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Epigenomic analysis of the HOX gene loci reveals mechanisms that may control canonical expression patterns in AML and normal hematopoietic cells

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Abstract

HOX genes are highly expressed in many acute myeloid leukemia (AML) samples, but the patterns of expression and associated regulatory mechanisms are not clearly understood. We analyzed RNA sequencing data from 179 primary AML samples and normal hematopoietic cells to understand the range of expression patterns in normal versus leukemic cells. HOX expression in AML was restricted to specific genes in the HOXA or HOXB loci, and was highly correlated with recurrent cytogenetic abnormalities. However, the majority of samples expressed a canonical set of HOXA and HOXB genes that was nearly identical to the expression signature of normal hematopoietic stem/progenitor cells (HSPCs). Transcriptional profiles at the HOX loci were similar between normal cells and AML samples, and involved bidirectional transcription at the center of each gene cluster. Epigenetic analysis of a subset of AML samples also identified common regions of chromatin accessibility in AML samples and normal CD34⁺ cells that displayed differences in methylation depending on HOX expression patterns. These data provide an integrated epigenetic view of the HOX gene loci in primary AML samples, and suggest that HOX expression in most AML samples represents a normal stem cell program that is controlled by epigenetic mechanisms at specific regulatory elements.

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Introduction

HOX gene expression is a common feature of acute myeloid leukemia (AML), and is thought to reflect “dysregulation” of HOX pathways that lead to abnormal self-renewal and the development of leukemia. Initial studies of HOX gene expression in human hematopoietic cells showed that expression is largely restricted to hematopoietic stem/progenitor cells (1–4), which are uniquely capable of long-term self-renewal. In addition, functional studies in mice demonstrated that expression of specific HOXA and HOXB genes can lead to expansion of long-term repopulating hematopoietic stem cells and a myeloproliferative phenotype (5–9). Mice lacking specific *Hox* genes also showed deficits in the repopulating ability of hematopoietic cells in competitive transplantation experiments (10–13), although these phenotypes have been variable across studies (14).

In AML patient samples, HOX gene expression is most closely associated with translocations involving *MLL*, which characteristically show high expression of HOXA genes (15). *HOXA9* in particular has been shown to be a “target” of *MLL* fusion oncoproteins (16–18), and is required for the survival and proliferation of *MLL*-positive leukemic cells (19,20). Other AML mutations have also been associated with either the presence or absence of HOX gene expression. *MLL* partial tandem duplications (PTDs) and *PICALM-MLLT10* gene fusions have been associated with high levels of HOXA gene expression (21–23), and NPMc mutations are associated with expression of both HOXA and HOXB cluster genes in human AML samples (24,25), and in mice expressing this mutation (26). In contrast, AMLs with the *PML-RARA* and *RUNX1-RUNX1T1* gene fusions (27,28) and mutations in *CEBPA* (29) have been associated with low or absent HOX gene expression.

Although AML-associated HOX expression phenotypes are often described as “aberrant”, the specific expression patterns reported in the literature are variable and involve multiple genes from either the HOXA or HOXB gene cluster (or both) (30,31). Most studies have relied on targeted gene expression measurements of only selected HOX genes, or they have focused on AMLs with canonical somatic mutations and/or cytogenetic abnormalities. In addition, although some studies have shown that HOX genes are expressed in both AML samples and normal hematopoietic cells (25), the precise patterns of expression in normal versus malignant hematopoietic cells remains unclear. As a result, a comprehensive view of HOX gene expression patterns in AML samples—and their relationships to normal hematopoietic cells—has not yet been established. In this study, we carried out an integrated analysis of HOX gene expression using RNA-sequencing data from 179 primary AML samples that have been previously characterized by either whole-genome or whole-exome sequencing. We compared the HOX expression phenotypes in these AMLs to data from normal bone marrow cells to study the HOX regulatory programs in normal and malignant hematopoiesis. Finally, we performed high-resolution bisulfite sequencing and chromatin accessibility profiling of selected AML samples to identify changes in DNA methylation and chromatin structure at *cis*-acting regulatory elements within the HOX loci that may be involved in HOX gene regulation in AML. These analyses provide an integrated, unbiased view of HOX gene expression, and have identified potential regulatory elements that may be

important for HOX gene expression in normal hematopoietic development, and in AML cells.

Materials and Methods

Clinical and genomic data from primary AML samples

All primary AML samples used in this study were from unfractionated bone marrow cells obtained at the time of diagnosis at the Washington University School of Medicine, using protocols approved by the Human Research Protection Office following informed consent in accordance with the Declaration of Helsinki. Clinical annotation, processing, and analysis of these samples has been described previously (32).

Normal hematopoietic cell populations

Hematopoietic cells from bone marrow aspirates were obtained from three volunteer donors using protocols approved by the Human Research Protection Office following informed consent. Aspirates were processed via ammonium-chloride-potassium red cell lysis, washed, and prepared for fluorescence-activated cell sorting (FACS). A portion of each sample was used to purify promyelocytes (CD14⁻, CD15⁺, CD16^{low/-}) (33), monocytes (CD14⁺), neutrophils (CD14⁻, CD15⁺, CD16⁺) (33), T-cells (CD33⁻, CD3⁺) and B-cells (CD33⁻, CD19⁺), with the remaining cells enriched for CD34⁺ cells using bead-based enrichment (MACS human CD34 MicroBead kit, Miltenyi Biotec). CD34-enriched samples were then further purified for CD34⁺ cells via FACS. The following antibodies were used for FACS: CD34-PE (PE-pool, Beckman Coulter, PN IM1459U), CD14-APC (BD, M5E2), CD15-FITC (BD, clone HI98), CD16-PE (BD, 3G8), CD33-APC (eBioscience, clone WM-53), CD3-V450 (eBioscience, clone OKT3), and CD19-PE (BD, clone HIB19). All samples were cryopreserved in Trizol LS (Life Technologies) for subsequent RNA extraction.

