Mammalian Trithorax and Polycomb-group homologues are antagonistic regulators of homeotic development


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Control of cell identity during development is specified in large part by the unique expression patterns of multiple homeobox-containing (Hox) genes in specific segments of an embryo. Trithorax and Polycomb-group (Trx-G and Pc-G) proteins in Drosophila maintain Hox expression or repression, respectively. Mixed lineage leukemia (MLL) is frequently involved in chromosomal translocations associated with acute leukemia and is the one established mammalian homologue of Trx. Bmi-1 was first identified as a collaborator in c-myc-induced murine lymphomagenesis and is homologous to the Drosophila Pc-G member Posterior sex combs. Here, we note the axial-skeletal transformations and altered Hox expression patterns of Mll-deficient and Bmi-1-deficient mice were normalized when both Mll and Bmi-1 were deleted, demonstrating their antagonistic role in determining segmental identity. Embryonic fibroblasts from Mll-deficient compared with Bmi-1-deficient mice demonstrate reciprocal regulation of Hox genes as well as an integrated Hoxc8-lacZ reporter construct. Reexpression of Mll was able to overcome repression, rescuing expression of Hoxc8-lacZ in Mll-deficient cells. Consistent with this, Mll and BMI-1 display discrete subnuclear colocalization. Although Drosophila Pc-G and Trx-G members have been shown to maintain a previously established transcriptional pattern, we demonstrate that MLL can also dynamically regulate a target Hox gene.

Acute leukemias with cytogenetic abnormalities at chromosome segment 11q23 have a poor prognosis and bear a translocation of the mixed lineage leukemia (MLL) gene (1). MLL, a large protein (3,972 aa), is homologous to Drosophila trithorax (trx) in several regions. The homology between MLL and trx provided important clues about MLL’s normal function. The highest homology resides within a carboxy-terminal domain termed the SET domain because of its presence in the Drosophila proteins Su(var)3–9, Enhancer of zeste, and trx (2). SET domains are found in an evolutionarily conserved family of proteins that maintain specific patterns of gene expression after the initiation of transcription during development. Two major families of SET-containing proteins, the Trithorax (trx-G) and Polycomb (Pc-G) groups, are chromatin-associated proteins that act antagonistically to alter chromatin structure to either promote or repress transcription, respectively (3–5). Some of the best understood downstream targets of the Pc-G and trx are the homeotic (HOM-C) genes in the Antennapedia and bithorax complexes. Drosophila trx mutants show loss or posteriorly shifted patterns of HOM-C gene expression that result in defects in segment identity, indicating trx is a positive regulator of HOM-C expression (6–8). In contrast, mutations in the Pc-G result in anterior shifts in HOM-C expression boundaries, indicating these proteins normally function as repressors of HOM-C expression (5). The data available suggest that Pc-G and trx-G proteins regulate gene expression by forming large multisubunit complexes at specific chromosomal sites. Subcellular localization studies show that trx and Pc-G proteins colocalize at many sites on polytene chromosomes, including the bithorax complex, the genes that are most affected by Pc-G and trx mutations (9). Further support for this concept comes from immunoprecipitation and fractionation studies that show that Pc-G members such as Polycomb and polyhomeotic are associated in multimeric protein complexes estimated to be in the 2–5 × 10⁶ Da size range (10).

Many aspects of homebox regulation appear to be conserved between Drosophila and mammals. In mammals, the clustered homeobox (Hox) genes are organized in linear arrays on four different chromosomes. As in the fly, their 3’ to 5’ organization in the genome parallels their rostral to caudal anterior expression boundaries in the developing embryo. Recently, mammalian homologs of the Pc-G proteins have been identified, including Bmi-1, Mph-1, EZH2, and M33 (11–15). Furthermore, some of these homologs have been shown to regulate Hox gene expression in vivo. Bmi-1 was first identified as a collaborator in c-myc-induced murine lymphomagenesis and is homologous to the Drosophila Pc-G member posterior sex combs (12, 13). Mice with homozygous disruption of Bmi-1, the mammalian homolog of Posterior sex combs, show anterior shifts in Hox expression boundaries (16, 17). Mll-deficient mice demonstrate altered Hox expression and abnormal segmental identity. Of note, the Mll heterozygous mice show an overt phenotype. Skeletal abnormalities were frequently noted in heterozygous mice. Mll−/+ newborns revealed frequent homeotic transformations, including posterior T12 → L1 transformations, as indicated by complete or partial loss of the T13 rib as well as anterior transformations (C7 → C6, T3 → T2). Homeotic defects were observed in +/+ animals whether the disrupted allele was of maternal or paternal origin, arguing they were the result of haploinsufficiency rather than parental imprinting (18). Gene dosage effects have been reported in trx mutants in Drosophila as well as in Bmi-1 mutant mice (19).

