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Runx1 is required for hematopoietic defects and leukemogenesis in *Cbfb-MYH11* knockin mice

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Abstract

CBFβ-SMMHC, the fusion protein generated by the chromosome 16 inversion fusion gene, *CBFB-MYH11*, is known to initiate leukemogenesis. However, the mechanism through which CBFβ-SMMHC contributes to leukemia development is not well understood. Previously it was proposed that CBFβ-SMMHC acts by dominantly repressing the transcription factor RUNX1, but we recently showed that CBFβ-SMMHC has activities that are independent of RUNX1 repression. In addition, we showed that a modified CBFβ-SMMHC with decreased RUNX1 binding activity accelerates leukemogenesis. These results raise questions about the importance of RUNX1 in leukemogenesis by CBFβ-SMMHC. To test this, we generated mice expressing *Cbfb-MYH11* in a *Runx1* deficient background, resulting from either homozygous *Runx1* null alleles ($Runx1^{-/-}$) or a single dominant negative *Runx1* allele ($Runx1^{+/lz}$). We found that loss of *Runx1* activity rescued the differentiation defects induced by *Cbfb-MYH11* during primitive hematopoiesis. During definitive hematopoiesis, RUNX1 loss also significantly reduced the proliferation and differentiation defects induced by *Cbfb-MYH11*. Importantly, *Cbfb-MYH11* induced leukemia had much longer latency in $Runx1^{+/lz}$ mice than in *Runx1* sufficient mice. These data indicate that *Runx1* activity is critical for *Cbfb-MYH11* induced hematopoietic defects and leukemogenesis.

Keywords

AML; *Runx1*; *Cbfb-MYH11*; CBFβ-SMMHC; Inversion 16

Introduction

Acute myeloid leukemia (AML) is often characterized by the presence of specific, recurrent chromosomal abnormalities, many of which involve transcription factors important for normal hematopoiesis[1]. Inversion of chromosome 16, inv(16)(p13;q22) or the related

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Conflict of Interest

The authors have no conflicts of interest to disclose.

Supplemental information is available at Leukemia's website.

t(16;16)(p13;q22) translocation, is found in nearly all patients with AML subtype M4 with eosinophilia[2] and generates a fusion gene between the core-binding factor beta gene, *CBFB,* and *MYH11*, the gene for smooth muscle myosin heavy chain (SMMHC)[3, 4]. The fusion gene, *CBFB-MYH11*, which encodes the CBFβ-SMMHC protein, has been shown to be necessary, but not sufficient for leukemogenesis[5, 6].

CBFβ-SMMHC is thought to initiate leukemogenesis by blocking normal hematopoietic differentiation through inhibition of the key hematopoietic transcription factor Runt-related protein 1 (RUNX1; AML1). RUNX1 is one of the three α subunits in the core binding factor (CBF) family, all of which have RUNT domains that mediate DNA binding and heterodimerization with the single β subunit in the CBF family, CBFβ[7]. Dimerization with CBFβ stabilizes binding of the α subunits to DNA and allows the α–β heterodimer to regulate gene expression through the transactivation domains of the α subunits[8]. CBFβ-SMMHC retains the RUNX binding domain from CBFβ, and also contains a second RUNX high affinity binding domain (HABD) in the SMMHC tail, resulting in a higher binding affinity for RUNX as compared to wildtype CBFβ[9]. In vitro work has shown that CBFβ-SMMHC may serve as a transcriptional repressor and that CBFβ-SMMHC may sequester RUNX1 in the cytoplasm[10–12]. Based on these findings, it has been proposed that CBFβ-SMMHC acts by dominantly repressing RUNX1.

Mouse models have provided evidence that CBFβ-SMMHC has dominant RUNX repressor activities in vivo. Previously, we generated *Cbfb-MYH11* knockin mice (*Cbfb*$^{+/MYH11}$) which express the fusion gene under the control of the endogenous *Cbfb* promoter. At embryonic day 12.5 (E12.5), heterozygous *Cbfb*$^{+/MYH11}$ embryos have a complete block in definitive hematopoiesis and severe central nervous system hemorrhaging, which contributes to lethality by E13.5[13]. This phenotype is very similar to that of *Runx1* null (*Runx1*$^{-/-}$) and *Cbfb* null (*Cbfb*$^{-/-}$)[14–18] mice, consistent with CBFβ-SMMHC acting as a dominant repressor of RUNX1 and CBFβ functions during embryogenesis.

More recent work has shown that CBFβ-SMMHC also has RUNX1-repression independent activities. We found that *Cbfb*$^{+/MYH11}$ embryos have defects in differentiation and gene expression during primitive hematopoiesis that are not seen in *Runx1*$^{-/-}$ or *Cbfb*$^{-/-}$ embryos[19]. In addition, we found that many genes are uniquely deregulated in *Cbfb*$^{+/MYH11}$ embryos (but not in *Runx1*$^{-/-}$ or *Cbfb*$^{-/-}$ embryos), and are expressed in leukemic cells from mice and humans. Together, these results imply that *Cbfb-MYH11*'s *Runx1*-repression independent activities play a role in leukemogenesis.

Our previous research using deletion mutants of the *Cbfb-MYH11* fusion gene has raised further questions about *Runx1*'s role in *Cbfb-MYH11* induced leukemogenesis. We generated knock-in mice expressing a CBFβ-SMMHC mutant lacking the HABD in the SMMHC tail. Consequently, the mutant CBFβ-SMMHC has lower RUNX1 binding affinity[20]. Unexpectedly, these mice showed accelerated leukemogenesis. We also generated knock-in mice with a deletion mutant of CBFβ-SMMHC lacking the C-terminal 95 amino acids (*Cbfb*$^{+/\Delta C95}$), a region not predicted to interact with RUNX1, but that contains multimerization and repression domains. These mice showed a rescue of the *Runx1*-repression independent defects in primitive hematopoiesis, and never developed leukemia

implying that CBFβ-SMMHC has activities independent of RUNX1 binding that are critical for mediating differentiation defects and leukemogenesis[21].

