

C/EBP β promotes BCR-ABL-mediated myeloid expansion and leukemic stem cell exhaustion

Yoshihiro Hayashi, MD^{1,2}, Hideyo Hirai, MD, PhD¹, Naoka Kamio, MD¹, Hisayuki Yao, MD, PhD¹, Satoshi Yoshioka, MD^{1,3}, Yasuo Miura, MD, PhD¹, Eishi Ashihara, MD, PhD^{1,4}, Yoshihide Fujiyama, MD, PhD², Daniel G. Tenen, MD^{5,6} and Taira Maekawa, MD, PhD¹

¹Department of Transfusion Medicine and Cell Therapy, Kyoto University Hospital, Kyoto 606-8507, Japan; ²Division of Gastroenterology and Hematology, Shiga University of Medical Science, Shiga 520-2192, Japan; ³Department of Hematology and Oncology, Kyoto University Hospital, Kyoto 606-8507, Japan; ⁴Department of Clinical and Translational Physiology, Kyoto Pharmaceutical University, Kyoto 607-8414, Japan; ⁵Cancer Science Institute, National University of Singapore, Singapore 117456, Singapore; and ⁶Harvard Stem Cell Institute, Boston, MA 02115, USA

Running title: C/EBP β drives BCR-ABL-mediated myeloid expansion

Corresponding author: Hideyo Hirai, M.D., Ph.D.

Department of Transfusion Medicine and Cell Therapy, Kyoto University Hospital,
54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

TEL: +81-75-751-3630; FAX: +81-75-751-4283; E-mail: hhirai@kuhp.kyoto-u.ac.jp

21 This work was partly supported by Grants-in-Aid for Scientific Research from the Japan
22 Society for the Promotion of Science and the Global COE Program ‘Center for Frontier
23 Medicine’ from the Ministry of Education, Culture, Sports, Science and Technology
24 (MEXT) of Japan, Takeda Science Foundation, the Kobayashi Foundation for Cancer
25 Research, and Senshin Medical Research Foundation.

Abstract

The BCR-ABL fusion oncoprotein accelerates differentiation and proliferation of myeloid cells during the chronic phase of chronic myeloid leukemia (CP-CML). Here, the role of C/EBP β , a regulator for 'emergency granulopoiesis', in the pathogenesis of CP-CML was examined. C/EBP β expression was upregulated in Lineage⁻ CD34⁺ CD38⁻ hematopoietic stem cells and myeloid progenitors isolated from bone marrow of patients with CP-CML. In EML cells, a mouse hematopoietic stem cell line, BCR-ABL upregulated C/EBP β , at least in part, through the activation of STAT5. Myeloid differentiation and proliferation induced by BCR-ABL was significantly impaired in C/EBP β -deficient bone marrow cells *in vitro*. Mice that were transplanted with BCR-ABL-transduced C/EBP β knockout bone marrow cells survived longer than mice that received BCR-ABL-transduced wild-type bone marrow cells. Significantly higher levels of leukemic stem cells were maintained in BCR-ABL-transduced C/EBP β -deficient cells than in BCR-ABL-transduced wild-type cells. These results suggest that C/EBP β is involved in BCR-ABL-mediated myeloid expansion. Further elucidation of the molecular mechanisms underlying the C/EBP β -mediated stem cell loss might reveal a novel therapeutic strategy for eradication of CML stem cells.

43 **Keywords:** C/EBP β ; BCR-ABL; chronic myeloid leukemia

44

Introduction

Chronic phase chronic myeloid leukemia (CP-CML) is characterized by massive proliferation and differentiation of myeloid cells.^{1, 2} In sharp contrast to acute myeloid leukemia with leukemic hiatus, both myeloid progenitors and mature granulocytes accumulate in the bone marrow, peripheral blood and spleen in CP-CML.¹ The myeloid expansion in CP-CML has been attributed to the BCR-ABL fusion protein resulting from a translocation between chromosomes 9 and 22.²⁻⁴ Experiments using transgenic mouse models have shown that the BCR-ABL-mediated leukemic status is reversed by suppression of BCR-ABL,⁵⁻⁷ suggesting that BCR-ABL is the sole cause of the myeloid expansion in CP-CML. In practice, inhibition of the BCR-ABL tyrosine kinase activity effectively controls the disease during the chronic phase in most cases.⁸⁻¹² Recent findings suggested a hierarchical organization of CML hematopoiesis, with CML stem cells giving rise to heterogeneous progeny.¹³⁻¹⁵ Accumulating clinical experiences, together with experimental data, have shown that leukemic stem cells in CP-CML are resistant to tyrosine kinase inhibitors (TKIs) and sometimes causes a relapse of the disease after discontinuation of TKI treatment.¹⁶⁻¹⁹ Progression of CML toward the accelerated phase and blast crisis is considered to be a consequence of further acquisition of genetic mutations, which makes

the disease more resistant to TKIs and results in an extremely poor prognosis.^{20, 21}

Therefore, a better understanding of the characteristics of leukemic stem cells, as well as the pathogenesis of CP-CML, are essential for establishing a novel therapeutic strategy for CML.

Granulopoiesis is a process in which hematopoietic stem cells give rise to mature granulocytes throughout life. Our previous findings revealed that a member of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors, C/EBP β , is required for 'emergency granulopoiesis', which is characterized by the accelerated differentiation and proliferation of granulocytic precursors in response to infections or cytokine stimulation.²² Myeloid expansion is a common feature of both emergency granulopoiesis and CP-CML. However, little is known regarding the role of C/EBP β in the pathogenesis of CP-CML. In this study, the effects of BCR-ABL on the expression and function of C/EBP β in BCR-ABL-induced myeloid expansion was determined and the therapeutic implications of these data are discussed.

Materials and methods

Primary human bone marrow cells and cell lines

Frozen samples of human bone marrow cells from healthy donors or untreated CP-CML patients were purchased from AllCells LLC (Emeryville, CA, USA). In all cases, written informed consent was obtained according to the Institutional Review Board or Human Subject Committee approved donor program. The characteristics of the patients are shown in Supplementary Table 1. EML cells²³ (a kind gift from Dr. Schickwann Tsai at the University of Utah, UT, USA) were maintained in Iscove modified Dulbecco's medium (IMDM) supplemented with 20% heat-inactivated horse serum and 15% BHK/MKL cell-conditioned medium.

Mice

C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan). C/EBP β knockout (KO) mice²⁴ were back crossed to C57BL/6 strain mice at least 8 times. Whenever C/EBP β KO mice were analyzed, wild-type (WT) littermates were used as control. All mice were maintained under specific pathogen-free conditions in the Institute of Laboratory Animals, Kyoto University. All experiments were performed according to the institutional guidelines

of Kyoto University.

Reagents

A STAT5 inhibitor (N'-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide) and Ly294002, a PI3K inhibitor, were purchased from Merck (Darmstadt, Germany). U0126, a MEK inhibitor, was purchased from Cell Signaling Technology (Danvers, MA, USA). Stock solutions of the STAT5 inhibitor, Ly294002, and U0126 were made in dimethyl sulfoxide (DMSO).

Plasmids

The pMSCVneo vector and pMSCV-internal ribosome entry site- green fluorescent protein (GFP) vector (MIG),²⁵ and their derivatives for the expression of BCR-ABL (p210), were kind gifts from Dr. Keiko Okuda (Kyoto Prefectural University of Medicine, Kyoto, Japan). Retroviruses expressing the constitutively-active STAT5 mutant (STAT5^{1*6})²⁶ and the dominant negative STAT5 mutant (STAT5^{Δ749})²⁷ were kind gifts from Dr. Toshio Kitamura (University of Tokyo, Tokyo, Japan).

Retrovirus infection

Plat-E packaging cells²⁸ were transfected with retrovirus vectors using FuGENE6 (Roche Diagnostics, Mannheim, Germany) as previously described.^{22, 28} Bone marrow cells were harvested from mice (4 to 8 weeks of age) treated with 5-fluorouracil (5-FU) (150 mg/kg, intraperitoneally). The bone marrow cells were cultured at 37 °C for 48 h in IMDM containing 15% FBS, 50 µM 2-mercaptoethanol, 50 ng/ml mouse stem cell factor (SCF), 50 ng/ml human thrombopoietin (TPO) (a kind gift from Kyowa Hakko Kirin Co., Ltd), 50 ng/ml mouse fms-like tyrosine kinase 3 ligand (FL), and 10 ng/ml mouse interleukin-6 (IL-6). First round retroviral infection was carried out using RetroNectin (Takara Bio, Otsu, Japan) in the same medium. For the second round infection, polybrene was added with the retroviral supernatant.

Bone marrow transplantation

Recipient C57BL/6 mice (8 to 10 weeks of age) were lethally irradiated (10 Gy). MIG or MIG-BCR-ABL-transduced bone marrow cells (0.5 to 1×10^5 GFP-positive cells per mouse) were injected into the tail vein of primary recipient mice. For radioprotection, 2×10^5 cells of freshly harvested whole bone marrow were co-transplanted. For secondary

transplantation, bone marrow cells were harvested from mice that received the primary transplants and 0.2 to 2×10^6 GFP-positive cells were intravenously injected into sublethally irradiated secondary recipient mice. The frequencies of leukemic stem cells were calculated using the L-Calc software (StemCell Technologies, Vancouver, Canada).

Methylcellulose colony-forming assay

MIG-BCR-ABL-transduced mouse bone marrow cells from C/EBP β KO or WT mice were subjected to a methylcellulose colony-forming assay using the cytokine-free medium Methocult 3231 (StemCell Technologies). The fluorescence images of the colonies were obtained using AxioCam MRm digital camera and Axio Vision software in combination with SteREO Lumar V12 microscope and Neolumar S objective lens (0.8 $\times$) (Carl Zeiss, Oberkochen, Germany).

Wright Giemsa staining

Smears of mice peripheral blood or bone marrow cells and cytopsin slides of EML cells or colony-forming cells were stained using a Diff-Quik kit (Sysmex, Kobe, Japan), a modified Wright Giemsa staining system. Images were obtained using DP71 digital camera and DP

Controller software in combination with CX41 microscope and PlanCN objective lens (40× / 0.65 numerical aperture) (Olympus, Tokyo, Japan).

