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ORIGINAL ARTICLE

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

RK Hyde[1,2], L Zhao, L Alemu and PP Liu[2]

CBFβ-SMMHC (core-binding factor β-smooth muscle myosin heavy chain), 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 (Runt-related protein 1), 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.

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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 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 β 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 in the SMMHC tail, resulting in a higher binding affinity for RUNX as compared with wild-type 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* knock-in 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. Taken together, these results imply that *Cbfb-MYH11*'s *Runx1*-repression-independent activities have 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 high-affinity

Oncogenesis and Development Section, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA. Correspondence: Dr RK Hyde, Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, 985870 Nebraska Medical Center, Durham Research Building I, Room 7008, Omaha, NE 68198, USA or PP Liu, Oncogenesis and Development Section, National Human Genome Research Institute, National Institutes of Health, 49 Convent Drive, Room 3A26, Bethesda, MD 20892, USA.
E-mail: kate.hyde@unmc.edu or pliu@mail.nih.gov
[1]Current address: Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE, USA.
[2]Co-corresponding authors.
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binding domain 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]

Taken 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]

MATERIALS AND METHODS

Animals

All animals used and the procedures performed in this study were approved by the National Human Genome Research Institute Animal Care and Use Committee. *Cbfb-MYH11* conditional knock-in (*Cbfb*$^{+/56M}$),[23] Runx1-knockout (*Runx1*$^{-/-}$),[15] *Runx1-lacZ*-knock-in (*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 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 (pIpC; InvivoGen, San Diego, CA, USA) was used to induce *Cbfb-MYH11* expression as described previously.[19] To accelerate leukemia development, mice were treated with ENU (*N*-ethyl-*N*-nitrosourea) 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 Diagnostics, Abbott Park, IL, USA).

Flow cytometry

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

Proliferation and cell number assays

Cell proliferation was determined by bromodeoxyuridine (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 reverse transcription-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 reverse transcription-PCR fragment was cloned in frame into pSV-β-gal (β-galactosidase) (Promega, Madison, WI, USA) and the reverse transcription-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 HEK293 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; Wang *et al.*[16]), β-gal (ab616; Abcam, Cambridge, MA, USA), RUNX1 (Active Motif, Carlsbad, CA, USA) and α-tubulin (Abcam). Quantification of western blots was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).[27]

MCSFR promoter assay

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

Statistical analysis

Student's *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 that 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 *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 with the blood from *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/-}$ and *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ littermates (Figures 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 with their *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/-}$ and *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ littermates (Figures 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 (Supplementary Figure 1A), consistent with previous reports.[19,28] Taken 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

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-gal, *lacZ* (*Runx1*^{lz}; Figure 1a). This allele has been shown to be non-functional, presumably because of the loss of the transactivation domain.[24] Interestingly, *Runx1*$^{+/lz}$ had defects not seen in *Runx1*$^{+/-}$ mice, but are able to