Together, these findings raise the possibility that *Runx1* may be dispensable for *Cbfb-MYH11* activity. To test this hypothesis, we generated mice expressing *Cbfb-MYH11* but lacking *Runx1* activity. We found that *Runx1* activity is required for *Cbfb-MYH11* induced differentiation defects during both primitive and definitive hematopoiesis. In addition, we found that insufficient *Runx1* activity results in delayed leukemogenesis. These results indicate that *Cbfb-MYH11* requires *Runx1* activity for leukemogenesis and validates current efforts to develop inhibitors of the CBFβ-SMMHC:RUNX1 interaction for the treatment of inv(16) leukemia[22].

Material and Methods

Animals

All animals used and the procedures performed in this study were approved by the National Human Genome Research Institute (NHGRI) Animal Care and Use Committee. *Cbfb-MYH11* conditional knockin (*Cbfb*$^{+/56M}$)[23], *Runx1* knockout (*Runx1*$^{-/-}$)[15], *Runx1-lacZ* knockin (*Runx1*$^{+/lz}$)[24], *β-actin-Cre recombinase* transgenic (*Actb-Cre*$^{+}$)[25] and *Mx1-Cre recombinase* transgenic (*Mx1-Cre*$^{+}$) [26] mice have been described previously. All mice were maintained on a mixed C57BL/6;129/SvEv background and were genotyped by polymerase chain reaction (PCR) with gene-specific primers (sequences available upon request) using tail-snip DNA prepared using Gentra Puregene Mouse Tail Kit (Qiagen, Venlo, the Netherlands). Polyinosine-polycytidylic acid (pI:pC) (InvivoGen, San Diego, CA, USA) was used to induce *Cbfb-MYH11* expression as described previously[19]. To accelerate leukemia development, mice were treated with *N-ethyl-N-nitrosourea* (ENU) as described previously[5]. All mice were observed for leukemia development for 12 months.

Peripheral Blood Counts

Peripheral blood was collected from adult mice and the complete blood counts were analyzed using an Abbott Cell-Dyn 3700 Hematology Analyzer (Abbott Park, IL, USA).

Flow Cytometry

Peripheral blood cells from embryos and lineage negative bone marrow cells were isolated and stained as described previously[19]. Data were acquired using a LSRII Flow Cytometer (BD Biosciences, SanJose, CA, USA) and analyzed using FlowJo software (Flowjo, LLC, Ashland, OR, USA).

Proliferation and Cell Number Assays

Cell proliferation was determined by BrdU incorporation using the BrdU Flow kit (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Cell number was determined using a hemocytometer to count the number of lineage depleted cells extracted from both femurs of a mouse of the indicated genotype and resuspended in phosphate buffered saline with 5% fetal bovine serum.

Construction of *Runx1-lz* Expression Plasmid

Partial *Runx1-lz* cDNA was generated by RT-PCR with RNA from thymus tissue of *Runx1*$^{+/lz}$ mice and forward and reverse primers (sequences available upon request) in the *Runx1* and *lacZ* domains, respectively, of the fusion gene. The RT-PCR fragment was cloned in frame into pSV-βgal (Promega, Madison, WI, USA) and the RT-PCR fragment with the remainder of the lacZ coding sequence was cloned into pCDNA-Runx1 to generate pCDNA-Runx1-lz. The coding frame of the final plasmid was verified by sequencing.

Immunoprecipitation and Western blot analysis

Cell lysates were prepared from primary mouse tissues or from 293HEK cells transfected with the indicated plasmids using Lipofectamine 2000 (LifeTechnologies, Grand Island, NY, USA). Immunoprecipitation and western blot analysis was performed as described previously[19, 22] using antibodies against CBFβ and CBFβ-SMMHC (Aviva Biosystems, San Diego, CA, USA and anti-CBFβ141[16]), β-galactosidase (ab616, Abcam, Cambridge, MA, USA), RUNX1 (Active Motif, Carlsbad, CA, USA), and α-Tubulin (Abcam, Cambridge, MA, USA). Quantification of western blots was performed using ImageJ software[27].

MCSFR Promoter Assay

The MCSFR promoter assay was performed as described previously[21].

Statistical Analysis

Student *t* tests were performed using Excel (Microsoft, Redmond, WA, USA) to assess the significance of the differences in the indicated cell populations between samples. Log rank test was used (http://bioinf.wehi.edu.au/software/russell/logrank/) to assess the significance of the differences between the survival curves in Figure 4.

Results

Runx1 is required for primitive hematopoietic defects induced by *Cbfb-MYH11*

Previously we showed that *Cbfb-MYH11* induces defects in primitive hematopoiesis that are absent in *Runx1* null embryos, suggesting such defects are *Runx1*-repression independent[19]. However, it is not clear if *Runx1* is completely dispensable for these defects. To test this possibility, we generated mouse embryos homozygous for a null allele of *Runx1* (*Runx1*$^{-/-}$; Figure 1A)[15] and heterozygous for a floxed allele of *Cbfb-MYH11* (*Cbfb*^{56M})[23]. The mice also harbor the *β-Actin Cre Recombinase* transgene (*Actb-Cre*$^{+}$)[25] which allows for expression of *Cbfb-MYH11* in all tissues of the embryo. Using cell surface staining for the differentiation markers Kit and Ter119, we found that peripheral blood from E10.5 *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{-/-}$ embryos had an increase in mature Kit^{-}, Ter119^{high} cells coupled with a decrease in the less mature Kit^{-}, Ter119^{low}; Kit^{+}, Ter119^{low} and Kit^{+}, Ter119^{-} cells as compared to the blood from *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/-}$ and *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ littermates (Figure 1B and D). We also performed staining for the cell surface marker Csf2rb, which we previously showed is expressed on immature primitive blood cells[19]. Consistent with the Kit and Ter119 staining, we found that *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{-/-}$ embryos had fewer immature Csf2rb^{+} cells in the peripheral

blood as compared to their *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/-}$ and *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ littermates (Figure 1C and E). We did not observe any differences in cell surface staining between *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/-}$ and *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ littermates. In addition, we did not observe any staining difference between *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ embryos, but did observe a more subtle differentiation defect in the primitive blood of *Runx1*$^{-/-}$ embryos (Supplemental Figure 1a), consistent with previous reports[19, 28]. Together, these results show that genetic deletion of *Runx1* partially rescued the primitive hematopoietic defects induced by *Cbfb-MYH11*, indicating that *Cbfb-MYH11* has a genetic requirement for *Runx1* for its blockage of primitive hematopoiesis.

