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Functional Specificity of CpG DNA-binding C*XX*C Domains in Mixed Lineage Leukemia*

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Background: The non-methyl-CpG DNA-binding C*XX*C domain is critical for MLL leukemia.
Results: Only the DNMT1 C*XX*C domain substitutes in an MLL leukemia model because of similar capacity to protect from CpG DNA methylation and H3K9 methylation.
Conclusion: Differential protection from specific CpG DNA methylation mechanistically distinguishes similar C*XX*C domains.
Significance: C*XX*C domains have specific nonredundant activities that impact downstream regulatory functions.

The MLL C*XX*C domain binds nonmethylated CpG-containing DNA and is essential for the oncogenic properties of MLL fusion proteins. To determine potential functional promiscuity of similar DNA binding domains, we replaced the MLL C*XX*C domain in the context of the leukemogenic MLL-AF9 fusion with C*XX*C domains from DNMT1, CGBP (CFP1), and MBD1, or with a methyl-CpG-binding domain (MBD) from MBD1. MLL(DNMT1 C*XX*C)-AF9 shows robust *in vitro* colony forming activity and *in vivo* leukemogenesis, similar to MLL-AF9. However, colony forming ability and leukemogenicity are abrogated in MLL-AF9 containing either the CGBP or MBD1 C*XX*C domains or the MBD1 MBD domain. Direct comparison of *in vitro* DNA binding affinity of the isolated C*XX*C or MBD domains demonstrated that MLL, DNMT1, and CGBP C*XX*C domains could each bind to unmethylated DNA but with differing affinity. In contrast, the isolated MBD1 C*XX*C and MBD1 MBD domains were unable to bind to the same DNA. However, all substituted domains still allowed targeting of the MLL fusions to the functionally important *Hoxa9* locus in primary bone marrow progenitor cells. In addition to DNA binding activity, it was critical that specific CpG residues in the *Hoxa9* locus were protected from methylation for leukemia development. This ultimately prevented histone 3 lysine 9 trimethylation (H3K9me3) of the locus and enabled *Hoxa9* expression. These were properties shared by MLL and DNMT1 C*XX*C domains but not by CGBP C*XX*C or the other swapped fusions tested. We demonstrate that similar C*XX*C domains can be mechanistically distinguished by specificity of CpG nucleotides preferentially protected from DNA methylation.

The mixed lineage leukemia (*MLL*)[2] gene was initially identified through its involvement in chromosome translocations that cause acute leukemia (1, 2). MLL leukemia patients have a relatively poor prognosis, so the development of specific targeted therapies would be beneficial (3, 4). *MLL* encodes a large multi-domain protein that is involved in positively maintaining *Hox* gene expression during development and hematopoiesis (5–7). *MLL* translocations encode leukemogenic MLL fusion proteins in which the amino-terminal domains of MLL are retained. These include a menin-LEDGF interacting motif (8), AT hooks which bind to AT-rich DNA (9), nuclear localization motifs, and the DNA-binding C*XX*C domain (10, 11).

DNA methylation occurs on position five on the cytosine ring of CpG dinucleotides (12). DNA methyl marks can inhibit the binding of transcriptional activators while recruiting co-repressors. Methyl-CpG binding domains (MBDs) are protein domains that specifically bind to methylated CpG DNA. They are present in the MBD family of repressor proteins, which include MeCP2 and MBD1, -2, -3, and -4 (13). One family member, MBD1, is present with the maintenance DNA methyltransferase enzyme DNMT1 at the replication fork during S phase of the cell cycle (14, 15). When DNMT1 adds a DNA methyl mark on newly replicated, hemimethylated DNA, MBD1 can bind directly to the newly methylated DNA. MBD1 then recruits the repressive histone H3K9 methyltransferase enzyme SETDB1 (16), thus coupling DNA methylation to histone modifications and chromatin remodeling.

Although promoter DNA methylation is most often associated with repressive chromatin states, unmethylated CpG DNA found in the promoters of genes is often associated with active transcription. The C*XX*C protein domain, similar to the MBD domain, also specifically recognizes CpG DNA, but only in the alternate, unmethylated state (17, 18). C*XX*C domains have highly conserved spacing of eight cysteine residues, which function to coordinate two zinc ions. This domain folds into a sad-

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[2] The abbreviations used are: MLL, mixed lineage leukemia; MBD, methyl-CpG binding domain; CGBP, CpG binding protein; DNMT1, DNA methyltransferase 1.

dle-like structure that centers over an unmethylated CpG residue when bound to DNA (19, 20). C*XX*C domains are present in several chromatin-associated proteins, including MLL, DNMT1, MBD1, and the transcriptional activator CpG binding protein CGBP/CFP1/C*XX*C-1. Most C*XX*C domains that have been studied show binding specificity for unmethylated CpG DNA, including those from CGBP (21, 22), MLL (17, 19, 20, 23), and DNMT1 (24). However, two of the three C*XX*C domains from MBD1 are unable to bind DNA in any state (25). Similar to MBD domains, C*XX*C domains have also been implicated in bridging DNA and histone epigenetic states. The MLL protein contains both the C*XX*C domain, which recognizes unmethylated DNA, and the histone methyltransferase SET domain, which methylates histone H3 tails at lysine 4 (H3K4), a mark of active transcription. Similarly, the C*XX*C domain-containing CGBP protein also helps direct H3K4 methylation patterns set by Setd1, another histone methyltransferase (26). It has recently been shown that the C*XX*C domain in DNMT1 acts as an auto-inhibitory domain that binds to unmethylated DNA, protecting it from DNMT1 DNA methyltransferase activity (24).

The C*XX*C domain of MLL was initially identified by its homology to DNMT1 (27) and was first shown to bind unmethylated DNA through electrophoretic mobility shift assays (17). Through deletion and point mutation experiments, the presence of the MLL C*XX*C domain was shown to be essential to MLL fusion protein function in colony formation assays (23). Further structural and functional work by our lab and others has shown the specific interaction between the MLL C*XX*C domain and unmethylated CpG DNA (19, 20). By mutating specific residues that are critical for the DNA binding activity of the MLL C*XX*C domain while keeping the structure of the domain intact, we showed that the DNA binding function of the MLL C*XX*C domain is essential for the ability of the MLL fusion protein to transform bone marrow cells and promote leukemia development (19). Additionally, MLL and MLL fusion proteins bind to the *Hoxa9* locus and protect specific CpG DNA in this region from becoming methylated (28). This protection against DNA methylation function of MLL contributes to keeping the chromatin of target genes in the open state to allow for active gene expression.

Although C*XX*C domains are defined by a particular clustering of cysteine residues, multiple amino acid differences exist between C*XX*C domains of different proteins. We tested our hypothesis that not all C*XX*C domains possess the necessary properties required for an MLL fusion protein to function as an oncogene and delineate the mechanism underlying C*XX*C domain functional specificity.

