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Cbfβ Reduces Cbfβ-SMMHC–Associated Acute Myeloid Leukemia in Mice

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Abstract

The gene encoding for core-binding factor β (CBFβ) is altered in acute myeloid leukemia samples with an inversion in chromosome 16, expressing the fusion protein CBFβ-SMMHC. Previous studies have shown that this oncoprotein interferes with hematopoietic differentiation and proliferation and participates in leukemia development. In this study, we provide evidence that Cbfβ modulates the oncogenic function of this fusion protein. We show that Cbfβ plays an important role in proliferation of hematopoietic progenitors expressing Cbfβ-SMMHC *in vitro*. In addition, Cbfβ-SMMHC–mediated leukemia development is accelerated in the absence of Cbfβ. These results indicate that the balance between Cbfβ and Cbfβ-SMMHC directly affects leukemia development, and suggest that CBF-specific therapeutic molecules should target CBFβ-SMMHC function while maintaining CBFβ activity. (Cancer Res 2006; 66(23): 11214-8)

Introduction

The core-binding factor (CBF) transcription factor is the most common target of chromosomal rearrangements in human acute myeloid leukemia (AML), including the fusion genes *CBFB-MYH11* and *RUNX1-ETO* (1). Moreover, *RUNX1* is frequently mutated in AML. CBF is a heterodimeric transcription factor that consists of a DNA binding α-subunit, encoded by one of three members of the RUNX family (*RUNX1, RUNX2*, and *RUNX3*), and a β-subunit encoded by the *CBFB* gene that increases DNA-binding affinity to the complex. In hematopoiesis, the CBF heterodimer Cbfβ:Runx1 regulates expression of genes with critical functions in differentiation of lymphoid and myeloid lineages. The Cbfβ:Runx3 complex is involved in B-cell maturation and the silencing of the *CD4* gene during T-cell maturation (2). Studies in the mouse have determined that $Cbfb^{-/-}$ and $Runx1^{-/-}$ embryos fail to develop embryonic definitive hematopoiesis and die at midgestation (3–6). This phenotype was rescued in $Cbfb^{-/-}$ mice expressing *Cbfb* from the hematopoietic specific promoters *Tie2* or *GATA1*, further underscoring the key role of Cbfβ during hematopoietic differentiation (7, 8).

Approximately 12% of AML patients present a chromosome 16 inversion [inv(16); ref. 9] that breaks and joins the first five exons of *CBFB* with the second half of the smooth muscle myosin heavy chain gene *MYH11* (10). The resulting *CBFB-MYH11* gene encodes the CBFβ-SMMHC fusion protein, which retains the Runx-binding domain from Cbfβ and multimerization domain from the myosin sequence. Studies in mice have shown that Cbfβ-SMMHC is a dominant inhibitor of CBF function because $Cbfb^{+/MYH11}$ heterozygous knock-in embryos expressing the fusion protein failed to develop definitive hematopoiesis (11), as was shown for the *Cbfb*- and *Runx1*-null embryos (3–6).

Induction of Cbfβ-SMMHC expression or Runx1-loss in adult bone marrow does not seem to affect the maintenance of long-term hematopoietic stem cells (12–14). However, Cbfβ-SMMHC expression reduces hematopoietic stem cell function by inhibiting multilineage repopulation and creating a myeloid progenitor predisposed to leukemia development (11).

Several lines of evidence suggest that Cbfβ-SMMHC may exert an incomplete block of CBF function. First, ectopic expression of the fusion protein in embryonic stem cells expressing one or both copies of *Cbfb* does not inhibit differentiation *in vitro* (15). Second, $Cbfb^{+/MYH11}$ knock-in hematopoietic stem cells expressing Cbfβ-SMMHC persist in the bone marrow of the chimeras (16). Third, retroviral insertional mutagenesis in $Cbfb^{+/MYH11}$ knock-in chimeras identified common insertions in the *Runx2* gene (17), suggesting that Cbfβ-SMMHC leukemic function is affected by levels of Runx proteins.

In this study, we test the hypothesis that Cbfβ modulates the Cbfβ-SMMHC effect in adult hematopoiesis and leukemogenesis. We used mice with a *Cbfb* knock-out allele and a conditional *Cbfb-MYH11* knock-in allele to study adult myeloid differentiation and leukemia progression. This study provides evidence that Cbfβ modulates hematopoietic differentiation and Cbfβ-SMMHC–mediated leukemia development, and suggests that CBFβ up-regulation may efficiently counteract differentiation defects in human AML with inv(16).