Rescue of *Cbfb-MYH11* induced primitive hematopoietic defects by the *Runx1*^{lz} allele

In order to confirm the requirement for *Runx1* activity by *Cbfb-MYH11*, we used a mouse model with a different *Runx1* allele in which exons 7 and 8 are replaced by the gene for β-galactosidase, *lacZ* (*Runx1*^{lz}; Figure 1A). This allele has been shown to be non-functional, presumably due to the loss of the transactivation domain[24]. Interestingly, *Runx1*$^{+/lz}$ had defects not seen in *Runx1*$^{+/-}$ mice, but are able to survive to adulthood, unlike *Runx1*$^{-/-}$ mice. This implies that expression of one *Runx1-lz* allele results in a decrease in RUNX1 activity more severe than in Runx1$^{+/-}$ mice, but not a complete loss of RUNX1 activity as in Runx1$^{-/-}$ mice. Consequently, it is thought that the *Runx1-lz* allele acts as semi-dominant negative. To confirm this, we examined the platelet counts in the peripheral blood of adult *Runx1*$^{+/+}$ and *Runx1*$^{+/lz}$ mice. Previous work has shown that homozygous loss of *Runx1* causes a significant decrease in peripheral blood platelets, but that loss of a single *Runx1* allele does not[14, 29]. We found that the number of platelets in the peripheral blood in *Runx1*$^{+/lz}$ mice was significantly decreased as compared to that in *Runx1*$^{+/+}$ mice (Supplemental Figure 2), indicating that the *Runx1-lz* allele indeed has dominant negative activity.

To test whether the *Runx1-lz* allele also acts as a dominant negative in the context of *Cbfb-MYH11*, we generated embryos expressing the fusion gene and a single *Runx1-lz* allele. We found that the peripheral blood from *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/lz}$ embryos had more mature Kit^{-}, Ter119^{high} cells and fewer immature Kit^{-}, Ter119^{low}; Kit^{+}, Ter119^{low}; and Kit^{+}, Ter119^{-} cells as compared to *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ littermates (Figure 2A and C). Similarly, *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/lz}$ embryos had fewer Csf2rb+ cells as compared to *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ littermates (Figure 2B and D). These results indicate that a single *Runx1-lz* allele causes sufficient reduction in *Runx1* activity to rescue *Cbfb-MYH11* induced defects during primitive hematopoiesis. In addition, because *Runx1*$^{+/lz}$ mice are not embryonic lethal, these results indicate that the *Runx1-lz* allele can be used to study *Cbfb-MYH11*'s requirement for *Runx1* during adult hematopoiesis.

Because RUNX1 is a transcription factor, it is possible that it is required for the expression of *Cbfb-MYH11*. To test this, we examined the level of CBFβ-SMMHC, the protein product of the *Cbfb-MYH11* fusion gene, in E12.5 embryos by western blot (Supplemental Figure 3A). As shown in Supplemental Figure 3B, the relative CBFβ-SMMHC protein levels in *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/lz}$ embryos was comparable to that in *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ embryos. This finding indicates that *Runx1* activity is not required for the

expression of CBFβ-SMMHC, implying that the *Runx1* requirement is related to the activity of the fusion protein.

Runx1 is required for *Cbfb-MYH11* induced proliferation defects in definitive hematopoiesis

We showed previously that expression of *Cbfb-MYH11* in adult mice causes defects in definitive hematopoiesis prior to leukemic transformation[19]. These "pre-leukemic" defects include an increase in lineage negative (lin^-) cells in the bone marrow shortly after induction of *Cbfb-MYH11* in $Cbfb^{+/56M}$ mice with *Cre* expressed from the *Mx-1* promoter, which allows for inducible expression of *Cbfb-MYH11* in nearly all adult blood cells[26] (Figure 3A and Supplemental Figure 4A). To test whether this increase in lin^- cells is due to increased proliferation, we performed bromodeoxyuridine (BrdU) staining on lin^- cells after pI:pC induction of *Cbfb-MYH11* in *Mx1-Cre*$^+$, $Cbfb^{+/56M}$ mice. One week after *Cbfb-MYH11* induction, *Mx1-Cre*$^+$, $Cbfb^{+/56M}$, $Runx1^{+/+}$ mice showed an increase of $BrdU^+$, lin^- cells. (Figure 3B and Supplemental Figure 4B). In contrast, *Mx1-Cre*$^+$, $Cbfb^{+/56M}$, $Runx1^{+/lz}$ mice showed significantly fewer $BrdU^+$, lin^- cells and no increase in total lin^- cell number after *Cbfb-MYH11* induction as compared to *Mx1-Cre*$^+$, $Cbfb^{+/56M}$, $Runx1^{+/+}$ mice (Figure 3A and B, Supplemental Figure 4A and B). These results indicate that *Cbfb-MYH11* induced proliferation and lin^- cell population increase require *Runx1* activity during adult hematopoiesis.

Runx1 is required for the induction of the pre-leukemia cell population by *Cbfb-MYH11*

Cbfb-MYH11 expression in adult hematopoietic cells also induces an abnormal population of pre-leukemic cells expressing the cell surface markers Il1rl1 and Csf2rb[19]. As shown in Figure 3C, *Mx1-Cre*$^+$, $Cbfb^{+/56M}$, $Runx1^{+/+}$ mice show an increase of $Il1rl1^+$,$Csf2rb^-$ and $Il1rl1^+$,$Csf2rb^+$ cells two weeks after induction of *Cbfb-MYH11* expression. Both of these populations were significantly reduced in *Mx1-Cre*$^+$, $Cbfb^{+/56M}$, $Runx1^{+/lz}$ mice, especially the $Il1rl1^+$,$Csf2rb^+$ cells (Figure 3C and Supplemental Figure 4C&D). These data suggest that the induction of abnormal pre-leukemic cells is impaired in *Mx1-Cre*$^+$, $Cbfb^{+/56M}$, $Runx1^{+/lz}$ mice.