EXPERIMENTAL PROCEDURES

Cloning and Purification of GST-tagged Proteins—Isolated C*XX*C or MBD domains were cloned into the pGex-4T1 vector. The designs of the domains were based off of our previously published structure using 57 amino acids of the MLL C*XX*C domain (1147–1203) (19). Primers for the C*XX*C domains were designed to include the same number of amino acids before and after the C*XX*C cysteines as were used from MLL C*XX*C and 75 amino acids for the MBD domain, as was used previously to solve the MBD domain structure (29). DNMT1 (NM_001379) C*XX*C includes amino acids 645–700; CGBP (NM_001101654) C*XX*C includes amino acids 161–217; MBD1 transcript variant 1 (NM_015846) C*XX*C-1 includes amino acids 168–224; MBD1 (NM_015846) MBD includes amino acids 1–75. Cloning details provided upon request. Briefly, lysates were sonicated, cell debris was pelleted, and soluble GST-tagged C*XX*C or MBD proteins were purified using glutathione affinity chromatography (Fluka 49739). The GST-tagged proteins were eluted in 50 mM Tris, pH 7.2, 400 mM sodium chloride, 50 μM zinc chloride, 1 mM DTT, and 10 mM glutathione (G4251, Sigma). Protein purity was verified by Coomassie Blue staining.

DNA Binding Experiments using Fluorescence Polarization—Unmethylated C-terminal fluorescein labeled DNA (5′ GGGTCGCGGGAG 3′, Integrated DNA Technologies) and the purified GST-tagged C*XX*C or MBD domains were dialyzed into fluorescence polarization buffer (50 mM Tris-HCl, 150 mM KCl, 1 mM DTT, 10 μM $ZnCl_2$). Fluorescein-labeled DNA was added to 96-well black COSTAR (Corning Life Sciences, Lowell, MA) plates. The proteins were separately mixed with fluorescein-labeled DNA and serially diluted 1:2 onto the DNA-containing COSTAR plates. A PHERAstar microplate reader (BMG Labtech, Durham, NC) was used to measure fluorescence polarization with excitation at 494 nm and emission at 525 nm. The DNA binding experiments were performed three times. To calculate K_d for each protein, anisotropy values (mA) were plotted *versus* the log of protein concentration (μM), and the resulting plots were fit to a one-site sigmoidal binding curve using Origin software (version 7.0, MicroCal, Northampton, MA).

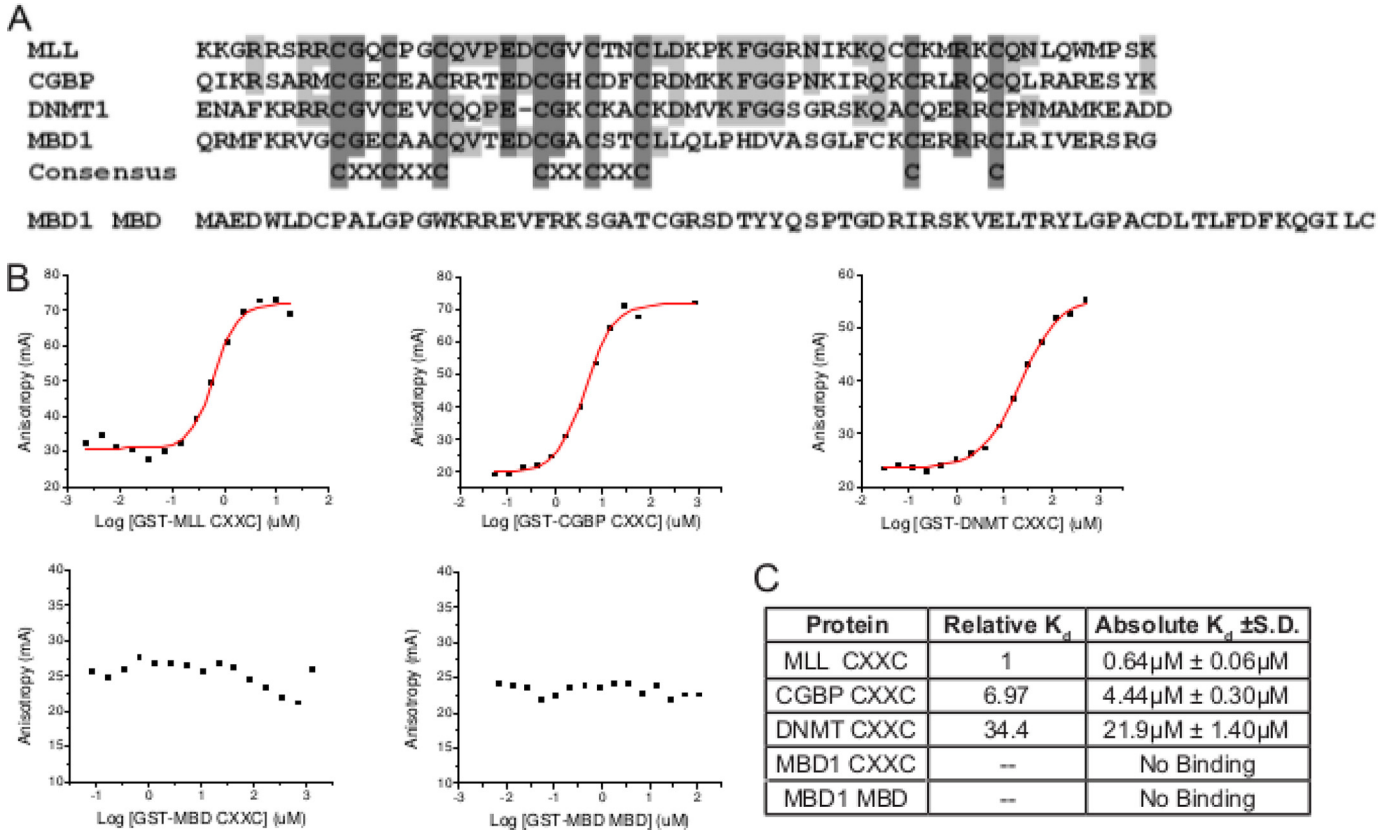
Cloning of MSCVneo-MLL-AF9-FLAG CXXC Domain Swap Constructs—Modified versions of our MSCVneo-MLL-AF9-FLAG construct were generated, which substituted the same domains used for the DNA binding experiments in place of the MLL C*XX*C domain. Cloning details will be provided upon request.

Cell Lines Used—A Phoenix-Eco cell line was obtained from Orbigen, Inc. (San Diego, CA) where it was tested for appropriate resistance to hygromycin and diphtheria toxin and negative for helper virus production. Stocks of early passage cells were frozen, and when used for experiments, were passaged less than three months.

Bone Marrow Colony Replating Assays and in Vivo Leukemia Experiments—Bone marrow colony assays and *in vivo* murine leukemia experiments were performed as described previously (19). All experiments using mice in this study were performed with the approval of and in accordance with the Loyola University Medical Center Institutional Animal Care and Use Committee, in accordance with federal guidelines.

