Global Analysis of H3K4 Methylation Defines MLL Family Member Targets and Points to a Role for MLL1-Mediated H3K4 Methylation in the Regulation of Transcriptional Initiation by RNA Polymerase II[▽][†]

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A common landmark of activated genes is the presence of trimethylation on lysine 4 of histone H3 (H3K4) at promoter regions. Set1/COMPASS was the founding member and is the only H3K4 methylase in *Saccharomyces cerevisiae*; however, in mammals, at least six H3K4 methylases, Set1A and Set1B and MLL1 to MLL4, are found in COMPASS-like complexes capable of methylating H3K4. To gain further insight into the different roles and functional targets for the H3K4 methylases, we have undertaken a genome-wide analysis of H3K4 methylation patterns in wild-type *Mll1*^{+/+} and *Mll1*^{−/−} mouse embryonic fibroblasts (MEFs). We found that Mll1 is required for the H3K4 trimethylation of less than 5% of promoters carrying this modification. Many of these genes, which include developmental regulators such as Hox genes, show decreased levels of RNA polymerase II recruitment and expression concomitant with the loss of H3K4 methylation. Although Mll1 is only required for the methylation of a subset of Hox genes, menin, a component of the Mll1 and Mll2 complexes, is required for the overwhelming majority of H3K4 methylation at Hox loci. However, the loss of MLL3/MLL4 and/or the Set1 complexes has little to no effect on the H3K4 methylation of Hox loci or their expression levels in these MEFs. Together these data provide insight into the redundancy and specialization of COMPASS-like complexes in mammals and provide evidence for a possible role for Mll1-mediated H3K4 methylation in the regulation of transcriptional initiation.

The mixed-lineage leukemia gene (*MLL1*) is involved in numerous translocations found in several human acute leukemias (2, 43, 53). Chromosomal translocations in the *MLL1* gene result in hematological malignancies, including acute myeloid and lymphoid leukemia. Although these cytogenetic abnormalities were discovered over 25 years ago, little is known about the biochemical functions of MLL1, its protein complexes, its translocation partners, and particularly, why these translocations result in leukemia. *MLL1* is one of at least six genes encoding histone methyltransferases in mammals that methylate histone H3 on lysine 4 (H3K4), a posttranslational mark primarily associated with promoters of active genes (49). Much of our knowledge of the implementation of this mark comes from studies of Set1/COMPASS in the yeast *Saccharomyces cerevisiae*. The first H3K4 methylase complex to be identified, COMPASS, was purified from yeast and contains Set1 and seven other polypeptides, named Cps60 to Cps15 according to their size in yeast (30). COMPASS is capable of mono-, di-, and trimethylating H3K4 (20, 42, 48, 56). Subsequently, it

was determined that six mammalian Set1 homologs, MLL1 to MLL4 (KMT2A to KMT2D) and human Set1A and human Set1B (KMT2F and KMT2G), are found in COMPASS-like complexes, all capable of methylating H3K4 (5, 15, 16, 23, 38, 49, 59).

We have recently shown that human Set1A/Set1B function most similarly to yeast COMPASS and mediate the bulk of the H3K4 trimethylation in mammalian cell extracts (57). In contrast, the members of the MLL family of proteins do not appear to individually mediate bulk changes in H3K4 methylation (9, 31, 57). A number of approaches have been used to investigate MLL1 and its target genes (3, 11, 31, 32, 44, 45). Collectively, however, there is no clear picture of whether members of the MLL family work globally on a common set of active genes (10–12) and/or more specifically on distinct subsets of targets (9, 32) to exert their regulatory activities. We have addressed this important issue for *Mll1*, the murine ortholog of human *MLL1*, by comparing gene expression profiles and genome-wide patterns of H3K4 trimethylation and dimethylation in *Mll1*^{+/+} and *Mll1*^{−/−} mouse embryonic fibroblasts (MEFs) to identify the set of genes that require Mll1-dependent methyltransferase activity. Our analyses have revealed that only a few hundred genes in MEF cells require Mll1 to maintain the appropriate levels of H3K4 methylation and gene expression. Although the *Hox* genes represent a

significant proportion of the target genes identified, only a subset of *Hox* genes is regulated by Mll1, while the Mll1/Mll2-interacting partner menin is required for essentially all H3K4 trimethylation across the *Hox* loci. In contrast, MLL3, the MLL3/MLL4 complex-interacting partner Pax transactivation domain-interacting protein (PTIP), and the Set1 complex have modest effects on H3K4 methylation or gene expression at the *Hox* loci. This suggests that each member of the Mll1-to-Mll4 family has distinct roles, with Mll1 and Mll2 being major regulators of *Hox* genes. Our work also reveals that many other regulators of transcription in development are Mll1-dependent target genes, which can further our understanding of the mechanistic role of MLL1 in development and in MLL1-mediated leukemogenesis. Furthermore, unlike H3K4 methylation by COMPASS, which correlates with the initiation of transcription and is subsequent to Pol II initiation, mammalian MLL1 functional targets require H3K4 methylation for recruitment of the basal transcription machinery and transcriptional initiation. This could be more important for specific classes of genes, including those lacking TATA or other core promoter elements that facilitate the recruitment of the basal transcription machinery (17).

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MATERIALS AND METHODS

Antibodies, cell lines, and siRNAs. The antibodies to dimethylated histone H3K4 (H3K4me2) (ab7766), trimethylated histone H3K4 (H3K4me3) (ab8580), histone H3 (ab1791), TATA-binding protein (TBP) (ab51841), and RNA Pol II C-terminal domain (ab5408; used for ChIP-chip experiment whose results are shown in Fig. 4) were purchased from Abcam. The rabbit anti-human RNA polymerase Rpb1 antibody (used in the experiment whose results are shown in Fig. 2) was generated by immunization with the synthetic peptide ERALRRTL QEDLVKDVLSNGC conjugated to keyhole lympet hemocyanin. All small interfering RNAs (siRNAs) were SMARTpool siRNAs from Dharmacon, Inc., and RNA interference analysis was done as previously described (57).

The *Mll1* and *Men1* wild-type and knockout MEF cell lines are gifts from Jay L. Hess (University of Michigan Medical School) and were described previously (13, 15). For the gene expression microarray analyses, RNA was extracted from *Mll1*$^{+/+}$ and *Mll1*$^{-/-}$ MEFs. Ten micrograms of RNA was labeled and hybridized to an Agilent microarray as described below. For the chromatin immunoprecipitation and hybridization microarray (ChIP-chip) analysis, approximately 3×10^8 to 5×10^8 cells were harvested and cross-linked with 1% formaldehyde. Preparation of ChIP-chip DNA was performed according to a published protocol (25).

The *PTIP*$^{+/+}$ and *PTIP*$^{-/-}$ cell lines were generated by infecting the simian virus 40 T antigen-immortalized PTIP conditional knockout MEF cell line *PTIP*$^{flox/flox}$ with retroviruses expressing vector or *Cre* as described previously (5). Primary *Mll3*$^{+/+}$ and *Mll3*$^{-/-}$ MEFs were isolated from wild-type or *Mll3*$^{-/-}$ littermate embryos at embryonic day 13.5 and immortalized by following the 3T3 protocol. The *Mll3* knockout mice were derived from embryonic stem cell line XM083 obtained from BayGenomics. The generation and characterization of *Mll3* knockout mice will be described in another manuscript.

ChIP-qPCR assay. Cells were fixed in 1% formaldehyde and sonicated in lysis buffer using a Bioruptor (Diagenode, Inc.). Sonicated lysates equivalent to 4×10^6 cells were subjected to ChIP analysis. ChIP products were analyzed by quantitative real-time PCR (qPCR) using SYBR green and a Bio-Rad MyIQ. The comparative cycle threshold method was used to determine expression levels relative to the level of input or total histone H3, which was then averaged over three independent experiments.

qRT-PCR assay. For quantitative reverse transcriptase PCR (qRT-PCR), RNA was isolated with RNeasy, treated with DNase I, and repurified with RNeasy. Hox expression assays were purchased from Applied Biosystems and included in a custom TaqMan array card arranged in an 8-by-48 format. Five reference controls were experimentally validated using geNorm. Actb and Tbp

were found to be stably expressed in all experiments and were chosen for use in normalization. Data analysis was performed using the comparative cycle threshold method.

