Bromodomain and Histone Acetyltransferase Domain Specificities Control Mixed Lineage Leukemia Phenotype

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Abstract

A critical unanswered question about mixed lineage leukemia (MLL) is how specific MLL fusion partners control leukemia phenotype. The MLL-cyclic AMP-responsive element binding protein–binding protein (CBP) fusion requires both the CBP bromodomain and histone acetyltransferase (HAT) domain for transformation and causes acute myelogenous leukemia (AML), often preceded by a myelodysplastic phase. We did domain-swapping experiments to determine whether unique specificities of these CBP domains drive this specific MLL phenotype. Within MLL-CBP, we replaced the CBP bromodomain or HAT domain with P300/CBP-associated factor (P/CAF) or TAF1q250 bromodomains or the P/CAF or GCN5 HAT domains, HAT, but not bromodomain, substitutions conferred enhanced proliferative capacity in vitro but lacked expression of myeloid cell surface markers normally seen with MLL-CBP. Mice reconstituted with domain-swapped hematopoietic progenitors developed different disease from those with MLL-CBP. This included development of lymphoid disease and lower frequency of the myelodysplastic phase in those mice developing AML. We conclude that both the CBP bromodomain and HAT domain play different but critical roles in determining the phenotype of MLL-CBP leukemia. Our results support an important role for MLL partner genes in determining the leukemia phenotype besides their necessity in leukemogenesis. Here, we find that subtleties in MLL fusion protein domain specificity direct cells toward a specific disease phenotype. (Cancer Res 2006; 66(20): 10032-9)

Introduction

Mixed lineage leukemia (MLL) is involved in chromosome translocations resulting in acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), or in leukemia of mixed immunophenotype (1). Approximately 70% of infant leukemia as well as over half of therapy-related leukemias arising secondary to chemotherapy treatment using DNA topoisomerase II-targeting drugs involve MLL rearrangements (1). MLL translocations result in the expression of fusion proteins with the NH2-terminal portion of MLL fused in-frame to one of >50 different partners proteins (1, 2). Notably, the particular partner gene fusion often correlates with a specific leukemia phenotype. For example, 95% of patients with MLL-AF4 present with ALL and 96% of MLL-AF9 have AML (3). Most patients with MLL-cyclic AMP-responsive element binding protein–binding protein (CBP) present with chronic myelomonocytic leukemia (CMML) before developing AML (4). MLL fusions all retain the same protein domains of MLL, so the partner proteins must contribute significantly to specify leukemia phenotype, although this has not been tested directly.

MLL is an ortholog of the Drosophila trithorax gene, which functions as a maintenance enhancer of homeotic gene expression during development. Mll−/− mice initially express Hoxa7 and Hoxc9, but this expression is not appropriately maintained later in development (5, 6). Mll−/− mice are embryonic lethal at day 10.5 and show defects in yolk sac hematopoiesis (5–7). MLL codes for a large, ubiquitously expressed nuclear protein with many functional domains (reviewed in ref. 8). The NH2-terminal portion of MLL contains three AT-hooks as well as a CpG DNA binding domain overlapping a transcriptional repression domain. Recently, the tumor suppressor Menin was found to interact with the NH2-terminal portion of MLL retained in the fusions. The COOH-terminal region of MLL is not present in the MLL-partner fusions critical for leukemogenesis. This region contains PHD fingers involved in protein-protein interactions, an activation domain, and a SET domain with histone methyltransferase activity.

The t(11;16)(q23p13.3) has been found almost exclusively in patients previously treated for a primary malignancy with DNA topoisomerase II inhibitors (4). This translocation results in an in-frame fusion of MLL to CBP (9), a global transcriptional coactivator with intrinsic histone acetyltransferase (HAT) activity (10, 11). CBP interacts with many different proteins, including transcription factors, signaling molecules, and nuclear hormone receptors (12). Through targeted acetylation of histones and transcription factors, CBP contributes directly to transcriptional regulation. We previously showed that the NH2 terminus of MLL fused to the bromodomain and HAT domain of CBP (MLL-CBP80/CBP140) is necessary and sufficient for imparting an enhanced proliferative capacity in vitro to murine hematopoietic progenitor cells (13). In vivo, MLL-CBP80/CBP140 caused a disease similar to that seen in humans with the t(11;16)(q23p13.3) (13).