Quantitative RT-PCR—RNA was isolated from week 1 methylcellulose colony assay cells, and cDNA was prepared. Real time PCR using Taqman probes or SYBR green was performed to measure *Hoxa9* and *MLL* fusion expression, respectively. Further details will be provided upon request.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation was performed using EZ-Magna ChIP A+G (Upstate/Millipore, Temecula, CA) with anti-FLAG, anti-H3K9me3 antibody, or control IgG on primary murine bone marrow progenitor cells expressing retroviral constructs. Results were analyzed by quantitative PCR in triplicate on an ABI 7300 real time PCR machine using iTaq SYBR Green Supermix with Rox (Bio-Rad).



Protein	Relative K_d	Absolute K_d ±S.D.
MLL CXXC	1	0.64μM ± 0.06μM
CGBP CXXC	6.97	4.44μM ± 0.30μM
DNMT CXXC	34.4	21.9μM ± 1.40μM
MBD1 CXXC	--	No Binding
MBD1 MBD	--	No Binding

FIGURE 1. **Relative binding affinities of isolated *CXXC* domains to unmethylated CpG-containing DNA.** *A*, *CXXC* domains are aligned to show amino acid sequence conservation: those identical in all or present in two or more are shaded in *dark* and *light gray*, respectively. The sequence of the MBD1 MBD is also shown. *B*, representative binding curves for fluorescein labeled DNA titrated with increasing concentrations of GST-tagged *CXXC* domains and measured by fluorescence polarization. *C*, absolute and relative K_d values for each of the GST-tagged *CXXC* domains. Absolute K_d values were determined from the fluorescence polarization titration curves. Relative K_d values were determined by comparison to GST-MLL *CXXC* (set to 1).

Enrichment was calculated as percent input chromatin. Primer sequences for *Hoxa9* DNA were as follows: 5′-CGGTGATTTAGGTAGTTTCCTGTTG-3′ and 5′-CACAGCGCCGAGGAAGAC-3′.

DNA Methylation Analysis—DNA was isolated from primary mouse bone marrow progenitor cells expressing MLL fusion constructs using DNeasy blood and tissue kit (Qiagen). DNA was bisulfite-treated and analyzed as described previously (19). Primers used for amplification were as follows: forward (outside), TYGAAATTYGYGGAGGAGGGTTTA; reverse (outside), CCCTACRATTATACCCAATCRAACCC; forward (inside), GTTAGGTTAYGYGTTTTTTGTT; reverse (inside), CCAACRATATAAAACRAATTCC.

RESULTS

To determine whether the functions of the MLL *CXXC* domain could be replaced by other *CXXC* domains or by the MBD meCpG-DNA binding domain, domain swap experiments were designed in the context of an oncogenic MLL-AF9 fusion protein to test for leukemogenic activity. As seen in the alignment, all *CXXC* domains contain the eight conserved cysteines, but there are various degrees of amino acid conservation between the domains at other positions (Fig. 1*A*). Furthermore, the MBD methyl CpG DNA-binding domain does not have any apparent amino acid sequence or structural similarity to the *CXXC* domains (29) (Fig. 1*A*).

Direct Comparison of DNA Binding Affinities for Isolated CXXC and MBD Domains—The DNA binding affinities of multiple CpG DNA binding domains to a specific DNA sequence have not been compared previously. Fluorescence polarization was used to measure the DNA binding affinities of several isolated CpG DNA binding domains (Fig. 1*A*). The DNA sequence used to measure the DNA binding affinities is derived from an MLL binding site in the *Hoxa9* locus, which contains two central CpG motifs and is protected from DNA methylation by MLL (28). We previously used isothermal titration calorimetry to demonstrate that the MLL *CXXC* domain binds to this specific nonmethylated DNA sequence (28). The other domains chosen for comparison with MLL vary in their CpG DNA binding capacity (Fig. 1*A*). Proteins were expressed as GST fusions in *Escherichia coli* and purified using glutathione agarose affinity chromatography (data not shown). Protein-DNA binding curves were generated using fluorescence polarization and show the approach of saturation of protein binding to DNA (Fig. 1*B*). From these curves, absolute and relative binding constants (K_d) were calculated (Fig. 1*C*). These results indicate that the MLL *CXXC* domain has the highest DNA binding affinity (0.64 μM) of the domains tested to this unmethylated DNA target. The CGBP *CXXC* domain has the second highest affinity at ~7-fold lower than MLL *CXXC*. DNMT1 *CXXC* could bind to this unmethylated DNA, but with a lower affinity: 34.4-fold

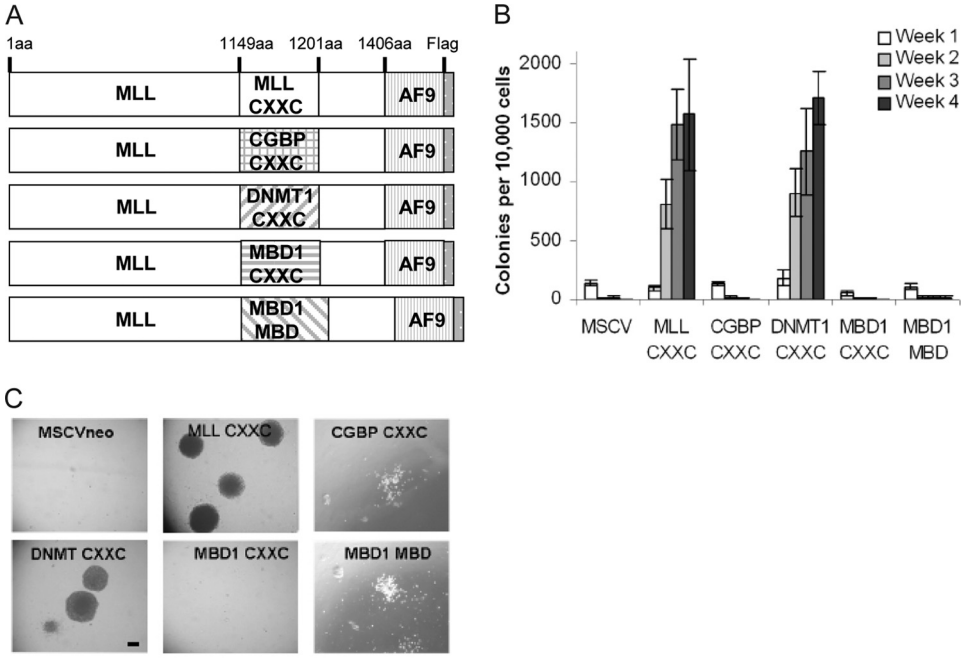


FIGURE 2. **Myeloid colony forming assays with bone marrow progenitor cells.** *A*, schematic of C*XX*C domain swaps into MLL-AF9-FLAG. *B*, average colony numbers for weeks 1–4 after plating primary mouse bone c-Kit+ progenitor cells expressing MLL-AF9 or MLL-AF9 with indicated C*XX*C domain swaps in methylcellulose, with *error bars* representing S.E. from 6–8 independent biological replicates. *C*, digital photographs (original magnification, 4×) showing colony morphologies at week 4 of the colony assay. *Scale bar* represents 250 μm. Pictures were taken through air on a Leica model DMIL microscope (Wetzlar, Germany), through a 4×/0.10 numerical aperture lens, with a Canon PowerShot S40 digital camera. Images were acquired with Canon ZoomBrowser EX (version 8).