Data analysis. All microarray data were analyzed by using R and the limma package. ChIP-chip experiments were performed on two different platforms, the commercially available Agilent mouse promoter arrays and the custom-designed mouse *Hox* tiling arrays. Two replicates were done on each platform. ChIP-chip data were median normalized within each array and aquantile normalized between arrays. Expression experiments were performed with two replicates on the Agilent mouse whole-genome platform. Expression data were loess normalized within each array and aquantile normalized between arrays (51). Dye swaps were performed for the expression data. After initial normalization and analysis, peaks were found in ChIP-chip data by using Splitter (http://zlab.bu.edu/splitter) with the following settings: Manual Cutoff, 1; Minrun, 5; and Maxgap, 200. Track files were created and viewed in the UCSC genome browser (18). Venn diagrams were created using Vennmaster (19) and VENNY (http://bioinfogp.cnb.csic.es /tools/venny/index.html).

Nomenclature for the MLL family. Two numbering systems are currently in use for the four MLLs. In this paper, MLL2 (HRX2) refers to the closest paralog of MLL1, and both are putative orthologs of *Drosophila melanogaster* Trx. MLL4 (ALR) is most similar to MLL3, and these two are putative orthologs of the *Drosophila* trithorax-related protein Trr. According to the recently adopted nomenclature for histone-modifying enzymes, MLL1 (MLL, HRX) is KMT2A, MLL2 (HRX2) is KMT2B, MLL3 is KMT2C, and MLL4 (ALR) is KMT2D (1).

Microarray data accession numbers. All ChIP-chip data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE18258.

RESULTS

Genome-wide analysis of Mll1-regulated genes. MLL1 has been shown to associate with most transcriptionally active genes, although Mll1-deficient cells show no bulk changes in H3K4 methylation (10–12). It is possible that loss of this specific methyltransferase activity can be compensated by other family members and that MLL1 activity is only required at a few specific sites. To define functional targets of the MLL family, we assessed H3K4me3 levels in *Mll1*$^{+/+}$ and *Mll1*$^{-/-}$ MEFs (13, 60) by performing ChIP-chip analysis on microarrays containing 16,327 mouse gene promoters. Peaks of H3K4me3 were detected at 10,041 genes in the *Mll1*$^{+/+}$ MEF cells. Reduction of H3K4me3 levels in the *Mll1*$^{-/-}$ MEFs was only observed at 525 promoters (Fig. 1A; also see Table S1 in the supplemental material), comprising roughly 5% of promoters harboring H3K4me3 peaks. This reflects a surprisingly small number of promoters affected by Mll1, considering its widespread association with active genes. We validated these results by repeating the ChIP experiment and by measuring H3K4me3 enrichments using qPCR. We chose 45 genes showing the largest decreases in H3K4me3 levels and 10 unchanged genes from the microarray analysis for validation by qPCR (Fig. 1B). The results of these "manual ChIP" analyses showed strong correspondence to changes observed in the ChIP-chip data, verifying the robustness of the microarray results.

To determine the effect of loss of Mll1-dependent H3K4me3, we performed gene transcriptional profiling in *Mll1*$^{+/+}$ and *Mll1*$^{-/-}$ MEFs. Out of 20,125 genes on the array, 2,265 genes were upregulated and 2,459 genes were downregulated upon loss of *Mll1* (Fig. 1C; also see Table S2 in the supplemental material). Comparing these results with the ChIP-chip data, of the 525 genes with reduced levels of H3K4me3 in the absence of Mll1, 299 (60%) also displayed a reduction in the levels of gene expression (Fig. 1D and E; also see Tables S3 and S4 in the supplemental material). Therefore,
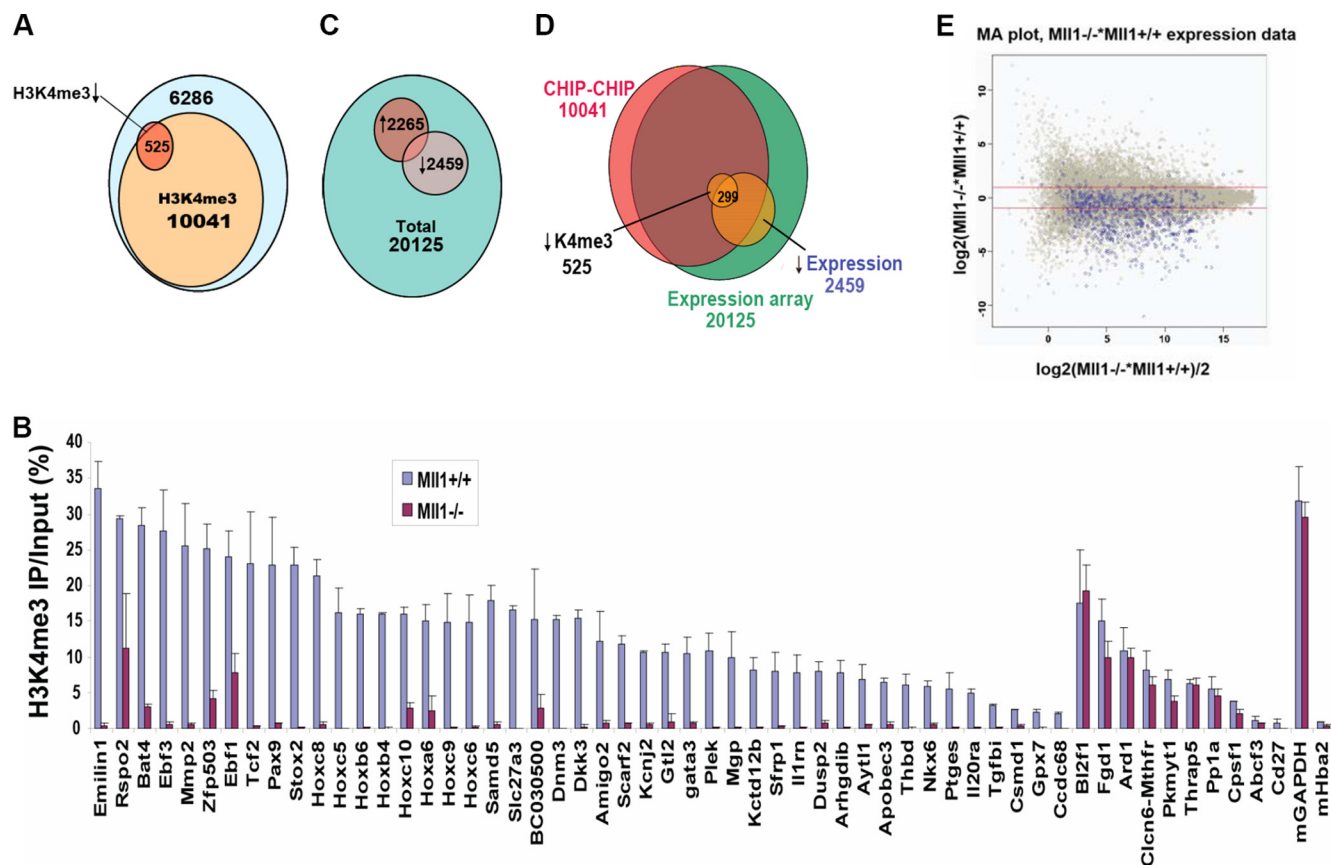
FIG. 1. Identification of genes requiring Mll1 for H3K4 methylation and gene expression. (A) Numbers of promoters with changes in H3K4me3 levels as assessed by ChIP-chip with H3K4me3 antibodies in $Mll1^{+/+}$ and $Mll1^{-/-}$ MEFs. Ten thousand forty-one out of 16,852 gene promoters had detectable peaks of H3K4me3 in wild-type MEFs. Only 525 (5%) of these genes showed significant loss of H3K4me3 in the absence of Mll1. (B) ChIP-qPCR analysis confirmed the H3K4me3 changes on 45 of the genes found by ChIP-chip analysis to be most changed for H3K4me3, as well as the ChIP-chip results for 10 unchanged genes. The *Hemoglobin* gene, inactive in fibroblasts (*Hba2*), was used as a negative control for H3K4me3, and the housekeeping gene *Gapdh* was used as a positive control for the presence of H3K4me3. Error bars show standard deviations. (C) Gene expression analysis of 20,125 genes was performed with RNA isolated from $Mll1^{+/+}$ and $Mll1^{-/-}$ fibroblasts. The expression of 2,265 genes is upregulated and that of 2,459 genes is downregulated in the absence of Mll1. (D) Overlap of H3K4me3 and gene expression. About 299 of the 525 genes with loss of H3K4me3 also have decreased expression in the absence of *Mll1*. (E) Microarray (MA) plot of the gene expression data, with the 525 genes with significantly reduced H3K4 methylation highlighted in blue. The *y* axis represents the intensity ratio of mutant over wild type, and the *x* axis represents the average intensity of each spot. The red lines indicate a twofold up- or downregulation of expression. Most of the genes dependent on Mll1 for H3K4me3 show reduced expression.

it appears that in these MEFs, approximately 3% of genes require Mll1's methyltransferase activity for gene expression.

