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Gata2 deficiency delays leukemogenesis while contributing to aggressive leukemia phenotype in *Cbfb-MYH11* knockin mice

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Abstract

Inversion of chromosome 16 (inv(16)) generates a fusion gene *CBFB-MYH11*, which is a driver mutation for acute myeloid leukemia (AML). Gene expression profiling suggests that *Gata2*, a hematopoietic transcription factor, is a top upregulated genes in preleukemic *Cbfb-MYH11* knockin mice and is expressed in human inv(16) AML. On the other hand, we have also identified recurrent monoallelic deletions of *GATA2* in relapsed human CBF-AML patients. To clarify the role of *Gata2* in leukemogenesis by *Cbfb-MYH11*, we generated conditional *Cbfb-MYH11* knockin mice with *Gata2* heterozygous knockout. *Gata2* heterozygous knockout reduced abnormal myeloid progenitors, which are capable of inducing leukemia in the *Cbfb-MYH11* mice. Consequently, *Cbfb-MYH11* mice with *Gata2* heterozygous knockout developed leukemia with longer latencies than those with intact *Gata2*. Interestingly, leukemic cells with *Gata2* heterozygous knockout gained higher number of mutations and showed more aggressive phenotype in both primary and transplanted mice. Moreover, leukemic cells with *Gata2* heterozygous knockout showed higher repopulating capacity in competitive transplantation experiments. In summary, reduction of *Gata2* activity affects mutational dynamics of leukemia with delayed leukemia onset in *Cbfb-MYH11* knockin mice, but paradoxically results in a more aggressive leukemia phenotype, which may be correlated with leukemia relapse or poor prognosis in human patients.

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Authorship

Contribution: S.S. designed and performed experiments, analyzed data, and wrote the paper; T.Z., and G.L. performed experiments; E.M.K. performed RNA sequencing and microarray data analysis; K.Y. performed analysis of whole exome sequencing; L.J.M. contributed Gata2 mouse model expertise; and P.P.L. designed the experiments, analyzed data, and wrote the paper.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

Introduction

Inversion of chromosome 16 (inv(16)) generates a *CBFB-MYH11* fusion gene, which causes acute myeloid leukemia (AML) subtype M4Eo.[1] The encoded fusion protein, CBFβ-SMMHC, contributes to the pathogenesis of *CBFB-MYH11* AML. Accumulating evidence has shown that *CBFB-MYH11* AML follows a 'two-hit' model for leukemogenesis,[2, 3] which states that AML is the consequence of collaborations between at least two broad classes of mutations; class I mutations that confer proliferative and/or survival advantage to cells (e.g. RAS signaling) and class II mutations that impair hematopoietic differentiation (e.g. *CBFB-MYH11*). We have shown that *Cbfb-MYH11* (the mouse-human hybrid version of *CBFB-MYH11* expressed in a mouse knockin model[4]) is necessary but not sufficient for leukemia and initiates leukemogenesis by blocking normal hematopoietic differentiation through inhibition of RUNX1, consistent with two-hit hypothesis.[4–6] In vitro studies have shown that CBFβ-SMMHC may serve as a transcriptional repressor and it may sequester RUNX1 in the cytoplasm.[7–9] Additionally, recent studies suggest CBFβ-SMMHC has roles not only for RUNX1 suppression but also for transcriptional activation of various genes in the process of leukemogenesis.[10, 11] Thus, although recent studies have gradually unraveled the pathogenesis of *CBFB-MYH11* AML, further investigation is still needed to better understand leukemogenesis and disease progression.

To further unveil the mechanism of pathogenesis for this type of leukemia, we performed gene expression profiling on *Cbfb-MYH11* knockin mouse embryos.[11] As shown below, we have also analyzed microarray and RNA-seq data to identify the most important dysregulated genes in *Cbfb-MYH11* pre-leukemic mice.[11, 12] Using these two datasets, we have identified *Gata2*, a critical hematopoietic transcription factor,[13] as one of six commonly dysregulated genes shared by these two datasets. These findings suggest *Gata2* may play an important role in the development of this type of leukemia.

Recently we also performed comprehensive somatic mutational analysis of CBF-AML patient samples, and identified deletions of a region on chromosome 3 in relapse samples from three patients.[14] Interestingly, the minimal overlapping region of these deletions includes the *GATA2* gene. This observation suggests that *GATA2* haploinsufficiency may contribute to relapse of CBF-AML. Recent studies have also shown that patients with advanced myeloid malignancy associated with *GATA2* deficiency have poor prognosis.[15]

From these findings, we proposed two hypotheses; 1) up-regulation of *GATA2* contributes to *CBFB-MYH11* leukemogenesis in the initiation phase; and 2) *GATA2* deficiency contributes to the relapse/evolution of CBF-AML. To test these hypotheses, we generated conditional *Cbfb-MYH11* knockin mice with heterozygous *Gata2* knockout. Our findings suggest that *Gata2* plays important but distinct roles in two different stages of *Cbfb-MYH11* leukemia: sufficient *Gata2* activity is important for *Cbfb-MYH11* induced leukemogenesis, while *Gata2* deficiency may contribute to the relapse of the disease.

Materials and Methods

Animals

All animal studies 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*$^{+/56M}$*Mx1-Cre*)[16, 17], mice harboring a *Gata2* exon 5 flanked by loxP sites (*Gata2*$^{+/f}$)[18] (obtained through the Mutant Mouse Resource and Research Center, mmrrc.org), mice harboring a *Runx1* exon 4 flanked by loxP sites (*Runx1*$^{f/f}$)[19], and R26R-Brainbow2.1 (*BB*$^{+/f}$) mice[20] harboring fluorescent protein have been previously described. The mice were backcrossed to C57BL/6 for >10 generations, then crossed to each other. All mice were genotyped by polymerase chain reaction (PCR) with gene-specific primers (Supplemental Table1) using tail-snip DNA. Eight- to 12-week-old mice were injected intraperitoneally with 250 mg of poly(I:C) (pIpC; InvivoGen) every other day for 3 doses to activate Cre-recombinase under the control of the Mx1 promotor, which induces lox-recombination in bone marrow (BM) cells. All mice were observed for leukemia development for 12 months. Bulk spleen cells (1×10^6) from *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-cre* or *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-cre* ($CD45.1^-CD45.2^+$) mice were transplanted into sublethally irradiated (650 rads) wildtype C57BL/6×129Sv ($CD45.1^+/CD45.2^+$) mice. For competitive transplantation, various numbers of c-Kit^+ leukemic cells from *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-cre* ($CD45.1^-CD45.2^+YFP^-$) were mixed with those of *BB*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-cre* ($CD45.1^-CD45.2^+YFP^+$; 3×10^5) and injected intravenously into sublethally irradiated wildtype mice ($CD45.1^+/CD45.2^+$).

