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# MLL Targets SET Domain Methyltransferase Activity to *Hox* Gene Promoters

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## Summary

**MLL, the human homolog of *Drosophila trithorax*, maintains *Hox* gene expression in mammalian embryos and is rearranged in human leukemias resulting in *Hox* gene deregulation. How MLL or MLL fusion proteins regulate gene expression remains obscure. We show that MLL regulates target *Hox* gene expression through direct binding to promoter sequences. We further show that the MLL SET domain is a histone H3 lysine 4-specific methyltransferase whose activity is stimulated with acetylated H3 peptides. This methylase activity is associated with *Hox* gene activation and H3 (Lys4) methylation at *cis*-regulatory sequences in vivo. A leukemogenic MLL fusion protein that activates *Hox* expression had no effect on histone methylation, suggesting a distinct mechanism for gene regulation by MLL and MLL fusion proteins.**

## Introduction

Rearrangements of the mixed lineage leukemia gene *MLL*, the human homolog of the *Drosophila* gene *trithorax*, are associated with aggressive lymphoid and myeloid acute leukemias in both children and adults. MLL and trithorax are members of an evolutionarily conserved family of proteins termed the *trithorax* group (trxG) that are positive regulators of gene expression during development whose activity is opposed by the repressive activity of *Polycomb* group (PcG) genes. In development, the trxG and PcG proteins are not required for initiation of gene activity but maintain transcriptional states through later stages of development (Brock and van Lohuizen, 2001).

In mammals, MLL positively regulates the clustered homeobox (*Hox*) genes, which specify segment identity (Yu et al., 1995). *Hox* gene expression initiates normally in *Mll* knockout mice but is not maintained past embryonic day 9.5, demonstrating the importance of MLL in the maintenance of *Hox* gene expression (Yu et al., 1998). *Hox* genes also play a key role in hematopoietic differentiation, and overexpression of individual *Hox* genes is leukemogenic in mice (Kroon et al., 1998; Magli et al., 1997). This suggests that deregulation of *Hox* gene expression is pivotal for MLL-associated leukemogenesis. Furthermore, *Hox* gene expression is upregulated in human leukemias carrying MLL rearrangements (e.g., *Hox a5*, *a7*, and *a9*; Armstrong et al., 2002; Rozovskaia et al., 2001; Yeoh et al., 2002), and transformation of murine bone marrow by MLL fusion proteins is *Hox* gene dependent (Ayton and Cleary, 2001b).

How MLL regulates *Hox* gene expression is poorly understood. The domain structure of MLL is complex, making it difficult to unravel the key components of MLL function. Domains that may have a role in MLL function include the AT hooks, which bind DNA, a region homologous to DNA methyl transferases (DNMT), the cysteine-rich PHD domain, and a highly conserved SET domain. The SET domain is found in many proteins now demonstrated to mediate lysine-directed histone methylation (Lachner and Jenuwein, 2002; Zhang and Reinberg, 2001; Jenuwein and Allis, 2001). These findings suggest a possible role for MLL in chromatin remodeling mediated by histone methylation. However, early studies of this domain in MLL did not reveal evidence of enzymatic activity, leaving its function enigmatic (Rea et al., 2000). Furthermore, rearrangements of MLL that occur in leukemia consistently delete the PHD and SET domains and replace these sequences with one of over 30 different translocation partners that in general share little sequence homology (Ayton and Cleary, 2001a).

Progress in understanding the mechanistic role of MLL in maintenance and gene regulation has also been slowed by a lack of known target binding sites for mammalian PcG or trxG homologs. To address these issues, we have focused our attention on how MLL regulates transcription of *Hox c8*. This target was chosen because it is tightly regulated by MLL and because it is the only *Hox* gene in which the sequences required for the correct initiation and maintenance of expression have been extensively mapped in vivo (Bradshaw et al., 1996; Shashikant et al., 1995). Our studies show that *Hox c8* is upregulated by MLL, supporting a transcriptional activating role for MLL. MLL binds directly to proximal promoter sequences but not to other regions of the *Hox c8* locus including the 5′ and 3′ enhancer sequences, suggesting that MLL-dependent regulatory elements in mammalian *Hox* genes are organized differently than in *Drosophila*. The *Hox c8* promoter is necessary and sufficient for MLL responsiveness and, along with the 5′ enhancer, exhibits differential histone acetylation and H3 (Lys4) methylation in $Mll^{+/+}$ as compared to $^{-/-}$ cells. Reexpression of MLL in null cells resulted in methylation of H3 (Lys4) at the *Hox c8* 5′ enhancer and promoter as well as at other *Hox* gene promoters. We demonstrate that H3 (Lys4) methylation is dependent on an intact MLL SET domain and that this methyltransferase activity is stimulated by H3 peptides that are acetylated at Lys9 or Lys14. Collectively, our experiments underscore the importance of a concerted series of histone and DNA

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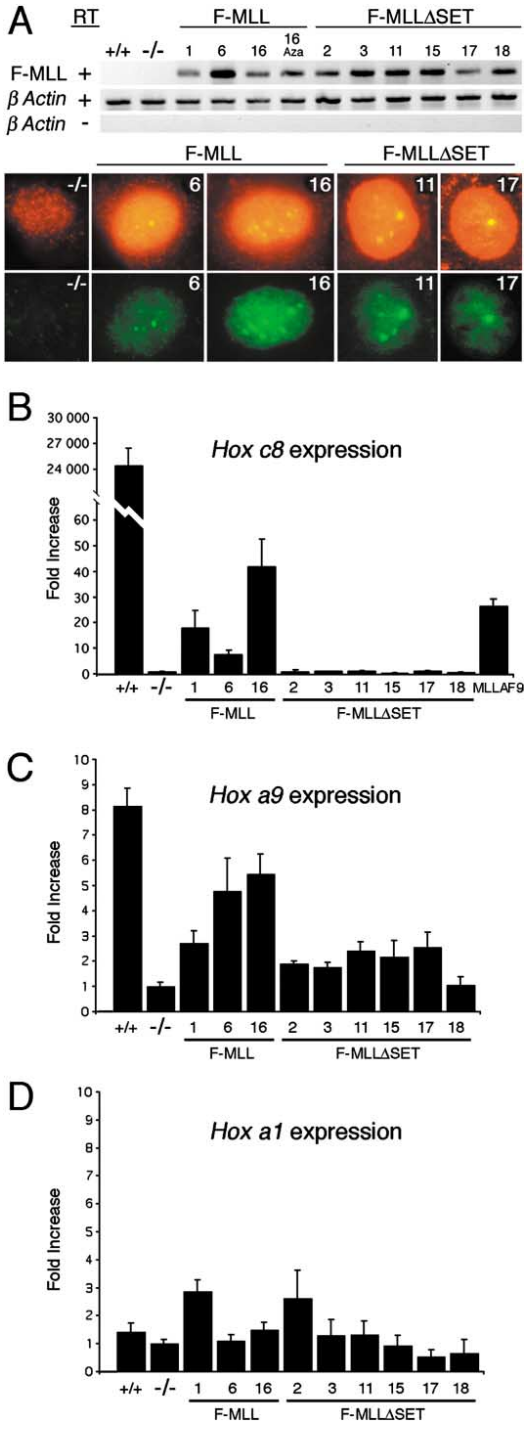


Figure 1. *MLL* Regulates *Hox* Expression in Fibroblasts

(A) *Mll*$^{-/-}$ clonal cell lines expressing wild-type (F-MLL #1, #6, and #16) or a deleted C-terminal SET domain (F-MLLΔSET #2, #3, #11, #15, #17, and #18) were analyzed by RT-PCR (top) and immunofluorescence (bottom). β-actin and samples lacking reverse transcriptase (−RT) are present as RT-PCR controls. FLAG expression was detected with polyclonal (middle, red) or monoclonal anti-FLAG antibody (bottom, green). +/+, *Mll*$^{+/+}$ fibroblasts; −/−, *Mll*$^{-/-}$ fibroblasts.

