Identification of CD25 as STAT5-Dependent Growth-Regulator of Leukemic Stem Cells in Ph+ CML

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Abstract

Purpose: In chronic myelogenous leukemia (CML), leukemic stem cells (LSC) represent a critical target of therapy. However, little is known about markers and targets expressed by LSCs. The aim of this project was to identify novel relevant markers of CML LSCs.

Experimental Design: CML LSCs were examined by flow cytometry, qPCR, and various bioassays. In addition, we examined the multipotent $CD25^+$ CML cell line KU812.

Results: In contrast to normal hematopoietic stem cells, $CD34^+/CD38^-$ CML LSCs expressed the IL-2 receptor alpha chain, IL-2RA (CD25). STAT5 was found to induce expression of CD25 in $Lin^-/Sca\text{-}1^+/Kit^+$ stem cells in C57Bl/6 mice. Correspondingly, shRNA-induced STAT5 depletion resulted in decreased CD25 expression in KU812 cells. Moreover, the BCR/ABL1 inhibitors nilotinib and ponatinib were found to decrease STAT5 activity and CD25 expression in KU812 cells and primary CML LSCs. A CD25-targeting shRNA was found to augment proliferation of KU812 cells *in vitro* and their engraftment *in vivo* in NOD/SCID-IL-2R$\gamma^{-/-}$mice. In drug-screening experiments, the PI3K/mTOR blocker BEZ235 promoted the expression of STAT5 and CD25 in CML cells. Finally, we found that BEZ235 produces synergistic antineoplastic effects on CML cells when applied in combination with nilotinib or ponatinib.

Conclusions: CD25 is a novel STAT5-dependent marker of CML LSCs and may be useful for LSC detection and LSC isolation in clinical practice and basic science. Moreover, CD25 serves as a growth regulator of CML LSCs, which may have biologic and clinical implications and may pave the way for the development of new more effective LSC-eradicating treatment strategies in CML.

Introduction

Chronic myelogenous leukemia (CML) is a hematopoietic stem cell (SC) disorder defined by the reciprocal translocation t(9;22) and the related oncoprotein, BCR/ABL1 (1–3). It is generally appreciated that BCR/ABL1 is a major driver responsible for initiation and evolution of CML (2–4). Correspondingly, the BCR/ABL1-targeting tyrosine kinase inhibitor (TKI) imatinib induces major cytogenetic and molecular responses in a majority of patients with chronic phase (CP) CML (4, 5). However, although long-term disease control can be achieved in many patients, imatinib is usually unable to eliminate CML. This phenomenon is best explained by intrinsic and acquired drug resistance in leukemic stem cells, LSC (6–12). The intrinsic form of resistance is common to all LSC fractions and is considered to be independent of BCR/ABL1. By contrast, the acquired form of TKI resistance is caused by newly acquired, subclone-specific defects, including BCR/ABL1 mutations (7–13).

The 'LSC hypothesis' is based on the observation that only a subset by leukemic progenitors exhibits long-term disease-propagating capacity (14–16). This concept has major implications for the development of curative treatment approaches (7–19). LSC research is currently focusing on LSC-specific targets and drugs capable of attacking LSCs (17–19). In CML and other leukemias, the development of such LSC-targeting concepts is a major challenge (17–19). Notably, many different factors, including multiple signaling cascades and the so-called SC niche, regulate the development and expansion of LSCs in CML (9–11, 17–19).

One important regulator of survival and growth of CML LSCs appears to be the transcription factor STAT5 (20–23). A number of previous and more recent studies have shown that BCR/ABL1 triggers STAT5 activity in CML cells (20–23). In addition, however, STAT5 expression and activation may be regulated independently of BCR/ABL1 in CML cells (11, 24). Especially in LSCs, STAT5 expression may be induced by BCR/ABL1-independent mechanisms. Recent data suggest that STAT5 triggers production of reactive oxygen species and clonal instability and thereby promotes the occurrence of BCR/ABL1 mutations (24).

CML LSCs are considered to represent a small subset of $CD34^{+}/CD38^{-}$ cells in the leukemic clone (7–10, 25–27). However, since normal bone marrow stem cells (BM SCs) also display this phenotype, additional markers need to be applied to differentiate normal BM SCs from CML LSCs. Recent studies have shown that CML LSCs specifically express IL-1RAP and dipeptidyl-peptidase IV, DPPIV = CD26 (28–30). As assessed by gene array analyses, CML LSCs may

express additional markers (30–32). One of these aberrant markers appears to be the low-affinity receptor for IL-2, CD25 (30–32). However, little is known about the functional role of CD25 in human CML LSCs and the mechanisms contributing to abnormal CD25 expression.

In this study, we show that expression of CD25 on CML LSCs is triggered by STAT5 and that CD25 acts as a negative regulator of LSC growth in CML. In addition, we show that BCR/ABL1 TKIs downregulate STAT5- and CD25 expression in LSCs, whereas the PI3K/mTOR blocker BEZ235 promotes CD25 expression.

Translational Relevance

Although chronic myelogenous leukemia (CML) is a stem cell disease, little is known about the expression and function of specific markers and targets in CML LSCs. We here describe that CD25 serves as a novel robust marker of CML LSCs that can be used for detection, enumeration, and isolation of LSCs in these patients. Moreover, CD25 was found to serve as a drug-inducible suppressor of LSC expansion, which may be relevant clinically and may pave the way for the development of new treatment strategies. Based on our work, a straightforward approach might be to combine BCR/ABL1 tyrosine kinase inhibitor that downregulates CD25 expression with targeted drugs promoting CD25 expression, such as the PI3K/mTOR blocker BEZ235. Indeed, these drug combinations produced highly synergistic antiproliferative effects on KU812 cells in the present study.

Materials and Methods

Reagents

A detailed description of reagents used in this study is provided in the Supplement. Monoclonal antibodies (mAb) used in this study are described in Supplementary Table S1.

Cell lines

The multipotent human BCR/ABL1+ cell line KU812 was kindly provided by Dr. K. Kishi (Niigata University, Niigata, Japan) in 1989; K562 cells and murine Ba/F3 cells expressing various BCR/ABL1 mutants (M244V, G250E, Q252H, Y253H, E255K, E255V, T315I, F317L, F317V, F359V, and H396P) or wild- type BCR/ABL1 were kindly provided by Dr. M. Deininger (Hunts- man Cancer Institute, University of Utah, Salt Lake City, UT) in 2013; and imatinib-resistant K562 cells (K562-R) were kindly provided by Dr. J. D. Griffin (Dana-Farber Cancer Center, Harvard Medical School, Boston, MA) in 1999. KCL-22 cells were purchased from the German Collection of Microorganism and Cell Culture (DSMZ) in 2010. The identity of KU812, K562, and K562- R cells was confirmed by DSMZ using nonaplex-PCR in 2010. All experiments were performed from these stocks, and cells were thawed from these stocks (or secondary stocks) every 1 to 3 months. Cell lines were maintained in RPMI 1640 medium, 10% fetal calf serum (FCS), and antibiotics at 37°C. K562-R cells were cultured in the presence of 1 μmol/L imatinib. Mouse M2-10B4 feeder cells were purchased from the American Type Culture Collection. Ecotropic retroviral packaging cell lines GP^{+}/E86 encoding for STAT5A-IRES-GFP, STAT5B-IRES-GFP (33), or the empty vector, and GP^{+}/E86 cells encoding for $p210^{BCR/ABL1}$-IRES- dsRED (23) were maintained in complete medium supplemented with 10% FCS as described (23, 33).

Patients and cell sampling

Sixty-three patients with BCR/ABL1+ CML (32 females, 31 males) were examined for expression of CD25 on $CD34^{+}/CD38^{-}$ CML LSCs and $CD34^{+}/CD38^{+}$ CML progenitor cells. The median age was 54 years (range, 18–86 years). Most patients were examined at diagnosis (before treated with BCR/ABL1 TKI). The patients' characteristics are shown in Supplementary Table S2. Peripheral blood (PB) and/or BM cells (iliac crest or sternum) were collected at diagnosis and in the follow-up. Control samples included normal/reactive BM and other myeloid neoplasms (Supplementary Table S3). All donors gave written informed consent, and all studies were

approved by the ethics committees of the Medical University of Vienna and the University of Veterinary Medicine Vienna.

Flow cytometry experiments

Phenotyping of $CD34^+/CD45^+/CD38^-$ (L)SCs and $CD34^+/CD45^+/CD38^+$ progenitor cells was performed on BM or PB cells (unseparated or Ficoll-isolated) by multicolor flow cytometry as described (34, 35) using fluorochrome-conjugated mAb shown in Supplementary Table S1. Flow cytometry was performed on a FACSCalibur (Becton Dickinson). A detailed description of the staining techniques is provided in the Supplement.

Retroviral infection of murine BM cells

Retroviral packaging GP^+/E86 cells encoding for STAT5A-IRES- GFP, STAT5B-IRES-GFP (33), or empty vector were mixed 1:1 with GP^+/E86 cells encoding $p210^{BCR/ABL1}$-IRES-dsRED (23) and seeded on gelatine (0.2%) precoated dishes. Single-cell suspensions from total wild-type BM cells of C57Bl/6N mice were prepared, and $3x10^6$ cells/mL were cocultured with GP^+/E86 cells in DMEM supplemented with murine IL-3 (25 ng/mL), IL-6 (50 ng/mL), stem cell factor, SCF (50 ng/mL), and polybrene (7 µg/mL) for 72 hours. Thereafter, BM cells were stained with mAb PC61 directed against CD25 and analyzed by flow cytometry using a BD FACS-Canto II FACS device and BD FACS Diva software (Becton Dickinson). Six-to-eight–week-old male C57Bl/6N mice (20 g) were purchased from the Jackson Laboratory.

Gene array analyses

KU812 cells transduced with a random (RDM) shRNA or a CD25 shRNA were subjected to RNA isolation and gene array analysis using Affymetrix technology. Details are provided in the Supplement. A complete set of mRNA data is available at Gene Expression Omnibus, accession number:

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token1⁄4orotekeollgdduf&acc1⁄4GSE60315.

Quantitative PCR and cytogenetics

RNA was isolated from CML cell lines, primary CML (stem) cells, and pooled colony-derived cells. CD25-, CD122-, CD132-, and BCR/ABL1 mRNA expression levels were measured by qPCR as reported (29, 35). Conventional cytogenetics and FISH were performed according to

published protocols (36). A detailed description of the methods applied is provided in the Supplement.

Transfection studies

Knockdown studies with shRNAs were performed following published techniques (37, 38). In CD25 knockdown studies, 3 shRNAs against human CD25 (clone #1: 5'-AAATCTGTTGTTGT-GACGAGG-3' ; clone #2: 5' -TTTCTGTTCTTCAGGTTGAGG-3' ; clone #5: 5'-TACTCTGTTGTAAATATGGAC-3'; Open Biosystems) expressed in pLKO.1 lentiviral vector containing the puromycine-resistance gene were applied. In select experiments ($CD34^+$ cells), the puromycine-resistance gene was replaced by mCherry. For knockdown of STAT5, an shRNA targeting STAT5A and STAT5B (5' -GCAGCAGACCATCATCCTG-3' ; ref. 37) expressed in pLKO.1 containing the puromycine-resistance gene was used. The shRNA-mediated knockdown of CD25 was induced in KU812 cells and primary $CD34^+$ CML cells, and knockdown of STAT5A/STAT5B in KU812 cells. Transfected KU812 cells were selected by exposure to puromycin (2 µg/mL), and transduced primary $CD34^+$ CML cells by sorting for mCherry. Knockdown was confirmed by flow cytometry (CD25) or Western blotting (STAT5) according to published techniques (39). To evaluate the functional consequence of the CD25 knockdown, CD25 shRNA (clones #1, #2, and #5)-transduced KU812 cells and KU812 transduced with random (RDM) shRNA were mixed at 1:1 and cultured. Cell mixtures were periodically examined for CD25 expression by flow cytometry using phycoerythrin (PE)-conjugated mAb 2A3 against CD25. To study proliferation of KU812 cells and primary $CD34^+$ CML cells transduced with CD25 shRNA (clone #2) or RDM shRNA, ^{3}H-thymidine uptake experiments were performed. In a separate set of experiments, CD25 was lentivirally expressed in K562, K562-R, and KCL-22 cells. A precision LentiORF IL-2RA (CD25) gene construct (GFP tag) and a control construct was obtained from GE Dharmacon. Virus production and transduction of cells were performed as described above, and GFP^+ cells were selected by cell sorting. Purified cells were applied in mixing experiments (1:1 of empty vector control-transduced cells and CD25-transduced cells) as described above and analyzed 3 times/week using APC-conjugated CD25 mAb BC96.