FACS

Peripheral blood cells, spleen and BM cells from mice were isolated and stained as previously described for flow cytometry assay.[21] Flow cytometry was performed using BD LSRII, and sorting was performed using BD ARIA-II. See Supplemental Methods for details regarding the antibodies used in this study.

Quantitative PCR

Quantitative PCR was performed by using Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. RNA and DNA were extracted from BM cells using RNA MiniPrep Plus kit (Zymo Reserch) or DNA mini kit (Qiagen). Exon5/6 primers were used to detect unexcised *Cbfb-MYH11* flox allele; *Gata2* unexcised primers were used to detect unexcised *Gata2* flox allele. Genomic control primers were used to as internal control for genomic DNA. Primers are listed in Supplemental Table 1.

Colony forming assay

Colony forming assay was performed by using BM cells 12 days after pIpC injection and MethoCult GF M3434 (STEMCELL Technologies) according to the manufacturer's instructions.

Apoptosis and cell cycle analysis

For apoptotic analysis, cells were stained for Annexin V and 7AAD according to the manufacturer's protocol (eBioscience). For cell cycle analysis, after staining surface markers, cells were fixed and stained for DAPI to evaluate DNA content. Then flow cytometry was performed and data was analyzed by FlowJo.

RNA-seq and whole exome sequencing

RNA-seq and whole exome sequencing (WES) were performed on messenger RNA (mRNA) and genomic DNA isolated from c-Kit$^+$ BM or spleen cells of end-stage leukemic mice. The RNA-seq data and WES data have been deposited at Gene Expression Omnibus and Sequence Read Archive, respectively (accession numbers GSE 130343 and SRP193773). Details on all of the above procedures and data analysis are provided in Supplemental Methods.

Statistical analysis

For all in vitro experiments, at least three samples were tested per condition or genotype in order to perform statistical analysis. For animal studies, the numbers of animals used in each condition and/or genotype were determined based on the estimations of effect sizes and desired power, which was set at 1. No randomization was used, since we compared animals based on their genotype. No samples were excluded from data analysis. The researchers were not blinded for the animal studies. Data were analyzed with Prism software (GraphPad Software, La Jolla, CA, USA). Results are expressed as mean ± standard error of the mean. Differences between 2 groups were tested with a Student t test. The survival times of mice were analyzed with the Kaplan-Meier method and log-rank test. A two-sided t-test value of $P < 0.05$ was considered statistically significant (in figure legends: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Results

Gata2 is upregulated in preleukemic hematopoietic cells in *Cbfb-MYH11* knockin mice

Cbfb-MYH11 is thought to contribute to leukemogenesis by affecting the expression of various genes.[10, 11] To identify candidate genes affected by *Cbfb-MYH11*, we screened the genes that differentially expressed in *Cbfb-MYH11* knockin mouse model using two different cohorts. The first cohort was composed of a microarray analysis of the peripheral blood cells in the *Cbfb-MYH11* conventional knockin (*Cbfb*$^{+/MYH11}$) embryos compared with those in wild-type (WT) littermates (GSE19194).[11] Top genes (24) were selected if they had fold change greater than 5 or less than −5 between *Cbfb-MYH11* and WT mice and had adjusted p-value < 0.001 (Supplemental Figure 1). The second cohort is an RNA-seq analysis of the c-Kit$^+$ BM cells from *Cbfb-MYH11* conditional knockin (*Cbfb*$^{+/56M}$) mice, collected at the preleukemic stage (2 weeks after treatment with pIpC) compared with c-Kit$^+$ BM cells from wild-type littermates (GSE102388).[12] Top genes (66) were selected if they had fold change greater than 3 or less than −3 and had adjusted p-value < 0.001 (Supplemental Figure 2). For both cohorts, most of the selected genes were upregulated in the cells from the *Cbfb*$^{+/56M}$ mice compared with those from WT mice. Interestingly, *Gata2*

was among 6 genes which were shared by these two cohorts as differentially expressed genes in *Cbfb-MYH11* knockin mice (Figure 1A and Supplemental Table 2). We also evaluated *Gata2* gene expression by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot in the conditional *Cbfb-MYH11* knockin mice (*Cbfb*$^{+/56M}$*Mx1-Cre*), which were injected with pIpC to induce the expression of *Cbfb-MYH11* through Cre-recombinase activation. At 12 days after pIpC treatment, before the development of leukemia, BM cells were collected from the mice. *Gata2* was expressed higher in *Cbfb*$^{+/56M}$*Mx1-Cre* preleukemic BM cells compared to WT BM cells at both mRNA and protein levels (Supplemental Figure 3A and 3B). CBFβ-SMMHC, the fusion protein encoded by *CBFB-MYH11*, is a dominant negative repressor of RUNX1.[1] Therefore, we evaluated *Gata2* gene expression in BM cells in the *Runx1*$^{f/f}$*Mx1-Cre* mice. Gata2 was also expressed higher in *Runx1*$^{f/f}$*Mx1-Cre* BM cells compared to WT BM cells, 2 weeks after pIpC treatments (Supplemental Figure 4A). We also analyzed a published ChIP-seq study of ME-1 (a human inv(16) leukemia cell line that expresses *CBFB-MYH11*) using anti- Runx1, CBFβ and SMMHC antibodies (GSE46044)[22], and the results suggested that *GATA2* was likely a transcriptional target of *CBFB-MYH11* (Supplemental Figure 4B). These findings suggest that both *Runx1* suppression and transcriptional activation by CBFβ-SMMHC can lead to higher *Gata2* expression in the *Cbfb-MYH11* knockin mice.