lower affinity than the MLL C*XX*C domain. We tested binding of the MBD1 C*XX*C domain that has the least amino acid identity to MLL C*XX*C. It was shown previously that the mouse isoform of this Mbd1 C*XX*C domain was unable to bind to DNA regardless of methylation status (25), and our current data with the human orthologous region also shows no DNA binding activity (Fig. 1, *B* and *C*). As expected, the MBD1 MBD was unable to bind to the unmethylated DNA. Thus, most of the C*XX*C domains were able to bind nonmethylated CpG-containing DNA but with significant differences in binding affinity.

Construction of MLL-AF9 Retroviral Constructs with Substituted CXXC or MBD Domain—Retroviral constructs were generated to produce FLAG-tagged MLL-AF9 fusion proteins with various CpG DNA binding domains replacing the MLL C*XX*C domain (Fig. 2*A*). Amino acids 1150 through 1201 of MLL, which contain the minimal C*XX*C domain, were deleted and replaced with other CpG-recognizing domains analyzed above (Fig. 1). The C*XX*C domain from DNA methyltransferase 1 (DNMT1 amino acids 649–697), CpG binding protein (CGBP/CFP1 amino acids 163–215), and methyl-CpG binding domain protein 1 (MBD1 amino acids 172–221), as well as the MBD from MBD1 (amino acids 1–75) were swapped into the MLL-AF9 fusion. Stable expression of all the MLL-AF9 fusion proteins was confirmed by Western blot and quantitative RT-PCR (data not shown). We predicted that the different CpG DNA binding domains would change the strength or specificity of MLL protection of DNA against methylation, which would affect the ability of MLL-AF9 to cause leukemia.

Ability of Other CXXC and MBD Domains to Functionally Replace MLL CXXC Domain in MLL-AF9 in Vitro Immortalization Assay—MLL fusion proteins, including MLL-AF9, confer increased proliferative capacity and immortalization when exogenously expressed in murine bone marrow progenitor cells, such that they can be serially replated in methylcellulose (19, 30). Vector-infected or non-leukemogenic mutant-infected progenitor cells typically form colonies only in the first week and then differentiate and die, similar to normal bone marrow progenitors. To determine whether the CpG binding domains from other proteins would affect this ability of MLL-AF9 to give an enhanced proliferative capacity to bone marrow progenitor cells, we performed i*n vitro* colony assays. Murine bone marrow c-Kit+ progenitor cells were isolated and infected with domain swap or wild type MLL-AF9 retroviral fusion genes (Fig. 2*A*). We observed significant differences between the ability of various CpG DNA binding domains to function in the context of an MLL-AF9 fusion protein (Fig. 2*B*). As expected, bone marrow cells expressing MLL-AF9 continue to proliferate and form many colonies through four sequential platings of the colony assay, whereas MSCVneo vector infected cells have very few, if any, colonies remaining. MLL(DNMT1 C*XX*C)-AF9 shows robust *in vitro* colony forming activity, similar to the oncogenic MLL-AF9 fusion. However, MLL(CGBP C*XX*C)-AF9, MLL(MBD1 C*XX*C)-AF9, and MLL(MBD1 MBD)-AF9 almost completely abrogated colony forming ability. Those few colonies that persisted for these constructs were often diffuse in nature and typical of differentiated bone marrow cells, with few cell numbers (Fig. 2*C*). The MLL-AF9 and MLL(DNMT1 C*XX*C)-AF9 cells formed