Bromodomains are frequently found in chromatin-associated proteins that also contain HAT domains (14). Bromodomains bind to acetylated lysines, with ligand selectivity due to conformational differences in the loops of the bromodomain (14, 15). The CBP bromodomain specifically binds acetylated Lys382 in the COOH terminus of p33, which is responsible for coactivator recruitment by p33 after DNA damage. The P300/CBP-associated factor (P/CAF) bromodomain, the first bromodomain found to bind acetylated lysines, binds to the histone tail of H4 acetylated at Lys8 and to HIV Tat-1 acetylated at Lys50 (14). TAF1q250, a subunit of transcription factor IID that recognizes and binds promoter DNA,
contains two tandem bromodomains. The TAF\(_d\)250 double bromodomains bind to histone H4 peptides diacetylated (K8/K16) or tetra-acetylated (K5, K8, K12, and K16) with a much higher affinity than to either a monoacetylated (K16) or a nonacetylated H4 peptide, highlighting the ligand specificity of bromodomain binding (16).

HATs transfer an acetyl group from CoA to a lysine residue of histone and nonhistone proteins. CBP belongs to a different family of HATs than P/CAF and GCN5 (17–19), and acetylates histone tails with different preferences (19–22). P/CAF, GCN5, and CBP also have unique nonhistone targets of acetylation (23). For example, acetylation of HMGA1 at K71 by GCN5/P/CAF stabilizes an enhanceosome, potentiating transcription of the -IFN gene (24). However, CBP acetylates HMGA1 K65, making the enhanceosome less stable and inhibiting transcription.

We undertook domain-swapping experiments in the context of MLL-CBP\(^{\text{Br}}\)/CBP\(^{\text{H}}\) to determine if there are specific, nonreplaceable roles of the CBP bromodomain and HAT domain in the immortalization and differentiation of hematopoietic progenitors. In the context of MLL-CBP, the CBP bromodomain was replaced with the P/CAF bromodomain or with the TAF\(_d\)250 double bromodomain. In addition, we replaced CBP HAT domain with the P/CAF or GCN5 HAT domain (Fig. 1A). We found clear differences in the in vitro proliferative capacity and in vivo leukemia phenotypes caused by the domain swap constructs. This work provides the first direct evidence that specific domains of MLL partner gene proteins are critical in determining the leukemia phenotype.

### Materials and Methods

**Plasmid construction.** Splicing by overlap extension was used to swap CBP domains using Pfu polymerase (primers available on request; Stratagene, La Jolla, CA; ref. 25). The swapped domains contain the following amino acids: GCN5 HAT domain, 494 to 651 (26); P/CAF HAT domain, 719 to 832 (14); and TAF\(_d\)250 double bromodomain, 1,352 to 1,638 (16). The product was subcloned into pFLAG CMV2 and fully sequenced at Loyola University Medical Center Sequencing Core (Maywood, IL). The CBP/switched fragments were used in a three-way ligation reaction with MIE or MSCVneo vector and the MLL NH\(_{2}\) terminus as described previously (13).

**Reverse transcription-PCR.** RNA was isolated using TriReagent or TriReagent BD (Sigma, St. Louis, MO), and cDNA was synthesized using a three-way ligation reaction with MIE or MSCVneo vector and the MLL NH\(_{2}\) terminus as described previously (13).