Cbfb-MYH11 expression also induces defects in the lin^- $Sca1^-$, Kit^+ myeloid progenitor compartment[19]. Consistent with our previous findings, *Mx1-Cre*$^+$, $Cbfb^{+/56M}$, $Runx1^{+/+}$ showed a decrease in the proportion of $Sca1^-$, Kit^+ cells 2 weeks after induction of *Cbfb-MYH11* (Fig. 3D, Supplemental Figure 4E). However, this decrease was not seen in *Mx1-Cre*$^+$, $Cbfb^{+/56M}$, $Runx1^{+/lz}$ mice indicating *Runx1* activity is required for *Cbfb-MYH11* induced decreases in myeloid progenitors.

Within the myeloid progenitor compartment, previous work showed that expression of *Cbfb-MYH11* results in an accumulation of $CD34^-$, $FC\gamma RII/III^-$ cells that have leukemia initiating activity[23]. Similarly, we saw a statistically significant increase in the proportion of $Sca1^-$, Kit^+, $CD34^-$, $FC\gamma RII/III^-$ cells in the bone marrow of *Mx1-Cre*$^+$, $Cbfb^{+/56M}$, $Runx1^{+/+}$ mice two weeks after induction of *Cbfb-MYH11* (Fig. 3D, Supplemental Fig. 4F). In contrast, 2 weeks after induction of *Cbfb-MYH11*, the proportion of the $Sca1^-$, Kit^+, $CD34^-$, $FC\gamma RII/III^-$ cells was significantly lower in *Mx1-Cre*$^+$, $Cbfb^{+/56M}$, $Runx1^{+/lz}$ mice (Fig 3D,

Supplemental Figure 4F). These results indicate that loss of Runx1 activity impairs *Cbfb-MYH11* induced accumulation of Sca1^{-}, Kit^{+}, CD34^{-}, FCγRII/III^{-} cells, implying that Runx1 may be important for *Cbfb-MYH11*'s ability to induce leukemia initiating cells and consequently, leukemia.

Runx1 is required for efficient leukemogenesis by *Cbfb-MYH11*

To test whether *Runx1* is required for *Cbfb-MYH11* induced leukemia, we treated adult *Mx1-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ mice (N=34), *Mx1-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/-}$ (N= 5), *Mx1-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/lz}$ mice (N=14), and their litter mate control mice (WT; *Mx1-Cre*$^{+}$, *Runx1*$^{+/lz}$; *Cbfb*$^{+/56M}$; *Mx1-Cre*$^{+}$; *Runx1*$^{+/lz}$; *Cbfb*$^{+/56M}$, *Runx1*$^{+/lz}$; N=33) with pI:pC to induce *Cbfb-MYH11* expression. The mice were also treated with the mutagen N-ethyl-N-nitrosourea to induce cooperating mutations. We monitored these mice for leukemia development and progression for 12 months. Mice from both experimental cohorts developed myeloid leukemias similar to what has been described previously[5, 23]. The leukemia development was significantly delayed ($p<0.0001$) in *Mx1-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/lz}$ mice as compared to *Mx1-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ mice (Figure 4). In addition, 21% (3 out of 14) of the *Mx1-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/lz}$ mice never developed leukemia during the 12 months observation, while 100% of *Mx1-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ mice died from leukemia within 5 months after pI:pC and ENU treatment. We did not observe any difference in leukemia development between *Mx1-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/-}$ and *Mx1-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ mice (Supplemental Figure 5), which is consistent with *Runx1-lz* allele having dominant negative activity. No leukemias were observed in the control mice, although solid tumors, including thymomas, were detected in some control mice upon dissection likely due to the ENU treatment. These data suggest that *Runx1* strongly enhances *Cbfb-MYH11* induced leukemogenisis.

RUNX1-βgal interacts with CBFβ-SMMHC and is more abundant than RUNX1 in the bone marrow

To explore the mechanism of *Runx1-lz*'s ability to block CBFβ-SMMHC activity, we tested whether the *Runx1-lz* protein product, RUNX1-βgal, is able to interact with CBFβ and CBFβ-SMMHC. HEK293 cells were transfected with constructs expressing RUNX1-βgal and either CBFβ or CBFβ-SMMHC. RUNX1-βgal containing complexes were immunoprecipitated with an antibody against β-galactosidase, and probed by western blot with an antibody against CBFβ. We found that RUNX1-βgal co-immunoprecipitated with both wildtype CBFβ and CBFβ-SMMHC (Figure 5A). β-galactosidase alone was not able to co-immunoprecipitate either wildtype CBFβ or CBFβ-SMMHC (data not shown) indicating that the RUNX1 portion of the RUNX1-βgal fusion protein is required for this interaction. These results indicate that RUNX1-βgal is capable of binding wildtype CBFβ and CBFβ-SMMHC, just as has been shown for wildtype RUNX1.

To understand how RUNX1-βgal is capable of acting as a dominant negative, we performed a reporter assay with the MCSFR reporter construct, which has been demonstrated before as a RUNX1 target[30]. In this assay, RUNX1-βgal was nonfunctional but did not display significant dominant negative activity (Supplemental Figure 6). We then examined the relative expression levels of RUNX1 and RUNX1-βgal in the bone marrow of adult

Runx1$^{+/+}$ and *Runx1*$^{+/lz}$ mice. In *Runx1*$^{+/lz}$ mice, we found that the intensity of the RUNX1-βgal band was 2.22 fold (S.D.= 0.61, N=4) greater than the wildtype RUNX1 band (Figure 5B). The overabundance of the RUNX1-βgal protein provides a potential explanation for its semi-dominant negative behavior: the non-functional RUNX1-βgal protein may be able to outcompete wildtype RUNX1 for CBFβ and DNA target binding, leading to reduction of functional CBFβ-RUNX1 heterodimers and RUNX1-DNA complexes.