Gata2 is highly expressed in hematopoietic stem/progenitor cells.[13] We therefore compared the expression levels of *Gata2* in the hematopoietic stem/progenitor compartments of wildtype and *Cbfb-MYH11* preleukemic BM cells. At 12 days after pIpC treatment, lineage negative BM cells from the wild type mice could be further divided into LSK ($Lin^-Sca\text{-}1^-c\text{-}Kit^+$), granulocyte-monocyte progenitors (GMPs) ($Lin^-CD34^+CD16/32^-$), common myeloid progenitors (CMPs) ($Lin^-CD34^+CD16/32^+$), and megakaryocyte-erythroid progenitors (MEPs) ($Lin^-CD34^-CD16/32^-$), as described previously (Supplemental Figure 5A).[12, 23, 24] *Cbfb*$^{+/56M}$*Mx1-Cre* preleukemic BM cells contained LSK, CMPs, and GMPs as well, and an abnormal myeloid progenitor population (AMP) ($Lin^-CD34^-CD16/32^{dim-+}$) instead of the MEPs in WT control (Supplemental Figure 5A). This AMP population is specific to *Cbfb*$^{+/56M}$*Mx1-Cre* mice and a putative leukemia initiating population in this leukemic mouse model.[12, 16] To confirm which populations are responsible for the *Gata2* high expression, LSKs, GMPs, CMPs, MEPs, and AMPs were sorted by FACS (Supplemental Figure 5B). qRT-PCR showed that LSKs had the highest *Gata2* gene expression in the control mice (Figure 1B), but *Gata2* was expressed higher in *Cbfb*$^{+/56M}$*Mx1-Cre* than WT control for every population examined except LSK (Figure 1B). Moreover, the highest level of *Gata2* expression was detected in the AMPs of the *Cbfb-MYH11* knockin mice. Considering that AMPs are specific to *Cbfb-MYH11* mice and have been reported as responsible for leukemia initiation, these data suggest *Gata2* has important roles in leukemogenesis during preleukemic phase of this mouse model.

Establishment of *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice

To determine the functional significance of *Gata2* in *Cbfb-MYH11* induced leukemia, we generated *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* and *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice.[16, 18] The *Gata2* knockout mice were injected with pIpC to induce the expression of *Cbfb-MYH11* and the knockout of *Gata2*. Quantitative and conventional PCR reactions were performed to

determine the excision efficiency of *Cbfb-MYH11* and *Gata2* flox alleles in BM cells 12 days after pIpC injection. The data confirmed that the excisions were almost complete for both genes (Supplemental Figures 6A, B and 7A, B). Moreover, qRT-PCR showed that the expression of the intact *Gata2* mRNA was reduced in *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice at 12 days after pIpC injection (Supplemental Figure 8A). These results revealed that appropriate induction of *Cbfb-MYH11* and knockout of *Gata2* in the hematopoietic cells occurred in the pIpC-treated *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice.

Heterozygous knockout of *Gata2* leads to reduced colony forming ability and smaller AMP in *Cbfb-MYH11* preleukemic BM cells

As we showed previously, activation of *Cbfb-MYH11* expression in the knockin mice causes increase of colony forming ability and emergence of the AMP population in the BM.[16] We were therefore interested in the effect of *Gata2* knockout on these phenotypes in the *Cbfb-MYH11* knockin mice. At 2 weeks after pIpC treatment (before leukemia initiation), in vitro colony formation assay demonstrated that *Gata2* knockout reduced colony forming ability of the *Cfb-MYH11* - expressing cells (Figure 2A). However, *Gata2* status had no effect on colony types formed by *Cbfb-MYH11* - expressing cells (Supplemental Figure 8B). The AMP population accumulated in both *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* and *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice (Figure 2B), but the AMP population was smaller in the *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice than in the *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice (Supplemental Figure 8C). The LK population was also decreased in *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice but the decrease did not reach statistical significance. Conversely, the LSK population, known to contain hematopoietic stem cells, was not different between these 2 groups of mice. These results suggest that *Gata2* deficiency reduced the colony forming ability and the size of the AMP population in *Cbfb-MYH11* knockin mice during early preleukemic phase. One the other hand, *Gata2*$^{+/f}$*Mx1-Cre* mice did not show any hematopoietic phenotype (Supplemental Figures 9A–D).

Heterozygous knockout of *Gata2* delays leukemia development in *Cbfb-MYH11* knockin mice

To test our hypotheses that increased expression of *Gata2* contributes to leukemogenesis by *CBFb-MYH11*, we observed leukemia development in *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* and *Gata2*$^{+/+}$ *Cbfb*$^{+/56M}$*Mx1-Cre* mice. Consistent with *Gata2* playing an important role during leukemogenesis, the number of c-Kit^{+} leukemic cells in the peripheral blood was significantly lower in *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice when compared to *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice at the same time points (Figure 2C). Hematocrit levels were also higher in *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice than in *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice (Figure 2D), which indicated that progression of anemia due to leukemia development was slower in the *Cbfb-MYH11* knockin mice with *Gata2* knockout. Eventually, both groups developed leukemia with similar phenotype. BM cells from each group showed two types of leukemic cells, immature cells with predominantly monocytic features and blast-like progenitors, as shown in a previous study (Supplemental Figure 10A and B).[16] Analysis of cell surface markers also showed similar phenotype between these two genotypes (Supplemental Figure 11). Various organs such as BM, spleen and liver were infiltrated with leukemic cells (Supplemental Figure 10A). Importantly, even though all the mice with the genotype of

Gata2$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* or *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* developed leukemia, *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemic mice had a longer survival than *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice (median survival, 215 vs 125 days; P=.0007; Figure 2E). As expected, *Gata2* gene expression in c-Kit^{+} leukemic cells was reduced in *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice than in *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice, but the expression of *Cbfb-MYH11* and *Runx1* was not different between these two groups of mice (Supplemental Figure 12A–C). Taken together, these results suggest that *Gata2* heterozygous knockout delays leukemogenesis by *Cbfb-MYH11* but does not change the morphology of the developed leukemia cells. Interestingly, despite the slower development of leukemia, there were more circulating white blood cells (WBCs) in the *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice at the end-stage than in the *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice (Figure 2G). Conversely, % of c-Kit^{+} cells, hematocrit and spleen weight were not different between the two groups (Supplemental Figure 13A–C). Since hyperleukocytosis is a well-known poor prognostic marker in human leukemia,[25] these findings led us to further characterizations of the leukemia cells of each genotype in the context of transplantation.

Leukemia developed faster in mice transplanted with *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells

To evaluate the characteristics of leukemic cells in both groups, we performed transplantations with spleen cells (1.0×10^{6} per recipient) isolated from end-stage leukemic mice (Figure 3A). Four different donors from each group showed similar characteristics including % of c-Kit^{+} cells in spleen (Supplemental Table 3). Surprisingly, despite slower development of leukemia, transplanted leukemic cells from *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice led to much faster leukemia development in the recipient mice than those from *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice. WBC count, % and number of c-Kit^{+} positive cells were higher in recipient mice transplanted with *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells than those transplanted with *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells at 4 weeks after transplantation (Supplemental Figure 14A–C). Hematocrit was lower in mice transplanted with *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells than those transplanted with *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells at the same time point (Supplemental Figure 14D). The above findings were reproducible among the 4 donors for each genotype (Supplemental Figure 14E–H). Furthermore, mice transplanted with *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells had a shorter survival than mice transplanted with *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells (median survival, 35.5 vs 83.5 days; P<.0001; Figure 3B). These findings revealed that leukemic cells from *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice showed more aggressive phenotype than those from *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice.

