regions (Fig. 2*C, black line*). MLL-ENL-ER also showed strong binding to *Meis1* (Fig. 2*D, black column*), but no binding was seen at *Hox a1* or at the silent *Hox c8* locus (Fig. 2*D*). In the absence of 4-OHT, MLL-ENL-ER binding was abolished at *Hox a9* (Fig. 2*C, white line*)

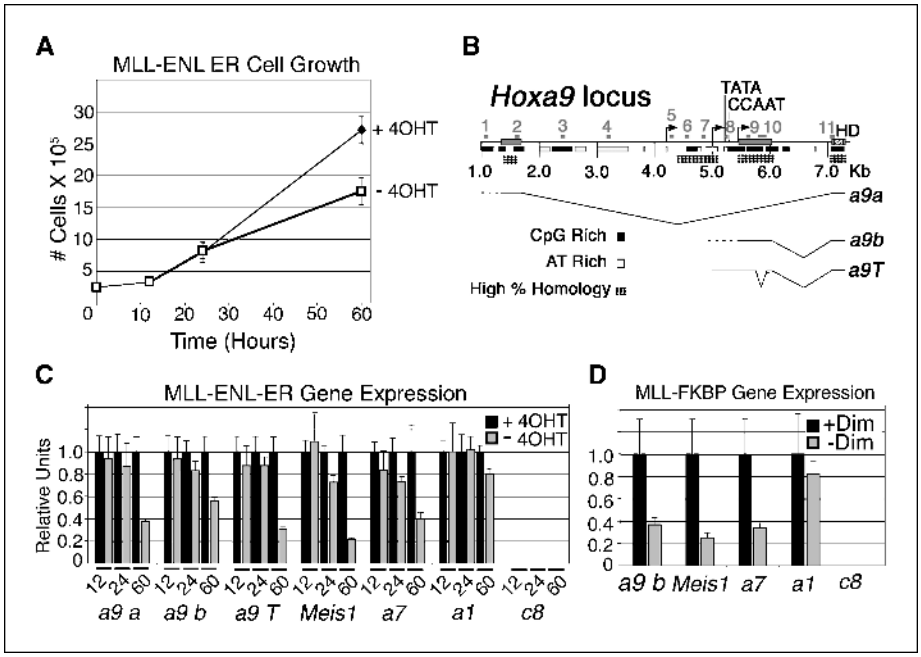

Figure 1. Inactivation of the MLL-ENL-ER and MLL-FKBP conditional oncoproteins reduces target gene expression. *A,* 4-OHT-dependent growth of MLL-ENL-ER cells. By 60 hours after 4-OHT withdrawal, cell numbers as assessed by trypan blue exclusion are reduced in the absence of 4-OHT (−, *white box*) compared with the presence of 4-OHT (+, *black diamond*). *Points,* average of three independent experiments. *B,* schematic of a 7-kb genomic region spanning the *Hox a9* locus. *Gray boxes,* exons. *Box with dashed lines,* homeodomain (*HD*); *arrows,* position of known transcription start sites. A previously identified MLL binding site (18) is marked as a putative TATA box. Distances in kilobases are marked below the line as well as the position of CpG rich (*black boxes*) and AT rich (*white boxes*) regions. *Dotted boxes,* regions of >80% sequence conservation between mouse and human. Three major transcripts from the *Hox a9* locus: an upstream transcript (*a9a*) and two downstream transcripts (*a9b* and *a9T*). The *a9T* transcript codes for a protein with no homeodomain. The positions of 11 different Taqman primer and probe sets (*gray numbers/boxes 1-11 above the line*) used for Real-time PCR quantification of the chromatin immunoprecipitation experiments. *C* and *D,* maintenance of expression of specific *Hox* gene transcripts is dependent on the inducible MLL-ENL-ER protein or on inducible dimerization of the MLL-FKBP protein. Taqman probes and Real-time PCR techniques were used to quantify expression of specific transcripts. Calculated values in cells with induction (+) are arbitrarily set to 1, and calculated values for noninduced (−) cells are presented as a reduction relative to the appropriate + control. *C,* expression of the *Hox* gene transcripts indicated was compared in MLL-ENL-ER cells grown with 4-OHT (+, *black columns*) compared with those grown in the absence of 4-OHT (−, *gray columns*) for 12, 24, or 60 hours. By 60 hours, in −4-OHT cells expression of all three *Hox a9* transcripts (*a9a, a9b*, and *a9T*) as well as *Meis1* and *Hox a7* transcripts is reduced to between 20% and 50% of the expression seen in +4-OHT cells. Expression of *Hox a1*, a gene that is not a target of MLL regulation, is only slightly reduced at 60 hours after 4-OHT withdrawal. *Hox c8* is not expressed in these cells. *Columns,* average of three independent experiments. *D,* similar to what was seen with MLL-ENL-ER cells, withdrawal of dimerizer from MLL-FKBP transform cells for 72 hours (− *Dim, gray columns*) reduces expression of *Hox a9b, Meis1*, and *Hox a7* to between 20% and 40% of that seen in cells grown in the presence of dimerizer (+ *Dim, black columns*) for the same time period. *Columns,* average of two independent experiments.

and at *Meis1* (Fig. 2*D, white column*). In the presence or absence of 4-OHT, chromatin immunoprecipitation with Ab1 produced only background signal across the *Hox a9* locus or at *Meis1* (Fig. 2*B*; data not shown), indicating that endogenous ER does not bind to *Hox* genes under these conditions and that Ab10 is specific for the MLL-ENL-ER fusion protein.