Materials and Methods

Generation of triple $Mx1Cre/Cbfb^{-/MYH11}$ transgenic mice. The design of the conditional $Cbfb^{+/MYH11}$ knock-in mice has previously been described (12), with the exception that monoclonal β-actin antibody (Sigma, St. Louis, MO) was used as western blot control. Expression of CBFβ-SMMHC was induced in 3-week-old mice by activation of Cre recombinase from the *Mx1Cre* transgene using one to three doses of polyinosinic-polycytidylic acid (pIpC) every other day (18). Heterozygous $Cbfb^{-/+}$ knockout mice were generously provided by Nancy Speck (Dartmouth Medical School, Hanover, NH; ref. 4). For this study, all mice were maintained in the 129SvEv strain. In the transplantation assays, 1×10^6 leukemic cells were transplanted into sublethally irradiated syngenic recipients as described elsewhere (19).

Molecular and cytology analysis. The Western blot, flow cytometry, and histopathology analyses were done as previously described (12). Fluorescence-activated cell-sorting analysis was done in peripheral blood of leukemic mice using FITC-c-kit and phycoerythrin-lineage antibodies (Lin+: B220, CD3, Gr1, and Mac1; all from BD Biosciences, San Jose, CA).

Colony forming assays. Mice with the genotypes $Cbfb^{+/56M}$, $Cre;Cbfb^{+/56M}$, $Cbfb^{-/56M}$, and $Cre;Cbfb^{-/56M}$ were injected with pIpC at weaning every other day. Two days after the second injection, bone marrow cells were

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harvested, and 1×10^4 WBC were plated in duplicate in methylcellulose supplemented with cytokines interleukin (IL)-3, IL-6, and stem cell factor and erythropoietin (Methocult-3434, Stem Cell Technologies, Vancouver, Canada) in 35-mm nontreated tissue culture dishes (Corning). The number of myeloid colonies was scored at day 7. Single colonies were harvested and either cytospun for cytology analysis or placed into lysis buffer for PCR analysis.

Statistical considerations. Differences in survival functions between groups were evaluated by Kaplan-Meier product limit survival analysis using the Tarone-Ware test to test the hypothesis of overall equivalence. In the presence of significant overall differences, pairwise comparisons were made between the noncontrol groups using Tarone-Ware tests with a Sidak adjustment to compensate for the additive type I error due to multiple comparisons.

Analysis of human AML samples. Patients had a diagnosis of primary AML, confirmed by cytologic examination of blood and bone marrow. After informed consent, bone marrow aspirates or peripheral blood samples were taken at diagnosis (n = 285) and processed for Affymetrix U133A GeneChip analysis (20). For PCR and sequence analyses, cDNA prepared from 50 ng of RNA was used for all PCR amplifications. The *CBFB* coding region was sequenced for 27 inv(16)$^+$ AML samples by cDNA amplification using the primers *CBFB-FOR* 5′-CAGAGAAGCAAGTTCGAGAACG-3′ with *CBFB-REV* 5′-GTTTGAGGTCATCACCACCAC-3′ and *CBFB-FOR* with *CBFB6* 5′-GTCTTGTTGTCTTCTTGCCAG-3′ (25 mmol/L deoxynucleotide triphosphate, 15 pmol primers, 2 mmol/L $MgCl_2$, Taq polymerase and 10× buffer; Invitrogen Life Technologies, Breda, the Netherlands). Cycling conditions for both primer sets consisted of a denaturing cycle for 5 minutes at 94°C followed by 30 cycles for amplification (1 minute 94°C, 1 minute 62°C, 1 minute 72°C), and a final extension cycle for 7 minutes at 72°C. PCR products were purified using the Multiscreen-PCR 96-well system (Millipore, Bedford, MA) followed by direct sequencing with *CBFB-FOR, CBFB-REV*, and *CBFB6* using an ABI-PRISM3100 genetic analyzer (Applied Biosytems, Foster City, CA).