Flow cytometric analysis

Flow cytometric analysis and cell sorting were performed with a FACSCalibur, FACSCanto II, or FACS Aria instrument (BD Biosciences, San Jose, CA, USA). As lineage markers for human bone marrow mononuclear cells, phycoerythrin (PE)-Cy5-conjugated anti-CD235, biotin-conjugated anti-CD3, CD4, CD8, CD11b, CD14, CD19, CD20 (eBioscience, San Diego, CA, USA), and anti-CD56 antibodies (Bio Legend, San Diego, CA, USA) were used and followed by staining with streptavidin-PE-Cy5. Cells were further stained with allophycocyanin (APC)-conjugated anti-CD34 (8G12), phycoerythrin (PE)-conjugated anti-CD38 (HIT2), fluorescein isothiocyanate (FITC)-conjugated anti-CD45RA (HI100) (all from BD Pharmingen, San Diego, CA, USA) and PE-Cy7-conjugated anti-CD123 antibodies (eBioscience) for definition of hematopoietic stem cells and myeloid progenitors. For the staining of mouse cells, PerCP-Cy5.5-conjugated anti-CD3, CD4, CD11b, B220, and Ter119 antibodies, and PE-Cy5.5-conjugated anti-CD8 and Gr-1 antibodies (all from eBioscience) were used as lineage markers. PE-Cy7-conjugated anti-Sca-1, PE-conjugated

anti-CD11b and APC-conjugated anti-c-kit antibodies (all from eBioscience) were used for definition of hematopoietic stem cells and myeloid cells. Data were analyzed with the FlowJo software (Tree Star, Ashland, OR, USA).

Real-time RT-PCR

Total RNA was extracted with an RNeasy Micro Kit (Qiagen, Valencia, CA, USA) and converted to cDNA using random primers. The cDNA was amplified using an Applied Biosystems Step One Plus thermal cycler. The following parameters were used: 95 °C for 20 sec, followed by 45 cycles at 95 °C for 1 sec and 60 °C for 20 sec. The following primers, and probes from the Universal Probe Library (Roche Applied Science, Mannheim, Germany) were used: for mouse C/EBP β , probe #55, and primers 5'-ATCGACTTCAGCCCCTACCT-3' and 5'-TAGTCGTCGGCGAAGAGG-3'; for mouse GAPDH, probe #80, and primers 5'-TGTCCGTCGTGGATCTGAC-3' and 5'-CCTGCTTCACCACCTTCTTG-3'; for human C/EBP β , probe #74, and primers 5'-CGCTTACCTCGGCTACCA-3' and 5'-ACGAGGAGGACGTGGAGAG-3'; and for human GAPDH, probe #60, and primers 5'-AGCCACATCGCTCAGACAC-3' and 5'-GCCCAATACGACCAAATCC-3'. Results were normalized by the level of GAPDH

mRNA.

Western blot analysis

Cells were diluted with equal amounts of Laemmli sample buffer and boiled at 100 °C for 10 min. Samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. A “Can Get” Signal immunoreactions enhancer kit (Toyobo, Osaka, Japan) was used to dilute the primary and secondary antibodies. Antibodies specific for C/EBP β (sc-150, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (sc-25778, Santa Cruz Biotechnology) were used as primary antibodies. Immunoreactive proteins were detected using horseradish peroxidase-conjugated anti-rabbit IgG (NA934V, GE Healthcare, Little Chalfont, UK) and visualized using enhanced chemiluminescence (ECL, GE Healthcare).

Statistics

Statistical analyses were performed using Student’s *t*-test. Survival of mice was analyzed using the log-rank test. *P* values < 0.05 were considered statistically significant.

Results

C/EBP β is upregulated in bone marrow hematopoietic stem cells and myeloid progenitors from patients with CP-CML

C/EBP β expression is upregulated or maintained in normal myeloid progenitors during emergency granulopoiesis, while the expression of all other C/EBP family members was downregulated.²² Therefore, the expression of C/EBP β in bone marrow cells of patients with CP-CML was examined. Among the lineage marker negative (Lin⁻) bone marrow cells, the frequency of CD34⁺ CD38⁻ hematopoietic stem cells (HSCs) was lower and the frequency of CD34⁺ CD38⁺ myeloid progenitors was higher in CP-CML bone marrow than in bone marrow from healthy donors (Figure 1a and b, Supplementary Figure S1). The CD34⁺ CD38⁺ population was further subdivided into common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), and megakaryocyte-erythrocyte progenitors (MEPs) based on the expression levels of CD123 and CD45RA²⁹ (Figure 1a). The frequency of GMPs was significantly lower and that of MEPs was significantly higher (approximately two-fold) in CP-CML bone marrow than in bone marrow from healthy donors (Figure 1c). The differences between normal and CP-CML bone marrow in the frequency of CD34⁺ CD38⁻ HSCs and of myeloid progenitors

were consistent with previous findings.^{13, 15, 30} The expression of C/EBP β in the purified progenitors was measured. The levels of C/EBP β mRNA in HSCs and all the myeloid progenitors from CP-CML bone marrow were significantly higher than levels in bone marrow from healthy donors (Figure 1d, healthy donors, $n = 6$; CP-CML, $n = 5$; GMP, $P < 0.01$; HSC, CMP, and MEPs, $P < 0.05$). These results suggest that C/EBP β is upregulated in HSCs and myeloid progenitors in patients with CP-CML.

BCR-ABL upregulates C/EBP β in EML cells, a mouse hematopoietic stem cell line

The presence of the BCR-ABL fusion protein is thought to be the only difference between normal hematopoiesis and BCR-ABL-mediated myeloid expansion in CP-CML.^{2, 4} To assess whether BCR-ABL could upregulate C/EBP β , the *BCR-ABL* gene was retrovirally introduced into a factor-dependent mouse hematopoietic stem cell line, EML cells, and the expression of C/EBP β was compared with EML cells transduced with a control vector (EML-control). After transduction with BCR-ABL, EML cells (EML-BCR-ABL) became factor-independent and could be cultured long term in the absence of stem cell factor-containing conditioned medium. The morphologies of the EML-control and EML-BCR-ABL cells were indistinguishable by Giemsa staining (Figure

231 2a, Day 0). The parent EML cells expressed c-kit but not CD11b. Flow cytometric
232 analysis of the transduced cells revealed that a small subset of EML-BCR-ABL expressed
233 c-kit at a slightly lower intensity and expressed CD11b weakly (Figure 2b, Day 0). Myeloid
234 differentiation of EML cells can be induced by the addition of interleukin-3, retinoic acid,
235 and granulocyte-macrophage colony stimulating factor (GM-CSF).²³ As shown in Figure 2a
236 (Day 5), myeloid differentiation of both EML-control and EML-BCR-ABL cells was
237 effectively induced. Lower c-kit and higher CD11b expression by EML-control cells was
238 observed 5 days after the induction of differentiation and the extent of the changes in the
239 expression of c-kit and CD11b was more evident in EML-BCR-ABL cells (Figure 2b).
240 These results suggest that BCR-ABL enhanced myeloid differentiation of immature cells
241 such as EML cells.

242 The amount of C/EBP β mRNA in undifferentiated EML-BCR-ABL cells was
243 1.87-fold higher than in undifferentiated EML-control cells (Figure 2c). When the c-kit⁺
244 CD11b⁻ fraction of the EML-control cells and EML-BCR-ABL cells was analyzed, a
245 significant difference was still observed 2.26-fold higher in EML-BCR-ABL cells (Figure
246 2d), suggesting that the upregulation of C/EBP β was not the result of contamination of
247 differentiated cells. The level of C/EBP β protein was 3.76-fold higher in EML-BCR-ABL

cells relative to EML-control cells (Figure 2e). When EML-BCR-ABL cells were treated with imatinib mesylate, the upregulation of C/EBP β by BCR-ABL was reduced (Figure 2f), while the level of C/EBP β in EML-control cells was not affected. These results suggest that C/EBP β is upregulated directly in response to signaling downstream of BCR-ABL.

STAT5 is involved in the BCR-ABL-mediated upregulation of C/EBP β

Various signaling pathways are activated by BCR-ABL, including the JAK/STAT, Raf/MEK/ERK, and PI3K/AKT pathways.³¹⁻³⁶ To elucidate the signaling pathways responsible for the upregulation of C/EBP β , each of the known downstream signaling pathways was inhibited. When EML-BCR-ABL cells were treated with the MEK inhibitor U0126 or the PI3K inhibitor Ly294002, C/EBP β expression was not affected (Figure 3a). In contrast, treatment with the STAT5 inhibitor (N'-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide) significantly reduced C/EBP β expression in EML-BCR-ABL cells (Figure 3b). A dominant negative STAT5 mutant, STAT5 $^{\Delta 749}$, was introduced into the EML-derived cell lines to inhibit STAT5. STAT5 $^{\Delta 749}$ significantly repressed the expression of C/EBP β in EML-BCR-ABL cells but had no effect in EML-control cells (Figure 3c). Conversely, when a constitutively-active

STAT5 mutant, STAT5^{1*6}, was retrovirally transduced into the parental EML cells (EML-CA-STAT5), C/EBP β mRNA levels were significantly greater compared to the level in EML cells transduced with a control vector (Figure 3d). These results suggest that STAT5 is involved in the BCR-ABL-mediated upregulation of C/EBP β .

C/EBP β regulates BCR-ABL-mediated proliferation and differentiation of myeloid cells *in vitro*

The upregulation of C/EBP β in the presence of BCR-ABL was confirmed using primary bone marrow cells. BCR-ABL was retrovirally introduced into bone marrow cells obtained from 5-FU-treated WT mice and RNA was extracted from purified c-kit⁺ Sca-1⁺ Lin⁻ (KSL) cells after the transduced cells were cultured for 2 days. The RNA was analyzed by quantitative RT-PCR. The expression of C/EBP β in BCR-ABL-transduced KSL cells was significantly greater than in control vector-transduced KSL cells ($n = 3$ each, $P < 0.01$) (Figure 4a).