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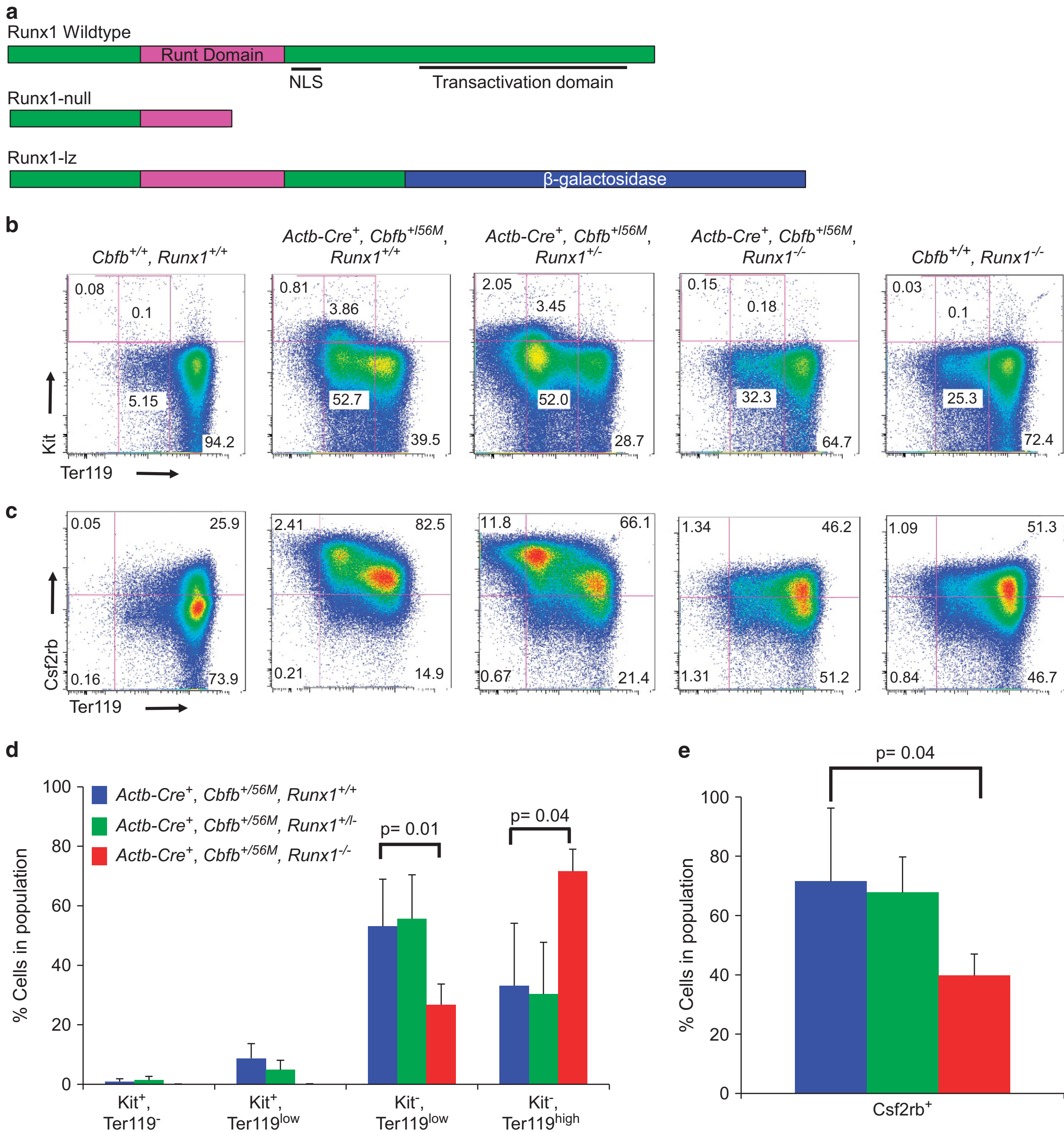

Figure 1. *Runx1* is required for *Cbfb-MYH11*-induced differentiation defects during primitive hematopoiesis. (**a**) Schematic representations of different *Runx1* alleles used.[15,24] (**b** and **c**) Representative FACS (fluorescence-activated cell sorting) plots and (**d** and **e**) bar graphs 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. $N \geqslant 3$ for each genotype.

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 with that in *Runx1*$^{+/+}$ mice (Supplementary 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 with *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ littermates (Figures 2a and c). Similarly, *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/lz}$