Discussion

It has been proposed that CBFβ-SMMHC, the protein product of the *Cbfb-MYH11* fusion gene, acts by binding and dominantly inhibiting the activity of RUNX1, which leads to changes in gene expression that contribute to leukemogenesis. However, recent studies using knock-in mouse models have demonstrated that CBFβ-SMMHC also has RUNX1-repression independent activities that contribute to abnormal differentiation and leukemia development in *Cbfb-MYH11* mice[19–21]. Notably we found that *Cbfb-MYH11* is able to induce defects in primitive hematopoiesis, a stage not significantly affected by *Runx1* knockout. Moreover, we found that mice expressing a modified CBFβ-SMMHC with reduced ability to bind RUNX1 developed leukemia faster than those expressing the full length CBFβ-SMMHC. These findings raised the possibility that CBFβ-SMMHC can induce leukemia through a RUNX1-independent mechanism. In this study we performed experiments to determine the importance of RUNX1 for leukemogenesis by CBFβ-SMMHC.

Using mouse models, we showed that *Runx1* is genetically required for the differentiation block induced by *Cbfb-MYH11* during primitive hematopoiesis, since this block is rescued in embryos either homozygous for a null allele of *Runx1* (*Runx1*$^{-/-}$) or with a single semi-dominant negative *Runx1-lz* allele (*Runx1*$^{+/lz}$). These results, together with our previous work[19–21], emphasize that CBFβ-SMMHC has important activities that don't involve repression of RUNX1. In fact, it is likely that CBFβ-SMMHC binds RUNX1 to form a transcriptional activator complex. We previously showed that the majority of the gene expression changes induced by the fusion protein are increases in expression[19]. Similarly, Mandoli et al showed that the majority of genes bound by CBFβ-SMMHC are positively regulated by the fusion protein[31]. CBFβ-SMMHC cannot bind DNA without a RUNX protein, so homozygous deletion of *Runx1* would severely impair target gene regulation by CBFβ-SMMHC. Because the Runx1-βgal protein encoded by the Runx1-lz allele is missing Runx1's transactivation domain, we would predict that expression of this mutant inhibits CBFβ-SMMHC activity because of its inability to activate transcription. However, it is also possible that the β-gal domain interferes with DNA binding. By electrophoretic mobility shift assay, we were unable to detect a Runx1-βgal:CBFβ complex bound to DNA (data not shown).

We also found that loss of Runx1 activity significantly rescues *Cbfb-MYH11* induced defects during definitive hematopoiesis. Expression of the dominant negative *Runx1-lz* allele markedly reduced the rate of proliferation and the number of pre-leukemic cells induced by *Cbfb-MYH11*. These data suggest that *Runx1* is also important for *Cbfb-MYH11* activities during definitive hematopoiesis. Importantly, we found that loss of Runx1 activity causes a

significant delay in *Cbfb-MYH11* induced leukemogenesis. Half of the *Cbfb-MYH11* mice with wildtype *Runx1* developed leukemia 2 months after pIpC injection, and all of them died from leukemia within 5 months. On the other hand, it took 5 months for half of the *Cbfb-MYH11* mice with *Runx1-lz* to develop leukemia and 21% of them were still alive after 12 months. It is likely that *Runx1* is required for efficient generation of the leukemia initiating population, since we observed a smaller population of lin^- cells in the *Cbfb-MYH11* mice with *Runx1-lz*. Our recent finding that the RUNX1 inhibitor Ro5-3335 decreases leukemic burden and increases survival of mice transplanted with CBFβ-SMMHC$^+$ leukemic cells[22], as well as a recent report of RUNX1 addiction of CBF leukemia cell lines in culture[32], suggest that RUNX1 also has a role during leukemic maintenance, in addition to its role during initiation.

Currently, we can only speculate how RUNX1 is required for the leukemogenesis induced by CBFβ-SMMHC. Chromatin immunoprecipitation experiments with the inv(16) cell line ME-1 imply that RUNX1 binds DNA with both the fusion protein and wildtype CBFβ and that each complex regulates transcription of different target genes[31], suggesting that direct interaction between RUNX1 and CBFβ-SMMHC is required. On the other hand, work with other leukemia fusion proteins shows that the CBFβ:RUNX1 dimer is required for the transcription of pro-survival factors in leukemia cells[32, 33]. It is possible that the CBFβ:RUNX1 dimer plays a similar role in inv(16) leukemia. In fact, Goyama et al propose that the accelerated leukemogenesis observed in mice expressing a *Cbfb-MYH11* mutant with reduced RUNX1 binding may be due to a concomitant increase in the CBFβ:RUNX1 dimer, and consequently increased expression of pro-survival target genes[20, 33]. Ben-Ami et al also demonstrated addiction to normal RUNX1 in leukemia cells with the other common CBF fusion protein, RUNX1-RUNX1T1 (also known as AML1-ETO)[32]. In fact, RUNX1 mutations are rare in CBF leukemia patients, while such mutations are common in non-CBF leukemia cases, implying a requirement for the CBFβ;RUNX1 dimer in CBF leukemias[34]. We showed that the non-functional RUNX1-βgal protein bound both CBFβ-SMMHC and wildtype CBFβ, so RUNX1-βgal would be expected to reduce the activity of both complexes.