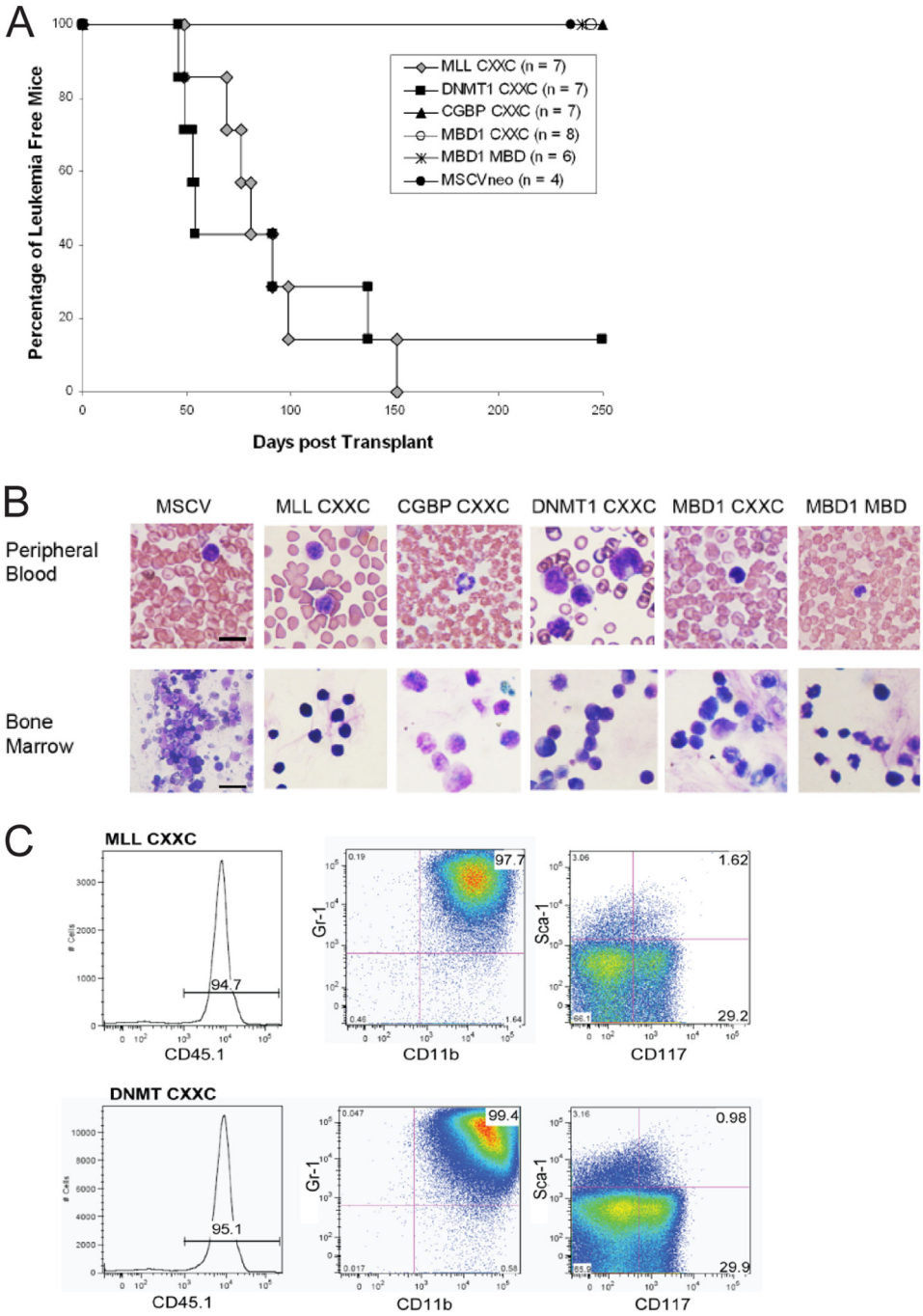


FIGURE 3. **Incidence of leukemia in mice expressing MLL-AF9 or MLL-AF9 with substituted *CXXC* domains.** *A*, survival curve of mice transplanted with bone marrow progenitor cells expressing MLL-AF9 or MLL-AF9 *CXXC* domain swap fusion proteins. *B*, peripheral blood smears and bone marrow samples taken from indicated mice at time of sacrifice (original magnification, 100×). *Scale bar* represents 12 μm. Pictures were taken on an Olympus BH-2 microscope (Tokyo, Japan), under a 100×/1.25 Numerical aperture oil immersion lens, with a Sony 3CCD camera (model DXC-76OMD). Images were acquired with Adobe Premier software (version 4.2.1) and processed using Adobe Photoshop CS3 (version 10.0). *C*, FACS profiles of bone marrow cells from leukemic mice. CD45.1 indicates transplanted cell population expressing the MLL-AF9 or domain swap construct. The *second* and *third panels* show the percentage of CD45.1 positive cells that express myeloid markers CD11b and Gr-1 and progenitor markers CD117 and Sca-1.

dense compact round colonies typical of bone marrow progenitor cells. Cell counts over the course of the replating experiment also reinforced this observation. MLL-AF9 and MLL(DNMT1 C*XXC*)-AF9 both conferred an ~870-fold expansion of cell numbers at each replating, whereas for the remaining constructs, the cell expansion ranged from 3- to 7-fold (data not shown). Cytospins of the bone marrow cells taken from week 3 or 4 of the colony assays also confirm that the transformed MLL-AF9 and MLL(DNMT1 C*XXC*)-AF9 cells primarily resemble leukemic blasts, whereas the other constructs produce a more heterogeneous differentiated bone marrow cell population (data not shown).

Because the domains from MBD1 were not able to function in MLL-AF9, our hypothesis that MLL C*XXC* must maintain the ability to bind to unmethylated DNA to transform bone marrow cells was supported. However, we expected that both CGBP and

DNMT1 C*XX*C domains would replace MLL C*XX*C domain in the context of the MLL-AF9 fusion because the isolated domains bound to the nonmethylated CpG-containing DNA *in vitro*. This capability did not strictly align with DNA binding affinity of the isolated C*XX*C domain, however, because the C*XX*C domain from CGBP had a higher affinity for DNA binding as compared with the DNMT1 C*XX*C domain (Fig. 1*B*). These results suggest that C*XX*C DNA binding affinity is not the only function of this domain necessary for MLL-AF9 transformation.

Ability of Other CXXC and MBD Domains to Functionally Replace MLL CXXC Domain in MLL-AF9 to Cause Leukemia in Vivo—In vivo murine studies were performed to determine whether the different CpG DNA binding domains would alter the ability of MLL fusion proteins to cause leukemia. Murine c-Kit+ progenitor cells isolated from B6/SJL CD45.1+ mice were infected with MLL-AF9 or domain swap constructs and then transplanted into irradiated C57Bl/6 recipient mice as described previously (19). The results of the leukemia assay correlate with the colony assay data in that all MLL-AF9 mice and all but one of the MLL(DNMT1 C*XX*C)-AF9 mice developed MLL acute myeloid leukemia, whereas none of the MSCVneo, MLL(CGBP C*XX*C)-AF9, MLL(MBD1 C*XX*C)-AF9, or MLL(MBD1 MBD)-AF9 mice developed leukemia (Fig. 3*A*). A small number of the mice that did not develop leukemia (one or two mice from each group, excluding MLL-AF9 and MLL (CGBP C*XX*C)-AF9) died from other cancers during the course of the experiment. However, these tumor cells did not express the CD45.1 marker from the donor cells; therefore, they were unrelated to MLL-AF9 and were likely a side effect of the radiation that the recipients received prior to transplant. Peripheral blood and bone marrow from leukemic mice show increased blast cells (Fig. 3*B*). Wright-Giemsa stained organ sections also show infiltration of leukemia cells into the spleen, liver, lungs, and kidneys of the MLL-AF9 and MLL(DNMT1 C*XX*C)-AF9 mice (data not shown). Bone marrow cells taken upon sacrifice from the leukemic or healthy mice were subjected to surface marker staining for multiple murine hematopoietic cell markers followed by flow cytometric analysis. All MLL-AF9 and MLL(DNMT1 C*XX*C)-AF9 mice with leukemia showed a high percentage of CD45.1+ cells, indicating that the leukemic cells had replaced the normal bone marrow cells of the mice (Fig. 3*C*), whereas the healthy mice only had a small population of CD45.1+ cells in their bone marrow. Nearly all of the CD45.1+ leukemia cells expressed the myeloid markers CD11b and Gr-1. Smaller proportions were positive for the progenitor markers Sca-1 and c-Kit (CD117). The cells were negative for lymphocyte and other hematopoietic cell markers (data not shown). The results of this leukemia assay corroborate the *in vitro* colony assay results and again emphasize that the DNMT1 C*XX*C domain can functionally replace the MLL C*XX*C domain to enable an MLL-AF9 fusion to cause leukemia. However, although the nonmethyl-CpG DNA binding function of MLL C*XX*C domain remains essential, the CGBP C*XX*C domain with similar DNA binding function is not sufficient for MLL-AF9 oncogenic activity.

Hoxa9 Expression in MLL-AF9 and Domain-swapped MLL-AF9-transduced Bone Marrow Progenitors—The MLL target gene *Hoxa9* is frequently up-regulated in MLL leukemias, and its overexpression is involved in disease progression (31, 32). To determine whether the MLL-AF9 C*XX*C domain swap proteins cause increased *Hoxa9* expression, RNA was isolated from bone marrow cells expressing the domain swap fusion proteins. *Hoxa9* transcript levels were assessed by quantitative RT-PCR (Fig. 4*A*). The MLL-AF9 and MLL(DNMT1 C*XX*C)-AF9 proteins cause overexpression of *Hoxa9*, whereas the other swap constructs that were non-transforming did not increase expression over the levels of MSCVneo-infected bone marrow cells. The lower levels of *Hoxa9* expression help explain why the MLL-AF9 fusion proteins with the CGBP C*XX*C, MBD1 C*XX*C, or MBD1 MBD domain were unable to transform bone marrow cells.