Gata2$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells had higher leukemia repopulating potential than *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells

To further characterize the advantage of leukemia repopulating capacity attributed to *Gata2* deficiency, we compared *BB*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* (*Gata2* WT) and *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* (Gata2 knockout) leukemia cells in competitive transplantation assays (Figure 3C).[26] Brainbow or *BB*$^{+/f}$ is a genetic cell-labeling technique by stochastic expression of distinct fluorescent proteins induced by Cre-recombinase activation.[20] *BB*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice developed leukemia with fluorescent protein expression (Supplemental Figure 15A),

which had similar latency and pathology as *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice (Supplemental Figure 15B). We sorted c-Kit^{+}, yellow fluorescent protein (YFP) positive spleen cells from an end-stage *BB*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mouse and c-Kit^{+}, YFP^{-} spleen cells from an end-stage *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mouse (Supplemental Figure 16A), which were then transplanted at various ratios (Supplemental Figure 16B) into CD45.1^{+}CD45.2^{+} recipient mice. As shown in Figure 3D, the YFP-, *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells were responsible for leukemia development in all recipients, even for those that received *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* and *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* cells at 1:100 ratio. Even though both YFP+ and YFP- populations were present in the recipient mice in the early weeks after transplantation, leukemia cells from the *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* (YFP-) mouse overwhelmed those from the *BB*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mouse by the end stage (Supplemental Figure 17). These findings suggest that *Cbfb-MYH11* leukemic cells with *Gata2* deficiency have higher leukemia repopulating capacity than those with WT *Gata2*.

Pathway analysis revealed difference in cell cycle status and apoptosis between *Gata2*$^{+/+}$ and *Gata2*$^{+/f}$ leukemic cells

To further investigate the difference between leukemic cells from *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice and those from *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice, we performed global gene expression profiling with RNA-seq (Supplemental Figure 18A and B). PCA plot and sample distance matrix showed a clear separation between *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* and *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells (Supplemental Figure 18C and D). Compared with *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* cells, 1579 genes in *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* cells were differentially expressed with P_{adj}<.05 and absolute fold changes ≥2; 845 genes were upregulated (cluster 1) and 734 genes were downregulated (cluster 2) in the *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice (Figure 4A). IPA upstream analysis showed *Gata2* was the second most important putative upstream transcription factors that affect these differentially expressed genes (DEGs) (Supplemental Figure 19). Gene ontology (GO) analysis showed that genes associated with cell cycle and mitosis were significantly enriched in cluster 1 (Supplemental Figure 20A). For cluster 2 the enriched GO terms are related to cell differentiation and regulation of apoptotic process. To validate these observations we performed cell cycle analysis, which revealed that G1 phase was reduced while S and G2 phases were increased in *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells (Figure 4B and Supplemental Figure 20B). Apoptosis assay using Annexin V revealed that apoptosis was reduced in *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells after short term incubation (Figure 4C). These observations suggest that *Gata2* deficiency results in cell cycle acceleration and reduction of apoptosis in *Cbfb-MYH11* induced leukemia.

Difference in cooperating mutations between *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* and *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice

The leukemic cells likely have additional somatic mutations, which may affect their phenotype. Therefore, we performed whole exome sequencing (WES) of c-Kit+ leukemic cells from the spleens of primary leukemic mice (*Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* and *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre*). DNA samples from CD3+ or CD19+ lymphocytes were used as germline controls. The average depth of sequencing coverage was 73X (Supplemental

Figure 21), and the identified somatic mutations are listed in Supplemental Table 4. The types of mutations in each mouse with variant allele frequency (VAF) above 0.1 are shown in Figure 5A. On average, 19.71 ± 4.78 (s.d.) somatic mutations were identified per mouse, with more mutations found in *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice than in *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice (23.25 ± 3.03 versus 15.0 ± 1.41, p= 0.0141) (Supplemental Figure 22A). Supplemental Table 5 lists nonsense, missense, and frameshift mutations in each mouse. On average, 11.6 ± 4.10 (s.d.) such mutations were identified per mouse, again with more mutations found in the *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice than in the *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice (14.5 ± 2.87 versus 7.67± 1.25, p= 0.0225) (Supplemental Figure 22B). By reviewing the variants for genes likely involved in leukemogenesis, we identified mutations in *Kras, Bcor, Trp53, Kit* and *Ptpn11* (Figure 5B), which are well known mutated genes in human leukemia.[27] The expression of these variants in the leukemia cells was confirmed by RNA-seq. For example, the *Bcor* (c.2941C>T; p.R981X, c.4393delG; p.V1465fs), *Kras* (c.34G>T; p.G12C), *Trp53* (c.508T>G; p.C170G), and *Kit* (c.2472T>G, p.N824K) mutations were observed in the corresponding leukemia RNA samples by RNA-seq (Supplemental Figure 22C–G). Importantly, these mutations (Kras p.G12C and Trp53 p.C170G) correspond to the mutations previously detected in human neoplastic patients (KRAS p.G12C and TP53 p.C176G, respectively) (Supplemental Figure 23A and B). In addition, the detected *Kit* mutation in *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* (c.2472T>G, p.N824K; in sample 11) corresponds to the *KIT* p.N822K mutation that is frequently observed in human leukemia patients and is also known as a poor prognostic marker in human *CBFB-MYH11* leukemia (Supplemental Figure 23C and D).[28] These observations suggest that our *Cbfb-MYH11* leukemic mouse model recapitulate the process of human leukemogenesis. Furthermore, preleukemic *Gata2* status may affect the mutational landscape in leukemic cells and also their disease phenotype in the mouse model.

Discussion

The understanding of the functional consequences of genetic events at early stages of transformation is critical for the understanding of leukemogenesis. Development of *CBFB-MYH11* AML is classically explained by a clonal evolution model which is featured with multiple stepwise cooperating mutations.[29, 30] The cooperating mutations in *CBFB-MYH11* leukemia are supposed to be class I mutations, e.g., mutations in receptor tyrosine kinases that provide proliferation or survival signal to hematopoietic cells, since *CBFB-MYH11* is a class II mutation [2]. Using pre-leukemic hematopoietic cells from *Cbfb-MYH11* knockin mice, gene expression profiling allowed us to identify 6 most upregulated genes at pre-leukemic stage. Among the human homologues of these 6 genes, *GATA2* and *CSF2RB2* are frequently expressed in *CBFB-MYH11* leukemia patients (Supplemental Table 2). Of these two genes, *Gata2* encodes a master regulator of hematopoiesis,[13] and a recent study demonstrated increased *Gata2* expression in preleukemic cells in the *Cbfb-MYH11* knockin mice.[31] To clarify the roles of *Gata2* in the *Cbfb-MYH11* leukemic mouse model, we established *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* (conditional *CBFB-MYH11* expression and *Gata2* heterozygous knockout) mice and evaluated them in both ‘preleukemic’ and ‘leukemic’ phases. From the perspective of class I and class II mutations for leukemogensis, this mouse

model is unique and interesting as it combined mutations of two transcription factors (both class II mutations) in a mouse model.