RNA-sequencing, processing, and analysis

Poly-A RNA-sequencing of normal bone marrow cells was performed using established methods (32). Additional RNA-seq data from normal CD34⁺ cells were obtained from GSE48173. All RNA-seq data were processed from the raw sequencing reads using the tophat (version 2.0) and cufflinks (version 2.2) following the recommended protocols for unstranded libraries (34). FPKM gene expression values were generated simultaneously across all samples (with the “cuffnorm” command) using the Gencode version 18 annotation. Clustering analysis was performed in R using Ward’s method (Murtagh and Legendre 2013).

Nanostring nCounter gene expression analysis

Orthogonal measurements of HOX expression in AML samples and normal CD34⁺ cells were obtained using a custom Nanostring nCounter codeset targeting 54 genes, including all HOXA-HOXD genes and additional controls for expression normalization and quality control. 100 ng of total RNA was used for each assay, and all experiments were performed in duplicate. Expression values were obtained via normalization to the control genes using the NanoStringNorm package in R (35).

Microarray gene expression analysis

Microarray data from the Affymetrix U133+2 array platform were downloaded from the GEO repository, and included datasets from AML samples (GSE10358), normal bone hematopoietic cells (GSE12662), and purified hematopoietic progenitors (GSE24006). Processed data for GSE10358 were used directly from the GEO repository, but all other data were reprocessed from raw images. Signal intensities and gene expression values for these samples were processed together with the RMA processing algorithm using the *oligo* package in R (36). Clustering analysis was performed in R as above.

Bisulfite sequencing and analysis

Bisulfite sequencing was performed using either whole-genome bisulfite-converted sequencing libraries generated with the Epigenome library preparation kit, or with the Agilent SureSelect Methyl-Seq kit (Agilent, Santa Clara, CA). Indexed sequencing was performed on Illumina HiSeq 2000 instruments and reads were mapped with BSMAP using default parameters (37). Methylation values for the HOX gene clusters were obtained using the Bis-SNP program with default parameters (38). Differential methylation analysis was performed on pooled methylation data using a chi-squared test of methylated vs. unmethylated counts for each AML type, and required a bonferroni-corrected p-value of 0.05 and minimum difference between any pooled dataset of 0.5 for significance. Smoothed methylation values were generated for visualization using the *BSSeq* R package (39).

Chromatin accessibility profiling (ATAC-seq)

Transposase-mediated chromatin accessibility profiling was performed using the Nextera library preparation kit as described in (40) using 50,000 viable cells per sample. Nextera libraries were size-fractionated into small (<300 bp) and large (300–800 bp) libraries and sequenced on separate lanes of Illumina 2500 instruments (two libraries per lane). Reads were mapped with the *bwa mem* program and resulting data were merged and filtered to retain unique, properly-paired reads. Peaks were identified using *homer* (41) and coverage profiles were generated using *bamCoverage* (42).

Data availability

RNA-seq expression data from AML samples are available from the TCGA portal (<https://tcga-data.nci.nih.gov/tcga/>). Raw RNA-seq data from normal bone marrow cells, as well as ATAC-seq and bisulfite sequencing data from AML samples, will be deposited in dbGaP (<http://www.ncbi.nlm.nih.gov/gap>). Processed ATAC-seq and methylation datasets from the HOX loci are available from the authors upon request.

Results

HOX gene expression patterns in AML samples are associated with recurrent AML mutations and a canonical pattern found in normal hematopoietic progenitors

To understand the range of HOX expression patterns in AML and their relationship to recurrent AML mutations and normal hematopoietic HOX expression, we studied poly-A selected RNA-sequencing data from 179 well-characterized AML samples (32) and 29

samples from normal bone marrow cells at different stages of development (from (43), and this study). We first compared gene expression levels defined by RNA-seq to microarray expression data, and also to data from a custom Nanostring assay targeting all HOX genes. All platforms demonstrated variable levels of expression of the HOXA and HOXB cluster genes, with *HOXA3-HOXA10* and *HOXB2-HOXB6* showing the greatest expression, and no detectable expression of HOXC and HOXD genes (Figures S1A–C). HOXA and HOXB gene expression was generally concordant across the expression platforms, although RNA-seq showed better agreement with orthogonal expression measurements from the Nanostring platform than array-based expression values, perhaps due to the design of the array probes that target the HOX genes (Figures S1D, S1E).

We next explored HOXA and HOXB expression patterns in the AML samples and their relationship to somatic mutations identified by whole genome and exome sequencing. Figure 1A shows unsupervised hierarchical clustering of RNA-seq gene expression for all HOXA and HOXB genes (and the HOX cofactor *MEIS1*) from all 179 AML samples, along with cytogenetic classification and mutational status of recurrently mutated genes organized into functional categories (see Table S1). This analysis resulted in distinct HOX expression groups that were characterized by the presence or absence of expression of genes in either the HOXA or HOXB gene clusters (or both), which were associated with recurrent chromosomal rearrangements and certain somatic mutations (Table 1). Although *MEIS1* was frequently expressed in samples with HOX gene expression, the overall correlation between expression levels of *MEIS1* and HOX genes was relatively low (Figure S1F). The first HOX expression group had essentially undetectable HOX expression at both gene clusters (Figure 1A, red; mean expression across both HOXA and HOXB genes: 0.25 FPKM; range: 0–6.8 FPKM), and was comprised of ~20% of the AML samples, including nearly all samples with the *RUNX1-RUNX1T1* and *PML-RARA* gene fusions (Figure S2A). A second group had primarily HOXA expression (Figure 1A, green), and included all of the samples with *MLL* translocations (Figure S2A); this is consistent with previous studies of HOX expression in AML, and the established role of HOXA genes in *MLL* leukemias. AMLs with other mutations in the *MLL* gene, including partial tandem duplications (PTDs, n=8), were also associated with HOX expression, although these cases tended to show expression of both HOXA and HOXB genes (Figure S2B). AMLs with *inv(16)*, resulting in the *CBFB-MYH11* gene fusion, defined a third group with low to moderate expression of HOXB genes (Figure 1A, blue), and little or no HOXA gene expression (Figure S2A). The last group demonstrated moderate to high expression of a canonical subset of genes in both the HOXA and HOXB gene clusters (Figure 1A, purple), including the *HOXA3-HOXA10* and *HOXB2-HOXB6* genes, although in some cases the 5' HOXB genes *HOXB8* and *HOXB9* were also expressed. The samples in this group were comprised of primarily normal karyotype AMLs, and included nearly all of the samples with the NPMc mutation (Figure S2C), although AMLs with wild type *NPM1* were also found in this set. Statistical analysis of mutations and mutation categories with respect to these HOX expression phenotypes demonstrated that NPMc mutations, mutations in genes involved in DNA methylation (specifically *DNMT3A* mutations), and recurrent gene fusions were significantly associated with one or another of the HOX expression patterns after correction for multiple comparisons (Table 1 and Table S2).