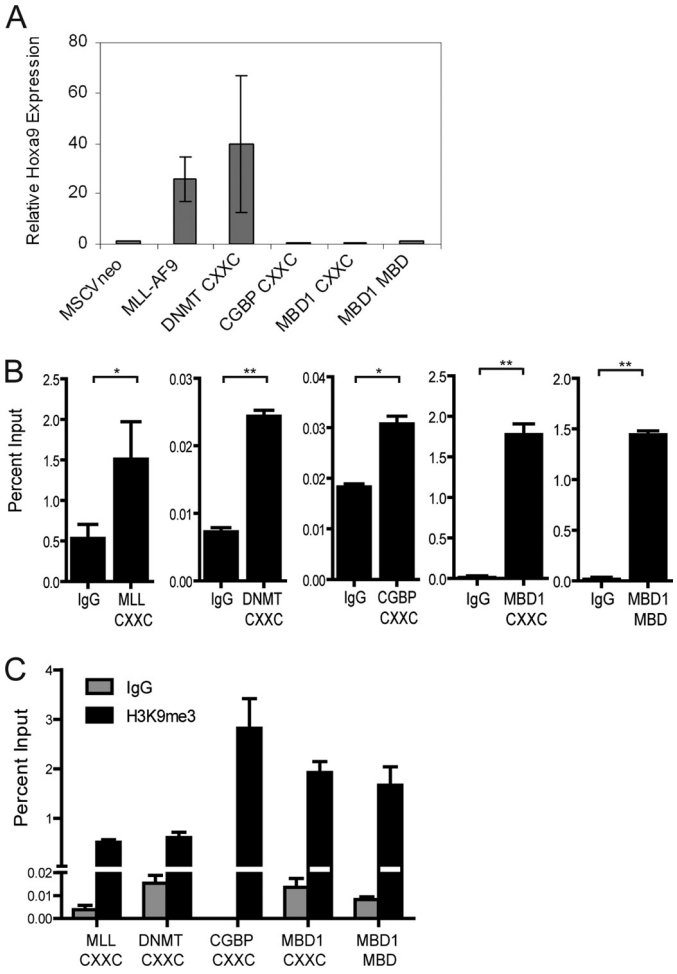


FIGURE 4. **Hoxa9 expression, fusion protein binding to the *Hoxa9* locus, and H3K9 methylation in bone marrow progenitor cells expressing MLL-AF9 or MLL-AF9 with substituted *CXXC* domains.** *A*, quantitative RT-PCR for *Hoxa9* in bone marrow cells expressing the indicated constructs. Shown are mean relative expression levels compared with *Hprt* from two independent experiments each done in triplicate, with *error bars* indicating S.D. *B* and *C*, chromatin immunoprecipitation assay from primary murine progenitor cells expressing the indicated constructs. Anti-FLAG or IgG (*B*) or anti-H3K9me3 or IgG (*C*) were used to immunoprecipitate chromatin, and quantitative PCR was performed with primers that amplify an MLL-binding region in the *Hoxa9* locus. Primer location indicated by *arrows* in the *Hoxa9* locus schematic shown in Fig. 5. Results are shown as average percent input from triplicate experiments with *error bars* representing S.E.

Chromatin Localization in MLL-AF9 and Domain-swapped MLL-AF9 Fusion Proteins—To determine whether MLL-AF9 domain swap constructs which do not cause overexpression of *Hoxa9* still bind to the locus, chromatin immunoprecipitation