Xenotransplantation assay

To investigate the functional role of CD25 in CML cells *in vivo*, KU812 cells transduced with

shRNA against CD25 (clone #2) or with RDM shRNA were injected intravenously into nonirradiated nonobese diabetic NOD/SCID-IL-2R$\gamma^{-/-}$ (NSG) mice (5 per group). Ten-to-twelve-week-old, female NSG mice (20–30 g) were purchased from the Jackson Laboratory. Cells were resuspended in 0.15 mL RPMI with 10% FCS and antibiotics and injected into the tail vein of NSG mice ($1x10^6$ cells/mouse). After 6 weeks, blood was drawn to analyze engraftment. Then, mice were sacrificed and BM cells (tibia, femur, pelvis) and spleens were isolated and prepared for immunohistochemistry and flow cytometry. The size and weight of spleens were measured in each animal. Then, splenic samples were prepared as follows: each spleen was cut into small pieces with a scalpel and mashed through a small-sized filter to obtain single-cell suspensions. Long bones were flushed to recover BM cells for flow cytometry in order to document engraftment. Multicolor flow cytometry was performed using mAb 515 against CD44 for detection of KU812 cells. TO-PRO-3 (Invitrogen) was used to exclude nonviable cells.

Long-term culture-initiating cell assay

To investigate the long-term growth of sorted $CD34^+/CD38^-/CD25^+$ LSCs and $CD34^+/CD38^-/CD25^-$ (presumably normal) SCs obtained from 2 patients with CP CML, a long-term culture-initiating cell (LTC-IC) assay was performed as described (29). A detailed description of the method applied is provided in the Supplement.

Statistical evaluations

To determine the level of significance in differences in CD25 expression and pSTAT5 levels in LSCs in various donor-groups or before and after drug exposure, the Student t test was applied. Results were considered to be significantly different, when $P < 0.05$. Drug combination experiments were performed following published guidelines (40). Drug interactions were determined by calculating combination index (CI) values using Calcusyn software (Calcusyn; Biosoft) as described previously (40, 41). A CI of less than 1 indicates synergy, a CI of 1 an additive effect, and a CI of more than 1 an antagonistic effect.

Results

Human CML LSCs express IL-2RA (CD25)

As assessed by flow cytometry, $CD34^+/CD38^-$ LSCs expressed CD25 in 50 of 54 patients with CML (92.6%; Fig. 1A and B). CD25 was detected on LSCs in patients with CP (40/43), accelerated phase (AP, 2/3), and blast phase (BP, 8/8). As expected, highly enriched CML LSCs expressed CD25 mRNA in all samples tested (Fig. 1C). $CD34^+/CD38^+$ CML progenitor cells expressed only trace amounts or no detectable CD25 (Fig. 1C and D). During treatment with imatinib (14 patients) or other TKIs (3 patients), the numbers of $CD25^+$ LSCs decreased in the BM compared to pretreatment values (Fig. 1E). CML LSCs did not express IL-2RB (CD122) or IL-2RG (CD132) at the mRNA level (Fig. 1C) or protein level (Table 1). In the normal/reactive BM, $CD34^+/CD38^-$ SCs did not express CD25 (Fig. 1B). Moreover, CD25 was not detectable on $CD34^+/CD38^-$ SCs in various control groups, including 13 of 14 patients with idiopathic cytopenia of unknown significance (ICUS), other classical myeloproliferative neoplasms (MPN), or mastocytosis (Fig. 1B).

Aberrant expression of CD25 on CML cells is restricted to LSCs

As assessed by multicolor flow cytometry, $CD25^+$ CML LSCs coexpressed two other well-established LSC antigens, IL-1RAP and DPPIV (CD26). In addition, $CD25^+$ CML LSCs coexpressed Siglec-3/CD33, CD44, CD52, and KIT/CD117 (Table 1; Supplementary Fig. S1). Among more mature myeloid cells in the CML clone, only basophils were found to express CD25, with slightly higher expression levels observed in CML basophils compared with normal basophils (Table 2). In all other myeloid lineages examined, including monocytes and mast cells, CD25 was not detectable in our CML patients. These data suggest that aberrant expression of CD25 in the CML clone is restricted to LSCs. In a subset of patients, we separated the $CD25^+$ and the $CD25^-$ SC fractions from the same samples to near homogeneity (>95% purity) by cell sorting. In these experiments, all $CD25^+$ LSCs (100%) contained BCR/ABL1 by FISH, whereas only 45% of the CD25 SCs expressed BCR/ABL1 (Supplementary Fig. S2; Supplementary Table S4). The long-term proliferative (LTC-IC) capabilities of $CD25^+$ CML LSCs and normal $CD25^-$ SCs obtained from the same patients are shown in Supplementary Fig. S3.

STAT5 activity contributes to the expression of CD25 in LSCs

Because CD25 is a STAT5 target gene, we applied shRNA against STAT5, which led to a substantial knockdown of STAT5 expression in KU812 cells (Supplementary Fig. S4). The STAT5 knockdown resulted in a decreased expression of CD25 at the mRNA and protein level (Fig. 2A). A STAT5-targeting drug, pimozide, was also found to downregulate CD25 expression in KU812 cells (Fig. 2B). A similar effect was seen with the MEK inhibitors PD0325901 and RDEA119 (Fig. 2C), whereas the PI3K/mTOR blocker BEZ235 did not inhibit CD25 expression. Rather, BEZ235, the mTOR-targeting drug RAD001, and the PI3K inhibitor LY294002, were found to upregulate expression of CD25 in KU812 cells (Fig. 2D) as well as in the other CML cell lines examined (Supplementary Fig. S5A). Similar results were obtained with primary CML LSCs. Again, the STAT5 blocker pimozide decreased CD25 expression (Fig. 3A), and BEZ235 and RAD001 were found to augment CD25 expression on $CD34^+/CD38^-$ CML LSCs (Fig. 3B). Finally, BEZ235 was found to upregulate CD25 expression on Ba/F3 cells containing various BCR/ABL1 mutants, including E255K, F317L, and T315I (Supplementary Fig. S5B). As expected, BEZ235 was also found to inhibit proliferation in these cells (Supplementary Fig. S5C). Together, these data suggest that expression of CD25 on CML LSCs is regulated by a signaling cascade involving STAT5, PI3K, and mTOR.

Effects of BCR/ABL1-targeting TKIs on expression of pSTAT5 and CD25 in CML cells

All three BCR/ABL1 TKIs tested (imatinib, nilotinib, and ponatinib) were found to inhibit the expression of pSTAT5 and of CD25 in KU812 cells (Fig. 3C). Nilotinib and ponatinib were also found to block CD25 expression in primary CML LSCs (Fig. 3D). However, the weaker BCR/ABL1 blocker imatinib showed no significant effect on expression of CD25 on primary CML LSCs (Fig. 3D). All three TKIs were found to downregulate expression of pSTAT5 in primary CML LSCs (Fig. 3E). Finally, we found that imatinib, nilotinib, and ponatinib downregulate the expression of CD25 in Ba/F3 cells expressing various mutant forms of BCR/ABL1, including E255K, G250E, F317V, and F317L (Supplementary Fig. S6).

Retroviral infection of murine SCs with STAT5 is followed by upregulation of CD25

Infection of BM stem cells of C57Bl/6 mice with a retroviral vector encoding either STAT5A or STAT5B, or coinfection of BCR/ABL1-p210 and STAT5, resulted in an enhanced expression of CD25 on $Lin^-/Sca\text{-}1^+/Kit^+$ (LSK) cells (Fig. 4). There was no difference in expression of CD25

on LSK cells when comparing cells infected with STAT5 alone with cells infected with both, STAT5 and BCR/ABL1 (Fig. 4). Interestingly, infection of BM cells with BCR/ABL1 alone was not followed by a major increase in expression of CD25 on LSK cells (Fig. 4). In line with this observation, infection with BCR/ABL1 alone also failed to substantially increase the expression of STAT5 in LSK cells (not shown). These data suggest that expression of CD25 in LSK cells is triggered by STAT5 activity in the absence and presence of BCR/ ABL1 in C57Bl/6 mice.

Identification of CD25 as a regulator of growth of CML cells

We next examined the potential biologic function of CD25 on CML LSCs. In initial experiments, we were unable to demonstrate any effects of IL-2 on growth of KU812 cells, K562 cells, or primary CML cells (Supplementary Fig. S7). In addition, preincubation with IL-2 did not alter responses of primary CML cells to the PI3K/mTOR inhibitor BEZ235 (Supplementary Fig. S7). Finally, we applied a random (RDM) shRNA and 3 different CD25-specific shRNAs (clone #1, #2, and #5) on KU812 cells. In these experiments, the knockdown of CD25 resulted in an increased proliferative capacity compared with cells transduced with an RDM shRNA (Fig. 5A). We also confirmed the growth advantage of CD25-negative KU812 cells *in vivo* in an NSG xenotransplantation model. NSG mice injected with CD25 shRNA–transfected (clone #2) KU812 cells showed significantly higher engraftment levels in the BM, PB, and spleen when compared with mice injected with control cells (Fig. 5B). In a next step, we transduced $CD34^+$ SC from 2 patients with CML CP (23%–30% of the $CD34^+$ cells expressed CD25; and >80% of the $CD34^+/CD38^-$ cells expressed CD25) with a specific CD25 shRNA (clone #2) or an RDM control shRNA. The knockdown of CD25 resulted in an enhanced proliferation of these $CD34^+$ CML cells (Fig. 5C). Finally, we expressed CD25 in the CD25-negative (or weakly CD25-positive) CML cell lines K562, K562-R, and KCL-22 by lentivirus-mediated transduction. In all three cell lines tested, transduction with CD25 resulted in decreased proliferation (Supplementary Fig. S8). Together, these data strongly suggest that CD25 acts as a "leukemia-suppressing" molecule in CML (stem) cells.

Identification of CD25-regulated target genes in KU812 cells

In an attempt to define the mechanism of CD25-induced growth inhibition in CML cells, we performed gene array analyses using KU812 cells transfected with RDM shRNA or CD25 shRNA (clone #2). In these analyses, a number of up- and down- regulated mRNA species were identified in CD25-depleted cells (Supplementary Table S5). Among the top upregulated gene

products, a number of mediators of cell growth, proliferation, or survival were identified, including several members of the DAZ- family, RAB27B, IGFBP7, SSX2, RASGEF1A, or IGF-1 (Supplementary Table S5A). Downregulated genes included several "tumor-suppressor" genes, genes involved in lymphocyte activation, inflammatory response, and cell signalling (Supplementary Table S5B). Together, these data suggest that multiple target genes may contribute to CD25-induced growth inhibition in CML cells. In a next step, we performed pathway analyses in order to detect additional genes or gene patterns relevant to CD25-induced growth inhibition. However, no additional genes or pathways potentially involved in CD25-induced suppression of SC growth could be identified (Supplementary Fig. S9). We also compared cell-cycle progression, mitosis, and apoptosis in shRNA-transduced versus RDM control shRNA-transduced KU812 cells. In these experiments, we were able to show that the shRNA-induced knockdown of CD25 resulted in a slightly increased number of mitotic cells compared with RDM-transduced cells (Supplementary Fig. S10). By contrast, we were unable to detect any changes in the percentage of apoptotic cells after CD25 shRNA transduction (Supplementary Fig. S10). Finally, shRNA-induced knockdown of CD25 did not alter cell-cycle progression in KU812 cells (Supplementary Fig. S10).

Identification of CD25 as a BEZ235-induced growth inhibitor: a rationale for the design of drug combinations with synergistic effects

In a final step, we asked whether CD25 can serve as a secondary "drug effector" blocking the growth of CML cells. The shRNA-induced knockdown of CD25 in KU812 cells was not followed by any changes in their responses to imatinib, nilotinib, or ponatinib (comparable IC_{50} values; Supplementary Fig. S11A). However, the shRNA-induced knockdown of CD25 resulted in a decreased response of KU812 cells to BEZ235 (Supplementary Fig. S11A). Since BEZ235 upregulates CD25 in KU812 cells, these data suggest that CD25 may serve as a secondary target mediating BEZ235-induced growth inhibition. Because we also noted that BCR/ABL1 TKIs decrease CD25 expression, we asked whether combined application of BEZ235 and BCR/ABL1 TKIs would result in cooperative growth-inhibitory effects. Indeed, combinations of BEZ235 and nilotinib and BEZ235 and ponatinib resulted in strong synergistic growth-inhibitory effects in KU812 cells (Supplementary Fig. S11B).