Results

Cbfβ is essential to maintain proliferation capacity of myeloid progenitor cells expressing Cbfβ-SMMHC. We have recently shown that bone marrow cells expressing Cbfβ-SMMHC accumulate abnormal myeloid progenitors able to form myeloid colonies *in vitro* (12). To assess whether this effect is dependent on the presence of Cbfβ, colony-forming unit (CFU) assays were done with bone marrow cells from heterozygous floxed (*Cbfb*$^{+/56M}$), hemizygous floxed (*Cbfb*$^{-/56M}$), heterozygous restored (*Cbfb*$^{+/MYH11}$), and hemizygous restored (*Cbfb*$^{-/MYH11}$) mice (Fig. 1). The switch from Cbfβ to Cbfβ-SMMHC expression (switching floxed to restored allele) was induced by pIpC-mediated Cre activation using the *Mx1Cre* transgene.

The number of myeloid and erythroid colonies was significantly reduced in hemizygous restored bone marrow progenitor cells expressing Cbfβ-SMMHC when compared with control groups (Fig. 2*A*). Considering that the loss of one *Cbfb* allele did not affect CFU formation (heterozygous floxed versus hemizygous floxed), these results indicate that the number of CFUs is markedly reduced at *Cbfb* levels below 50%. In addition, the size of the colonies was markedly reduced in hemizygous restored progenitors when compared with controls (Fig. 2*B*). The Cre-lox–mediated excision in CFUs from hemizygous restored plates was confirmed by PCR analysis (data not shown). Cytology analysis of day-7 CFUs revealed the presence of all myeloid forms, with a small but consistent increase of blastlike immature cells in hemizygous restored colonies (Fig. 2*C*). These results revealed that the loss of Cbfβ in the context of Cbfβ-SMMHC significantly reduces the proliferation capacity of bone marrow myeloid progenitors *in vitro*.

Hemizygous bone marrow cells expressing Cbfβ-SMMHC show higher susceptibility to AML. The *Cbfb*$^{+/MYH11}$ restored mice succumb to AML with a median latency of 5 months (12). This latency is dose dependent as mice induced with three pIpC doses developed AML with a median latency of 5 months, mice induced with one pIpC dose had AML with a median latency of 8 months, and uninduced mice remained disease-free.

To test whether the presence of Cbfβ has an effect in Cbfβ-SMMHC–mediated AML, we compared heterozygous restored and hemizygous restored mice after treatment with three doses of pIpC. In the absence of Cbfβ, 100% of mice with bone marrow cells expressing Cbfβ-SMMHC developed AML with a significant