We next compared HOXA and HOXB expression in AML to RNA-seq-based HOX expression from normal hematopoietic progenitors (CD34⁺; n=20) and myeloid cells at various stages of development, including promyelocytes (n=3), monocytes (n=3), and neutrophils (n=3). As expected, HOX genes were expressed in CD34⁺ cells (Figure 1B). Expression was substantially lower in promyelocytes, and nearly absent in more mature myeloid cells (see Figure S3A and S3B), consistent with previous studies of developmental HOX expression during hematopoiesis (1–3,44). Expression in CD34⁺ cells was also restricted to the HOXA and HOXB genes, with no expression of HOXC and HOXD genes (Figure S4A). Remarkably, this pattern was highly similar to the common HOXA/HOXB signature observed in the majority of the normal karyotype AML samples. The presence of this normal stem cell signature in AML was supported by array-based HOX expression values, and analysis of additional microarray expression data from progenitor cell populations within the CD34⁺ compartment (45) suggested that this HOX expression pattern is enriched in CD34⁺/CD38⁻ hematopoietic stem/progenitors and multipotent progenitors (Figure S4B). This observation suggests that HOX gene expression in many AML samples may not be dysregulated *per se*, but rather reflects a normal stem/progenitor cell pattern that is “captured” in AML cells.

HOX gene expression patterns correlate with patient outcomes

HOX gene expression patterns have been shown to be associated with patient outcomes in some studies (27,46,47). We therefore compared patient survival across the HOX expression phenotypes identified by our clustering analysis. Indeed, overall patient survival was significantly longer in the groups with no HOX expression or HOXB expression alone, compared to those with HOXA or with the HOXA/HOXB signatures (Figure 1C). Since these expression patterns were closely correlated with recurrent cytogenetic abnormalities that have well-established relationships to patient outcome, we conducted a separate survival analysis on 184 intermediate risk AML samples from this and a previously published study (48). HOX expression phenotypes were obtained from unsupervised clustering of microarray-based HOX gene expression values from all samples included in this study (Figure S5), and the overall patient survival was compared between samples with normal karyotype or intermediate risk cytogenetic abnormalities and different HOX expression groups. Importantly, this analysis also showed that HOX expression phenotypes were significantly associated with overall survival, with no HOX expression having longer survival compared to samples that express HOXA alone, or both HOXA and HOXB genes (Figure 1D). Since the AML samples in this set did not have favorable risk cytogenetic findings, the lack of HOX expression in AML appears to predict good survival regardless of the cytogenetic risk status.

Transcriptional activity at the HOXA and HOXB gene clusters occurs in a canonical fashion that is shared between AML samples and normal hematopoietic progenitors

The coordinated expression of specific “sets” of genes in the HOXA or HOXB clusters in both AML and normal hematopoietic progenitors suggests that these genes may be coordinately regulated in both normal and malignant hematopoietic cells. Indeed, we observed expression of the same HOXA and HOXB genes across all AMLs in which a specific HOX cluster was expressed (*i.e.* the same HOXA genes tend to be highly expressed

in AMLs with *MLL* translocations vs. those with the HSPC-like signature; Figure 1A), as well as normal progenitors (although the expression levels of some genes were significantly different among AML samples with different HOX expression patterns, and between AML samples and normal CD34⁺ cells, see Figures S3A and S3B). To determine whether these characteristic expression patterns extended beyond summarized RNA-seq expression values (*i.e.*, gene-based FPKM values), we compared the raw RNA-seq signals and isoform usage across the HOXA and HOXB loci in normal CD34⁺ cells, and in AMLs with different HOX expression phenotypes and mutation profiles. Figure 2A shows the median RNA-seq signal (normalized read depth) at the HOXA locus for normal CD34⁺ cells, AMLs with *MLL* translocations, and AMLs with the HOXA/HOXB HSPC-like expression signature (with and without NPMc mutations). Samples in each group displayed a similar HOXA coverage profile, with the highest transcriptional activity localized to the central region within the locus (*HOXA5-HOXA10*). Likewise, the RNA-seq coverage signal at the HOXB locus was greatest within a region between *HOXB2-HOXB6*, and was similar between CD34⁺ cells and AMLs with the HSPC-like expression pattern, regardless of the presence of the NPMc mutation (Figure 2B). *Inv(16)/CBFB-MYH11* AMLs, which showed low level HOXB expression, demonstrated a highly similar transcriptional profile, but with lower coverage depth. Transcriptional activity at both loci was detected on both strands, with expression levels of HOX antisense transcripts that were highly correlated with coding gene expression at both HOXA and HOXB gene clusters (Figure S6A), and which demonstrated similar mutation-specific expression patterns (Figure S6B). Analysis of transcript isoform usage at the HOXA and HOXB loci also showed that the dominant isoforms of the highly expressed HOXA and HOXB genes were shared across all AML types and normal CD34⁺ cells (Figure 2C). These findings imply that transcriptional activity at the HOXA and HOXB loci occurs in a canonical, bidirectional fashion in normal hematopoietic progenitors, and in a subset of AML samples.

