KIT with D816 mutations cooperates with *CBFB-MYH11* for leukemogenesis in mice

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KIT mutations are the most common secondary mutations in inv(16) acute myeloid leukemia (AML) patients and are associated with poor prognosis. It is therefore important to verify that *KIT* mutations cooperate with *CBFB-MYH11*, the fusion gene generated by inv(16), for leukemogenesis. Here, we transduced wild-type and conditional *Cbfb-MYH11* knockin (KI) mouse bone marrow (BM) cells with *KIT* D816V/Y mutations. *KIT* transduction caused massive BM Lin− cell death and fewer colonies in culture that were less severe in the KI cells. D816Y *KIT* but not wild-type *KIT* enhanced proliferation in Lin− cells and led to more mixed lineage colonies from transduced KI BM cells. Importantly, 60% and 80% of mice transplanted with KI BM cells expressing D816V or D816Y *KIT*, respectively, died from leukemia within 9 months, whereas no control mice died. Results from limiting dilution transplantations indicate higher frequencies of leukemia-initiating cells in the leukemia expressing mutated *KIT*. Signaling pathway analysis revealed that p44/42 MAPK and Stat3, but not AKT and Stat5, were strongly phosphorylated in the leukemia cells. Finally, leukemia cells carrying *KIT* D816 mutations were sensitive to the kinase inhibitor PKC412. Our data provide clear evidence for cooperation between mutated *KIT* and *CBFB-MYH11* during leukemogenesis. (*Blood*. 2012;119(6):1511-1521)

Introduction

Chromosome 16 inversion is one of the most frequent chromosomal translocations found in acute myeloid leukemia (AML), occurring in 10% to 15% of AML cases, especially those in subtype M4Eo.[1] This translocation fuses the *CBFB* gene with the *MYH11* gene, resulting in a protein product that fuses the first 165 amino acids of core binding factor β (CBFβ) to the coiled-coil region of the smooth muscle myosin heavy chain.[2] In mouse models, the *CBFB-MYH11* fusion gene predisposes mice to AML but requires cooperating mutations for leukemogenesis.[3,4] In humans, mutations in receptor tyrosine kinase genes *KIT* and *FLT3* and in proto-oncogenes *N-RAS* and *K-RAS* have been observed in up to 70% of inv(16) AML patients,[5-7] and probably serve as cooperating factors during leukemogenesis.[8-10]

KIT (CD117) is a member of the type 3 subclass of transmembrane receptor tyrosine kinases, with 5 immunoglobulin-like domains in the extracellular region. It has a negative regulatory juxtamembrane domain and a split adenosine triphosphate–binding and phosphotransferase tyrosine kinase domain.[11] KIT is a receptor that is specific for stem cell factor (SCF). The interaction between KIT and SCF is crucial for the development of hematopoietic, gonadal, and pigment stem cells.[11-13] Genetic mutations that disrupt the expression of *KIT* are associated with piebaldism, a disorder featured by loosing pigmentation of the skin,[13] whereas overexpression or constitutive activation of *KIT* is associated with tumorigenesis.[11,14] *KIT* is expressed in almost 80% of human AML cases.[15-17]

Mutations in the *KIT* gene are especially common in inv(16) AML, compared with other AML subtypes,[18] occurring in 10% to 45% of the cases.[6,7,19] The most common *KIT* mutations are deletion and insertion mutations in exon 8[20,21] and the point mutations in exon 17.[21,22] Exon 8 mutations are in the extracellular domain and cause spontaneous receptor dimerization and activation of the downstream signaling pathways without SCF.[23] The codon 816 in exon 17 is a mutation hot spot in inv(16) AML,[18,21,22] and the most common substitutions, D816V and D816Y, cause constitutive activation of *KIT*.[24] These *KIT* mutations have been reported to adversely affect overall survival of inv(16) AML patients[21]; however, the real prognostic value of *KIT* mutations for inv(16) AML remains controversial.[21,25,26]

The D816 mutant forms of KIT are resistant to inhibition by the kinase inhibitor imatinib in vitro.[27,28] Similarly, results from clinical studies suggest that the kinase inhibitor imatinib has limited clinical benefit to patients with CBF leukemia carrying *KIT* D816 mutations.[29] Conversely, studies in cell lines and primary neoplastic mast cells show that another kinase inhibitor, PKC412, is a promising candidate for blocking D816 mutation,[28,30] with demonstrated activity in a patient with mast cell leukemia with the D816V *KIT* mutation.[31]

So far, the potential cooperation between mutant *KIT* and *CBFB-MYH11* has not been verified, and no in vivo or in vitro models exist for such studies. Given the high frequency and the potential prognostic value of the *KIT* mutations in inv(16) AML, it is important to develop an in vivo model not only to confirm the contribution of mutant *KIT* to leukemogenesis by *CBFB-MYH11*

but also to test and develop effective therapeutic approaches for this frequent clinical combination.

Methods

Animals

Conditional *Cbfb-MYH11* knockin mice (*Cbfb*[+/56m]) and wild-type (WT) littermates were genotyped as described previously[32] and crossed with Mx1-Cre [*Tg(Mx1-Cre)*] transgenic mice (The Jackson Laboratory). Six- to 8-week-old *Cbfb*[+/56m]; *Tg(Mx1-Cre)* mice and their littermate controls were treated with 250 μg of poly-IC (pI:pC; Invivogen) intraperitoneally every other day for 3 times to induce the expression of *Cbfb-MYH11*. Two weeks later, the mice were given 5-fluorouracil at 150 mg/kg body weight using intraperitoneal injection, and 48 hours later bone marrow (BM) cells were harvested from tibia and femur. C57BL/6 × 129/SvEv F1 hybrid mice were sublethally irradiated (650 cGy) and used as recipients for transplantation. All animals used and the procedures performed in this study were approved by the National Human Genome Research Institute Animal Care and Use Committee.

Production of stable retroviral cell lines

Phenix packaging cells were transfected with vectors carrying MSCV-*KIT* D816V, *KIT* D816Y, WT *KIT*, or green fluorescent protein (GFP) alone[28] using a calcium transfection kit from Invitrogen. Two days after transfection, the supernatants were harvested and incubated with either the ecotropic packaging cell line GP+E-86[33] that was derived from mouse NIH3T3 cells, or 293T cells for 2 days. Then, single GFP+ GP+E-86 and 293T cells were sorted to a 96-well plate and 2 to 3 weeks later, the cells were transferred to 24-well plates and later 6-well plates for expansion. Viral titers were measured,[34] and the clones with titers more than 1×10^6 particles/mL from GP+E-86 cells were expanded for transduction of mouse bone marrow cells.

Retroviral transduction of bone marrow cells

Bone marrow cells were harvested and cultured in DMEM (Invitrogen) supplemented with 20% FBS (HyClone Laboratories) and cytokines (IL-6, FLT3L, SCF, and Thrombopoietin at 100 ng/mL and IL-3 at 10 ng/mL, PeproTech) for 2 days. They were then transduced by retroviral supernatants of *KIT* D816V, D816Y, WT *KIT*, and GFP controls together with 8 μg/mL Polybrene (Sigma-Aldrich) or coculture with stable retrovirus-producing lines of WT *KIT* or the D816 mutants. Two days later, BM cells were harvested and sorted by FACS for GFP+ BM cells.

Transplantation of BM cells to recipient mice

GFP+ BM cells (1×10^6) were transplanted to recipient mice using retro-orbital injection. Two to 4 weeks later, peripheral blood (PB) was taken from mice for FACS analysis of the GFP+ cells in PB. For limiting dilution transplantation of leukemia cells, 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2, 10, and 1 live leukemia spleen cell(s) from terminal leukemic mice [4 donors of *Cbfb*[+/56m]; *Tg(Mx1-Cre)/KIT 816*[Y/V] and 2 donors of *Cbfb*[+/56m]; *Tg(Mx1-Cre)*] were transplanted to recipient mice using retro-orbital injection. They were monitored daily for any abnormal behavior or physiologic changes, and FACS analysis of PB cells was done every 2 weeks after transplantation.