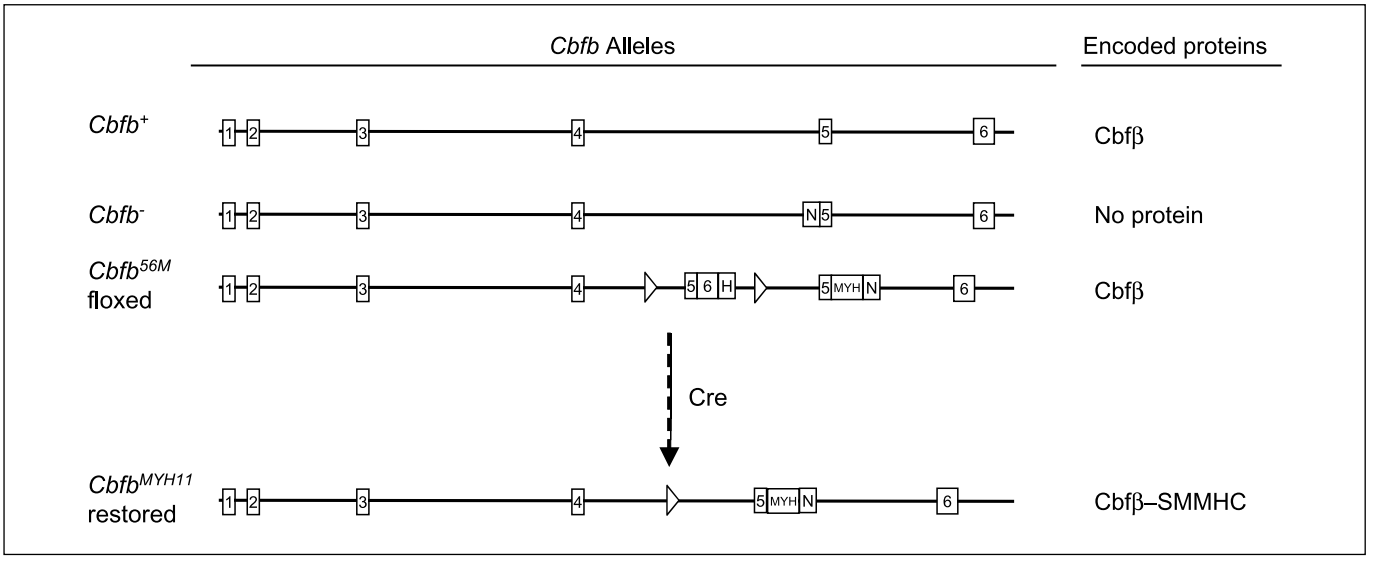

Figure 1. *Cbfb* alleles used in this study. Exons 1 to 6 of the *Cbfb* gene are shown in boxes, and the encoded protein is shown on the right. The *Cbfb* knock-out allele includes a neomycin (*N*) gene fused to the 3′-end of exon 5 (4). The floxed *Cbfb*^{56M} allele includes exons 5 and 6 and a *hygromycin* gene (*H*) between loxP sites (*triangle*), followed by exon 5 fused to the 3′ *MYH11* sequence and a neomycin gene (12). Upon Cre-mediated loxP deletion, Cbfβ-SMMHC is induced in the *Cbfb*^{MYH11} restored allele.