(B) *Hox c8* expression measured by real-time PCR is increased in *Mll*$^{-/-}$ cells that express F-MLL and in *Mll*$^{-/-}$ cells that express the leukemogenic fusion protein MLLAF9 but not in those that express F-MLLΔSET.

(C) Compared to *Mll*$^{-/-}$ cells, *Hox a9* expression is increased in *Mll*$^{+/+}$ cells and in *Mll*$^{-/-}$ cells that express F-MLL.

(D) *Hox a1* expression is not increased significantly in the various cell lines relative to *Mll*$^{-/-}$ cells.

modifications in the regulation and maintenance of target genes during mammalian development and provide a framework for comparing mechanisms of epigenetic forms of gene regulation by MLL and MLL fusion proteins.

## Results

### MLL Dynamically and Selectively Regulates *Hox* Gene Expression

MLL primarily regulates genes expressed posteriorly in the embryo, which are 5′ in the *Hox* clusters (Hanson et al., 1999). For example, *Hox c8* and *Hox a9* are *Mll* dependent, but *Hox a1* is Mll independent. In fibroblast lines established from embryonic day 10.5 *Mll*$^{+/+}$ and $^{-/-}$ embryos, *Hox c8* was expressed at 24,000 times higher levels in *Mll*$^{+/+}$ compared with $^{-/-}$ cells as assessed by quantitative PCR (Figure 1B). In contrast, *Hox a1* was expressed at comparable levels in both cell types (Figure 1D) similar to results in embryos.

We next examined if *Hox* gene expression could be upregulated in *Mll*$^{-/-}$ cells by stably transfecting a human MLL expression construct fused to an amino-terminal FLAG epitope tag (F-MLL) (Yamamoto et al., 1993). Expressing clones were identified by immunofluorescence and RT-PCR, and three independent clones were selected for further study (F-MLL #1, F-MLL #6, and F-MLL #16) (Figure 1A). Quantitative RT-PCR was performed on *Mll*$^{+/+}$ cells, *Mll*$^{-/-}$ cells, and on the three *Mll*$^{-/-}$F-MLL-expressing lines. Although *Hox c8* was expressed at very low levels in *Mll*$^{-/-}$ cells, expression of MLL increased *Hox c8* expression 6- to 50-fold in the three different lines (Figure 1B). Similar results were obtained for *Hox a9*. *Hox a9* was expressed at levels 8-fold higher in *Mll*$^{+/+}$ compared with *Mll*$^{-/-}$ cells (Figure 1C), and its expression was also upregulated 3- to 6-fold by MLL reexpression (Figure 1C). Conversely, *Hox a1* was not significantly upregulated by MLL expression in *Mll*$^{-/-}$ cells (Figure 1D). These results show that MLL activates expression of specific *Hox* genes in fibroblasts.

### MLL Binds Specifically to the *Hox c8* Promoter

To identify an MLL binding site, we analyzed the *Hox c8* gene because its regulatory sequences have been mapped using transgenes in vivo (Bradshaw et al., 1996; Shashikant et al., 1995). These sequences include a 400 bp 5′ enhancer element required for the proper temporal and tissue-specific initiation of transcription, the proximal promoter, the coding region, and a 3′ enhancer element required for the maintenance of late expression of *Hox c8* expression in vivo (Bradshaw et al., 1996; Hanson et al., 1999). The *Hox c8* 3′ enhancer was therefore an attractive possibility to be an MLL-dependent maintenance element, but this hypothesis had not been directly tested.

We first mapped DNase hypersensitive sites across

the *Hox c8* locus. One strong site (HSS 21.2) and two weaker sites (HSS 17.0 and 17.4) were identified in the 3′E, but these were present in both $Mll^{+/+}$ and $^{-/-}$ cells, indicating that they were MLL independent (data not shown). Therefore, we tested the ability of specific regions of the *Hox c8* locus to confer MLL responsiveness in functional assays. Given its apparent role in maintenance, portions of the *Hox c8* 3′ enhancer were cloned 5′ to the *Hox c8* promoter, and the activity of these constructs was tested in stable cotransfection assays in the presence or absence of MLL. Two larger fragments from the 3′ enhancer at coordinates 16.6–19.6 and from 19.6–21.6 exhibited MLL-dependent expression (data not shown). These fragments were further subdivided, ultimately revealing three 400 bp fragments of the 3′ enhancer that were responsive to MLL at coordinates 18.6–19.0, 19.0–19.4, and 20.4–20.8 (shown as black bars in Figure 4). The latter fragment is close to the 21.2 HSS.

To detect MLL binding sites in this region, chromatin immunoprecipitation (ChIP) (Orlando, 2000) was performed with antibodies against F-MLL. To improve sensitivity of the ChIP assay, we first performed anti-FLAG ChIP on F-MLL-expressing cells that also contain multiple copies of the stably integrated *Hox c8* transgene, *c8* exon 1 (c8ex1). The c8ex1 construct contains all the sequences necessary for proper *Hox c8* expression (5′ enhancer, promoter, and 3′ enhancer) and also expresses the first exon of *Hox c8* as a fusion with *lacZ* (Bradshaw et al., 1996) (Figure 2A). Previous experiments showed this construct was expressed in $Mll^{+/+}$ but not $^{-/-}$ cells and was MLL responsive when stably integrated into genomic DNA (Hanson et al., 1999). Surprisingly, anti-FLAG ChIP did not reveal MLL binding at the 3′ enhancer sequences including those that enhanced responsiveness to MLL (Figure 2A). Signal was also not detected using primers located at position 21.6–21.8, just 3′ to the distal HSS (data not shown), at the 5′ enhancer, or in intergenic regions (Figure 2A). Instead, ChIP revealed that MLL bound specifically to proximal promoter sequences within 200 bp of the transcription start site (Figure 2A).

We then applied anti-FLAG ChIP with quantitative PCR detection to confirm that MLL bound to a localized region of the endogenous *Hox c8* proximal promoter. Two independent lines (F-MLL #1 and #6) showed that sequences within 200 bp of the endogenous *Hox c8* promoter were enriched in the ChIP (Figure 2B). No binding was detected 400 bp 5′ from the *Hox c8* promoter (Figure 2B). No binding was detected near the *Hox a1* promoter, which is not regulated by MLL, suggesting that MLL binding is promoter specific in addition to being tightly localized (Figure 2B).