Analysis of PB and leukemia samples from transplanted mice

The PB cells were stained with anti–mouse antibodies for CD3, CD4, CD8, B220, CD117 (c-KIT) Gr-1, Mac-1, and Sca-1 (BD Biosciences). Blood smears were performed at the same time. Mice were killed at the end point according to our protocol. Leukemic spleen cells were stained with anti–human CD117 antibody (BD Biosciences). Histology sections of spleen, liver, kidney, lung, brain, and sternum were analyzed to confirm the leukemia filtration by a mouse pathologist at the Diagnostic & Research Service Branch of the National Institutes of Health (L.B.).

Colony-forming assay of BM cells after transduction with retroviral vectors

BM cells were harvested from WT and *Cbfb*[+/56m]; *Tg(Mx1-Cre)* littermates 2 weeks after pI:pC treatment. BM cells were then transduced with the retroviruses and sorted by FACS. GFP+ BM cells were cultured in M3434 methocellulose (StemCell Technologies) for colony formation assay following the manufacturer's protocol. On days 7 and 12, colonies were classified, and colony numbers were counted according to manufacturer's protocol.

Treatment of primary leukemia cells with PKC412

Primary leukemia cells were seeded in 24-well culture plates (1×10^6 live cells/mL), and PKC412 (LC Laboratories) was added to each well at final concentrations of 0.01, 0.1, and 1μM (dissolved in DMSO). The cells were incubated at 37°C for 17 hours and then were harvested and stained with trypan blue. Live cells were counted using a hemacytometer, and the numbers were normalized relative to control DMSO-treated samples.

Western blot analysis of leukemia samples

Spleen tissue from leukemic mice (frozen at −80°C) was homogenized in radioimmunoprecipitation assay lysis buffer (Millipore) with protease inhibitors (Roche Applied Science), and protein concentration was determined (DC Protein Assay; Bio-Rad Laboratories). Proteins were separated by 4% to 12% SDS-PAGE gel (Invitrogen) electrophoresis. The proteins were then transferred to polyvinylidene difluoride membranes (Invitrogen) for Western blot analysis. Antibodies to regular and phosphorylated p44/42 MAPK, Stat3 (S717, Y705), Stat5 (Y694), and AKT (S473; Cell Signaling Technology) were used to probe the membranes. The ECL detection kit and Hyperfilm ECL (GE Healthcare) films were used to detect the bound antibodies.

Mitotic cell counting of spleen sections from leukemia mice

Mitotic cell counts were taken at 3 areas: at each end of the spleen and in the middle. In each of the 3 areas, the mitotic cells were counted in 10 fields at ×600 magnification. For the average mitoses of each area, the numbers were added and divided by 10. For the overall average for the spleen, the 3 areas were added; the number divided by 3, and the result was rounded up or down to the nearest integer.

BrdU and annexin V staining of transduced/leukemia BM cells

Forty-eight hours after retroviral transduction, BM cells in culture were treated with BrdU (10μM; BD Biosciences) for 30 minutes and then harvested and stained with antibodies against CD3ε, CD4, CD8, B220, TCRβ, NK1.1, Gr1, Mac1; KIT, and Sca-1 (BD Biosciences). After staining, cells were separated into 2 aliquots and following the staining protocol for annexin V (BD Biosciences) and BrdU. A BD LSRII flow cytometer was used to acquire the cells, and FlowJo Version 9.4.10 software (TreeStar) was used for data analysis. Because the GFP+/lin−/KIT+/Sca-1+ cell population was very small, we gated on lin−/GFP+ (transduced cells) and lin−/GFP− (untransduced cells) cell populations for annexin V and BrdU analysis.

Leukemic mice were given BrdU (1 mg) via the intraperitoneal injection 1 hour before euthanasia. BM cells were harvested from femurs and stained as described in the preceding paragraph.

Clonality analysis

EcoRI-digested genomic DNA from *KIT* leukemia spleen cells was separated with gel electrophoresis and transferred to Hybond XL nylon membranes (GE Healthcare) following the manufacturer's protocol. A probe of 600-bp GFP sequence was labeled with α-^{32}P-dCTP using Rediprime II random labeling system (GE Healthcare) and hybridized to the membranes overnight. After wash, the membranes were exposed to phosphor screen for 3 to 4 hours and then read by a FLA-5100 phosphorimager/fluorescent scanner using software IR FLA-5000 series Version 1.01 (Fujifilm).

Results

Generation of stable retrovirus-producing lines

Single GFP$^+$ cells transduced with the retroviral vectors were sorted into the wells of 96-well plates, expanded, and then virus titers were measured. Two to 12 lines of each virus were selected with their titers more than 1×10^6 particles/mL. These lines were derived from either PGE86 cells or Ecopack-293 cells. We compared the efficiency of 1 cell line from Ecopack-293 and 1 cell line from PGE86 of D816Y with similar titer (1×10^7/mL), for transducing WT BM cells. The transduction efficiency for Ecopack-293 D816Y was 10% and that for PGE86 D816Y was 40%. Therefore, PGE86-producing cell lines have been used for our subsequent experiments.

Transduction efficiency of BM cells by retrovirus

We found that coculturing of BM cells with stable virus-producing cell lines gave robust and efficient transduction, resulting in 34% to 77% of the transduced BM cells being GFP$^+$ (supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). The transduction efficiency of *Cbfb$^{+/56m}$; Tg(Mx1-Cre)* BM cells varied from 8% to 78.6% among different experiments using different *KIT* vectors (supplemental Table 1; supplemental Figure 1). The efficiency was determined not only by the titers of the retroviruses but also the state of the BM cells after culturing with cytokines for 2 days.

Effects of *KIT* overexpression on colony-forming ability of bone marrow cells in vitro

To assess the effect of coexpressing *CBFB-MYH11* and mutant *KIT* on hematopoiesis, BM cells were transduced with retroviral vectors for WT and D816Y/V-mutated *KIT* and then plated for colony-forming assay. We found that the total colony numbers were decreased ($P < .01$) in all *KIT*-transduced BM cells, from both WT and *Cbfb$^{+/56m}$; Tg(Mx1-Cre)* mice (Figure 1A). Expression of *Cbfb-MYH11* increased the number of total colonies, as we reported previously.[32] Significantly, the effect of colony number reduction by *KIT* was attenuated by *Cbfb-MYH11*: 7-fold reduction in WT BM cells versus 1.6-fold reduction in *Cbfb-MYH11* BM cells (Figure 1B). In addition, there was a decrease in the number of BFU-E (compared with GFP-transduced BM cells; $P < .01$) and an increase of CFU-GEMM (compared with those transduced with WT *KIT*; $P = .01$) in cultures of *Cbfb$^{+/56m}$; Tg(Mx1-Cre)* BM cells transduced with D816V/Y (Figure 1C). These results suggest that D816 KIT mutants may cause an accumulation of early myeloid progenitors.

KIT expression is toxic to lin$^-$ BM cells

Both WT and *Cbfb$^{+/56m}$; Tg(Mx1-Cre)* lin$^-$ BM cells exhibited greatly increased cell death after transduction with human *KIT* (both WT and 816 mutants; Figure 1D). Consistent with the data in Figure 1B, *KIT*-transduced *Cbfb$^{+/56m}$; Tg(Mx1-Cre)* lin$^-$ BM cells showed less cell death compared with WT lin$^-$ BM cells ($P = .05$; Figure 1D), especially with the 816 mutants (Figure 1E). However, there is no significant difference in cell death between WT KIT and 816 mutant KIT, for either *Cbfb$^{+/56m}$; Tg(Mx1-Cre)* or WT lin$^-$ BM cells. Overall, the data suggest that *KIT* is toxic to lin$^-$ BM cells, and this effect is more severe against WT than against *Cbfb$^{+/56m}$; Tg(Mx1-Cre)* cells.

D816Y mutants enhance the proliferation of lin$^-$ BM cells

We assessed the proliferation rate of KIT-transduced lin$^-$ BM cells by BrdU staining. For both WT and *Cbfb$^{+/56m}$; Tg(Mx1-Cre)* cells, there is a significant increase of proliferation by D816Y KIT transduction than by WT KIT transduction (Figure 1F). In contrast, the D816V mutation did not affect the proliferation rate of the transduced cells.