Epigenetic profiling identifies potential regulatory regions in the HOX gene loci of AML cells and normal hematopoietic progenitors

The close association between HOX expression phenotypes and recurrent *PML-RARA*, *RUNX1-RUNX1T1* and *MLL* gene fusions in AML suggests that they may have direct effects on gene regulation at the HOX loci. These fusion products are thought to function via direct interactions with target gene loci, and/or recruitment of transcription factors and/or epigenetic regulators (17,49,50). We therefore performed whole-genome and targeted bisulfite sequencing and transposase-mediated chromatin accessibility profiling (ATAC-seq) on AML samples with these mutations to identify potential regulatory elements within or near the HOX gene loci that may be the targets for such interactions. We selected four sets of AML samples with characteristic HOX expression patterns, including *MLL* translocations (*t(11;19)/MLL-ELL*; n=2), normal karyotype AMLs with NPMc mutations that exhibit the HSPC-like HOX pattern (n=3), and AMLs with *RUNX1-RUNX1T1* (n=3) or *PML-RARA* (n=3) gene fusions (Figure 3A); similar data were also obtained for normal CD34⁺ cells from previously published datasets (51).

Bisulfite sequencing of the AML samples resulted in high-confidence methylation values for 2,893 and 2,419 CpG dinucleotides at the HOXA and HOXB loci, respectively, which were

relatively hypomethylated in the samples with HOX expression (Figure 3B). Differential methylation analysis identified 427 (14.7%) and 458 (18.9%) differentially methylated CpGs among the four AML sets at the HOXA and HOXB loci, respectively, which clearly distinguished each group of AML samples upon hierarchical clustering (Figures 3C). Chromatin accessibility also correlated with HOX expression, and was increased in the *MLL-ELL* and normal karyotype samples at HOXA gene promoters (Figure 3D); genome-wide ATAC-seq signals were similar across all the samples (data not shown), suggesting that these findings were not due to differences in assay performance across this set of samples. Analysis of the methylation profiles and chromatin accessibility at the HOXA and HOXB loci demonstrated remarkable similarity across all of the samples, but also identified regions with differences that correlated with HOX expression. This was most apparent in a 38 kbp region within the HOXA locus, where the normal karyotype, HSPC-like, *MLL-ELL* AMLs, and normal CD34⁺ cells were largely unmethylated; they also contained regions of accessible chromatin at gene promoters and intergenic loci between the *HOXA5* and *HOXA10* genes (Figure 4A, shaded region). Similar findings were observed in a ~50 kbp region at the HOXB locus that was largely unmethylated, and that contained more accessible chromatin in both the normal karyotype AML cases with HSPC-like HOX expression, and CD34⁺ cells (Figure 4B, shaded region). We also observed similar methylation patterns at the same loci in array-based methylation data from an extended set of AML samples with these mutations (see Figures S7A and S7B), implying that these differences are not limited to this small set of samples. These data suggest that epigenetic regulatory activity is concentrated within distinct regions in both HOX loci.

Although there were characteristic epigenetic differences at these loci that correlated with HOX expression, samples with the same expression patterns showed very similar methylation and chromatin accessibility profiles. For example, AMLs with *PML-RARA* and *RUNX1-RUNX1T1* had the same patterns of hypermethylation. Further, regions with accessible chromatin in the *MLL-ELL* and normal karyotype AMLs were largely shared, or overlapped with DNase hypersensitive sites found in normal CD34⁺ cells. Interestingly, several non-promoter-associated regions with accessible chromatin were shared across all AML samples (regardless of HOX expression) and normal CD34⁺ cells, suggesting that these may be common regulatory elements involved in the activation or repression of HOX gene expression in both normal and AML cells. Regions at both the HOXA and HOXB loci showed remarkable similarity in the ATAC-seq signals in all of the AML samples, but differed dramatically in their methylation states (Figure 5A, 5B). Further analysis of these regions revealed that they occurred near binding sites for the epigenetic regulator CTCF in a variety of cell types, including embryonic stem cells and the K562 cell line (52). These findings suggest that epigenetic regulation of HOX gene expression in both normal and malignant hematopoietic cells may be orchestrated by CTCF (and perhaps other factors) at defined regulatory elements within the HOX loci.

Discussion

Understanding the regulatory mechanisms controlling self-renewal pathways in hematopoietic cells could provide insights into the biology of normal hematopoietic stem cells, as well as the events that initiate and sustain leukemia. HOX transcription factors have

been proposed to be master regulators of self-renewal in HSPCs, and are expressed in the majority of AML samples, which has led to the hypothesis that dysregulation of these transcription factors promotes abnormal self-renewal in AML. Indeed, previous studies have described the remarkable variability in HOX expression in primary AML samples, as well as mutations and clinical outcomes that are associated with the presence or absence of HOX expression in AML (25,27,46).

In this study, we conducted an integrated epigenetic analysis of the HOX loci in primary AML samples to understand HOX expression patterns and epigenetic regulation in AML. We found that HOX expression phenotypes in AML fall into four broad categories, including little to no expression, expression of either the HOXA or HOXB cluster genes alone, or a canonical pattern with expression of genes from both clusters that was nearly identical to the expression signature in normal HSPCs. These patterns were tightly linked to AML-subtype-defining mutations and were associated with patient survival, which is consistent with previous studies associating HOX expression and stem cell signatures with patient outcomes (27,45,53). In addition, we found that the transcriptional profiles, isoform usage, and noncoding transcript expression were similar across all AML samples (regardless of mutation status or subtype) and normal CD34⁺ cells, suggesting that each locus is regulated in a canonical fashion as a single unit, and not at the level of individual genes. Epigenetic analysis also identified distinct regions in each gene cluster that display the hallmarks of regulatory elements, which appear to be coordinating HOX expression in both normal and leukemic hematopoietic cells.