To understand the role of C/EBP β in BCR-ABL-mediated myeloid expansion, BCR-ABL and GFP were retrovirally introduced into bone marrow cells obtained from 5-FU-treated C/EBP β KO mice and their properties were compared to

282 BCR-ABL-transduced bone marrow cells from WT mice. The transduced cells were first
283 subjected to cytokine-free semisolid methylcellulose culture. All the colonies formed on
284 day 7 were GFP-positive and the numbers of colonies were equivalent between transduced
285 bone marrow cells from WT and KO mice (Figure 4b and c), suggesting that the
286 frequencies of clonogenic progenitors were similar. The majority of C/EBP β KO
287 cell-derived colonies were smaller in size (Figure 4b) and the cell number per C/EBP β KO
288 cell colony was significantly lower than WT cell colonies ($P < 0.01$) (Figure 4d). These
289 findings suggest that the loss of C/EBP β impaired BCR-ABL-mediated cell proliferation.
290 Having observed a difference between WT and C/EBP β KO cells in proliferative capacity
291 in the presence of the BCR-ABL fusion protein, the morphologies of the colony-forming
292 cells were assessed by Giemsa staining (Figure 4e). The BCR-ABL-transduced C/EBP β
293 KO cell colonies contained more immature myeloid cells with larger nuclei and basophilic
294 cytoplasm, whereas the BCR-ABL-transduced WT cell colonies contained more mature
295 neutrophilic granulocytes and macrophages (Figure 4e). Flow cytometric analysis revealed
296 that the BCR-ABL-transduced C/EBP β KO cells gave rise to a significantly higher
297 proportion of c-kit⁺ cells and a lower proportion of CD11b⁺ cells, relative to
298 BCR-ABL-transduced WT cells (%c-kit⁺ cells = $6.7 \pm 1.0\%$ vs. $2.0 \pm 0.2\%$, $P =$

0.01; %CD11b⁺ cells = 17.9 ± 4.4% vs. 41.3 ± 8.8%, $P = 0.039$) (Figure 4f and g). In addition, BCR-ABL-transduced C/EBPβ KO cells-derived colonies could be replated more than three times in cytokine-free medium, whereas BCR-ABL-transduced WT cells stopped growing after being replated only twice (Figure 4h and Supplementary Figure S2). These results suggest that the C/EBPβ deficiency abolished the BCR-ABL-mediated proliferation and differentiation of myeloid cells.

Loss of C/EBPβ delays BCR-ABL-mediated myeloid expansion *in vivo*

BCR-ABL-transduced bone marrow cells from C/EBPβ KO mice or WT mice were transplanted into lethally irradiated recipient mice to clarify the role of C/EBPβ in the *in vivo* myeloproliferation induced by BCR-ABL. After transplantation of transduced cells, increases in neutrophilic granulocytes were observed in the peripheral blood of mice having received either WT cells or KO cells (Figure 5a). Flow cytometric analysis of peripheral blood also revealed that over 70% of nucleated cells were CD11b⁺ in both WT and KO transplant recipients (Figure 5b). In mice transplanted with BCR-ABL-transduced C/EBPβ KO cells, the increase in white blood cell count (Figure 5c) and the development of splenomegaly (Figure 5d) were delayed compared to mice receiving transplants of WT

BCR-ABL-transduced cells. The median survival of mice transplanted with BCR-ABL-transduced WT cells was 19 days. In contrast, the median survival of mice transplanted with BCR-ABL-transduced C/EBP β KO cells was 31 days, significantly longer than mice receiving transplants of BCR-ABL-transduced WT cells ($P = 0.0005$) (Figure 5e). These results suggest that C/EBP β is involved in BCR-ABL-induced enhanced myelopoiesis *in vivo*.

BCR-ABL-induced loss of self-renewing hematopoietic/leukemic stem cells was attenuated in the absence of C/EBP β

In vitro experiments revealed that C/EBP β KO bone marrow cells retained more c-kit⁺ immature cells and could be replated more times after transduction with BCR-ABL (Figure 4f-h, Supplementary Figure S2), suggesting that C/EBP β is involved in the BCR-ABL-mediated loss of self-renewing hematopoietic/leukemic stem cells. Consistent with these observations, BCR-ABL-transduced C/EBP β KO cells had given rise to a higher proportion of c-kit⁺ cells than BCR-ABL-transduced WT cells on day 19 after transplantation ($16.0 \pm 2.6\%$ vs. $5.5 \pm 4.6\%$, $P = 0.01$) (Figure 6a and b).

Serial transplantation experiments were carried out to determine the role of

333 C/EBP β in the loss of self-renewal capacity. When 2×10^6 GFP $^{+}$ bone marrow cells from
334 the primary recipients were transplanted into sublethally irradiated secondary recipients, all
335 the mice having received either BCR-ABL-transduced WT cells or BCR-ABL-transduced
336 KO cells developed a myeloproliferative status, reminiscent of the first transplantation
337 (Figure 6c). After transplantation of 1×10^6 GFP $^{+}$ bone marrow cells from the primary
338 transplant recipients, one of the four recipient mice that received a secondary transplant of
339 BCR-ABL-transduced WT cells developed a myeloproliferative status and four out of five
340 recipient mice that received a secondary transplant of BCR-ABL-transduced C/EBP β KO
341 cells developed a myeloproliferative disorder (Figure 6d). On day 38 after the secondary
342 transplantation of 1×10^6 GFP $^{+}$ bone marrow cells, BCR-ABL-transduced C/EBP β KO
343 cells achieved a significantly higher degree of chimerism in the peripheral blood of the
344 secondary recipients than BCR-ABL-transduced WT cells ($n = 8$ each, $P = 0.013$) (Figure
345 6e). None of the mice that received less than 0.5×10^6 GFP $^{+}$ bone marrow cells from the
346 primary recipients developed a myeloproliferative status. Based on these transplantation
347 experiments, the estimated frequencies of leukemia-initiating cells in bone marrow of
348 primary recipient mice transplanted with BCR-ABL-transduced WT cells and
349 BCR-ABL-transduced C/EBP β KO cells were 1 in 1 404 129 and 1 in 683 773,

350 respectively (Figure 6f). These results suggest that enhanced C/EBP β expression induced
351 by BCR-ABL was involved in the loss of the self-renewal potential of leukemic stem cells.
352

Discussion

Proliferation and differentiation of myeloid cells are unique to CP-CML. This is the first report demonstrating that the BCR-ABL-mediated myeloid expansion in CP-CML is promoted by C/EBP β , a regulator of 'emergency granulopoiesis'.

One of the major findings of this study is the upregulation of C/EBP β by BCR-ABL in CP-CML. In EML cells or in immature mouse hematopoietic cells, BCR-ABL upregulated C/EBP β and accelerated the differentiation of these cells (Figure 2b-e and Figure 4a). In contrast, previous reports showed that, in 32Dcl3 cells transduced with BCR-ABL, C/EBP β was downregulated and granulocytic differentiation was blocked.^{37, 38}

One explanation for the discrepancy between the observations in EML cells and 32Dcl3 cells may be due to the differentiation status. 32Dcl3 cells give rise only to neutrophilic granulocytes in the presence of G-CSF,³⁹ while EML cells are multipotent and can give rise to monocytic, erythroid and lymphoid lineages, in addition to the granulocytic lineage,²³ suggesting that EML cells represent more immature hematopoietic cells than 32Dcl3 cells.

The higher C/EBP β expression in 32Dcl3 than in EML cells (Supplementary Figure S3) also supports this idea, as C/EBP β is upregulated during myeloid differentiation.^{40, 41}

Taking into account the previous findings that HSCs are the target cell population for

BCR-ABL during chronic phase,⁴ the data from EML cells are most likely to reflect conditions of CP-CML. Consistent with our observations, Minami et al. found that C/EBP β is one of the markedly upregulated genes in a pluripotent hematopoietic cell line transduced with BCR-ABL.⁴² Most importantly, C/EBP β is upregulated in purified HSCs and myeloid progenitors obtained from patients with CP-CML, as shown in Figure 1d. The differences in BCR-ABL-mediated regulation of C/EBP β in EML cells and in 32Dcl3 cells also suggested the involvement of cell context-dependent machinery. Guerzoni *et al.* described a downregulation of C/EBP β associated with the progression of CML toward a blast crisis,³⁷ whereas our study focused on the upregulation of C/EBP β in the chronic phase. The changes in the BCR-ABL-mediated regulation of C/EBP β during the progression of CML may be a consequence of genetic or epigenetic changes, which result in a cell context similar to 32Dcl3 cells, in terms of the regulation of C/EBP β .

BCR-ABL activates a number of signaling cascades through its tyrosine kinase activity. STAT5 is a well known target of BCR-ABL. STAT5 is phosphorylated by JAK2⁴³ and is thought to activate target genes to induce or maintain CP-CML.^{33, 44} Our current data strongly suggest that C/EBP β resides downstream of STAT5. Transplantation of BCR-ABL-transduced STAT5 KO bone marrow cells resulted in delayed progression of a

387 myeloproliferative disorder in mice,³³ a phenotype highly similar to our CP-CML model
388 using C/EBP β KO cells. The similarities between the behavior of BCR-ABL-transduced
389 STAT5 KO cells and BCR-ABL-transduced C/EBP β KO cells strongly suggested that these
390 two molecules act sequentially in the same pathway. STAT5 and C/EBP β are both essential
391 for cytokine-induced granulopoiesis,^{22, 45-47} suggesting that STAT5 and C/EBP β may also
392 interact when emergency granulopoiesis is stimulated. There has been no evidence, thus
393 far, to demonstrate that STAT5 directly regulates C/EBP β . The consensus binding site for
394 STAT5 (TTCN₃GAA) is not found in the proximal promoter region of C/EBP β . Recently,
395 Zhang *et al.* showed that STAT3 regulates C/EBP β transcription through binding to the IL-6
396 response element II (CTGGGA) located at -1180 base pairs in the C/EBP β promoter.⁴⁸
397 However, our preliminary analysis of the 2000 base pairs proximal promoter region of
398 C/EBP β by reporter assay failed to identify the positive regulatory elements which respond
399 to STAT5 (data not shown). In addition, specific binding of STAT5 to the particular
400 regulatory element was not observed in published chromatin immunoprecipitation
401 sequencing data analyzing T cells stimulated with interleukin-2 (GSE12346 (ref. 49) and
402 GSE26553 (ref. 50)). These data suggest that STAT5 might upregulate C/EBP β through
403 binding to regulatory elements outside this IL-6 response element II or through other

indirect mechanisms. Elucidation of the direct or indirect interactions between STAT5 and C/EBP β is necessary for further understanding of BCR-ABL-mediated myeloid expansion and emergency granulopoiesis.