In this study, we showed that there is a genetic requirement for *Runx1* activity for *Cbfb-MYH11* induced defects during hematopoiesis and leukemogenesis. This work provides important insight into the mechanism of leukemogenesis associated with CBFβ-SMMHC and validates current efforts to develop inhibitors of the CBFβ-SMMHC: RUNX1 interaction for the treatment of inv(16) AML.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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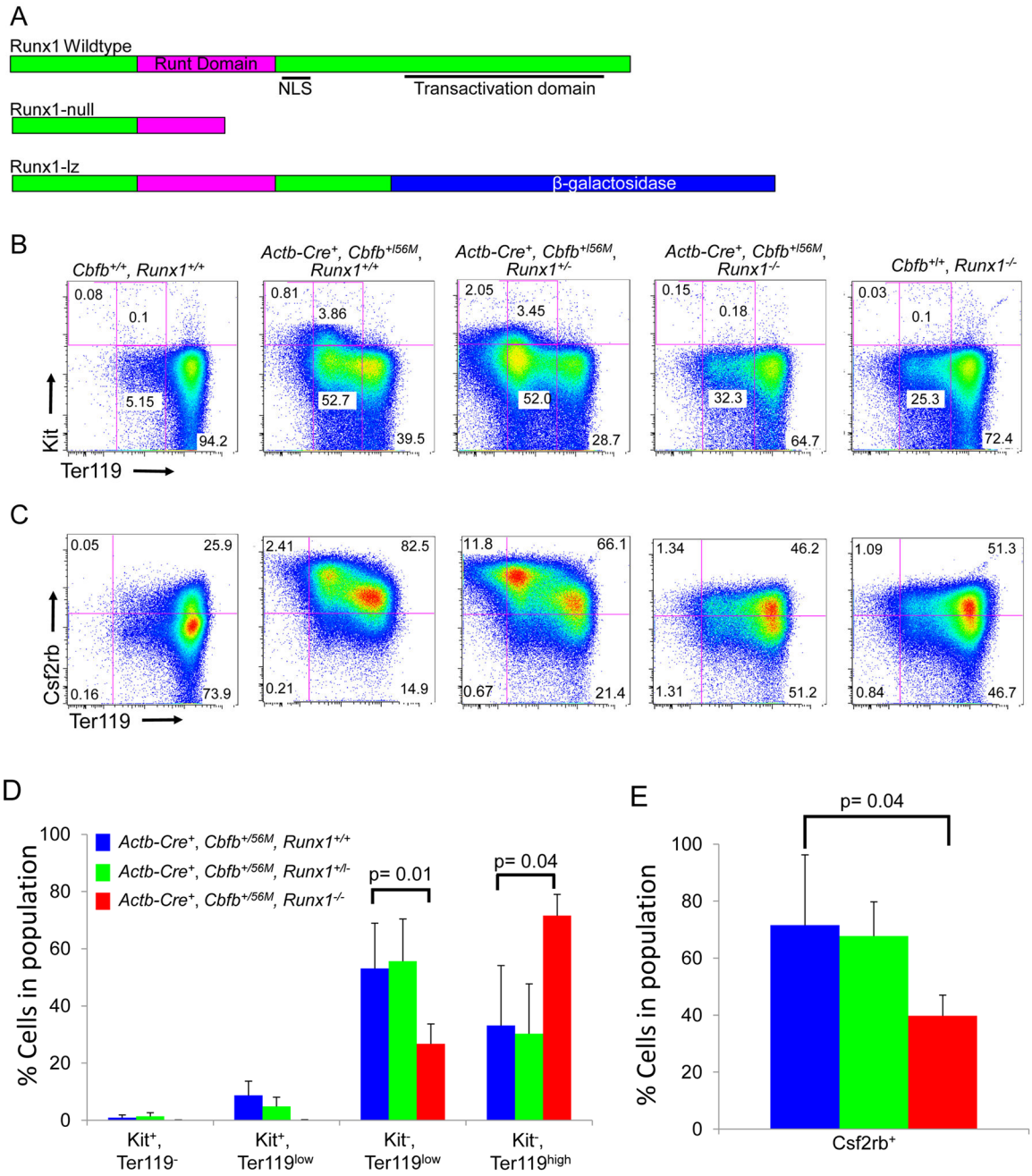


Figure 1. *Runx1* is required for *Cbfb-MYH11* induced differentiation defects during primitive hematopoiesis

(A) Schematic representations of different *Runx1* alleles used[15, 24]. (B and C) Representative FACS plots and (D and E) bar graphs of Terr119, Kit, and Csf2rb staining of peripheral blood cells from E10.5 embryos of the indicated genotype. Percentage of cells in each gate is given. Brackets in D and E indicate the samples being compared with the associated p-values. N≥3 for each genotype.