MLL-FKBP has a very similar binding pattern as MLL-ENL-ER at *Hox* genes. Using an antibody to the hemagglutinin tag on the MLL-FKBP protein, chromatin immunoprecipitation experiments reveal that MLL-FKBP binds with similar peaks in the upstream and downstream coding regions of *Hox a9* (Fig. 2*E*) and also to the coding region of *Meis1* (Fig. 2*F*). MLL-FKBP binds weakly to the *Hox a9* locus and at *Meis1* in monomeric form (Fig. 2*E* and *F*, *white line* and *white column*), but binding is greatly enhanced by induction of MLL-FKBP dimerization (Fig. 2*E* and *F, black line* and *black column*). Similar to MLL-ENL-ER, MLL-FKBP does not bind to *Hox a1* or *Hox c8* (Fig. 2*F*).

We then examined how wild-type MLL binding is affected by either fusion protein using chromatin immunoprecipitation with an antibody specific for MLL^C. In both MLL-ENL-ER and MLL-FKBP cells in the presence of either 4-OHT or dimerizer, respectively, Mll binds across the *Hox a9* locus (Fig. 2*G* and *I, black line*) and to the *Meis1* coding region (Fig. 2*H* and *J*, *black column*) with a distribution similar to the fusion proteins. Also similar to the fusion proteins, no Mll binding was detected at *Hox a1* or *Hox c8* (Fig. 2*H* and *J*). Interestingly, in the presence of either MLL-ENL-ER or MLL-FKBP, Mll binding is 5- to 15-fold higher compared with binding in the absence of either fusion protein (Fig. 2*G-J, white*). In general, binding of Mll in the absence of either fusion protein is in the range of background (<0.05) and is considered negligible. Although binding of Mll to target genes is not as high as that seen in $Mll^{+/+}$ MEF cells (20, 26), the signal is still well in the range of significance. This suggests that rather than disrupting the ability of wild-type Mll to bind to target genes, MLL fusion protein activity results in increased Mll at binding target genes. This may reflect direct recruitment by MLL fusion proteins or an indirect mechanism involving preferential association of Mll to transcriptionally active loci (26).

MLL fusion proteins induce multiple histone modifications at target loci. We next used chromatin immunoprecipitation to identify histone modifications altered by the presence or absence of MLL fusion proteins at the *Hox* loci. MLL-ENL-ER cells grown without 4-OHT for 24 hours showed a slight reduction in target gene expression (decreased to about 80%; Fig. 1*C*). Chromatin immunoprecipitation analysis in these cells revealed only slight changes in histone modifications (data not shown). The largest