Role of H3K4me3 in gene regulation. In the yeast *S. cerevisiae*, loss of the sole H3K4 methyltransferase (COMPASS) has little effect on the recruitment of RNA polymerase II (Pol II) and the expression of the majority of genes (21, 30, 37). COMPASS is recruited to active genes by polymerase-associated factor (the Paf1 complex), and H3K4me3 only marks actively transcribed genes (21, 55). This relationship was also observed in a reconstituted transcription system containing the mammalian homologs of these factors (39). However, given the reduced expression of genes in the absence of Mll1-dependent methylation, we investigated the recruitment of Pol II to Mll1-regulated genes by performing Pol II ChIP on two neighboring genes, *Il20ra* (whose H3K4 trimethylation is dependent on Mll1) and *Pex7* (whose H3K4 trimethylation remains unchanged after loss of Mll1) (Fig. 2A and B). Primers spanning different regions of each gene were used to measure H3K4me3

levels and Pol II occupancy. While *Pex7* had unchanged H3K4me3 and Pol II levels, both H3K4me3 and Pol II were markedly reduced at the *Il20ra* gene in the $Mll1^{-/-}$ cells (Fig. 2B and C). Based on the model by Vermeulen and colleagues that H3K4me3 recruits TFIID through the TAF3 plant homeodomain (PHD) finger (54), we performed TBP ChIP on the *Pex7* and *Il20ra* genes and found that TBP recruitment to *Il20ra* is significantly reduced in the $Mll1^{-/-}$ cells (Fig. 2D). The requirement of Mll1 for recruitment of the basal transcriptional machinery and Pol II reveals a difference between the role of the mammalian Mll1 methyltransferase and that of the related Set1/COMPASS in yeast, where the primary function of H3K4me3 appears to occur after Pol II recruitment.

Mll1 targets transcriptional regulators. Analysis of the 299 genes that require Mll1 for both H3K4 trimethylation and transcription reveals an abundance of transcriptional regulators (~55), cellular signaling pathways (~30), and signal transduction components (~30) that play key roles in regulating
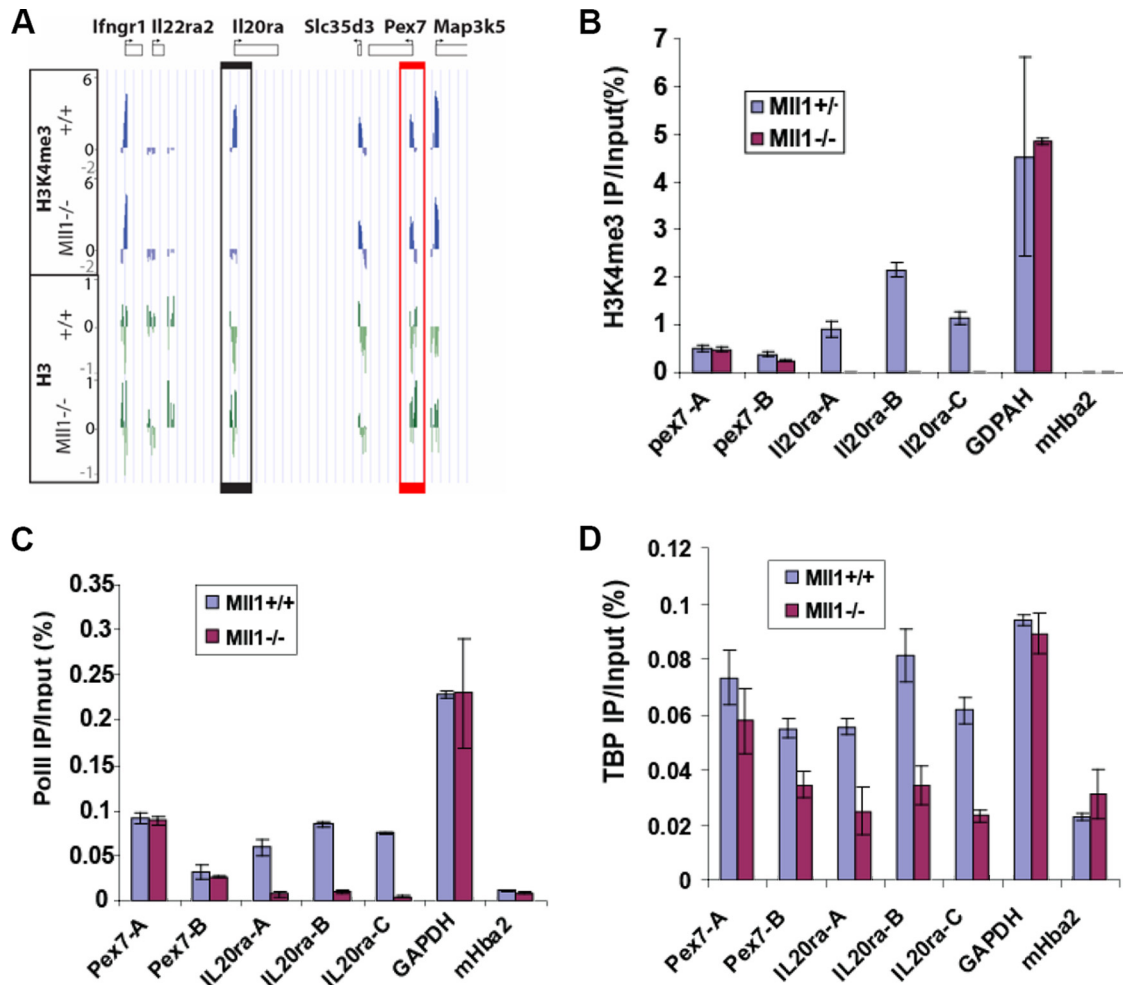
FIG. 2. Mll1 is required for the recruitment of the basal transcription machinery to chromatin. (A) The H3K4me3 level in the absence of Mll1 as determined by ChIP-chip analysis is dramatically decreased at the *Il20ra* promoter region (black box) but not at the neighboring *Pex7* gene (red box). Enrichment profiles for H3K4me3 and total H3 expressed as $\log_2$ ChIP/input. (B) ChIP-qPCR with H3K4me3 antibodies was used to validate the results shown in panel A, using three primer sets spanning the transcription start site. (C and D) The results of ChIP-qPCR with Pol II antibodies (C) or TBP antibodies (D) show that RNA polymerase and TBP recruitment to *Il20ra* are significantly decreased in the absence of Mll1. Pol II and TBP recruitment on the *Pex7 and Gapdh* genes, however, did not change in the absence of Mll1. The murine *Hba2* gene (mHba2), which is not transcribed in these cells, is used as a negative control. Error bars show standard deviations.

developmental processes (see Table S4 in the supplemental material). For example, downregulated expression of genes encoding multiple extracellular (*Dkk3*, *Sfrp1*, *Sfrp2*, and *Frzb*) modulators and one intracellular (*Frat2*) modulator of the Wnt pathway are revealed. While the gene expression of representatives of many of the major classes of transcription factors is affected upon loss of *Mll1* (e.g., *Pax9*, *Pitx2*, *MafB*, *Egr3*, *Foxc1*, *Stat5a*, *Sox5*, and *Zic*), members of the largest class (24 genes), including 11 members of the clustered *Hox* genes, encode homeobox-containing proteins. These observations are interesting in light of the established role of Mll1 and *Hox* genes in regulating hematopoiesis and the leukemogenic process (6, 22, 35, 36). Furthermore, the Wnt pathway plays an important role in specifying mesoderm differentiation toward a hematopoietic fate, in part through regulation of *Hox* genes (26, 28), suggesting that *Mll1* might also modulate signaling pathways, such as Wnt, that have an impact upon *Hox* gene regulation.