816-KIT transduced BM cells contribute to all lineages in PB

WT BM cells transduced with WT, *KIT* D816 mutations, or GFP vector alone were transplanted to irradiated congenic C57BL/6 × 129/SvEv F1 hybrid mice. GFP$^+$ cells were found in the PB as early as 1 week after transplantation, which remained detectable 12 months after transplantation (the end point of the experiments). This result indicates that the retroviral vectors transduced not only progenitor cells but also hematopoietic stem cells. T (CD3$^+$ or CD4$^+$ or CD8$^+$), B (B220$^+$), and myeloid cells (Mac1$^+$ or Gr1$^+$) derived from the transplanted cells were all detectable in the recipient mice (Figure 2A-B), suggesting that the *KIT* D816 mutations do not impair the differentiation potential of WT BM cells.

The *Cbfb$^{+/56m}$; Tg(Mx1-Cre)* BM cells also were transduced with WT or D816 Y/V KIT retroviruses and then transplanted into recipient mice. In the PB of the transplanted mice, GFP$^+$ cells also contributed to all lineages (Figure 2C-D and supplemental Figure 2). However, when leukemia started to develop (Figure 2E), GFP$^+$ cells were mostly Mac1$^+$, Gr-1$^+$, or Mac1$^+$Gr-1$^+$ myeloid cells with few or no B and T cells (Figure 2E), suggesting blockage of the lymphoid differentiation during leukemic transformation. This result correlates with previously published studies demonstrating blockage of T- and B-cell development by *CBFB-MYH11*[32,35] and suggests that the *KIT* D816 mutations do not impair the contribution of WT BM cells to mature blood cells in the PB in the early stages. However, we cannot rule out hidden differentiation defects in the bone marrow.

KIT D816Y/V mutants cooperate with *Cbfb-MYH11* for leukemia initiation

We monitored the disease progression with FACS analysis of GFP$^+$ cells in the PB. In mice that received WT BM cells transduced with *KIT*, the percentages of GFP$^+$ cells dropped greatly 3 months after transplantation (Table 1). In mice that received the transduced *Cbfb$^{+/56m}$; Tg(Mx1-Cre)* BM cells, the decreasing of GFP$^+$ cells was not as dramatic as in the WT BM cells at 12 weeks (Table 2). However, for those mice that did not eventually develop leukemia, the percentages of GFP$^+$ cells in PB also dropped to very low levels. These in vivo findings are consistent with the in vitro data demonstrating higher toxicity of human KIT expression to WT BM cells (Figure 1D-E).

The transplanted mice were observed for up to 12 months after transplantation. By 5 months after transplantation, 70% mice transplanted with *Cbfb$^{+/56m}$; Tg(Mx1-Cre)/KIT^{D816Y}* (N = 13) and 50% mice transplanted with *Cbfb$^{+/56m}$; Tg(Mx1-Cre)/KIT^{D816V}* (N = 10) BM cells died from leukemia (Figure 3A). Sixty to 80% of mice died from leukemia in these 2 groups within a year (Figure 3A). Alternatively, no mice transplanted with *Cbfb$^{+/56m}$; Tg(Mx1-Cre)/KIT* or *Cbfb$^{+/56m}$; Tg(Mx1-Cre)/GFP* BM cells developed leukemia during the 1 year period (N = 18; Figure 3A). In addition, WT BM cells transduced with *KIT$^{D816Y/V}$* did not cause leukemia within the 12 months after transplantation (N = 15; Figure 3A). These results suggest that *KIT$^{D816Y/V}$* does not cause
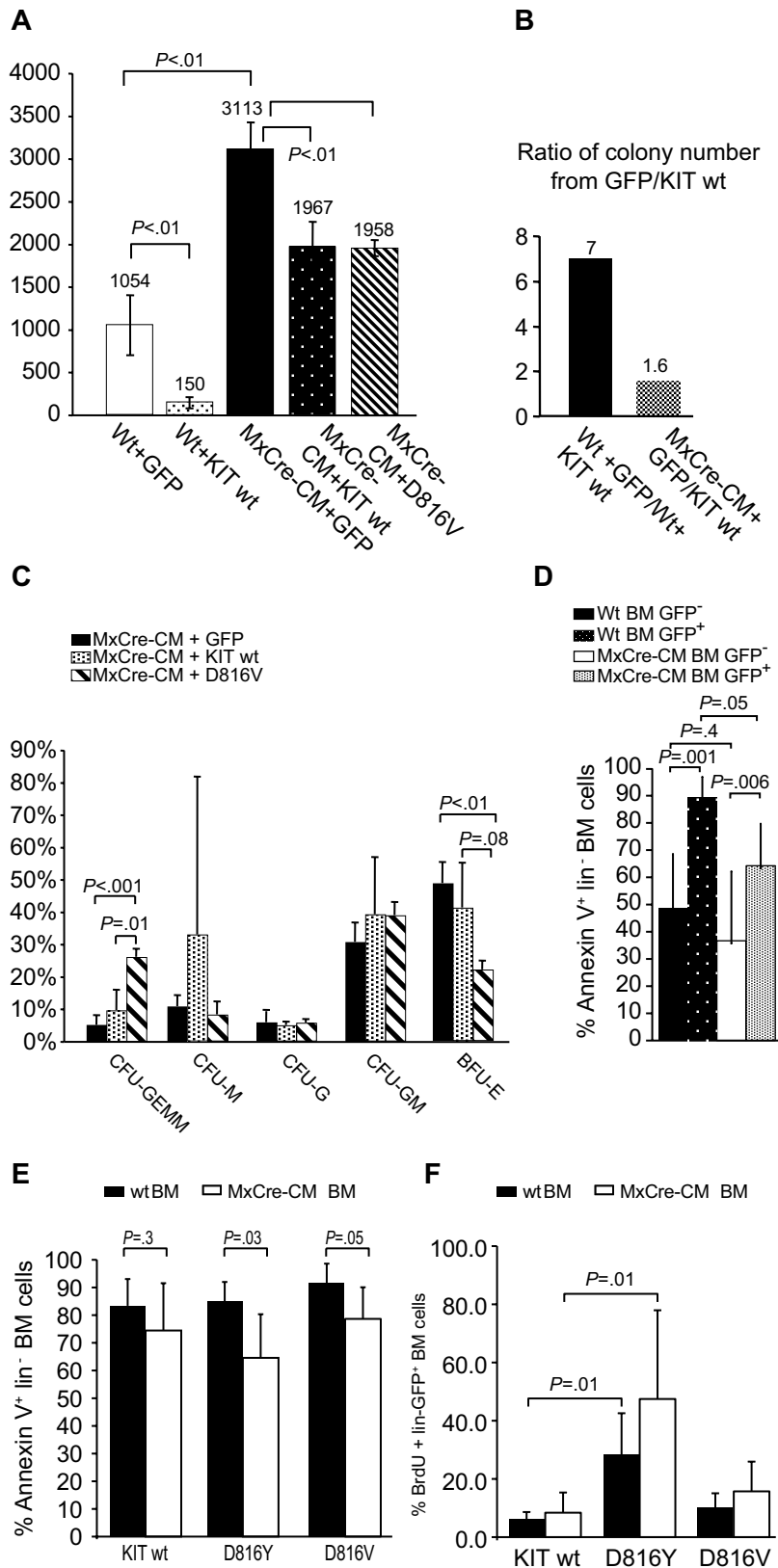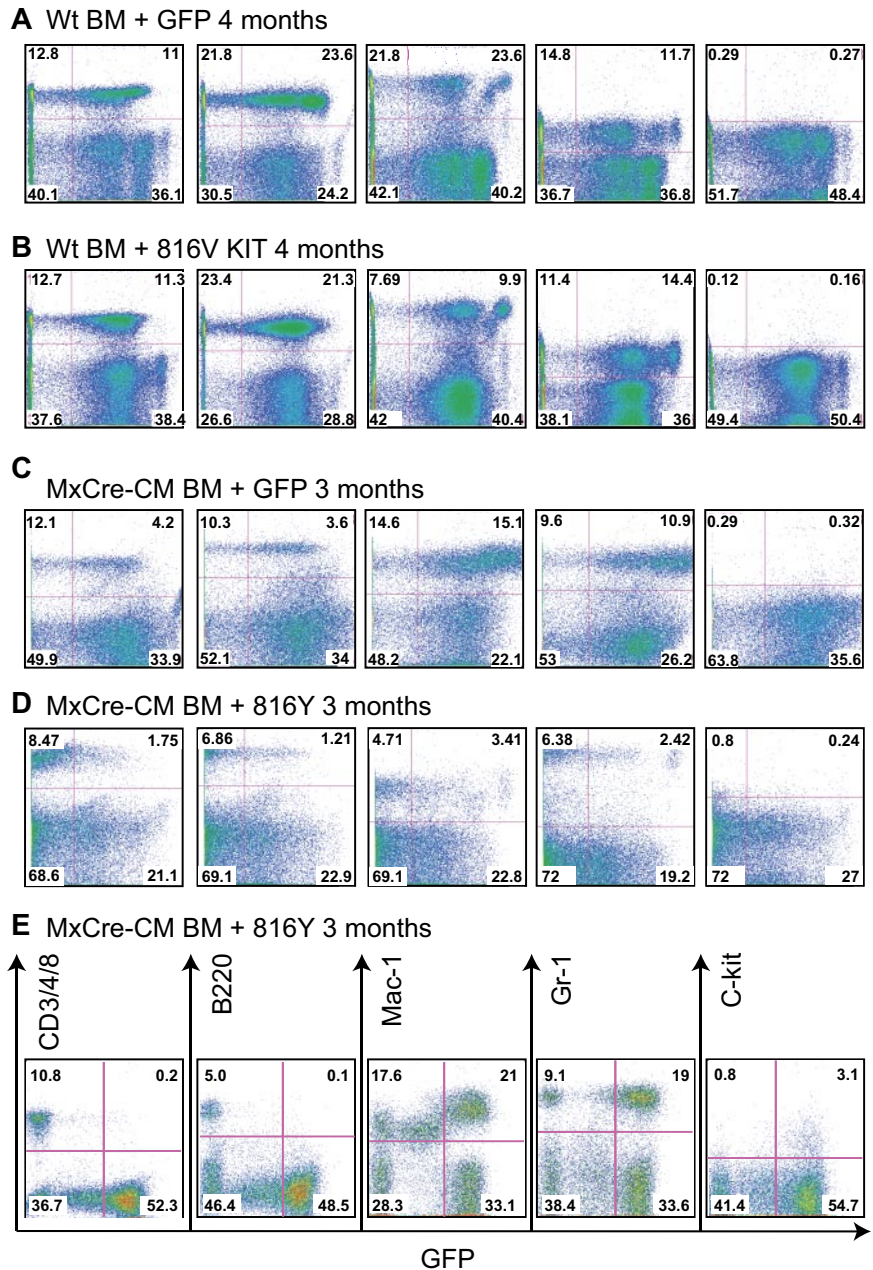
Figure 1. Differentiation potential of BM cells transduced with D816Y/V KIT and control vectors. (A) Total numbers of colonies from 10^5 cultured WT (wt) or *Cbfb$^{+/56m}$; Tg(Mx1-Cre)* BM cells transduced with retroviral vectors containing WT *KIT*, *KIT^{D816V}* or GFP and sorted for GFP expression. Wt + GFP indicates WT BM cells transduced with GFP vector alone; Wt + KIT wt, WT BM cells transduced with WT *KIT* vector; and MxCre-CM + GFP, *KIT* wt, or D816Y, *Cbfb$^{+/56m}$; Tg(Mx1-Cre)* BM cells transduced with GFP, Wt *KIT*, or *KIT^{D816V}* vector, respectively. The numbers above the bars are average colony numbers for the group. (B) Ratio of total colony numbers from 10^5 cultured WT or *Cbfb$^{+/56m}$; Tg(Mx1-Cre)* (MxCre-CM) BM cells transduced with GFP versus those transduced with WT *KIT*. (C) Types of colonies formed from transduced *Cbfb$^{+/56m}$; Tg(Mx1-Cre)* BM cells. CFU indicates colony-forming unit; G, granulocyte; E, erythrocyte; MM, monocyte and megakaryocyte; and GM, granulocyte and monocyte. All BM cells were sorted for GFP$^+$ cells before plating. (D) Comparison of annexin V$^+$ cell numbers (percentage) between the *KIT* (both WT and 816 mutants)–transduced (GFP$^+$) and untransduced (GFP$^-$) BM lin$^-$ cell populations, for both WT and *Cbfb$^{+/56m}$; Tg(Mx1-Cre)*. (E) Comparison of annexin V$^+$ cell numbers (percentage) between the WT and *Cbfb$^{+/56m}$; Tg(Mx1-Cre)* BM lin$^-$ cell populations that were transduced with WT *KIT* or *KIT$^{816Y/V}$*. (F) BrdU$^+$ cells in *KIT* (WT and 816 mutants)–transduced BM cells.