**Bromodomain binding assay.** Purified protein (35 µL) on FLAG M2 bead slurry was incubated with 20 nmol of a biotinylated version of the acetylated peptide of interest. The biotinylated histone H4 peptide was acetylated at K5, K8, K12, and K16 (Upstate Biotechnology, Charlottesville, VA). The pS3 acetylated K382 peptide is Biotin-GQTSRBBK-Ack-LMKKTE (30); the HIV Tat-1 acetylated K50 peptide is Biotin-GISYGR-AcK-KRRQRRRP (15). The samples were rocked at room temperature for 2 hours, washed four times with 1 mL of NET-N buffer containing protease inhibitor cocktail (Sigma), incubated with 0.5 µCi [\(^{3}H\)Acetyl CoA, 48 µL of purified protein on a slurry of anti-FLAG M2 beads in HAT assay buffer [50 mmol/L HEPES (pH 8.0), 10% glycerol, 0.1 mmol/L EDTA, 1 mmol/L DTT, 0.2 mmol/L sodium butyrate], and processed as described previously (29).

**Bromodomain binding assay.** Purified protein (35 µL) on FLAG M2 bead slurry was incubated with 20 nmol of a biotinylated version of the acetylated peptide of interest. The biotinylated histone H4 peptide was acetylated at K5, K8, K12, and K16 (Upstate Biotechnology, Charlottesville, VA). The pS3 acetylated K382 peptide is Biotin-GQTSRBBK-Ack-LMKKTE (30); the HIV Tat-1 acetylated K50 peptide is Biotin-GISYGR-AcK-KRRQRRRP (15). The samples were rocked at room temperature for 2 hours, washed four times with 1 mL of NET-N buffer containing protease inhibitor cocktail, resuspended in 50 µL of NET-N, and loaded onto nitrocellulose using a slot blot apparatus. The membrane was blocked at 4°C in 5% milk/PBS-0.05% Tween 20 (PBS-T), washed three times in PBS-T for 10 minutes each, and incubated at room temperature in streptavidin-horseradish peroxidase (diluted 1:1,000 in PBS; Amersham Biosciences, Piscataway, NJ) for 1 to 2 hours. The membrane was then washed three times for 20 minutes each in PBS-T. Binding was visualized using enhanced chemiluminescence.

**Retrovirus production and myeloid colony assay.** Retrovirus was produced in Phoenix-Eco cells (Garry Nolan, Stanford University, Stanford, CA) using standard procedures (31), concentrated at 500 × g using Centricon YM-100 filters, and then frozen in aliquots at –80°C. Retroviruses were titered on NIH3T3 cells. Myeloid colony assay was essentially done as...
described previously (13, 32) with the following exceptions. Bone marrow cells were harvested from 5- to 6-week-old C57BL/6 mice (Taconic, Hudson, NY) and enriched for hematopoietic progenitors using StemSep (Stemcell Technologies, Vancouver, BC, Canada).

**Flow cytometry.** Cells were blocked with anti-CD16/32 clone 93 and then stained using the following anti-mouse antibodies (clone in parentheses; all antibodies were from eBioscience, San Diego, CA): CD45.1 (A20), B220 (RA3-6B2), CD117 (2B8), Ly-6A/E (Sca-1; D7), Gr-1 (RB6-8C5), CD11b (Mac-1; M1/70), CD3 (145-2C11), CD4 (GK1.5), and CD8 (53-6.7). RBCs were lysed using fluorescence-activated cell sorting (FACS) lysing buffer (Becton Dickinson, Franklin Lakes, NJ). Data were collected on a Becton Dickinson FACSCalibur and analyzed using CellQuest or FlowJo software.

**Tumorigenicity assay.** Bone marrow from 3- to 4-week-old B6.SJL mice (Taconic) was harvested, enriched for hematopoietic progenitors, and transduced with MIE constructs as described previously (13, 32). Six-week-old C57BL/6 mice were lethally irradiated with 9 Gy of γ-radiation. Mice were reconstituted with 3 × 10^4 transduced B6.SJL (CD45.1) cells and 1 × 10^5 to 2 × 10^5 untreated C57BL/6 (CD45.2) mononuclear cells. Mice were anesthetized with isoflurane (Abbott Animal Health, Abbott Park, IL) before retro-orbital injection of cells in PBS (100 μL). Reconstitution was examined by CD45.1 staining of peripheral blood. Mice were monitored for disease by observation, periodic complete blood counts (CBC; Hemavet 850), blood smears, and flow cytometric analysis. Animals were maintained in a barrier environment according to Institutional Animal Care and Use Committee-approved protocols. Tissues were fixed in 10% formalin and stained with H&E (Loyola University Tissue Core Facility).