In the analysis of the 'preleukemic' stage in the *Cbfb-MYH11* mice, we found that *Gata2* was upregulated, especially in a putative leukemia initiating population[16] in the bone marrow. *Runx1* deletion in murine hematopoietic cells also led to higher *Gata2* expression (Supplemental Figure 4A). Moreover, *GATA2* may be a direct transcriptional target of CBFβ-SMMHC (Supplemental Figure 4B). Heterozygous knockout of *Gata2* in the *Cbfb-MYH11* mice delayed leukemia onset, suggesting that *Gata2* has a significant role in the initiation of *Cbfb-MYH11* induced leukemia. Interestingly, despite slower development of leukemia, the *Cbfb-MYH11* induced leukemia cells in the *Gata2* deficient mice showed more aggressive phenotype at the 'leukemic' stage (higher WBC counts in the primary mice, higher leukemia repopulating potential through transplantation, accelerated cell cycle and reduced apoptosis) than leukemia cells with intact *Gata2* gene. These findings are consistent with the previous report of recurrent deletions of *GATA2* in relapsed human CBF-AML patients.[14] Thus, *Gata2* deficiency in preleukemic phase seems to have contrasting roles in *Cbfb-MYH11* leukemia: it represses or delays leukemogenesis from normal hematopoietic progenitors, but increases the repopulating potential of the leukemia cells (Supplemental Figure 24).

In order to understand the molecular mechanisms underlying this functional dichotomy we performed whole exome sequencing and RNA-seq of the leukemia cells. Interestingly, we found that $Gata2^{+/f}Cbfb^{+/56M}Mx1\text{-}Cre$ leukemic cells had more somatic mutations than $Gata2^{+/+}Cbfb^{+/56M}Mx1\text{-}Cre$ leukemia cells, suggesting that preleukemic cells with *Gata2* deficiency may needed more mutations for leukemia transformation, which explains why leukemia development was slower in $Gata2^{+/f}Cbfb^{+/56M}Mx1\text{-}Cre$ mice. Although we could not see a clear difference in the number of somatic mutations between human CBF-AML samples with and without *GATA2* deficiency (Supplemental Figure 25A) in our previous study,[14] in another dataset[32] *CBFB-MYH11* AML samples with lower GATA2 expression had relatively higher number of somatic mutations, although the increase did not reach statistical significance (Supplemental Figure 25B and C). On the other hand, RNA-seq analysis revealed a large number of genes whose expression levels were altered in the $Gata2^{+/f}Cbfb^{+/56M}Mx1\text{-}Cre$ leukemia cells as compared to $Gata2^{+/+}Cbfb^{+/56M}Mx1\text{-}Cre$ leukemia cells. The upregulated pathways included cell cycle, cell division, DNA replication, and cell proliferation. These gene expression changes were consistent with phenotypic observations of cell cycle changes and reduced apoptosis of the $Gata2^{+/f}Cbfb^{+/56M}Mx1\text{-}Cre$ leukemia cells, which could be associated with more aggressive leukemia in the $Gata2^{+/f}Cbfb^{+/56M}Mx1\text{-}Cre$ mice. It should be pointed out that these observed phenotype in the $Gata2^{+/f}Cbfb^{+/56M}Mx1\text{-}Cre$ leukemic cells may be due to additional mutations in these cells rather than *Gata2* knockout itself.

Although we have not seen any hematopoietic phenotype in heterozygous *Gata2* knockout ($Gata2^{f/+}Mx1\text{-}cre$) mice, which is consistent with previous studies (Supplemental Figure 9),[33, 34] *Gata2* may affect multiple aspects of hematopoietic cells in transplantation studies, especially in the presence of the *Cbfb-MYH11* fusion gene. Previous studies suggested *Gata2* knockout contribute to reduced hematopoietic stem cell activity.[35, 36] Nevertheless,

higher leukemia repopulating potential and aggressive phenotype in the *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemic cells were observed in our experiment, likely due to modifying interaction between *Gata2* and *Cbfb-MYH11*. In addition, the aggressive phenotype of the *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells may be related to the additional mutations observed in the *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells rather than *Gata2* deficiency itself. Our findings also raised a possibility that dosage of *Gata2* was not that critical for leukemic cells to proliferate once the disease was initiated. Moreover, drug resistant cancer stem cells responsible for disease relapse are thought to be dormant in the previous studies.[37] Therefore, the observed aggressive phenotype of the *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells may not necessarily be related to CBF leukemia relapse in human patients.

Recent genomic sequencing studies in AML patients revealed the presence of preleukemic stem cells, harboring only a few founding mutations and capable of persisting during remission, which thus serve as a potential reservoir for subsequent leukemia relapse.[38, 39] In fact, relapsed leukemia often arises from such preleukemic clones.[40, 41] Our observation may reflect the process of leukemogenesis in human leukemic patients, in which such delayed leukemic transformation could contribute to relapse or poor prognosis.

GATA2 deficiency, a hereditary leukemia predisposition syndrome, is caused by germline heterozygous mutations in the *GATA2* gene and is characterized by bone marrow failure and immunodeficiency with systemic features.[42] The *Gata2* deficiency in our leukemic mouse model is more like a ‘somatic’ mutation (conditional heterozygous knockout), therefore we have to interpret the results of this study carefully in comparison with the human patients bearing germline *GATA2* deficiency. Interestingly, we could find no CBF-AML in human germline *GATA2* deficiency patients reported in the literature, even though CBF-AML can be found in 10–15% of leukemia patients without *GATA2* deficiency.[43, 44] Although our mouse model might not be a precise model for leukemia with germline *GATA2* deficiency, the fact that leukemic cells with *Gata2* deficiency have more aggressive phenotype than those without it may be consistent with the clinical observation that patients with advanced hematological malignancy and germline *GATA2* deficiency have poor prognosis.[15]