Figure 2. Lineage contribution of *KIT*-transduced bone marrow cells in transplanted mice. PB cells were collected at the indicated times after transplantation from the recipient mice, stained for the indicated markers, and analyzed by FACS. (A) WT BM cells transduced with GFP. (B) WT BM cells transduced with KIT^{D816}. (C) $Cbfb^{+/56m}$; *Tg(Mx1-Cre)* BM cells transduced with *GFP*. (D) $Cbfb^{+/56m}$; *Tg(Mx1-Cre)* BM cells transduced with KIT^{D816Y}. (E) PB from a mouse that was starting to develop leukemia that was transplanted with $Cbfb^{+/56m}$; *Tg(Mx1-Cre)* BM cells expressing KIT^{D816Y}. CD3/4/8 indicates combination staining of CD3, CD4, and CD8.

A Wt BM + GFP 4 months

B Wt BM + 816V KIT 4 months

C MxCre-CM BM + GFP 3 months

D MxCre-CM BM + 816Y 3 months

E MxCre-CM BM + 816Y 3 months

leukemia by itself but cooperates with *Cbfb-MYH11* for leukemogenesis in this mouse model.

The morphology of the leukemia cells showed a progenitor cell appearance with large nuclei and scant cytoplasm, and mitotic cells were easily detectable, suggesting active proliferation of these cells (Figure 3B). The mitotic cell count from the spleen sections of leukemia mice showed a significant increase in the $Cbfb^{+/56m}$; *Tg(Mx1-Cre)*/KIT^{816Y} mice (N = 5) than the $Cbfb^{+/56m}$; *Tg(Mx1-Cre)* mice

(N = 5; Figure 3C). There were significantly higher percentages ($P = .05$) of lin⁻ BM cells stained positive for BrdU from leukemic mice carried *KIT* mutations (48% of the GFP⁺/lin⁻ cells; N = 5) than from leukemic mice with only *Cbfb-MYH11* (28% of the c-kit⁺/lin⁻ cells; N = 5). FACS staining showed that they were mainly c-kit⁺ and lineage-negative cells, with minimal myeloid differentiation (Figure 3D). The leukemia cells infiltrated BM, spleen, liver, kidneys, lungs, heart, and meninges of the spinal cord

Table 1. GFP level at different time points after transplantation with WT BM cells

Vector	Animal sample size, N	Mean ± SD GFP, %			
		3 wk	12 wk	6 mo	12 mo
GFP	4	61 ± 30	50 ± 29	58 ± 36	47 ± 25 (N = 3)
KIT wt	5	24 ± 8	3 ± 2	5 ± 5	4 ± 3.7 (N = 3)
816Y	3	21 ± 7	0.7 ± 0.2	0.4 ± 0.6	1.5 ± 0.8
816V	6	35 ± 10	1.6 ± 0.9	0.8 ± 0.6	2.5 ± 1.0

Table 2. GFP level at different time points after transplantation with MxCre-CM BM cells (excluding mice that developed leukemia)

Vector	Animal sample size, N	Mean ± SD GFP, %				
		3 wk	8 wk	12 wk	6 mo	12 mo
GFP	4	36 ± 7		30 ± 12	2 ± 2	3 ± 0.4 (N = 2)
KIT wt	5		24 ± 7	33 ± 8	0.8 ± 0.8	2.4 ± 0.7 (N = 4)
816Y	9		48 ± 14	37 ± 29 (N = 7)	3 ± 2 (N = 4)	0.8 ± 0.1 (N = 2)
D816V	9		38 ± 7	42 ± 10	4 ± 6	3.5 ± 2.9 (N = 5)

in all mice examined (supplemental Figure 3). In addition, leukemia cells infiltrated the brain tissue in 2 of the *Cbfb*[+/56m]; *Tg(Mx1-Cre)/KIT*[816Y] mice.