Discussion

Although LSCs are an emerging new target of therapy, little is known about disease-specific markers and targets expressed in CML LSCs. We here show that CML LSCs aberrantly express CD25 and that STAT5 triggers the expression of CD25 on LSCs. Moreover, we show that shRNA-induced CD25-depletion in CML cells is associated with enhanced proliferation *in vitro* and enhanced engraftment of KU812 cells in NSG mice. These data suggest that CD25 is a novel biomarker of CML LSCs and that it acts as a 'leukemia-suppressing' molecule, which may have clinical and diagnostic implications.

Recently, gene array studies have shown that CML LSCs express CD25 mRNA in excess over normal SCs (30–32). In the current study, we confirmed these results at the mRNA and protein level. In particular, CD25 was found to be expressed on CML LSCs in almost all patients tested, independent of the phase of disease. The levels of CD25 on LSCs varied from donor to donor, but CD25 was detectable in >90% of all patients with CML. By contrast, in most control samples tested, normal $CD34^+/CD38^-$ BM SCs did not express CD25. After successful treatment with imatinib, the numbers of $CD25^+$ SCs in the BM decreased substantially in our CML patients. However, in a few patients entering a major molecular response (BCR/ABL1 <0.1%), $CD34^+/CD38^-$ SCs still expressed CD25, even when CD26 and IL-1RAP were no longer detectable. This phenomenon may have several explanations. First, these cells may represent an early (BCR/ABL1-negative) phase of LSC evolution (42, 43). An alternative explanation would be that normal SCs in these patients were in an activated state and therefore expressed CD25. Recent data suggest that LSCs in patients with acute myeloid leukemia express CD25 (44). In the present study, we asked whether CD25 is specifically expressed on CML LSCs among MPNs. However, we were neither able to detect CD25 on SCs in patients with JAK2-mutated MPNs nor in patients with systemic mastocytosis, which was an unexpected result, because STAT5 activation occurs downstream of JAK2 V617F and KIT D816V. One explanation may be that additional (disease- or cell-specific) factors, apart from STAT5, are required for induction of CD25 expression in LSCs. We therefore asked whether aberrant expression of CD25 is detectable in various lineages in the CML clone. However, we were unable to detect substantial amounts of CD25 in other cell types in our CML patients, including $CD34^+/CD38^+$ progenitor cells and more mature myeloid cells in the CML clone. These data suggest that aberrant expression of CD25 is confined to the LSC fraction in CML.

A number of previous studies have shown that CD25 is a STAT5 target gene (45, 46). Other studies have shown that BCR/ABL1 directly triggers STAT5 activation (20–23). In the present study, we were able to show that expression of CD25 on CML LSCs is dependent on STAT5 activity. First, the shRNA-induced knockdown of STAT5 resulted in a decreased expression of CD25. Furthermore, the STAT5-targeting drug pimozide was found to inhibit expression of CD25 in KU812 cells and primary CML LSCs. Finally, infection of murine BM SCs with STAT5A or STAT5B resulted in an enhanced expression of CD25. An interesting observation was that CD25 expression on murine CML LSCs (LSK cells) was induced by STAT5 rather than by BCR/ABL1, contrasting recently published results (32). One explanation for these discrepant data would be differences in the transfection assay or transfection efficacy. Another explanation may be that BCR/ABL1 triggers STAT5 activation rather than STAT5 production in LSK cells.

Imatinib and other BCR/ABL1 TKIs remain the standard of treatment in patients with Ph^+ CML (4, 5, 47). However, the potency of nilotinib and ponatinib against BCR/ABL1 clearly exceeds the potency of imatinib (48–50). We asked whether exposure to TKIs would result in a decreased expression of activated STAT5 and CD25. In KU812 cells, all three TKIs were found to inhibit the expression of pSTAT5 and expression of CD25, without major differences in IC_{50} values. In primary patient-derived LSCs, however, ponatinib was the most potent compound, followed by nilotinib, whereas imatinib showed only little if any effect. These data may be explained by the fact that ponatinib and nilotinib are more potent inhibitors of BCR/ABL1. Alternatively, CD25 expression in LSCs is dependent not only on BCR/ABL1 and STAT5 but also on other targets recognized by ponatinib and nilotinib, but not imatinib.

Next, we explored the functional role of CD25 in CML LSCs. In a proliferation assay, IL-2 did not modulate growth of CML cells even when applied at high concentrations. In line with this observation, CML LSCs and KU812 cells stained negative for IL-2RB (CD122) and IL-2RG (CD132). Recent data suggest that CD25 expression on LSK cells correlates with their proliferative capacity *in vivo* (32). By contrast, we observed a growth-promoting effect of various CD25 shRNAs in KU812 cells. In fact, the shRNA-induced knockdown of CD25 was found to be associated with an enhanced proliferation *in vitro* and with a significantly increased engraftment of KU812 cells in NSG mice. These data suggest that CD25 is involved in the regulation of growth of KU812 cells. Moreover, we were able to show that lentivirus-mediated expression of CD25 in the CD25-negative cell lines K562, K562-R, and KCL-22 leads to reduced proliferation. Finally, we were able to show that a knockdown of CD25 in primary $CD34^+$ CML stem- and

progenitor cells is associated with enhanced growth. All in all, these data suggest that CD25 is a negative regulator of growth of CML stem cells.

We next attempted to explore mechanisms underlying the CD25-mediated growth inhibition. In these analyses, slightly different numbers in mitotic KU812 cells were found, whereas we were unable to detect any major differences in cell-cycle distribution when comparing CD25 shRNA-transduced cells with RDM shRNA–transduced cells. Alternatively, CD25 may serve as a receptor for an as yet unknown negative regulator of stem cell survival or proliferation. In order to address these questions, we performed gene array studies on shRNA-transduced KU812 cells. In these studies, we were able to identify a number of regulated genes that might be responsible for the different growth kinetics (mitotic rate) in KU812 cells. These genes include several members of the DAZ family, RAB27B, IGFBP7, SSX2, RASGEF1A, and IGF-1. We hypothesize that these genes act together to promote growth in CD25-depleted CML cells. In fact, no other critical genes or pathways that could explain the CD25-mediated growth inhibition in KU812 cells were identified in this study.

Because CD25 expression in CML cells may be associated with reduced growth, we screened for drugs that would upregulate CD25 expression in CML cells. Whereas most drugs were found to downregulate the expression of STAT5 and CD25 in CML cells, the PI3K/mTOR blocker BEZ235, the mTOR-targeting drug RAD001 (everolimus), and the PI3K inhibitor LY924002 were found to upregulate CD25 expression. We were therefore interested to learn whether drug-induced upregulation of CD25 contributes to the antineoplastic effects of these two agents. Indeed, we found that shRNAs against CD25 reduce the sensitivity of KU812 cells against BEZ235, suggesting that CD25 may act as secondary drug effector of BEZ235. Based on these observations, we were interested to know whether BEZ235-induced upregulation of CD25 may enhance the effects of BCR/ABL1 TKIs that were found to downregulate CD25 expression. To address this question, we performed drug combination experiments and found that BCR/ABL1 TKIs synergize with BEZ235 in producing growth inhibition in CML cells. Although it remains unknown whether CD25 is indeed a critical target mediating drug synergy, our data suggest that these two types of drugs utilize different mechanisms of action, which may explain synergistic effects. A related hypothesis would be that TKI-induced CD25 downregulation in CML LSCs is part of an escape mechanism, and that this escape is disrupted by addition of PI3K/mTOR blockers.

Together, our data show that CML LSCs aberrantly express CD25 and that STAT5 triggers

expression of CD25 in CML LSCs. Moreover, our data show that CD25 expression in LSCs is functionally relevant and associated with decreased proliferative capacity. These data may have implications for the biology of CML and should facilitate LSC detection and isolation. Moreover, CD25 may serve as a potential indirect drug target of drug therapy.

Disclosure of Potential Conflicts of Interest

T.L. Holyoake reports receiving speakers bureau honoraria from Ariad and Novartis, and commercial research grants from Bristol-Myers Squibb, Constellation Pharmaceuticals, Novartis, and Roche. P. Valent is a consultant/advisory board member for Ariad, Bristol-Myers Squibb, Novartis and Pfizer, and reports receiving commercial research support from Ariad and Novartis. No potential conflicts of interest were disclosed by the other authors.

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Tables

Table 1

Expression of surface antigens on $CD34^+/CD38^-$ and $CD34^+/CD38^+$ cells in patients with CML and in normal/reactive BM

		CP CML		AP CML		BP CML		normal/reactive BM	
CD	antigen	$CD34^+/CD38^-$	$CD34^+/CD38^+$	$CD34^+/CD38^-$	$CD34^+/CD38^+$	$CD34^+/CD38^-$	$CD34^+/CD38^+$	$CD34^+/CD38^-$	$CD34^+/CD38^+$
CD25	IL-2RA	++	+/−	++	+	++	++	−	+/−
CD26	DPPIV	++	+/−	++	+/−	+	+/−	−	−
CD33	Siglec-3	++	++	++	++	+	+	+	++
CD44	Pgp1	++	++	++	++	++	++	++	++
CD52	Campath-1	+	+/−	n.t.	n.t.	+	+	+	+
CD122	IL-2RB	−	−	n.t.	n.t.	−	−	−	−
CD132	IL-2RG	−	−	n.t.	n.t.	n.t.	n.t.	−	−
CD117	KIT/SCFR	++	++	++	++	n.t.	n.t.	++	++
n.c.	IL-1RAP	+	+	n.t.	n.t.	++	++	−	+/−

Abbreviations: IL-1RAP, IL-1 receptor accessory protein; IL-2RB, IL-2 receptor beta; IL-2RG, IL-2 receptor gamma; n.c., not yet clustered; n.t., not tested; SCFR, stem cell factor receptor; ++, strongly expressed in a majority of cases; +, clear expression in majority of cases; +/−, expression in minority of cases or weak expression in majority of cases; −, no expression in a vast majority of cases.

Table 2

Expression of CD25, CD122 and CD132 on various cell types in CML and comparison to cells obtained from healthy donors[a]

Types of cell	Expression of:	CD25	CD122	CD132
Control				
$CD3^+$	T cells	+/−	+/−	+
$CD19^+$	B cells	+/−	−	+/−
$CD14^+$	Monocytes	−	−	+/−
$CD123^+$	Basophils	+	−	+/−
CML				
$CD3^+$	T cells	+/−	+/−	+/−
$CD19^+$	B cells	+/−	−	+/−
$CD14^+$	Monocytes	−	−	−
$CD123^+$	Basophils	++	−	+/−

Abbreviation: CML, chronic myeloid leukemia.

[a]Expression of CD25, CD122, and CD132 was examined on PB or BM cell subsets by multicolor flow cytometry using monoclonal antibodies shown in Supplementary Table S1. Cells were analyzed in freshly obtained PB or BM samples (CML, n = 5; healthy controls, n = 5). Expression of CD25, CD122, and CD132 was determined as median fluorescence intensity (MFI) and is expressed as staining-index (SI), according to the formula: SI = MFI (test mAb)/MFI (isotype-control mAb). Results were scored as follows:

SI 0–1.3, −

SI 1.3–3, +/−

SI 3–10, +

SI >10, ++.