Although HOX expression in AML is typically described as dysregulated, the similarity between normal and leukemic HOX expression patterns we observed suggests that most HOX expression in AML in fact reflects a normal stem cell state that is “captured” in the transformed cells. With this view, non-HSPC-like expression patterns (e.g., no expression, or the expression of genes from only one cluster) truly represent the dysregulated state in AML. The absence of HOX gene expression could potentially occur because of direct suppression of the HOX gene loci by oncogenic fusions, such as *PML-RARA* and *RUNX1-RUNX1T1*, or it may result from the inactivation of a common pathway required for maintenance of HOX expression. Alternatively, AMLs with non-HSPC-like expression patterns may simply originate from a more differentiated hematopoietic progenitor—one that has already downregulated one or both of the HOX gene clusters as part of normal development. If this is the case, and if AML cells truly require self-renewal as part of their defining biology, then the AMLs with no HOX expression may have their self-renewal signals provided by novel mechanisms. Indeed, both *PML-RARA* and *RUNX1-RUNX1T1* are known to “reprogram” myeloid progenitors to have a self renewal phenotype (54,55). We suspect that the other AML cases with no HOX expression (and neither of these fusions) may have alternative self-renewal pathways that have not yet been recognized. Regardless, the fact that AMLs with the same HOX expression profiles have similar patient outcomes suggests that they indeed share some fundamental biological property that correlates with responses to current, established therapies.

In addition to the striking differences in expression patterns at the HOX loci, the levels of HOX expression were also somewhat variable among the AML samples. This could be due

to true differences in expression levels, or to more technical issues, such as the fraction of cells in samples that were part of the malignant clone. Some genes did show statistically different expression levels among AML types, but the range of expression values overlapped, arguing against systematic differences in transcript abundance across AMLs with different mutations and HOX expression phenotypes (the notable exception being AML samples with *inv(16)*, which all showed low level expression of only the HOXB genes). The range of HOX expression values in normal CD34⁺ cells was similar to that observed in AML samples with the stem cell pattern, supporting the notion that the HOX genes are regulated in similar fashion in normal and malignant HSPCs. It is also interesting to note that the HOX expression patterns we observed always involved multiple genes. This suggests that functional studies based on overexpression of a single gene should be interpreted with caution, since this approach may not accurately model HOX transcription factors in normal or leukemic cells, where multiple HOX genes are always expressed together.

The findings presented here also provide evidence for distinct regulatory regions within the HOXA and HOXB gene clusters that may be acting as “locus control regions” in hematopoietic cells. These regions contain open chromatin in AML samples and normal cells, regardless of HOX expression, and appear to be at the center of epigenetic differences associated with HOX expression, including DNA methylation and chromatin accessibility. It is not yet clear whether these regions are also targets for certain transcription factors or oncogenic mutations, although we observed canonical epigenetic patterns that appear to be associated with only the presence or absence of HOX expression (rather than with specific mutations). These candidate regulatory regions are located within or near CTCF binding sites, which may be organizing higher order chromatin structures that are involved in locus regulation. Experimental evidence from other systems has shown that chromatin structure at the HOX loci is dynamic throughout development and cellular differentiation (56–58); similar regulatory pathways may be involved with AML. However, additional experiments will be necessary to understand whether direct interactions with oncogenic gene fusions (or other mutant genes) play a direct role in guiding such processes, or whether additional factors (or the HOX transcription factors themselves) are involved in regulating HOX gene expression. These studies may identify “upstream” factors involved in regulating HOX expression in normal cells and in AML, and may point to novel approaches for disrupting HOX gene expression in AML for therapeutic purposes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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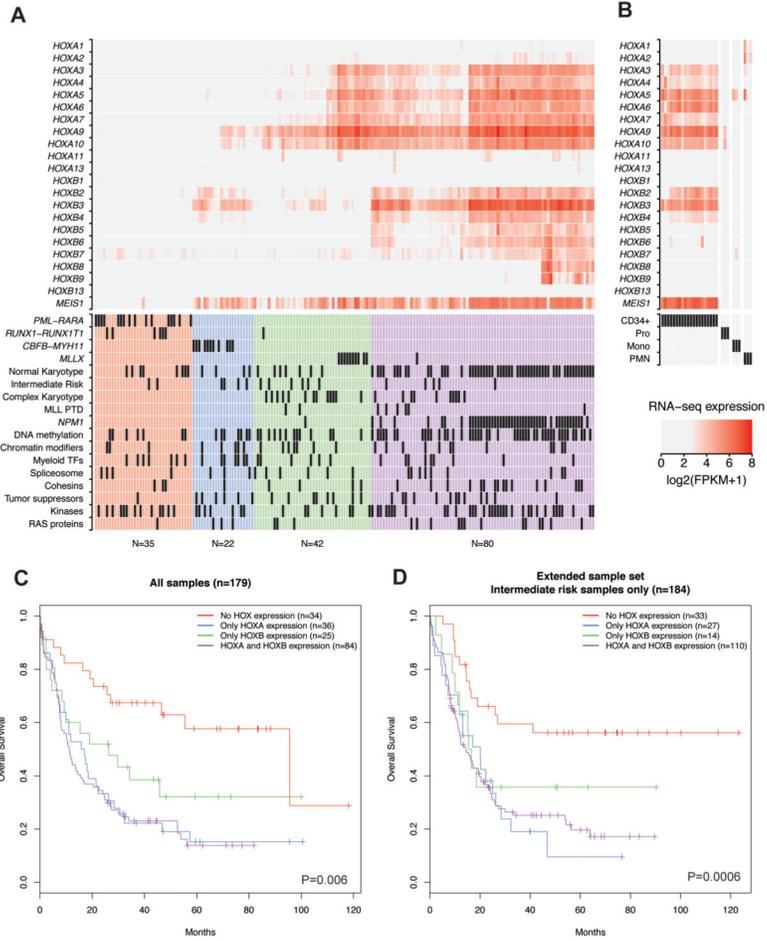