To confirm that the transplanted GFP^{+} cells expressed the human KIT proteins encoded by the retroviral vectors, we stained the cells for the human KIT protein by FACS analysis. Human KIT protein was detected on the surface of some of the GFP[+] leukemic spleen cells (Figure 4A) from mice transplanted with *Cbfb*[+/56m]; *Tg(Mx1-Cre)/KIT*[D816Y/V] bone marrow cells. Human *KIT* also was detected on the surface of nonleukemic spleen cells from mice transplanted with *Cbfb*[+/56m]; *Tg(Mx1-Cre)/KIT* bone marrow cells (Figure 4B). The human leukemia cell line ME-1 was used as a positive control for human KIT staining (Figure 4C). However, human KIT was not readily detectable on the PB cells before or after leukemia development.

We also performed Western blot using leukemia tissue from different mice and confirmed that the human KIT protein was expressed abundantly in the *Cbfb*[+/56m]; *Tg(Mx1-Cre)/KIT*[D816Y] but not the *Cbfb*[+/56m]; *Tg(Mx1-Cre)* leukemic cells (Figure 4D).

Accelerated leukemia progression and enrichment of leukemia-initiating cells by the *KIT*[D816Y/V] mutants

To see the effect of *KIT*[D816Y/V] mutants on disease progression, the life span of mice transplanted with leukemia cells carrying *Cbfb-MYH11* and *KIT* D816 mutants [*Cbfb*[+/56m]; *Tg(Mx1-Cre)/KIT*[D816Y/V]] or *Cbfb-MYH11* alone [*Cbfb*[+/56m]; *Tg(Mx1-Cre)*] was measured. As shown in Figure 5A, mice transplanted with leukemia cells carrying *Cbfb-MYH11* and *KIT* D816 mutants had a much shorter average life span of 3.2 weeks (N = 31, from 4 donors) compared with 6.2 weeks (N = 19, from 3 donors) for mice transplanted with *Cbfb-MYH11* leukemia cells.

We then did limiting-dilution transplantation using leukemia cells (total live splenocytes with > 80% of GFP^{+} leukemia cells) that carried *Cbfb*[+/56m]; *Tg(Mx1-Cre)/KIT*[D816Y/V] from 4 different donor mice (Figure 5B). For 1 donor (3422-1), all recipient mice died from leukemia with only 10 cells transplanted. For the other 3 donors, all mice that received 1000 cells died from leukemia as well. In contrast, 10[5] cells were needed for the *Cbfb*[+/56m]; *Tg(Mx1-Cre)* leukemia cells (from 2 donors) to achieve 100% leukemia development in the recipient mice (Figure 5B). Moreover, the latencies were much longer for mice transplanted with *Cbfb*[+/56m]; *Tg(Mx1-Cre)* leukemia cells (7-15 weeks) than those with the *Cbfb*[+/56m]; *Tg(Mx1-Cre)/KIT*[D816Y/V] leukemic cells (2-10 weeks).

Overall, the data showed that leukemia development in the recipient mice transplanted with *Cbfb*[+/56m]; *Tg(Mx1-Cre)/KIT*[D816Y/V] leukemia cells was faster and required fewer starting cells. This suggests a higher leukemia-initiating cell (LIC) frequency in the *Cbfb*[+/56m]; *Tg(Mx1-Cre)/KIT*[D816Y/V] leukemic cells.

Clonality analysis of leukemia cells

We performed Southern blot hybridization using DNA from secondarily transplanted mice and found that every tested founder was a specific clone and that the secondarily transplanted mice derived from each founder carried the same retroviral insertions

(Figure 5C). The copy number of the insertions did not correlate with the latency of leukemia development. One mouse with only 1 copy of the mutant *KIT* transgene developed leukemia within 3 months, whereas another mouse with multiple copies developed leukemia ∼ 5 months after transplantation. Furthermore, the monoclonal nature of the first mouse suggests that additional events play important roles in transformation, whereas the polyclonal leukemia emergence in the second mouse suggests that coexpression of *KIT* D816 mutations and *CBFb-MYH11* may be sufficient to initiate leukemogenesis.

Cbfb-MYH11/KIT D816 mutant but not *Cbfb-MYH11* leukemia cells are sensitive to PKC412

Primary leukemia cells with or without *KIT* D816 mutations [*Cbfb*[+/56m]; *Tg(Mx1-Cre)/KIT*[D816Y], N = 4; *Cbfb*[+/56m]; *Tg(Mx1-Cre)*, N = 5] were treated with protein kinase inhibitor PKC412 at various concentrations in culture. *Cbfb*[+/56m]; *Tg(Mx1-Cre)/KIT*[D816Y] leukemia cells showed a dose-dependent decrease in cell viability after PKC412 treatment for 17 hours, reaching more than 50% reduction at 1μM (Figure 5D). Alternatively, the viability of *Cbfb*[+/56m]; *Tg(Mx1-Cre)* leukemia cells did not change significantly after PKC412 treatment. The data suggest that *KIT* D816 mutations sensitize the *Cbfb-MYH11* leukemia cells to this protein kinase inhibitor.

MAPK and Stat3 pathways are activated in leukemic cells

KIT[D816 V/Y] mutations cause constitutive activation of *KIT* that can then activate several downstream signaling pathways that are potentially involved in the transformation of *Cbfb-MYH11* BM cells.[28,36,37] Therefore, we checked the downstream signaling pathways of *KIT* using leukemia cells from our mouse models. We found that p44/42 MAPK was strongly phosphorylated in the *Cbfb*[+/56m]; *Tg(Mx1-Cre)/KIT*[D816Y] leukemic samples that we examined (Figure 6). Interestingly, p44/42 MAPK also was phosphorylated in *Cbfb-MYH11* leukemia mouse tissue without the transduced *KIT* mutants. In addition, serine but not tyrosine phosphorylation of Stat3 was strongly activated in all leukemia cells tested. In contrast, the phosphorylation level of Stat5 and AKT was low in most of the leukemia samples, especially those with *Cbfb*[+/56m]; *Tg(Mx1-Cre)/KIT*[D816Y] (Figure 6).