Mll1 specialization at the *Hox* loci. *Mll1*, like its *Drosophila* homolog *Trithorax*, is well-known as an important regulator of *Hox* gene expression. We therefore focused our attention on the changes in H3K4me3 levels at the promoters of the *Hox* genes by utilizing ChIP-chip on a promoter array. Comparison of the H3K4 trimethylation patterns of $Mll1^{+/+}$ and $Mll1^{-/-}$ MEF cells revealed that Mll1 does not exert a common function in all four *Hox* clusters or for all genes within a cluster. Instead it appears to exert a more selective role in targeting the *Hox* clusters and genes. For example, the *Hoxc* complex is strongly affected by loss of Mll1 in these cells, with nearly all of the promoters showing markedly reduced H3K4me3 levels in the $Mll1^{-/-}$ cells (Fig. 3A). In sharp contrast, promoters in the *Hoxd* complex are largely insensitive to the loss of *Mll1*. The *Hoxa* and *Hoxb* clusters display a mixed pattern where a few of the promoters (e.g., *Hoxa6* and *Hoxb4*) display Mll1-dependent H3K4 trimethylation (Fig. 3A). We also performed qRT-
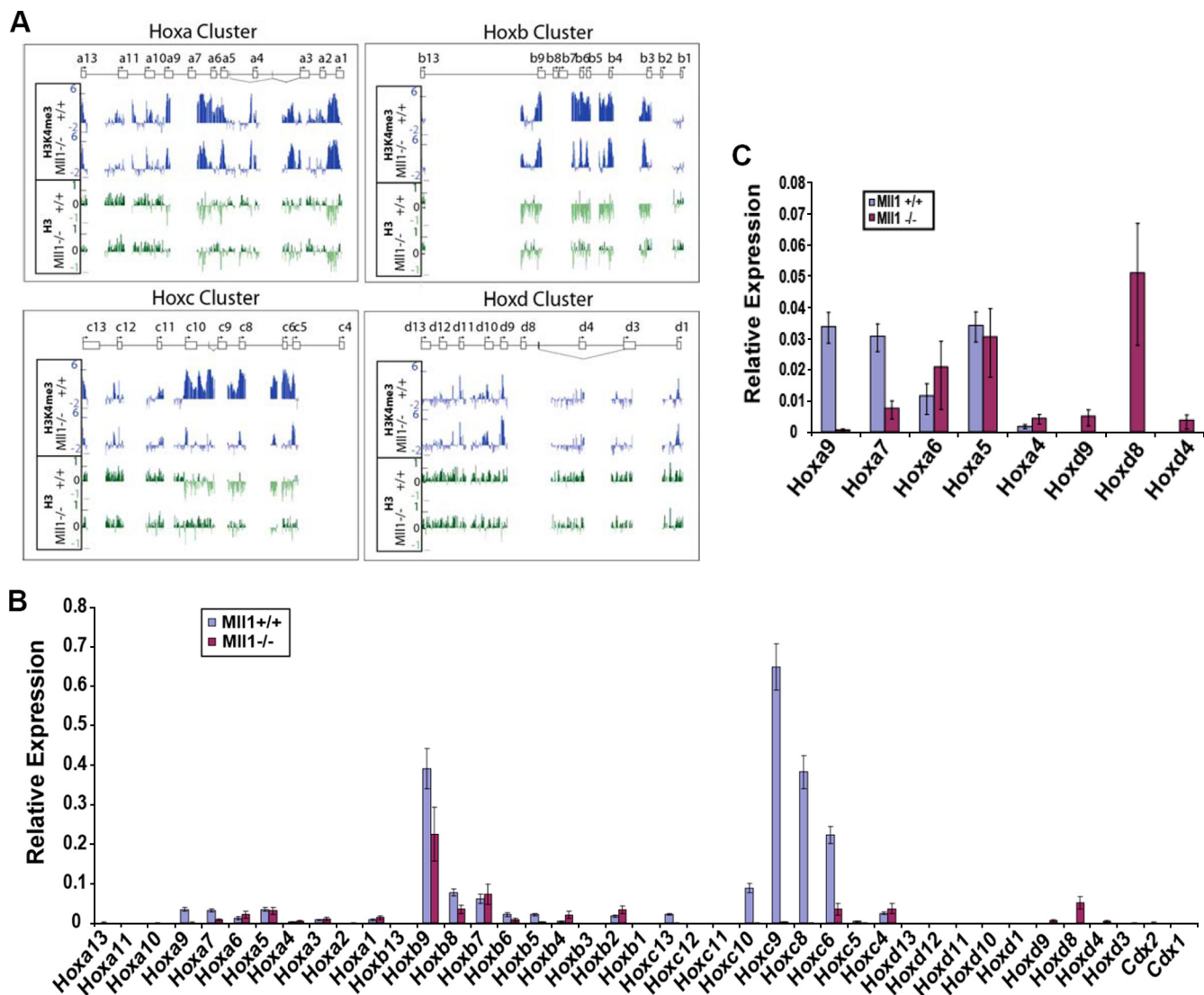
FIG. 3. Mll1 is required for the H3K4 methylation and transcription of a subset of *Hox* genes. (A) Distribution of H3K4me3 and total H3 across 8-kb regions of each *Hox* gene promoter (5.5 kb before the transcription start site and 2.5 kb after the transcription start site) on the Agilent promoter array as determined by ChIP. Each individual *Hox* gene is represented as a box with an arrow indicating the major transcription start site and the direction of transcription. Vertical lines represent small alternative exons, and diagonal lines represent alternative splicing events. Reductions in H3K4me3 can be seen at several locations across the *Hoxa*, *Hoxb*, and *Hoxc* clusters. In contrast, *Hoxd* genes show little loss of H3K4me3 in *Mll1⁻/⁻* cells. (B) qRT-PCR analysis of the expression of *Hox* genes in *Mll1⁺/⁺* and *Mll1⁻/⁻* fibroblasts normalized to the average value of reference genes *Actb* and *Tbp*. (C) Rescaling of the RT-PCR data presented in panel B. Shown are selected *Hoxa* and *Hoxd* genes, both of which have relatively low levels of expression in these fibroblasts. Some *Hoxd* genes that are not expressed in wild-type MEFs are expressed in the *Mll1⁻/⁻* MEFs. Error bars show standard deviations.

PCR analysis of the 39 murine *Hox* genes in *Mll1⁺/⁺* and *Mll1⁻/⁻* MEFs to assess the role of Mll1 in *Hox* gene expression (Fig. 3B and C). Consistent with the analyses from the promoter array and transcriptional profiling, Mll1 has a major role at *Hoxc* in these cells. Several genes from the *Hoxa* and *Hoxb* clusters show reduced levels of expression in the absence of Mll1, although the expression levels of the *Hoxa*, *Hoxb*, and *Hoxd* genes are relatively low in these fibroblasts.

The presence of Mll1 activity at *Hox* promoters led us to more systematically investigate whether Mll1 affected other regions throughout the *Hox* clusters. To this end, we utilized a high-density tiling microarray, designed in-house, that cov-