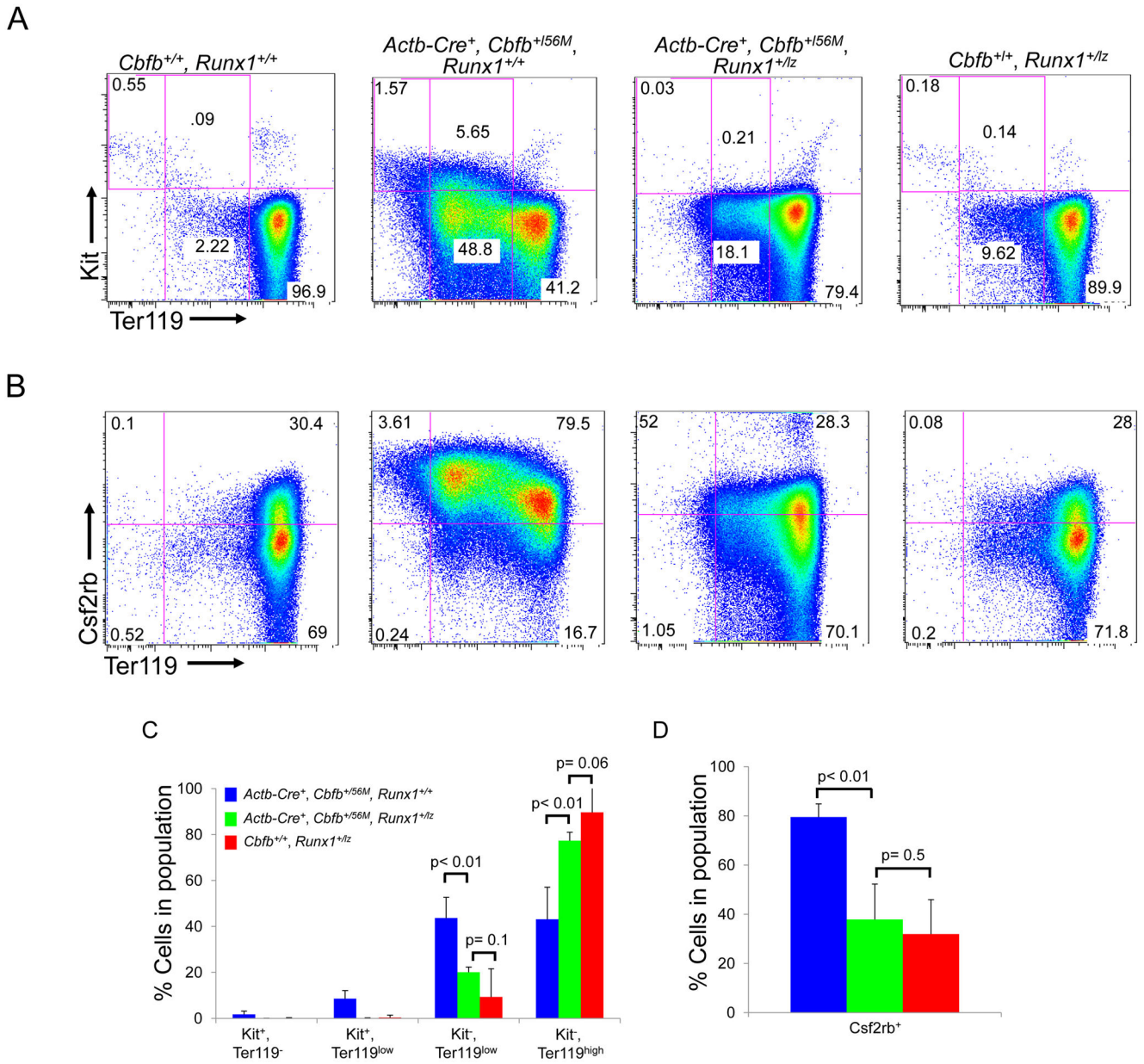
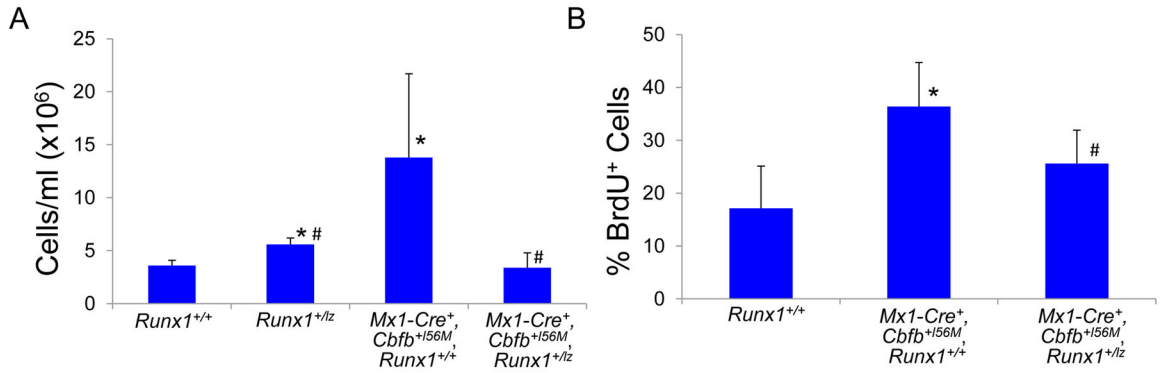


Figure 2. Expression of one *Runx1-lz* allele rescues *Cbfb-MYH11* induced defects in primitive blood differentiation

(A and B) Representative FACS plots and (C and D) bar graphs of Terr119, Kit, and Csf2rb staining of peripheral blood cells from E10.5 embryos of the indicated genotype. Percentage of cells in each gate is given. Brackets in C and D indicate the samples being compared with the associated p-values. N≥3 for each genotype.



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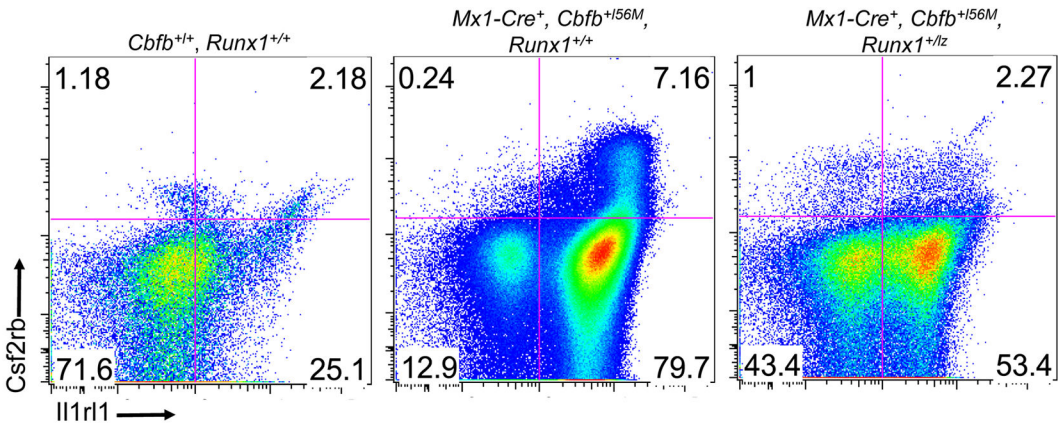

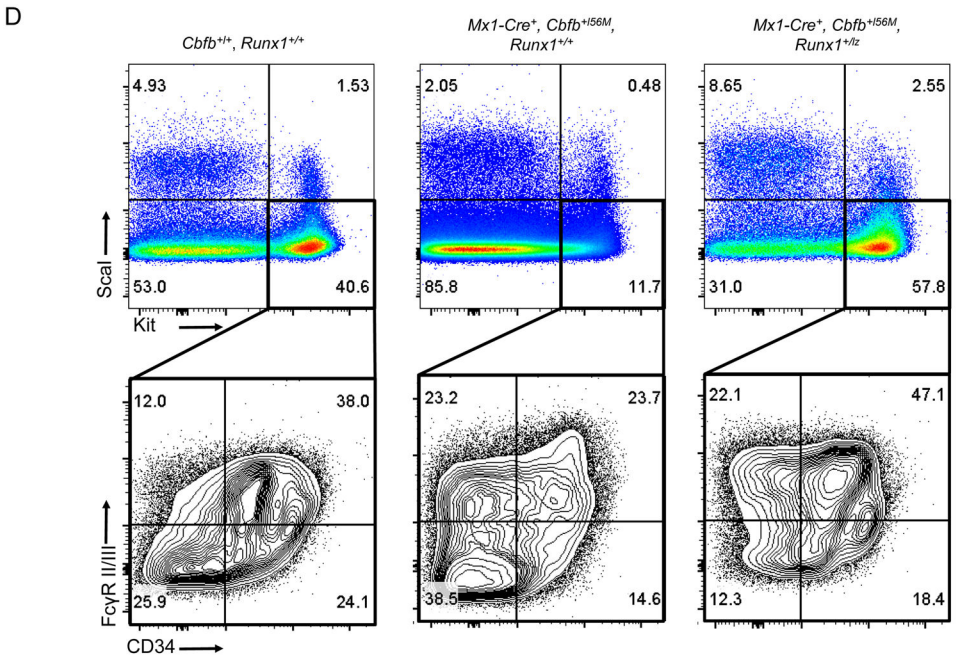

Figure 3. *Runx1* activity is required for *Cbfb-MYH11* induced defects during definitive hematopoiesis