Discussion

KIT mutations have been found in leukemia[5,38-40] and solid tumors, such as gastrointestinal stromal tumor,[41] breast cancer,[42] and lung cancer.[43] Two specific mutations affecting the aspartic acid residue 816 of KIT, D816Y and D816V, have been reported in CBF AML cases and are potential indicators of poor clinical outcomes.[6,21,25,44] Here, we report a mouse leukemia model that coexpresses the human D816V/Y-KIT and a knockin allele of *Cbfb-MYH11* (*Cbfb*[+/56m]). Similar to what we reported previously, *Cbfb-MYH11* by itself or with WT
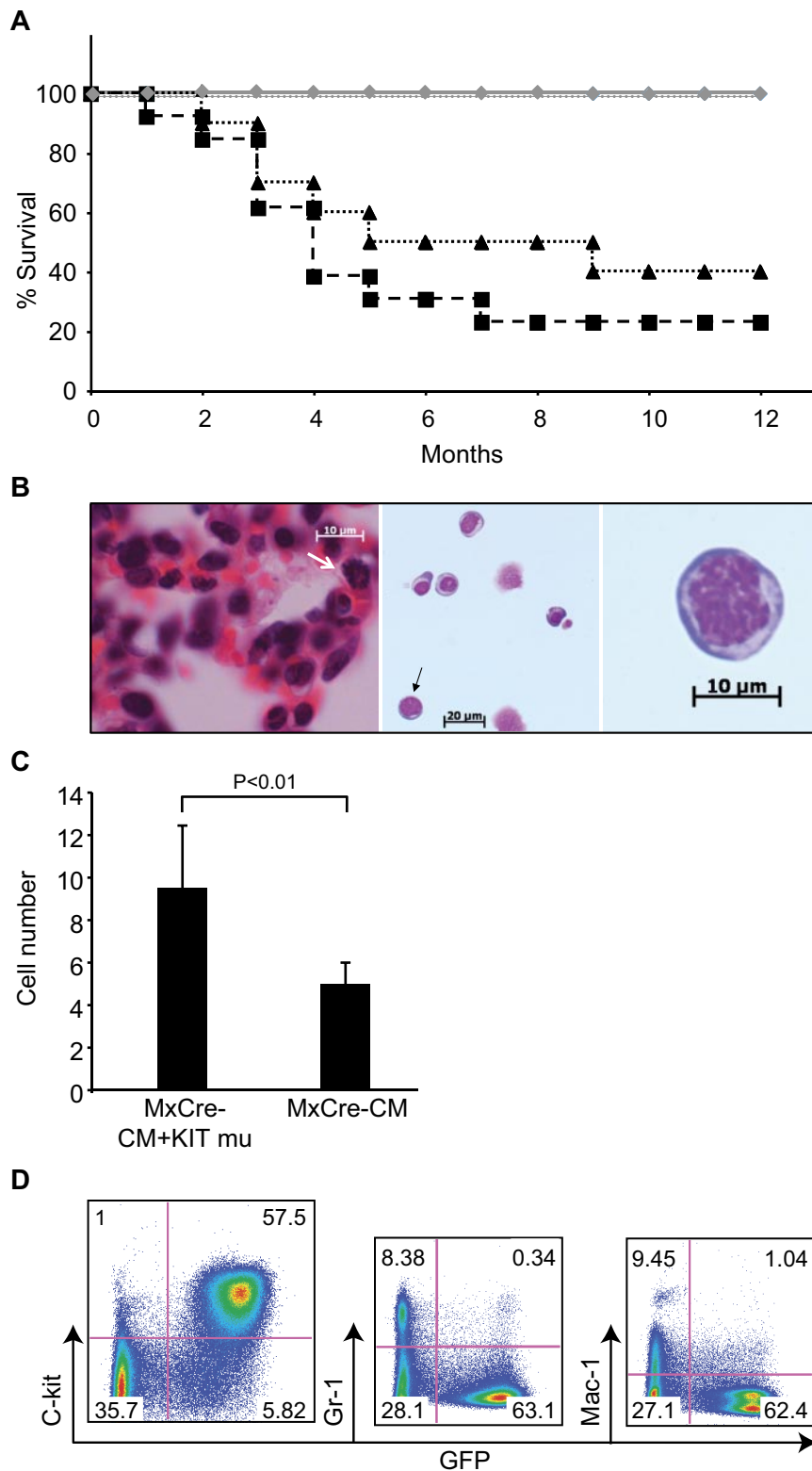
Figure 3. Leukemia development in mice transplanted with *Cbfb*[+/56m]; Tg(Mx1-Cre)/*KIT*[D816Y/V] BM cells. (A) Survival curves of transplanted mice. Black dashed line with squares indicate mice transplanted with *Cbfb*[+/56m]; Tg(Mx1-Cre)/*KIT*[D816Y] BM cells (N = 13); black dotted line with triangles, mice transplanted with *Cbfb*[+/56m]; Tg(Mx1-Cre)/*KIT*[D816V] BM cells (N = 10); gray line with diamonds, mice transplanted with *Cbfb*[+/56m]; Tg(Mx1-Cre)/*KIT* or *Cbfb*[+/56m]; Tg(Mx1-Cre)/GFP BM cells (N = 18); and gray dotted line, mice transplanted with *KIT* (both WT and D816 mutants)–transduced WT BM cells (N = 15). (B) H&E stained lung tissue (left) and Wright Giemsa–stained leukemia cells in PB (middle and right, respectively) from a *Cbfb*[+/56m]; Tg(Mx1-Cre)/*KIT*[D816Y] leukemic mouse. White arrow in the left panel indicates a cell in mitosis. Black arrow in the middle panel indicates a cell that has been enlarged in the right panel. (Imager D2, Zeiss; 20×, 40×, and 63× plan-Apochromat objective lenses; AxioVision 4.8 acquision software; AxioCam HRC, Zeiss). (C) Mitotic cell count from leukemic spleen sections of *Cbfb*[+/56m]; Tg(Mx1-Cre) mice (MxCre-CM; N = 3) and *Cbfb*[+/56m]; Tg(Mx1-Cre)/*KIT*[D816Y] (MxCre-CM + KIT mu; N = 3) mice. (D) FACS analysis of PB cells from a leukemic mouse transplanted with *Cbfb*[+/56m]; Tg(Mx1-Cre)/*KIT*[D816Y] BM cells.
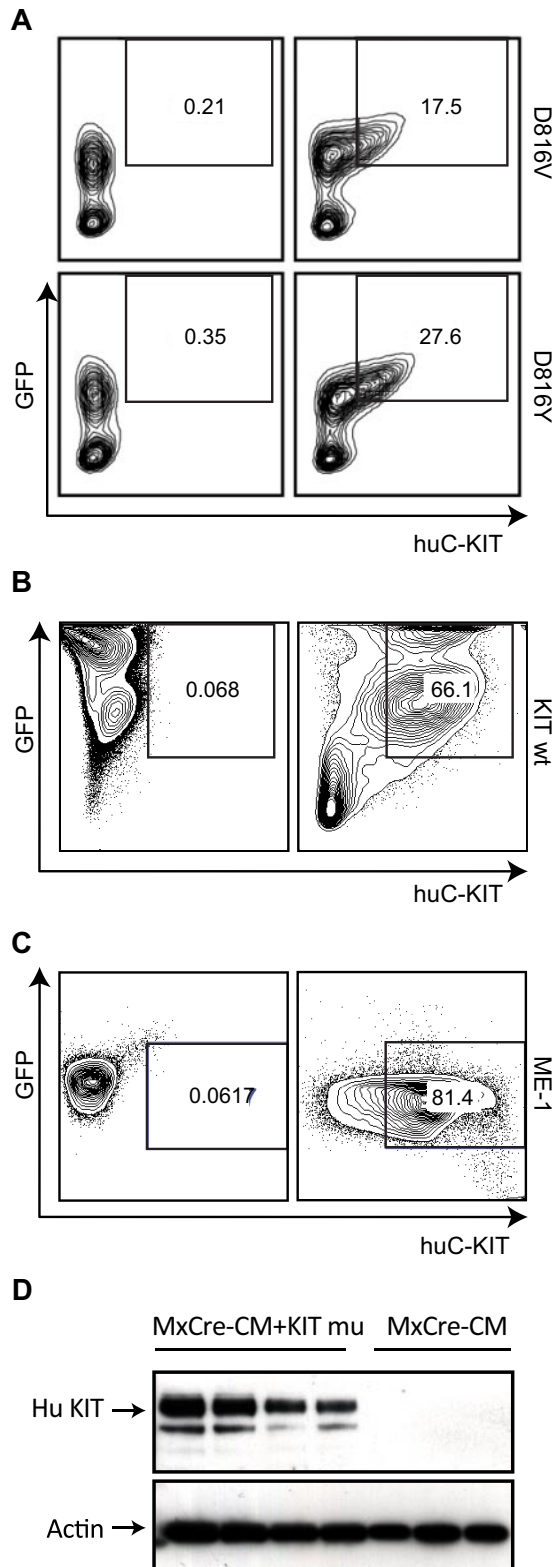
Figure 4. Expression of human KIT protein in the transplanted mice. (A) Leukemic cells from spleens of mice transplanted with *Cbfb*[+/56m]*; Tg(Mx1-Cre)/KIT*[D816V] or *Cbfb*[+/56m]*; Tg(Mx1-Cre)/KIT*[D816Y] BM cells. (B) Splenocytes from a mouse (nonleukemic) transplanted with *Cbfb*[+/56m]*; Tg(Mx1-Cre)/KIT* wt BM cells. (C) Leukemia cell line ME-1. The cells in panels A-C were stained with an anti–human KIT antibody and analyzed by FACS. In panels A through C, the cells in the left-hand panels were unstained and those in the right-hand panels were stained with the anti–human KIT. Cells in the boxes are GFP[+] and human KIT[+]. (D) Western blot of leukemic spleen cells from *Cbfb*[+/56m]*; Tg(Mx1-Cre)/KIT*[D816Y] (MxCre-CM + KIT mu) and *Cbfb*[+/56m]*; Tg(Mx1-Cre)* (MxCre-CM) mice using the anti–human KIT antibody. Actin was probed as the loading control.