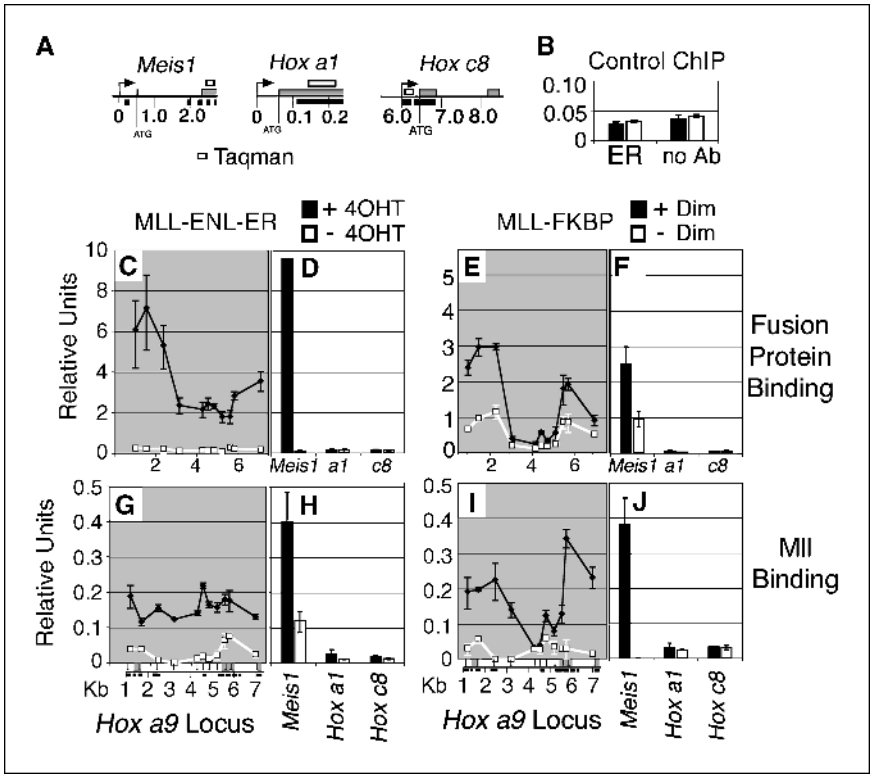

Figure 2. MLL-ENL-ER, MLL-FKBP and MII all bind directly to a large overlapping domain of the *Hox a9* locus. *A,* positions of Taqman primer probe sets (*white boxes*) at the *Meis1, Hox a1*, and *Hox c8* loci used for quantification of the chromatin immunoprecipitation experiments. *Arrows,* positions of transcription start sites; *gray boxes,* exons; *black boxes,* CpG-rich regions. Distances are marked in kilobases below each schematic. *B,* endogenous ER does not bind to the *Hox a9* locus. A control chromatin immunoprecipitate with an antibody (ER) that immunoprecipitates endogenous estrogen receptor (Ab1, reference; see Materials and Methods) but not the mutant receptor on MLL-ENL-ER produces the same background signal as a no-antibody (*no Ab*) control. This indicates that the signal generated with the Ab10 anti-ER antibody is specific to the MLL-ENL-ER protein (see below). Signal was quantified using all 11 probes (see Fig. 1*B*) at the *Hox a9* locus. Only the results for probe 2 are shown. *C-J,* MLL-ENL-ER, MLL-FKBP, and MII all have increased binding in the presence (+, *black*) of 4-OHT or dimerizer relative to the absence (−, *white*) of 4-OHT or dimerizer. Chromatin immunoprecipitation experiments were all quantified using the Taqman primer/probes sets as in (*A*) and across 7 kb of the *Hox a9* locus (see Fig. 1*B* and the schematics at the bottom of *G* and *I*). Distances across *Hox a9* are in kilobase. Exons at *Hox a9* are between 1 and 2 kb, 5 and 6 kb, and at 7 kb. *Points,* average of six Real-time PCR reactions. Two other independent chromatin immunoprecipitation experiments gave results similar to those shown. *C* and *D,* in the presence (+, *black*) but not in the absence (−, *white*) of 4-OHT, MLL-ENL-ER binds across *Hox a9* and at *Meis1* but not at the nontarget *Hox a1* or the silent *Hox c8* locus. Chromatin immunoprecipitation was done using an antibody (Ab10, reference) that recognizes the mutant receptor on MLL-ENL-ER. *E* and *F,* MLL-FKBP binds across *Hox a9* and at *Meis1* in the presence (+, *black*) of dimerizer (*Dim*) but only weakly in its absence (−, *white*). No binding is seen at the nontarget *Hox a1* or the silent *Hox c8* locus. Chromatin immunoprecipitation was done with an anti-hemagglutinin antibody to the hemagglutinin tag on the MLL-FKBP protein as in Martin et al. (21). *G* and *H,* in MLL-ENL-ER cells, in the presence (+, *black*) but not in the absence (−, *white*) of 4-OHT, MII binds across *Hox a9* and at *Meis1* but not at the nontarget *Hox a1* or the silent *Hox c8* locus. *I* and *J,* in MLL-FKBP cells, in the presence (+, *black*) but not in the absence (−, *white*) of dimerizer, MII binds across *Hox a9* and at *Meis1* but not at the nontarget *Hox a1* or the silent *Hox c8* locus. Chromatin immunoprecipitation was done using an antibody to MLL^C, which does not recognize MLL fusion proteins.

change in gene expression occurred at 60 hours; therefore, histone modifications were analyzed in detail at this time point with and without the MLL-ENL-ER fusion protein. We also analyzed changes in histone modifications upon MLL-FKBP dimerization to determine whether this type of fusion protein has similar or distinct effects on histone modifications.

At the *Hox a9* locus, global histone H3 acetylation (AcH3) is decreased roughly 2- to 5-fold in the absence of 4-OHT (Fig. 3*A, black* versus *white line*) and 2-fold in the absence of dimerizer (Fig. 3*B, black* versus *white line*) in MLL-ENL-ER and MLL-FKBP cells, respectively. Similar results were seen for global histone H4 acetylation (AcH4) in MLL-ENL-ER cells in the absence of 4-OHT (Fig. 3*C, black* versus *white line*), although there was no change in acetylation in the downstream coding region of *Hox a9*. Interestingly, withdrawal of dimerizer had almost no effect on global histone H4 acetylation in MLL-FKBP cells (Fig. 3*D, black* versus *white line*). These results indicate that both fusion proteins have an effect on H3 acetylation, but only MLL-ENL-ER has an effect on H4 acetylation.

Histone H3 Lys^4 trimethylation has been reported to be associated primarily with the promoter regions of actively expressed genes (27–29). In keeping with another recent study of *Hox* loci (29), we found histone H3 Lys^4 trimethylation extended across the *Hox a9* locus in both MLL-ENL-ER cells and in MLL-FKBP cells (Fig. 4*A* and *C*) with levels of Lys^4 trimethylation in the upstream (between 1 and 3 kb) and downstream (between 5 and 7 kb) coding regions that closely correlated with peaks of MLL-ENL-ER and MLL-FKBP binding (Fig. 4*A* and *C* compared with Fig. 2*C* and *E*). Although *Hox a9* expression is reduced by the absence of 4-OHT or dimerizer in MLL-ENL-ER or MLL-FKBP cells, respectively, (Fig. 1), we were unable to detect a parallel reduction in Lys^4 trimethylation (Fig. 4*A* and *C, black* versus *white lines*) at the *Hox a9* locus. However, we detected a small (about 2-fold) reduction in Lys^4 trimethylation at the *Meis1* locus in both MLL-ENL-ER and MLL-FKBP cells in the absence of 4-OHT or dimerizer (Fig. 4*B* and *D, black* versus *white columns*). No significant Lys^4 trimethylation is detected at the silent *Hox c8* locus (Fig. 4*B* and *D*) and no change is seen at the *Hox a1* locus (Fig. 4*B* and *D*).