To determine if the *Hox c8* promoter was sufficient for MLL responsiveness in the absence of enhancer sequences, a construct with only the *Hox c8* promoter driving luciferase expression (c8Luc) was tested in transient transfection assays (Figure 2C). Normalized c8Luc expression was 11.3-fold above background in $Mll^{+/+}$ cells but only 2.5-fold over background in $Mll^{-/-}$ cells (Figure 2C). Importantly, cotransfection of F-MLL with c8Luc into $Mll^{-/-}$ cells increased expression to 41-fold over background and to 106-fold when F-MLL was cotransfected with c8Luc into $Mll^{+/+}$ cells (Figure 2C).

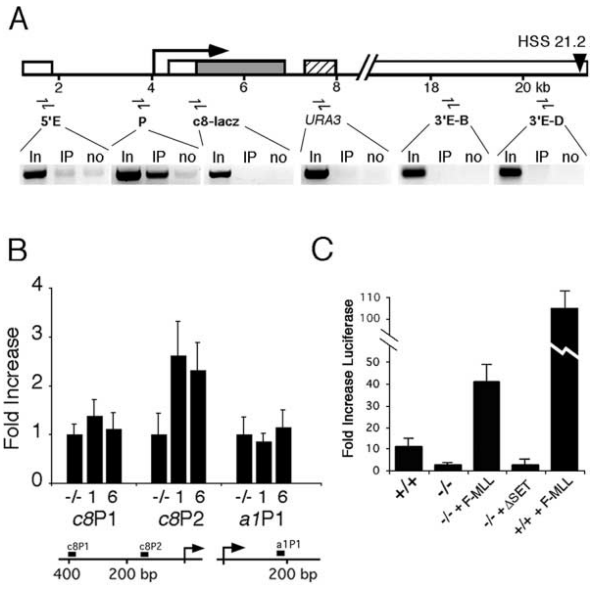


Figure 2. MLL Binds the *Hox c8* Promoter

(A) F-MLL binds specifically near the *Hox c8* promoter on a transgene. A physical map of the *c8ex1* transgene that contains all the sequences necessary to recapitulate *Hox c8* expression in vivo is shown at the top. The first exon of *Hox c8* is fused to *lacZ* (*c8-lacZ*). An $Mll^{-/-}$ cell line expressing FLAG-tagged MLL and containing the *c8ex1* construct (FMLL #16) was analyzed by ChIP using an anti-FLAG antibody. F-MLL binds specifically to the promoter (P) but not to sequences in the 5′ enhancer (5′E), 3′ enhancer (3′E-B and -D), or other regions of the construct (c8lacZ and URA3). PCR primer positions are shown. 3′E-B and -D represent positions of MLL-dependent enhancer activity in the 3′ enhancer (see Figure 4). In, input chromatin (~2% total); IP, anti-FLAG ChIP; no, no antibody control ChIP.

(B) F-MLL binds specifically near the endogenous *Hox c8* promoter. An anti-FLAG ChIP was performed on F-MLL #1 (1), F-MLL #6 (6), and $Mll^{-/-}$ (−/−) cells and analyzed using real-time PCR. The position of the probes (*c8*P1, *c8*P2, and *a1*P1) relative to the transcriptional start of *Hox c8* and *Hox a1* is shown below the graph. Enrichment was detected only with the *c8*P2 probe.

(C) The *Hox c8* promoter is sufficient for MLL activation. A luciferase reporter construct driven solely by the *Hox c8* promoter (c8Luc) was transfected into $Mll^{+/+}$ and $Mll^{-/-}$ cells with or without F-MLL or a F-MLL construct with a deletion of the C-terminal SET domain (ΔSET). The c8Luc construct expressed at levels significantly above background luminescence only when transfected into $Mll^{+/+}$ cells (11.3-fold) or when cotransfected with F-MLL into $Mll^{-/-}$ cells (41-fold) or $Mll^{+/+}$ cells (106-fold). Cotransfection of ΔSET with c8Luc into $Mll^{-/-}$ cells was the same as with c8Luc alone (2.5-fold above background) indicating that the SET domain is necessary for MLL activity.

Therefore, the *Hox c8* promoter alone is sufficient to confer MLL responsiveness.

### MLL Controls Histone Acetylation of *Hox c8* Promoter and Enhancer Sequences

Given the close link between histone acetylation and CpG methylation of DNA (Jones et al., 1998; Nan et al., 1998), we explored the role of histone acetylation and DNA methylation in *Hox c8* regulation. $Mll^{-/-}$ cells were treated with the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA), the DNA methylation inhibitor 5-Azacytidine (Aza), or both. As measured by quantitative PCR, *Hox c8* expression is upregulated about 24-fold (±3.3-

fold) with TSA treatment, 4-fold (±1.6-fold) with Aza treatment, and 29-fold (±6.2-fold) when treated with both TSA and Aza. Aza also upregulated expression of *Hox c8* in cells expressing exogenous MLL in an additive, rather than cooperative, manner. This increase in *Hox c8* expression was not due to an effect on the levels of F-MLL expression, as F-MLL expression levels are unaffected by Aza treatment (see Figure 1A, RT-PCR). These findings suggest that histone acetylation plays an important role in regulating *Hox c8* expression and that histone acetylation and DNA methylation are independently regulated.

ChIP using antibodies to acetylated histones was performed to assess the acetylation status of histones H3 and H4 at the promoter, intergenic region, and 5′ and 3′ enhancer sequences of *Hox c8* (Figure 3A). These studies showed the *Hox c8* locus was globally acetylated in the $^{+/+}$ cells. In contrast, this locus was hypoacetylated in the *Mll*$^{-/-}$ cells in the 5′ enhancer, promoter, and intergenic regions (Figure 3A). Importantly, expression of exogenous MLL in *Mll*$^{-/-}$ cells reestablished histone H3 and H4 acetylation at the 5′enhancer of *Hox c8,* but less of an increase was seen in the proximal promoter region and intergenic regions (Figure 3A). This result was surprising because MLL-dependent acetylation changes occur at the 5′ enhancer in the absence of direct MLL binding. To extend this result, we assayed local acetylation changes at the 3′ enhancer to determine which regions exhibited MLL-dependent acetylation. As shown in Figure 4, MLL-dependent histone acetylation occurs only at sites in the 3′ enhancer which are MLL-dependent activators of transcription (shown as black bars in Figure 4). Sequences outside the domain defined by HSS 17.0 and HSS 21.2 are acetylated in all three cell lines and are therefore MLL independent (Figures 3A and Figure 4).