**Results**

**Domain swap constructs produce functional proteins.** All constructs (Fig. 1A) were sequenced (data not shown) and then used in transient transfections to confirm that proteins of the expected size were produced (Fig. 1B; data not shown). Proteins were immunoprecipitated using anti-FLAG antibody or antibodies against NH₂-terminal portions of MLL (amino acids 310-400 and amino acids 1,100-1,400) followed by immunoblotting using anti-FLAG antibody (28). Proteins were assessed for specific acetyl lysine binding and HAT activity to ensure that the bromodomains and HAT domains remained functional in their new context. The swapped MLL fusions still bound peptides representing known binding targets for the included bromodomains (Fig. 1C). As expected, MLL-TAF2BCBP bound to tetra-acetylated histone H4 peptide (16) and MLL-P/CAFBCBP bound HIV Tat-1 acetylated K50 (14). Replacing the HAT domain of CBP with either the P/CAF or GCN5 HAT domains did not affect the ability of the CBP bromodomain to bind to acetylated (K382) p53 (Fig. 1C; ref. 30). Therefore, in the context of MLL fusions, the bromodomain modules retained the ability to bind to their wild-type targets. To test whether the HAT domains acetylated histone targets in the context of MLL fusions, in vitro HAT assays were done. The CBP HAT domain could acetylate all four core histones but preferentially acetylated H3 and H4. P/CAF and GCN5 HAT domains preferentially acetylated H3 and H4, respectively (Fig. 1D). Thus, in the context of the domain-swapped MLL fusions, the HAT domains retained enzymatic activity.

**Swapped bromodomain and HAT domains alter myeloid immortalization in vitro.** We addressed whether the specific functions provided by the CBP bromodomain and the CBP HAT domain were unique in their ability to provide an enhanced proliferative capacity to MLL-CBP-transduced hematopoietic progenitors in an in vitro myeloid colony assay or would other similar domains suffice. Cells were transduced using retroviruses of similar titer (data not shown), and cells from the colony assay were analyzed by RT-PCR to confirm the expression of mRNA from each of the transduced constructs (Fig. 2A). Replacing the HAT domain
of CBP with either that from P/CAF or GCN5 allowed the cells to proliferate into at least the fourth passage similar to MLL-CBP (Fig. 2B; Supplementary Fig. S1). In contrast, cells transduced with either the MLL-TAF2/CBP£ or MLL-P/CAF£/CBP£ grew well into the second or third passage, respectively, but then ceased dividing. The in vitro colony morphology was not predictive of future rephatating potential. Both tightly compact and looser cell colonies were observed at all passages in the positive control, MLL-CBP£/CBP£ (Supplementary Fig. S1). In addition, RT-PCR was done on cytokine-dependent cell lines generated from the myeloid colony assay. The immortalized cells continue to express mRNA from their respective constructs (data not shown). Our results imply a specific requirement for the CBP bromodomain in providing hematopoietic progenitors with an enhanced proliferative capacity. In contrast, different HAT domains in addition to the CBP HAT domain could also provide an increased proliferative capacity to progenitors in vitro (Fig. 2B).

Although HAT domain-swapped constructs conferred increased proliferative capacity, flow cytometry of cells from at least two independent colony assay experiments revealed differences in cell surface marker expression compared with MLL-CBP£/CBP£ cells. MLL-CBP£/CBP£ cells express progenitor cell markers CD117 and CD34 as well as myeloid cell markers (Mac-1 and Gr-1) but do not express other cell lineage markers, including B220, CD3, and TER119 (Fig. 3; data not shown). MLL-CBP£/GCN5H and MLL-CBP£/P/CAF£-transduced cells express high levels of hematopoietic progenitor cell surface markers CD117, Sca-1, and CD34 and are negative for lineage-specific markers (Fig. 3; data not shown). Although this phenotype can also be associated with mast cells, cytospin cells did not display granules or any other mast cell features (Fig. 3). Rather, these cells may reflect an activated population of hematopoietic stem cells (HSC) that was previously shown to be capable of reconstitution in a murine transplantation model (33). These differences indicate that the specific HAT domain plays a role in determining the phenotype of cells; the presence of the CBP HAT domain allows the cells to express more differentiated myeloid markers while still retaining hallmarks of progenitor cells. In contrast, cells expressing the P/CAF HAT or GCN5 HAT domains did not induce expression of the more committed lineage markers. This suggests that these other HAT domains do not potentiate expression of myeloid lineage cell surface markers when fused to MLL but still provide an enhanced proliferation capacity similar to the CBP HAT domain.