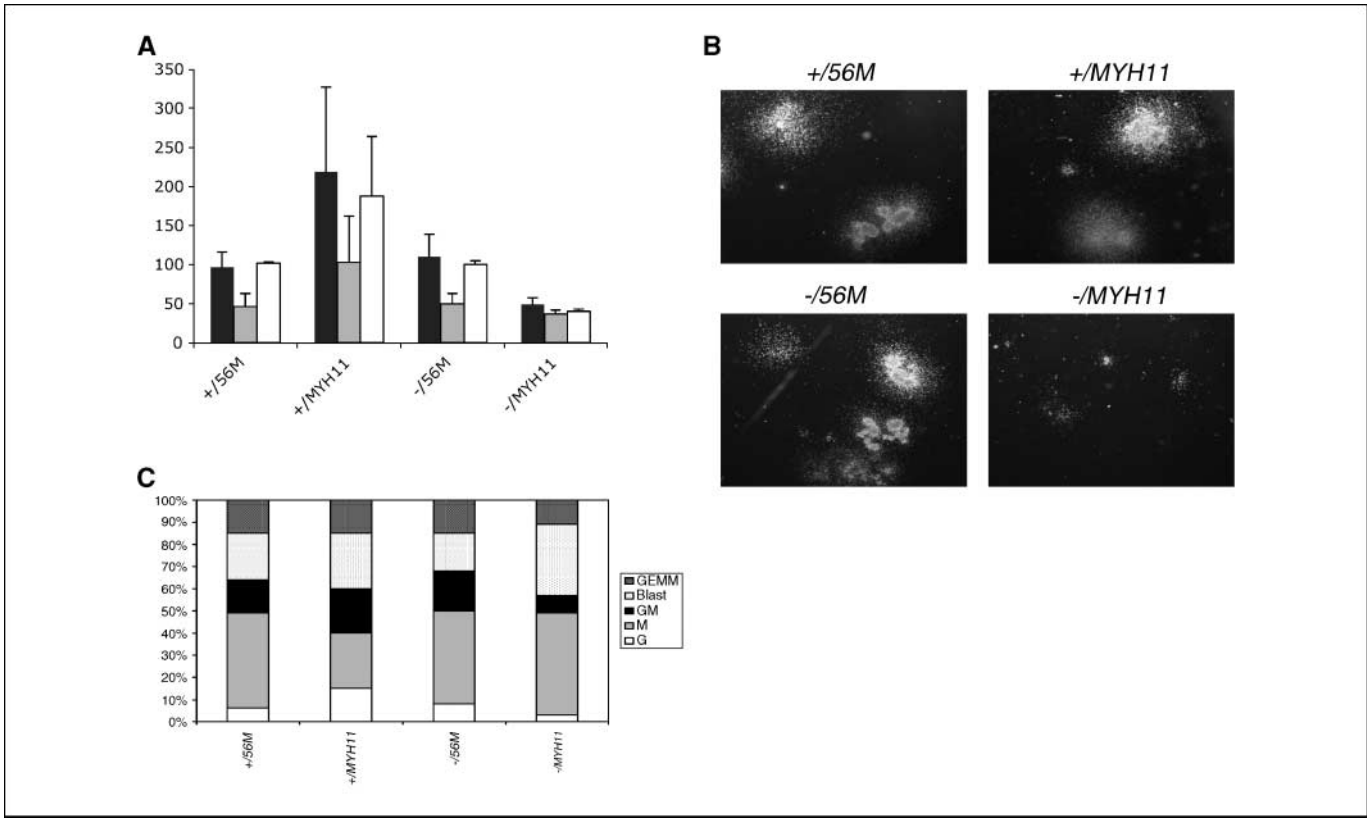

Figure 2. Cbfβ modulates bone marrow myeloid proliferation *in vitro*. Colony forming assays in methylcellulose cultures using 1×10^4 bone marrow progenitor cells with heterozygous floxed (*+/56M*), heterozygous restored (*+/MYH11*), hemizygous floxed (*−/56M*), or hemizygous restored (*−/MYH11*) *Cbfb* genotypes. *A,* erythroid colonies were scored at day 4 (*gray*) and myeloid colonies at day 4 (*black*) and day 7 (*white*). *Columns,* number of colonies from three independent experiments, each in duplicate. *B,* representative images of colony size (amplification, ×50). *C,* histogram representation of cytology analysis of CFU-GEMM, CFU-Blast, CFU-GM, CFU-M, and CFU-G.

acceleration of disease onset (median latency of 1.5 ± 0.5 months; $P < 0.00001$; Fig. 3*A*). Surprisingly, uninduced *Mx1Cre/Cbfb*$^{-/56M}$ mice also developed AML with similar latency to that of induced group (Fig. 3*A*; *red dashed line,* uninduced; *red solid line,* induced). It has previously been reported that Cre expression from the *Mx1Cre* transgene is leaky in mice not treated with pIpC (21). In our study, all AML samples from induced and uninduced groups exhibited deletion of the floxed sequence by PCR analysis (data not shown) and Cbfβ-SMMHC expression was detected by Western blot analysis (Fig. 3*B*). Furthermore, secondary transplantation of *Cbfb*$^{-/MYH11}$ AML cells derived from induced or uninduced mice produced leukemia in sublethally irradiated recipients with a median latency of 6 weeks (data not shown). These results indicate that Cbfβ-SMMHC–induced AML development is accelerated in the absence of Cbfβ.

Disease pathology is dictated by Cbfβ-SMMHC. The hemizygous restored leukemic *Cbfb*$^{-/MYH11}$ mice presented an increase in WBC count (mean, 41.7 ± 74.7 × 10^6/mL) when compared with healthy littermates (mean, 4×10^6/mL). As previously described in heterozygous restored leukemic mice, the leukemic cells from the hemizygous mice included predominant blastlike and myelomonocytic cells (Fig. 3*C, top row*) with the characteristic cell-surface profile c-kit$^+$/Lin$^-$ (Fig. 3*D*). Disease was also marked by progressive anemia and infiltration into other organs, including the spleen and liver. Histology analysis of these organs confirmed the disruption of splenic architecture marked by infiltration of leukemic cells (Fig. 3*C, middle row*) and focal infiltrations of leukemic cells into the interstitial spaces of the liver (Fig. 3*C, bottom row*). Compromise of the thymus and lymph nodes was not observed. Taken together, the pathology of disease in hemizygous and heterozygous mice was similar, suggesting that the AML phenotype was determined by Cbfβ-SMMHC.

The wild-type *CBFB* allele is not a frequent target of mutations in inv(16) AML. To assess whether *CBFB* is frequently altered in human CBF AML samples, expression and mutation analyses of *CBFB* were undertaken. Sequence analysis of the *CBFB* coding region in a panel of 29 inv(16) AML samples identified no mutations. Expression analysis of *CBFB* in a panel of 285 human AML samples indicated that inv(16) AML samples had a 40% reduction in *CBFB* transcript when compared with CD34$^+$ bone marrow cells (relative value, 0.4 ± 0.08), as expected by the expression of one *CBFB* allele. The *CBFB* levels in t(8;21) and non-CBF cytogenetic groups were unchanged [t(8;21) relative value, 0.9 ± 0.23; non-CBF relative value, 1.0 ± 0.32]. These results indicate that the remaining *CBFB* allele is not frequently altered in inv(16) AMLs.