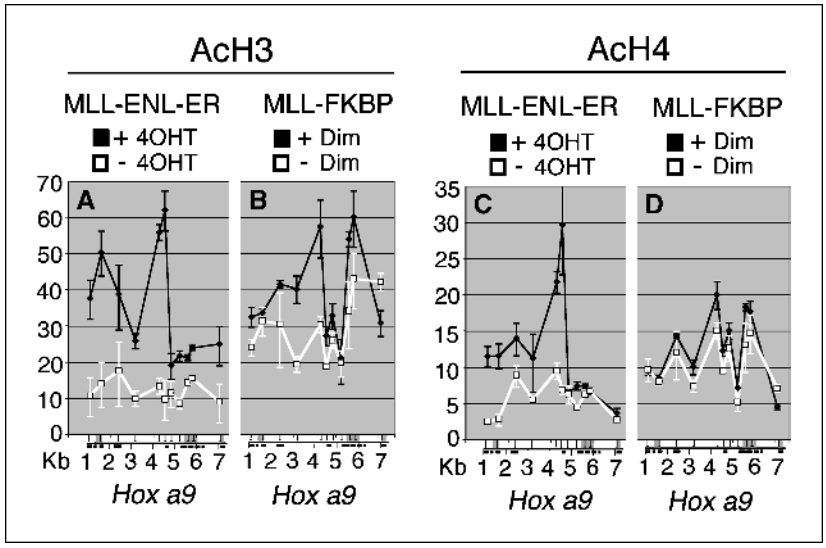

Figure 3. MLL-ENL-ER and MLL-FKBP fusion proteins are associated with an increase of acetylation at the *Hox a9* locus. *A,* acetylation of histone H3 (AcH3) is increased across *Hox a9* in the presence (+, *black*) relative to the absence (−, *white*) of MLL-ENL-ER. *B,* similarly, acetylation of histone H3 (AcH3) is increased across *Hox a9* in the presence (+, *black*) relative to the absence (−, *white*) of MLL-FKBP. *C,* acetylation of histone H4 (AcH4) is increased in the upstream region of *Hox a9* in the presence (+, *black*) relative to the absence (−, *white*) of MLL-ENL-ER. *D,* acetylation of histone H4 (AcH4) across *Hox a9* is not significantly affected by the MLL-FKBP protein (*black* versus *white*).

Histone H3 Lys^{79} methylation is associated with activation of genetic loci (30, 31). Histone H3 Lys^{79} methylation closely paralleled the pattern of Lys^{4} trimethylation and fusion protein binding across the entire *Hox a9* locus (Fig. 4*E* and *G*). Importantly, in the presence of 4-OHT, there is a large (5- to 7-fold) difference in Lys^{79} methylation at *Hox a9* in MLL-ENL-ER cells compared with MLL-FKBP cells (Fig. 4*E* and *F, black* versus *G* and *H*). Withdrawal of 4-OHT causes a large reduction of Lys^{79} methylation in MLL-ENL-ER cells down to the levels observed in MLL-FKBP cells (Fig. 4*E* and *F, white* versus *G* and *H*). Although withdrawal of dimerizer in MLL-FKBP cells has a strong effect on both gene expression (Fig. 1*D*) and MLL-FKBP binding (Fig. 2*E* and *F*), dimerization has minimal effects on Lys^{79} methylation at *Hox a9* (Fig. 4*G, black* versus *white line*) and only a minor (about 2-fold) effect at *Meis1* (Fig. 4*H, black* versus *white column*). Lys^{79} methylation levels are equivalently low at *Hox a1* in both MLL-ENL-ER cells and in MLL-FKBP cells (Fig. 4*F* and *H*), and there is no Lys^{79} detected at *Hox c8* (Fig. 4*F* and *H*). Another cell line transformed by the MLL-AF9 fusion protein also shows H3 Lys^{79} dimethylation levels at *Hox a9* equivalent to those seen in MLL-ENL-ER cells (Supplementary Fig. S1), whereas a population of neutrophils from mouse bone marrow does not (Supplementary Fig. S1). This suggests that the MLL-AF9 fusion protein has a similar effect on H3 Lys^{79} dimethylation. Taken together, these results suggest that H3 Lys^{79} dimethylation is important for transcriptional activation via MLL-ENL-ER and probably by MLL-AF9, given the high homology between ENL and AF9. In contrast, dimerized MLL-FKBP activates transcription by a mechanism that apparently does not involve increases in Lys^{79} methylation.

Our analysis of histone modifications following withdrawal of 4-OHT or dimerizer is likely to detect only histone modifications with relatively rapid turnover. In an effort to measure more proximate effects of MLL-ENL-ER on histone modifications, we created a "ground state" of low gene activity where most active histone marks had been erased by prolonged culture in the absence of 4-OHT. We then tested if adding back MLL-ENL-ER fusion protein activity at this point would increase transcription and, if so, what histone modifications are affected. Cells cultured without 4-OHT (and without GM-CSF) for 12 days ceased proliferating (13% of cells in S phase compared with 36% at day 2) but remained