Domain swapping changes disease phenotype in vivo. Several groups have shown that MLL fusion constructs exhibiting an enhanced proliferative capacity in vitro cause leukemia in mice in vivo (32, 34–36). All of the domain swap constructs were used to transduce hematopoietic progenitors for reconstitution of lethally irradiated congenic mice to determine their leukemogenic potential. Mice were assessed for evidence of disease by frequent observation, CBC analysis, and flow cytometry of peripheral blood. Sick mice were sacrificed, and tissues were harvested for flow cytometry and H&E staining and analysis.

Previously, we found that both full-length MLL-CBP and MLL-CBP£/CBP£ caused a preleukemic phase in mice before progressing to AML (13). Here, we confirmed these results for the FLAG-tagged MLL-CBP£/CBP£ construct. MLL-CBP£/CBP£ caused myeloid leukemia in all mice preceded by a myeloproliferative phase in the majority of them (Table 1). The expression of domain swap mRNAs was confirmed by RT-PCR analysis of murine blood taken before any sign of illness (Fig. 4A). Further, because the fusion constructs used in vivo also expressed EGFP, we assessed the level of reconstitution by flow cytometry and could analyze the GFP$^+$ cells separately from the GFP$^-$ cells. More peripheral blood GFP$^+$ (transduced) cells expressed the hematopoietic progenitor marker CD117 (c-kit) than GFP$^-$ (nontransduced) cells. Example flow cytometry data are presented from a representative MLL-CBP£/GCN5H mouse (Fig. 4B). All of the constructs proved capable of causing hematopoietic disease in mice (Table 1). Mice with leukemia exhibited >20% blasts in their bone marrow as well as leukemic infiltrates often found in the liver, kidney, lungs, spleen, nodes, and thymus (Fig. 4C; Supplementary Fig. S2). The median

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**Figure 3.** Myeloid colony assay cell phenotype. Representative FACS data showing the differences in cell phenotypes at the end of the fourth passage of the myeloid colony assay. White curve, isotype controls; dark curve, staining with specified antibodies. Cells from colonies were stained with Wright-Giemsa. Images taken at ×1,000 magnification under oil immersion. Bar, ~16 μm.
latency was shortest for MLL-CBP<sup>B</sup>CBP<sup>H</sup> (291 days; Table 2). This corresponds with the in vitro result that MLL-CBP<sup>B</sup>CBP<sup>H</sup> caused the greatest enhanced proliferative capacity in the myeloid colony assay. The percentage of reconstitution does not account for different latencies between mice. For example, at 12 weeks after reconstitution, one MLL-CBP<sup>B</sup>CBP<sup>H</sup> mouse had 75% of nucleated peripheral blood cells expressing CD45.1 but had a longer latency (323 days) than another MLL-CBP<sup>B</sup>CBP<sup>H</sup> mouse with 45% CD45.1 cells (137 days). The latency for the MLL-CBP<sup>B</sup>CBP<sup>H</sup> is longer in this study than previously reported; this variation is likely due to slight modifications of our protocol. Most interestingly, the disease phenotypes varied between the domain swap groups (Table 1; Supplementary Fig. S3). As discussed, the MLL-CBP<sup>B</sup>CBP<sup>H</sup> mice all developed AML (10 of 10) and 80% experienced an increase in the percentage of monocytes before having an increase in total WBC count. However, in contrast to MLL-CBP, <35% of domain swap mutants underwent any detectable preleukemic phase. In sharp contrast to all of the other mouse cohorts, almost half of the TAF<sub>i</sub>250 double bromodomain-swapped mice developed lymphoid disease. Lymphoblastic lymphoma repeatedly occurred in MLL-TAF<sub>i</sub>250<sup>B</sup>CBP<sup>H</sup> mice (Table 1; Supplementary Figs. S2 and S3).