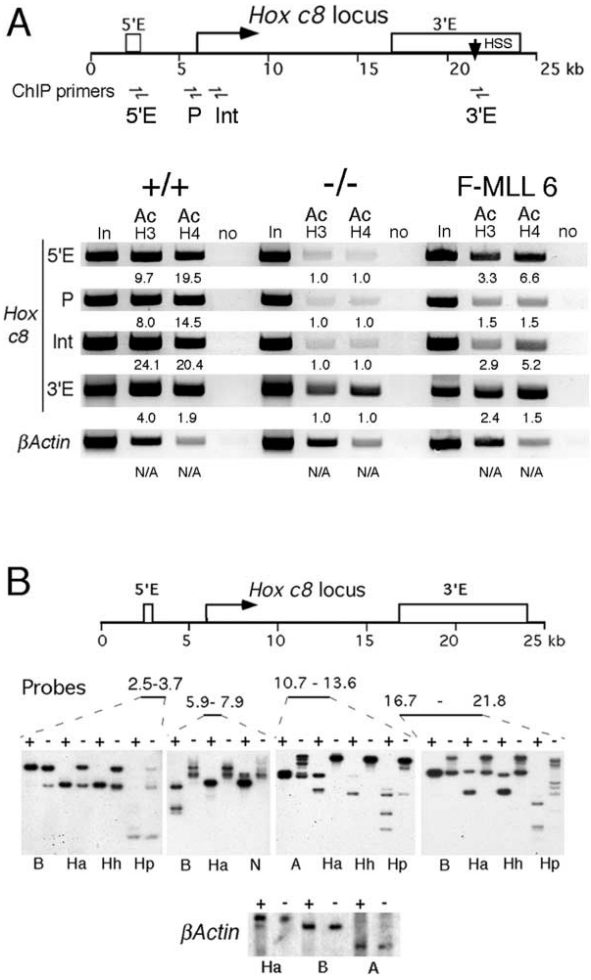


Figure 3. *Hox c8* Is Differentially Histone Acetylated and DNA Methylated in *Mll* Mutant Cells

(A) A physical map of the *Hox c8* locus with ChIP PCR primer positions is shown at the top of the figure. Acetylated histone H3 (AcH3) and H4 (AcH4) ChIP at the *Hox c8* locus reveals histone hypoacetylation across the 5′ enhancer (5′E), promoter (P), and intergenic (Int) regions in *Mll*$^{-/-}$ (−/−) versus *Mll*$^{+/+}$ (+/+) cells. Expression of F-MLL in the F-MLL #6 cell line increases histone acetylation in the 5′ enhancer relative to the $^{-/-}$ cells. The 3′ enhancer (3′E) at position 21.6 remains acetylated in all three cell lines. Acetylated ChIP was compared to input (In, ~2% total chromatin) and a no antibody control ChIP (no).

(B) Southern blot analysis of the *Hox c8* locus in *Mll* $^{+/+}$ and $^{-/-}$ cells with methylation-sensitive restriction enzymes reveals global CpG methylation in *Mll*$^{-/-}$ cells. Enzymes used were BstUI (B), HaeII (Ha), HhaI (Hh), HpaII (Hp), AvaI (A), and NaeII (N). The probe positions relative to the *Hox c8* locus are shown (top). The DNA from all four regions is resistant to digestion by higher $M_r$ bands in *Mll*$^{-/-}$ cells (−) compared with *Mll*$^{+/+}$ cells (+).

## *Hox c8* Is Globally Methylated in *Mll* Null Fibroblasts

Methylation of CpG dinucleotides in the vicinity of promoters and enhancers is a well-established mechanism for silencing gene expression (Bird, 2002; Bird and Wolffe, 1999; Siegfried et al., 1999). We next examined the CpG methylation status of the *Hox c8* locus in *Mll*$^{-/-}$ versus $^{+/+}$ cells by Southern analysis using a series of methylation-sensitive restriction enzymes. The entire *Hox c8* locus, including promoter, 5′ enhancer, and intergenic sequences, is extensively CpG methylated in *Mll*$^{-/-}$ compared with $^{+/+}$ cells (Figure 3B). Despite exhibiting high levels of histone acetylation, the 3′ enhancer region is extensively CpG methylated (Figure 3B). Reintroduction and expression of exogenous MLL failed to reverse CpG methylation in *Mll*$^{-/-}$ cells (data not shown), suggesting that this modification is regulated independently of histone acetylation and is not under direct MLL control.

## The MLL SET Domain Is Responsible for Histone H3 (Lys4) Methylation at *Hox* Loci In Vivo

The function of the SET domain, the most highly conserved domain between MLL and trx, has remained enigmatic. To explore the role of the SET domain in *Hox* gene regulation, we established six cell lines that express F-MLL lacking the C-terminal SET domain while retaining the PHD fingers and CBP interaction domain (F-MLLΔSET, Figure 1A). Immunofluorescence studies confirmed that the deletion of the SET domain did not affect localization of the protein in the nucleus, as it was still expressed with a punctate pattern at comparable levels as F-MLL (Figure 1A). *Hox c8* was not upregulated in any of the F-MLLΔSET lines indicating that the SET domain is necessary for MLL-mediated activation of *Hox c8* (Figure 1B). Whereas wild-type MLL increased expression from the *Hox c8* promoter by more than 40-

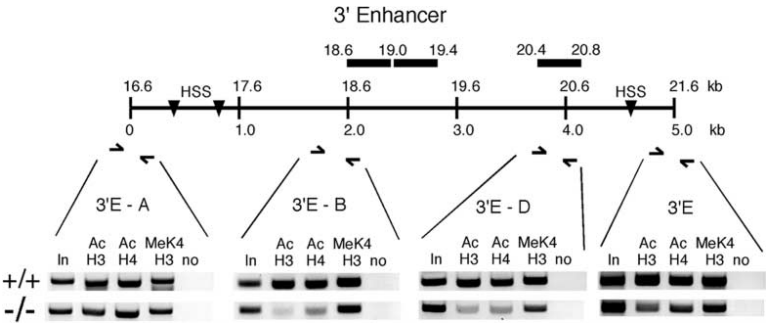


Figure 4. The *Hox c8* 3′ Enhancer Exhibits MLL-Dependent Histone Acetylation

The line represents the first 5 kb of the 3′ enhancer of *Hox c8*. In vivo coordinates in kb relative to the rest of the *Hox c8* locus are above, and the distances in kb from start of the 3′E are below. The thick black bars at coordinates 18.6–19.0, 19.0–19.4, and 20.4–20.8 represent the three smallest fragments that exhibit MLL-dependent activation in stable transfection assays. DNase hypersensitivity sites (HSS) are shown at positions 17.0, 17.4, and 21.2. The 3′ enhancer was subjected to ChIP analysis with antibodies directed against acetylated H3 (AcH3), acetylated H4 (AcH4), and Lys4-methylated H3 (MeK4), and compared to input DNA (In) and a no antibody control (no) using the primer pairs indicated. 3′E-B and 3′E-D show MLL-dependent histone acetylation. No difference in H3 MeK4 was detected.

fold, cotransfected F-MLLΔSET had no effect on the expression of the *Hox c8* reporter (Figure 2C). As this construct retains both the PHD fingers and CBP interaction domain, these data indicate that these regions alone are not sufficient for target gene activation. Instead, the data demonstrate that the SET domain is absolutely necessary for MLL-mediated activation of the *Hox c8* promoter.

Several SET domain-containing proteins exhibit histone methyltransferase (HMT) activity directed at either histone H3 or H4 (Lachner and Jenuwein, 2002). Because H3 Lys9 methylation is correlated with repression of gene transcription and heterochromatin assembly (Jenuwein and Allis, 2001; Lachner and Jenuwein, 2002; Zhang and Reinberg, 2001), one possible model for MLL activity might be that the protein inhibits H3 lysine 9 methylation, perhaps mediated by PcG-repressive complexes. Alternatively, since the closest MLL homolog in yeast, SET1, is an H3 (Lys4) HMT (Lachner and Jenuwein, 2002), MLL could regulate H3 (Lys4) methylation directly through its own SET domain (see below).

