

## MYELOID NEOPLASIA

# RUNX1 and CBF $\beta$ -SMMHC transactivate target genes together in abnormal myeloid progenitors for leukemia development

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## KEY POINTS

- *Runx1* is required for leukemia-initiating cells in *Cbfb-MYH11* mice.
- RUNX1 recruits CBF $\beta$ -SMMHC to target genes.

**Inversion of chromosome 16 is a consistent finding in patients with acute myeloid leukemia subtype M4 with eosinophilia, which generates a *CBFB-MYH11* fusion gene. It is generally considered that CBF $\beta$ -SMMHC, the fusion protein encoded by *CBFB-MYH11*, is a dominant negative repressor of RUNX1. However, recent findings challenge the RUNX1-repression model for CBF $\beta$ -SMMHC-mediated leukemogenesis. To definitively address the role of *Runx1* in *CBFB-MYH11*-induced leukemia, we crossed conditional *Runx1* knockout mice (*Runx1<sup>fl/fl</sup>*) with conditional *Cbfb-MYH11* knockin mice (*Cbfb<sup>+ /56M</sup>*). On *Mx1-Cre* activation in hematopoietic cells induced by poly (I:C) injection, all *Mx1-CreCbfb<sup>+ /56M</sup>* mice developed leukemia in 5 months, whereas no leukemia developed in *Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+ /56M</sup>* mice, and this effect was cell autonomous. Importantly, the abnormal myeloid progenitors (AMPs), a leukemia-initiating cell population induced by *Cbfb-MYH11* in the bone marrow, decreased and disappeared in *Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+ /56M</sup>* mice. RNA-seq analysis of AMP cells showed that genes associated with proliferation, differentiation blockage, and leukemia initiation were differentially expressed between *Mx1-CreCbfb<sup>+ /56M</sup>* and *Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+ /56M</sup>* mice. In addition, with the chromatin immunocleavage sequencing assay, we observed a significant enrichment of RUNX1/CBF $\beta$ -SMMHC target genes in *Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+ /56M</sup>* cells, especially among downregulated genes, suggesting that RUNX1 and CBF $\beta$ -SMMHC mainly function together as activators of gene expression through direct target gene binding. These data indicate that *Runx1* is indispensable for *Cbfb-MYH11*-induced leukemogenesis by working together with CBF $\beta$ -SMMHC to regulate critical genes associated with the generation of a functional AMP population. (*Blood*. 2020;136(21):2373-2385)**

## Introduction

Inversion of chromosome 16, *inv(16)*, is associated with acute myeloid leukemia (AML) subtype M4 with eosinophilia, which generates the *CBFB-MYH11* fusion gene.<sup>1</sup> CBF $\beta$ -SMMHC, encoded by *CBFB-MYH11*, comprises most of core-binding factor  $\beta$  (CBF $\beta$ , 1-165 amino acids of a total of 187 amino acids) and the C-terminal coiled-coil domain of the smooth muscle myosin heavy chain (SMMHC).<sup>2,3</sup> Studies have shown that CBF $\beta$ -SMMHC is necessary, but not sufficient for leukemogenesis.<sup>4,5</sup>

CBF $\beta$ -SMMHC is thought to initiate leukemogenesis by blocking normal hematopoietic differentiation through inhibiting the function of RUNX1, which is 1 of the 3  $\alpha$ -subunits in the CBF family (RUNX1, RUNX2, and RUNX3), all of which have a RUNT domain that mediates DNA binding and heterodimerization with CBF $\beta$ .<sup>6</sup> Binding with CBF $\beta$  leads to the stabilization of the

RUNX-DNA interaction and gene expression regulation through the transactivation domain of RUNX proteins.<sup>7</sup> CBF $\beta$ -SMMHC retains the RUNX-binding domain from CBF $\beta$  and also contains a second RUNX high-affinity binding domain in the SMMHC tail, resulting in a higher binding affinity for RUNX1 than wild-type CBF $\beta$ .<sup>8</sup> In vitro studies have shown that CBF $\beta$ -SMMHC may serve as a transcriptional repressor by sequestering RUNX1 in the cytoplasm.<sup>9-11</sup> In vivo, *Cbfb-MYH11* heterozygous knockin mice (*Cbfb<sup>+ /MYH11</sup>*) have a complete block in definitive hematopoiesis and central nervous system hemorrhaging at embryonic day 12.5 (E12.5), which contributes to lethality by E13.5.<sup>12</sup> This phenotype is similar to that of *Runx1* null (*Runx1<sup>-/-</sup>*) and *Cbfb* null (*Cbfb<sup>-/-</sup>*) mice,<sup>13-17</sup> suggesting that CBF $\beta$ -SMMHC acts as a dominant repressor of RUNX1 and CBF $\beta$  functions during embryogenesis. However, subsequent studies challenge the RUNX1 repression model for CBF $\beta$ -SMMHC-mediated

leukemogenesis, including (1) loss-of-function mutations in *RUNX1* are common in human AML, but they have not been found in *inv(16)* AML<sup>18-21</sup>; (2) *Cbfb*<sup>+/MYH11</sup> embryos have differentiation defects and abnormal gene expression during primitive hematopoiesis that are not seen in *Runx1*<sup>-/-</sup> or *Cbfb*<sup>-/-</sup> embryos<sup>22</sup>; (3) there are both upregulated and downregulated genes in CBFβ-SMMHC-expressing cells<sup>23</sup>; (4) many genes are uniquely deregulated in *Cbfb*<sup>+/MYH11</sup> embryos but not in *Runx1*<sup>-/-</sup> or *Cbfb*<sup>-/-</sup> embryos and are expressed in leukemic cells from mice and humans<sup>22</sup>; (5) knock-in mice expressing a high-affinity binding domain-deleted CBFβ-SMMHC developed leukemia faster than mice expressing full-length CBFβ-SMMHC, although hematopoietic defects associated with *Runx1* inhibition were partially rescued<sup>24</sup>; and (6) in the *Cbfb*-*MYH11* expressing ME-1 cells, 89% of CBFβ-SMMHC binding regions colocalize with *RUNX1* binding sites.<sup>25</sup>

More importantly, our previous work demonstrated that *RUNX1* activity is required for *Cbfb*-*MYH11*-induced differentiation defects during both primitive and definitive hematopoiesis, and insufficient *Runx1* activity (*Runx1*<sup>+/-</sup>) delays *Cbfb*-*MYH11*-induced leukemia development in mice.<sup>26</sup> Together, these results suggested that CBFβ-SMMHC does not simply repress *RUNX1*. Instead, current data imply that *Runx1* is required for *CBFB*-*MYH11* leukemogenesis.

However, this previous work was inconclusive because the *Runx1*<sup>+/-</sup> mice used in the previous study contained an artificial *Runx1*-Iz allele and 1 wild-type *Runx1* allele, which still retains some *Runx1* function.<sup>26</sup>

To definitely address the functional importance of *Runx1* in *Cbfb*-*MYH11*-induced leukemia, we used a Cre-based conditional *Runx1* knockout mice (*Runx1*<sup>fl/fl</sup>)<sup>27</sup> and generated *Cbfb*-*MYH11* knockin mice with homozygous *Runx1* deletion. We found that the resulting *Runx1*<sup>-/-</sup> *Cbfb*<sup>+/MYH11</sup> mice could not develop leukemia, and the likely reason was dysregulation of critical genes in the abnormal myeloid progenitor (AMP) population, including those associated with proliferation, differentiation blockage, and maintenance of leukemia-initiating ability of such cells. In addition, our results suggested that, besides acting as a dominant negative repressor of *RUNX1*, CBFβ-SMMHC also acts as a coactivator of *RUNX1* target genes. These results indicate that *Runx1* is indispensable for leukemia development induced by *Cbfb*-*MYH11*, and the *RUNX1*-CBFβ-SMMHC interaction could be a good target for developing novel treatments of *inv(16)* leukemia.

## Methods

### Animals

All animals used in this study were approved by the National Human Genome Research Institute Animal Care and Use Committee, and all the procedures performed followed relevant National Institutes of Health guidelines and regulations. *Cbfb*-*MYH11* conditional knockin (*Cbfb*<sup>+/56M</sup>)<sup>28</sup> *Mx1*-Cre mice,<sup>29</sup> and *Runx1* conditional knockout (*Runx1*<sup>fl/fl</sup>) mice<sup>27</sup> have been described previously. All mice were genotyped by polymerase chain reaction (PCR) with gene-specific primers (supplemental Table 1 available on the *Blood* Web site) using tail-snip DNA prepared with DNeasy Blood & Tissue Kit (Qiagen). Eight- to 12-

week-old mice and their littermate controls were injected intraperitoneally with 250 μg poly (I:C) (plpC; InvivoGen) to induce the expression of *Cbfb*-*MYH11* and/or knockout of *Runx1* every other day for 3 doses. For the noncompetitive transplantation assay, C57BL/6 (CD45.1) × 129/SvEv F1 mice were irradiated (550 rads × 2 times) and then injected through tail vein with 1 million bone marrow cells from indicated donor mice. The recipient mice were then treated with plpC as above 9 weeks after transplantation to induce the expression of *Cbfb*-*MYH11* and deficiency of *Runx1*. All mice were observed for leukemia development for 12 months after plpC injections.