**Discussion**

MLL has >50 different partner genes, but although MLL fusions all retain the same portion of MLL, they do not cause the same type of leukemia. We hypothesized that specific characteristics of the partner genes determine the disease phenotype. We chose MLL-CBP to test this hypothesis for several reasons. Much is known about the functional specificity of CBP domains, and there is a strong correlation of the t(11;16)(q23;p13.3) with the development of an myeloproliferative phase that progresses to AML, which is not observed with other MLL fusions. We focused our study on the bromodomain and HAT domain of CBP because we previously showed that they are necessary and sufficient, in the context of MLL fusion, to provide an enhanced proliferative capacity to murine hematopoietic progenitors in vitro; in vivo, the disease observed in mice seems to recapitulate the human disease phenotype (13).

The results of the myeloid colony assays support a model in which very specific characteristics of minimally required partner gene domains determine the phenotype of MLL fusion-immortalized cells. Substituting the HAT domain of CBP with either HAT domain in the context of a MLL fusion still resulted in an enhanced proliferative capacity of bone marrow progenitor cells. Although

**Table 1. Summary of hematopoietic disease development in reconstituted mice**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Myeloid</th>
<th>Lymphoid</th>
<th>Progenitor*</th>
<th>Nonhematopoietic</th>
<th>Not analyzed $^1$</th>
<th>Myeloproliferative phase (%) $^2$</th>
</tr>
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<tr>
<td>MLL-CBP&lt;sup&gt;B&lt;/sup&gt;CBP&lt;sup&gt;H&lt;/sup&gt;</td>
<td>10/10</td>
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<td>0/10</td>
<td>0/10</td>
<td>1</td>
<td>80</td>
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<tr>
<td>MLL-CBP&lt;sup&gt;B&lt;/sup&gt;P/CAF&lt;sup&gt;H&lt;/sup&gt;</td>
<td>7/9</td>
<td>1/9</td>
<td>0/9</td>
<td>1/9</td>
<td>4</td>
<td>33</td>
</tr>
<tr>
<td>MLL-CBP&lt;sup&gt;B&lt;/sup&gt;GCN5&lt;sup&gt;H&lt;/sup&gt;</td>
<td>5/9</td>
<td>1/9</td>
<td>1/9</td>
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<td>3/13</td>
<td>6</td>
<td>23</td>
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<td>5/12</td>
<td>0/12</td>
<td>1/12</td>
<td>4</td>
<td>16.7</td>
</tr>
</tbody>
</table>

*Mice whose cells did not stain, by flow cytometry, for either myeloid or lymphoid markers. The bone marrow cells were positive for progenitor cell surface markers CD117 and/or Sca-1.

$^1$ Number of mice that could not be analyzed due to death without previous symptoms, and the resulting destruction of tissues before samples could be collected.

$^2$ Mice with an increase in the percentage myeloid cells without an increase in the total WBC count.

**Figure 4.** Characteristics of reconstituted mice. **A,** RT-PCR done on peripheral blood of mice to determine expression of MLL fusion constructs. **B,** flow cytometry of blood of one mouse showing that differences could be observed between transduced and nontransduced cells in vivo. **C,** bone marrow from mice showing >20% of blast cells present. Blast cells have scant blue cytoplasm and large nuclei. Photos taken at $\times 1,000$ magnification under oil immersion. Bar, 12 μm.
the swapped HAT domains have different substrate preferences for their effector activity, the P/CAF and GCN5 HATs were able to provide some function that maintained the extended growth of progenitors. Although it is not clear what may be the critical in vivo targets of CBP HAT activity when tethered to MLL, one property of the HAT domains may give insight into why the substitution of these domains could still function similarly. The structural flexibility of one loop in the P/CAF HAT domain may allow greater diversity in substrates and provide some redundancy with the CBP HAT domain function (37). The GCN5 HAT domain also contains an analogous loop to the P/CAF HAT domain and would likely behave similarly.