Our present data showed that myeloid expansion was delayed when BCR-ABL was transduced into C/EBP β KO bone marrow cells, clearly suggesting the involvement of C/EBP β in the enhanced myelopoiesis observed in patients with CP-CML. The effects of BCR-ABL on the self-renewing potential of HSCs are still controversial. Schemionek *et al* recently reported that BCR-ABL partially impaired long term HSCs.⁵¹ In the present study, a phenotypically and functionally immature status was maintained in a greater fraction of BCR-ABL-transduced C/EBP β KO cells than in BCR-ABL-transduced WT cells both *in vitro* and *in vivo*. These data suggested that C/EBP β is involved in the BCR-ABL-mediated loss of self-renewing potential of the HSCs. From the therapeutic point of view, repression of C/EBP β in CP-CML patients would delay the progression of the disease, although leaving leukemic stem cells relatively intact. This strategy might be effective for patients with BCR-ABL mutations that are resistant to tyrosine kinase inhibitors, as the role of C/EBP β in the pathogenesis of CP-CML should be common to all BCR-ABL mutants. Alternatively, upregulation of C/EBP β in leukemic stem cells might induce exhaustion of

the leukemic stem cells, leading to a complete cure for the disease. Actually, Guerzoni *et al.* showed that transduction of C/EBP β promotes differentiation of BCR-ABL expressing cells.^{37, 52} The effects of upregulation of endogenous C/EBP β on leukemic stem cells should be determined in the future. Further understanding of the regulation of the self-renewal and differentiation of leukemic stem cells in CP-CML may lead to identification of novel therapies for CML based on the regulation of C/EBP β .

The leukocytosis observed during infections (=emergency granulopoiesis) is sometimes called a 'leukemoid' reaction because of the great increase in the number of myeloid cells with a 'left shift' in the shape of the nucleus.⁵³ Here, a molecular link between leukemoid reactions and CP-CML was identified. C/EBP β fine tunes the proliferation and differentiation of HSCs and CML leukemic stem cells in response to their respective upstream signals. Further elucidation of the molecular mechanisms that regulate the self-renewal and differentiation of stem cells in CP-CML and emergency granulopoiesis will facilitate the development of novel strategies for the treatment of CML.

Acknowledgments

We thank Dr. Toshio Kitamura (University of Tokyo, Tokyo, Japan) and Dr. Keiko Okuda (Kyoto Prefectural University of Medicine, Kyoto, Japan) for providing us with STAT5 mutant vectors and BCR-ABL-expressing vectors, respectively. We are grateful to Mikiko Katakami, Yoko Nakagawa, and Yoshiko Manabe for their excellent technical assistance. This work was partly supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science and the Global COE Program ‘Center for Frontier Medicine’ from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, Takeda Science Foundation, the Kobayashi Foundation for Cancer Research, and Senshin Medical Research Foundation.

447 **Conflict of Interest**

448 The authors declare no conflict of interest.

449

450 Supplementary information is available at the Leukemia's website

451

References

1. Cotta CV, Bueso-Ramos CE. New insights into the pathobiology and treatment of chronic myelogenous leukemia. *Ann Diagn Pathol* 2007; **11**: 68-78.
2. Ren R. Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. *Nat Rev Cancer* 2005; **5**: 172-183.
3. Sawyers CL. Chronic myeloid leukemia. *N Engl J Med* 1999; **340**: 1330-1340.
4. Melo JV, Barnes DJ. Chronic myeloid leukaemia as a model of disease evolution in human cancer. *Nat Rev Cancer* 2007; **7**: 441-453.
5. Huettner CS, Zhang P, Van Etten RA, Tenen DG. Reversibility of acute B-cell leukaemia induced by BCR-ABL1. *Nat Genet* 2000; **24**: 57-60.
6. Huettner CS, Koschmieder S, Iwasaki H, Iwasaki-Arai J, Radomska HS, Akashi K, *et al.* Inducible expression of BCR/ABL using human CD34 regulatory elements

results in a megakaryocytic myeloproliferative syndrome. *Blood* 2003; **102**:
3363-3370.

7. Koschmieder S, Gottgens B, Zhang P, Iwasaki-Arai J, Akashi K, Kutok JL, *et al.*
Inducible chronic phase of myeloid leukemia with expansion of hematopoietic stem
cells in a transgenic model of BCR-ABL leukemogenesis. *Blood* 2005; **105**:
324-334.

8. Goldman JM, Melo JV. Chronic myeloid leukemia--advances in biology and new
approaches to treatment. *N Engl J Med* 2003; **349**: 1451-1464.

9. Kimura S, Naito H, Segawa H, Kuroda J, Yuasa T, Sato K, *et al.* NS-187, a potent
and selective dual Bcr-Abl/Lyn tyrosine kinase inhibitor, is a novel agent for
imatinib-resistant leukemia. *Blood* 2005; **106**: 3948-3954.

10. Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N, *et al.*
Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N*

486 *Engl J Med* 2006; **355**: 2408-2417.

- 487
- 488 11. Kantarjian HM, Giles F, Gattermann N, Bhalla K, Alimena G, Palandri F, *et al.*
489 Nilotinib (formerly AMN107), a highly selective BCR-ABL tyrosine kinase
490 inhibitor, is effective in patients with Philadelphia chromosome-positive chronic
491 myelogenous leukemia in chronic phase following imatinib resistance and
492 intolerance. *Blood* 2007; **110**: 3540-3546.

- 493
- 494 12. Hochhaus A, Baccarani M, Deininger M, Apperley JF, Lipton JH, Goldberg SL, *et*
495 *al.* Dasatinib induces durable cytogenetic responses in patients with chronic
496 myelogenous leukemia in chronic phase with resistance or intolerance to imatinib.
497 *Leukemia* 2008; **22**: 1200-1206.

- 498
- 499 13. Jamieson CH, Ailles LE, Dylla SJ, Muijtjens M, Jones C, Zehnder JL, *et al.*
500 Granulocyte-macrophage progenitors as candidate leukemic stem cells in
501 blast-crisis CML. *N Engl J Med* 2004; **351**: 657-667.

- 503 14. Passegue E, Weisman IL. Leukemic stem cells: where do they come from? *Stem*
504 *Cell Rev* 2005; **1**: 181-188.
- 505
- 506 15. Bruns I, Czibere A, Fischer JC, Roels F, Cadeddu RP, Buest S, *et al.* The
507 hematopoietic stem cell in chronic phase CML is characterized by a transcriptional
508 profile resembling normal myeloid progenitor cells and reflecting loss of quiescence.
509 *Leukemia* 2009; **23**: 892-899.
- 510
- 511 16. Michor F, Hughes TP, Iwasa Y, Branford S, Shah NP, Sawyers CL, *et al.* Dynamics
512 of chronic myeloid leukaemia. *Nature* 2005; **435**: 1267-1270.
- 513
- 514 17. Copland M, Hamilton A, Elrick LJ, Baird JW, Allan EK, Jordanides N, *et al.*
515 Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in
516 primary CML but does not eliminate the quiescent fraction. *Blood* 2006; **107**:
517 4532-4539.
- 518
- 519 18. Mahon FX, Rea D, Guilhot J, Guilhot F, Huguet F, Nicolini F, *et al.* Discontinuation

of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. *Lancet Oncol* 2010; **11**: 1029-1035.

19. Corbin AS, Agarwal A, Loriaux M, Cortes J, Deininger MW, Druker BJ. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J Clin Invest* 2011; **121**: 396-409.

20. Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, *et al*. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* 2001; **293**: 876-880.

21. Branford S, Rudzki Z, Walsh S, Grigg A, Arthur C, Taylor K, *et al*. High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. *Blood* 2002; **99**: 3472-3475.

537

538 22. Hirai H, Zhang P, Dayaram T, Hetherington CJ, Mizuno S, Imanishi J, *et al.*
539 C/EBPbeta is required for 'emergency' granulopoiesis. *Nat Immunol* 2006; **7**:
540 732-739.

541

542 23. Tsai S, Bartelmez S, Sitnicka E, Collins S. Lymphohematopoietic progenitors
543 immortalized by a retroviral vector harboring a dominant-negative retinoic acid
544 receptor can recapitulate lymphoid, myeloid, and erythroid development. *Genes Dev*
545 1994; **8**: 2831-2841.

546

547 24. Screpanti I, Romani L, Musiani P, Modesti A, Fattori E, Lazzaro D, *et al.*
548 Lymphoproliferative disorder and imbalanced T-helper response in C/EBP
549 beta-deficient mice. *Embo J* 1995; **14**: 1932-1941.

550

551 25. Hawley RG, Lieu FH, Fong AZ, Hawley TS. Versatile retroviral vectors for
552 potential use in gene therapy. *Gene Ther* 1994; **1**: 136-138.

553

- 554 26. Onishi M, Nosaka T, Misawa K, Mui AL, Gorman D, McMahon M, *et al.*
555 Identification and characterization of a constitutively active STAT5 mutant that
556 promotes cell proliferation. *Mol Cell Biol* 1998; **18**: 3871-3879.
- 557
- 558 27. Moriggl R, Gouilleux-Gruart V, Jahne R, Berchtold S, Gartmann C, Liu X, *et al.*
559 Deletion of the carboxyl-terminal transactivation domain of MGF-Stat5 results in
560 sustained DNA binding and a dominant negative phenotype. *Mol Cell Biol* 1996;
561 **16**: 5691-5700.
- 562
- 563 28. Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient
564 packaging of retroviruses. *Gene Ther* 2000; **7**: 1063-1066.
- 565
- 566 29. Manz MG, Miyamoto T, Akashi K, Weissman IL. Prospective isolation of human
567 clonogenic common myeloid progenitors. *Proc Natl Acad Sci U S A* 2002; **99**:
568 11872-11877.
- 569
- 570 30. Diaz-Blanco E, Bruns I, Neumann F, Fischer JC, Graef T, Roskopf M, *et al.*

571 Molecular signature of CD34(+) hematopoietic stem and progenitor cells of patients
572 with CML in chronic phase. *Leukemia* 2007; **21**: 494-504.

573

574 31. Shuai K, Halpern J, ten Hoeve J, Rao X, Sawyers CL. Constitutive activation of
575 STAT5 by the BCR-ABL oncogene in chronic myelogenous leukemia. *Oncogene*
576 1996; **13**: 247-254.