Currently, approximately half of the CBF-AML patients relapse after initial treatment, and survival post-relapse is poor.[45, 46] Better understanding of how leukemia develop and relapse will lead to better treatments of the leukemia patients. Our findings further highlight the complex dynamic clonal evolution and the contribution from multiple gene mutation events, including from those that are unexpected.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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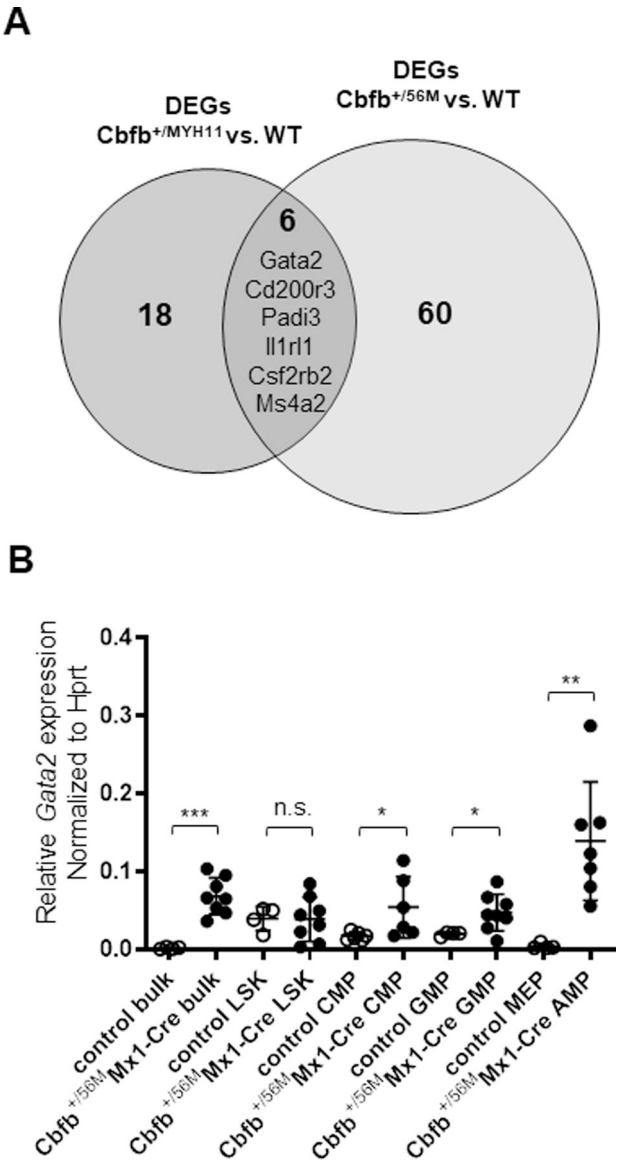


Figure 1. Analysis of *Gata2* expression in preleukemic *Cbfb*$^{+/56M}$*Mx1-Cre* mice

(A) The Venn diagram of common upregulated genes in the *Cbfb*$^{+/MYH11}$ embryos and c-kit + cells in the *Cbfb*$^{+/56M}$*Mx1-Cre* mice. (B) Relative levels of *Gata2* mRNA in sorted bulk, LSK, CMP, GMP, MEP and AMP populations in BM cells at 12 days after pIpC injection. Each dot represents data derived from one sample and all measurements were made in triplicates. Values are presented relative to those of *Hprt* mRNA (n=4–8 for each group). *p<0.05; **p<0.01; ***p<0.001.

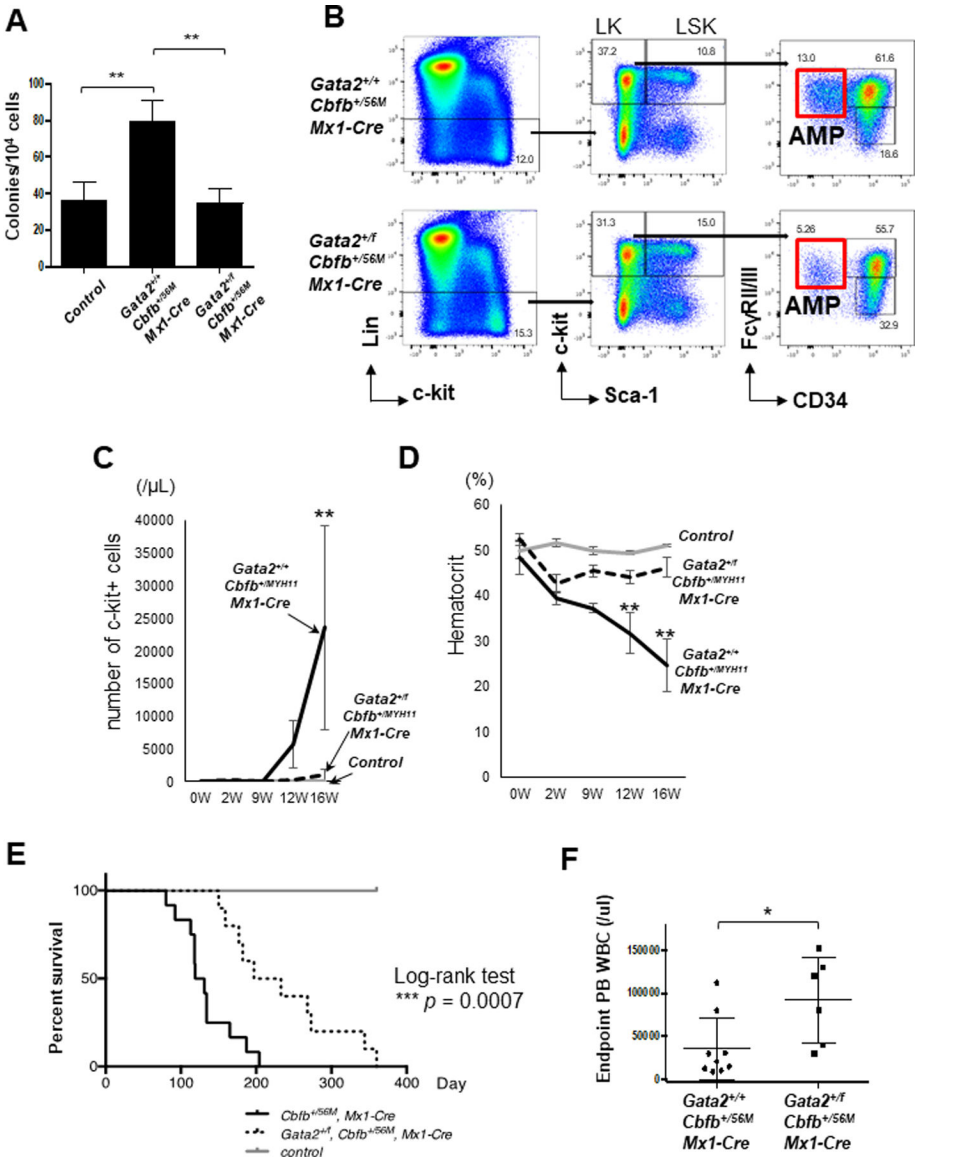


Figure 2. *Gata2* heterozygous knockout delays leukemogenesis induced by *Cbfb-MYH11*