### Plasmids

*RUNX1* cDNA sequence was cloned into pmCherry-C1 and pcDNA3.1(+) vector (Addgene), respectively. *CBFB* and *CBFB*-*MYH11* cDNA sequences were cloned into the PGEM vector (Promega) and pEGFP-C1 vector (Addgene), respectively. Truncated *RUNX1*-related plasmids were created on related *RUNX1* plasmids using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent).

### Flow cytometry

Peripheral blood cells and spleen and bone marrow cells from mice were harvested and stained as described previously.<sup>23</sup> See the supplemental Data (available on the *Blood* Web site) for detail for the antibodies used in this study.

### Western blot analysis

Western blot analysis was performed with standard protocols. More details are described in the supplemental Methods.

### Immunofluorescence

Immunofluorescence staining of transfected 293T cells and bone marrow cells from mice was performed with standard protocols. More details are described in the supplemental Methods.

### CSF1R promoter reporter assay

The *CSF1R* promoter reporter assay was performed as described previously.<sup>30</sup>

### Quantitative PCR

Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) according to manufacturer's instruction. *Runx1* unexcised primers were used to detect unexcised *Runx1* flox allele, and exon 5/6 primers were used to detect unexcised *Cbfb*-*MYH11* flox allele. Genomic control primers were used as internal controls for genomic DNA. Primers are listed in supplemental Table 1.

### RNA sequencing, ChIC sequencing, and single-cell RNA sequencing

Mice were injected with plpC, and 2 to 3 weeks later, the AMP population in the bone marrow cells of these mice were sorted out with a BD FACSAria IIIu cell sorter (BD Biosciences). mRNA from the AMP cells was extracted and subjected to RNA sequencing (RNA-Seq). Paraformaldehyde (1%)-fixed AMP cells were subjected to chromatin immunocleavage sequencing (ChIC-seq) as described<sup>31</sup>; the 10X genomics chromatin platform<sup>32</sup> was used to capture isolated AMP cells, and all steps of single-cell RNA sequencing were performed according to manufacturer's instructions. Detailed procedures and data analysis are provided in supplemental Methods.

## Statistical analysis

Data were analyzed using Graphpad Prism. Results are expressed as mean  $\pm$  standard error of the mean (SEM). Differences between the 2 groups were tested with the Student t test. The differences in survival times of mice were analyzed with the Kaplan-Meier method and log-rank test.  $P < .05$  was considered statistically significant.

## Results

### Truncated RUNX1 protein in the *Runx1<sup>fl/fl</sup>* mice was functionally defective at the molecular and cellular levels

To determine whether RUNX1 is required during *Cbfb*-*MYH11* leukemogenesis, we crossed a Cre-based conditional *Runx1* knockout mouse strain (*Runx1<sup>fl/fl</sup>*)<sup>27</sup> with Mx1-Cre-based conditional *Cbfb*-*MYH11* knockin mice (*Mx1-CreCbfb<sup>+/-56M</sup>*)<sup>28</sup> to generate *Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+/-56M</sup>* mice. The conditional *Runx1* knockout allele deletes *Runx1* exon 4 (amino acids 158-192 of mouse RUNX1, equivalent to amino acids 171-205 of human RUNX1; supplemental Figure 1) after Cre-mediated recombination; however, a truncated RUNX1 protein could be detected in the bone marrow cells (supplemental Figure 2). Although it had been shown that a C-terminal truncation of human RUNX1 to amino acid 170 was sufficient to disrupt DNA and CBF $\beta$  binding in vitro,<sup>33</sup> whether this internally truncated RUNX1 protein was functional remained to be determined.

As the nuclear localization sequence in RUNX1 was partially disrupted by the exon 4 deletion (supplemental Figure 1A),<sup>34</sup> the localization of the truncated RUNX1 was examined. Indeed, unlike RUNX1 in wild-type and *Mx1-CreCbfb<sup>+/-56M</sup>* mice, which was mainly present in the nuclear fraction, the truncated RUNX1 was found in both cytoplasmic and nuclear fractions in plpC-treated *Runx1<sup>fl/fl</sup>Mx1-Cre* and *Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+/-56M</sup>* bone marrow cells (Figure 1A; supplemental Figure 2). Similarly, in transfected 293T cells the truncated RUNX1 was located in both cytoplasm and nuclei (Figure 1B).

We then determined whether the truncated RUNX1 interacted with CBF $\beta$  and CBF $\beta$ -SMMHC. As expected, when full-length RUNX1 was cotransfected with CBF $\beta$ , the localization of CBF $\beta$  was changed compared with cells transfected with CBF $\beta$  alone, from both cytoplasm and nuclear localization to mainly nuclear, suggesting interaction between RUNX1 and CBF $\beta$  (Figure 1C). In contrast, when the truncated RUNX1 was cotransfected with CBF $\beta$ , the localization of CBF $\beta$  did not change compared with cells transfected with CBF $\beta$  alone, suggesting the truncated RUNX1 could not interact with CBF $\beta$  (Figure 1C). Similar subcellular localization changes were observed when CBF $\beta$ -SMMHC was cotransfected with truncated RUNX1 (Figure 1D). The lack of interaction between the truncated RUNX1 and CBF $\beta$ /CBF $\beta$ -SMMHC was also confirmed with endogenous proteins. As shown in Figure 1A, in *Mx1-Cre, Cbfb<sup>+/-56M</sup>* bone marrow cells, clear colocalization signals were observed between RUNX1 and CBF $\beta$ /CBF $\beta$ -SMMHC in the nuclei, whereas in *Runx1<sup>fl/fl</sup>Mx1-Cre* and *Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+/-56M</sup>* bone marrow cells, the truncated RUNX1 was located in both cytoplasm and nuclei, and much less colocalization signal was observed between the truncated RUNX1 and CBF $\beta$ /CBF $\beta$ -SMMHC. All together, these results suggested that the truncated RUNX1 lost the ability to interact with CBF $\beta$  and CBF $\beta$ -SMMHC.

We also tested the ability of the truncated RUNX1 to transactivate target genes in the presence of CBF $\beta$  or CBF $\beta$ -SMMHC in a reporter assay, in which the expression of luciferase was driven by the promoter of *CSF1R*.<sup>35</sup> As can be seen in Figure 1E-F, the truncated RUNX1 was not able to transactivate *CSF1R* luciferase activity as RUNX1 did, whether in the absence or presence of the CBF $\beta$  proteins. Taken together, these results confirm that the truncated RUNX1 is a loss of function mutant.