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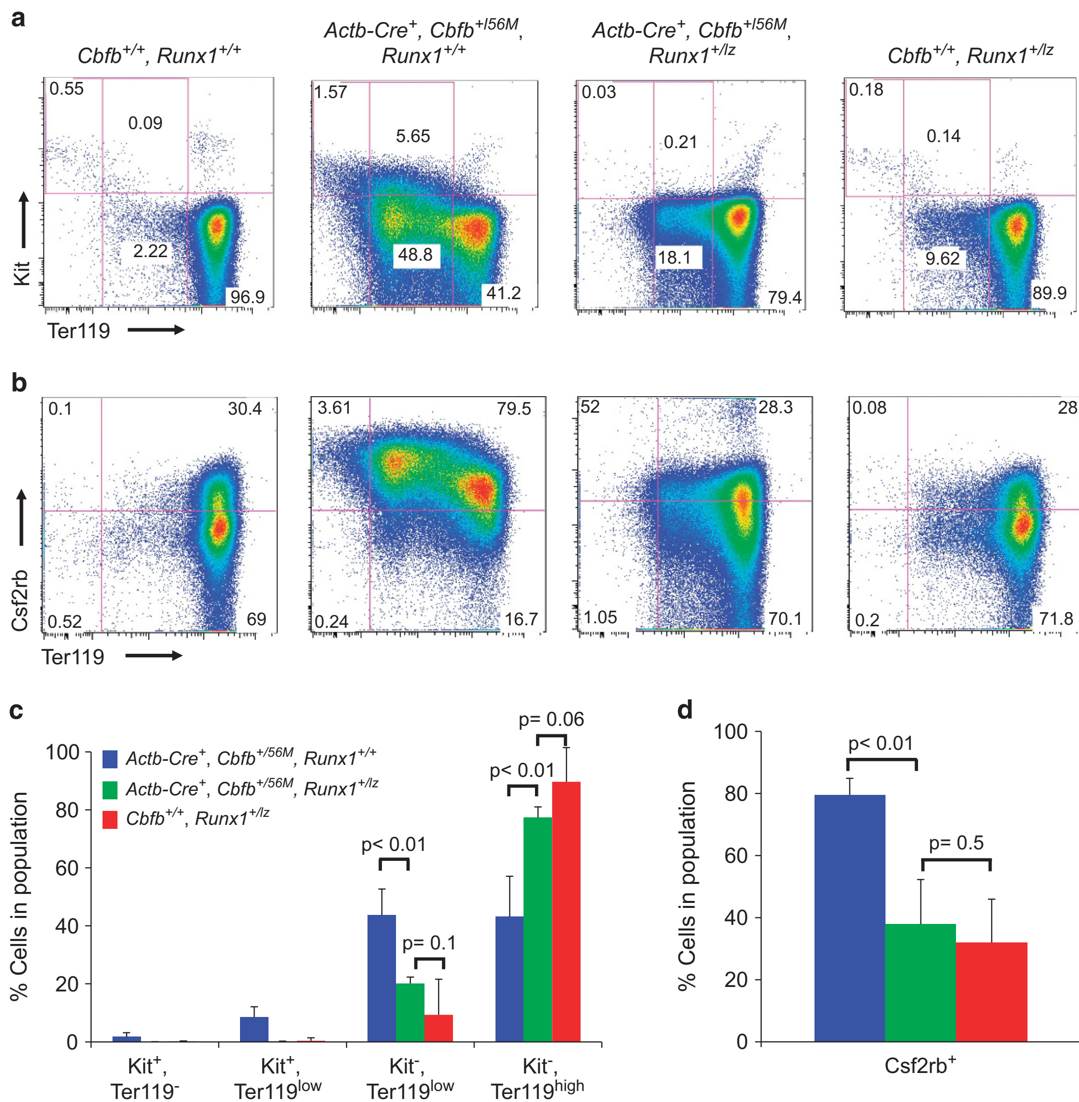

Figure 2. Expression of one *Runx1*^{lz} allele rescues *Cbfb-MYH11*-induced defects in primitive blood differentiation. (**a** and **b**) Representative FACS (fluorescence- activated cell sorting) plots and (**c** and **d**) bar graphs of Terr119, Kit and Csf2rb staining of peripheral blood cells from 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. $N \geqslant 3$ for each genotype.

embryos had fewer Csf2rb+ cells as compared with *Actb-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ littermates (Figures 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 embryonically 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 (Supplementary Figure 3A). As shown in Supplementary 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 before leukemic transformation.[19] These 'preleukemic' defects include an increase in lin^{-} cells in the bone marrow shortly after induction of *Cbfb-MYH11* in *Cbfb*$^{+/56M}$ mice with *Cre* expressed from the *Mx1* promoter, which allows for inducible expression of *Cbfb-MYH11* in nearly all adult blood cells[26] (Figure 3a and Supplementary Figure 4A). To test whether this increase in lin^{-} cells is due to increased proliferation, we performed BrdU staining on lin^{-} cells after pIpC 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 Supplementary 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 with *Mx1-Cre*$^{+}$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ mice (Figures 3a and b and Supplementary Figures 4A and B). These results indicate