Figurelegends

Figure 1

$CD34^+/CD38^-$ stem cells in CML express CD25

A, CD25 expression on $CD34^+/CD38^-$ LSCs in 3 patients with CP CML, 3 in AP, and 3 in BP. Black histograms represent isotype-matched control antibodies, and red histograms represent CD25 expression. Patient numbers (#) refer to Supplementary Table S2. B, CD25 expression on $CD34^+/CD38^-$ LSCs in patients with CML (CP, n = 43; AP, n = 3; BP, n = 8), SCs in normal/reactive BM (n = 24), idiopathic cytopenia (ICUS, n = 14), JAK2 V617F+ MPNs (n = 6), and indolent mastocytosis (ISM, n = 7). Results show the percentage of $CD25^+$ SCs. Horizontal bars indicate median values in each group. C, qPCR analysis of CD25 (left), CD122 (middle), and CD132 (right) mRNA expression in sorted $CD34^+/CD38^-$ LSCs, $CD34^+/CD38^+$ progenitors, $CD34^+$ cells, and total mononuclear cells (MNC) obtained from 3 patients with CML. mRNA levels are expressed as a percentage of ABL1 and represent the mean ± SD from three experiments. D, expression of CD25 on $CD34^+/CD38^-$ LSCs (left) and $CD34^+/CD38^+$ cells (right) in 3 patients with CML. Black open histograms represent isotype-matched control antibodies, red histograms CD25 expression on $CD34^+/CD38^-$ LSCs (left), and blue histograms CD25 expression on $CD34^+/CD38^+$ progenitors (right). E, percentage of $CD25^+$ SCs at diagnosis, after therapy for at least 9 months in all patients (n = 17, top), and at the time of major molecular response (MMR) and complete cytogenetic response (CCyR) (n = 12, bottom). Patients were treated with imatinib (400 mg/day, n = 14), dasatinib (100 mg/day, n =1), or nilotinib (2x 400 mg/day; n = 2).

Figure 2

Expression of CD25 on CML cells is dependent on STAT5 activity

A, KU812 cells transduced with shRNA against STAT5 or a random control (RDM) shRNA were analyzed for CD25 expression by qPCR (left) and flow cytometry (right). Results are expressed as a percentage of RDM control and represent mean ± SD from three (qPCR) or five (flow cytometry) experiments. Asterisk (*): $P < 0.05$ compared with RDM control. B, expression of CD25 mRNA levels (left) and CD25 surface

(right) in KU812 cells after incubation in medium (control) or pimozide (1, 5, and 10 µmol/L) at 37°C for 24 hours. mRNA levels were quantified by qPCR, and surface expression by flow cytometry. Results are expressed as a percentage of ABL (qPCR) or percentage of medium control (flow cytometry), and represent the mean ± SD from five (qPCR) and four (flow cytometry) experiments. Asterisk (*): $P < 0.05$ compared with untreated cells (control). C, flow cytometric evaluation of CD25 expression on KU812 cells after incubation in control medium or with the MEK inhibitors PD0325901 or RDEA119 (each 0.1–3 µmol/L) at 37°C for 24 hours. Results are expressed as a percentage of control and represent the mean ± SD from 3 experiments. Asterisk (*): $P < 0.05$ compared with untreated cells (control). D, CD25 expression on KU812 cells after incubation in control medium, BEZ235, RAD001, or LY294002 (each 0.03–3 µmol/L) at 37°C for 24 hours. Results represent the mean ± SD from three independent experiments. Asterisk (*): $P < 0.05$ compared with untreated cells (control).

Figure 3

Expression of CD25 on LSCs is dependent on STAT5 activity

A, CD25 expression on $CD34^+$/ $CD38^-$ LSCs was analyzed by flow cytometry after incubation in control medium, pimozide (10 or 50 µmol/L), or DMSO at 37°C for 24 hours. Results show CD25 expression (median fluorescence intensity) on $CD34^+/CD38^-$ LSCs as a percentage of control and represent the mean ± SD from three independent experiments. Asterisk (*): $P < 0.05$ compared with untreated cells (control). B, CD25 expression on $CD34^+/CD38^-$ LSCs after incubation in control medium, BEZ235, or RAD001 (each 1 µmol/L) at 37°C for 24 hours. Results show CD25 expression on $CD34^+/CD38^-$ LSCs as a percentage of control and represent the mean ± SD from five independent experiments. C, expression of pSTAT5 (top) and CD25 (bottom) in KU812 cells after incubation in control medium or TKIs (imatinib, nilotinib, ponatinib, each 0.01–1 µmol/L) at 37°C for 4 hours (pSTAT5) or 24 hours (CD25). Results are expressed as staining index (SI; pSTAT5) or as a percentage of control (CD25), and represent the mean ± SD from at least three independent experiments. Asterisk (*): $P < 0.05$ compared with untreated cells (control). D, CD25 expression on $CD34^+/CD38^-$ LSCs after incubation in control medium or TKIs (imatinib, nilotinib, or ponatinib, each 1 µmol/L) at 37°C for 24 hours. Results show CD25 expression on $CD34^+/CD38^-$ LSCs as a

percentage of medium control and represent the mean ± SD of five independent experiments. Asterisk (*): $P < 0.05$ compared with untreated cells. E, expression of pSTAT5 in $CD34^+/CD38^-$ LSCs after incubation in control medium, BEZ235, RAD001 (left), or TKIs (imatinib, nilotinib, ponatinib, 1 μmol/L each; right) at 37°C for 4 hours. Results represent the mean ± SD from five independent experiments.

Figure 4

Infection of STAT5A/B is followed by upregulation of CD25 in mouse LSK cells

CD25 expression in $Lin^-/Sca\text{-}1^+/Kit^+$ LSK cells after infection of murine C57Bl/6 BM cells with retroviral constructs encoding $p210^{BCR/ABL1}$ (red bars), STAT5A/GFP (left, green bar) or STAT5B/GFP (right, green bar), or combinations of $p210^{BCR/ABL1}$ and STAT5/GFP isoforms (blue bars). BM cells were infected as described in the text. Expression of CD25 on LSK cells was analyzed by multicolor flow cytometry using an antibody against CD25. Results are expressed as median fluorescence intensity (CD25 expression) and represent the mean ± SD from five independent experiments. Asterisk (*) indicates $P < 0.05$ compared with $p210^{BCR/ABL1}$-infected cells.

Figure 5

Evaluation of CD25 as a functional target on CML cells

A, CD25 shRNA–transduced cells (shRNA clones #2, #1, #5 as indicated) were mixed 1:1 with KU812 cells transduced with an RDM shRNA and cultured at 37°C. Expression of $CD25^+$ cells was analyzed 3 times a week by flow cytometry using the PE-conjugated mAB 2A3 against CD25. Results are expressed as a percentage of $CD25^+$ cells and represent the mean ± SD from at least 4 independent experiments. Asterisk (*) indicates $P < 0.05$ compared with CD25 expression on day 0. B, NSG mice were injected with CD25 shRNA (clone #2) or RDM shRNA–transduced KU812 cells (5 mice/group). After 6 weeks, NSG mice were analyzed for engraftment of $CD44^+$ cells (expressed as %) in the BM, PB, and spleen by flow cytometry using an antibody against CD44 (top). The spleen weight, spleen size, and white blood count (WBC) are shown in the bottom plots. Results represent the mean ± SD from five mice per group. Asterisk (*): $P < 0.05$ compared with control mice. The images in the very right plots show the spleen size of NSG mice (#1–5: NSG mice injected with KU812 cells transduced with RDM shRNA; #6–9: NSG mice

injected with KU812 cells transduced with CD25 shRNA). C, primary $CD34^+$ stem/progenitor cells from 2 patients with CML CP were transduced with a CD25 shRNA (clone #2) or an RDM control shRNA. After 24 hours (recovery time), ^{3}H-thymidine uptake was measured in highly enriched (sorted for mCherry) transduced cells. Results are expressed as a percentage of control (^{3}H-thymidine uptake in RDM shRNA–transduced cells = 100%) in three independent experiments (one experiment with cells from patient #51 and two experiments with cells from patient #50).

References

1. Rowley JD. A new consistent chromosomal abnormality in chronic mye- logenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature 1973;243:290–3.

2. Faderl S, Talpaz M, Estrov Z, O'Brien S, Kurzrock R, Kantarjian HM. The biology of chronic myeloid leukemia. N Engl J Med 1999;341: 164–72.

3. Melo JV, Barnes DJ. Chronic myeloid leukaemia as a model of disease evolution in human cancer. Nat Rev Cancer 2007;7:441–53.

4. Druker BJ. Perspectives on the development of a molecularly targeted agent. Cancer Cell 2002;1:31–6.

5. Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N; IRIS Investigators. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. N Engl J Med 2006;355:2408–17.

6. Copland M, Jørgensen HG, Holyoake TL. Evolving molecular therapy for chronic myeloid leukaemia–are we on target? Hematology 2005;10: 349–59.

7. Jiang X, Zhao Y, Smith C, Gasparetto M, Turhan A, Eaves A, et al. Chronic myeloid leukemia stem cells possess multiple unique features of resistance to BCR-ABL targeted therapies. Leukemia 2007;21:926–35.

8. Valent P. Emerging stem cell concepts for imatinib-resistant chronic mye- loid leukaemia: implications for the biology, management, and therapy of the disease. Br J Haematol 2008;142:361–78.

9. Kavalerchik E, Goff D, Jamieson CH. Chronic myeloid leukemia stem cells. J Clin Oncol 2008;26:2911–5.

10. 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.

11. Hamilton A, Helgason GV, Schemionek M, Zhang B, Myssina S, Allan EK, et al. Chronic myeloid leukemia stem cells are not dependent on Bcr-Abl kinase activity for their survival. Blood 2012;119:1501–10.

12. Jiang X, Forrest D, Nicolini F, Turhan A, Guilhot J, Yip C, et al. Properties of CD34þ CML stem/progenitor cells that correlate with different clinical responses to imatinib mesylate. Blood 2010;116:2112–21.

13. La Ros ee P, Deininger MW. Resistance to imatinib: mutations and beyond. Semin Hematol 2010;47:335–43.

14. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature 1994;367:645–8.

15. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 1997;3:730–7.

16. Dick JE. Stem cell concepts renew cancer research. Blood 2008;112: 4793–807.

17. Misaghian N, Ligresti G, Steelman LS, Bertrand FE, B€asecke J, Libra M, et al. Targeting the leukemic stem cell: the Holy Grail of leukemia therapy. Leukemia 2009;23:25–42.

18. Essers MA, Trumpp A. Targeting leukemic stem cells by breaking their dormancy. Mol Oncol 2010;4:443–50.

19. Valent P. Targeting of leukemia-initiating cells to develop curative drug therapies: straightforward but nontrivial concept. Curr Cancer Drug Targets 2011;11:56–71.

20. Shuai K, Halpern J, ten Hoeve J, Rao X, Sawyers CL. Constitutive activation of STAT5 by the BCR-ABL oncogene in chronic myelogenous leukemia. Oncogene 1996;13:247–54.

21. Nieborowska-Skorska M, Wasik MA, Slupianek A, Salomoni P, Kitamura T, Calabretta B, et al. Signal transducer and activator of transcription (STAT)5 activation by BCR/ABL1 is dependent on intact Src homology (SH)3 and SH2 domains of BCR/ABL1 and is required for leukemogenesis. J Exp Med 1999;189:1229–42.

22. Sillaber C, Gesbert F, Frank DA, Sattler M, Griffin JD. STAT5 activation contributes to growth and viability in Bcr/Abl-transformed cells. Blood 2000;95:2118–25.

23. Hoelbl A, Schuster C, Kovacic B, Zhu B, Wickre M, Hoelzl MA, et al. Stat5 is indispensable for the maintenance of bcr/abl-positive leukaemia. EMBO Mol Med 2010;2:98–110.

24. Warsch W, Grundschober E, Berger A, Gille L, Cerny-Reiterer S, Tigan AS, et al. STAT5 triggers BCR-ABL1 mutation by mediating ROS production in chronic myeloid leukaemia. Oncotarget 2012;3:1669–87.

25. Petzer AL, Eaves CJ, Lansdorp PM, Ponchio L, Barnett MJ, Eaves AC. Characterization of primitive subpopulations of normal and leukemic cells present in the blood of patients with newly diagnosed as well as established chronic myeloid leukemia. Blood 1996;88:2162–71.

26. Eisterer W, Jiang X, Christ O, Glimm H, Lee KH, Pang E, et al. Different subsets of primary chronic myeloid leukemia stem cells engraft immuno- deficient mice and produce a model of the human disease. Leukemia 2005;19:435–41.

27. Heaney NB, Pellicano F, Zhang B, Crawford L, Chu S, Kazmi SM, et al. Bortezomib induces apoptosis in primitive chronic myeloid leukemia cells including LTC-IC and NOD/SCID repopulating cells. Blood 2010;115: 2241–50.