### *Runx1* was required for *Cbfb*-*MYH11*-induced leukemia

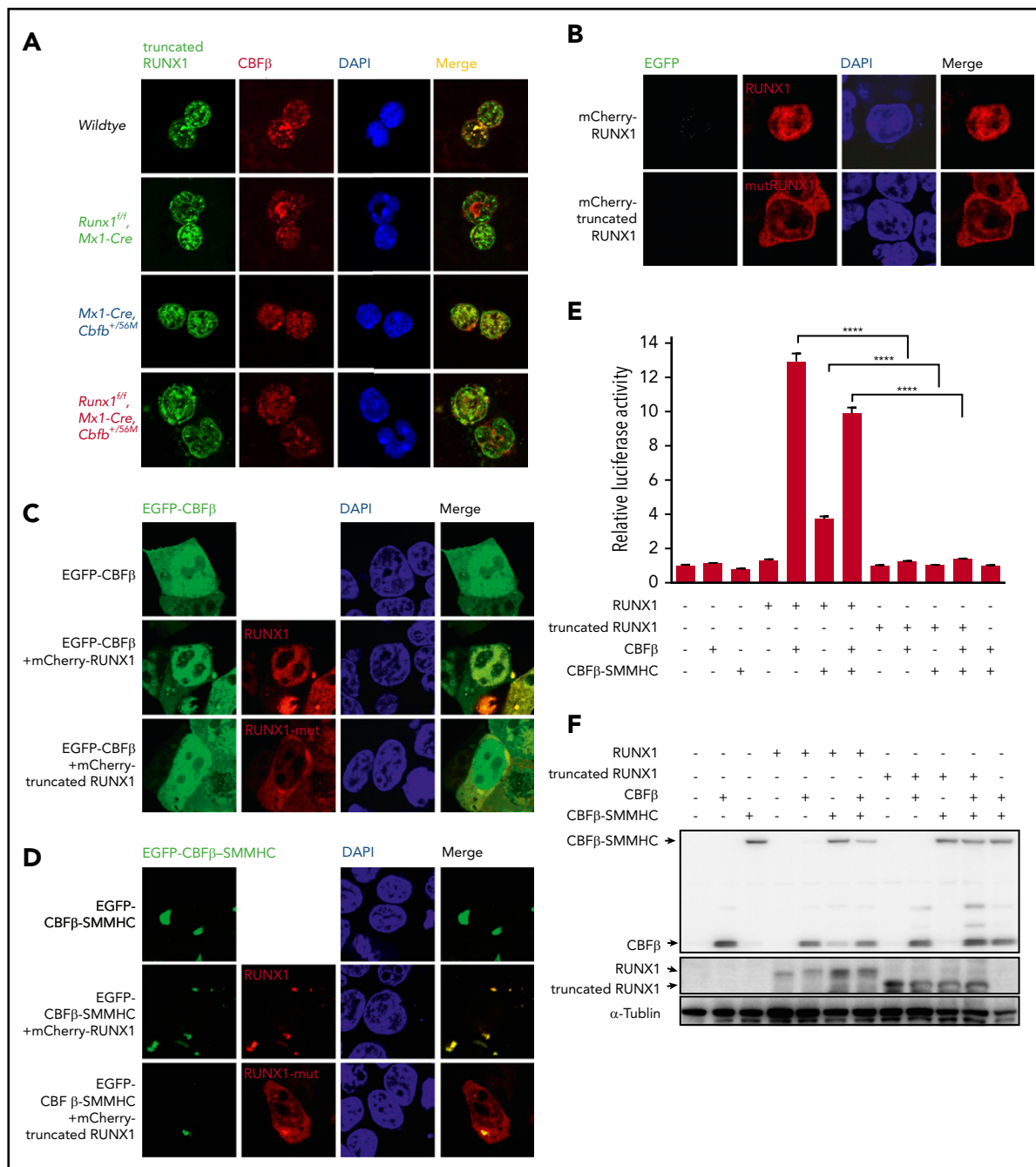
To explore the effect of *Runx1* knockout on *Cbfb*-*MYH11*-induced leukemia, we treated adult (8-12 weeks old) *Mx1-CreCbfb<sup>+/-56M</sup>* ( $n = 9$ ), *Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+/-56M</sup>* ( $n = 11$ ), and *Runx1<sup>fl/fl</sup>Mx1-Cre* ( $n = 6$ ) mice, as well as their littermate control mice (wild type; *Mx1-Cre* or other genotypes without *Mx1-Cre*,  $n = 7$ ) with plpC to induce *Mx1-Cre* expression. The excisions of *Runx1* and *Cbfb*-*MYH11* flox alleles in bone marrow cells of these mice 4 months after plpC treatment were nearly complete, as determined by quantitative PCR (supplemental Figure 3A).

As expected, *Mx1-CreCbfb<sup>+/-56M</sup>* mice succumbed to AML with a median survival of 114 days (Figure 2A-D; supplemental Figure 4). Of the 11 *Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+/-56M</sup>* mice, 3 of them died around 2 weeks after plpC treatment. The exact cause of death was not clear but all 3 developed pancytopenia, especially severe anemia and thrombocytopenia (supplemental Figure 5A), with no signs of leukemia (supplemental Figure 5B-C). In addition, transplantation of spleen cells from these mice did not lead to leukemia development in the recipient mice (supplemental Figure 5D).

The remaining 8 *Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+/-56M</sup>* mice were free of hematopoietic malignancy 12 months after plpC injection, with nearly complete excisions of *Runx1* and *Cbfb*-*MYH11* flox alleles in bone marrow cells at 12 months after plpC injection (supplemental Figure 3B). This phenotype was different from that observed in *Runx1<sup>+/-56M</sup>Mx1-CreCbfb<sup>+/-56M</sup>* mice, ~80% of which developed leukemia, albeit with longer latency than *Mx1-CreCbfb<sup>+/-56M</sup>* mice.<sup>26</sup> As expected, no leukemia was observed in the *Runx1<sup>fl/fl</sup>Mx1-Cre* mice or the control mice (Figure 2A). The *Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+/-56M</sup>* mice did develop nonlethal thrombocytopenia (supplemental Figure 4A) and mild myeloproliferative changes (Figure 2B,D; supplemental Figure 4B-C), which were likely caused by *Runx1* knockout,<sup>27,36</sup> as similar phenotypes were also observed in *Runx1<sup>fl/fl</sup>Mx1-Cre* mice (Figure 2B,D; supplemental Figure 4). These results suggest that *Runx1* is required for *Cbfb*-*MYH11*-induced leukemogenesis.

### The requirement of *Runx1* for *Cbfb*-*MYH11* leukemogenesis was cell autonomous

To determine whether *Runx1* is required in the hematopoietic cells or in the surrounding microenvironment for *Cbfb*-*MYH11* leukemogenesis, we transplanted bone marrow cells from *Mx1-CreCbfb<sup>+/-56M</sup>*, *Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+/-56M</sup>*, and *Runx1<sup>fl/fl</sup>Mx1-Cre* mice into lethally irradiated recipient mice, which were injected with plpC 9 weeks later to induce the expression of *Cbfb*-*MYH11* and knockout of *Runx1*. Efficient engraftment of donor cells was confirmed in all recipient mice before plpC treatment (supplemental Figure 6A). Mice transplanted with cells from *Mx1-CreCbfb<sup>+/-56M</sup>* mice died of leukemia with a median survival of



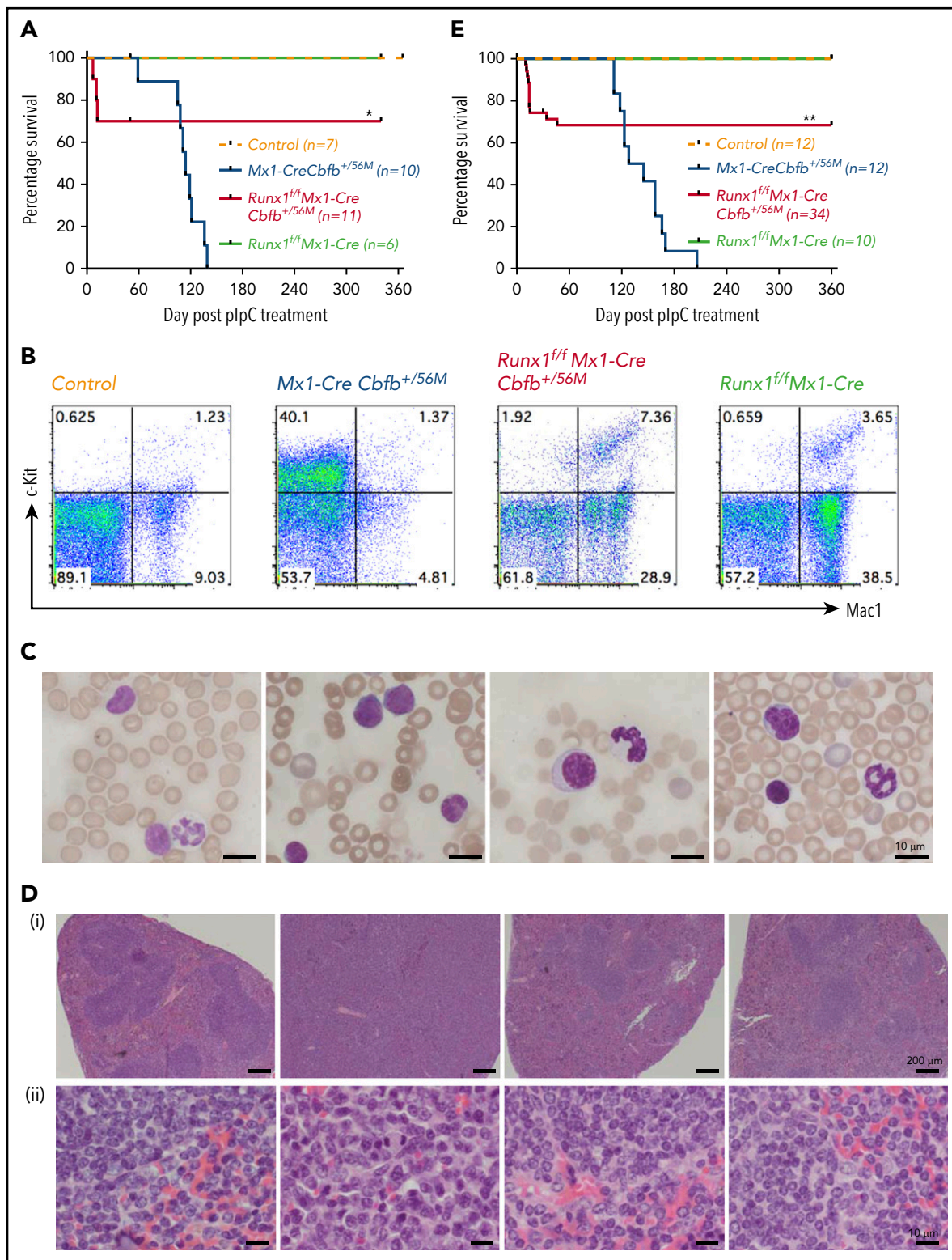
**Figure 1. The mutated RUNX1 protein in *Runx1<sup>fl/fl</sup>* mice was nonfunctional.** (A) Immunofluorescent staining of bone marrow cells from the indicated mice 2 weeks after plpC treatment to detect the colocalization between RUNX1 or the mutated RUNX1 and CBFβ/CBFβ-SMMHC. (B-D) 293T cells were transfected with the indicated plasmids, and immunofluorescence staining was performed to detect the localization of the indicated proteins. The labels on the left side of the pictures indicate the plasmids transfected into the cells and the labels on top of the pictures indicate the observed proteins at the appropriate microscope filter settings. (E-F) Luciferase reporter assay in 293T cells transfected with a *CSF1R* promoter-driven luciferase reporter plasmid and plasmids encoding the indicated proteins. (E) Relative activities (mean ± SEM) were determined based on 3 independent experiments. \*\*\*\**P* < .0001. (F) Representative expression levels of the transfected proteins for this reporter assay by western blot analysis.