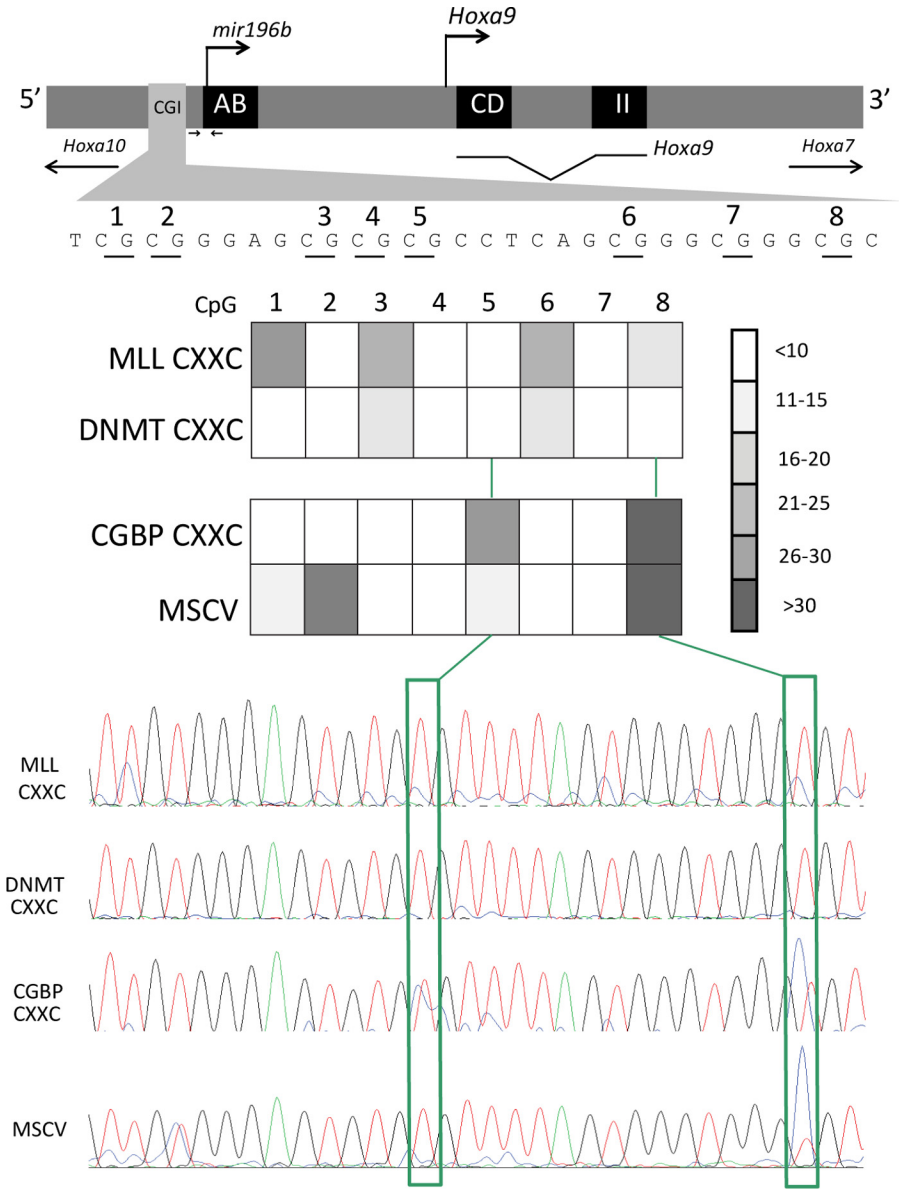


FIGURE 5. **Differential DNA methylation in bone marrow progenitor cells expressing MLL-AF9 or MLL-AF9 with substituted *CXXC* domains.** *Top*, schematic representation of the *Hoxa9* locus. CpG island (*CGI*) containing eight CpG residues dependent on Mll for protection from DNA methylation are located upstream of *mir196b* and *Hoxa9* transcription start sites. Location of primers used for ChIP is indicated by *arrows*. *Middle*, heat map depicting DNA methylation levels in primary bone marrow progenitor cells expressing the MLL *CXXC*, DNMT *CXXC*, or CGBP *CXXC* domains swapped into MLL-AF9 or control MSCVneo vector. Methylation levels represent the average of five experiments. CpG 1–8 correspond to the CpGs shown in schematic. *Bottom*, representative chromatograms of sequenced PCR products from bisulfite-treated genomic DNA isolated from primary MLL-AF9 bone marrow cells with substituted *CXXC* domains. CpGs correspond to those in schematic and heat map.

assays were performed using bone marrow cells expressing the MLL-AF9 domain swap constructs. All of the MLL-AF9 *CXXC* domain swap proteins show significant binding to the *Hoxa9* locus, regardless of their ability to activate transcription of the locus (Fig. 4*B*). This suggests that, similar to what we found previously for MLL-AF9 (19), the MLL *CXXC* domain is not essential for MLL-AF9 proteins to localize to the *Hoxa9* locus.

Transforming MLL Fusions Protect a Specific Subset of CpG Sequences from DNA Methylation—Although both DNMT- and CGBP-*CXXC* domain swapped MLL-AF9 fusion proteins were able to bind the *Hoxa9* locus, only DNMT *CXXC* functioned to cause leukemia. Our previous studies demonstrated that MLL and MLL fusion proteins protect specific CpG sequences from methylation. We hypothesized that differential ability to protect CpG DNA methylation may be the mechanism underlying functional differences. DNA was isolated from bone marrow progenitor cells expressing the MLL fusions and assessed for DNA methylation status using bisulfite sequencing. Different DNA methylation patterns were observed when comparing transforming fusions with the non-transforming fusion (Fig. 5). In particular, the CGBP *CXXC* domain-containing fusion did not efficiently protect CpG-5 and CpG-8 from methylation, whereas both MLL-AF9 and the DNMT1 *CXXC*-containing MLL-AF9 abrogated methylation of this region. Conversely, CGBP more effectively protected CpG-6 and -7 from methylation than the transforming fusion proteins. This

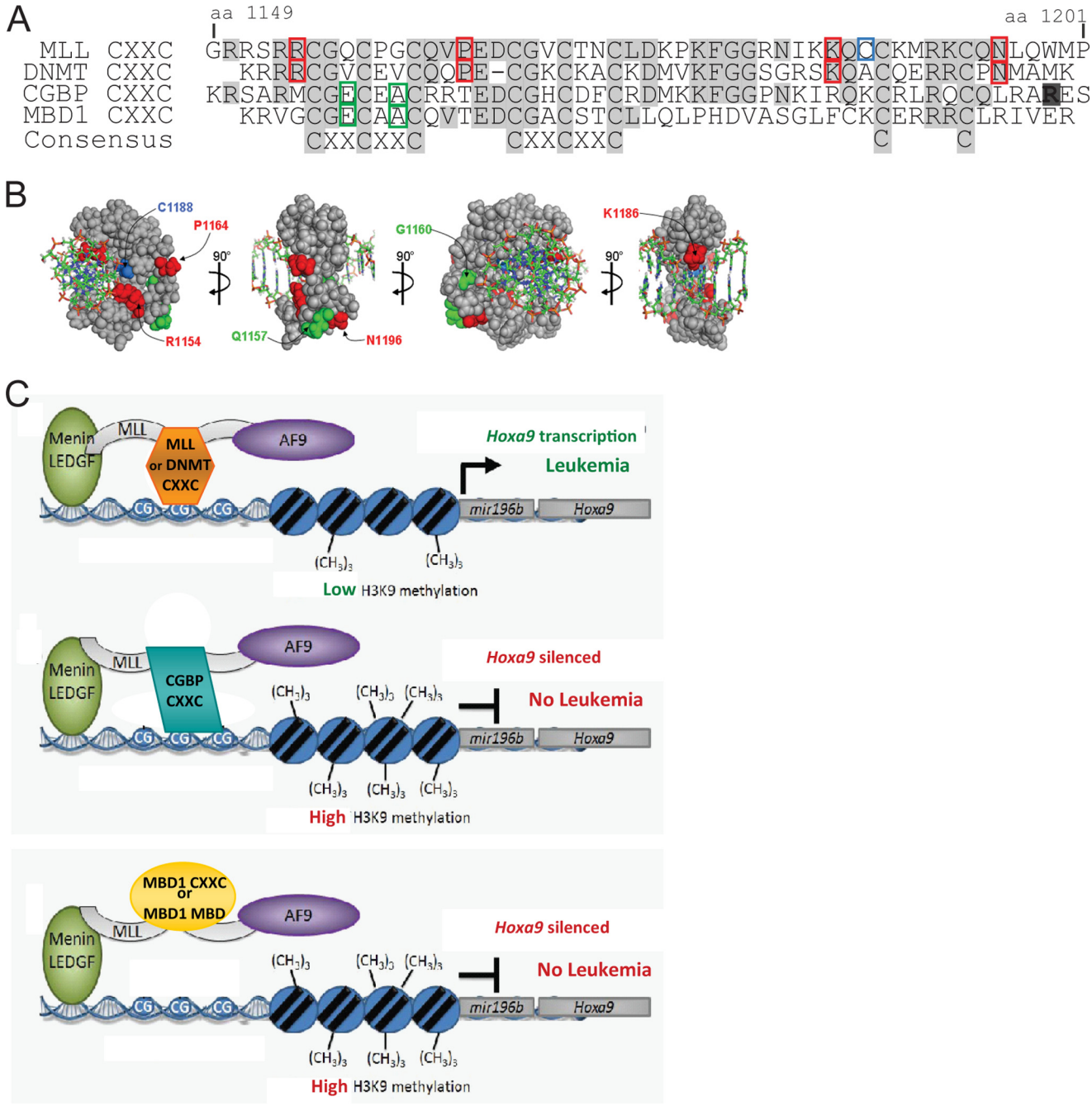


FIGURE 6. **Model of MLL-AF9 *CXXC* domain functional specificity in leukemogenesis.** *A*, amino acid alignment to show sequence conservation between different *CXXC* domains. Identical residues to MLL are shaded in *gray*, whereas *boxed residues* highlight differences. *Red* residues are identical in MLL and DNMT1 but not present in CGBP or MBD1 *CXXC*. *Green* residues are conserved in CGBP and MBD1 but not present in MLL *CXXC*. *B*, solution structure of MLL *CXXC* domain in complex with CpG DNA (Protein Data Bank code 2KKF). The MLL *CXXC* domain is shown as a space-filling model in contact with the ball and stick structure of DNA, in four orientations. The same color scheme is used as described in *A*. *C*, model of functional specificity of CpG DNA binding domains in the context of MLL fusion proteins, adapted from Ref. 19.

suggests that transforming ability may depend on the methylation status of specific CpG nucleotides.