28. J€ara s M, Johnels P, Hansen N, Agerstam H, Tsapogas P, Rissler M, et al. Isolation and killing of candidate chronic myeloid leukemia stem cells by antibody targeting of IL-1 receptor accessory protein. Proc Natl Acad Sci 2010;107:16280–5.

29. Herrmann H, Sadovnik I, Cerny-Reiterer S, Ru€licke T, Stefanzl G, Willmann M, et al. Dipeptidylpeptidase IV (CD26) defines leukemic stem cells (LSC) in chronic myeloid leukemia. Blood 2014;123:3951–62.

30. Gerber JM, Gucwa JL, Esopi D, Gurel M, Haffner MC, Vala M, et al. Genome-wide comparison of the transcriptomes of highly enriched nor- mal and chronic myeloid leukemia stem and progenitor cell populations. Oncotarget 2013;4:715–28.

31. Bruns I, Czibere A, Fischer JC, Roels F, Cadeddu RP, Buest S, et al. The hematopoietic stem cell in chronic phase CML is characterized by a transcriptional profile resembling normal myeloid progenitor cells and reflecting loss of quiescence. Leukemia 2009;23:892–9.

32. Kobayashi CI, Takubo K, Kobayashi H, Nakamura-Ishizu A, Honda H, Kataoka K, et al. The IL-2/CD25 axis maintains distinct subsets of chronic myeloid leukemia-initiating cells. Blood 2014;123:2540–9.

33. Warsch W, Kollmann K, Eckelhart E, Fajmann S, Cerny-Reiterer S, Ho€lbl A, et al. High STAT5 levels mediate imatinib resistance and indicate disease progression in chronic myeloid leukemia. Blood 2011;117:3409–20.

34. Florian S, Sonneck K, Hauswirth AW, Krauth MT, Schernthaner GH, Sperr WR, et al. Detection of molecular targets on the surface of CD34þ/CD38 stem cells in various myeloid malignancies. Leuk Lymphoma 2006;47: 207–22.

35. Herrmann H, Cerny-Reiterer S, Gleixner KV, Blatt K, Herndlhofer S, Rabitsch W, et al. CD34(þ)/CD38( ) stem cells in chronic myeloid leukemia express Siglec-3 (CD33) and are responsive to the CD33-target- ing drug gemtuzumab/ozogamicin. Haematologica 2012;97:219–26.

36. Brothman AR, Persons DL, Shaffer LG. Nomenclature evolution: changes in the ISCN from the 2005 to the 2009 edition. Cytogenet Genome Res 2009;127:1–4.

37. Scheeren FA, Naspetti M, Diehl S, Schotte R, Nagasawa M, Wijnands E, et al. STAT5 regulates the self-renewal capacity and differentiation of human memory B cells and controls Bcl-6 expression. Nat Immunol 2005;6: 303–13.

38. Hoermann G, Cerny-Reiterer S, Pern e A, Klauser M, Hoetzenecker K, Klein K, et al. Identification of oncostatin M as a STAT5-dependent mediator of bone marrow remodeling in KIT D816V-positive systemic mastocytosis. Am J Pathol 2011;178:2344–56.

39. Peter B, Cerny-Reiterer S, Hadzijusufovic E, Schuch K, Stefanzl G, Eisenwort G, et al. The pan-Bcl-2 blocker obatoclax promotes the expression of Puma, Noxa, and Bim mRNA and induces apoptosis in neoplastic mast cells. J Leukoc Biol 2014;9:95–104.

40. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984;22:27–55.

41. Gleixner KV, Ferenc V, Peter B, Gruze A, Meyer RA, Hadzijusufovic E, et al. Polo-like kinase 1 (Plk1) as a novel drug target in chronic myeloid leukemia: overriding imatinib resistance with the Plk1 inhibitor BI 2536. Cancer Res 2010;70:1513–23.

42. Valent P, Bonnet D, De Maria R, Lapidot T, Copland M, Melo JV, et al. Cancer stem cell definitions and terminology: the devil is in the details. Nat Rev Cancer 2012;12:767–75.

43. Valent P, Bonnet D, Wo€hrer S, Andreeff M, Copland M, Chomienne C, et al. Heterogeneity of neoplastic stem cells: theoretical, functional, and clinical implications. Cancer Res 2013;73:1037–45.

44. Saito Y, Kitamura H, Hijikata A, Tomizawa-Murasawa M, Tanaka S, Takagi S, et al. Identification of therapeutic targets for quiescent, chemotherapy- resistant human leukemia stem cells. Sci Transl Med 2010;2:17ra9.

45. Lin JX, Leonard WJ. The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. Oncogene 2000;19:2566–76.

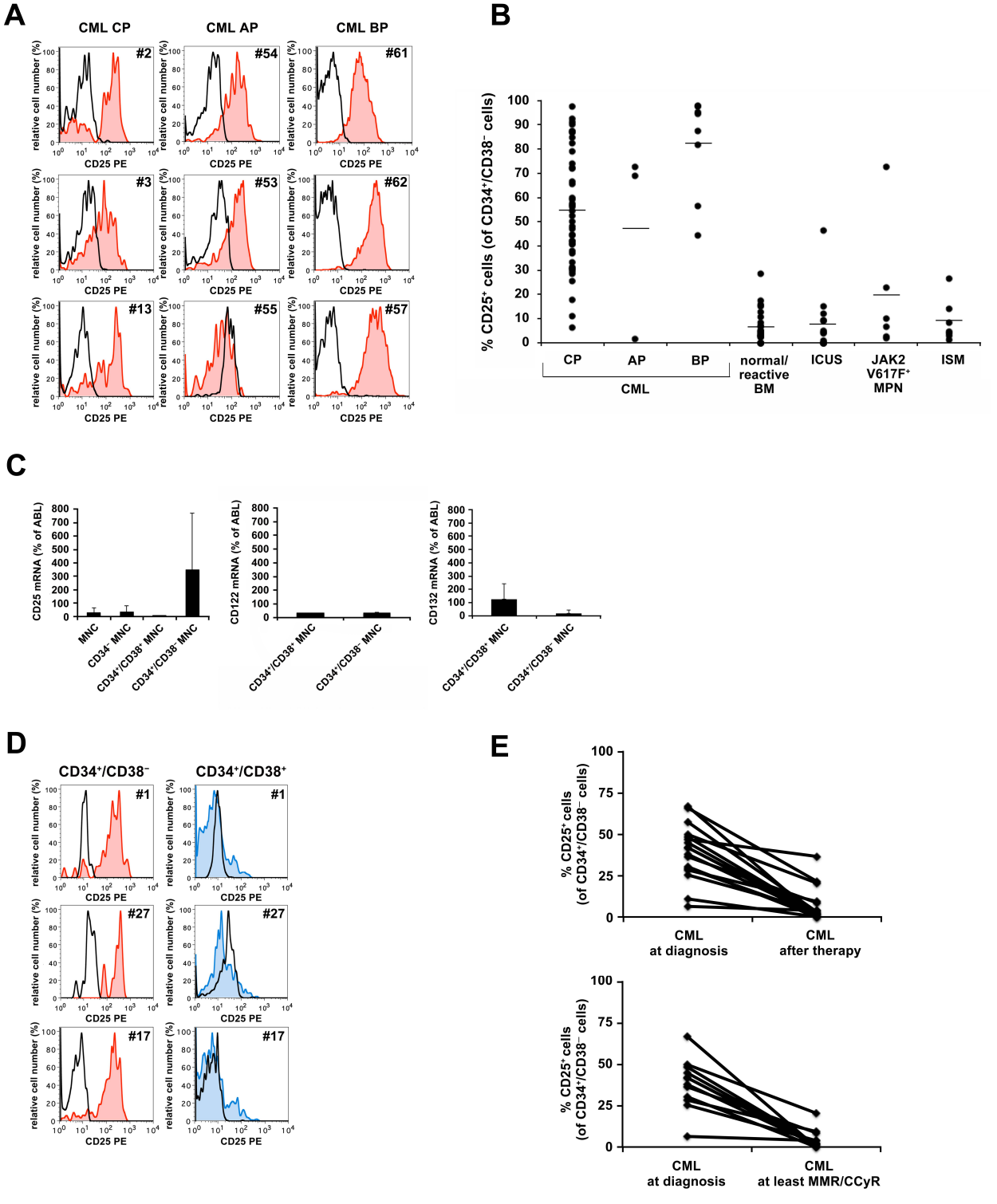
46. Cohen AC, Nadeau KC, Tu W, Hwa V, Dionis K, Bezrodnik L, et al. Cutting edge: decreased accumulation and regulatory function of CD4þ CD25(high) T cells in human STAT5b deficiency. J Immunol 2006; 177:2770–4.

47. Mahon FX. Is going for cure in chronic myeloid leukemia possible and justifiable? Hematology Am Soc Hematol Educ Program 2012;2012: 122–8.

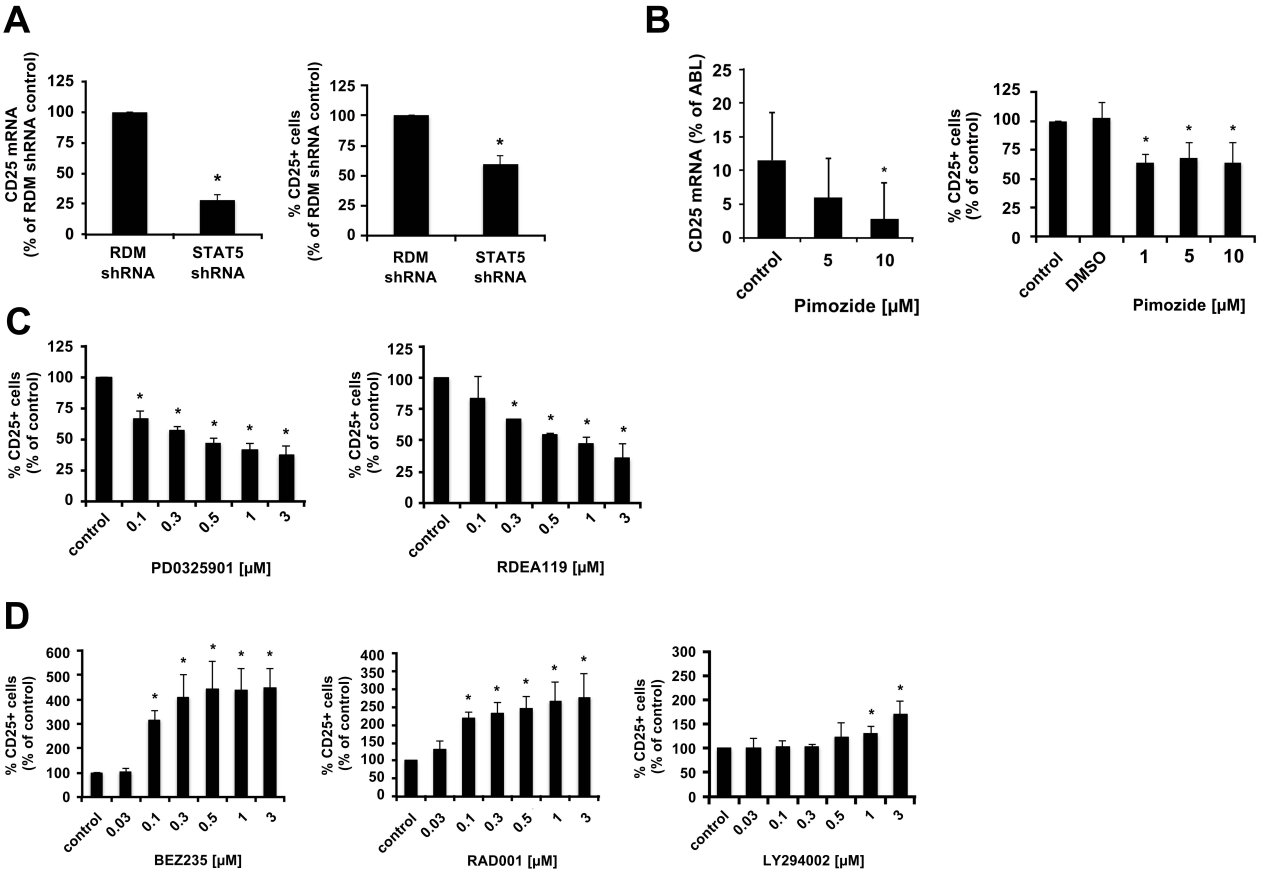
48. Cortes JE, Kantarjian H, Shah NP, Bixby D, Mauro MJ, Flinn I, et al. Ponatinib in refractory Philadelphia chromosome-positive leukemias. N Engl J Med 2012;367:2075–88.