136.5 days (Figure 2E; supplemental Figure S6B-C). In contrast, 30% of mice transplanted with cells from *Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+/56M</sup>* mice died shortly after plpC treatment (Figure 2E) with pancytopenia, including severe anemia and thrombocytopenia (supplemental Figure 6B-C), whereas the remaining 70% of the mice transplanted with *Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+/56M</sup>* cells were free of leukemia up to 1 year after plpC injection (Figure 2E), with nearly complete excisions of *Runx1* and *Cbfb-MYH11* flox alleles in bone marrow cells at 12 months after plpC injection (supplemental Figure 6D). These

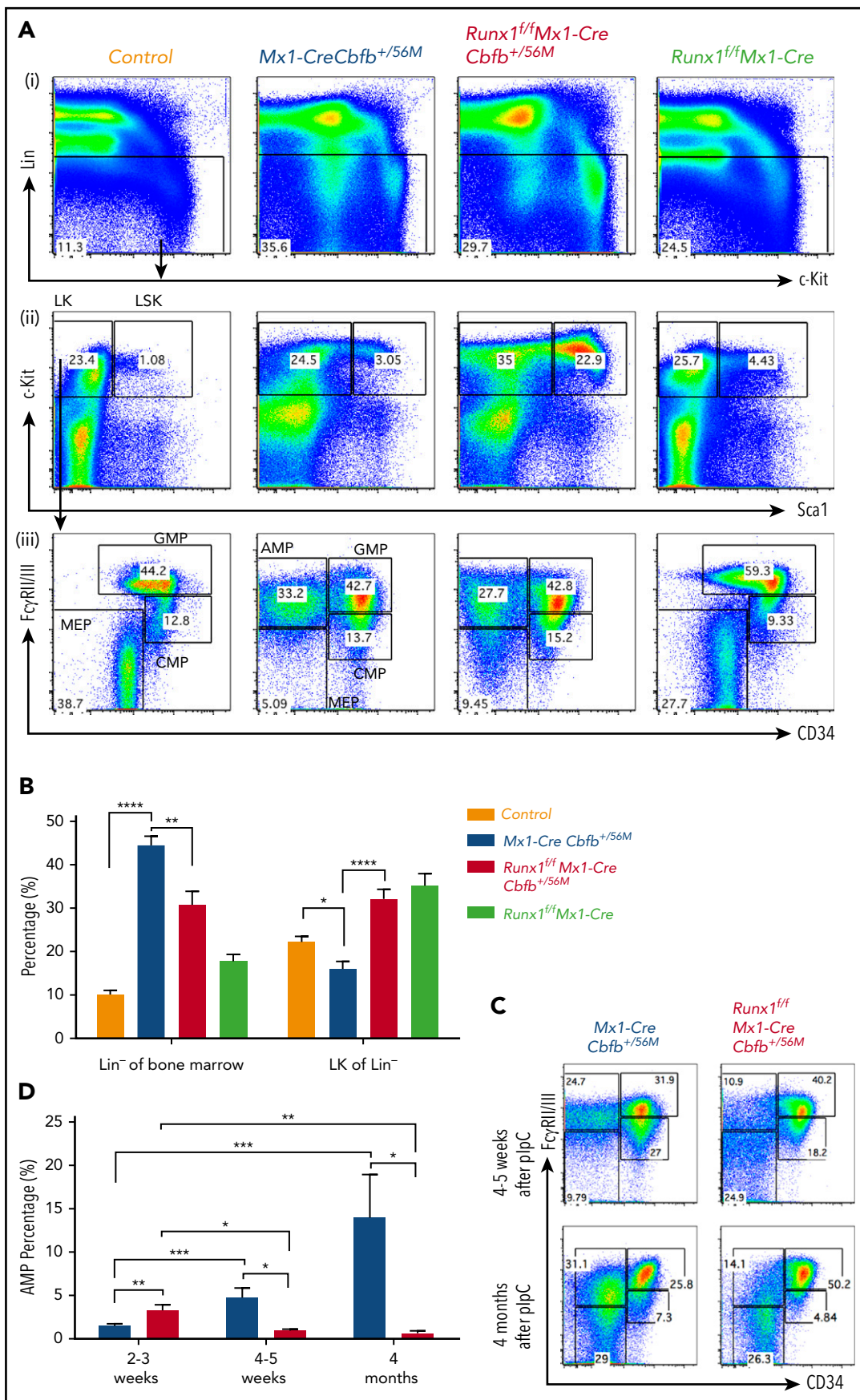
results suggest that the requirement of *Runx1* for *Cbfb-MYH11* leukemogenesis is cell autonomous.

### AMP cells could not initiate leukemia in the *Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+/56M</sup>* mice

We then explored the potential mechanism of the *Runx1* requirement for *Cbfb-MYH11*-induced leukemia development. As we showed previously, *Cbfb-MYH11* expression in adult mice leads to aberrant hematopoiesis before leukemic transformation



**Figure 2. Runx1 is required for Cbfb-MYH11-induced leukemia.** (A) Mice of the indicated genotype were treated with plpC to induce the expression of Cbfb-MYH11 and/or Runx1 deficiency, and leukemia development in these mice was monitored for 1 year. Kaplan-Meier survival curves of these mice are shown. (B-D) Mice of the indicated genotypes were treated with plpC and then killed 4 months after the last dose of plpC for analysis. (B) Representative fluorescence-activated cell sorting (FACS) plots of c-Kit<sup>+</sup> and Mac1<sup>+</sup> cells in the peripheral blood of these mice. (C) Representative Wright-Giemsa-stained peripheral blood smears from these mice. (D) Representative hematoxylin and eosin-stained spleen sections (i, 50 $\times$ ; ii, 400 $\times$ ) from these mice. (E) Kaplan-Meier survival curves of recipient mice (3-5 per donor mouse) after noncompetitive transplantation assay. \* $P < .05$  and \*\* $P < .01$ , each comparing the Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+/56M</sup> group with the Mx1-CreCbfb<sup>+/56M</sup> group.



**Figure 3.**

and these abnormalities include increased lineage negative (Lin<sup>-</sup>) cells and decreased myeloid progenitor cells (LK, Lin<sup>-</sup>/c-Kit<sup>+</sup>/Sca1<sup>-</sup>) in the bone marrow.<sup>22,28</sup> These changes were partially rescued by *Runx1* knockout (Figure 3A-B). Interestingly, we found that the AMP population (LK/CD34<sup>-</sup>/FcyRIII/III<sup>+</sup>), which was absent in control and *Runx1<sup>fl/fl</sup>Mx1-Cre* mice (Figure 3A; supplemental Figure 7A), was larger in the *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* mice compared with that in *Mx1-CreCfbf<sup>+56M</sup>* mice shortly after plpC treatment (2-3 weeks). However, the AMP population decreased 4 to 5 weeks after plpC treatment and disappeared after 4 months (Figure 3C-D). On the other hand, the AMP population, but not common myeloid progenitors (CMP, LK/CD34<sup>+</sup>/FcyRIII/III<sup>+</sup>), granulocyte-macrophage progenitor (GMP, LK/CD34<sup>+</sup>/FcyRIII/III<sup>high</sup>), and megakaryocyte-erythroid progenitor (MEP, LK/CD34<sup>-</sup>/FcyRIII/III<sup>-</sup>; Figure S7B), grew larger with time in the *Mx1-CreCfbf<sup>+56M</sup>* mice, and eventually led to leukemia (Figure 3C-D). These results suggested that *Runx1* was required for the generation and maintenance of a functional AMP population and the decrease/disappearance of this AMP population was likely responsible for the failure of leukemia development in the *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* mice.