KIT does not cause leukemia within 12 months after transplantation. In contrast, coexpressing *Cbfb-MYH11* and D816Y or D816V *KIT* led to leukemia development in 60% to 80% of the mice in 9 months (Figure 3). Therefore, our data demonstrate cooperation between *Cbfb-MYH11* and mutant *KIT* during leukemogenesis. Moreover, in the presence of the mutant *KIT*, the disease progression was more aggressive, and the number of LICs was increased, as shown by shortened life span of secondarily transplanted mice and by limiting dilution transplantation.

KIT is a potent growth factor for hematopoietic progenitor cells, and its expression is normally decreased in more differentiated cells.[45] The reduced colony numbers observed in our study (Figure 1A) would have resulted from massive cell death caused by the expression of *KIT*, either WT or mutant, from the retroviral vectors (Figure 1D). This demonstrates that *KIT* is toxic to BM cells. However, *Cbfb-MYH11*–expressing BM cells were more resistant to the *KIT* toxicity (Figure 1D-E), allowing the cells coexpressing both to survive and undergo leukemic transformation. Expressing the D816Y mutant in BM cells also led to an increased proliferation (Figure 1F) that also may have contributed to leukemia development in these mice. In addition, BM lin^{−} leukemia cells coexpressing D816Y/V KIT and *Cbfb-MYH11* proliferated faster than leukemia cells with only *Cbfb-MYH11* (48% vs 28%), which correlated with more aggressive disease and higher LIC frequency for the *Cbfb*[+/56m]*; Tg(Mx1-Cre)/KIT*[D816V/Y] leukemia.

Leukemia did take 2 to 9 months to develop in the recipient mice transplanted with *Cbfb*[+/56m]*; Tg(Mx1-Cre)* BM cells expressing mutant KIT transgenes. Such protracted and prolonged latency would suggest that additional mutations are required for full leukemic transformation. Clonality analysis showed that some leukemia samples had as few as 1 copy of *KIT* integration, whereas others had multiple copies of *KIT* insertion. These observations also suggest that additional events might contribute to leukemogenesis, especially those with only 1 copy of *KIT*. Moreover, we used pI:pC to induce the expression of *Cbfb-MYH11*, which is not 100%. This also may have contributed to the long latency and incomplete penetrance of leukemia.

Interestingly, overexpression of mutant *KIT*, but not the WT *KIT*, in the BM cells derived from *Cbfb*[+/56m]*; Tg(Mx1-Cre)* mice led to significant increases of CFU-GEMM, which contains early myeloid progenitor cells (Figure 1C). This effect may lead to an expansion of immature myeloid progenitors in the bone marrow, the potential target cells for transformation by *Cbfb-MYH11*. Although we did not investigate accumulation of myeloid cells in the BM of transplanted mice, we did observe Mac1^{+}/Gr1^{+}/GFP^{+} myeloid cells in the PB in early leukemic mice (Figure 2E). Therefore, this expansion might have contributed to the accelerated leukemia development and increased numbers of LICs in the mice coexpressing *Cbfb-MYH11* and mutant *KIT* (Figures 3A and 5).

It has been proposed that SCF and *KIT* play important roles for the survival and proliferation of cancer stem cells.[46] Our data support the notion that activated KIT proteins increase the proliferation of transduced BM cells and leukemia cells (Figures 1F and 3C) and could be a mechanism for the observed higher frequencies of LICs (Figure 5). KIT activation may provide a proliferative or survival signal to the LICs in our model, through signaling pathways such as MAPK and Stat3 that were strongly activated in the *Cbfb-MYH11* leukemia cells carrying *KIT* D816Y/V mutations. Interestingly, MAPK and Stat3 also were phosphorylated in *Cbfb-MYH11* leukemia cells without mutant *KIT*. These data suggest that the MAPK and Stat3 pathways play important roles in *Cbfb-MYH11* leukemia cells, with or without *KIT* mutations.

Figure 5. Accelerated leukemia development and increased LICs by *KIT*[D816V/Y]**.** (A) Disease latency in secondarily transplanted mice with leukemic cells of *Cbfb*[+/56m]*; Tg(Mx1-Cre)* (black diamonds) and *Cbfb*[+/56m]*; Tg(Mx1-Cre)/KIT*[D816V/Y] (gray squares). Each recipient mouse was given 10^6 leukemia cells. (B) Leukemia incidences from limiting dilution transplantation with *Cbfb*[+/56m]*; Tg(Mx1-Cre)/KIT*[D816Y] leukemia cells (N = 4; black lines) and *Cbfb*[+/56m]*; Tg(Mx1-Cre)* leukemia cells (N = 2; gray lines). Numbers of cells injected per mouse are shown on x-axis. N = 5 in each dose group. (C) Genomic Southern blot hybridization for clonality analysis. Each lane is from one secondarily transplanted mouse. Panels A, B, and C are 3 different donors (lanes not labeled had degraded DNA). (D) Leukemia cell viability in culture after PKC412 treatment. Gray line indicates *Cbfb*[+/56m]*; Tg(Mx1-Cre)/KIT*[D816Y] mice (N = 4); and black line, *Cbfb*[+/56m]*; Tg(Mx1-Cre)* mice (N = 5; *$P < .01$).
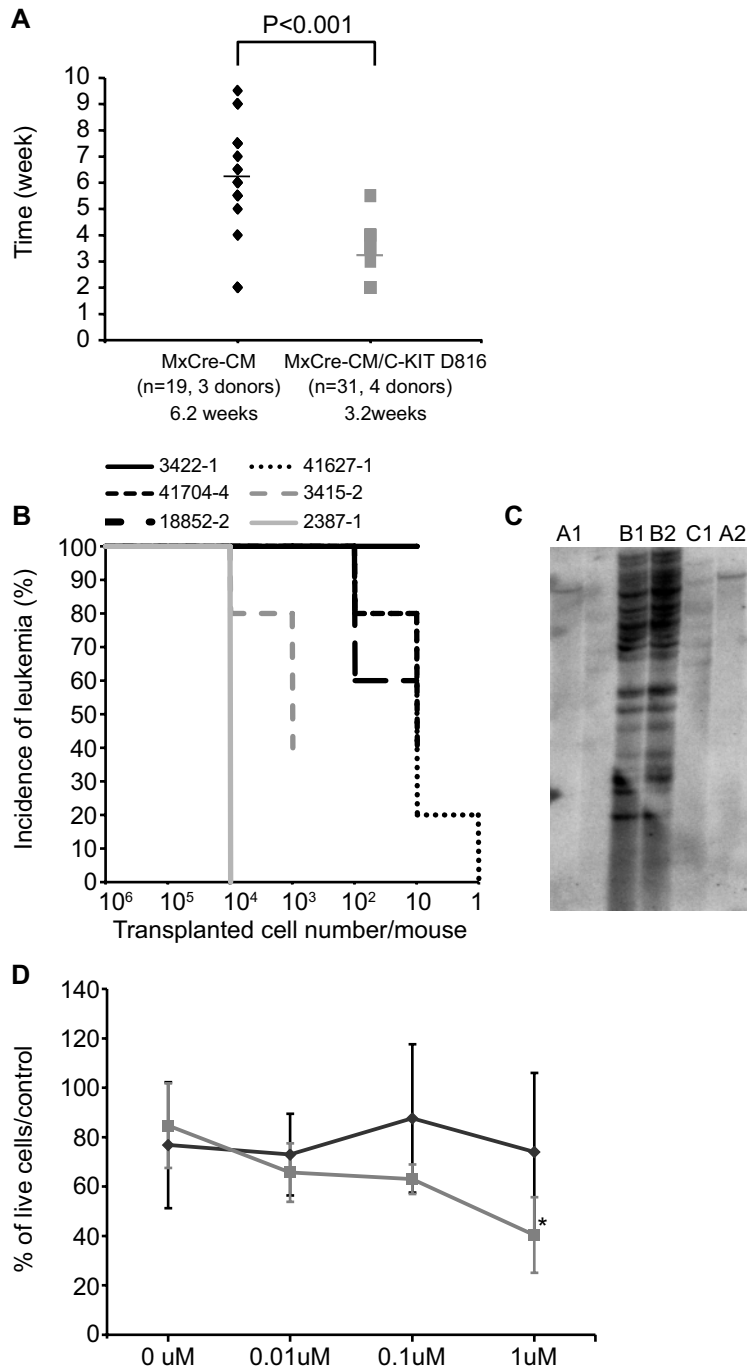
PKC412 is a broad protein kinase inhibitor that has been reported to be effective for *KIT*[D816] mutations.[28,30] PKC412 reduced viability of *Cbfb*[+/56m]*; Tg(Mx1-Cre)/KIT*[D816Y] leukemia cells, whereas it did not affect *Cbfb*[+/56m]*; Tg(Mx1-Cre)* leukemia cells (Figure 5D). This suggested that even though the leukemia cells from both genotypes had activated MAPK and Stat3, the causative signal(s) in the *KIT*[D816Y] leukemia cells were not activating mutations in tyrosine kinase genes. Inhibitors of MAPK and Stat3 pathways might be useful for treating CBF leukemia and should be tested in the future.