ers the entire region of the murine *Hoxa*, *Hoxb*, and *Hoxd* complexes. These arrays were probed with DNA obtained with H3K4me3, H3K4me2, RNA Pol II, and total H3 antibodies from *Mll1⁺/⁺* and *Mll1⁻/⁻* cells (Fig. 4 and 5). The results of our analyses reveal that the regions of H3K4 methylation dependent on Mll1 are quite extensive, including not just promoters but also coding and some intergenic regions (Fig. 4). At several *Hox* genes, particularly the *Hoxa6-Hoxa9* and *Hoxb5-Hoxb8* genes, the levels of both H3K4me3 and H3K4me2 decrease (Fig. 4), consistent with the ability of MLL1 to mediate di- and trimethylation of H3K4 in vitro (57). However, some regions showing loss of
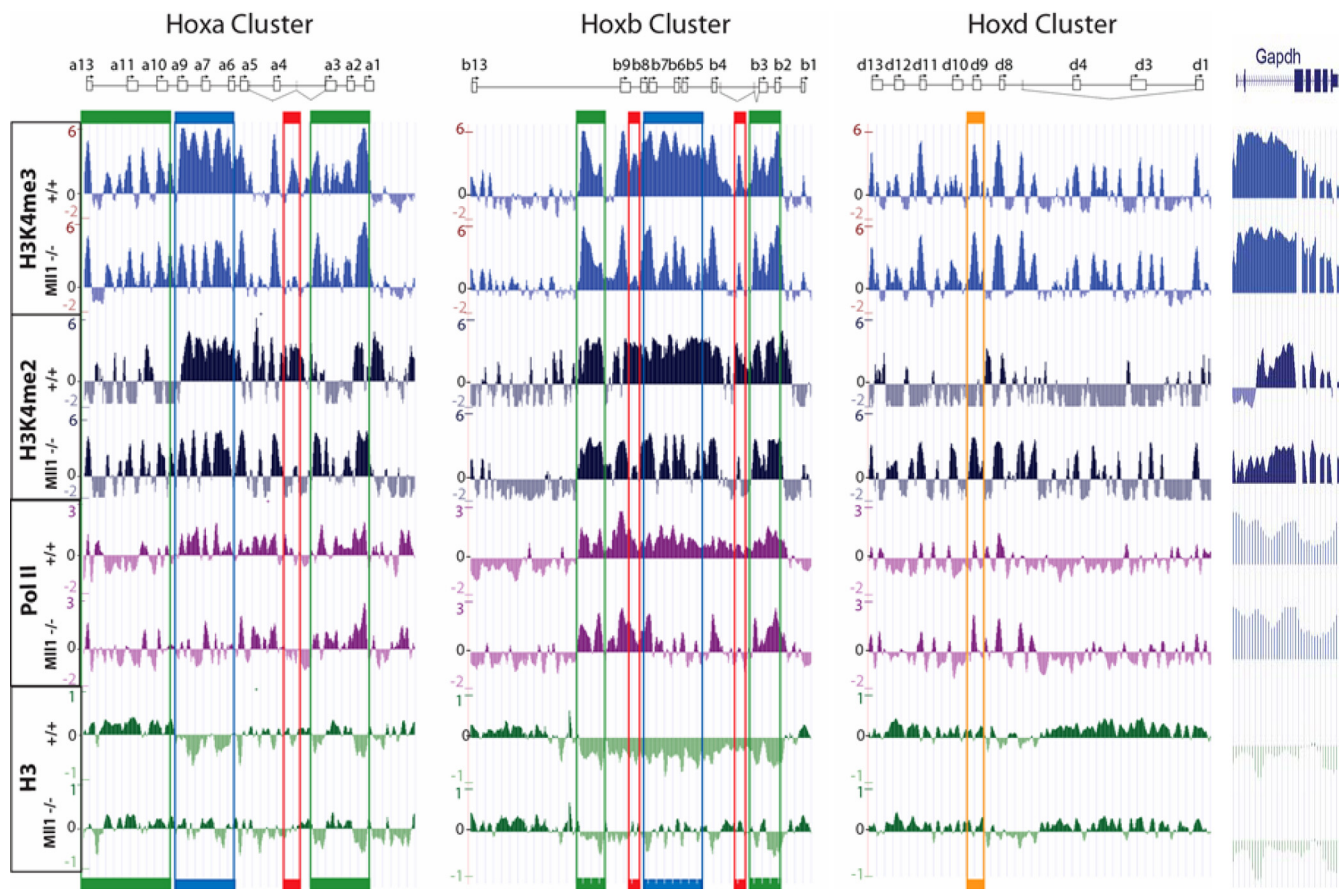
FIG. 4. H3K4 methylation is broadly lost at some coding and intergenic regions of *Hox* genes in the absence of *Mll1*. H3K4me3, H3K4me2, RNA Pol II, and total H3 profiles were determined across the *Hoxa*, *Hoxb*, and *Hoxd* loci by using a custom Agilent tiling array in the presence and absence of *Mll1*. Blue boxes indicate regions showing large reductions in both H3K4me3 and Pol II occupancy in the absence of Mll1. Green boxes indicate regions with unchanged H3K4 methylation and unchanged RNA Pol II levels. The orange box indicates a region in the *Hoxd* locus which shows little change in the H3K4me3 level but notable increases in H3K4me2 and Pol II occupancy in the *Mll1*⁻/⁻ cells. Red boxes indicate three regions of interest that correspond to the location of putative alternative promoters and enhancers regulated by Mll1. Also shown is the *Gapdh* gene, which is a control region on the *Hox* tiling array. Schematics for the *Hox* clusters are described in the Fig. 3A legend.

H3K4me3 in the absence of Mll1 still have residual peaks of this modification (Fig. 5). Sometimes, the region in between the H3K4me3 peaks and the H3K4me2 pattern "inverts" in the mutant, with valleys of H3K4me2 in the wild-type showing peaks of this modification, while peaks of H3K4me2 in the wild type decrease in the mutant. Together these results suggest some redundancy or interplay among different H3K4 methyltransferases at the Hox loci.

Intriguingly, some genes that exhibit no significant or detectable decrease of H3K4me3 levels in the *Mll1*⁻/⁻ cells show increased levels of H3K4me2. This is clearly observed for nearly all members of the *Hoxd* complex (for example, see the results for the *Hoxd9* cluster in Fig. 4). These changes in dimethylation appear significant, as *Hox* genes and other loci showing selectively increased H3K4me2 frequently displayed increased expression (Fig. 4; also see Table S2 in the supplemental material). For example, expression of *Hoxd9*, *Hoxd8*, and *Hoxd4* is not detectable in *Mll1*⁺/⁺ MEFs, but in the absence of Mll1, expression is observed for these genes (Fig. 3C).

Several regions of the *Hox* complexes with large reductions in H3K4 methylation in *Mll1*⁻/⁻ MEFs also have significant drops in RNA Pol II levels (Fig. 4). This is in agreement with the hypothesis that in mammalian cells, MLL1-mediated H3K4 methylation helps to recruit RNA Pol II, in contrast to the established roles for COMPASS in yeast. Accordingly, those regions not dependent on Mll1 for H3K4 trimethylation have normal levels of Pol II (Fig. 4). Furthermore, some regions with increased H3K4me2 (e.g., *Hoxd9*) also have increased levels of Pol II (Fig. 4), thus showing a correlation between changes in H3K4 methylation and Pol II recruitment in mammals.

ChIP with an antibody that recognizes all forms of histone H3 shows no large-scale differences between *Mll1*⁺/⁺ and *Mll1*⁻/⁻ MEFs, although regions showing loss of both H3K4me3 and RNA Pol II generally have low levels of H3 in wild-type cells and a modest increase in H3 levels in the *Mll1*⁻/⁻ cells (Fig. 4). This is consistent with lower nucleosome density at actively transcribed genes that is due to nucleosome eviction (34). For example, in the *Hoxd* locus, where there are
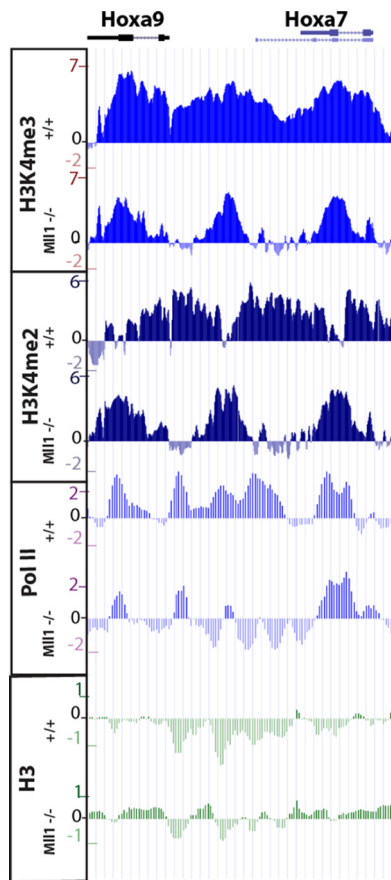
FIG. 5. Enlargement of the results for the Hoxa9-Hoxa7 region shown in Fig. 4. Some regions showing loss of H3K4me3 in the absence of Mll1 still have residual peaks of this modification. In the region in between the H3K4me3 peaks, the H3K4me2 pattern "inverts" in the mutant, with valleys of H3K4me2 in the wild-type now showing peaks of this modification. Accordingly, peaks of H3K4me2 in the wild type decrease in the mutant. Together, these results suggest some redundancy or interplay among different H3K4 methyltransferases at the Hox loci.

generally lower levels of transcription, we find higher levels of H3 and lower levels of RNA Pol II than at *Hoxa* and *Hoxb*.

Identifying new features of *Hox* complexes. The organization and regulation of clustered *Hox* genes in vertebrates is a diverse and complex process, with alternative promoters and splicing, tissue-specific enhancers, global/long-range regulatory inputs, and the presence of noncoding RNA transcripts. While H3K4me3 is a common landmark for promoter regions of active genes and might serve to identify novel promoters within the *Hox* complexes, it might reveal additional features which participate in gene regulation. Therefore, we carefully examined results from the high-density tiling arrays for differences in H3K4me3 levels across the entirety of the complexes. In the intergenic region between *Hoxb3* and *Hoxb4*, there is a region that is highly methylated at H3K4 in *Mll1*$^{+/+}$ cells that has greatly reduced H3K4 methylation in *Mll1*$^{-/-}$ cells (Fig. 4). This region precisely corresponds to a previously identified alternative promoter for *Hoxb3* in the nervous system (46), indicating that this promoter is also recognized in MEF cells. A similar change in pattern is seen at the corresponding location

in the *Hoxa* locus where a peak of H3K4me3 in the intergenic region between *Hoxa4* and *Hoxa3* is reduced in *Mll1*$^{-/-}$ cells (Fig. 4). This suggests that this region corresponds to an alternative promoter for *Hoxa3*, similar to the alternative promoter at *Hoxb3*, which has been conserved following the duplication and divergence of the vertebrate clusters.