### Gene expression changes in the AMP population in *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* mice

To understand how the AMP population disappeared in *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* mice, we performed RNA-seq with AMP cells isolated from *Mx1-CreCfbf<sup>+56M</sup>* and *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* mice (2-3 weeks after plpC treatment) to profile gene expression changes between them. Principal component analysis showed a clear separation of the AMP cells in *Mx1-CreCfbf<sup>+56M</sup>* mice from those in the *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* mice (Figure 4A). Compared with *Mx1-CreCfbf<sup>+56M</sup>* mice, 858 genes were identified as differentially expressed genes (DEGs; *q* value < 0.01, absolute fold change ≥ 2) in *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* mice, with 69.7% upregulated genes and 30.3% downregulated genes (Figure 4B; supplemental Table 2).

Gene set enrichment analysis (GSEA)<sup>37</sup> was performed with the entire RNA-seq data of the AMP cells isolated from *Mx1-CreCfbf<sup>+56M</sup>* and *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* mice. As shown in supplemental Table 3, only 6 gene sets were enriched (with FDR < 0.25) in the AMP cells in the *Mx1-CreCfbf<sup>+56M</sup>* mice. Interestingly, genes involved in regulating cell cycle were significantly enriched in these gene sets, including G2M checkpoint genes, MYC targets, and E2F targets. On the other hand, these cell cycle-related gene sets were not enriched in the AMP cells from the *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* mice. In fact, no gene sets were identified in the *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* AMP cells with FDR < 0.25. These findings may suggest that RUNX1 is a major driver of cell cycle in the AMP cells.

We also performed GSEA analysis of the DEGs between AMP cells isolated from *Mx1-CreCfbf<sup>+56M</sup>* and *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* mice. The data showed that several curated gene sets were

significantly enriched. As shown in Figure 4C-D and supplemental Table 4, the upregulated DEGs in *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* cells were negatively correlated with the FISCHER\_G2\_M\_CELL\_CYCLE gene set (cell cycle genes with peak expression in G2/M check point, such as CDK1) and positively correlated with the BROWN\_MYELOID\_CELL\_DEVELOPMENT\_UP gene set (genes with potential to regulate myeloid cell growth and differentiation), suggesting that the disappearance of the AMP population in *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* mice was at least in part through inhibition of proliferation and promotion of differentiation. Moreover, the upregulated DEGs in *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* cells were positively correlated with the GAL\_LEUKEMIC\_STEM\_CELL\_DN gene set (Figure 4E; supplemental Table 4), which contained DEGs downregulated in leukemic stem cells, suggesting that the AMP population in *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* mice lost the leukemia initiating ability. In addition, gene ontology analysis showed that downregulated DEGs were associated with terms such as cell differentiation and mitotic cell cycle (supplemental Figure 8A), whereas the upregulated DEGs were associated with immune response (supplemental Figure 8B), consistent with the finding that RUNX1 can negatively regulate inflammatory cytokine production.<sup>38</sup>

IPA upstream analysis showed that GATA2 was the most significant putative upstream transcription factor that regulated the DEGs in the AMP cells of the *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* mice (supplemental Figure 8C), suggesting that RUNX1 functions in part through other key hematopoietic transcription factors, such as GATA2, consistent with our previous observation that *Gata2* deficiency delays leukemogenesis by *Cfbf-MYH11*.<sup>39</sup>

### RUNX1 and CBFβ-SMMHC mainly activated their target genes in AMPs

To assess the role of *Runx1* for *Cfbf-MYH11*-induced gene expression changes, we performed ChIC-seq assays<sup>31</sup> on the AMP populations isolated from *Mx1-CreCfbf<sup>+56M</sup>* and *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* mice with RUNX1, CBFβ, SMMHC, H3K27ac, and H3K27me3 antibodies and identified their binding regions using the cLoops clustering algorithm.<sup>40</sup> A significantly lower number of binding sites or genes assigned to the binding sites of RUNX1 and SMMHC was observed in the *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* samples than in the *Mx1-CreCfbf<sup>+56M</sup>* samples (supplemental Figure 9A; supplemental Table 5). In contrast, the numbers of binding sites for CBFβ, H3K27ac, or H3K27me3 were not significantly changed between the 2 genotypes (supplemental Figure 9A). These findings suggested that RUNX1 was required for CBFβ-SMMHC, but not CBFβ, to interact with DNA targets.

Although the number of RUNX1 peaks in *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* samples was reduced by only 67% compared with that identified in *Mx1-CreCfbf<sup>+56M</sup>* samples, most of the detected RUNX1 peaks (4279 of the total 5844 peaks) were observed in both sets of samples and the intensity of these peaks was much lower in the *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>* samples than in

**Figure 3. *Runx1* is required for the maintenance of the AMP population.** (A-D) The indicated groups of mice were treated with plpC to induce the expression of *Cfbf-MYH11* and/or *Runx1* deficiency. At certain time points after plpC treatment, the mice were killed, and flow cytometry assays were performed. (A) Representative FACS plots of bone marrow cells from mice treated with plpC for 2 to 3 weeks gated on single cells (i), Lin<sup>-</sup> cells (ii), and LK cells (iii) are shown for the intensities of the indicated antibodies. (B) Bar graphs showing the percentages (mean ± SEM) of Lin<sup>-</sup> of bone marrow and LK fraction of Lin<sup>-</sup> compartments in mice of the indicated genotypes as showed in panel A. (C) Representative FACS plots of bone marrow cells gated on LK cells from mice treated with plpC for 4 to 5 weeks and 4 months. (D) Bar graph showing the percentages (mean ± SEM) of the AMP population in the bone marrow of mice of indicated genotypes treated with plpC for 2 to 3 weeks, 4 to 5 weeks, and 4 months as showed in panels A and C. \**P* < .05, \*\**P* < .01, \*\*\**P* < .001, \*\*\*\**P* < .0001. All genotypes in this figure are color-coded the same way (blue for *Mx1-CreCfbf<sup>+56M</sup>*, red for *Runx1<sup>fl/fl</sup>Mx1-CreCfbf<sup>+56M</sup>*, and green for *Runx1<sup>fl/fl</sup>Mx1-Cre*).

the *Mx1-CreCfbf*<sup>+/-56M</sup> samples (supplemental Figure 9B). The RUNX1 peaks unique to *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> samples were most likely identified because of the setting and high sensitivity of the peak-calling algorithm. As seen in supplemental Figure 9B, lower right panel, these peaks have much lower intensities than the other 2 types of peaks in supplemental Figure 9B, and they were not really unique to *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> samples because similar peaks were also identified in the *Mx1-CreCfbf*<sup>+/-56M</sup> samples. These observations suggest that these RUNX1 peaks in the *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> samples were background/nonspecific binding of the RUNX1 antibody.

Among the RUNX1 binding sites identified in the AMP cells from mice of these 2 genotypes, ~69.4% of them were only observed in *Mx1-CreCfbf*<sup>+/-56M</sup> cells (supplemental Figure 9B). Of these binding sites unique to *Mx1-CreCfbf*<sup>+/-56M</sup> cells, besides the RUNX (Runt) motif, GATA2 (GATA) motifs were also significantly enriched (supplemental Figure 9C), suggesting RUNX1 and GATA2 co-occupy many genomic regions to regulate the expression of nearby genes.<sup>25</sup> All together, these results suggest that RUNX1 functions in part through GATA2 to regulate *Cfbf*-*MYH11*-induced leukemia.