Although the cells expressing domain-swapped HATs have an extended growth capacity, they express different cell surface markers than those observed on MLL-CBP/P-CAF transduced cells. We found that the CBP HAT domain was required for the expression of more differentiated cell surface markers in vitro, whereas the P/CAF and GCN5 HAT domains resulted in cells expressing only more primitive cell surface markers, indicating perhaps a stronger block in differentiation than seen with the CBP HAT constructs. This suggests a mechanism for the specific myelodysplastic syndrome before acute leukemia in CBP-containing MLL fusions: promoting expression of myeloid differentiation markers. Intriguingly, in muscle cell terminal differentiation, CBP HAT activity is essential for activation of late genes in muscle cell differentiation and cell fusion, and it was hypothesized that P/CAF/GCN5 was important at earlier stages (38).

For increased proliferative capacity in the myeloid colony assay, the CBP bromodomain was absolutely required in the MLL fusion. The decreased proliferative capacity of the bromodomain-swapped constructs is reminiscent of what we previously reported with the NH2 terminus of MLL fused only to the HAT domain of CBP (13). Because all of the domain swap constructs generate proteins that retain properties expected of their isolated domains, it is unlikely that simple problems with protein synthesis, folding, or function explain our results. Neither the P/CAF nor the TAFq250 double bromodomain was able to compensate for the loss of the CBP bromodomain. The CBP bromodomain could be vital to the formation of a protein complex by recruiting specifically acetylated transcription factors or better targeting the MLL fusion to a subset of MLL target genes with a specific pattern of acetylated histone tails. Clearly, there is a requirement for a unique function provided by the CBP bromodomain in the context of the MLL-CBP fusion.

In our in vitro model, MLL-CBP/P-CA

The myeloproliferative phase was not observed as frequently with the other constructs; therefore, a specific function provided by the CBP bromodomain and/or HAT domain more efficiently drives this phenotype. Our in vitro data correlate well with these findings. In vitro, HAT domain-swapped constructs did not coexpress myeloid/granulocyte differentiation markers and progenitor markers, whereas this was observed with MLL-CBP. The induced expression of myeloid differentiation markers along with progenitor markers is a phenotype caused more efficiently by the CBP HAT than the other HAT domains. Unlike the results of the in vitro myeloid colony assay, in vivo mice with the HAT domain swapped with either the GCN5 HAT or P/CAF HAT predominantly lead to leukemia cells expressing myeloid surface markers, such as CD11b and Gr-1. However, these mice lack the myeloproliferative phase of the disease. The difference may be due to in vivo selection and expansion of those cells expressing myeloid markers, whereas in vitro these cells may constitute a very small fraction of the cells and cannot proliferate well in the provided growth conditions. The stromal environment in the mice may be more supportive for these cells to proliferate and transform. The monoclonality or oligoclonality observed in the MLL mouse leukemia models supports the idea that few cells are selected for and seed the leukemogenic process (13, 32). The development of leukemia and myeloproliferative disease is likely two separable events, and our domain swap mutants lack the specificity provided by the CBP HAT domain required to efficiently cause myeloproliferative disease.

Although the HAT domain of CBP has proven crucial to MLL-CBP-induced leukemia, not all fusions possessing a HAT domain require it to be oncogenic (13). For example, MOZ-TIF2 does not require HAT activity but does require an interaction with CBP for transformation (39). In the MOZ-CBP fusion, the HAT domain of CBP is required for in vitro cell growth and inhibition of terminal cell differentiation into macrophages (40). Like MLL-CBP, MOZ-TIF2, MORF-CBP, and MOZ-CBP are associated with AML (41, 42). Because CBP plays an essential role in these fusions, it suggests that CBP may have a role not only in leukemogenic processes but also in directing the disease toward a myeloid phenotype.