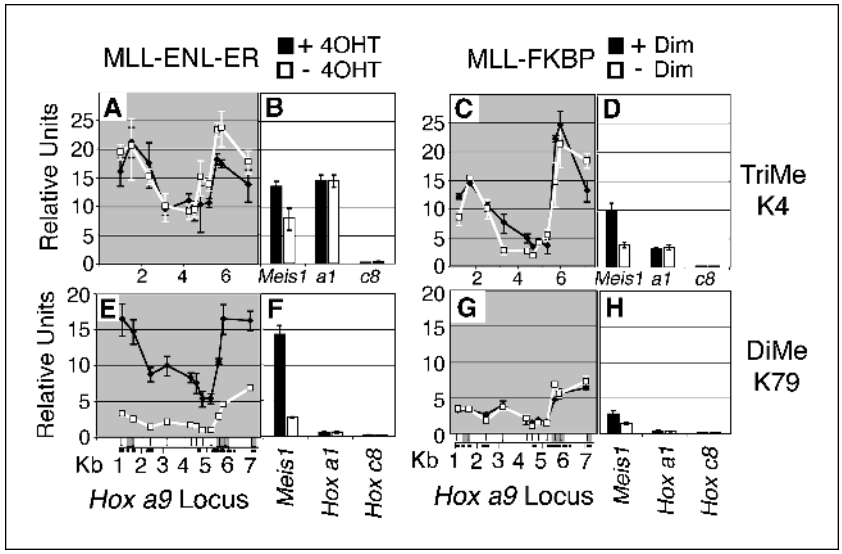

Figure 4. The MLL-ENL-ER fusion protein specifically increases histone H3 Lys^{79} dimethylation (*DiMeK79*). *A-D,* histone H3 trimethylation of Lys^{4} (*TriMeK4*) is unaltered at *Hox a9* and *Hox a1*, but a 2-fold reduction of TriMeK4 is observed at *Meis1* in both MLL-ENL-ER cells (*B*) and in MLL-FKBP cells (*D*) in the absence (−, *white*) compared with the presence (+, *black*) of 4-OHT or dimerizer, respectively. No significant TriMeK4 is seen at the silent *Hox c8* locus. *E* and *F,* histone H3 dimethylation of Lys^{79} (*DiMeK79*) is drastically reduced at *Hox a9* and at *Meis1* in MLL-ENL-ER cells in the absence (*white*) of 4-OHT compared with the presence (*black*). No significant DiMeK79 is seen at *Hox c8*, and only very low levels (about 0.5 unit) are seen at *Hox a1. G* and *H,* no change in DiMeK79 is seen in MLL-FKBP cells except for a slight (about 2-fold) reduction at *Meis1* in the absence of dimerizer (*white column, H*). Because signal strength depends on the kinetics of antibody-epitope recognition, it is not possible to compare absolute amounts of two different proteins in chromatin immunoprecipitations done with different antibodies. For example, it is not possible to determine whether or not there are higher levels of H3 acetylation versus H4 acetylation.

viable and nonapoptotic as evidenced by propidium iodide cell cycle analysis (data not shown) and lack of caspase-3 cleavage (Supplementary Fig. S2). Cytospin preparations of these cells showed a heterogeneous mixture of myeloblasts, maturing myeloid cells, and macrophages (data not shown). Cells deprived of 4-OHT showed target gene expression between 18% and 60% relative to T60 + 4-OHT cells from Fig. 1*C* (Fig. 5*A, white columns*). Cells supplanted with 4-OHT for 48 hours showed up-regulation of target gene expression including *Hox a7, Hox a9b, Hox a9T*, and *Meis1* (Fig. 5*A, black columns*). This increase in transcription was paralleled by an increase in binding of MLL-ENL-ER to both the *Hox a9* and *Meis1* loci in the presence of 4-OHT (Fig. 5*B*, *black line* and *column*).

Chromatin immunoprecipitation showed histone H3 Lys^4 trimethylation remaining at the levels seen in previous experiments (Fig. 4*A-D*; data not shown). Conversely, H3 Lys^{79} dimethylation was drastically reduced at *Hox a9* and *Meis1* in cells grown without 4-OHT (Fig. 5*C, white line* and *white box*). Importantly, treatment of these MLL-ENL-ER cells with 4-OHT for 48 hours increases H3 Lys^{79} dimethylation ~3-fold (Fig. 5*C, black line* and *black box*), showing that MLL-ENL-ER binding is highly associated with increases in H3 Lys^{79} dimethylation.

Chromatin immunoprecipitation experiments also revealed increased histone acetylation across the *Hox a9* locus after a 48-hour treatment with 4-OHT (data not shown). Chromatin immunoprecipitation with antibodies recognizing specific acetylated residues were done to determine if specific lysines were preferentially acetylated by fusion protein activation. Calculated values are expressed as a fold difference to emphasize the differences seen (Fig. 5*D*). Only the results for probe 2 from the upstream exon of *Hox a9* (Fig. 1*B*) are shown (Fig. 5*D*). In the presence of the MLL-ENL-ER fusion protein, histone H3 at Lys^9 is increased 3-fold (Fig. 5*D, black column*; K9). Acetylation of histone H4 Lys^5, Lys^8, and Lys^{12} increased ~1.8-fold (Fig. 4*D, black columns*; K5, K8, and K12). No effect was seen for histone H3 Lys^{14} or Lys^{27} acetylation or for H4 Lys^{16} acetylation. Lys^4 trimethylation also showed no significant change (Fig. 5*D*, K4). In contrast, histone H3 Lys^{79} dimethylation showed a 3-fold increase (Fig. 5*D*, K79). Taken together, these results show that binding of MLL-ENL-ER up-regulates target gene expression in association with acetylation of specific lysines on histone H3 as well as methylation of histone H3 Lys^{79}.

Discussion

The large number of different translocations involving MLL has made it challenging to define how this diverse group deregulates *Hox* gene expression. The division of Mll fusion partners into two broad groups, transcriptional activators and dimerizers, suggests that there are at least two different mechanisms for MLL fusion protein–mediated activation of *Hox a9* and *Meis1*. In the experiments outlined here, we sought to define the genomic site of action and histone modifications affected by the two groups of fusion partners as represented by the MLL-ENL and MLL-FKBP fusion proteins and how these compare with wild-type MLL.