As most DEGs (56.1%) between *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> and *Mx1-CreCfbf*<sup>+/-56M</sup> mice, especially downregulated DEGs (72.7% vs 48.8% of upregulated DEG), were RUNX1 target genes (Figure 5A-B), we wondered how RUNX1 regulated the expression of these DEGs. We intersected the binding sites for RUNX1, CBFβ, and SMMHC with Intervene,<sup>41</sup> and we considered the CBFβ and SMMHC co-occupied binding sites as CBFβ-SMMHC binding sites (the overlapped region between blue and green in supplemental Figure 9D). Interestingly, almost all such CBFβ-SMMHC binding sites (95.7%) were colocalized with RUNX1 binding sites, reinforcing the importance of RUNX1 for CBFβ-SMMHC in leukemogenesis. The RUNX1 and CBFβ-SMMHC co-occupied binding sites were then considered as RUNX1/CBFβ-SMMHC binding sites and genes assigned to these binding sites as target genes of RUNX1/CBFβ-SMMHC. Both upregulated and downregulated DEGs in *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> mice contained RUNX1/CBFβ-SMMHC target genes (Figure 5A,C). Interestingly, a significantly higher percentage of DEGs downregulated in *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> mice were RUNX1/CBFβ-SMMHC target genes than upregulated DEGs (21.5% vs 14.7%; Figure 5A,C), suggesting RUNX1/CBFβ-SMMHC was more responsible for the downregulated DEGs in *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> mice. Correspondingly, RUNX1 binding enrichment at the transcription start sites (TSSs) was reduced for both downregulated and upregulated DEGs in *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> cells (Figure 5D-E). On the other hand, reduced CBFβ and SMMHC bindings were only observed at TSSs of downregulated but not upregulated DEGs in *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> cells (Figure 5D-E). In addition, reduced H3K27ac modification was only observed at TSSs of downregulated DEGs in the absence of *Runx1*, which likely correlated with decreased expression of these genes in *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> cells. Conversely, reduced H3K27me3 modification was observed at TSSs of upregulated DEGs in the absence of *Runx1*, which likely correlated with increased expression of these genes in *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> cells (Figure 5D-E). Taken together, our results suggest that instead of acting as a transcription repressor, the transcription activation role of RUNX1/CBFβ-SMMHC is more prevalent in *Cfbf*-*MYH11*-induced leukemia.

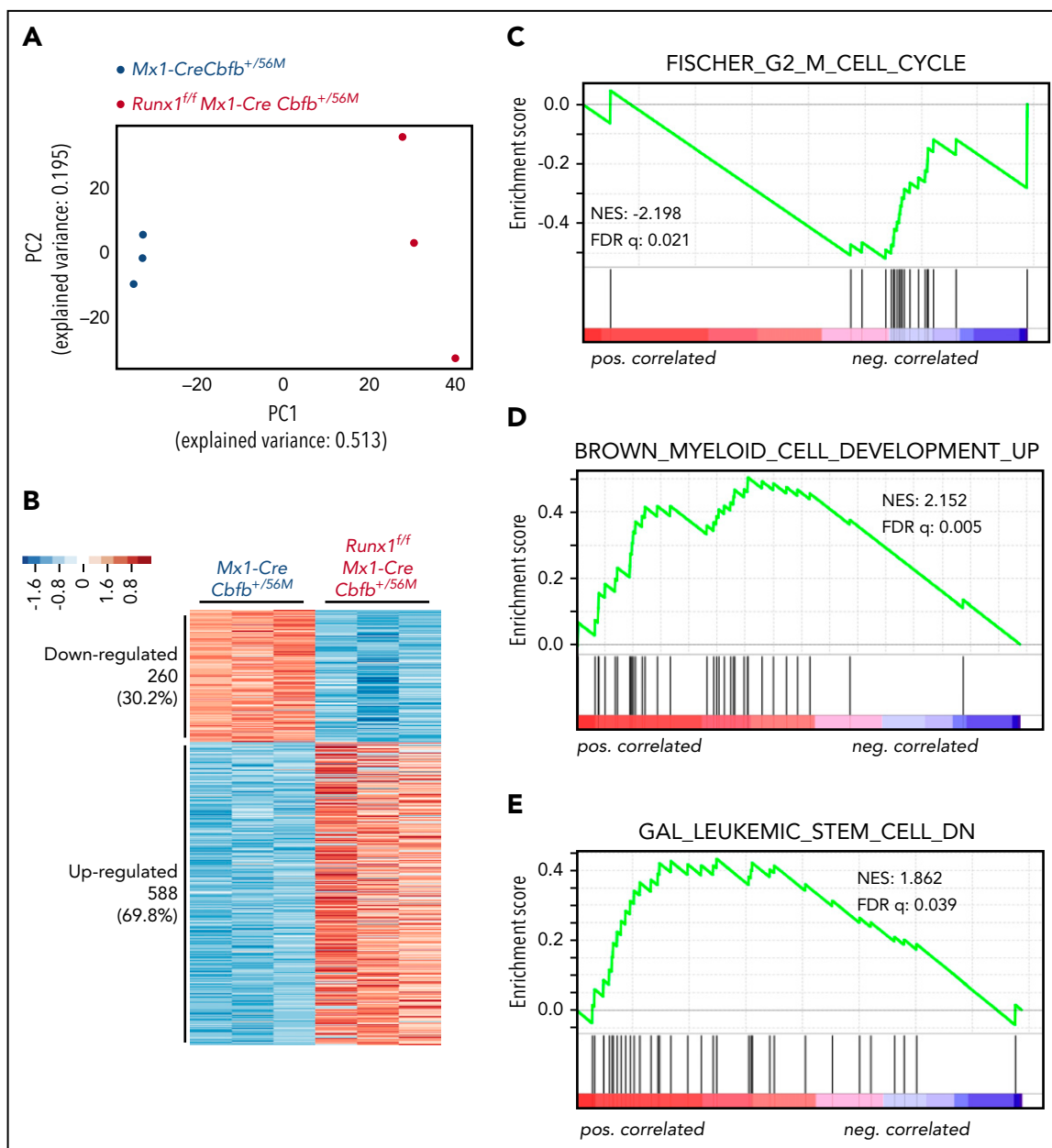
## Single-cell RNA-seq identified the AMP population in the *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> mice as homogenous and distinct from that in the *Mx1-CreCfbf*<sup>+/-56M</sup> mice

To explore the heterogeneity of the AMP population and determine which subpopulation was affected by *Runx1* knockout, we performed single-cell RNA-sequencing (scRNA-seq) with AMP cells from *Mx1-CreCfbf*<sup>+/-56M</sup> and *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> mice. In the first set of experiment, 14 092 cells were captured, including 6456 cells from a *Mx1-CreCfbf*<sup>+/-56M</sup> mouse and 7636 cells from a *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> mouse (supplemental Figure 10A). Clustering of pooled cells from both mice with uniform manifold approximation and projection<sup>42</sup> allowed the separation of the cells into 4 clusters (Figure 6A), with most of the cells belonged to clusters 2 (42.05%) and 3 (54.3%). Further analysis revealed that the two smaller clusters, cluster 0 (1.11%) and cluster 1 (2.53%), contained neutrophil cells (expressing *Elane* and *Mpo*) and B cells (expressing *Cd79A* and *Cd19*), respectively (supplemental Figure 10B), which were not considered in subsequent data analyses. We then determined the distribution of the cells from those 2 mice. Interestingly, cluster 2 was mainly composed of cells from the *Mx1-CreCfbf*<sup>+/-56M</sup> mouse and cluster 3 was mainly composed of cells from the *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> mouse (Figure 6B-C). These 2 clusters could not be subdivided further meaningfully, suggesting that the AMP cells were very homogenous in both mice. In the repeat experiment with another 2 mice, only 3 clusters were observed with 2 of them being the small contaminating B cells and neutrophils (Figure 6D; supplemental Figure 10B). Again, the main cluster (cluster 2 in Figure 6D) could be subdivided into 2 distinct, homogenous subclusters according to their genotypes, as observed in the first experiment (Figure 6E). Several genes were specifically expressed in *Mx1-CreCfbf*<sup>+/-56M</sup> or *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> cells in both experiments (Figure 6C,F; supplemental Figure 10C; supplemental Table 6). The differences observed between these 2 experiments were most likely because of different mice used that were harvested at different times after plpC injections. Taken together, these results suggest that the AMPs in the *Runx1*<sup>fl/fl</sup>*Mx1-CreCfbf*<sup>+/-56M</sup> mice are homogenous and distinct from those in the *Mx1-CreCfbf*<sup>+/-56M</sup> mice at the transcriptome level, further confirms that *Runx1* was required for the generation and maintenance of a functional AMP population.

## Discussion

Recent studies challenged the RUNX1 repression model and suggested a RUNX1 repression independent model for CBFβ-SMMHC-mediated leukemogenesis.<sup>18-22,24,25,30</sup> Especially interesting was our previous finding that a moderate reduction of *Runx1* activity (*Runx1*<sup>+/-lz</sup>) delayed *Cfbf*-*MYH11*-induced leukemia,<sup>26</sup> which suggested that not only CBFβ-SMMHC did not repress RUNX1, but it actually required RUNX1 for leukemogenesis. However, the mouse model used in our previous study was imperfect because the *Runx1*<sup>+/-lz</sup> mice retained 1 wild-type *Runx1* allele with normal RUNX1 function, in addition to an artificial *Runx1-lz* fusion allele.<sup>26</sup> In this study, we performed the experiments with an inducible homozygous *Runx1* knockout model, which should retain no functional RUNX1 (Figure 1).