To assess the role of MLL on histone methylation in vivo, we analyzed both H3 (Lys4) and H3 (Lys9) methylation across the *Hox c8* locus. Standard PCR showed comparably low levels of Lys9 methylation in all three cell lines (Figure 5A). However, quantitative PCR revealed a 3-fold enrichment for Lys9 methylation at the *Hox c8* promoter in *Mll*$^{-/-}$ cells relative to *Mll*$^{+/+}$ cells or the F-MLL-expressing line (Figure 5B). This suggests that low levels of Lys9 methylation at the promoter are involved in repressing the *Hox c8* locus and may be dynamically influenced by MLL, perhaps through promotion of Lys9 acetylation. In contrast, significant MLL-dependent changes were observed for H3 (Lys4) methylation. The *Hox c8* locus was globally H3 (Lys4) methylated in *Mll*$^{+/+}$ cells and was hypomethylated in $^{-/-}$ cells at the 5′ enhancer, promoter, and intergenic regions (Figure 5A). Importantly, reexpression of MLL in *Mll*$^{-/-}$ cells induces H3 (Lys4) methylation at the endogenous *Hox c8* promoter and 5′ enhancer, even though MLL binding was detected only at the promoter (Figures 5A, 2A, and 2B). Quantitative PCR using probes within 400 bp of the promoters of *Hox c8* showed 5- to 20-fold enrichment of H3 (Lys4) methylation in MLL expression compared with *Mll*$^{-/-}$ cells (Figure 5D). In addition, no enrichment of H3 (Lys4) methylation was seen in cells expressing MLL lacking the SET domain (F-MLLΔSET #11 and #17, Figure 5D), suggesting Lys4 methylation at the *Hox c8* promoter is MLL SET domain dependent.

Interestingly, the 3′ enhancer exhibited no local changes in histone methylation (Figures 4 and 5A), indicating that another chromatin modifier besides MLL is responsible for mediating H3 (Lys4) methylation within this region. Western blot analysis of extracts prepared from *Mll*$^{+/+}$ and *Mll*$^{-/-}$ cells showed comparable levels of H3 (Lys4) and (Lys9) methylation (Figure 5C), indicating that MLL does not globally affect histone methylation in mammalian cells.

### The MLL SET Domain Has Intrinsic H3 (Lys4) Methyltransferase Activity

To determine if MLL has intrinsic HMT activity, the MLL SET domain was expressed in bacteria and tested for its ability to methylate histones as well as a series of H3 peptides. Although previously no methyltransferase activity was detected for the MLL SET domain (Rea et al., 2000), under our expression and HMT assay conditions, the MLL SET domain has robust histone methyltransferase activity toward recombinant H3, core histones, and unmodified H3 peptides (Figures 6A and 6B). Although yeast Set1 is capable of di- and trimethylation (Santos-Rosa et al., 2002), little, if any, HMT activity by MLL was observed on H3 peptides where Lys4 is dimethylated (Figure 6B). Similar results were observed for Set7/9 (Nishioka et al., 2002; Wang et al., 2001) and are confirmed by our data (see Figure 6B, inset). Whether certain mammalian methyltransferases lack the ability to catalyze conversion of di- to trimethylation remains to be firmly established. Together, our data strongly suggest that the MLL SET domain exhibits H3 (Lys4)-specific HMT activity both in vitro and in vivo. Interestingly, MLL SET domain Lys4 methyltransferase activity was increased significantly on H3 peptides acetylated at Lys9 or Lys14 (Figure 6C). In keeping with our ChIP analyses, these data suggest the intriguing possibility that MLL preferentially methylates acetylated target loci in vivo.

### Histone H3 (Lys4) Methylation of *Hox* Genes Is a General Mechanism of MLL Function

Given the apparent importance of *Hox a7* and *a9* in MLL-induced leukemia (Ayton and Cleary, 2001b), we also examined whether these loci were regulated by MLL. *Hox a9* was expressed at roughly 8-fold higher levels in