Mll−/− embryos were embryonic lethal and showed severe developmental abnormalities, including abnormal ganglia, absence of the maxillary branch of the first branchial arch, small fore and hind limbs, and pooling of erythroid precursors in the coelomic cavity (20). Viable embryos could be recovered up to embryonic day 10.5 (E10.5). Histologic sections of the Mll null embryos showed extensive apoptotic cell death that was most marked in the first branchial arch, somites, and liver. These

Abbreviations: MLL, mixed lineage leukemia; RT, reverse transcription; E10.5, embryonic day 10.5; MEF, murine embryonic fibroblast.

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findings suggest that MLL or downstream target genes of MLL play a major role in regulating both cell proliferation and survival in the developing embryo. The Mll knockout indicated that MLL is required for the maintenance rather than the initiation of gene expression in early embryogenesis. Genes downstream of Mll including Hoxa7 are activated appropriately in the absence of Mll but require Mll for sustaining their expression (20). Given that both MLL and Bmi-1 are involved in hematopoietic malignancies, we chose to examine whether Bmi-1 and Mll are antagonistic in the same developmental regulatory pathways.

Materials and Methods

Mll1y2 and Bmi-1y2 mice have been described (18, 19). Mll-mutant mice used in this study have been backcrossed for more than 10 generations on a C57BL6/C3H background. Bmi-1-mutant mice have been maintained since their initial production on an FVB background. Mice heterozygous for both Mll and Bmi-1 were mated to obtain animals for this study. Genotyping was done by using tail DNA (18, 19) as described. Skeletons were prepared as described (21).

The Hoxc8 probe is a 408-bp reverse transcription (RT)–PCR product (nucleotides 274–682) cloned into the HinII site of pBS-SK. The plasmid was linearized with KpnI and an antisense transcript synthesized with T3 RNA polymerase. In situ hybridization was as described (22).

Bmi-1 cDNA was cloned by RT-PCR using mouse fibroblast RNA and together with a 5' HA tag was cloned into pUHD15–1 expression vector (www.zmbh.uni-heidelberg.de/bujard/homepage.html). The MLL expression plasmid is described (23, 24) with the modification of a 5' FLAG epitope.

Murine embryonic fibroblast (MEF) lines were established from E10.5 C57BL6/C3H embryos and were immortalized by transfection with a Polyoma virus expression plasmid (25). Cells were plated in 6-well plates and were transfected with 1 μg of total DNA by using Lipofectamine Plus (GIBCO/BRL). Stable transfectants were selected in media containing 135 μg/ml hygromycin. Twenty to thirty colonies were typically obtained from each transfection. RNA was isolated with RNeasy miniprep columns (Qiagen, Chatsworth, CA) and was treated for 15 min with DNase I (GIBCO/BRL, amplification grade). RNA (400 ng) was used for RT-PCR. Sequences of the oligonucleotide primers will be provided on request. 293 cells were transiently transfected by using Lipofectamine (GIBCO/BRL). Cells were permeabilized and stained with mouse M2 FLAG monoclonal antibody (Sigma) and rabbit anti-HA antibody (Santa Cruz Biotechnology) at 10 μg/ml. Secondary detection was with goat anti-mouse Alexa 488 (Molecular Probes) and donkey anti-mouse Cy3 conjugates (Jackson ImmunoResearch) at 1:250. Images were acquired on a Zeiss Molecular Dynamics laser scanning confocal microscope, and fluorescence intensities were adjusted by using Molecular Dynamics IMAGE SPACE software.

Results

Mll heterozygous and Bmi-1 heterozygous mice were mated to obtain double mutant mice, and the skeletal phenotype was characterized. Abnormalities in the cervical spine of Bmi-1y2 mice include a widened and split first cervical vertebra (C1), a small C2 suggestive of C2 → C3 transformation, and an ectopic rib associated with C7, indicative of C7 → T1 transformation (Fig. 1a and b). Mll1y2 cervical skeletons are notable for frequent widening of C2 (Fig. 1c). Compound double-mutant
mice (Bmi-1−/−, Mll+/−) corrected several Mll- and Bmi-1-deficient phenotypes (Fig. 1d; Table 1), of particular note being the widened/split C1 and C2 → C3 transformation of Bmi-1 deficiency. Thus, a balanced loss of these homeotic regulators results in a normalization of segment specification. The frequent abnormalities of the sternum, sternebrae, and thoracolumbar spine found in the individual gene mutants were not reduced in the double mutants (data not shown). This suggests that Mll and Bmi-1 do not universally co-regulate the same homeotic genes at every segmental level.