49. Kantarjian H, Giles F, Wunderle L, Bhalla K, O'Brien S, Wassmann B, et al. Nilotinib in imatinib-resistant CML and Philadelphia chromosome-pos- itive ALL. N Engl J Med 2006;354:2542–51.

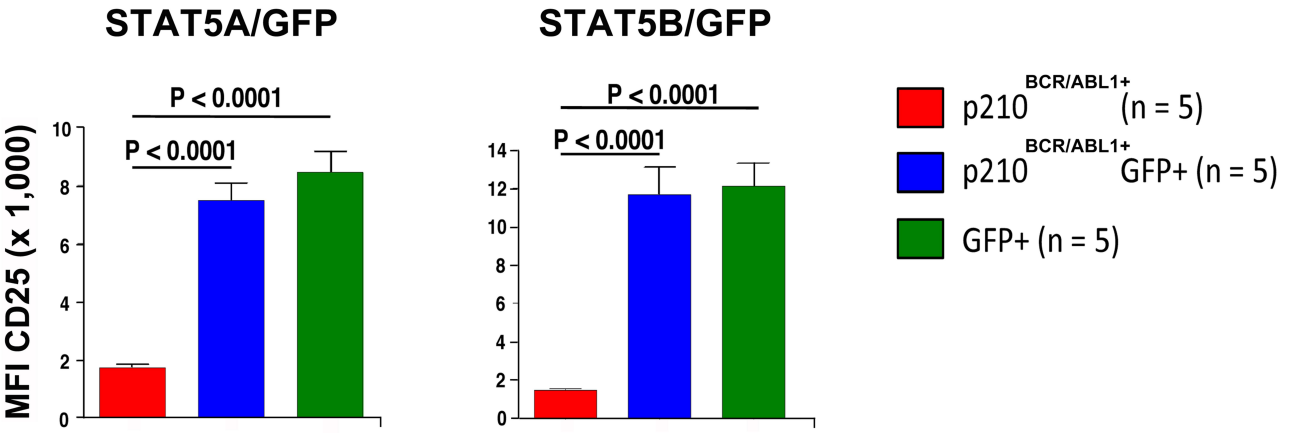
50. Saglio G, Kim DW, Issaragrisil S, le Coutre P, Etienne G, Lobo C, et al. ENESTnd Investigators. Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia. N Engl J Med 2010;362:2251–9.



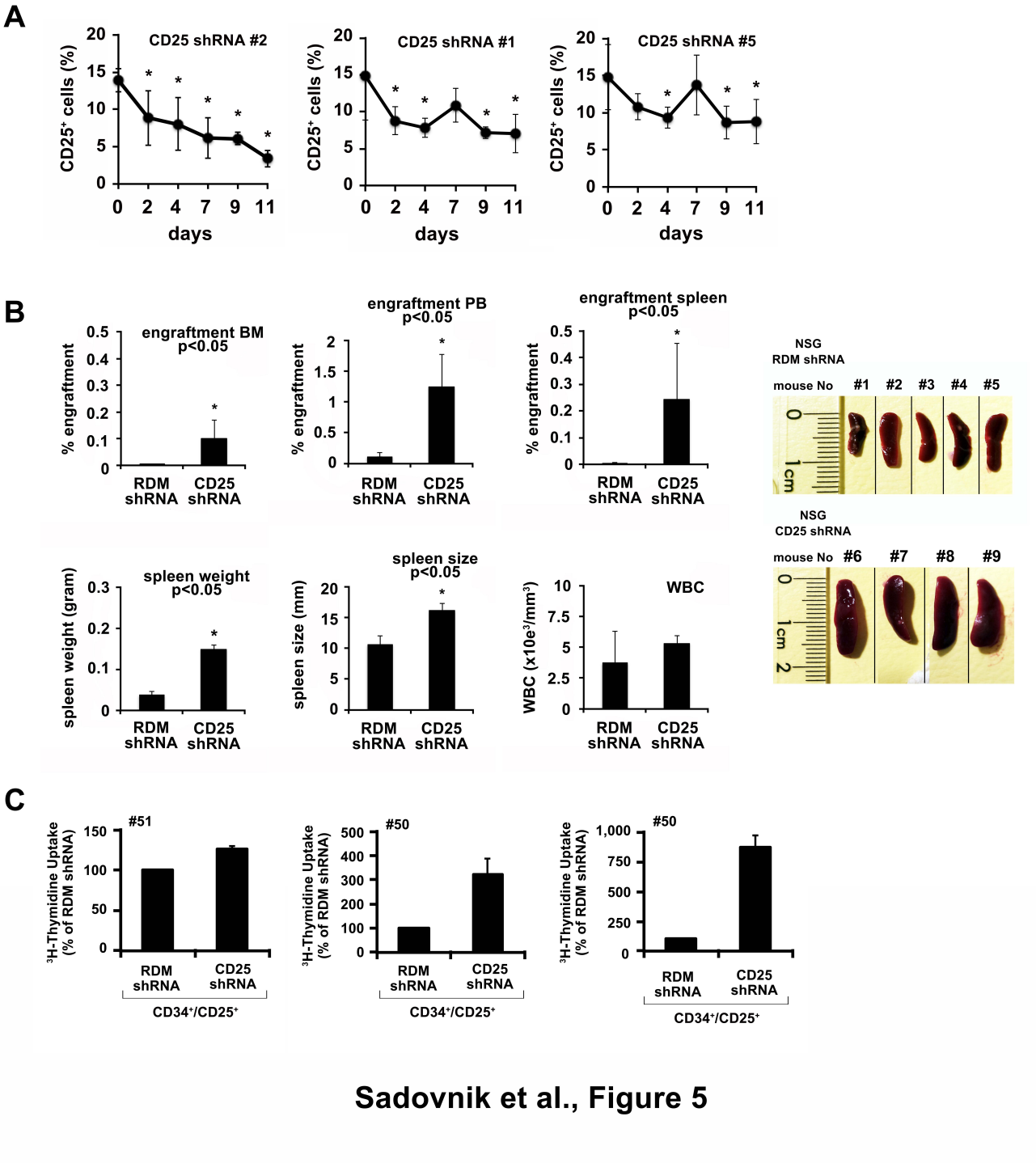
Sadovnik et al., Figure 1



Sadovnik et al., Figure 2



Sadovnik et al., Figure 4



Sadovnik et al., Figure 5

Supplementary Data to Manuscript:

Identification of CD25 as STAT5-Dependent Growth-Regulator of Leukemic Stem Cells in Ph+ CML (Sadovnik et al.)

Methods

Reagents

Pimozide, polybrene and RNase I were purchased from Sigma Aldrich (San Louis, MO, USA) and RDEA119 and PD0325901 from ChemieTek (Indianapolis, IN, USA). Imatinib, nilotinib and BEZ235 were kindly provided by Dr.E.Buchdunger and Dr.P.W.Manley (Novartis, Basel, Switzerland). Ponatinib and RAD001 (everolimus) were purchased from Selleck Chemicals (Houston, TX, USA) and LY294002 from Merck Millipore (Billerica, MA, USA). Stock solutions of drugs were prepared by dissolving in dimethyl-sulfoxide (DMSO) (Merck, Darmstadt, Germany). RPMI 1640 medium, Dulbecco's modified eagle medium (DMEM), fetal calf serum (FCS), and puromycin were purchased from PAA Laboratories (Pasching, Austria), ^{3}H-thymidine from Amersham (Buckinghamshire, United Kingdom), propidium iodide (PI) from eBioscience (San Diego, CA, USA), recombinant human (rh) interleukin-2 (IL-2) from R&D Systems (Minneapolis, MN, USA) or ImmunoTools (Friesoythe, Germany), rh IL-3 and rh IL-6 from Novartis (Vienna, Austria), rh stem cell factor (SCF) from Peprotech (Rocky Hill, NJ, USA), rh thrombopoietin (TPO) and rh Flt3/Flk-2 ligand (FL) from Sigma (St. Louis, MO, USA), and recombinant murine IL-3, murine IL-6 and murine SCF from R&D Systems (Minneapolis, USA). Stem Span medium, Myelocult H5100 medium, and Methocult H4434 medium (medium supplemented with methylcellulose and cytokines) were purchased from StemCell technologies (Vancouver, Canada). Stem Span medium was supplemented with rh IL-3 (50 ng/ml), rh IL-6 (50 ng/ml), rh SCF (50 ng/ml), rh TPO (25 ng/ml) and rh FL (5 ng/ml). A characterization of monoclonal antibodies (mAb) used in our flow cytometry experiments is provided in Supplementary Table S1.

Patients, diagnostic evaluation and follow-up examinations

A total number of 63 patients with CML (32 females, 31 males) were examined. The median age was 54.04 years (range: 18-86 years). Diagnoses and the phase of CML were established according to the classification provided by the World Health Organization (WHO) (1). Prognostication at diagnosis and risk-stratification were performed according to the Sokal Score, Hasford Score, and EUTOS Score (2-4). Peripheral blood (PB) and/or bone marrow (BM) cells (iliac crest or sternum) were collected at diagnosis as well as in the follow-up. Karyotyping was performed at diagnosis and in defined time-intervals (3-month-intervals in the first year; and in 3-12 month-intervals after the first year) following the recommendations of the European Leukemia Net (ELN) (5,6). BCR/ABL1 mRNA levels were determined in PB cells using *ABL* as a reference gene and the International Scale (IS) for standardized quantification (7-9). BCR/ABL1 was measured in 3-12 month-intervals. The patients´ characteristics are shown in Supplementary Table S2. Of the 63 patients examined, 52 had chronic phase (CP) CML, 3 accelerated phase (AP) CML, and 8 were suffering from blast phase (BP) of CML (Supplementary Table S2). Patients were treated with imatinib (400 mg daily per os) and other tyrosine kinase inhibitors (TKI) according to available guidelines (5,6). In case of resistance or intolerance against imatinib, patients received a second-generation TKI (nilotinib or dasatinib). Hydroxyurea was used on demand for cytoreduction. All patients provided written informed consent before PB or BM samples were collected. The control patients´ cohorts are shown in Supplementary Table S3A (normal BM), Supplementary Table S3B (idiopathic cytopenia), Supplementary Table S3C (systemic mastocytosis) and Supplementary Table S3D (myeloproliferative neoplasm). All studies, including storage of cells, drug incubation experiments and the examination of LSCs were approved by the local ethics committee of the Medical University of Vienna.

Cell sampling and storage

A total number of 180 cell samples, including 124 CML samples and 56 control samples were analyzed. Control samples were from patients with reactive or normal BM (Non-Hodgkins´s or Hodgkin´s Lymphoma without BM involvement, n=14; monoclonal gammopathy of unknown significance, n=1; solid tumors without BM

involvement, n=2; suspected mastocytosis, n=7), idiopathic cytopenia of undetermined significance, ICUS (n=14), myeloproliferative neoplasms, MPN (n=6), and indolent systemic mastocytosis, ISM (n=7). The patients´ characteristics are shown in Supplementary Table S3A-S3D. Stabilizer-free heparin (Biochrom AG, Berlin, Germany) was used as anticoagulant. Freshly obtained samples (PB, BM) were subjected to flow cytometry analysis and isolation of mononuclear cells (MNCs) using Ficoll gradient centrifugation. Isolated MNCs were either used immediately or were frozen in liquid nitrogen until used. Frozen MNCs were thawed using DNAse type I, 100 U/mL (Sigma Aldrich, St. Louis, MO, USA) to prevent cell clumping.