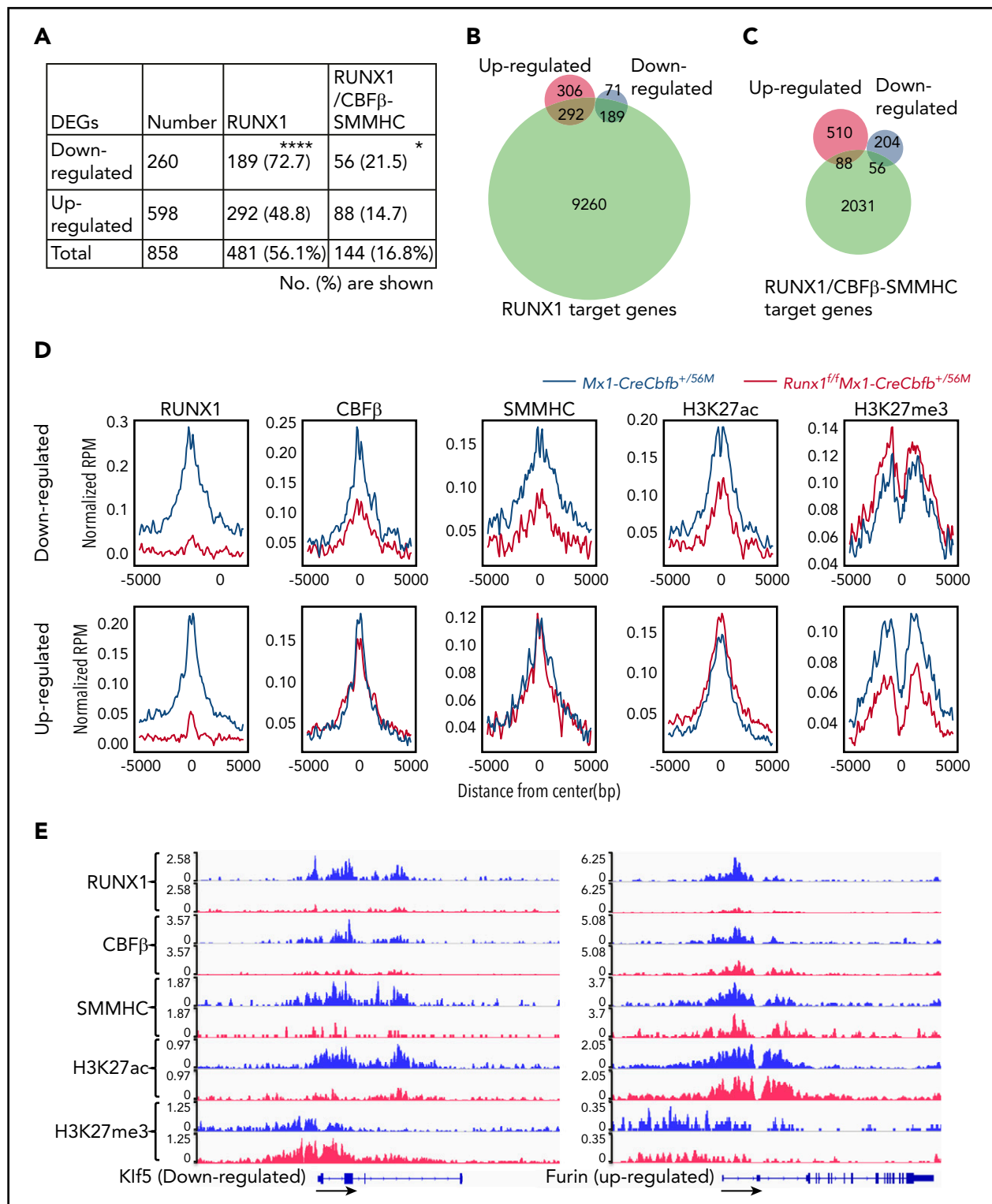


**Figure 4. *Runx1* is required for the regulation of critical genes to maintain functional AMPs for leukemogenesis by *Cbfb-MYH11*.** (A-G) RNA-seq was performed on AMP cells isolated from *Mx1-CreCbfb<sup>+/56M</sup>* and *Runx1<sup>ff</sup>Mx1-CreCbfb<sup>+/56M</sup>* mice 2 to 3 weeks after plpC treatment. N = 3 for each genotype. (A) Two-dimensional principal component analysis plots showing clear separation between these 2 genotype groups. (B) Heatmap representation of identified DEGs between these 2 groups. Numbers and percentages of DEGs in each of the 2 expression clusters (down- and upregulated) are listed on the left. (C-E) GSEA (Preranked) identified curated genes sets significantly enriched in these DEGs, including (C) the FISCHER\_G2\_M\_CELL\_CYCLE gene set, which is negatively correlated with DEGs upregulated in *Runx1<sup>ff</sup>Mx1-CreCbfb<sup>+/56M</sup>* cells; (D) the BROWN\_MYELOID\_CELL\_DEVELOPMENT\_UP gene set, which is positively correlated with DEGs upregulated in *Runx1<sup>ff</sup>Mx1-CreCbfb<sup>+/56M</sup>* cells; and (E) the GAL\_LEUKEMIC\_STEM\_CELL\_DN gene set, which is positively correlated with DEGs upregulated in *Runx1<sup>ff</sup>Mx1-CreCbfb<sup>+/56M</sup>* cells.

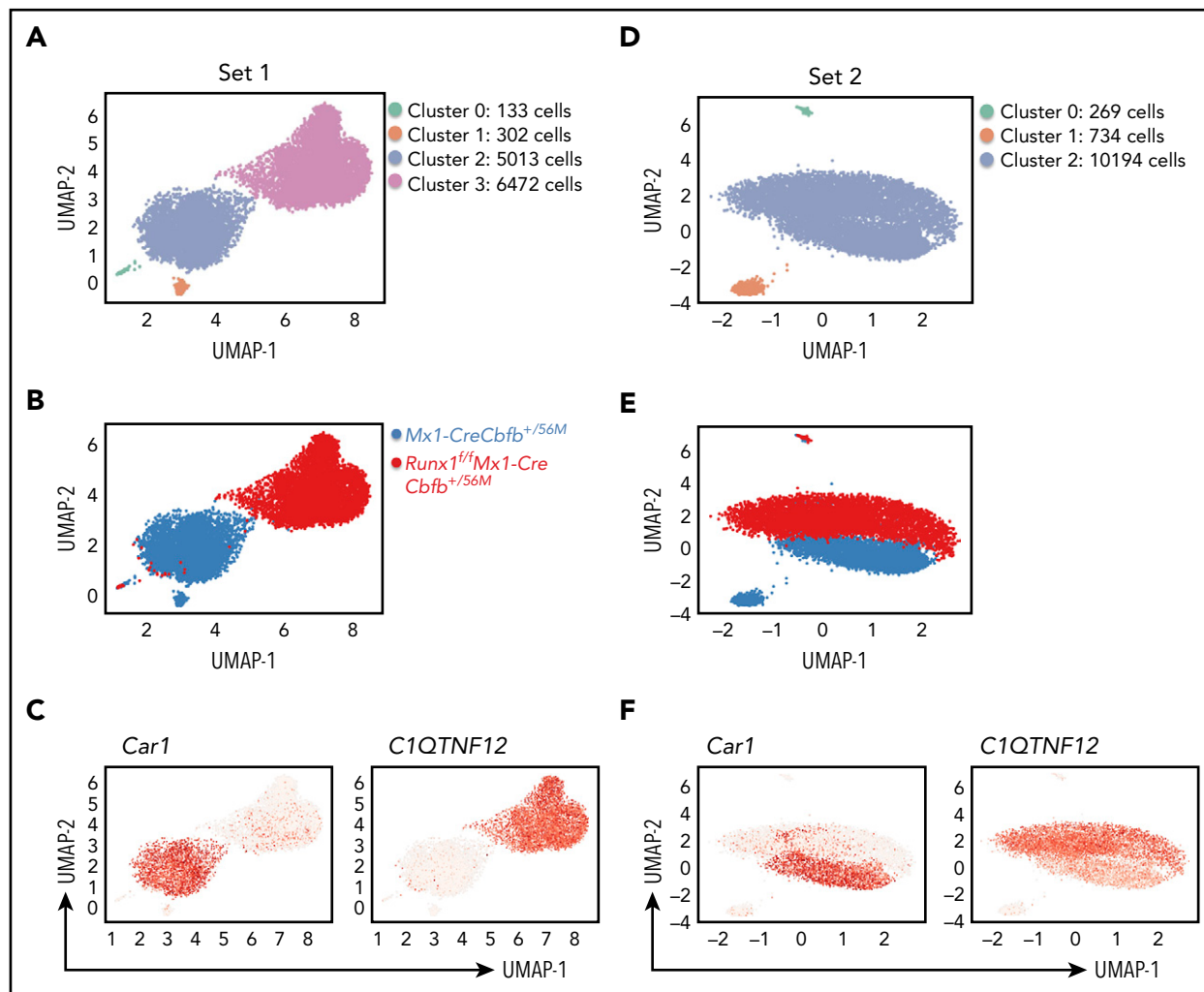
Strikingly, leukemia did not develop in any mice with *Runx1* knockout and *Cbfb-MYH11* knockin (*Runx1<sup>ff</sup>Mx1-CreCbfb<sup>+/56M</sup>*; Figure 2A; supplemental Figure 5), indicating that RUNX1 was required for leukemogenesis by CBF $\beta$ -SMMHC. We also found that the AMPs, a population unique to *Cbfb-MYH11*-expressing mice and able to induce leukemia,<sup>28</sup> decreased and then disappeared in *Runx1<sup>ff</sup>Mx1-CreCbfb<sup>+/56M</sup>* mice shortly after plpC treatment (Figure 3A,C,D; supplemental Figure 7B). These results suggest that leukemogenesis from these AMPs requires RUNX1. It is possible that other cell populations in the bone marrow, especially hematopoietic stem cells and multipotent