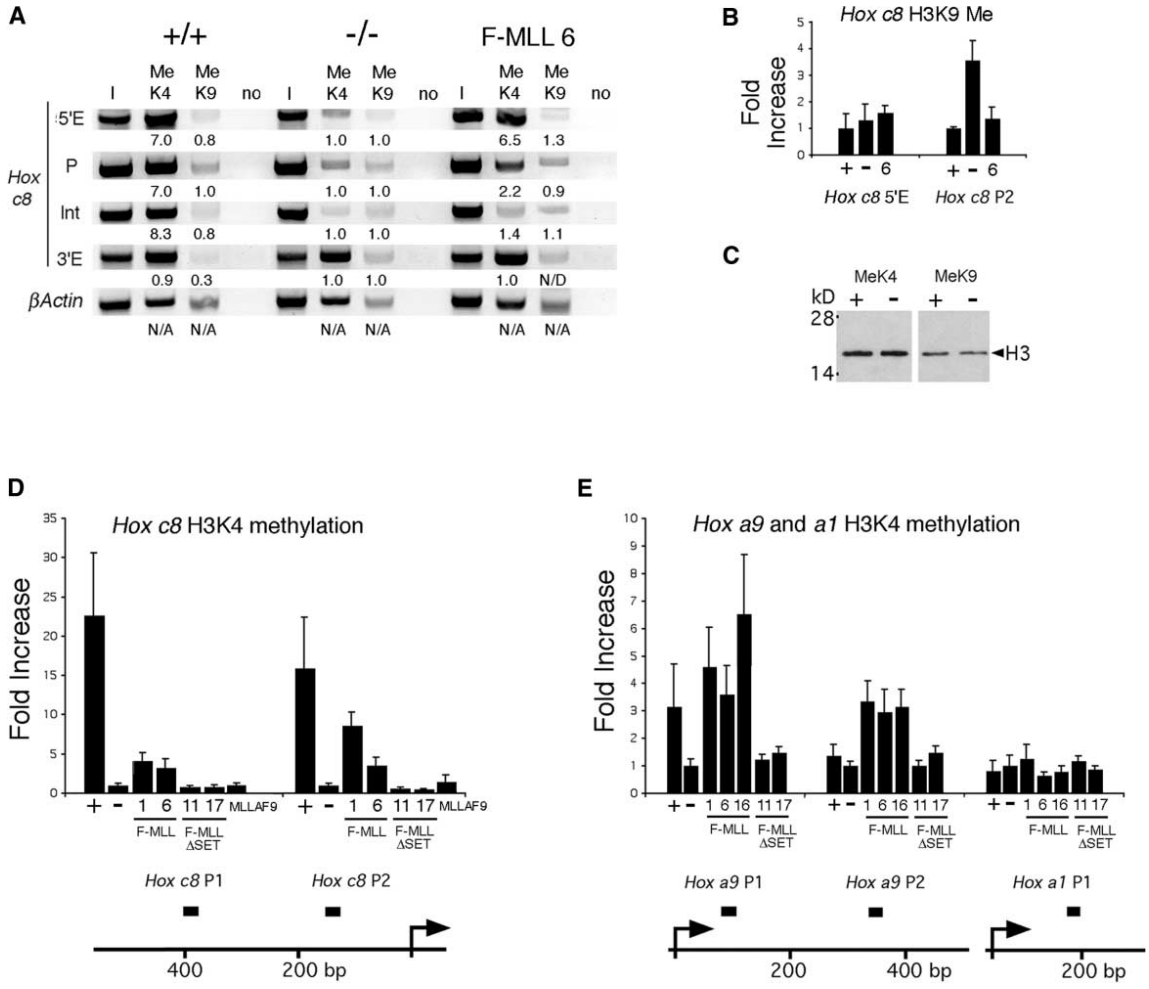


Figure 5. MLL Is Required for H3 (Lys4) Methylation at *Hox c8*

(A) Histone H3 is hypomethylated at Lys 4 across the 5′ enhancer, promoter, and intergenic regions in *Mll*$^{-/-}$ versus $^{+/+}$ cells. Expression of F-MLL (FLAG-tagged MLL) in the $^{-/-}$F-MLL #6 cell line increases H3 (Lys4) methylation in the 5′ enhancer and promoter regions relative to the $^{-/-}$ cells. The 3′ enhancer at position 21.6 remains H3 (Lys 4) methylated in all three cell lines. There is no evidence of enrichment for histone H3 lysine 9 methylation (MeK9) in any of the cell lines. PCR primer positions are as shown in Figure 4A.

(B) Quantification of H3MeK9 ChIP using real-time PCR. H3 Lys9 methylation is increased at the *Hox c8* promoter (c8P2) in *Mll*$^{-/-}$ (−) cells relative to *Mll*$^{+/+}$ (+) and F-MLL #6 (6) cells. No changes in H3 Lys9 methylation are seen at the 5′E.

(C) Western blot of *Mll*$^{+/+}$ versus *Mll*$^{-/-}$ extracts probed with antibodies specific to either H3 MeK9 or H3 MeK4. Overall levels of histone methylation are the same in each cell line, so MLL does not affect global histone methylation.

(D) Quantification of H3MeK4 ChIP at the *Hox c8* locus using real-time PCR. *Mll*$^{+/+}$, F-MLL #1 (1), and F-MLL #6 (6) cells are enriched for H3MeK4 around the *Hox c8* promoter relative to *Mll*$^{-/-}$ cells. No significant H3MeK4 enrichment is seen in the F-MLLΔSET lines #11 (11) and #17 (17) or in *Mll*$^{-/-}$ cells that express the MLLAF9 leukemogenic fusion protein (MLLAF9). The positions of the probes are shown below the graph.

(E) Quantification of H3MeK4 ChIP at the *Hox a9* and *Hox a1* loci in different cell lines using real-time PCR. *Mll*$^{+/+}$, F-MLL #1, F-MLL #6, and F-MLL #16 cells are enriched for H3MeK4 around the *Hox a9* proximal promoter relative to *Mll*$^{-/-}$ cells. No significant H3MeK4 enrichment is seen in the F-MLLΔSET lines #11 and #17. No relative differences in H3MeK4 enrichment were seen at *Hox a1* in any cell line. The position of the probes for each gene is shown below the graph.

*Mll*$^{+/+}$ compared with $^{-/-}$ cells (Figure 1C). Reexpression of MLL upregulated *Hox a9* expression by 3- to 6-fold (F-MLL #1, F-MLL #6, and F-MLL #16) (Figure 1C). Similar to *Hox c8*, H3 (Lys4) methylation at *Hox a9* was increased in the range of 3- to 6-fold in *Mll*$^{+/+}$ cells relative to *Mll*$^{-/-}$ cells (Figure 5E). As was seen at the *Hox c8* locus, no significant H3 Lys4 methyl enrichment was seen in cells expressing SET domain-deleted forms of MLL (F-MLLΔSET #11 and #17) (Figure 5E). Similar results were seen with *Hox a7*, whose expression and Lys4 methylation is also tightly regulated by MLL (data not shown). As previously noted, *Hox a1* expression is not regulated by MLL and neither is H3 (Lys4) methylation of the locus (Figure 5E). Therefore, we propose that induction of H3 (Lys4) methylation may be a general mechanism of MLL-mediated transcriptional regulation.

### MLL-AF9 Fusions Activate *Hox c8* but Do Not Cause Histone (Lys4) Methylation

Leukemogenic MLL fusions delete the SET domain yet apparently activate *Hox* genes in hematopoietic cells. Yet as we have shown above, deletion of the SET domain

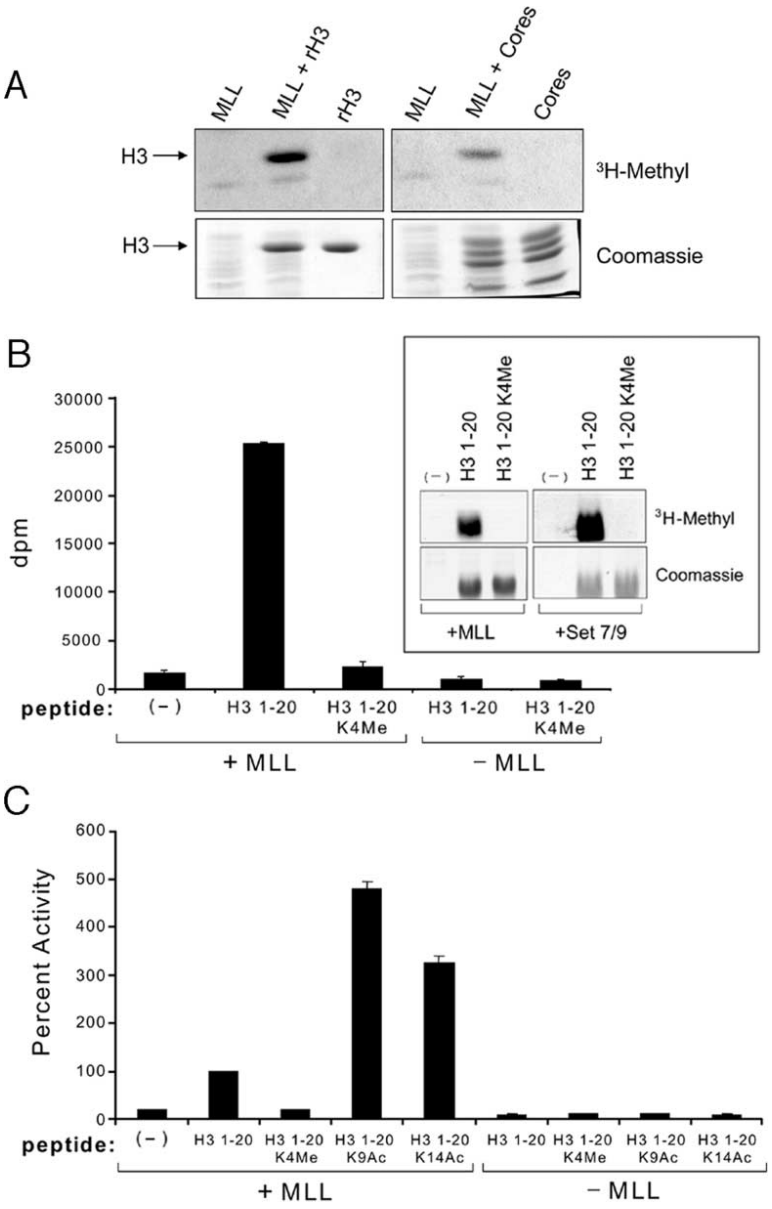


Figure 6. The MLL SET Domain Has Intrinsic H3 (Lys4) HMT Activity that Is Stimulated by Acetylation

(A) MLL expressed in bacteria was incubated with recombinant *Xenopus* H3 (rH3) or chicken core histones (Cores) along with S-adenosyl-L-[methyl-$^3$H]methionine ($^3$H-SAM) and analyzed by SDS-PAGE, Coomassie staining, and fluorography ($^3$H-Methyl).