577

578 32. Ilaria RL, Jr., Van Etten RA. P210 and P190(BCR/ABL) induce the tyrosine
579 phosphorylation and DNA binding activity of multiple specific STAT family
580 members. *J Biol Chem* 1996; **271**: 31704-31710.

581

582 33. Ye D, Wolff N, Li L, Zhang S, Ilaria RL, Jr. STAT5 signaling is required for the
583 efficient induction and maintenance of CML in mice. *Blood* 2006; **107**: 4917-4925.

584

585 34. Steelman LS, Pohnert SC, Shelton JG, Franklin RA, Bertrand FE, McCubrey JA.
586 JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and
587 leukemogenesis. *Leukemia* 2004; **18**: 189-218.

588

589 35. Kharas MG, Fruman DA. ABL oncogenes and phosphoinositide 3-kinase:
590 mechanism of activation and downstream effectors. *Cancer Res* 2005; **65**:
591 2047-2053.

592

593 36. Quintas-Cardama A, Cortes J. Molecular biology of bcr-abl1-positive chronic
594 myeloid leukemia. *Blood* 2009; **113**: 1619-1630.

595

596 37. Guerzoni C, Bardini M, Mariani SA, Ferrari-Amorotti G, Neviani P, Panno ML, *et*
597 *al.* Inducible activation of CEBPB, a gene negatively regulated by BCR/ABL,
598 inhibits proliferation and promotes differentiation of BCR/ABL-expressing cells.
599 *Blood* 2006; **107**: 4080-4089.

600

601 38. Schuster C, Forster K, Dierks H, Elsasser A, Behre G, Simon N, *et al.* The effects of
602 Bcr-Abl on C/EBP transcription-factor regulation and neutrophilic differentiation
603 are reversed by the Abl kinase inhibitor imatinib mesylate. *Blood* 2003; **101**:
604 655-663.

605

606 39. Steinman RA, Tweardy DJ. Granulocyte colony-stimulating factor receptor mRNA
607 upregulation is an immediate early marker of myeloid differentiation and exhibits
608 dysfunctional regulation in leukemic cells. *Blood* 1994; **83**: 119-127.

609

610 40. Du Y, Campbell JL, Nalbant D, Youn H, Bass AC, Cobos E, *et al.* Mapping gene
611 expression patterns during myeloid differentiation using the EML hematopoietic
612 progenitor cell line. *Exp Hematol* 2002; **30**: 649-658.

613

614 41. Bjerregaard MD, Jurlander J, Klausen P, Borregaard N, Cowland JB. The in vivo
615 profile of transcription factors during neutrophil differentiation in human bone
616 marrow. *Blood* 2003; **101**: 4322-4332.

617

618 42. Minami Y, Stuart SA, Ikawa T, Jiang Y, Banno A, Hunton IC, *et al.*
619 BCR-ABL-transformed GMP as myeloid leukemic stem cells. *Proc Natl Acad Sci U*
620 *SA* 2008; **105**: 17967-17972.

621

- 622 43. Xie S, Wang Y, Liu J, Sun T, Wilson MB, Smithgall TE, *et al.* Involvement of Jak2
623 tyrosine phosphorylation in Bcr-Abl transformation. *Oncogene* 2001; **20**:
624 6188-6195.
- 625
- 626 44. Hoelbl A, Schuster C, Kovacic B, Zhu B, Wickre M, Hoelzl MA, *et al.* Stat5 is
627 indispensable for the maintenance of bcr/abl-positive leukaemia. *EMBO Mol Med*
628 2010; **2**: 98-110.
- 629
- 630 45. Feldman GM, Rosenthal LA, Liu X, Hayes MP, Wynshaw-Boris A, Leonard WJ, *et*
631 *al.* STAT5A-deficient mice demonstrate a defect in granulocyte-macrophage
632 colony-stimulating factor-induced proliferation and gene expression. *Blood* 1997;
633 **90**: 1768-1776.
- 634
- 635 46. Kimura A, Rieger MA, Simone JM, Chen W, Wickre MC, Zhu BM, *et al.* The
636 transcription factors STAT5A/B regulate GM-CSF-mediated granulopoiesis. *Blood*
637 2009; **114**: 4721-4728.
- 638

- 639 47. Akagi T, Saitoh T, O'Kelly J, Akira S, Gombart AF, Koeffler HP. Impaired response
640 to GM-CSF and G-CSF, and enhanced apoptosis in C/EBPbeta-deficient
641 hematopoietic cells. *Blood* 2008; **111**: 2999-3004.
- 642
- 643 48. Zhang H, Nguyen-Jackson H, Panopoulos AD, Li HS, Murray PJ, Watowich SS.
644 STAT3 controls myeloid progenitor growth during emergency granulopoiesis. *Blood*
645 2010; **116**: 2462-2471.
- 646
- 647 49. Liao W, Schones DE, Oh J, Cui Y, Cui K, Roh TY, *et al.* Priming for T helper type 2
648 differentiation by interleukin 2-mediated induction of interleukin 4 receptor
649 alpha-chain expression. *Nat Immunol* 2008; **9**: 1288-1296.
- 650
- 651 50. Yang XP, Ghoreschi K, Steward-Tharp SM, Rodriguez-Canales J, Zhu J, Grainger
652 JR, *et al.* Opposing regulation of the locus encoding IL-17 through direct, reciprocal
653 actions of STAT3 and STAT5. *Nat Immunol* 2011; **12**: 247-254.
- 654
- 655 51. Schemionek M, Elling C, Steidl U, Baumer N, Hamilton A, Spieker T, *et al.*

656 BCR-ABL enhances differentiation of long-term repopulating hematopoietic stem
657 cells. *Blood* 2010; **115**: 3185-3195.

658

659 52. Guerzoni C, Ferrari-Amorotti G, Bardini M, Mariani SA, Calabretta B. Effects of
660 C/EBPalpha and C/EBPbeta in BCR/ABL-expressing cells: differences and
661 similarities. *Cell Cycle* 2006; **5**: 1254-1257.

662

663 53. Sakka V, Tsiodras S, Giamarellos-Bourboulis EJ, Giamarellou H. An update on the
664 etiology and diagnostic evaluation of a leukemoid reaction. *Eur J Intern Med* 2006;
665 **17**: 394-398.

666

Figure Legends

Figure 1. C/EBP β expression in hematopoietic stem and progenitor cells from the bone marrow of CP-CML patients.

(a) Flow cytometric analysis of lineage marker negative (Lin⁻) bone marrow cells from healthy donors and CP-CML patients identified CD34⁺ CD38⁻ hematopoietic stem cells (HSC) and CD34⁺ CD38⁺ myeloid progenitors (upper panels). Myeloid progenitors were further subdivided into common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP) and megakaryocyte-erythrocyte progenitors (MEP) based on the expression of CD45RA and CD123 (lower panels). The data shown are representative of six independent experiments. (b) Frequency of CD34⁺ CD38⁻ HSCs within the Lin⁻ fraction of bone marrow cells. Error bars indicate SD (healthy donors, $n = 6$; CP-CML, $n = 5$; $*P < 0.05$). (c) Percentage of CMPs, GMPs, and MEPs in the Lin⁻ CD34⁺ CD38⁺ fraction. Error bars indicate SD (healthy donors, $n = 6$; CP-CML, $n = 5$; $*P < 0.001$). (d) C/EBP β mRNA levels in purified HSCs, CMPs, GMPs, and MEPs. Results were normalized to the expression level of HSCs from healthy donors (healthy donors, $n = 6$; CP-CML, $n = 5$; $*P < 0.05$; $**P < 0.01$).

Figure 2. Effects of BCR-ABL on the expression of C/EBP β in EML cells.

(a) Wright Giemsa staining of pMSCVneo vector-transduced EML cells (EML-control) and BCR-ABL-containing pMSCVneo vector-transduced EML cells (EML-BCR-ABL) before (Day 0) and after (Day 5) the induction of myeloid differentiation (scale bar, 20 μ m; original magnification, 400 $\times$). (b) Flow cytometric analysis of c-kit and CD11b expression in EML-control and EML-BCR-ABL cells (Day 0 and Day 5 after myeloid differentiation). Numbers in each quadrant indicate the percentage of live cells. (c) C/EBP β mRNA levels in EML-control and EML-BCR-ABL cells. Results were normalized to the expression level of control. Error bars indicate SD from duplicate samples. Results are representative of three independent experiments. $*P = 0.011$. (d) C/EBP β mRNA levels in c-kit $^{+}$ CD11b $^{-}$ fraction of the EML-control cells and EML-BCR-ABL cells. Results were normalized to the expression level of control. Error bars indicate SD from duplicate samples. $*P < 0.01$. (e) C/EBP β protein levels in EML-control and EML-BCR-ABL cells. Results were normalized to the expression level of control. Error bars indicate SD from duplicate samples. Results are representative of three independent experiments. $*P < 0.01$. (f) C/EBP β mRNA levels in EML-control and EML-BCR-ABL cells with or without a 48 h treatment with imatinib mesylate (100 nM). Dimethyl sulfoxide (DMSO) was used as the control. Results were

normalized to the expression level of control. Error bars indicate SD from duplicate samples. Results are representative of three independent experiments. $*P = 0.016$.

Figure 3. Involvement of BCR-ABL downstream signaling pathways in the upregulation of C/EBP β .

Changes in C/EBP β mRNA in EML-BCR-ABL cells 24 h after treatment with the PI3K inhibitor Ly294002 (2.5 μ M), the MEK inhibitor U0126 (25 μ M) (**a**), or a STAT5 inhibitor (40 μ M) (**b**). DMSO was used as the control. Results were normalized to the expression level of control. Error bars indicate SD from duplicate samples. Results are representative of two independent experiments. $*P < 0.01$. (**c**) C/EBP β mRNA in EML-control and EML-BCR-ABL cells transduced with a dominant negative STAT5 mutant, STAT5 Δ^{749} . Results were normalized to the expression level of control. Error bars indicate SD from duplicate samples. Results are representative of two independent experiments. $*P < 0.05$. (**d**) C/EBP β mRNA levels in EML cells transduced with a constitutively-active STAT5 mutant (CA-STAT5: STAT5 1*6) or empty-vector. Results were normalized to the expression level of control. Error bars indicate the SD from duplicate samples. Results are representative of two independent experiments. $*P < 0.01$.