progenitors were also affected by RUNX1 loss; however, we decided to focus on the AMPs because of their dynamic change in the *Runx1<sup>ff</sup>Mx1-CreCbfb<sup>+/56M</sup>* mice and the fact that they are considered as the leukemia-initiating cells.

To address how *Runx1* cooperated with *Cbfb-MYH11* for leukemogenesis, we first performed bulk RNA-seq of the AMPs from *Mx1-CreCbfb<sup>+/56M</sup>* and *Runx1<sup>ff</sup>Mx1-CreCbfb<sup>+/56M</sup>* mice to determine the effect of *Runx1* knockout on transcriptome. We found that genes associated with proliferation, differentiation blockage, and maintenance of leukemia-initiating ability were



**Figure 5. Target gene upregulation is a major function of CBF $\beta$ -SMMHC in leukemogenesis.** (A-D) ChIP-seq was performed on AMP cells isolated from *Mx1-CreCfb<sup>+/56M</sup>* and *Runx1<sup>fl/fl</sup>Mx1-CreCfb<sup>+/56M</sup>* mice 2 to 3 weeks after plpC treatment. (A) Number (percentage) of the downregulated or upregulated DEGs occupied by RUNX1 and RUNX1/CBF $\beta$ -SMMHC complex. \* $P < .05$ , \*\*\*\* $P < .0001$ , between down- and upregulated DEGs by  $\chi^2$  test. (B-C) Venn diagrams representing the overlap of DEGs between *Mx1-CreCfb<sup>+/56M</sup>* and *Runx1<sup>fl/fl</sup>Mx1-CreCfb<sup>+/56M</sup>* AMPs (up- or downregulated in the *Runx1<sup>fl/fl</sup>Mx1-CreCfb<sup>+/56M</sup>* AMPs, same as below) and RUNX1 target genes (B) or RUNX1/CBF $\beta$ -SMMHC target genes (C) in AMPs from *Mx1-CreCfb<sup>+/56M</sup>* cells. (D) Average binding profile of indicated proteins at the TSSs of the DEGs. (E) ChIP-seq analyses of RUNX1, CBF $\beta$ , SMMHC, H3K27ac and H3K27me3 binding at the *Klf5* and *Furin* genes in AMP cells from *Mx1-CreCfb<sup>+/56M</sup>* (blue) and *Runx1<sup>fl/fl</sup>Mx1-CreCfb<sup>+/56M</sup>* mice (red). Direction of the gene was indicated by black arrows.



**Figure 6. Runx1 is required for Cbfb-MYH11-induced generation of functional AMP population.** Uniform manifold approximation and projection plots of AMP single cells from *Mx1-CreCfb<sup>+/56M</sup>* and *Runx1<sup>fl/fl</sup>Mx1-CreCfb<sup>+/56M</sup>* mice at 2 to 3 weeks after plpC treatment. (A-C) the first set of experiments. (D-F) The second set of repeat experiments. (A,D) Clusters based on gene expression patterns. (B,E) Distributions of the cells of the 2 genotypes among the clusters. (C,F) Feature plot depicting the expression of genes (*Car1* and *C1QTNF12*) across different cell populations (red, high; gray, low).

differentially expressed between *Mx1-CreCfb<sup>+/56M</sup>* and *Runx1<sup>fl/fl</sup>Mx1-CreCfb<sup>+/56M</sup>* AMPs (Figure 4), suggesting these genes were required for the generation and function of the AMPs. We also performed ChIC-seq to determine the effect of *Runx1* knockout on target gene binding and histone modifications. Results from the ChIC-seq assay imply that RUNX1 is required for the recruitment of CBF $\beta$ -SMMHC to target genes in AMPs and that RUNX1 and CBF $\beta$ -SMMHC work together to both activate and inhibit, especially activate, target genes expression. Of the DEGs between *Mx1-CreCfb<sup>+/56M</sup>* and *Runx1<sup>fl/fl</sup>Mx1-CreCfb<sup>+/56M</sup>* AMPs, a significantly higher percentage of DEGs downregulated in *Runx1<sup>fl/fl</sup>Mx1-CreCfb<sup>+/56M</sup>* AMPs were RUNX1/CBF $\beta$ -SMMHC target genes compared with upregulated DEGs. In addition, CBF $\beta$  and SMMHC bindings were reduced at TSSs only for DEGs downregulated in *Runx1<sup>fl/fl</sup>Mx1-CreCfb<sup>+/56M</sup>* cells, although RUNX1 binding was reduced at TSSs of both downregulated and upregulated DEGs in *Runx1<sup>fl/fl</sup>Mx1-CreCfb<sup>+/56M</sup>* cells. As expected, reduced H3K27ac modification was observed at TSS of downregulated DEGs in the *Runx1<sup>fl/fl</sup>Mx1-CreCfb<sup>+/56M</sup>* cells, correlating with decreased expression of these genes. Together, the RNA-seq

and ChIC-seq data demonstrate that RUNX1 and CBF $\beta$ -SMMHC more frequently function as a transcription activator complex and possibly recruit other proteins, such as GATA2, histone acetyl transferases and/or histone deacetylases<sup>10</sup> to promoters of many target genes, inducing H3K27ac modification and eventually leading to enhanced genes expression. These results are consistent with previous findings that most of the gene expression changes induced by CBF $\beta$ -SMMHC are increases in expression.<sup>25,43</sup>

Interestingly, 30% of *Runx1<sup>fl/fl</sup>Mx1-CreCfb<sup>+/56M</sup>* mice, in both primary and transplant models, died around 2 weeks after plpC treatment with no signs of leukemia, but multilineage pancytopenia with especially severe thrombocytopenia and anemia, which were not observed in *Mx1-CreCfb<sup>+/56M</sup>* or *Runx1<sup>fl/fl</sup>Mx1-Cre* mice. The exact cause of death was not clear, probably because of a combined effect of genetic changes (loss of *Runx1* and expression of *Cbfb-MYH11*) and plpC treatment. *Runx1* is known to be important for megakaryocytic differentiation while plpC treatment can suppress erythroid development,<sup>44</sup> and it may induce cell death through its effector, type I interferons.<sup>45</sup>

Although beyond the scope of this paper, it will be interesting to try another Cre system, such as tamoxifen-inducible CreER, to determine how important plpC treatment is for this early lethality phenotype in the *Runx1<sup>fl/fl</sup>Mx1-CreCbfb<sup>+/-56M</sup>* mice.

In this study, we show that *Runx1* is required for *Cbfb-MYH11*-induced leukemogenesis. In addition, we provide important insight into the mechanism of leukemogenesis associated with CBFβ-SMMHC (ie, the fusion protein acts as a transactivator of gene expression in the presence of RUNX1). These results therefore validate current efforts to develop inhibitors of RUNX1-CBFβ-SMMHC interaction for the treatment of inv(16) AML.<sup>46,47</sup>

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## Authorship

Contribution: T.Z. designed and performed experiments, analyzed data, and wrote the paper; Y.C. analyzed the bulk RNA-seq, ChIC-seq, and scRNA-seq data and wrote the paper; G.R., L.Z., R.K.H., G.L., D.F., and L.A. performed experiments; K.Z. contributed to the ChIC-seq

experimental design and data analysis; and P.P.L. designed the experiments, analyzed data, and wrote the paper.

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## Footnotes

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\*T.Z. and Y.C. contributed equally to this study.

For original data, please contact [pliu@nih.gov](mailto:pliu@nih.gov). The data from RNA-seq, ChIC-seq and single cell RNA-seq have been deposited in GEO with accession no. GSE152573.

The online version of this article contains a data supplement.

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