Figure 1.
 A) Patterns of HOX gene expression in primary AML samples. HOXA and HOXB gene expression values from RNA-seq data ($\log_2(\text{FPKM}+1)$) are shown for 179 primary AML samples following unsupervised hierarchical clustering of the samples based solely on the expression of the genes shown, which identified four patterns defined by the presence or absence of HOXA and/or HOXB genes. The mutation status and cytogenetic category for each AML is shown in the table below the heatmap (32). B) HOXA and HOXB gene expression in normal hematopoietic cells at different developmental stages from RNA-seq data, including CD34⁺ progenitors (n=20), promyelocytes (Pro, n=3), monocytes (Mono, n=3), and neutrophils (PMN, n=3). C) Kaplan-Meier analysis of overall patient survival stratified by the HOX expression groups identified in panel A (n=179, P=0.006 across all groups; performed using Cox proportional hazard regression). D) Survival analysis of 184 AML patients with intermediate risk cytogenetic status based on HOX expression phenotype from microarray expression (data obtained from (48); see Figure S5; P=0.0006, Cox proportional hazard regression).

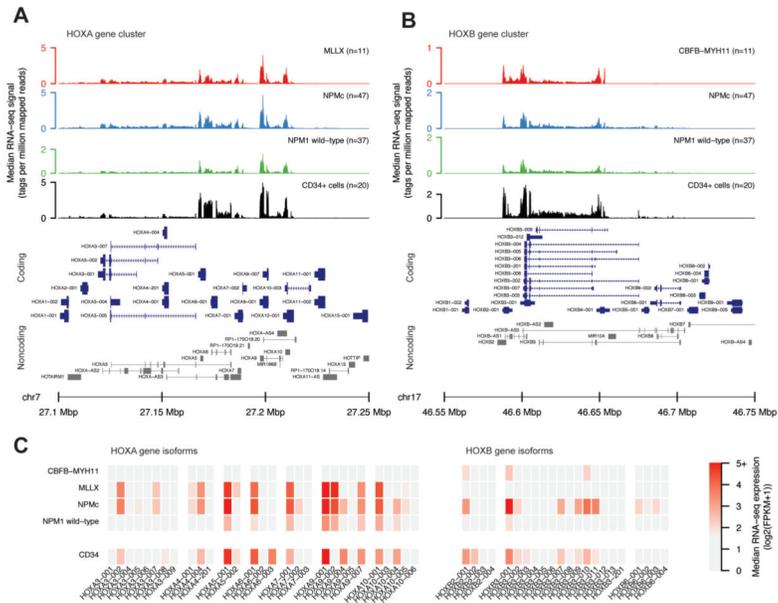


Figure 2. Transcriptional profiles of the HOXA and HOXB gene clusters from different AML subtypes. A) and B) show the median normalized RNA-seq read depth from the HOXA and HOXB loci, respectively, from AMLs with *MLL* translocations (*MLLX*, n=11), Normal Karyotype and NPMc mutations (n=47), Normal Karyotype without NPMc mutations (n=33), and normal CD34⁺ cell (n=20). Protein-coding genes (in blue) and noncoding transcripts (in gray) are shown below each plot. Note difference in scale of the Y axis across the sample types, indicating different expression levels despite the similarity in transcriptional patterns. C) Median expression (in FPKM) of transcript isoforms for expressed genes in the HOXA and HOXB clusters for AMLs in the indicated mutation or cytogenetic category and CD34⁺ cells.