718

719 **Figure 4.** BCR-ABL-mediated in vitro colony formation in the absence of C/EBP β .

720 **(a)** C/EBP β mRNA levels in c-kit⁺ Sca-1⁺ Lin⁻ cells from WT bone marrow cells
721 transduced with a control MIG vector or a MIG-BCR-ABL vector ($n = 3$ each, $P < 0.01$).

722 **(b)** Colonies formed by BCR-ABL-transduced C/EBP β KO bone marrow cells and WT
723 bone marrow cells after culture for 7 days in cytokine-free methylcellulose (scale bar, 1
724 mm). Colony numbers **(c)**, cell numbers per colony **(d)**, and Wright Giemsa staining (scale
725 bar, 20 μ m; original magnification, 400 $\times$) **(e)** of the colony-forming cells are shown. Error

726 bars indicate SD from triplicate cultures. Results are representative of three independent
727 experiments. $*P < 0.01$. **(f)** Flow cytometric analysis of the cells forming primary colonies

728 by day 7. Numbers in each quadrant indicate the percentage of live cells. **(g)** Frequency of

729 c-kit⁺ cells and CD11b⁺ cells in the cells forming primary colonies by day 7. Results are
730 representative of two independent experiments. $*P < 0.05$. **(h)** Serial colony-replating of 1

731 $\times 10^4$ BCR-ABL-transduced C/EBP β WT and KO bone marrow cells. The colonies were
732 counted and collected on day 10 (2nd) or 14 (3rd), respectively. Error bars indicate SD

733 from triplicate cultures. Results are representative of three independent experiments. $*P <$
734 0.01.

735

736 **Figure 5.** C/EBP β deficiency alters the BCR-ABL-dependent myeloproliferative status *in*
737 *vivo*.

738 Wright Giemsa staining (scale bar, 20 μ m; original magnification, 400 $\times$) (a), 20 days
739 post-transplantation, and flow cytometric analysis (b), 28 days post-transplantation, of
740 peripheral blood from recipient mice transplanted with BCR-ABL-transduced WT cells or
741 BCR-ABL-transduced C/EBP β KO cells. Numbers in quadrants indicate the percentages
742 within peripheral blood nucleated cells. (c) Peripheral white blood cell count of recipients
743 after transplantation. Data are mean $\pm$ SD ($n = 4$ each; $*P < 0.05$). (d) Splenomegaly
744 observed in recipients at day 19 after transplantation (scale bar, 10 mm). Spleen weights are
745 presented as mean $\pm$ SD (WT, $n = 9$; KO, $n = 6$; $*P < 0.01$). (e) Survival of recipients
746 transplanted with BCR-ABL-transduced WT cells or BCR-ABL-transduced C/EBP β KO
747 cells (WT, $n = 12$; KO, $n = 11$; $*P = 0.0005$).

748

749 **Figure 6.** Involvement of C/EBP β in BCR-ABL-mediated loss of immature hematopoietic
750 cells.

751 Flow cytometric analysis of GFP $^{+}$ bone marrow cells from recipients 19 days after

transplantation of BCR-ABL-transduced WT cells or BCR-ABL-transduced C/EBP β KO cells (**a** and **b**). Numbers in quadrants indicate the percentages within GFP⁺ bone marrow cells (WT, $n = 2$; KO, $n = 3$; $*P = 0.01$). (**c** and **d**) Survival of secondary transplantation recipients. 2×10^6 (**c**; WT, $n = 7$; KO, $n = 7$) or 1×10^6 BCR-ABL-transduced GFP⁺ cells (**d**; WT, $n = 4$; KO, $n = 5$) from the first recipients were transplanted. (**e**) GFP⁺ cell chimerism on day 38 after the secondary transplantation of BCR-ABL-transduced WT cells or BCR-ABL-transduced C/EBP β KO cells ($n = 8$ each; $*P = 0.013$). (**f**) The frequencies of leukemia-initiating cells in bone marrow of primary recipient mice transplanted with BCR-ABL-transduced WT cells or BCR-ABL-transduced C/EBP β KO cells.