Transforming MLL Fusions Protect from Histone 3 Lysine 9 Trimethylation—CpG methylation has been shown to enable recruitment of protein complexes that ultimately cause histone modifications associated with silent chromatin such as HeK9me3 (16). To determine whether MLL-AF9 domain swap fusion proteins that do not cause leukemia enable increased H3K9me3, ChIP assays were performed (Fig. 4*C*). We found that an increased level of H3K9me3 at the *Hoxa9* locus correlated with inability of the swapped fusion proteins to activate *Hoxa9* expression, to transform bone marrow progenitors *in vitro*, and cause leukemia *in vivo*.

DISCUSSION

We have explored the functional specificity of the *CXXC* domain in MLL leukemia. For the first time, the DNA binding affinities of *CXXC* domains from multiple proteins have been directly measured and compared with each other for binding to the same CpG-containing DNA sequence. MLL *CXXC* binds

with highest affinity to the unmethylated CpG DNA, followed by CGBP C*XX*C, and then DNMT1 C*XX*C. MBD1 C*XX*C and MBD domains were unable to bind to unmethylated DNA. MBD1 MBD domain sequence is unrelated to C*XX*C domains and was previously shown to bind only methylated CpG-containing DNA; therefore, its lack of binding was expected (33). One of the MBD1 splice variants contains three C*XX*C domains (34). Previous DNA binding studies of the murine Mbd1 C*XX*C domains demonstrated that the first two C*XX*C domains from Mbd1 were unable to bind DNA regardless of its methylation status (25), which we also found to be true of the first C*XX*C domain from human MBD1, which has the least identity to MLL of the C*XX*C domains studied here. These different C*XX*C or MBD domains were substituted in place of the MLL C*XX*C domain in the context of an MLL-AF9 fusion, and the mutant fusion proteins were tested for leukemogenic activities *in vitro* and *in vivo*. The C*XX*C and MBD domains from MBD1, which are unable to interact with unmethylated CpG DNA, were incapable of replacing the MLL C*XX*C domain in promoting MLL-AF9 oncogenicity. This confirmed our hypothesis that DNA binding is an essential function of the MLL C*XX*C domain in MLL-AF9 promotion of *Hoxa9* overexpression and development of acute leukemia. However, an intriguing finding from this study is that the DNMT1 C*XX*C domain, which has a lower DNA binding affinity compared with that of the MLL C*XX*C domain, is able to replace MLL C*XX*C in MLL-AF9, whereas the CGBP C*XX*C domain, which has a higher DNA binding affinity compared with the DNMT1 C*XX*C domain, is not functional in MLL-AF9. Overall, these results suggest that for a C*XX*C domain to function in the context of an MLL-AF9 fusion protein, DNA binding activity to relevant target DNA sequence is required, but a high DNA binding affinity alone is not sufficient. Additional properties of the C*XX*C domain must also contribute to function.

Our ChIP studies show that MLL-AF9 is able to localize to *Hoxa9* regardless of which CpG-binding domain is present. Additional MLL targeting domains, including menin/LEDGF binding domains and the AT hooks, would likely contribute to the chromatin binding ability of the fusion protein. Although all domain swapped proteins bound to the *Hoxa9* locus, they differed in the specific CpG residues protected from DNA methylation. CGBP cells had demethylated CpG-6 and -7, which agrees with the previous finding that CGBP C*XX*C preferentially binds a CpGG motif (22). Arginine 213 of CGBP (highlighted in Fig. 6*A*) forms two hydrogen bonds with the guanosine nucleotide base following the CpG dinucleotide. In contrast, MLL and DNMT contain nonpolar amino acids in the same site (Fig. 6*A*) and are associated with preferentially demethylated CpG-5 and -8. Therefore, it may be of critical importance that CpG-5 and -8 remain unmethylated for transformation of hematopoietic progenitors by MLL fusion proteins. C*XX*C domain specificity likely determines preferential gene targets affected by proteins containing these structurally similar domains. Our data also support the ability of the non-transforming fusions to enable proteins with H3K9 methyltransferase activity access to this critical locus. It has been previously shown that the MBD1 MBD domain binds to co-repressors HP1 and Suv39h1, a histone H3K9 methyltransferase. This would act to further enforce transcriptional repression of gene targets by linking DNA and histone modifications (35). In addition, a region of MBD1 that includes the MBD domain and its first two C*XX*C domains is able to bind the chromatin assembly factor-1 and the histone methyltransferase SETDB1 to provide histone H3K9 trimethylation on newly replicated, DNA-methylated chromatin (16). Our data also suggest that CGBP C*XX*C allows recruitment of repressive H3K9 methyltransferase activity to *Hoxa9*, whereas MLL and DNMT C*XX*C domains protect against this effect.

Eight structural cysteines are conserved among all C*XX*C domains. Additional residues are identical between the two transforming C*XX*C domains from MLL and DNMT1 (Fig. 6, *A* and *B*, highlighted in *red*) but not the other C*XX*C domains, suggesting they may be essential to MLL C*XX*C function. *Green* residues are identical between CGBP and MBD1 C*XX*C domains but are not present in MLL or DNMT1, which suggests that the amino acids at these positions in CGBP or MBD1 may be inhibitory to C*XX*C function in MLL-AF9. These differences could influence DNA binding affinity or protein-protein interactions.

The MLL C*XX*C domain is a critical functional domain in MLL fusion proteins. The C*XX*C DNA binding function is essential to promote an unmethylated DNA state, which helps to keep target genes activated (19). In this study, we have shown that of the domains tested, only the DNMT1 C*XX*C domain can functionally replace MLL C*XX*C to provide critical functions required of the C*XX*C domain in a leukemogenic MLL fusion protein (Fig. 6*C*). C*XX*C domains from different proteins share some but not all functional characteristics. MLL fusion proteins require a C*XX*C domain that can both bind to unmethylated DNA and provide the ability to prevent both specific CpG DNA methylation and histone H3K9 methylation. This allows critical target loci such as *Hoxa9*, to remain active. The MLL C*XX*C domain remains a potentially tractable region for targeted therapies to be developed to treat MLL leukemia.

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