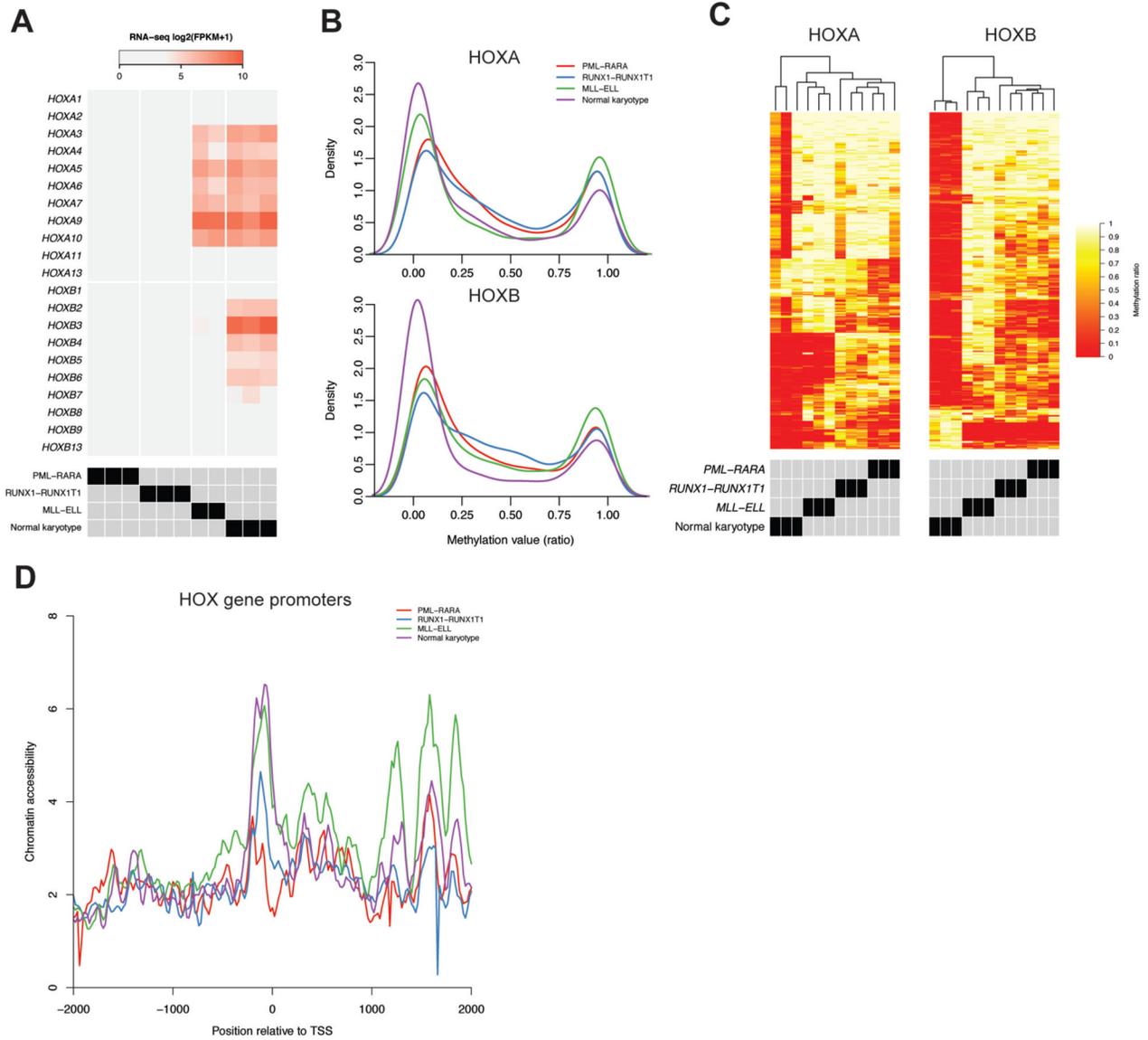


Figure 3. Epigenetic analysis of the HOX loci in primary AML samples with characteristic HOX expression patterns. A) RNA-seq expression of the HOX genes from the samples used for epigenetic analysis, including *PML-RARA*, *RUNX1-RUNX1T1*, *MLL-ELL*, and normal karyotype AMLs with the HSPC-like HOX expression pattern and NPMc mutations (n=3 each). B) Methylation distributions from bisulfite sequencing of the HOX loci in primary AML samples, showing skewing towards less methylation in samples with HOXA expression (*MLL-ELL* and normal karyotype) and HOXB expression (normal karyotype only). C) Clustering of methylation values identified from differential methylation analysis of CpGs at the HOXA and HOXB locus between each AML set. D) Aggregate chromatin accessibility profiles at HOX gene promoters from each AML type. Each curve shows the mean normalized ATAC-seq signal across all HOXA and HOXB gene promoters from the indicated AML types (n=3 each), which demonstrates that AMLs with HOX expression

(*MLL-ELL* and normal karyotype samples) have more open chromatin compared to those without HOX expression.

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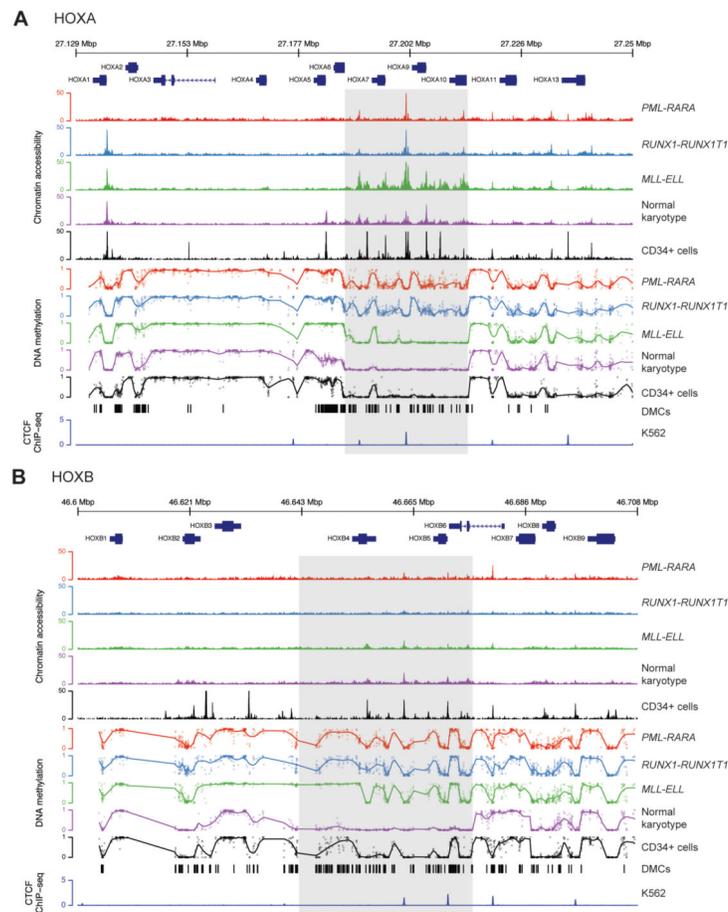


Figure 4.

Integrated epigenomic profiles of the HOXA and HOXB gene clusters in primary AML samples and normal CD34⁺ cells. Panels A and B show DNA methylation and transposase-mediated chromatin accessibility (ATAC-seq) profiles from primary AML samples at the HOXA and HOXB loci, respectively. Methylation values reflect the coverage-weighted mean methylation ratio across all samples in each group. Chromatin accessibility values represent the median number of tags per 1 kbp per million mapped tags across each AML set (n=3 each). The bottom tracks show differentially methylated CpGs (DMCs) at each locus, and the ChIP-seq signal for CTCF from the K562 cell line from the ENCODE consortium (UT Austin) (52), respectively.