Overall, our results show that both the HAT domain and the bromodomain of CBP play a critical role in determining the phenotype of the hematopoietic disease. Because all of the constructs caused hematopoietic disorders in mice, the overlapping functions of the domains facilitate the immortalization of hematopoietic precursors, but the unique binding and acetylation activities of the bromodomain and HAT domain, respectively, are critical to the differentiation potential of these precursors and the development of a preleukemic phase.

An inducible knock-in model of Mll-Cbp was recently created (43). Only after treatment with an agent to induce additional mutations, such as irradiation or N-nitroso-N-ethylurea, the mice experienced a myeloproliferative phase before progressing to AML. These results suggested that MLL-CBP alone is not sufficient to cause disease but requires the accumulation of additional mutational events. This correlates well with the relatively long latency to leukemia development in our retroviral transduction model of MLL-CBP and the domain-swapped mutants.

Others have shown by retroviral transduction that some, but not all, oncogenes are capable of imparting self-renewal capacity to committed hematopoietic progenitors. For example, MOZ-TIF2 and MLL-ENL, but not BCR-ABL, had this effect in vitro and were able to cause leukemia when used to reconstitute irradiated mice. In these experiments, the leukemias had identical phenotype regardless of whether a HSC or more committed myeloid

Table 2. Disease latency in mice

<table>
<thead>
<tr>
<th>Construct</th>
<th>Median latency (d)</th>
<th>Average latency (d) ± SD</th>
<th>Range (d)</th>
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<tbody>
<tr>
<td>MLL-CBP/P-CAF (n = 11)</td>
<td>291</td>
<td>277.91 ± 121.82</td>
<td>83-474</td>
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<tr>
<td>MLL-CBP/GCN5 (n = 12)</td>
<td>329</td>
<td>334.25 ± 39.28</td>
<td>290-394</td>
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<tr>
<td>MLL-CBP/P-CAF (n = 14)</td>
<td>359.5</td>
<td>312.38 ± 140.45</td>
<td>68-508</td>
</tr>
<tr>
<td>MLL-TAFq250-CAF (n = 16)</td>
<td>355</td>
<td>318.38 ± 103.31</td>
<td>148-441</td>
</tr>
<tr>
<td>MLL-P-CAF/GCN5 (n = 19)</td>
<td>397</td>
<td>374.84 ± 113.41</td>
<td>74-482</td>
</tr>
<tr>
<td>Vector (n = 6)</td>
<td>401.5</td>
<td>346.67 ± 147.03</td>
<td>68-449</td>
</tr>
</tbody>
</table>
phenotype of disease...ultimately a proliferating population of translocation-containing cells would...to expand and express some myeloid differentiation markers. The critical role in the development of CMML phase by allowing cells to...whole patient basis to the resultant leukemia. It is likely that both mechanisms are important in vivo.

In conclusion, we have clearly shown that, in the MLL-CBP fusion protein, the CBP bromodomoid and HAT domains contribute critical functional specificity to the immortalization potential of murine hematopoietic progenitors in vitro and the phenotype of disease in vivo. The CBP HAT domain may play a critical role in the development of CMMI phase by allowing cells to expand and express some myeloid differentiation markers. The proliferating population of translocation-containing cells would have the opportunity to acquire additional mutations, ultimately resulting in AML. The cooperation between the CBP bromodomoid and HAT domain seems to be vital to the transformation of cells possessing the translocation. The CBP bromodomoid may help recruit MLL-CBP to a subset of MLL target genes within chromatin containing acetylated histones or with acetylated transcription factors also present at that locus. Thus, the CBP HAT domain would be brought in close proximity to a subset of MLL targets, altering expression patterns that affect cell differentiation and immortalization. Alternatively, the bromodomoid may be critical in recruiting additional proteins to MLL target loci and forming a complex on MLL-CBP that may include substrates for the HAT domain or can target substrates of the HAT domain, although several groups have investigated the potential of HAT inhibitors for therapeutic purposes, using these inhibitors in combination with blocking the CBP bromodomoid from binding to acetylated lysines may also benefit patients (46, 47). We have begun to explore MLL target gene expression in MLL-CBP mice compared with domain swap mice. Further work will clarify the pathways involved and how these affect disease phenotype.

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