(A) Total colonies produced from in vitro erythroid and myeloid differentiation assays with BM cells from the indicated genotypes (n=5 for each group). (B) Representative FACS plots of bone marrow cells gated on total cells (left plots), Lin- cells (middle plots), LK cells and AMP cells (right plots). (C) Numbers of c-Kit+ cells in the peripheral blood at the indicated time points after pIpC injection (n=5–6 for each group). (D) Hematocrit levels in the peripheral blood at the indicated time points after pIpC injection (n=5–6 for each group). (E) Kaplan-Meier curves of mice with the indicated genotypes during 12-month observation of leukemia development. Black line: *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre*, n=12; dotted line: *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre*, n=10; grey line: control mice, n=17. The *P*-values were calculated with log-rank test. The difference between *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice and *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice was highly significant (P=0.0007). (F) Peripheral blood

white blood cell count at the end points for the mice with the indicated genotypes. Each dot represents data derived from one mouse (n=6–9 for each group). *p<0.05; **p<0.01.

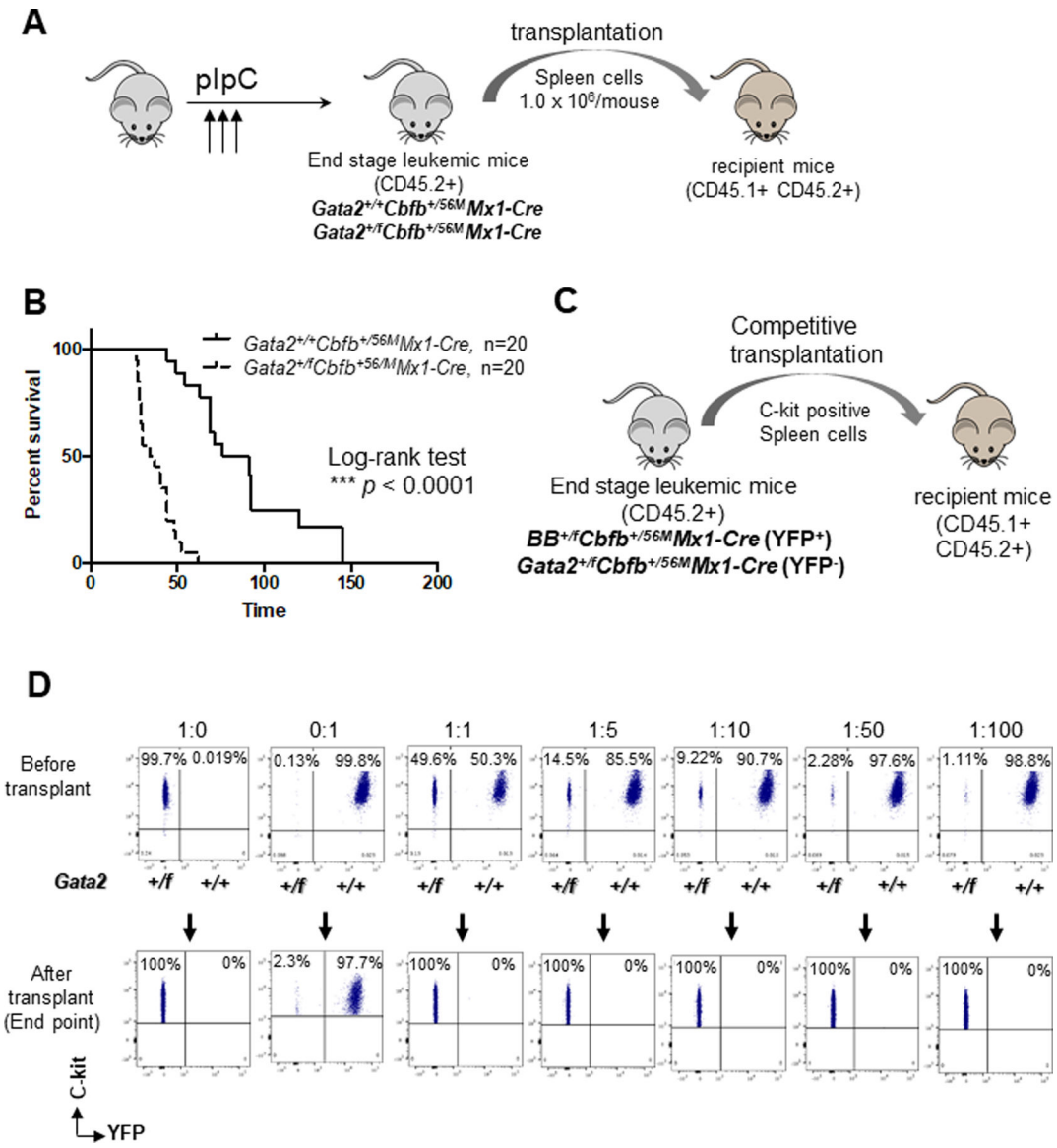


Figure 3. Accelerated leukemia development in recipient mice transplanted with *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells

(A) Experimental design. Equal numbers (1 million) of donor spleen cells obtained from end point mice (CD45.2+) were transplanted into sublethally irradiated WT recipient mice (CD45.1+CD45.2+). (B) Kaplan-Meier survival curves of transplanted mice. Black line: *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre*, n=20; dotted line: *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre*, n=20. The p-values were calculated with log-rank test. The difference between *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice and *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice was highly significant (P<0.0001).(C) For competitive transplantation, spleen cells from donor mice (CD45.2+; *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre*) and competitor mice (CD45.2+; *BB*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre*) were mixed in various ratios and injected through the tail vein of irradiated recipient mice (CD45.1+ CD45.2+). The efficacy of BM reconstitution was determined in peripheral blood (PB) at 4, 8, 12, and 16 weeks post-transplantation and BM at endpoint by FACS. (D) Representative FACS plots of cells injected samples immediately after sorting and recipients' peripheral

blood samples at endpoints. Leukemic cells from *BB*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* (Gata2 wildtype) mice were c-Kit+YFP+; those from *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice were c-Kit +YFP-.