Flow cytometry analysis

Phenotyping of $CD34^+/CD45^+/CD38^-$ stem cells (SCs) and $CD34^+/CD45^+/CD38^+$ progenitor cells was performed in 54 patients with CML. Whole BM and/or PB samples or MNCs were used (n=116). Multicolor flow cytometry was performed as described (10,11) using fluorochrome-conjugated mAb shown in Supplementary Table S1. Flow cytometry was performed on a FACSCalibur (Becton Dickinson, San José, CA, USA). All staining reactions were controlled by isotype-matched control antibodies and expressed as percentage of positive cells. In a select group of healthy (non-leukemic) controls (n=5) and CML patients (n=5), expression of CD25, CD122 and CD132 on $CD19^+$ B cells, $CD3^+$ T cells, $CD14^+$ monocytes, and $CD123^+$ basophils (lineage controls) was analyzed by multicolor flow cytometry. Results of these analyses are shown in Table 2. In addition, we analyzed the expression of CD25 on $CD34^+/CD38^-$ SCs in the follow-up of 17 CML patients treated with imatinib (400 mg per os daily, n=14), nilotinib (2 x 400 mg per os daily; n=2) or dasatinib (100 mg per os daily, n=1) according to the guidelines of the ELN (5,6). In 4 patients with CML, LSCs ($CD34^+/CD38^-$) and progenitor cells ($CD34^+/CD38^+$) were purified to homogeneity by cell sorting on a FACSAria (Becton Dickinson) as described (11). In a separate series of experiments, $CD34^+/CD38^-/CD25^+$ LSCs and $CD34^+/CD38^-/CD25^-$ (residual normal) stem cells were sorted from MNC of 5 CML patients by flow cytometry (for FISH analyses and LTC-IC). For transfection studies, $CD34^+$ CML stem- and progenitor cells were purified by MACS technology from MNC in 2 patients (CML CP) as described (12). Enriched $CD34^+$ cells (23-30% of the cells

expressed CD25) were transduced with a short hairpin RNA (shRNA) against CD25 (clone #2) or a random (RDM) control shRNA. Four days after transduction, shRNA-transfected cells were enriched by cell sorting (sorted for the mCherry tag of the shRNA construct) on a FACSAria before being analyzed for their proliferative potential. To evaluate potential mechanisms underlying CD25 expression in CML cells, KU812 cells were incubated in control medium or in medium supplemented with imatinib, nilotinib, ponatinib, pimozide, PD0325901, RDEA119, BEZ235, RAD001, or LY294002 (0.01-3 µM) at 37°C for 24 hours. In addition, we incubated K562, imatinib-resistant K562 cells (K562-R), and KCL-22 cells with BEZ235, RAD001, and LY294002 (0.01-3 µM) at 37°C for 24 hours. After incubation, cells were stained with PE-conjugated mAb 2A3 against CD25 (15 minutes) and analyzed by flow cytometry. To further evaluate the effects of various drugs on CD25 expression, Ba/F3 cells expressing various BCR/ABL1 mutants were incubated in medium supplemented with imatinib, nilotinib, ponatinib or BEZ235 (0.01-5 µM) at 37°C for 24 hours. After incubation, cells were stained with a rat anti-mouse PE-conjugated mAb 3C7 against CD25 (15 minutes) and analyzed by flow cytometry. For staining of cytoplasmic molecules, KU812 cells or CML MNCs were incubated in control medium or in medium supplemented with various compounds at 37°C for 4 hours. After incubation, CML MNCs were stained with mAb HI30 against CD45, mAb 581 against CD34 and mAb HIT2 against CD38 (15 minutes). Then CML cells were fixed in 4% formaldehyde (15 minutes) and permeabilized in methanol (-20°C, 15 minutes). Thereafter, CML cells were incubated with mAb 47 (pY694) against pSTAT5 (30 minutes) and analyzed on a FACSCanto (Becton Dickinson). KU812 cells were first fixed in formaldehyde and permeabilized in methanol (-20°C) and then stained with mAb 47 against pSTAT5 and analyzed on a FACSCalibur. For cell cycle studies, KU812 cells were either transduced with a control RDM shRNA or a specific CD25 shRNA (clone #2). Cells were then resuspended in 300 µL permeabilization buffer (0.1% Na-acetate and 0.1% Triton X-100) and RNase I (1:100). Thereafter, PI was added and cell cycle distribution was analyzed on a FACSCalibur. For flow cytometric determination of apoptosis, cells were examined as described (13). In brief, cells were fixed in 4% formaldehyde and permeabilized in methanol as reported. Thereafter, cells were washed and incubated with a PE-conjugated mAb C92-605 against active

caspase-3 for 30 minutes. After washing, cells were analyzed on a FACSCalibur, and the percentage of apoptotic (active caspase-3^+) cells was determined.

Measurement of ^{3}H-thymidine uptake in CML cells

To determine the growth-inhibitory effects of various drugs, KU812, K562, Ba/F3 cells (BCR/ABL1 wild type and various BCR/ABL1 mutants) ($1x10^4$ cells/well), or primary CML MNCs ($1x10^5$ cells/well) were incubated in control medium or in various concentrations of BEZ235 (0.001-10 µM), imatinib (0.01-1 µM), nilotinib (0.0001-1 µM), or ponatinib (0. 05-1 nM) alone or in combination in 96-well culture plates (TPP, Trasadingen, Switzerland) at 37°C for 48 hours. The effects of IL-2 (either direct incubation or pre-incubation of cells with IL-2: 100-2,000 ng/ml) on proliferation were tested in KU812 cells, K562 cells as well as in primary CML MNCs (5 patients). In another set of experiments, primary $CD34^+$ cells (purity >98%) from 2 patients with CP CML (23-30% $CD25^+$ cells) were transduced with a specific CD25 shRNA (clone #2) or a RDM control shRNA and cultured in Stem Span medium supplemented with cytokines. After 4 days, shRNA-transduced cells were enriched by cell sorting ($mCherry^+$) and incubated for 24 hours (recovery time after sorting). After incubation of cells with drugs, cytokines, or shRNA, 0.5 µCi ^{3}H-thymidine was added (37°C, 16 hours). Cells were then harvested on filter membranes (Packard Biosciences, Meriden, CT) in a Filtermate 196 harvester (Packard Bioscience). Filters were air-dried, and the bound radioactivity was counted in a ß-counter (Top-count NXT, Packard Bioscience). All experiments were performed in triplicates.

Analysis of STAT5 expression by Western blotting (WB)

Untransduced KU812 cells and KU812 cells transduced with STAT5 shRNA or RDM shRNA (see text in main manuscript) were analyzed by WB as described (14) using a mAb against STAT5 (clone 89/STAT5, BD Bioscience, San Jose, CA) and a polyclonal antibody against Akt (Cell Signaling Technology, Danvers, MA). Antibody-reactivity was made visible by donkey-anti-rabbit IgG or sheep-anti-mouse IgG (both from GE Healthcare, Buckinghamshire, UK) and Lumingen PS-3 detection reagent (Thermo Scientific, Rockford, IL).

Gene Array Analyses

To define differences in mRNA expression patterns in KU812 cells transduced with RDM shRNA or a shRNA against CD25 (clone #2), gene array analyses were performed. Total RNA was extracted from CML cells using RNeasy Micro-Kit (Qiagen) and used (150 ng total RNA) for Gene Chip analyses. Preparation of terminal-labeled cRNA, hybridization to genome-wide human Gene 2.0 ST Arrays (Affymetrix, Santa Clara, CA, USA) and scanning of arrays were carried out according to the manufacturer's protocols (https://www.affymetrix.com). Robust Multichip Average (RMA) signal extraction and normalization were performed using GeneChip Expression Console Version 1.3.1. Differences in mRNA expression levels were calculated as normalized fluorescence signal ratio of KU812 CD25 shRNA cells versus KU812 RDM shRNA cells. A complete data set is available at Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=orotekeollgdduf&acc=GSE60315. In addition, this data set was analyzed for enriched pathways following CD25 depletion by shRNA (compared to RDM shRNA). Genes which are at least 2 fold regulated were included in this analysis using www.pantherdb.org. To determine statistical differences in enriched pathways, statistical analysis and Bonferroni correction were applied essentially as described (15).

Quantitative PCR (qPCR) and cytogenetics

To confirm expression of CD25, CD122, and CD132 mRNA in KU812 cells and CML LSCs, qPCR was performed as reported (11) using the following primers: CD25 forward: GGACTGCTCACGTTCATCATGG, CD25 reverse: GCTTTGAATGTGG CGTGTGG; CD122 forward: GGGCTCTGCAGGACACTTCC, CD122 reverse: GGAGCAGCTCACAGGTTTGG; CD132 forward: TTGGAGCACTTGGTGCA GTA, CD132 reverse: CCATCCACACTAGGCAAGGA. Total RNA was extracted from KU812 cells, sorted $CD34^+/CD38^-$ LSCs, $CD34^+/CD38^+$ progenitor cells, $CD34^-$ cells, and MNCs from four patients with CML using the RNeasy Micro Kit (Qiagen, Hilden, Germany). RNA was also extracted from pooled colonies obtained from LTC-IC cultures (grown from $CD34^+/CD38^-/CD25^+$ LSCs and $CD34^+/CD38^-/CD25^-$ stem cells). BCR/ABL1 was detected using the following primers: BCR/ABL1 forward:

TCCGCTGACCATCAATAAGGA, BCR/ABL1 reverse: CACTCAGACCCTGAGG CTCAA. ABL served as a reference gene: ABL forward: TGTATGATTTTGTGGC CAGTGGAG, ABL reverse: GCCTAAGACCCGGAGCTTTTCA. Conventional cytogenetics and FISH were performed according to published protocols (16). Conventional cytogenetics was performed in all patients with CML at diagnosis and in the follow-up (first year: 3 month intervals; and after the first year in 3-12 month-intervals and in case of suspected progression/relapse). At least 20 metaphases were examined whenever possible. Karyotypes were reported according to available guidelines (16). Results were expressed as percentage of Ph-chromosome-positive metaphases. In case of questionable results or poor growth, fluorescence in situ hybridization (FISH) analysis was performed. FISH was also performed to confirm the presence of BCR/ABL1 in sorted $CD25^+$ and $CD25^-$ fractions of $CD34^+/CD38^-$ stem cells in three patients with CML. A summary of FISH results is shown in Supplementary Table S4. FISH was performed on cytospin slides using a triple color-staining approach with one probe specific for ABL1 (red) and two probes (green and blue) specific for BCR (Kreatech, Amsterdam, The Netherlands) as described (16).

Long term culture-initiating cell (LTC-IC) assay

LTC-ICs were prepared using highly purified, sorted $CD34^+/CD38^-/CD25^+$ LSCs and $CD34^+/CD38^-/CD25^-$ (presumably normal) stem cells in 2 patients with CP CML and irradiated (60 Gy) murine M2-10B4 feeder cells (ATCC, Manassas, VA, USA) essentially as described (12). Suspension cultures were maintained in Myelocult H5100 Medium according to the manufacturer´s protocol (StemCell Technologies). Before being cultured in the LTC-IC assay, CML MNCs were separated (sorted) into $CD34^+/CD38^-/CD25^+$ LSCs and $CD34^+/CD38^-/CD25^-$ stem cells from the same samples (patients) and cultured separately in the LTC-IC assay (1,000 cells/mL/well). After 3 weeks in liquid culture, cells were recovered and transferred to methylcellulose (CFU) cultures (Methocult H4434) prepared according to the manufacturer´s protocol (StemCell Technologies). CFU-GM and BFU-E formation was assessed on day 14 using an inverted microscope as described (12). The percentage of BCR/ABL1 mRNA in isolated, pooled, CFU-GM colonies (10 colonies/pool) was determined by qPCR.