We searched for target genes co-regulated by Mll and Bmi-1 by assessing the expression patterns of the Hox clusters. Specifically, we analyzed Mll+/−, Bmi-1−/− double mutant embryos to determine whether Bmi-1 is the specific Pc-G member responsible for repressing the anterior limit of Hox expression in Mll mutants and conversely whether Mll is required for the ectopic Hox expression observed in Bmi-1 mutants. Importantly, a one-segment posterior shift of Hoxc8 observed in E9.5 and E12.5 Mll+/− embryos (Fig. 2 B and F) and a one-segment anterior shift in Bmi-1−/− embryos (Fig. 2 C and G) were normalized in the double mutants (Fig. 2 D and H). This result indicates that Mll and BMI-1 exert their effects in the segments immediately adjacent to the normal boundary of Hoxc8 expression and can be unmasked when the opposing factor is reduced or absent. Of note, the intensity of staining for Hoxc8 transcripts was reproducibly higher in Bmi-1−/− embryos and lower in Mll+/− and Mll+/−, Bmi-1−/− embryos, suggesting a dominance of Mll in transcriptional control.

To further explore the effects of MLL versus BMI-1, we examined the expression of endogenous Hox genes in MEFs. Mll-deficient, as compared with wild-type MEFs, displayed either no or markedly decreased expression of the Hox c cluster (c4, 5, 6, 8, 9) and the Hox a cluster (a3, 4, 5, 7, 9, 10) (Fig. 3a). In contrast, Bmi-1-deficient MEFs demonstrated elevated levels of Hoxc6 and c8 transcripts but no alteration of Hoxa5,9,10 expression (Fig. 3b). This indicates that selected Hox, including c8, but not all Hox genes, are reciprocally regulated by MLL and BMI-1.

We next wished to reconstitute in a chromatin environment the differential regulation of a physiologic target gene, Hoxc8, by the Pc-G and Trx-G members Mll and Bmi-1. Studies of Hoxc8

![Fig. 2.](image)

**Fig. 2.** Rescue of Hoxc8 deregulation in Mll/Bmi-1 double-mutant embryos. (A–D) Hoxc8 whole-mount in situ hybridization of E9.5 embryos. (E–H) In situ hybridization sections of E12.5 embryos. Arrows indicate the wild-type boundary of expression: specifically, the anterior edge of somite 14 (A–D) and the 12th prevertebral body (E–H). A posterior shift and decreased expression of Hoxc8 were observed in Mll+/− embryos (B and F). An anterior shift of Hoxc8 boundaries in somites and presomitic mesoderm (arrowhead in C) was seen in Bmi-1−/− embryos (C and G). The Mll+/−, Bmi-1−/− double mutants (D and H) restored the anterior boundaries to wild-type positions (A and E) but displayed decreased levels of expression.

### Table 1. Bmi/Mll skeletal analysis

<table>
<thead>
<tr>
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<th>Mll+/−, historical*</th>
<th>Mll+/−</th>
<th>Bmi−/−, historical*</th>
<th>Bmi−/−</th>
<th>Mll+/− Bmi−/−</th>
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<tr>
<td>Widened/split C1</td>
<td>N.R.</td>
<td>14/22</td>
<td>6/8</td>
<td>0/5</td>
<td>0/8</td>
</tr>
<tr>
<td>C → C3</td>
<td>0/22</td>
<td>0/8</td>
<td>N.R.</td>
<td>4/5</td>
<td>2/8</td>
</tr>
<tr>
<td>C7 → T1</td>
<td>0/22</td>
<td>0/8</td>
<td>9/15</td>
<td>4/5</td>
<td>0/8</td>
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N.R., not reported.

*Mice used in this study were compared to historical controls (18, 19) to rule out strain-dependent modifiers of phenotype.
expression during embryogenesis have identified critical regula-
tory regions upstream and downstream of the gene (26, 27). The 5' element is necessary and sufficient for correct temporal and tissue-specific activation of Hoxc8 expression but by itself is unable to direct expression to the correct anterior boundaries beyond E9 and fails to maintain expression beyond E12. The 3' regulatory element is required for appropriate anterior bound-
aries and maintains expression throughout embryogenesis. Consequently, we used a reporter construct in which lacZ expression is directed by the Hoxc8 promoter, 5' and 3' enhancer elements (Fig. 4a). As might be predicted, Mll status did not affect the expression of an extrachromosomal construct in transient expression assays (data not shown). In contrast, genomic integration of the Hoxc8-lacZ gene in stable transfectants provided a