Our experiments show that both *Hox a9b*, which encodes full-length Hox a9, and *Hox a9T*, which encodes a form of Hox a9 lacking a homeodomain, are up-regulated by MLL fusion proteins. Because Hox a9T lacks the homeodomain, it might be expected to be a dominant-negative inhibitor of the Hox a9 function, but

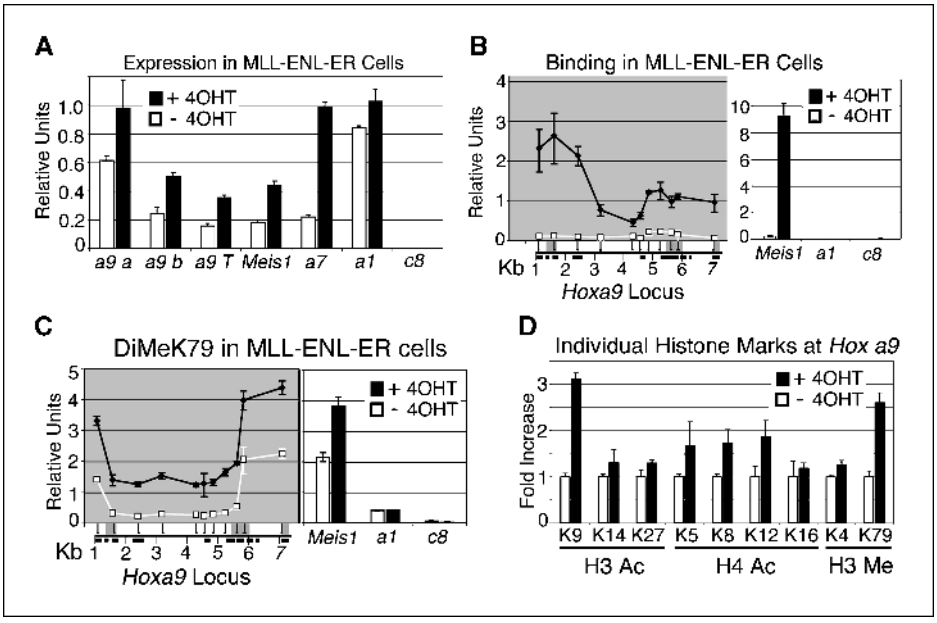

Figure 5. Reactivation of MLL-ENL-ER results in an upregulation of *Hox* gene expression and an increase in histone H3 Lys^{79} dimethylation and Lys^9 acetylation. *A,* expression of all three *Hox a9* transcripts (*a9a, a9b*, and *a9T*) and *Meis1* and *Hox a7* are increased by adding back 4-OHT (+, *black*) to cells grown for 12 days in the absence of 4-OHT (–, *white*). 4-OHT was added back for a 48-hour period. Only a slight change is seen in *Hox a1* expression, and *Hox c8* is not expressed. Gene expression is quantified relative to expression in +4-OHT cells at 60 hours from Fig. 1*C*. *B,* binding of MLL-ENL-ER in these cells is increased at *Hox a9* and *Meis1* in the presence (+, *black*) but not in the absence (–, *white*) of 4-OHT. *C,* histone H3 dimethylation of Lys^{79} (*DiMeK79*) is increased 2- to 3-fold across *Hox a9* and at *Meis1* in cells that have 4-OHT added back (+, *black*) compared with those grown in the absence of 4-OHT (–, *white*). No change is seen at *Hox a1*. *D,* chromatin immunoprecipitation for individual lysine modifications shows a 3-fold increase in acetylation of histone H3 at Lys^9 (*K9*) in the presence (+, *black*) compared with the absence (–, *white*) of 4-OHT. A small increase (~1.5-fold) of histone H4 Lys^5 (*K5*), Lys^8 (*K8*), and Lys^{12} (*K12*) is also seen. For comparison, a lack of change in histone H3 Lys^4 trimethylation (*K4*) and a 3-fold change in Lys^{79} dimethylation (*K79*). Quantifications were calculated using probe 2 (Fig. 1*B*) from the upstream exon of *Hox a9*. Similar quantification results were found for the entire *Hox a9* locus (data not shown).

additional experiments will be needed to assess the role of this isoform in transformation.

We found the MLL-ENL-ER and dimerized MLL bind across a broad region of the *Hox a9* locus spanning noncoding sequences as well as promoter and 3′ coding regions. This closely corresponds to the localization of RNA polymerase II as well as wild-type MLL (26, 32). Given the homology of MLL to the yeast Set1 proteins, which are associated with progressive RNA polymerase II and other experiments that show defects in polymerase II positioning in $Mll^{-/-}$ cells (26), this raises the possibility that MLL fusion proteins also associate with RNA polymerase II. The finding that the wild-type MLL is still associated with *Hox a9* and *Meis1* in the presence of MLL-ENL and MLL-FKBP (in fact, at higher levels in the presence of the fusion protein) raises the possibility that MLL contributes to transcriptional activation of target loci in these transformed cells. Whether wild-type Mll is directly recruited by MLL fusion proteins or whether it simply binds to transcriptionally active loci remains unclear. Experiments to test the significance of wild-type Mll in transformation are currently under way.