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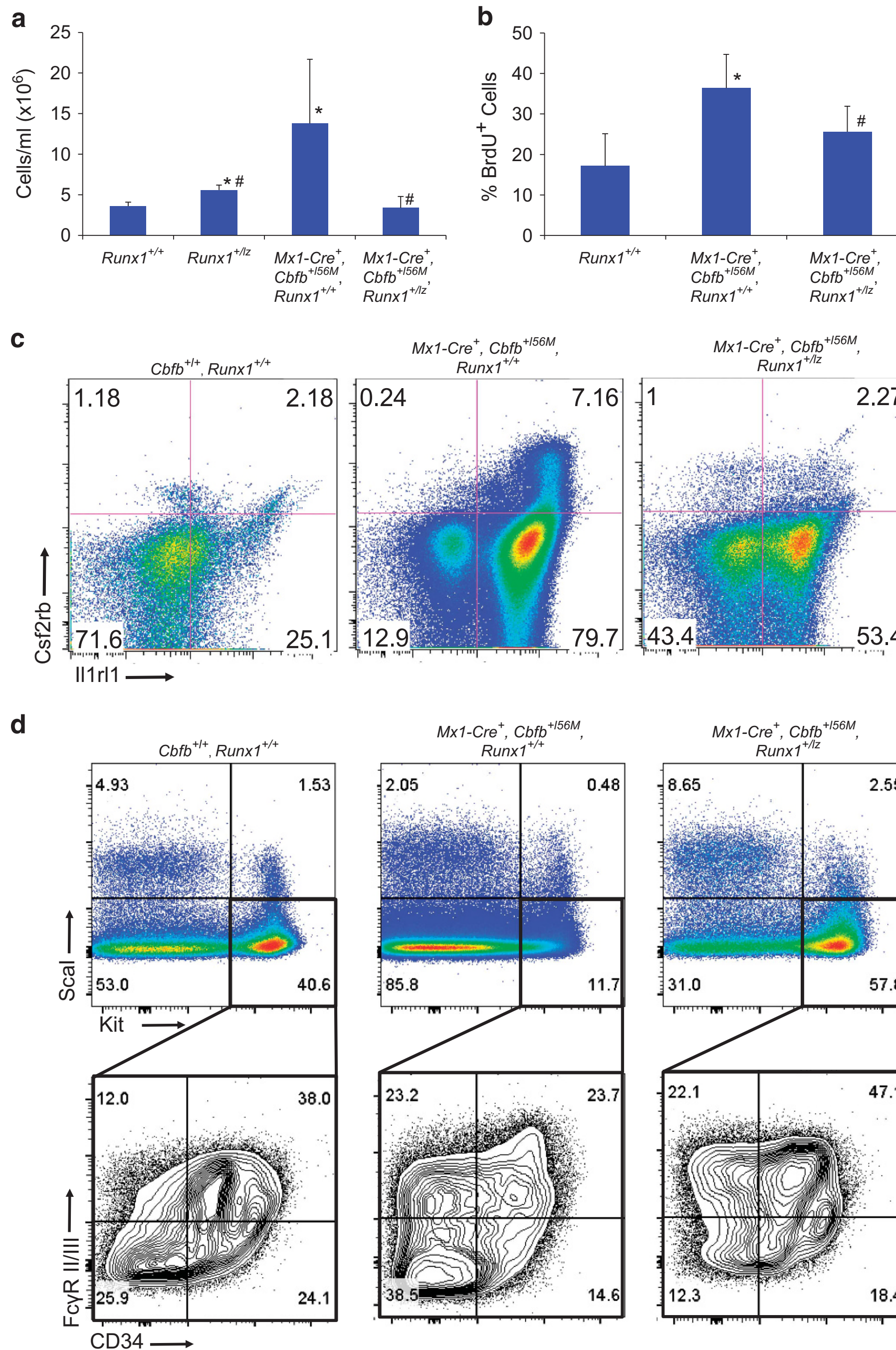