Another region of interest on the *Hoxb* tiling array is located between *Hoxb9* and *Hoxb8* (Fig. 4). While there is no evidence for alternative promoters in this location, this region is known to contain an enhancer for *Hoxb8* that directs expression in mesodermal tissues (4). Genome-wide data suggest that monomethylation of H3K4, not trimethylation, typically marks enhancers (14), but this may vary for specific genes. The selective changes in H3K4 methylation that we observed upon loss of Mll1 not only point out the potentially unique and complex organization of the *Hox* loci but illustrate that novel noncoding functional elements within the *Hox* clusters might be discovered by analyzing histone modification changes in different genetic and developmental states.

The role of other MLL family members at *Hox* loci. Our studies have uncovered a specialized or selective requirement for Mll1 in *Hox* regulation. However, Mll1 might play a larger role in regulating these genes which is masked by functional compensation from the other five H3K4 methylases. Based on common and distinct components of the protein complexes they participate in, the MLL family can be divided into two groups, Mll1/Mll2 and Mll3/Mll4 (Fig. 6B). Mll1 and Mll2 share the highest similarity to the *Drosophila Hox* gene regulator Trithorax and can form complexes with the tumor suppressor menin. Menin has previously been shown to be important for the recruitment of MLL1 to target genes (33). We performed H3K4me3 ChIP-chip analyses on a *Hox* tiling array of genes from wild-type and *Men1*$^{-/-}$ MEFs. In contrast to the loss of Mll1, the absence of menin results in a profound reduction in H3K4 methylation across the entirety of the *Hoxa*, *Hoxb*, and *Hoxd* clusters (Fig. 6A). The results of RT-PCR analyses also show that menin is required for the expression of all the detectably expressed *Hox* genes in these cells (Fig. 6C). Together, these results reveal that in MEFs, Mll1 and Mll2 function as the major H3K4 trimethylases at *Hox* loci and are key regulators of *Hox* gene expression.

The other two MLL family members, MLL3 and MLL4, were recently found to form complexes with PTIP, the nuclear receptor coactivator Ncoa6, and the H3K27 demethylase UTX (5, 16, 38). To explore the roles of this subfamily in *Hox* regulation, we performed H3K4me3 ChIP-chip on the *Hox* tiling array using *Mll3*$^{-/-}$ and *PTIP*$^{-/-}$ fibroblasts. The loss of Mll3 exerted few or no changes in H3K4me3 levels across the *Hox* clusters (Fig. 7A). RT-PCR analysis of *Hox* genes from *Mll3*$^{+/+}$ and *Mll3*$^{-/-}$ fibroblasts showed modest and variable changes in *Hox* gene expression in these cells (Fig. 7B). We also performed RT-PCR analysis of *Hox* gene expression in *PTIP*$^{+/+}$ and *PTIP*$^{-/-}$ fibroblasts and saw few changes in the expression of *Hox* genes with the loss of PTIP, a unique component of the MLL3/MLL4 complexes shown to be required for H3K4 methylation at a Pax-dependent reporter gene (Fig. 8A) (38). Furthermore, RNA interference-mediated knockdown of a unique component of the Set1A/Set1B complex, the Wdr82 protein, resulted in few or no changes in *Hox* expression (Fig. 8B). We did not analyze Ash1, the remaining enzyme
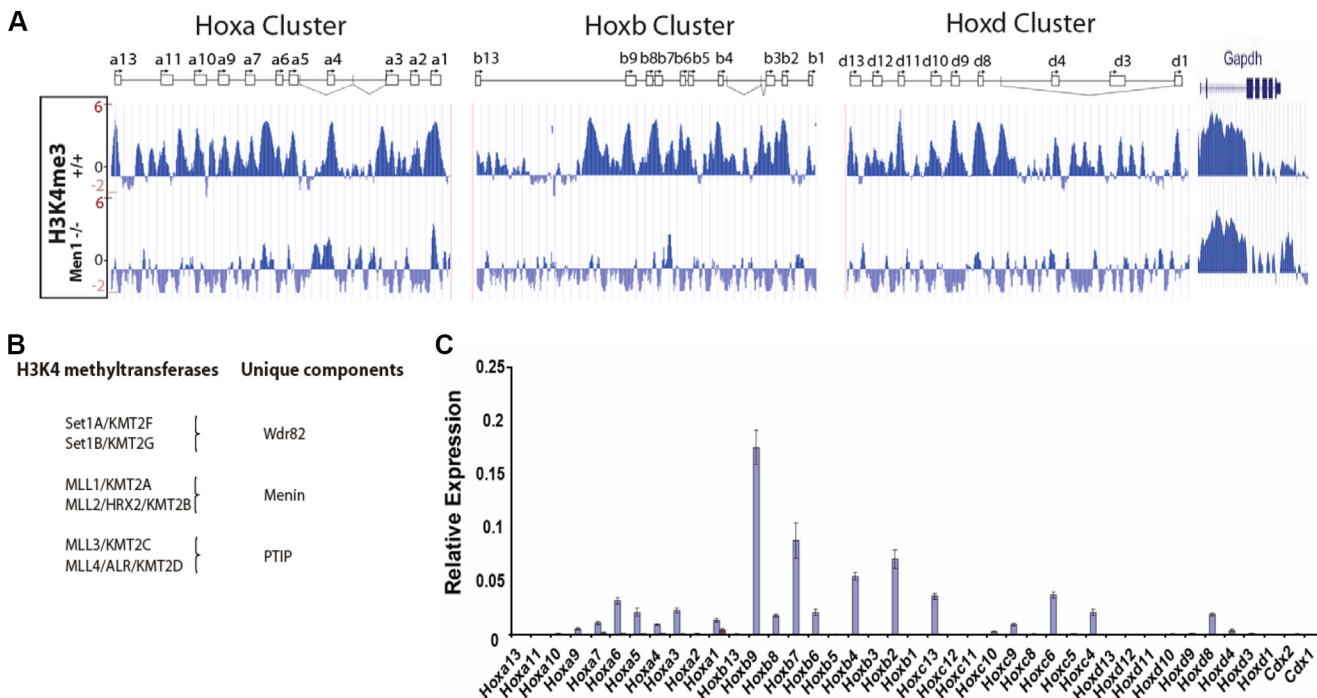
FIG. 6. Menin, a common component of Mll1 and Mll2 complexes, is required for the expression of nearly all Hox genes and for H3K4 trimethylation at Hox loci. (A) H3K4me3 ChIP profiles from $Men1^{+/+}$ and $Men1^{-/-}$ fibroblasts show that the vast majority of H3K4 methylation at *Hox* loci depends on menin. Schematics for the *Hox* clusters are described in the Fig. 3A legend. (B) Schematic of the association of subunits unique to Mll1/Mll2, Mll3/Mll4, and Set1A/Set1B complexes. Menin is found in Mll1/Mll2 but not other H3K4 methyltransferase complexes, PTIP is unique to Mll3/Mll4 but not other H3K4 methyltransferase complexes, and WDR82 is unique to the Set1A/Set1B but not the Mll1-to-Mll4 complexes. Alternate names for MLL2 and MLL4 are included to avoid confusion over alternative naming systems. (C) qRT-PCR analysis of *Hox* gene expression shows near total loss of *Hox* gene expression in $Men1^{-/-}$ fibroblasts. Error bars show standard deviations.

reported to methylate H3K4 in mammals; Ash1 has been shown previously to be present at *Hox* genes but did not appear to affect H3K4 methylation levels (10). Together, the results of these analyses suggest that the Set1A/Set1B and Mll3/Mll4 complexes have relatively minor roles in controlling Hox expression in MEFs, whereas they provide support for the menin-containing Mll1/Mll2 complexes in mediating the majority of H3K4 trimethylation at *Hox* loci and serving as key regulators of *Hox* transcription.

DISCUSSION

We have shown that a small but important subset of genes requires Mll1 for H3K4 trimethylation and gene expression. Although Mll1 is known as a major regulator of *Hox* gene transcription, we find a highly specialized role for Mll1 within the *Hox* clusters, affecting a subset of *Hox* genes. In addition to *Hox* genes, Mll1 is also a key regulator of other master regulators of transcription. Indeed, while the manuscript was under review, one of the 299 genes that we classify as functional targets of Mll1 was reported as an Mll1-regulated gene required for neurogenesis (27). Investigation of this set of Mll1-regulated genes could also potentially reveal additional effectors of leukemogenesis. The correlation between Mll1-dependent H3K4 methylation and Pol II recruitment reveals a key distinction between Mll1 and the yeast Set1/COMPASS.