We examined the histone modifications affected by the two types of MLL fusion proteins with the thought that these "footprints" would provide insights into their mechanisms of action. These experiments showed both MLL-ENL or MLL-FKBP association with target loci result in an increase in histone H3 acetylation. However, only MLL-ENL increases histone H4 acetylation. These acetylation increases could be caused by the direct recruitment of HAT complexes by the fusion proteins. For example, the NuA4 complex reportedly interacts with the MLL translocation partner AF9, which is homologous to ENL (33). These data also imply dimerized MLL fusion proteins involve different coactivators than those involving transcriptional activators. Characterization of MLL fusion protein complexes will help determine if recruitment of other HATs is important for fusion protein activity.

Our studies also revealed important effects of MLL fusion proteins on histone methylation. Lys^4 methylation seems one important regulator of transcriptional activation at MLL target genes. Deletion of the MLL SET domain methyltransferase eliminates the ability of MLL to activate *Hox* gene expression (17). In addition, *in vitro* transcription experiments with purified MLL complexes also provide strong evidence that Lys^4 trimethylation plays an important role in transcriptional activation that is synergistic with histone acetylation (20). In our experiments, we found that the *Hox a9* locus and other targets implicated in leukemogenesis were extensively trimethylated at histone H3 Lys^4. This is consistent with many studies implicating histone H3 Lys^4 trimethylation in transcriptional activation, possibly through promoting RNA polymerase II elongation (31, 34, 35). High-level histone H3 Lys^4 methylation was seen both in the presence or absence of fusion proteins. Because of the way the experiments were done, it is possible, in fact likely, that in contrast to acetylation, methylation changes would not be observed over a short time period, as this tends to be a long-lasting modification with low turnover. This does not negate the potential importance of this modification in transformation by MLL fusion proteins and the potential significance of the wild-type MLL protein in the process.

The most dramatic histone modification affected by MLL fusion proteins was histone H3 Lys^{79} methylation. This modification is mediated by Dot1, a non-SET containing histone methyltransferases (36). Lys^{79} methylation closely tracks with Lys^4 methylation (and activation) and seems to be a mark of successful transcription elongation (36). Lys^{79} dimethylation was rapidly reduced in the absence of the MLL-ENL fusion protein and was rapidly increased in association with a bound MLL-ENL protein. Conversely, although MLL-FKBP activates *Hox a9*, it had no effect on Lys^{79} methylation at this locus. This suggests that dimerized MLL fusion proteins may activate transcription through other mechanisms (e.g., primarily through histone acetylation).

Our results both support and challenge the recent finding that the MLL-AF10 fusion protein activates *Hox a9* by recruiting Dot1 and that the methyltransferase activity seems required for transformation (37). The authors of this article suggested that the *Hox* loci are either methylated at Lys^4 in the presence of MLL or methylated at Lys^{79} in the presence of MLL fusion proteins. The actual situation seems more complex. We did find that Lys^{79} methylation is influenced by MLL-ENL and probably also by MLL-AF9 (at least transformation by MLL-AF9 is accompanied by high-level Lys^{79} methylation at *Hox a9*). However, we also found that the fusion proteins influenced histone acetylation. Most importantly, our data show wild-type MLL remains localized, in fact at increased levels, at target loci, and that *Hox a9* and other targets show high, rather than low levels, of histone H3 Lys^4 trimethylation. In aggregate, the data suggest that histone H3 lysine methylation plays a role in some but not all MLL fusion protein transcriptional deregulation. In addition, in contrast to published models for transformation, wild-type MLL and Lys^4 trimethylation also may be involved in MLL fusion protein activity. The finding that the high level expression of unrearranged forms of MLL occur in myelodysplasia and leukemia also suggests a role for MLL in transformation (38). Experiments to assess the dependence of transformation on MLL are in progress.

Acknowledgments

Received 3/28/2005; revised 7/26/2005; accepted 10/4/2005.

Grant support: NIH grant CA78815 and CA92251 and Leukemia and Lymphoma Society of America's Specialized Center of Research.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Golub TR, Slonim DK, Tamayo P, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 1999; 286:531–7.
2. Thorsteinsdottir U, Mamo A, Kroon E, et al. Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. Blood 2002;99:121–9.
3. Nakamura T, Largaespada DA, Shaughnessy JD, Jr., Jenkins NA, Copeland NG. Cooperative activation of Hoxa and Pbx1-related genes in murine myeloid leukaemias. Nat Genet 1996;12:149–53.
4. Moskow JJ, Bullrich F, Huebner K, Daar IO, Buchberg AM. Meis1, a PBX1-related homeobox gene involved in myeloid leukemia in BXH-2 mice. Mol Cell Biol 1995;15: 5434–43.
5. Schnabel CA, Jacobs Y, Cleary ML. Hox A9-mediated immortalization of myeloid progenitors requires functional interactions with TALE cofactors Pbx and Meis. Oncogene 2000;19:608–16.
6. Kroon E, Krosl J, Thorsteinsdottir U, Baban S, Buchberg AM, Sauvageau G. Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b. EMBO J 1998;17:3714–25.
7. Kim MH, Chang HH, Shin C, Cho M, Park D, Park HW. Genomic structure and sequence analysis of human HOXA-9. DNA Cell Biol 1998;17:407–14.
8. Fujimoto S, Araki K, Chisaka O, Araki M, Takagi K, Yamamura K. Analysis of the murine Hoxa-9 cDNA: an alternatively spliced transcript encodes a truncated protein lacking the homeodomain. Gene 1998;209:77–85.