Figure 3. *Runx1* activity is required for *Cbfb-MYH11*-induced defects during definitive hematopoiesis. (**a**) Graph of the number of the lin$^-$ cells per ml from the bone marrow of adult mice of the indicated genotypes 2 weeks after the induction of *Cbfb-MYH11* expression. (**b**) Graph of the percentage 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. $^*P \leqslant 0.05$ as compared with *Runx1*$^{+/+}$ mice. $^\#P \leqslant 0.05$ as compared with *Mx1-Cre*$^+$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ mice. (**c**) Representative FACS (fluorescence-activated cell sorting) plots of Il1rl1 and Csf2rb and (**d**) Kit, Sca1, 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. $N \geqslant 3$ for each genotype.

that *Cbfb-MYH11*-induced proliferation and lin$^-$ cell population increase require *Runx1* activity during adult hematopoiesis.

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

Cbfb-MYH11 expression in adult hematopoietic cells also induces an abnormal population of preleukemic 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 2 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 Supplementary Figures 4C and D). These data suggest that the induction of abnormal preleukemic 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

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decrease in the proportion of Sca1$^-$, Kit$^+$ cells 2 weeks after induction of *Cbfb-MYH11* (Figure 3d and Supplementary Figure 4E). However, this decrease was not seen in *Mx1-Cre*$^+$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/lz}$ mice, indicating that *Runx1* activity is required for *Cbfb-MYH11*-induced decreases in myeloid progenitors.

Within the myeloid progenitor compartment, previous work showed that the expression of *Cbfb-MYH11* results in an accumulation of CD34$^-$, FCγRII/III$^-$ cells that have leukemia-initiating activity.[23] Similarly, we saw a statistically significant increase in the proportion of Sca1$^-$, Kit$^+$, CD34$^-$, FCγRII/III$^-$ cells in the bone marrow of *Mx1-Cre*$^+$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ mice 2 weeks after induction of *Cbfb-MYH11* (Figure 3d and Supplementary Figure 4F). In contrast, 2 weeks after induction of *Cbfb-MYH11*, the proportion of the Sca1$^-$, Kit$^+$, CD34$^-$, FCγRII/III$^-$ cells was significantly lower in *Mx1-Cre*$^+$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/lz}$ mice (Figure 3d and Supplementary 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 pIpC to induce *Cbfb-MYH11* expression. The mice were also treated with the mutagen ENU 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 with *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, whereas 100% of *Mx1-Cre*$^+$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$ mice died from leukemia within 5 months after pIpC 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 (Supplementary 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 because of the ENU treatment. These data suggest that *Runx1* strongly enhances *Cbfb-MYH11*-induced leukemogenesis.

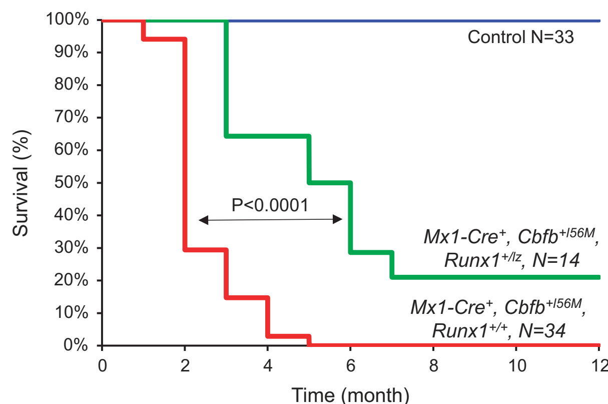

Figure 4. Impaired *Runx1* activity delays leukemogenesis by *Cbfb-MYH11*. Kaplan–Meier curve of mice with different genotypes during 12-month observation of leukemia development. Green line: *Mx1-Cre*$^+$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/lz}$, $N=14$; red line: *Mx1-Cre*$^+$, *Cbfb*$^{+/56M}$, *Runx1*$^{+/+}$, $N=34$; blue line: control mice (*Runx1*$^{+/+}$, *Runx1*$^{+/lz}$ and *Cbfb*$^{+/56M}$), $N=33$. The *P*-values were calculated with log-rank test. The difference between any two survival curves in the panel was highly significant ($P<0.0001$).