(B) MLL in the presence of $^3$H-SAM methylates an unmodified H3 peptide (1–20) but not a dimethylated H3 peptide (1–20 K4Me) as shown by filter binding assays. The inset represents the same reaction products analyzed by SDS-PAGE, Coomassie staining, and fluorography ($^3$H-Methyl). Set7/9, a known Lys4 methyltransferase, was used as a positive control.

(C) Filter binding assays demonstrate that MLL HMT activity is stimulated by H3 peptides that are acetylated at Lys9 or Lys14.

in F-MLL abrogates *Hox c8* activation and histone methylation. To determine if *Hox c8* could be activated by the MLL-AF9 fusion protein, *Mll*$^{-/-}$ cells were infected with a MSCV-based retrovirus containing MLL-AF9. This same virus was used to transform murine bone marrow and produce leukemia in transplanted mice (data not shown). Expression of MLL-AF9 caused 25-fold activation of *Hox c8*, comparable to that seen with F-MLL (Figure 1B). As shown in Figure 5D, ChIP assays showed no difference in H3 (Lys4) methylation, indicating that the MLL-AF9 fusion activates *Hox c8* by a mechanism other than MLL-mediated Lys4 methylation.

## Discussion

### MLL Binds to the Proximal Promoter of *Hox c8*

In *Drosophila*, trxG and PcG proteins bind maintenance elements that act at a distance of tens of kilobases from promoters (Brock and van Lohuizen, 2001), but increasing evidence indicates that they may also act directly on promoters (Orlando et al., 1998; Breiling et al., 2001; Saurin et al., 2001). Here we show that MLL does not bind the *Hox c8* 3′ enhancer under our assay conditions, a surprising result given that these sequences confer increased responsiveness to MLL and were required for maintenance in vivo (Bradshaw et al., 1996; Shashikant et al., 1995). Instead, MLL binds to a tightly localized region of the *Hox c8* promoter, suggesting that MLL exerts its activity through interactions at the promoter near the transcription start site. However, our data do not exclude the possibility of transient interactions between promoter-bound MLL with the 5′ and 3′ enhancers, as histone methylation and acetylation at the 5′enhancer and acetylation at the 3′ enhancer are MLL dependent.

It remains unclear how MLL is recruited to target promoter elements. MLL binding may be influenced by a combination of specific DNA sequences or be governed

by interaction with basal transcription factors or coactivators. For example, MLL interacts with CBP, HDACs, and the core SWI/SNF component INI-1, all of which have been found to be associated with proximal promoters of target genes (Ernst et al., 2001; Rozenblatt-Rosen et al., 1998). MLL may also recognize specific patterns of histone modifications as suggested by our results that the MLL SET domain prefers to methylate acetylated H3 peptides (Figure 6C) and that the trx SET domain binds more preferentially to acetylated histones (Katsani et al., 2001).

### MLL Regulates Histone Acetylation and H3 (Lys4) Methylation at *Hox* Genes

PcG and trxG proteins regulate transcription in part by modulating steady-state levels of histone acetylation at target genes. In *Drosophila*, H4 hyperacetylation is required for trx-dependent maintenance in vivo by *Fab-7* (Cavalli and Paro, 1999). PcG and trxG proteins associate with histone acetylases and deacetylases in vivo (Simon and Tamkun, 2002; Ernst et al., 2001). Curiously, MLL interacts with histone deacetylases via the DNMT domain (Xia and Zeleznik-Le, 2001; Birke et al., 2002) raising the question of whether net acetylation or deacetylation best describes MLL-mediated gene regulation. At the *Hox c8* locus, our data reinforces the view that MLL promotes histone acetylation at the 5′ and 3′ enhancers and thus functions as a coactivator at certain target promoters (Poux et al., 2002).

Site-specific histone methylation plays a major role in transcriptional regulation (Jenuwein and Allis, 2001; Lachner and Jenuwein, 2002; Zhang and Reinberg, 2001). H3 Lys4 methylation, for example, disrupts binding of the nucleosome remodeling and deacetylase (NuRD) repressor complex to H3 tails, thereby preventing targeted histone deacetylation catalyzed by the NuRD complex (Nishioka et al., 2002; Zegerman et al., 2002). PcG complexes associate with HDACs (Chang et al., 2001; Tie et al., 2001; van der Vlag and Otte, 1999), raising the intriguing possibility that MLL SET domain-induced H3 (Lys4) methylation at target promoters may similarly inhibit binding of PcG complexes and therefore prevent histone deacetylation of target loci. If so, H3 (Lys4) methylation may serve as a more stable epigenetic mark that prevents establishment of PcG-mediated silencing. Although we have shown that the MLL SET domain is required for activation and Lys4 methylation of target loci, the SET domain also interacts with members of the SWI/SNF remodeling complex (Fry and Peterson, 2002; Rozenblatt-Rosen et al., 1998) which also likely contributes to the activity of this domain.

Recently, the PcG protein E(Z) has been shown to methylate Lys9 and/or Lys27 in a pathway leading to transcriptional repression (Czermin et al., 2002; Müller et al., 2002; Kuzmichev et al., 2002; Cao et al., 2002). Together, the findings that MLL and E(Z) both possess methyltransferase activity that either activates (Lys4) or represses (Lys9/Lys27) transcription, respectively, provides an attractive model explaining the long-standing functional antagonism noted between trxG and PcG proteins.

The finding of extensive CpG methylation of *Hox* loci in *Mll*$^{-/-}$ cells and reports that H3 methylation at Lys9 is required for DNA methylation raise the additional possibility that MLL SET domain-induced H3 (Lys4) methylation plays a role in protecting target *Hox* genes from DNA methylation (Lachner and Jenuwein, 2002). It is noteworthy that MLL contains a region of DNMT homology that, at least in vitro, selectively binds to unmethylated CpG-rich DNA (Birke et al., 2002). Thus, MLL may antagonize methylated CpG binding proteins that recruit HDAC activity to methylated DNA (reviewed in Wade, 2001).