Figure 1

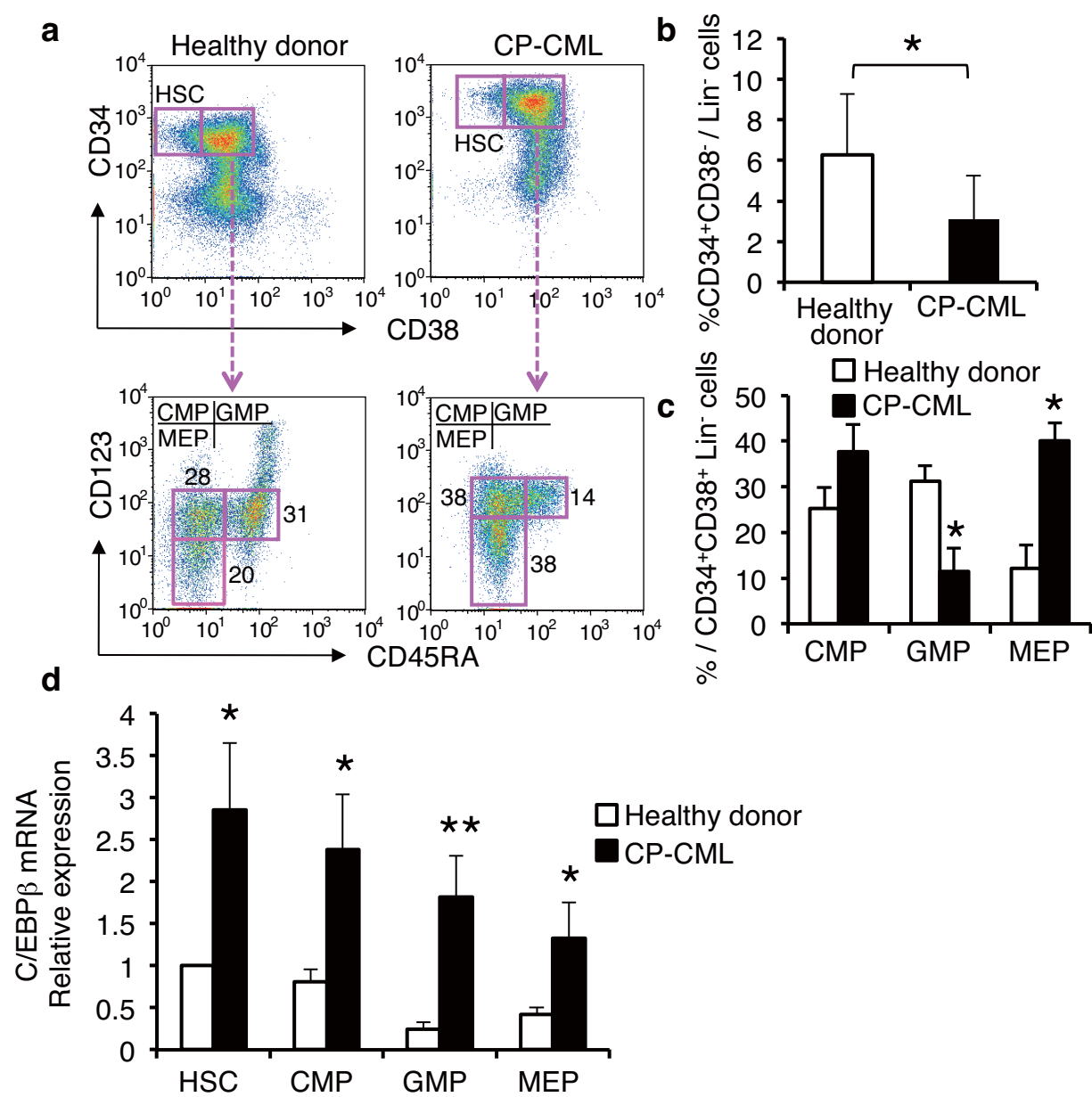


Figure 2

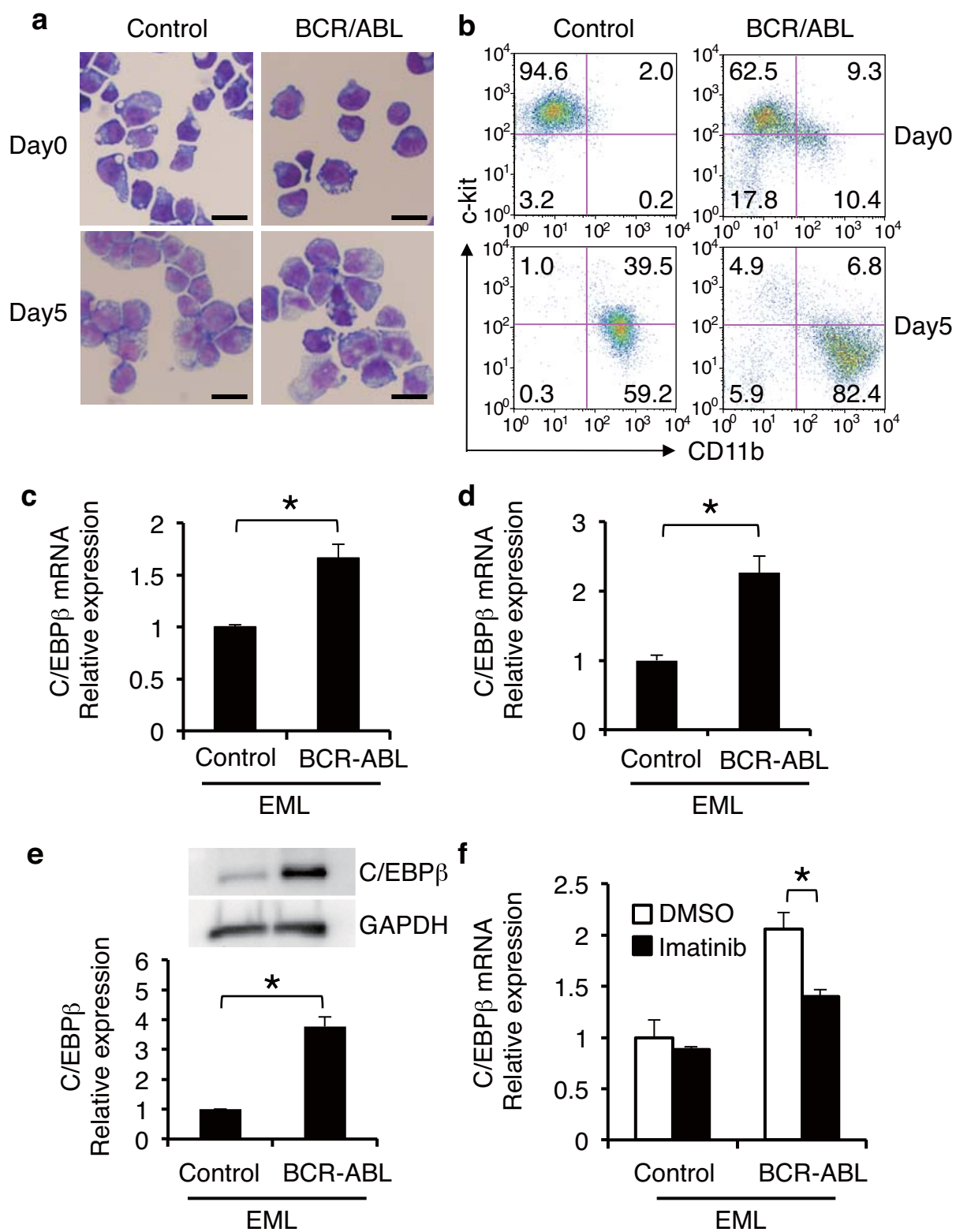


Figure 3

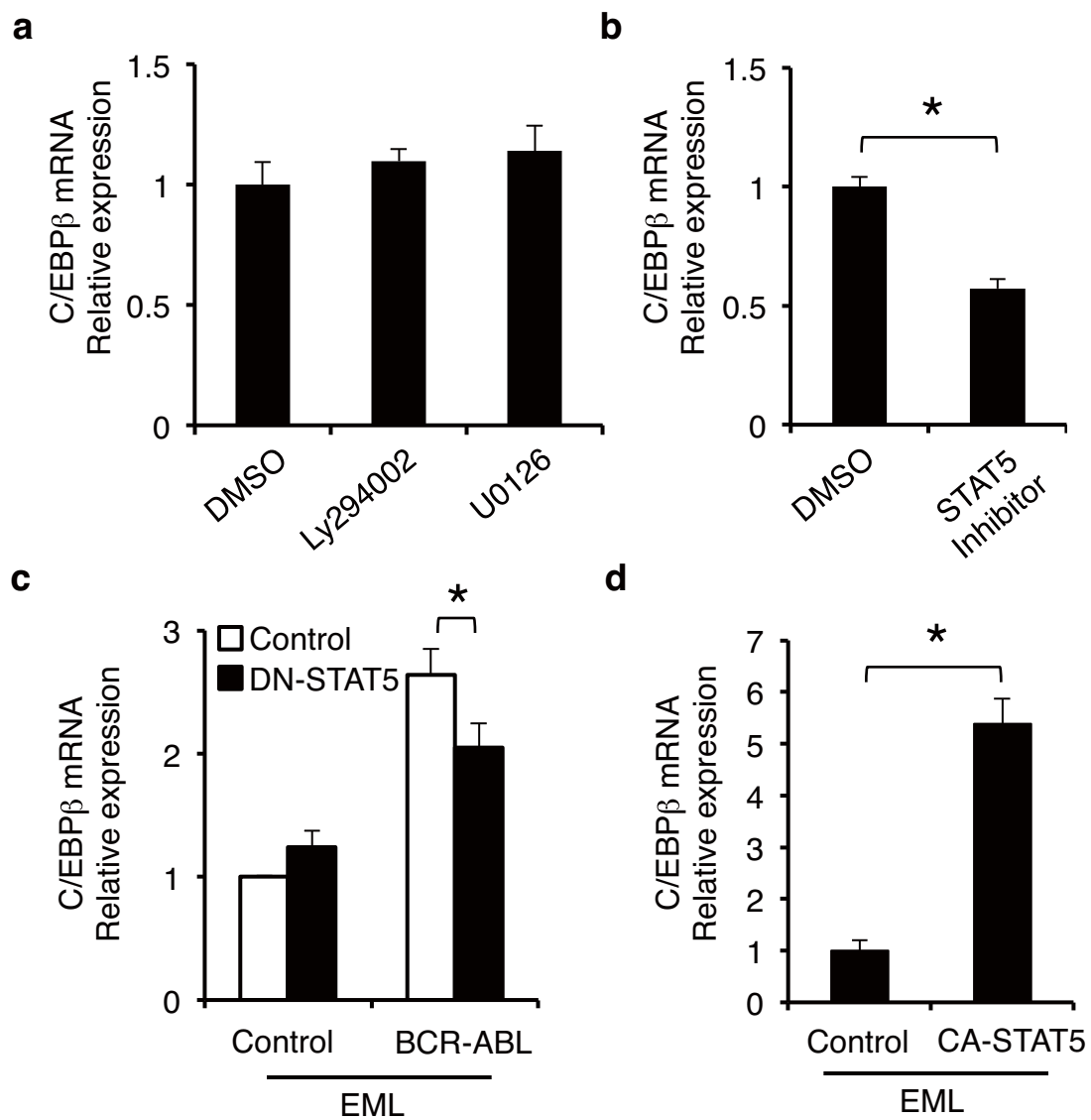


Figure 4

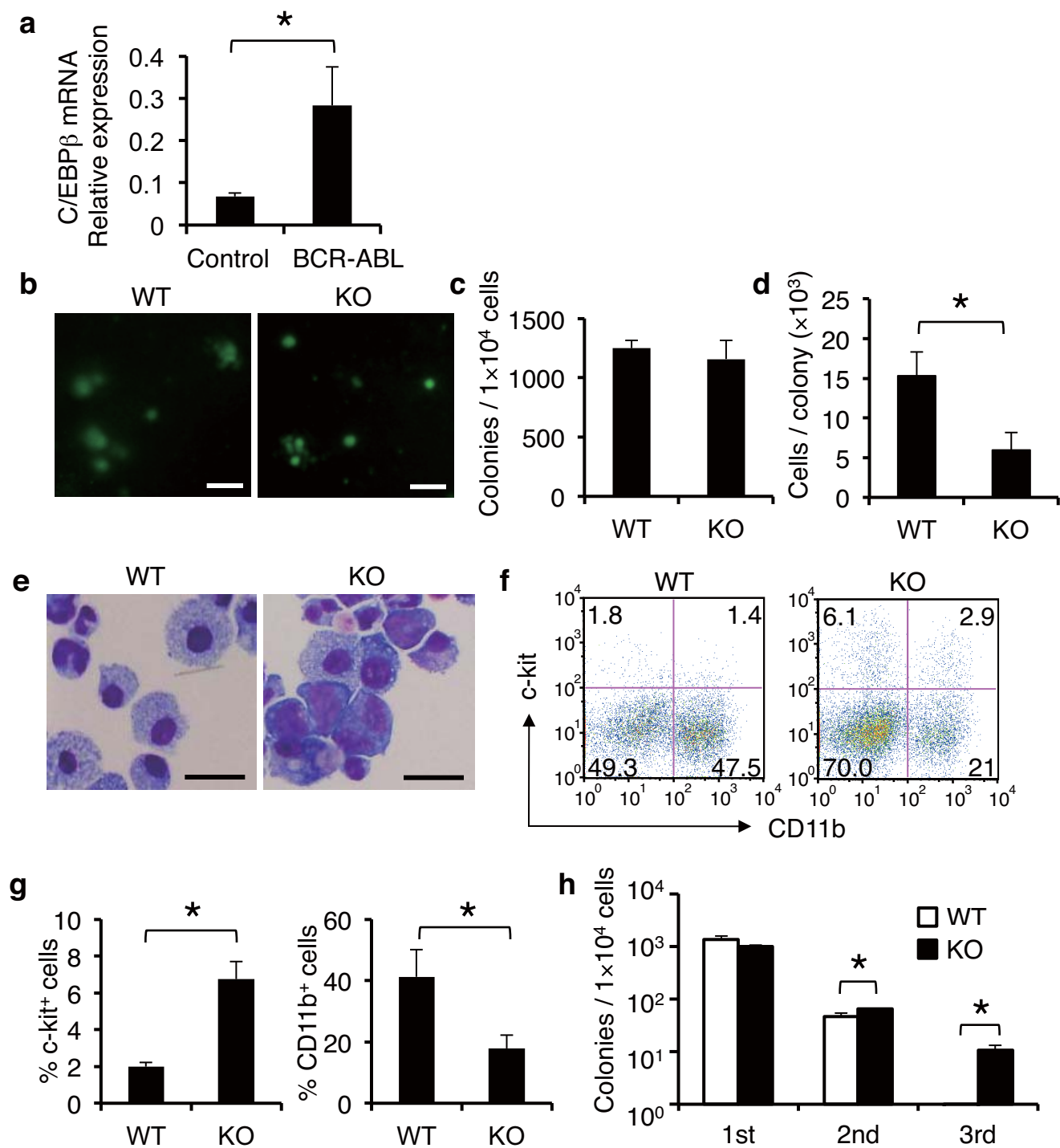


Figure 5

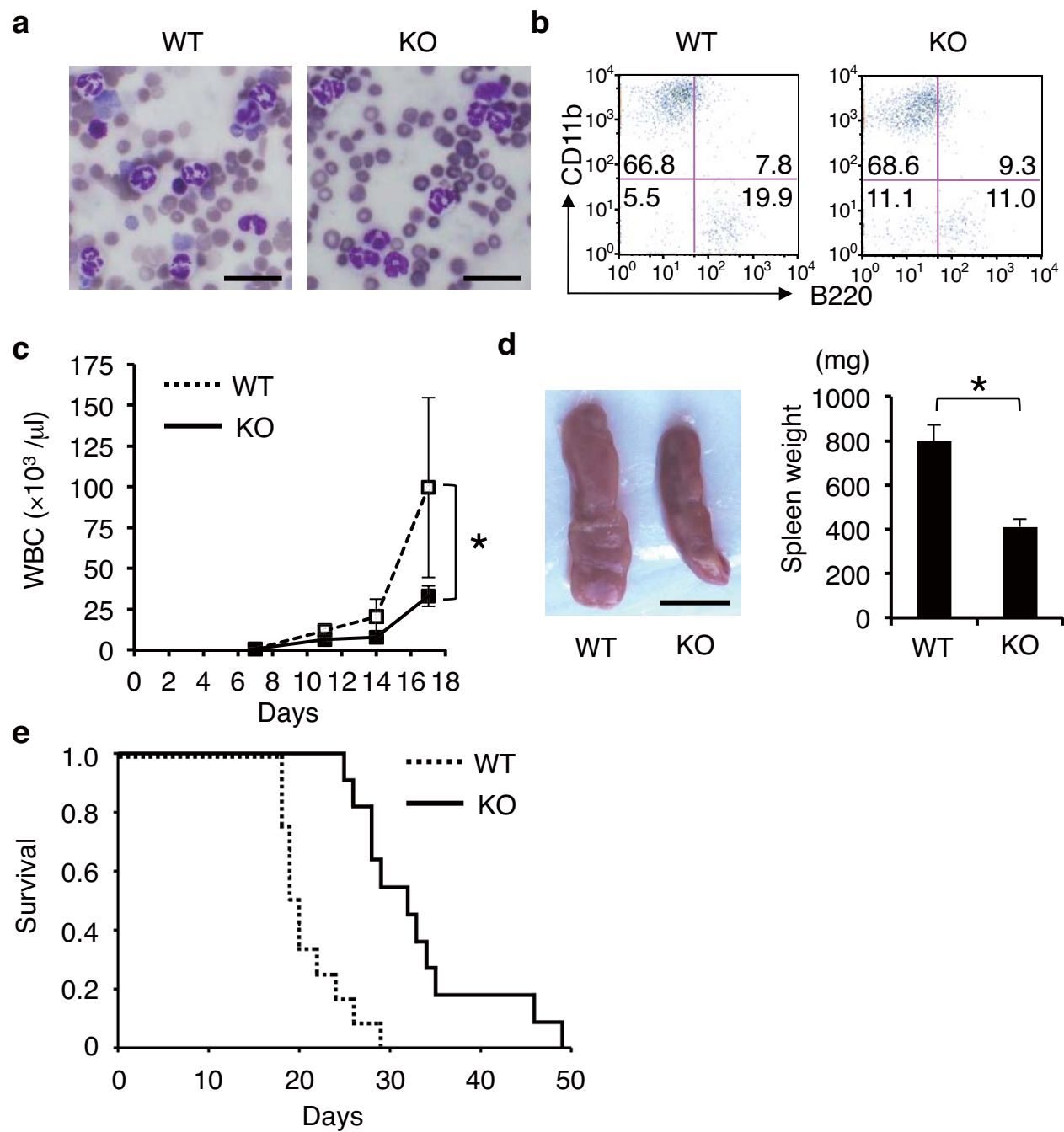
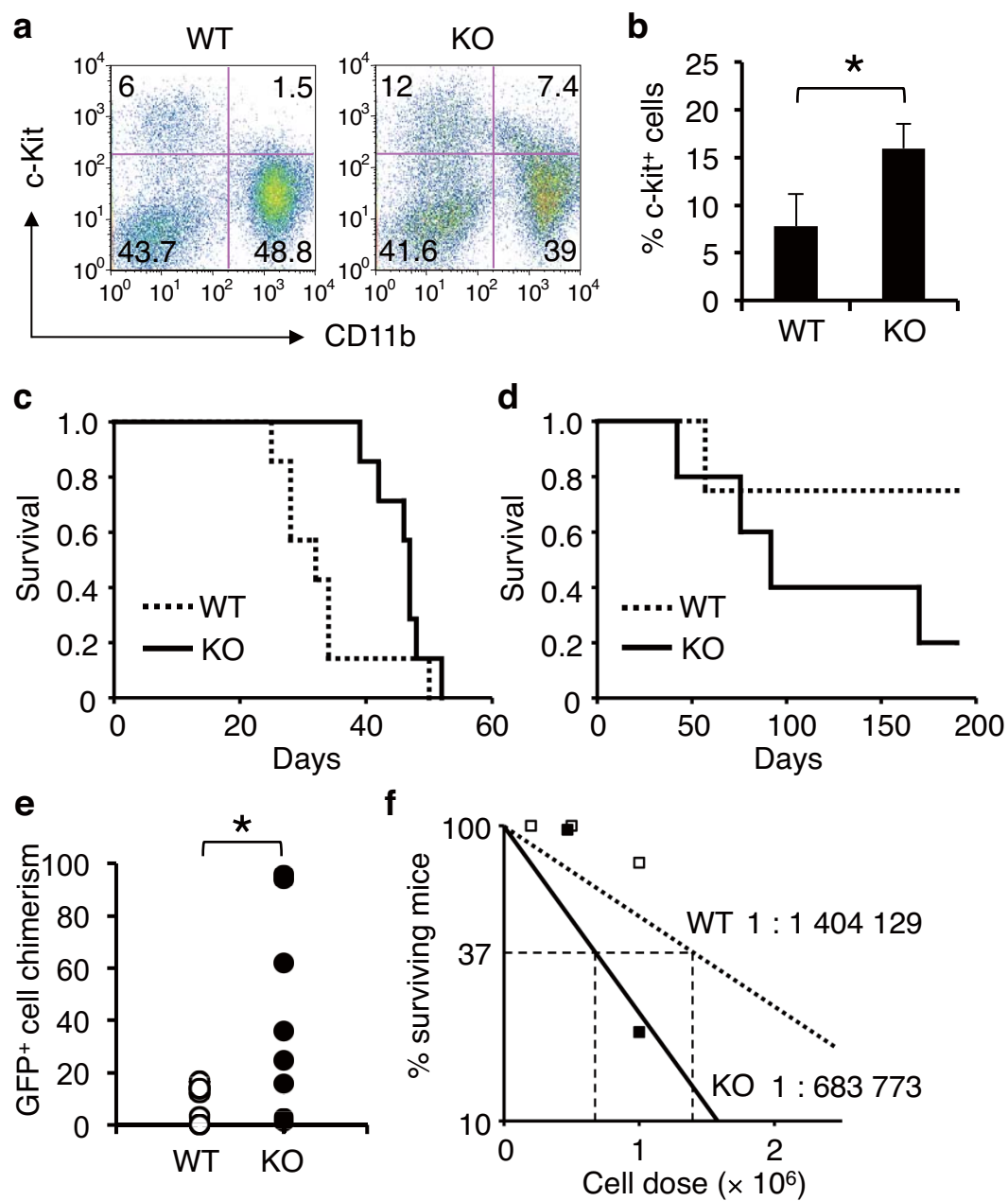


Figure 6



Supplementary Figure Legend

Figure S1. Frequency of myeloid progenitors in bone marrow of healthy donors and patients with CP-CML.

Frequency of CD34⁺ CD38⁺ myeloid progenitors within the Lin⁻ fraction of bone marrow cells. Error bars indicate SD (healthy donors, $n = 6$; CP-CML, $n = 5$; $*P < 0.05$).

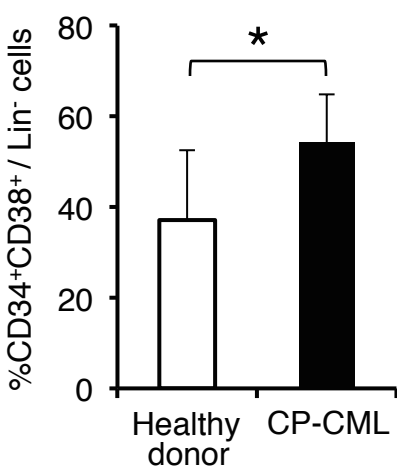
Figure S2. BCR-ABL-transduced C/EBP β KO cells could be replated at least up to 5 times.

Serial colony-replating of 1×10^4 BCR-ABL-transduced C/EBP β WT and KO bone marrow cells. The colonies were counted and collected on day 10 (2nd) or 14 (3rd, 4th, and 5th), respectively. Error bars indicate SD from triplicate cultures.