RUNX1-β-gal 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-β-gal, is able to interact with CBFβ and CBFβ-SMMHC. HEK293 cells were transfected with constructs expressing RUNX1-β-gal and either CBFβ or CBFβ-SMMHC. RUNX1-β-gal-containing complexes were immunoprecipitated with an antibody against β-gal, and were probed by western blot with an antibody against CBFβ. We found that RUNX1-β-gal co-immunoprecipitated with both wild-type CBFβ and CBFβ-SMMHC (Figure 5a). β-Gal alone was not able to co-immunoprecipitate either wild-type CBFβ or CBFβ-SMMHC (data not shown), indicating that the RUNX1 portion of the RUNX1-β-gal fusion protein is required for this interaction. These results indicate that RUNX1-β-gal is capable of binding wild-type CBFβ and CBFβ-SMMHC, just as has been shown for wild-type RUNX1.

To understand how RUNX1-β-gal 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-β-gal was non-functional but did not display significant dominant-negative activity (Supplementary Figure 6). We then examined the relative expression levels of RUNX1 and RUNX1-β-gal in the bone marrow of adult *Runx1*$^{+/+}$ and *Runx1*$^{+/lz}$ mice. In *Runx1*$^{+/lz}$ mice, we found that the intensity of the RUNX1-β-gal band was 2.22-fold (s.d. = 0.61, $N=4$) greater than the wild-type RUNX1 band (Figure 5b). The overabundance of the RUNX1-β-gal protein provides a potential explanation for its semi-dominant-negative behavior: the non-functional RUNX1-β-gal protein may be able to outcompete wild-type 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

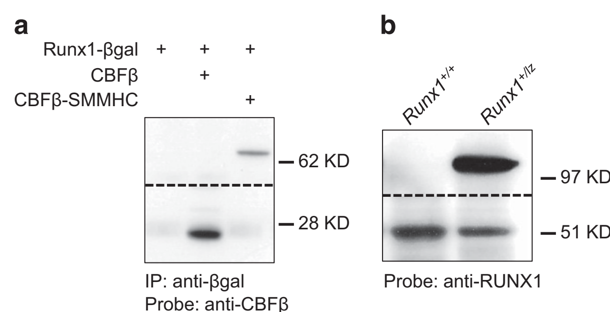

Figure 5. RUNX1-β-gal binds CBFβ and CBFβ-SMMHC and is present at higher levels than RUNX1 in the bone marrow. (**a**) Co-immunoprecipitation with anti-β-gal and western blot with anti-CBFβ141[16] of lysate from HEK293 cells transfected with plasmids expressing the indicated proteins. Data shown are 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 are from two different regions of the same blot. The dashed line indicates the transition between the two sections.

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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, as 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 do not 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.*[31] showed that the majority of genes bound by CBFβ-SMMHC are positively regulated by the fusion protein. 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-β-gal 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-β-gal: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 preleukemic 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 wild-type *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, as 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 wild-type 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 prosurvival factors in leukemia cells.[32,33] It is possible that the CBFβ:RUNX1 dimer has a similar role in inv(16) leukemia. In fact, Goyama *et al.*[33] 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 prosurvival target genes.[20] Ben-Ami *et al.*[32] also demonstrated addiction to normal RUNX1 in leukemia cells with the other common CBF fusion protein, RUNX1-RUNX1T1 (also known as AML1-ETO). In fact, RUNX1 mutations are rare in CBF leukemia patients, whereas 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-β-gal protein bound both CBFβ-SMMHC and wild-type CBFβ, so RUNX1-β-gal 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.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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