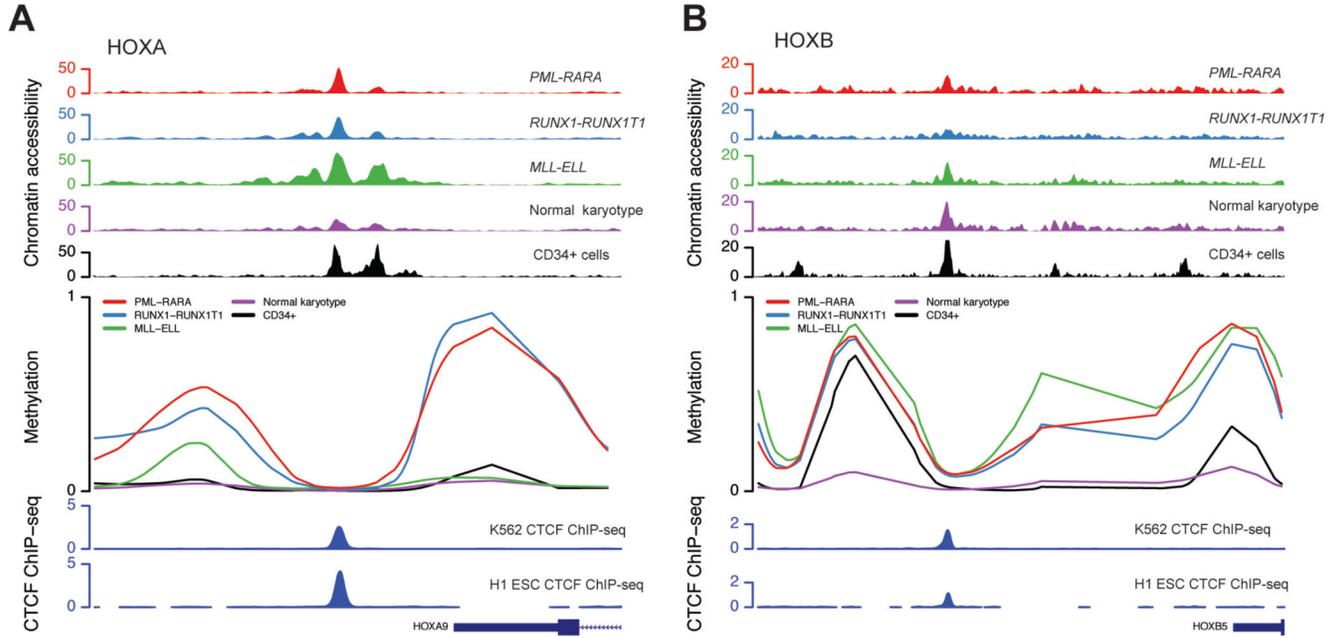


Figure 5. Differential methylation at common chromatin-accessible regions within the HOXA (panel A) and HOXB (panel B) gene clusters. Each panel shows chromatin accessibility and methylation at chromatin-accessible sites present in all AML samples and normal CD34⁺ cells, with common chromatin accessibility patterns and hypermethylation in samples without expression of nearby HOX genes. These loci occur near previously identified CTCF binding events in H1 hESCs and the K562 erythroleukemia cell line from the ENCODE consortium (UT Austin), (52).

Table 1

Association of clinical variables and somatic mutation categories* with HOX expression patterns in primary AML samples.

Variable	No HOX expression (n=35)	HOXA expression (n=42)	HOXB expression (n=22)	HOXA and HOXB expression (n=80)	Adjusted P-value#
White blood cell count; x 10 ⁹ /l, mean (range)	29 (0.4–224)	23 (0.8–115)	37 (1–172)	46 (0.6–297)	0.04
Bone marrow blasts; %, mean (range)	69 (34–100)	70 (30–100)	59 (33–90)	71 (32–100)	0.05
Monocytic differentiation; n, (%)	1 (1.8)	16 (28.6)	11 (19.6)	28 (50.0)	0.03
CD34 marker positive; n, (%)	20 (57.1)	33 (78.6)	20 (9.1)	39 (48.8)	0.01
Cytogenetic categories; n (%)					1.00E-10
Normal karyotype	8 (10.6)	7 (9.3)	4 (5.3)	56 (74.7)	
Complex cytogenetics	0	13 (56.5)	0	10 (43.4)	
PML-RARA	16 (100)	0	0	0	
RUNX1-RUNX1T1	6 (85.7)	1 (14.3)	0	0	
CBFB-MYH11	0	0	11 (100)	0	
MLL-X fusions	0	10 (90.9)	1 (9.1)	0	
Somatic mutation categories; n, (%)					
NPMc	0	1 (2.1)	0	47 (97.9)	0.005
DNA methylation	7 (9.6)	13 (17.8)	7 (9.6)	46 (63.0)	0.005
Tumor suppressors	1 (3.6)	8 (28.6)	6 (21.4)	13 (46.4)	N.S.
Kinases	13 (20.6)	8 (12.7)	7 (11.1)	35 (55.6)	N.S.
Myeloid transcription factors	7 (24.1)	6 (20.7)	7 (24.1)	9 (31.0)	N.S.
Chromatin modifiers	3 (11.1)	7 (25.9)	5 (18.5)	12 (44.4)	N.S.
Cohesins	3 (12.5)	4 (16.7)	1 (4.2)	16 (66.7)	N.S.
Spliceosome	4 (18.2)	6 (27.3)	3 (13.6)	9 (40.9)	N.S.
RAS pathway	1 (5)	3 (15)	3 (15)	13 (65.0)	N.S.

* See Table S1 for the specific genes included in each mutation category.

Significance testing performed via ANOVA or chi-squared test, followed by bonferroni correction for multiple comparisons.

N.S = not significant.