Differences between MLL and COMPASS methyltransferases. Most of the work on the implementation of H3K4 methylation comes from the yeast *S. cerevisiae*, where a single methyltransferase complex, Set1/COMPASS, is recruited to genes by the ubiquitination of histone H2B, requiring prior recruitment of Pol II and the PAF1 complex (21, 48, 55). Work in mammalian systems has primarily focused on MLL1, which is targeted by reciprocal translocations with heterogeneous partner genes fused in-frame to *MLL1*, encoding fusion proteins and associated with both acute lymphoblastic and myeloblastic leukemia (43, 53). Recent work by our lab indicates that although COMPASS and MLL1 form very similar complexes, they could have different functions (24, 57). COMPASS contains a unique subunit, Cps35, which mediates the recruitment of COMPASS to chromatin by ubiquitinated histone H2B (24). A human Set1/COMPASS complex containing a Cps35 homolog (WDR82) is also regulated by H2B ubiquitination and mediates the vast majority of H3K4 trimethylation in mammals (57). In contrast, $Mll1^{-/-}$ fibroblasts show no observable loss of H3K4 methylation in assays of bulk histones (31, 57). This may be explained by our detailed biochemical studies, which indicate that the MLL1 complex lacks a Cps35-like subunit (57), suggesting that it is recruited to chromatin by a different mechanism than Set1/COMPASS. In further support of these differences, the Set1/COMPASS in yeast is not important for Pol II occupancy on promoters, while the loss of Mll1 in MEFs results in reductions in Pol II occupancy on Mll1's functional targets.

The relationship between H3K4 methylation and gene activation could be explained by Vermeulen and colleagues' ob-
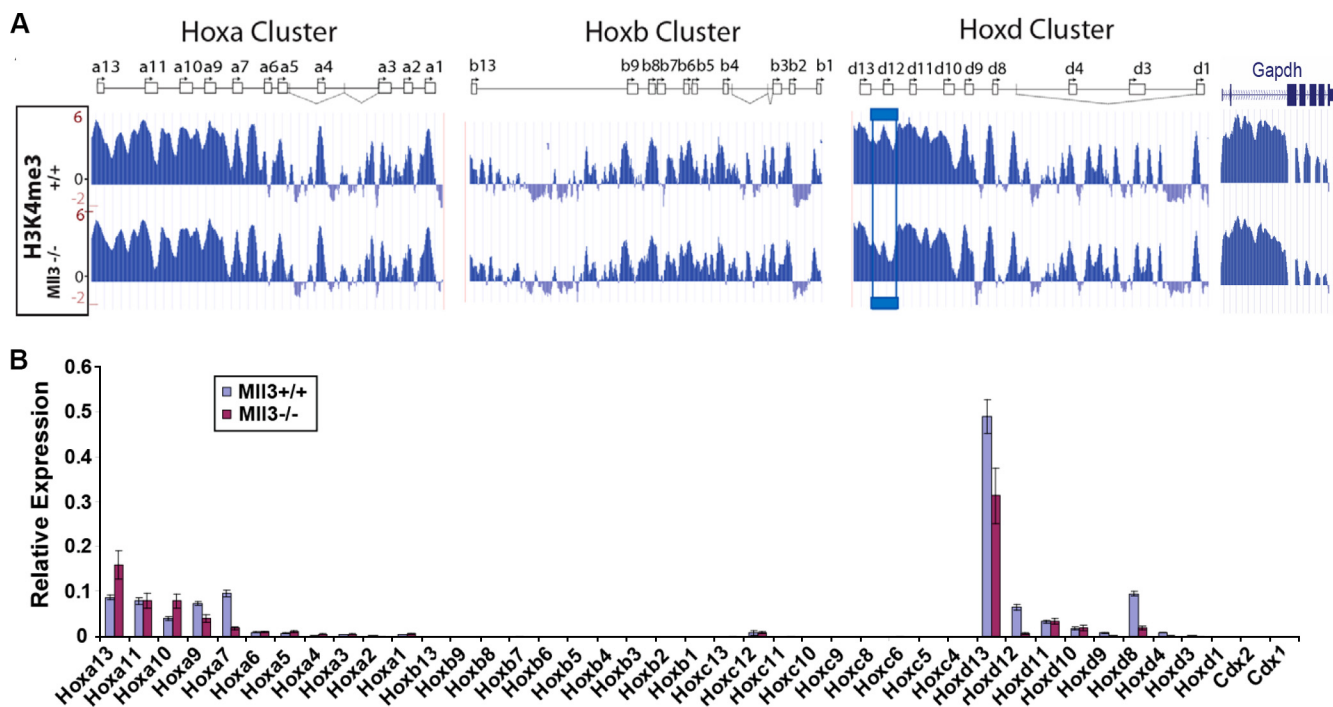
FIG. 7. Mll3 is not a major H3K4 histone methyltransferase at Hox loci in fibroblasts. (A) H3K4me3 ChIP profiles from $Mll3^{+/+}$ and $Mll3^{-/-}$ fibroblasts show relatively minor effects on the *Hox* gene H3K4me3 pattern by Mll3. A blue box outlines the results for the *Hoxd12* gene, where a significant reduction in H3K4me3 can be observed in Mll3$^{-/-}$ fibroblasts. Schematics for the *Hox* clusters are described in the Fig. 3A legend. (B) qRT-PCR analysis of *Hox* gene expression reveals minor effects of Mll3. Hoxd12 shows reductions in both H3K4me3 and gene expression in $Mll3^{-/-}$ fibroblasts. Error bars show standard deviations.

servation that the PHD domain of TAF3 is required for TFIID and, thereby, Pol II recruitment and transcriptional initiation (54). Similarly, several nucleosome remodeling complexes, such as NURF and chromodomain helicases, harbor H3K4me3 binding domains which could indirectly aid in the recruitment of the basal transcriptional machinery (41, 50, 58). ING proteins, bearing PHD fingers that have been shown to bind methylated H3K4, are found in many MYST family histone acetyltransferase complexes, so there appear to be multiple pathways for activating gene expression through H3K4 methylation (7, 40, 47). A requirement for MLL1-dependent H3K4 methylation could be restricted to a subset of genes lacking canonical core promoter elements (17). Interestingly, *Hox* genes are mostly TATA-less, suggesting that TFIID recruitment to these genes could be enhanced by prior methylation of H3K4 by MLL family proteins.

The MLL family and *Hox* gene regulation. Mll1 is well known as an important regulator of *Hox* gene expression during development, and misexpression of *Hox* genes can lead to leukemia (6, 60). Previous studies have individually found that some Hox genes are affected and some other Hox genes are not affected by the loss of Mll1 or Mll2 (8, 9); however, a *Hox*-wide analysis of Mll1 and Mll2 regulation has not previously been fully addressed. Here, we have shown that Mll1 is responsible for diverse and selective changes in the patterns of H3K4 methylation in *Hox* complexes and that this affects the levels of gene expression of a fraction of the *Hox* genes. Our results open the possibility that specific subsets of *Hox* genes are differentially regulated by distinct members of the MLL

family. Our analyses of $Men1^{+/+}$ and $Men1^{-/-}$ MEFs implicate Mll2 as the other major agent of H3K4 trimethylation at these genes. Furthermore, our results with $Mll3^{-/-}$, $PTIP^{-/-}$, and *Wdr82* knockdown cells further indicate that other MLL family members contribute to different subsets of targets than Mll1/2.

In these studies, we have taken advantage of the availability of fibroblast cell lines (MEFs) derived from knockout mouse embryos, some of which were derived by other laboratories. Isolation of MEFs from embryos of different ages and using different methods of cellular transformation will result in different *Hox* expression patterns in the resulting MEF lines. Although we cannot directly compare the regulation of a particular Hox gene in *Mll1* MEFs with the same *Hox* gene in *Men1* or *Mll3* MEFs, we can compare, for example, $Mll1^{+/+}$ to $Mll1^{-/-}$ MEFs because paired MEF cells are always derived from the same litter under identical conditions. Furthermore, by performing *Hox*-wide analysis with MEFs, we are able to obtain an overall picture of Hox gene regulation by the MLL family of proteins.