9. Dintilhac A, Bihan R, Guerrier D, Deschamps S, Pellerin I. A conserved non-homeodomain Hoxa9 isoform interacting with CBP is co-expressed with the 'typical' Hoxa9 protein during embryogenesis. Gene Expr Patterns 2004;4:215–22.

10. Yeoh EJ, Ross ME, Shurtleff SA, et al. Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. Cancer Cell 2002;1:133–43.

11. Rozovskaia T, Feinstein E, Mor O, et al. Upregulation of Meis1 and HoxA9 in acute lymphocytic leukemias with the t(4:11) abnormality. Oncogene 2001;20:874–8.

12. Armstrong SA, Staunton JE, Silverman LB, et al. MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. Nat Genet 2002;30:41–7.

13. Hess JL. MLL: a histone methyltransferase disrupted in leukemia. Trends Mol Med 2004;10:500–7.

14. Ernst P, Mabon M, Davidson AJ, Zon LI, Korsmeyer SJ. An Mll-dependent Hox program drives hematopoietic progenitor expansion. Curr Biol 2004;14:2063–9.

15. Ernst P, Fisher JK, Avery W, Wade S, Foy D, Korsmeyer SJ. Definitive hematopoiesis requires the mixed-lineage leukemia gene. Dev Cell 2004;6:437–43.

16. Yu BD, Hess JL, Horning SE, Brown GA, Korsmeyer SJ. Altered Hox expression and segmental identity in Mll-mutant mice. Nature 1995;378:505–8.

17. Milne TA, Briggs SD, Brock HW, et al. MLL targets SET domain methyltransferase activity to Hox gene promoters. Mol Cell 2002;10:1107–17.

18. Nakamura T, Mori T, Tada S, et al. ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. Mol Cell 2002;10:1119–28.

19. Ernst P, Wang J, Huang M, Goodman RH, Korsmeyer SJ. MLL and CREB bind cooperatively to the nuclear coactivator CREB-binding protein. Mol Cell Biol 2001;21:2249–58.

20. Dou Y, Milne T, Tackett AJ, et al. Physical Association and Coordinate Function of the H3 K4 methyltransferase MLL1 and the H4 K16 acetyltransferase of MOF. Cell 2005;121:873–85.

21. Martin ME, Milne TA, Bloyer S, et al. Dimerization of MLL fusion proteins immortalizes hematopoietic cells. Cancer Cell 2003;4:197–207.

22. So CW, Lin M, Ayton PM, Chen EH, Cleary ML. Dimerization contributes to oncogenic activation of MLL chimeras in acute leukemias. Cancer Cell 2003;4:99–110.

23. Jenuwein T, Allis CD. Translating the histone code. Science 2001;293:1074–80.

24. Zeisig BB, Milne T, Garcia-Cuellar MP, et al. Hoxa9 and Meis1 are key targets for MLL-ENL-mediated cellular immortalization. Mol Cell Biol 2004;24:617–28.

25. Frank SR, Schroeder M, Fernandez P, Taubert S, Amati B. Binding of c-Myc to chromatin mediates mitogen-induced acetylation of histone H4 and gene activation. Genes Dev 2001;15:2069–82.

26. Milne TA, Dou Y, Martin ME, Brock HW, Roeder RG, Hess JL. MLL associates specifically with a subset of transcriptionally active target genes. Proc Natl Acad Sci U S A 2005;102:14765–70.

27. Schneider R, Bannister AJ, Myers FA, Thorne AW, Crane-Robinson C, Kouzarides T. Histone H3 lysine 4 methylation patterns in higher eukaryotic genes. Nat Cell Biol 2004;6:73–7.

28. Santos-Rosa H, Schneider R, Bannister AJ, et al. Active genes are tri-methylated at K4 of histone H3. Nature 2002;419:407–11.

29. Bernstein BE, Kamal M, Lindblad-Toh K, et al. Genomic maps and comparative analysis of histone modifications in human and mouse. Cell 2005;120:169–81.

30. Shanower GA, Muller M, Blanton JL, Honti V, Gyurkovics H, Schedl P. Characterization of the grappa gene, the *Drosophila* histone H3 lysine 79 methyltransferase. Genetics 2005;169:173–84.

31. Im H, Park C, Feng Q, et al. Dynamic regulation of histone H3 methylated at lysine 79 within a tissue-specific chromatin domain. J Biol Chem 2003;278:18346–52.

32. Guenther MG, Jenner RG, Chevalier B, et al. Global and Hox-specific roles for the MLL1 methyltransferase. Proc Natl Acad Sci U S A 2005;102:8603–8.

33. Doyon Y, Selleck W, Lane WS, Tan S, Cote J. Structural and functional conservation of the NuA4 histone acetyltransferase complex from yeast to humans. Mol Cell Biol 2004;24:1884–96.

34. Krogan NJ, Dover J, Wood A, et al. The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. Mol Cell 2003;11:721–9.

35. Zhang K, Siino JS, Jones PR, Yau PM, Bradbury EM. A mass spectrometric "Western blot" to evaluate the correlations between histone methylation and histone acetylation. Proteomics 2004;4:3765–75.

36. Feng Q, Wang H, Ng HH, et al. Methylation of H3-lysine 79 is mediated by a new family of HMTases without a SET domain. Curr Biol 2002;12:1052–8.

37. Okada Y, Feng Q, Lin Y, et al. hDOT1L links histone methylation to leukemogenesis. Cell 2005;121:167–8.

38. Papenhausen PR, Griffin S, Tepperberg J. Oncogene amplification in transforming myelodysplasia. Exp Mol Pathol 2005;79:168–75.