Discussion

Endogenous expression of Cbfβ and Cbfβ-SMMHC from the *Cbfb* allele of conditional knock-in mice creates a leukemia precursor that progresses to AML in a multistep process (12). Although the fusion protein is thought to act as a dominant factor in differentiation and transformation (11, 12, 16), the role of Cbfβ in

Cbfβ-SMMHC–mediated leukemia is not clear. Here we showed that the capacity of Cbfβ-SMMHC to induce AML in mice is modulated by Cbfβ.

The presence of Cbfβ is critical for embryonic definitive hematopoiesis (4) and for *in vitro* myeloid differentiation from *Cbfb*$^{-/-}$ embryonic stem cells (15). Our study indicates that Cbfβ is necessary for *in vitro* myeloid-erythoid differentiation of bone marrow hematopoietic progenitors. In addition, because colonies were drastically reduced in the absence of Cbfβ but not in the presence of Cbfβ-SMMHC, our results support the hypothesis that Cbfβ-SMMHC may have an incomplete effect in differentiation. As Cbfβ and Cbfβ-SMMHC compete for binding with Runx proteins in bone marrow cells, basal levels of Cbfβ:Runx1 complex in hematopoietic progenitors expressing Cbfβ-SMMHC may be critical for proliferation of myeloid progenitors and delayed transformation.

Endogenous expression of Cbfβ-SMMHC and Cbfβ in bone marrow induces AML with a median latency of 5 months (12). We observed that upon Cre-lox–mediated switch from Cbfβ to Cbfβ-SMMHC expression in progenitor cells lacking a wild-type *Cbfb* allele, AML latency was shortened to 6 weeks. These results strongly suggest that Cbfβ-SMMHC function is enhanced by Cbfβ loss. Surprisingly, a similar AML latency was observed between induced and uninduced groups. Probably, a small progenitor population may have undergone Cre/lox deletion due to "leaky" Cre expression from the *Mx1Cre* transgene (21), and thus becoming