The four *Hox* clusters in vertebrates arose from an ancestral *Hox* cluster by successive duplication and divergence of the ancestral cluster (29). A large body of work has indicated that there is both functional redundancy and specialization among *Hox* paralogs in different clusters. *Hoxa* and *Hoxb* are generally more similar to each other and differ from *Hoxd* and *Hoxc* with respect to the numbers of genes, patterns of expression, and positions of conserved regulatory elements within the complexes. Although there are also four MLL family proteins in mammals, we do not see a major role of Mll3/Mll4 complexes
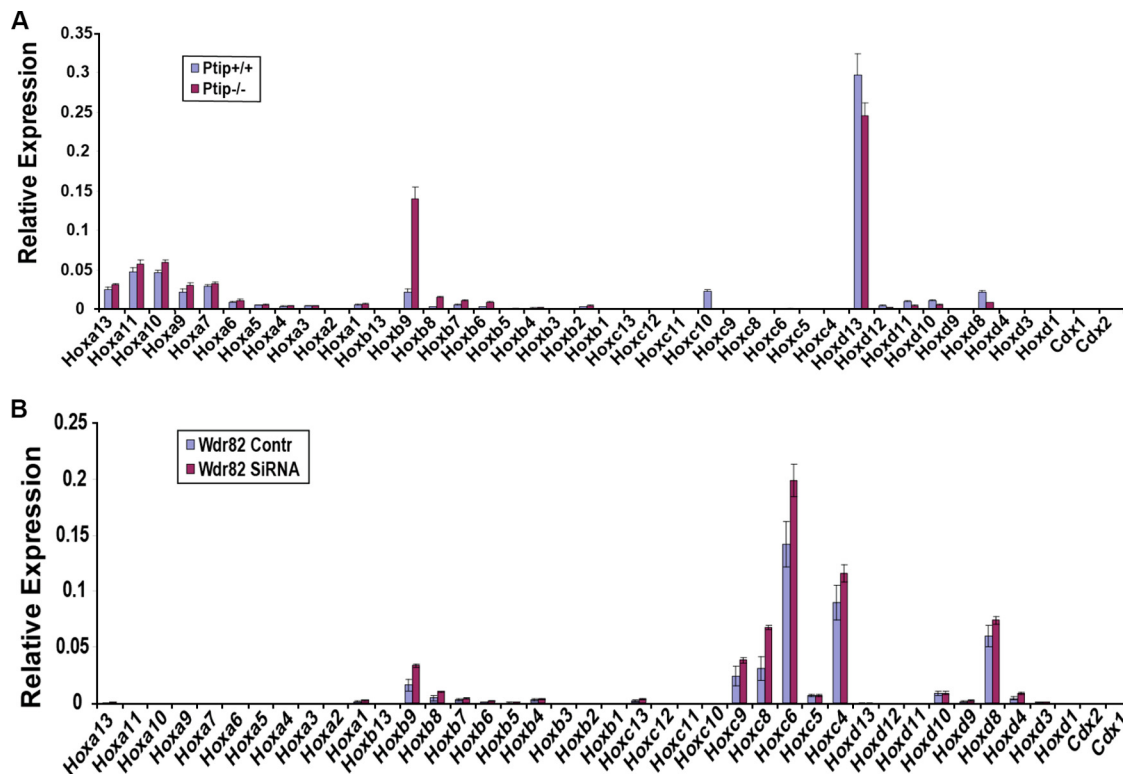
FIG. 8. qRT-PCR analysis of Hox gene expression in the presence and absence of H3K4 methylation regulators. (A) Results for RNA from *PTIP*^{+/+} and *PTIP*^{−/−} fibroblasts. (B) Results for RNA from wild-type fibroblasts treated with an siRNA to *Wdr82* or a nontargeting control siRNA. Error bars show standard deviations.

in *Hox* gene regulation in fibroblasts. Mll3 has been shown to activate *Hoxc8* in an Ap2-dependent manner (52). We also see a requirement for Mll3 in *Hoxd12* H3K4 trimethylation and gene expression in our cells (Fig. 7). Therefore, the potential for combinations of functional compensation and specialization between MLL-related proteins and the different *Hox* loci is enormous.

Additional functions of MLL1-mediated methylation. H3K4me3 has been strongly correlated with promoters of genes. In our analysis of the *Hox* loci, we observe a broad distribution of H3K4me3 across the entire complex, a result that has been previously observed (3). In the *Mll1*^{−/−} cells, we observed loss of H3K4me3 from a subset of *Hox* promoters, coding regions, and intergenic regions, suggesting that Mll1 has multiple inputs into the regulation of these genes. Some of the intergenic regions methylated by Mll1 are notable because they correspond to known or suspected alternative promoters and tissue-specific enhancers. Another intriguing aspect revealed by our study is whether Mll1-mediated methylation has distinct functions at enhancers versus promoters versus intergenic regions. One potential role for intergenic methylation is in the transcription of noncoding RNAs. In humans, a noncoding RNA transcribed in *Hoxc* helps to repress genes in *Hoxd*. The study of functional noncoding RNAs is a rapidly growing field, in large part due to the growth of resources for genome-wide analysis.

In light of the importance of *Hox* genes in development and disease processes, our current level of knowledge of regulatory components and their organization in the clusters is limited to a small subset of elements identified by focused functional assays. The correlation between H3K4 methylation patterns and specific gene activity dependent upon Mll1 illustrates the potential of systematically repeating our analyses with all of the MLL family members to uncover novel features of the *Hox* clusters in different cellular and developmental states. This could yield a wealth of novel regulatory elements for *Hox* genes that, in combination with the results of functional studies, have the potential to rapidly expand our mechanistic understanding of *Hox* gene regulation in development and disease.

MLL1 target genes and leukemia. MLL1's role in regulating *Hox* gene expression during development and the role of the translocation-associated MLL1 fusion proteins in leukemia may be tightly linked. The MLL1 portion of the fusion protein may lead to altered recruitment of complexes to *Hox* genes, resulting in altered gene expression as a key part of the multistep leukemogenic pathway (6, 12). Some of the Mll1-target genes identified by our analyses may be candidates for involvement in this process. For example, we found decreases in expression and H3K4 trimethylation at the loci of genes encoding extracellular (*Dkk3*, *Sfrp1*, *Sfrp2*, and *Frzb*) and intracellular (*Frat2*) proteins known to control the Wnt signaling pathway (Table 1; also see Table S4 in the supplemental material). Wnt signaling itself plays an important role in regulating *Hox* genes and other targets during hematopoietic differentiation (26, 28). Hence, in addition to the direct interactions we have described, Mll1 may have indirect inputs into modulating *Hox* expression in leukemia via the Wnt pathway.

TABLE 1. Top 20 genes with the K4me3 decreasing and transcriptional downregulation in the absence of Mll1

Gene	Product or other description	Expression level			ChIP-chip peak score ($\log_2$)	ChIP-qPCR fold change ($\log_2$)
		Mll1$^{-/-}$/Mll1$^{+/+}$ ($\log_2$)	P value	Adjusted P value		
Thbd	Thrombomodulin	−11.10	5.70E−05	1.39E−03	−4.75	−6.18
Kctd12b	Potassium channel tetramerization domain containing 12b	−8.08	5.86E−03	2.26E−02	−3.86	−5.46
Hoxc9	Homeobox C9	−6.53	7.14E−08	3.67E−04	−4.25	−6.04
Rspo2	R-Spondin 2 homolog (Xenopus laevis)	−6.52	4.64E−07	3.67E−04	−4.10	−1.37
Mgp	Matrix Gla protein	−6.50	5.69E−06	5.00E−04	−4.09	−6.25
Bnc1	Basonuclin 1	−8.28	1.91E−04	2.61E−03	−2.10	
Plek	Pleckstrin	−5.74	1.21E−06	3.67E−04	−4.41	−6.09
Tcf2	HNF1 homeobox B	−4.59	7.09E−06	5.43E−04	−5.28	−6.27
C4bp	Complement component 4 binding protein	−6.88	3.60E−07	3.67E−04	−2.99	
Pax9	Paired box gene 9	−6.42	1.36E−07	3.67E−04	−3.43	−5.03
9030425E11Rik	RIKEN cDNA 9030425E11 gene	−7.63	1.18E−07	3.67E−04	−2.16	
Il20ra	Interleukin 20 receptor alpha	−6.57	1.01E−06	3.67E−04	−3.20	−4.78
Abi3bp	ABI gene family, member 3 (NESH) binding protein	−7.06	6.97E−03	2.56E−02	−2.56	
Dkk3	Dickkopf homolog 3 (Xenopus laevis)	−5.95	4.85E−06	4.81E−04	−3.49	−5.80
Mafb	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)	−6.37	5.14E−06	4.85E−04	−3.01	
Ebf3	Early B-cell factor 3	−5.10	5.32E−02	1.11E−01	−4.26	−5.52
Tgfbi	Transforming growth factor, beta induced	−5.85	8.74E−07	3.67E−04	−3.51	−4.27
Pitx2	Paired-like homeodomain transcription factor 2	−6.83	6.69E−03	2.49E−02	−2.23	
Sfrp1	Secreted frizzled-related protein 1	−5.18	1.18E−03	7.62E−03	−3.79	
Enpp3	Ectonucleotide pyrophosphatase/ phosphodiesterase 3	−6.62	8.58E−03	2.96E−02	−2.30	

Since a large portion of the Mll1 target genes are known to be important transcriptional regulators of developmental processes, it would be valuable to analyze these regulators and their downstream targets in other contexts to determine whether they are also part of the gene regulatory network linked to leukemogenesis.

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