(A) Graph of the number of the lineage negative (lin-) cells per ml from the bone marrow of adult mice of the indicated genotypes 2 weeks after the induction of *Cbfb-MYH11* expression. (B) Graph of percentage of lin-cells that were BrdU+ in the bone marrow of adult mice of the indicated genotypes 1 week after the induction of *Cbfb-MYH11* expression. * indicates p≤0.05 as compared to $Runx1^{+/+}$ mice. # indicates p≤0.05 as compared to $Mx1\text{-}Cre^{+}$, $Cbfb^{+/56M}$, $Runx1^{+/+}$ mice. (C) Representative FACs plots of Il1rl1 and Csf2rb and (D) Kit, ScaI, CD34 and FcRγII/III staining of lin-bone marrow from adult mice of the indicated genotypes 2 weeks after the induction of *Cbfb-MYH11* expression. N≥3 for each genotype.

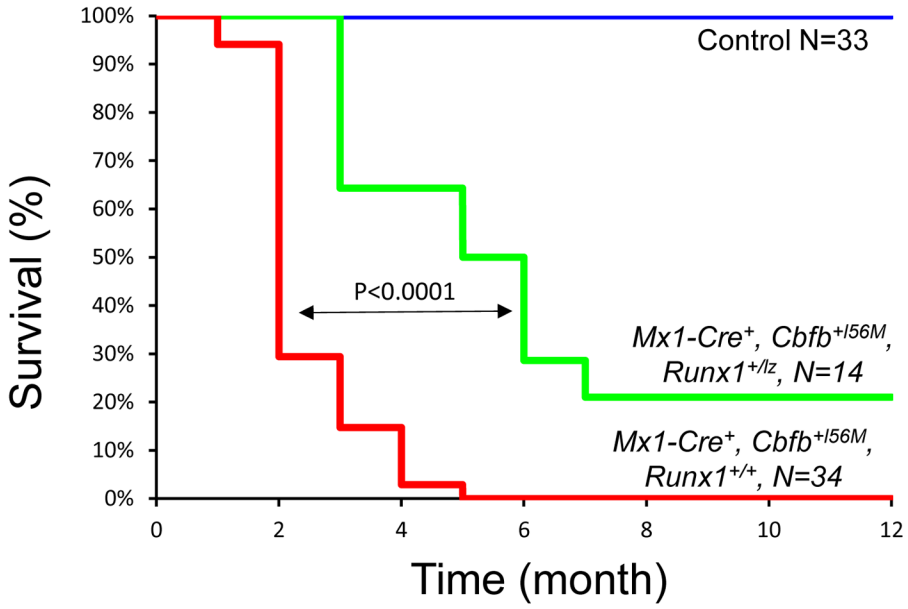

Figure 4. Impaired *Runx1* activity delays leukemogenesis by *Cbfb-MYH11*

Kaplan-Meier curve of mice with different genotypes during 12-month observation of leukemia development. Green line: *Mx1-Cre*$^{+}$, *Cbfb*$^{+l56M}$, *Runx1*$^{+/lz}$, N=14; red line: *Mx1-Cre*$^{+}$, *Cbfb*$^{+l56M}$, *Runx1*$^{+/l+}$, N=34; blue line: control mice (*Runx1*$^{+/+}$, *Runx1*$^{+/lz}$, and *Cbfb*$^{+l56M}$), N=33. The p values were calculated with log rank test. The difference between any two survival curves in the panel was highly significant (p<0.0001).

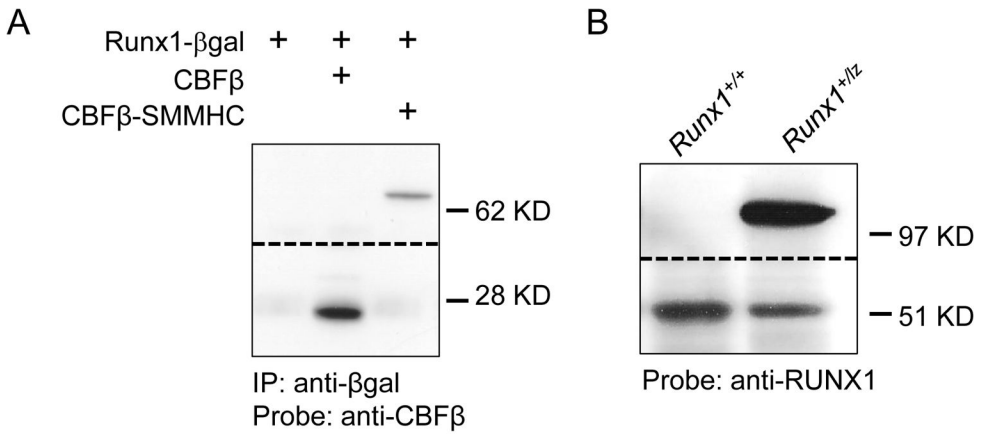

Figure 5. RUNX1-βgal binds CBFβ and CBFβ-SMMHC and is present at higher levels than RUNX1 in the bone marrow

(A) Co-immunoprecipitation with anti-βgal and western blot with anti-CBFβ141[1616] of lysate from 293HEK cells transfected with plasmids expressing the indicated proteins. Data shown is from two different regions of the same blot. The dashed line indicates the transition between the two sections. (B) Representative western blot of bone marrow of adult mice of the indicated genotypes probed with anti-RUNX1. Data shown is from two different regions of the same blot. The dashed line indicates the transition between the two sections.