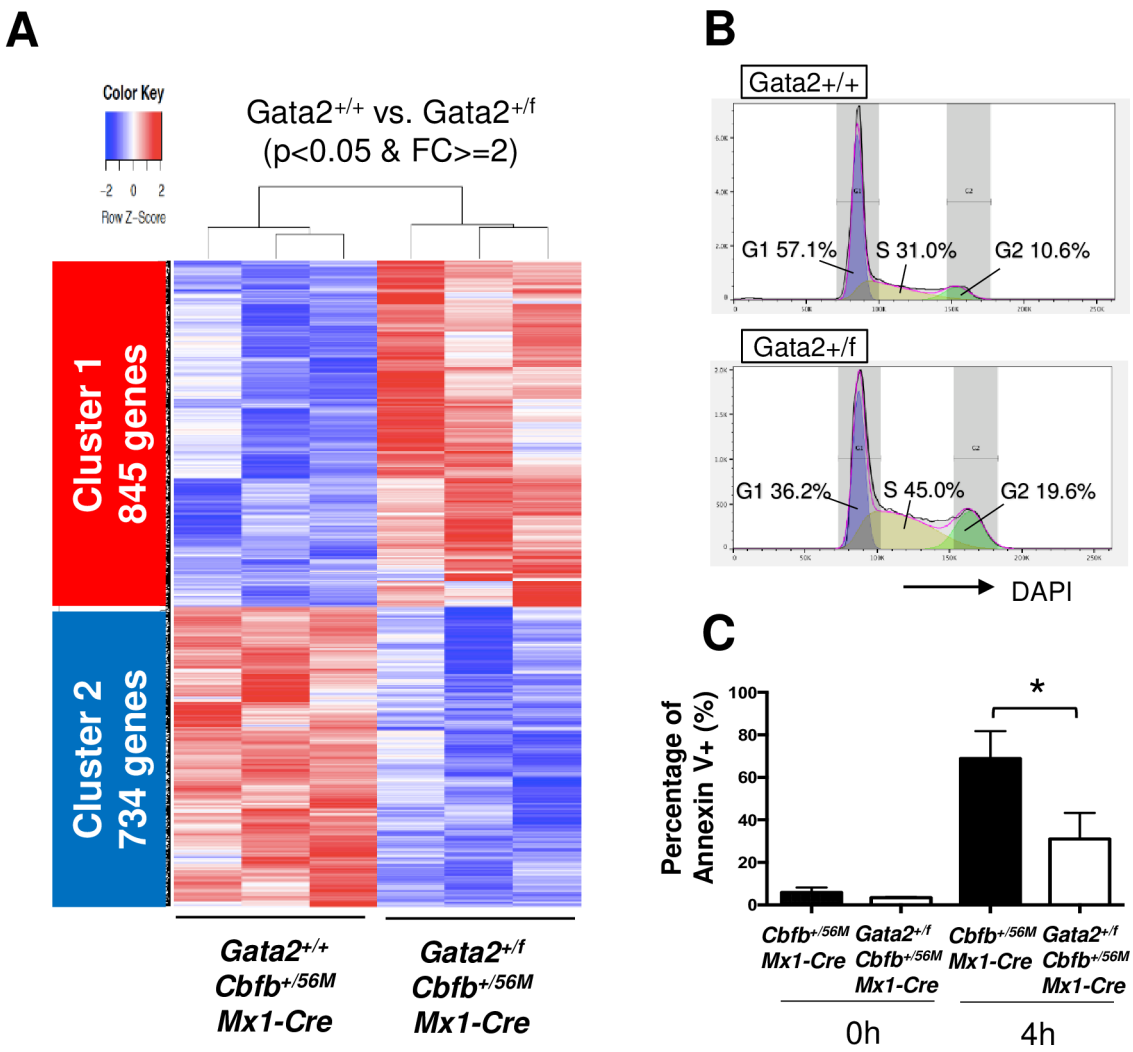
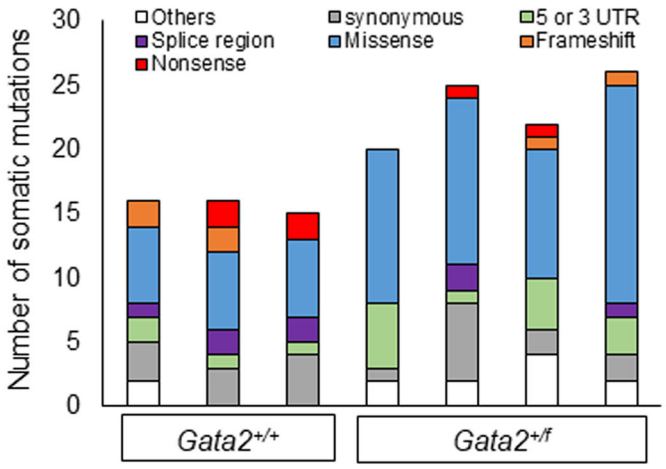


Figure 4. Gene expression profile of *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* leukemia cells

(A) Heatmap shows differentially expressed genes (DEGs) between c-kit+ BM cells from *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice and *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice at the end points. Cluster 1 is up-regulated genes in *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre*, and cluster 2 is down-regulated genes in *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre*. Only DEGs with p<0.05 and FC>=2 are shown. (B) Representative data of cell cycle analysis using DAPI staining of c-Kit+ BM cells from endpoint leukemic mice. Data was analyzed by FlowJo. (C) Bar graph showing percentage of Annexin V positive c-Kit+ leukemic cells of the indicated genotypes after 4 hours incubation (n=4 for each group). *p<0.05.

A



B

Genotype	Sample No.	Annotations
Gata2+/+	01	***Kras*** **(c.34G>T; p.Gly12Cys)** ***Bcor*** **(c.3555delG; p.Lys1186fs)**
	03	***Bcor*** **(c.2941C>T; p.Arg981*)**
	05	**-**
Gata2+/f	07	***Kras*** **(c.34G>T; p.Gly12Cys)**
	09	***Trp53*** **(c.508T>G; p.Cys170Gly)**
	11	***Kit*** **(c.2472T>G; p.Asn824Lys)** ***Bcor*** **(c.4393delG; p.Val1465fs)**
	13	***Ptpn11*** **(c.1484C>A; p.Pro495His)**

Figure 5. Profiling somatic mutations in leukemic cells from *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* and *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice

(A) Bar graphs showing the numbers and types of somatic mutations (with VAF>0.1) in c-Kit+ leukemic cells in 3 *Gata2*$^{+/+}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice and 4 *Gata2*$^{+/f}$*Cbfb*$^{+/56M}$*Mx1-Cre* mice. Others: upstream, downstream, and non-coding exons. (B) Case-specific mutations that are likely to contribute to leukemia pathogenesis.