Here, we used human *KIT* cDNA constructs that have been considered problematic in mouse models in previous reports.[47,48] Xiang et al reported that human *KIT*[D816V] did not transduce mouse cells well,

probably because the protein was blocked at the endoplasmic reticulum in a species-specific manner in mouse cells and did not induce disease in mice.[47] Conversely, mouse *KIT*[D814V] or a human-mouse hybrid *KIT*[D816V] protein with mouse extracellular or transmembrane domains functioned properly and could induce rapid fatal myeloproliferative diseases.[47] In a more recent study, HyC-*KIT*[N822K] and D816V, as well as juxtamembrane mutants HyC-*KIT*[571 + 14] and 557-558Del, could transform murine 32D cells to cytokine-independent growth, and coexpressing human–mouse hybrid *KIT*[N822K] and the AML1-ETO fusion gene led to the development of fatal AML.[48] In several other reports, however, human *KIT*[D816V] was found to be functional in mouse cells in culture,[28,49,50] and induced factor-independent growth of Ba/F3 in 2 of the studies.[28,49]
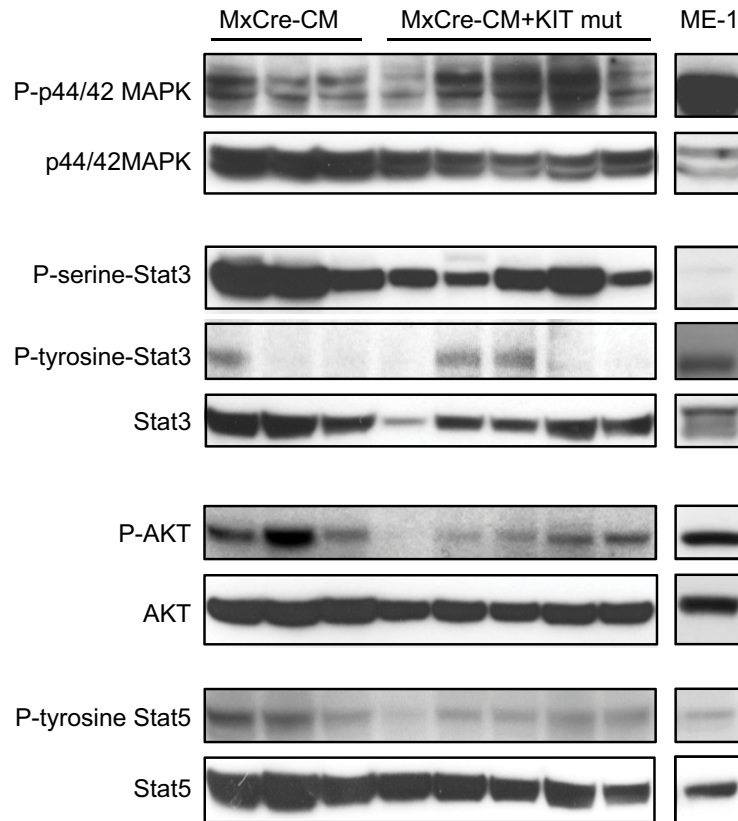
Figure 6. Phosphorylation status of MAPK/Stat3/Stat5/Akt in leukemia cells. Western blot analysis of leukemia cells from *Cbfb$^{+/56m}$; Tg(Mx1-Cre)/KIT^{D816Y}* (MxCre-CM + KIT mut) mice and *Cbfb$^{+/56m}$; Tg(Mx1-Cre)* mice (MxCre-CM) and human ME-1 cells for the indicated proteins and their phosphorylated products.

During our experiments, we also encountered significant problems with low transduction efficiency with human *KIT* constructs. However, we found that producing viral particles using the mouse packaging cell line GP+E-86[33] rather than using human cells, such as 293, significantly improved the efficiency of transducing mouse BM cells. In contrast, our observation that the human KIT proteins could be easily detected by Western blot, but only in a small fraction of splenic leukemia cells by FACS (Figure 4), suggests a similar trafficking defect of the human *KIT* protein in mouse cells as described by Xiang et al.[47] Consistent with their results, human *KIT* D816 mutations alone could not induce leukemia or other blood-related diseases in our mouse model. Nonetheless, our data unequivocally demonstrated cooperation between human *KIT*$^{D816V/Y}$ and *CBFB-MYH11* for leukemogenesis. Our data suggest that even though these human KIT D816 mutants do not translocate to the plasma membrane efficiently, they do have oncogenic activity. We found that the mutant KIT proteins not only were less toxic to *Cbfb-MYH11* BM cells but also enhanced the proliferation of these BM cells and must have contributed to leukemogenesis. It is possible that murine versions of the KIT mutants or human–mouse hybrid KIT mutants have higher oncogenic activities and may have worked more efficiently. A comparison between them, which is beyond the scope of this manuscript, will be interesting to perform.

In this mouse model, we have proved that leukemic cells with *Cbfb-MYH11* and *KIT* D816Y/V mutations cause more aggressive disease compared with leukemic cells carry only *Cbfb-MYH11*, which is likely because of increased LICs in the *Cbfb$^{+/56m}$; Tg(Mx1-Cre)/KIT$^{D816V/Y}$* leukemia samples. Cell surface staining indicated that GFP$^+$ leukemia cells are mostly CD117$^+$Sca-1$^-$lin$^-$

cells, suggesting that future treatment should aim at this early progenitor or stem-like cell population.

Authorship

Contribution: L.Z., D.G.G., and P.P.L. designed the research; L.Z., J.J.M., L.A., M. Kirby, S.A., M. Kench, S.H.-M., L.B., and Y.K. performed the research; L.Z., J.J.M., and Y.K. collected the data; L.Z. and P.P.L. analyzed the data; D.G.G. contributed the *KIT* vectors; and L.Z. and P.P.L. wrote the paper.

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