![Fig. 3. Hox expression in MEFs.](image)

**Fig. 3.** Hox expression in MEFs. (a) MEF lines prepared from Mll+/+ and Mll−/− embryos were examined for their expression pattern of Hoxa and c genes by semiquantitative RNA RT-PCR analysis. Mll (AT-hooks) region is present in the disrupted (−/−) as well as wild-type (+/+ alleles whereas Mll (exon 3) is deleted, but β-galactosidase is inserted in the (−/−) alleles. RT signifies the presence (+) or absence (−) of reverse transcriptase. (b) Hoxa and c expression in Bmi-1+/+ versus Bmi-1−/− MEFs.

![Fig. 4.](image)

**Fig. 4.** Expression of Hox c8-lacZ is reserved by MLL. (a) MEFs from Mll+/+ and Mll−/− embryos were transfected with a Hoxc8-lacZ construct, demonstrating integration site independent regulation of Hoxc8 in stable transfectant clones. (b) Expression of human MLL (hMLL) results in the expression of the integrated Hoxc8-lacZ reporter but not the endogenous Hoxc8 locus.
chromatin context that proved permissive for MLL regulation. The Hoxc-8-lacZ reporter, like the endogenous Hoxc8 locus, was repressed in Mll-deficient MEFs (Fig. 4a). However, four of five Mll+/− clones expressed the stably integrated Hoxc8-lacZ reporter (Fig. 4a).

One model holds that Pc-G activity increases the probability of forming a repressed chromatin conformation. Consequently, we asked whether MLL overexpression would antagonize the tendency for repression by an unopposed Pc-G, presumably Bmi-1, in Mll−/− cells. Cells transfected with an MLL expression vector were found to now express Hoxc8-lacZ whereas the endogenous Hoxc8 locus could not be activated (Fig. 4b).

To assess whether MLL and BMI-1 show any evidence for colocalization that would enable them to regulate shared loci, laser confocal immunomicroscopy was used. Transient cotransfection of MLL and Bmi-1 into mammalian 293 cells demonstrated a nuclear localization for both proteins that consists of numerous discrete speckles, as well as 5–10 larger aggregates per cell (Fig. 5 A and B). Exact alignment of doubly stained larger aggregates was observed whereas colocalization was also noted for the majority of the smaller speckles (Fig. 5C). We also verified localization in stable transfectants, where MLL and BMI-1 expression is similar to endogenous levels. In this case, the vast majority of MLL and BMI-1 was present as discrete speckles (Fig. 5 D and E), over half of which displayed colocalization (Fig. 5F).

**Discussion**

Initiation of Hox expression in Drosophila is under the control of the segmentation genes, which function to activate or repress specific Hox gene transcription in the appropriate segments (5). These factors are short-lived, and maintenance of the established patterns is controlled by the Trx and Pc-G members. Double mutants of Trx-G and Pc-G in Drosophila were noted to restore a wild-type phenotype (28). Substantial data supports the view that these ubiquitously expressed homeotic regulators act differentially on specific promoters in specific cell types to maintain the initially established transcriptional state (2–5). Axial Hox expression patterns in early mouse development argues that MLL may be critical for maintenance rather than initiation of Hox transcription (20). However, Hox transcription continues to be modulated throughout mammalian development. Hematopoietic cells in particular demonstrate ongoing activation and repression of Hox expression at each maturation point and in all hematopoietic lineages (29). The relative expression of Pc-G transcripts in early hematopoietic precursors has been shown to change during maturation with both up- and down-regulation of individual Pc-G genes (30).

The axial–skeletal transformations and altered Hox expression patterns of Mll-deficient and Bmi-1-deficient mice were normalized when both Mll and Bmi-1 were deleted, demonstrating their antagonistic role in determining segmental identity. Moreover, we noted that selected Hox, including c8, but not all Hox genes, are reciprocally regulated by MLL and BMI-1. An experimental model is needed to further refine cis-acting elements and pursue their differential regulation by an MLL or Bmi-1 associated multimeric protein complex. We sought a target gene regulated reciprocally by the Trx-G member Mll and selected Pc-G member Bmi-1, as they are both involved in oncogenesis. Mll-deficient and Bmi-1-deficient MEFs provide an easily obtainable and replenishable source of null cells that were assessed for their expression of the clustered Hox genes. Hox-c8 proves an attractive candidate to further define critical regulatory sequences as it was clearly reciprocally affected by the status of Mll and Bmi-1.

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