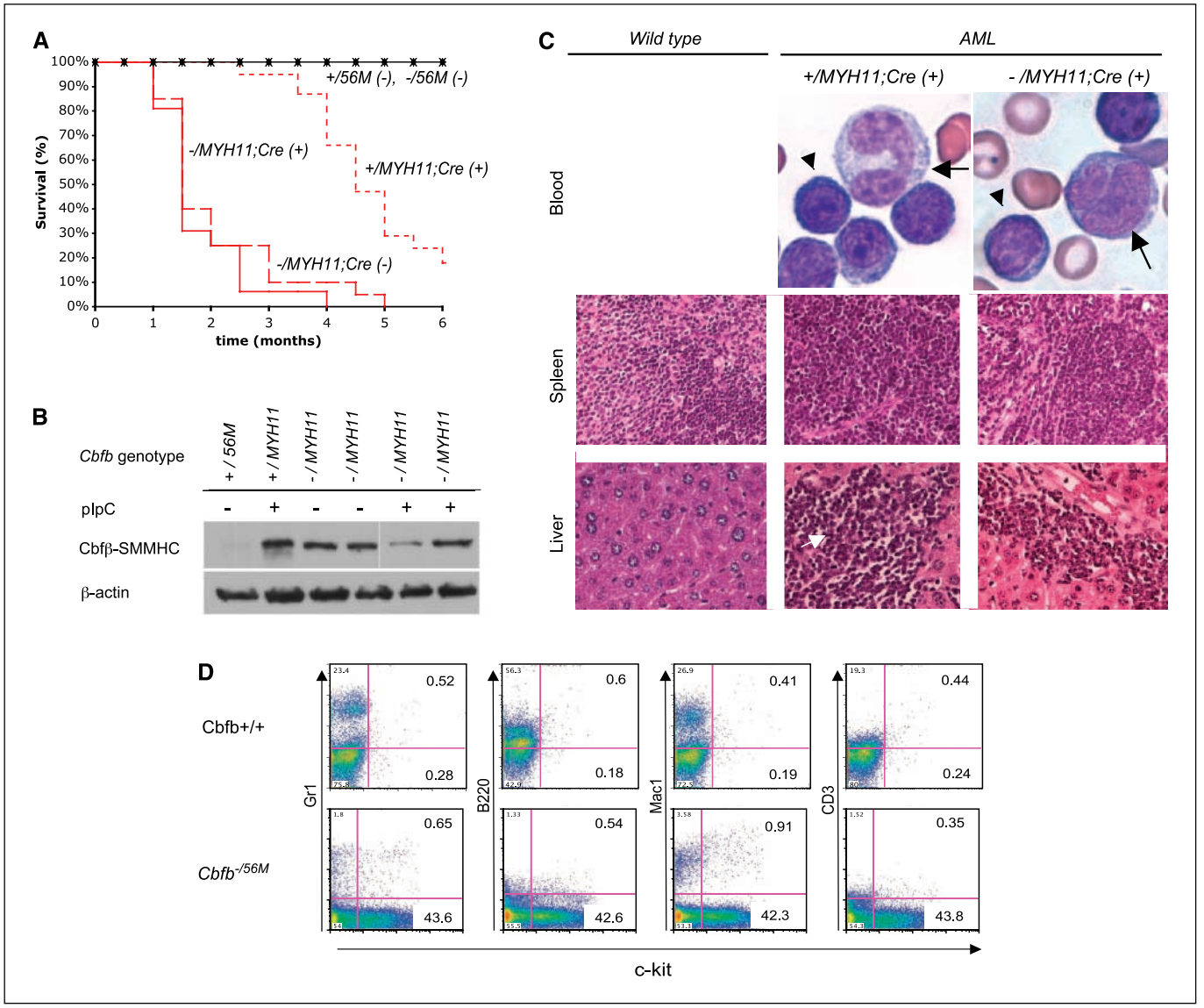

Figure 3. Loss of Cbfβ accelerates Cbfβ-SMMHC–mediated AML. *A,* Kaplan-Meier survival curve of mice expressing Cbfβ-SMMHC in the presence or absence of Cbfβ. Mice induced with pIpC (+) heterozygous restored [*red dotted line, +/MYH11;Cre (+)*; *n* = 38], uninduced (–) heterozygous floxed [*black line with star mark, +/56M (–)*; *n* = 20], uninduced hemizygous floxed [*black line with circle mark, –/56M (–)*; *n* = 15], untreated hemizygous restored [*red dashed line, –/MYH11; Cre (–)*; *n* = 20], or treated hemizygous restored [*red solid line, –/MYH11;Cre (+)*; *n* = 16]. *B,* Western blot analysis of Cbfβ-SMMHC and β-actin in AML samples derived from restored *Cbfb* mice induced (+) with pIpC or uninduced (–). The *Cbfb* genotype of the AML cells is shown on the top. *C,* disease pathology analysis depicting an increase of immature leukemic cells (*top row*; *triangle,* blastlike; *arrow,* myeloid form; magnification, ×1,000), disruption of spleen architecture (*middle row*; magnification, ×100), and the presence of infiltrating leukemia cells (*white arrow*) in the liver (*bottom row*; magnification, ×100). Cells analyzed from wild-type control (*left column*) and leukemic mice expressing Cbfβ-SMMHC in the presence (*middle column*) or absence (*right column*) of Cbfβ. *D*, FACS analysis of leukemic cells from hemizygous restored mice (*bottom*) compared with wild type control (*top*) using lineage markers (Gr1, B220, Mac1, CD3) and a progenitor marker (c-kit).

a leukemia precursor. Importantly, all AML samples presented Cre-mediated deletion, suggesting that transformation is due to the Cbfβ to Cbfβ-SMMHC switch. Furthermore, the finding that *CBFb* is not frequently lost in human AML argues against its role as an inv(16) cooperating tumor suppressor in AML. Rather, our results suggest that increase in the Cbfβ-SMMHC-to-Cbfβ ratio reduced proliferation of myeloid progenitors while increasing their susceptibility to neoplastic transformation, although the underlying mechanism is unclear. However, we cannot rule out the possibility that *Cbfb* loss in bone marrow could induce AML. The generation of conditional *Cbfb* knock-out alleles will provide a critical tool to directly address this possibility using a genetic approach.

Finally, these findings have important implications on the design of targeted therapies. One potential avenue is the identification of drugs that inhibit the fusion protein. Although candidate molecules should act to disrupt Cbfβ-SMMHC:Runx1 binding, it will be critical that CBFβ:Runx1 binding remains unaltered.

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