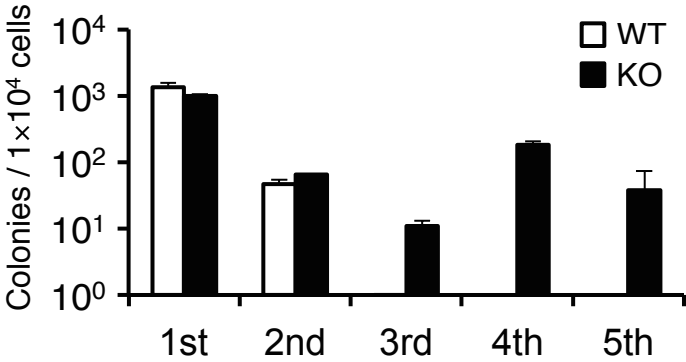
Figure S3. C/EBP β expression in EML cells and 32Dcl3 cells.

C/EBP β mRNA levels in EML cells and 32Dcl3 cells. Results were mean values of two independent experiments and normalized to the expression level of EML cells. Error bar indicates SD. $*P < 0.01$.

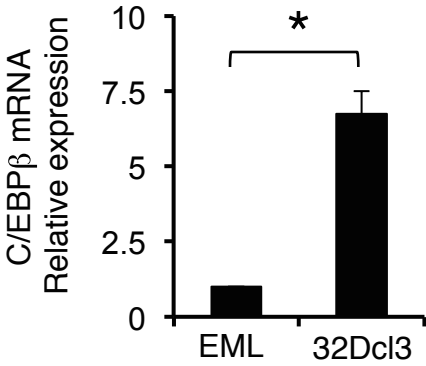
Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3



Supplementary Table 1. The characteristics of the patients

Patient ID	Gender / Age	Race	Disease stage	Previous treatment	Complete Blood Count			
					WBC ($10^9/L$)	RBC ($10^{12}/L$)	HGB (g/L)	PLT ($10^9/L$)
147742	M / 42	Asian	CP	No	16.1	3.68	121	98
06-620	F / 66	Caucasian	CP	No	155.6	3.08	100	1147
2008032001	NA	Caucasian	CP	No	NA	NA	NA	NA
EBO-BM0129	M / NA	Asian	CP	No	76.8	4.03	112	1116
160568	M / NA	Asian	CP	No	NA	NA	NA	NA

CP, chronic phase; WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin; PLT, platelet. NA indicates not available.