### MLL and Leukemia

Given our demonstration that transcriptional activation and HMT activity reside in the MLL SET domain, one possible mechanism for conversion of MLL into an oncogene is that separation of MLL from the SET domain converts the protein from an activator to a repressor. This mechanism is analogous to that proposed for both PML-RAR and more recently AML-ETO, both implicated in human myeloid leukemias which are converted from their normal role of transcriptional activators to repressors by fusion to their respective translocation partners (Lin et al., 1999; Wang et al., 1998). However, both clinical observations and experimental results make this mechanism unlikely for MLL. Instead, most data suggest that MLL target genes, such as *Hox a7* and *a9* which are consistently expressed in leukemias with MLL rearrangements, are persistently activated by MLL fusion proteins so that their normal downregulation during differentiation cannot occur (Pineault et al., 2002). As a first step toward exploring this possibility, we have found that the MLL fusion protein MLL-AF9 strongly activated expression of endogenous *Hox c8* through a mechanism that does not involve H3 Lys4 methylation and does not require wild-type MLL. Determining to what extent the histone code is altered, if at all, between MLL and leukemogenic MLL fusion proteins, and determining whether these changes require continual presence of the fusion protein or are "hit and run" will have important implications for the development of targeted therapies for MLL-associated leukemia. The finding that the MLL central SET domain is sufficient for MTase activity may also be particularly relevant for oncogenesis because homologous domains are found in several proteins altered in human tumors that are probably histone methyltransferases (Huang, 2002).

Taken together, our data provide compelling in vivo evidence that MLL regulates specific *Hox* target loci by direct binding that, in turn, modulates levels of histone H3 (Lys4) methylation by targeting the intrinsic SET domain H3 (Lys4) methyltransferase activity to promoters. Moreover, these data underscore the importance of histone methylation as a component of epigenetic gene regulation with far-reaching implications for human biology and disease.

## Experimental Procedures

### Cells

All experiments used mouse embryo fibroblasts (MEFs) prepared from day 10.5 wild-type or *Mll*$^{-/-}$ mice (a gift of Dr. Stanley Korsmeyer). *Mll*$^{-/-}$ MEFs were transfected with full-length *MLL* that contained an N-terminal FLAG tag (F-MLL) (a gift of Dr. Masao Seto) or the same construct with a stop codon introduced in an upstream

HpaI site (F-MLLΔSET). Bulk cultures were subjected to drug selection, and individual colonies positive for expression were isolated by immunofluorescence.

### Immunofluorescence and RT-PCR

Cell lines were fixed with 1% paraformaldehyde for 10 min at 37°C and then probed with an M2 anti-FLAG monoclonal (Sigma) or a rabbit anti-FLAG polyclonal (Sigma) followed by the appropriate fluorescent-conjugated secondary antibody. cDNA for RT-PCR was made using the Invitrogen Superscript kit. See below for real-time PCR quantification of *Hox* gene expression.

### Probes and Primers

Probes and primer pairs for all experiments are available as online supplemental data at http://www.molecule.org/cgi/content/full/10/5/1107/DC1.

### Stable Transfection Assays and Retroviral Transduction

The *Hox c8* promoter (coordinates 5906–6440) was cloned into a vector containing a luciferase reporter. These constructs were transferred to *Mll* null fibroblasts with a plasmid expressing hygromycin under the control of a CMV promoter, selected for 3 weeks and assayed for luciferase activity (details upon request). A leukemogenic MLL-AF9 cDNA was introduced into $Mll^{-/-}$ cells using a modified MSCV-based vector (Pear et al., 1998) that expresses GFP. Cells were harvested for quantitative RT-PCR and ChIP assays 2 weeks following transduction.

### Chromatin Immunoprecipitation (ChIP) and Standard PCR Detection

Polyclonal antibodies specific for modified histones were purchased from Upstate Biotechnology. Immunoprecipitation conditions for histones or for FLAG ChIP using antibodies from Sigma (M2 or polyclonal) followed the protocol recommended by Upstate. ChIP DNA was detected using standard PCR and staining with ethidium bromide after electrophoresis in 1.5% agarose gels. Bands were visualized with a Bio-Rad Gel Doc 2000 and quantified using the volume tools function in the Bio-Rad Quantity One software, normalized to a β-actin control.

### Real-Time PCR Quantification of Genomic DNA ChIP and *Hox* Gene Expression

Real-time PCR was performed in triplicate using Taqman probes and the ABI prism 7700 sequence detection system. Details of quantification are available as online supplemental data at http://www.molecule.org/cgi/content/full/10/5/1107/DC1.

### Methylation Sensitivity Assays

DNA was prepared from $Mll^{+/+}$ and $^{-/-}$ fibroblasts with DNAzol (GIBCO-BRL), digested with the methylation-sensitive enzymes and analyzed by Southern blotting with probes described in the online supplemental data at http://www.molecule.org/cgi/content/full/10/5/1107/DC1.

### 5-Aza-2′deoxycytidine (5′Aza-dC) and Trichostatin-A (TSA) Treatment

Exponentially growing cells were exposed to no drugs, 150 nM 5′Aza-dC, 300 nM TSA, or both for 24 hr. RNA was prepared using Trizol (GIBCO-BRL) according to the manufacturer's directions. Real-time PCR was performed as described.

### Western Blots

Protein extracts were prepared from $Mll^{+/+}$ cells and $Mll^{-/-}$ cells using RIPA lysis buffer plus protease inhibitors. Following electrophoresis, blots were probed with primary antibodies specific to either methylated H3 Lys4 or Lys9 (Upstate Biotechnology) and visualized using an Amersham ECL detection kit.

### Cloning and Expression of the MLL SET Domain

An MLL C-terminal fragment encoding amino acids 3592–3969 was amplified by PCR from pCDNA3-FLAG-MLL and subcloned. The resulting expression plasmid (pCAL-n-MLL) was expressed in the BL21-Gold (DE3) *E. coli* strain (Stratagene) following induction with 0.4 mM isopropyl-D-thiogalactoside for 3 hr at 20°C. Cells (2 ml) were collected, frozen at −80°C, lysed in 200 μl of lysis buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 1 mM magnesium acetate, 2 mM $CaCl_2$, 1 mM imidazole, plus protease inhibitors), sonicated on ice, and clarified by centrifugation. Supernatants and insoluble pellets together were resonicated, and then clarified supernatants were stored at −80°C after addition of glycerol to a final concentration of 10%. Full-length Set9 cDNA, a generous gift from Dr. Danny Reinberg, was cloned into pET28 (Novagen) and expressed as described above.

### Methyltransferase Assays

Two microliters of bacterial lysate was incubated with various histone substrates in the presence of 1.0 μCi of S-adenosyl-L-[methyl-$^3$H]methionine (Amersham Pharmacia Biotech) in methyltransferase buffer (final concentration of 25 mM Tris-HCL [pH 8.0] and 5% glycerol) for 30 min at 30°C in a total volume of 20 μl Histone substrates include 2 μg recombinant *Xenopus* H3, 10 μg chicken core histones, 10 μg of H3 unmodified peptide (1–20, ARTKQTARKSTGGKAP RKQL), 10 μg of H3 peptide dimethylated at Lys4 (1–20 K4Me), or 10 μg of H3 peptide acetylated at Lys9 (1–20 K9Ac) or Lys14 (1–20 K14Ac). Then 10 μl of the reaction volume was spotted on Whatman P-81 paper, washed 4 × 10 min with 50 mM $NaHCO_3$, and counted by liquid scintillation. The remainder of the reaction was analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography.

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