Supplementary Tables and Figures

Supplementary Table S1

Specification of monoclonal antibodies (mAb) used in this study

CD	antigen	clone	isotype	conjugate	reactivity	company*
n.c.	isotype control	MOPC-21	mouse, IgG1	PE	human	BD Biosciences
n.c.	isotype control	20102	mouse, IgG2A	PE	human	R&D Systems
n.c.	isotype control	133303	mouse, IgG2B	PE	human	R&D Systems
n.c.	isotype control	MOPC-21	mouse, IgG1	Alexa647	human	BD Biosciences
n.c.	isotype control	MOPC-21	mouse, IgG1	APC	human	BD Biosciences
CD3	TcR	UCHT1	mouse, IgG1	APC	human	BD Biosciences
CD14	LPSR	TÜK4	mouse, IgG2A	FITC	human	Dako
CD19	B4	4G7	mouse, IgG1	FITC	human	BD Biosciences
CD25	IL-2RA	2A3	mouse, IgG1	PE	human	BD Biosciences
CD25	IL-2RA	BC96	mouse IgG1	PE	human	eBioscience
CD25	IL-2RA	BC96	mouse IgG1	APC	human	BioLegend
CD26	DPPIV	M-A261	mouse, IgG1	PE	human	BD Biosciences
CD33	Siglec-3	WM53	mouse, IgG1	PE	human	BD Biosciences
CD34	HPCA-1	581	mouse, IgG1	FITC	human	BD Biosciences
CD34	HPCA-1	581	mouse, IgG1	PE	human	BD Biosciences
CD38	T10	HIT2	mouse, IgG1	APC	human	BD Biosciences
CD44	Pgp1	515	mouse, IgG1	PE	human	BD Biosciences
CD45	LCA	2D1	mouse, IgG1	PerCP	human	BD Biosciences
CD45	LCA	HI30	mouse, IgG1	V500	human	BD Biosciences
CD52	Campath-1	HI186	mouse, IgG2B	PE	human	BioLegend
CD117	SCFR/KIT	104D2	mouse, IgG1	PE	human	BD Biosciences
CD122	IL-2RB	Mik-β3	mouse, IgG1	PE	human	BD Biosciences
CD123	IL-3RA	AC145	mouse, IgG2A	APC	human	Miltenyi Biotec
CD132	IL-2RG	AG184	mouse, IgG1	PE	human	BD Biosciences
n.c.	IL-1RAP	89412	mouse, IgG1	PE	human	R&D Systems
n.c.	pSTAT5	47(pY694)	mouse, IgG1	Alexa647	human	BD Biosciences
n.c	caspase-3	C92-605	mouse, IgG1	PE	human	BD Biosciences
n.c.	isotype control	A95-1	rat, IgG2b	PE	mouse	BD Pharmingen
CD25	IL-2RA	3C7	rat, IgG	PE	mouse	BD Pharmingen
CD25	IL-2RA	PC61	rat, IgG	APC	mouse	BD Pharmingen
n.c.	Sca-1 or Ly6A/E	D7	rat, IgG	PE Cy7	mouse	BD Pharmingen
CD117	c-kit	2B8	rat, IgG	PE Cy5	mouse	eBioscience
CD11b	integrin alpha-M	M1/70	rat, IgG	biotin	mouse	BD Biosciences
CD3e	CD3e	145-2C11	rat, IgG	biotin	mouse	BD Biosciences
CD45R	B220	RA3-6B2	rat, IgG	biotin	mouse	BD Biosciences
n.c.	Ter119	Ter119	rat, IgG	biotin	mouse	BD Biosciences
n.c.	GR-1 or LygC/Ly6G	RB6-8C5	rat, IgG	biotin	mouse	BD Biosciences

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CD, cluster of differentiation; PE, phycoerythrin; FITC, fluorescein isothiocyanate; PerCP, peridinin chlorophyll protein; APC, allophycocyanin; Cy, cyanine dye; Alexa647, alexa fluor 647; n.c., not (yet) clustered; BD, Becton Dickinson; Ig, immunoglobulin; TcR, T cell receptor; LPSR, lipopolysaccharide-related antigen; IL-2RA, interleukin-2 receptor alpha chain; DPPIV, dipeptidyl-peptidase IV; HPCA-1, human precursor cell antigen-1; LCA, leukocyte common antigen; SCFR, stem cell factor receptor; IL-2RB, interleukin-2 receptor beta chain; IL-3RA, interleukin-3 receptor alpha chain; IL-2RG, interleukin-2 receptor gamma chain; IL-1RAP, interleukin-1 receptor accessory protein; p, phosphorylated; STAT5, signal transducer and activator of transcription 5.

Company Location: BD Bioscience, San José, CA, USA; R&D Systems, Minneapolis, MN, USA; Dako, Glostrup, Denmark; eBioscience, San Diego, CA, USA; Miltenyi Biotec, Bergisch Gladbach, Germany; BioLegend, San Diego, CA, USA.

Supplementary Table S2

Patients´ characteristics – chronic myeloid leukemia (CML)

No	diagnosis	karyotype	age (yrs)	gender (m/f)	WBC (G/L)	Hb (g/dL)	Plt (G/L)	basophil PB (%)	blasts PB (%)	blasts BM (%)	BCR/ABL1 (%)	Samples used at D/FU (months after therapy; TKI)	MR/CR
1	CML CP	46,XY,t(9;22)	49	m	232.75	9.3	1062	6	3	1	39.269	D	n.a.
2	CML CP	46,XX,t(9;22)	42	f	181.87	14.1	148	4	1	2	67.485	D	n.a.
2	CML FU	46,XX	44	f	6.43	12.5	200	0	0	1	0.188	FU (24 months; IM)	NMR/CCyR
3	CML CP	46,XY,t(9;22)	37	m	396	9.3	225	4	5	3	18.44	D	n.a.
3	CML FU	46,XY	45	m	6.54	14.2	204	1	0	2	0.07	FU (139 months; IM)	MMR/CCyR
4	CML CP	46,XX,t(9;22)	22	f	26.58	13.4	354	5	0	1	60.051	D	n.a.
4	CML FU	46,XX	23	f	4.48	11	210	0	0	1	0.076	FU (14 months; IM)	MMR/CCyR
5	CML CP	46,XY,t(9;22)	56	m	271.49	9.2	607	8	2	2	43.205	D	n.a.
5	CML FU	46,XY	57	m	4.58	11.8	235	2	0	1	0.116	FU (12 months; IM)	NMR/CCyR
6	CML CP	46,XX,t(9;22)	35	f	193.84	11.5	222	4	1	2	100	D	n.a.
6	CML FU	46,XX	38	f	2.79	12.6	122	0	0	1	0.006	FU (45 months; IM)	MMR/CCyR
7	CML CP	46,XY,t(9;22)	28	m	96.83	12.9	174	2	1	1	58.88	D	n.a.
7	CML FU	46,XY,t(9;22)	33	m	3.39	12.8	135	0	0	1	0.002	FU (64 months; IM)	MMR/PCyR
8	CML CP	46,XY,t(9;22)	85	m	45.74	12.4	330	2	1	2	37.752	D	n.a.
9	CML CP	46,XX,t(9;22)	62	f	43.52	11.7	265	19	1	4	13.62	D	n.a.
9	CML FU	46,XX	70	f	4.76	12.2	232	0	0	1	0	FU (98 months; IM)	CMR/CCyR
10	CML CP	46,XX,t(9;22)	46	f	47	11.1	591	2	0	1	80	D	n.a.
10	CML FU	46,XX	50	f	6.2	13.3	234	0	0	2	0.01	FU (57 months; IM, NIL)	MMR/CCyR
11	CML CP	46,XY,t(9;22)	33	m	190.69	9.7	750	4	3	8	38.867	D	n.a.

No	diagnosis	karyotype	age (yrs)	gender (m/f)	WBC (G/L)	Hb (g/dL)	Plt (G/L)	basophil PB (%)	blasts PB (%)	blasts BM (%)	BCR/ABL1 (%)	Samples used at D/FU (months after therapy; TKI)	MR/CR
12	CML CP	46,XX,t(9;22)	67	f	37.6	10.5	1375	3	1	4	100	D	n.a.
12	CML FU	46,XX	69	f	7.05	12.2	225	1	0	1	0	FU (20 months; IM, NIL)	CMR/CCyR
13	CML CP	46,XY,t(9;22)	62	m	46.54	11.8	419	4	1	2	51.478	D	n.a.
13	CML FU	46,XY	63	m	5.66	12.6	198	1	0	1	0.076	FU (11 months; IM)	MMR/CCyR
14	CML CP	46,XX,t(9;22)	84	f	67.64	12.8	305	2	0	1	42.294	D	n.a.
15	CML CP	46,XY,t(9;22)	56	m	48.12	13.9	217	1	0	1	67	D	n.a.
15	CML FU	46,XY	60	m	8.15	11.4	268	0	0	2	0.21	FU (43 months; IM)	NMR/CCyR
16	CML CP	46,XX,t(9;22)	67	f	332.22	10.3	568	4	4	4	98	D	n.a.
17	CML CP	46,XY,t(9;22)	47	m	55.74	13.9	250	1	1	3	45.667	D	n.a.
17	CML FU	46,XY	50	m	4.94	13.7	176	1	0	1	0.003	FU (35 months; IM)	MMR/CCyR
18	CML CP	46,XY,t(9;22)	51	m	174.2	11.6	275	1	1	1	69.884	D	n.a.
18	CML FU	46,XY	52	m	5.5	13.2	184	1	0	2	0.627	FU (9 months; IM)	NMR/CCyR
19	CML CP	46,XY,t(2;10),t(9;22)	72	m	112.5	11.9	394	13	6	4	79	D	n.a.
20	CML CP	46,XX,t(9;22)	45	f	184	10.1	700	1	1	1	45.037	D	n.a.
20	CML FU	46,XX	46	f	3.36	10.8	148	0	0	1	0.027	FU (50 months, IM)	MMR/CCyR
21	CML CP	46,XX,t(9;22)	74	f	34.22	12.8	911	6	0	1	72.343	D	n.a.
22	CML CP	46,XX,t(9;22)	22	f	230.57	10.4	720	5	1	1	50.089	D	n.a.
23	CML CP	46,XY,t(9;22)	64	m	23.9	13.3	164	1	0	1	61.3	D	n.a.
24	CML CP	complex, t(9;22)	54	m	290.43	12.4	441	3	2	2	63.192	D	n.a.
24	CML FU	46,XY	57	m	5.91	13.1	319	1	0	1	0.029	FU (36 months; DAS)	MMR/CCyR
25	CML CP	46,XY,t(9;22)	65	m	38.39	11.8	311	5	0	1	57.164	D	n.a.
26	CML CP	46,XY,t(9;22)	65	m	30.58	15.2	216	3	0	1	40	D	n.a.
26	CML FU	46,XY	70	m	7.2	13.4	227	0	0	1	0.006	FU (63 months; IM)	MMR/CCyR

No	diagnosis	karyotype	age (yrs)	gender (m/f)	WBC (G/L)	Hb (g/dL)	Plt (G/L)	basophil PB (%)	blasts PB (%)	blasts BM (%)	BCR/ABL1 (%)	Samples used at D/FU (months after therapy; TKI)	MR/CR
27	CML CP	46,XX,t(9;22)	42	f	61.2	13.5	319	3	0	1	92.56	D	n.a.
27	CML FU	46,XX	48	f	4.89	11.3	194	1	0	1	0.008	FU (61 months; IM)	MMR/CCyR
28	CML CP	46,XY,t(9;22)	74	m	48.41	13.1	321	1	1	1	22.897	D	n.a.
29	CML CP	n.t.	43	f	113.7	12.3	514	5	3	2	34.27	D	n.a.
30	CML CP	46,XY,t(9;22)	65	m	27.75	14.7	114	7	1	1	34.055	D	n.a.
31	CML CP	46,XX,t(9;22)	68	f	40.22	12.1	413	5	n.t.	2	21.851	D	n.a.
32	CML CP	46,XY,t(9;22)	71	m	49.8	14.1	320	4	1	1	38.655	D	n.a.
33	CML CP	46,XY*	54	m	32.8	12.7	434	2	n.t.	3	76.909	D	n.a.
34	CML CP	46,XY,t(9;22)	18	m	175.8	8.7	282	3	2	1	51.578	D	n.a.
35	CML CP	46,XY,t(9;22)	71	m	179.1	11.1	83	4	1	n.t.	4.507	D	n.a.
36	CML CP	46,XY,t(9;22)	65	m	273.59	7.7	470	8	10	6	78.903	D	n.a.
37	CML CP	46,XX*	67	f	46.7	10.6	313	n.t.	n.t.	2	n.t.	D	n.a.
38	CML CP	46,XX,t(9;22)	57	f	39.11	12.1	150	n.t.	n.t.	1	38.662	D	n.a.
39	CML CP	n.t.	73	f	10.4	4.4	942	n.t.	n.t.	n.t.	47.979	D	n.a.
40	CML CP	46,XX,t(9;22)	27	f	348	9.6	213	7	6	<5	n.t.	D	n.a.
41	CML CP	46,XX,t(9;22)	27	f	415	7	406	2	1	<5	n.t.	D	n.a.
42	CML CP	46,XY,t(9;22)	40	m	320	10.4	507	4	2	<5	n.t.	D	n.a.
43	CML CP	46,XX,t(9;22)	57	f	365.6	8.8	516	5	8	<5	n.t.	D	n.a.
44	CML CP	46,XY,t(9;22)	50	m	282	10.8	632	9	5	<5	n.t.	D	n.a.
45	CML CP	46,XX,t(9;22)	51	f	99.8	10,5	202	2	1	<5	n.t.	D	n.a.
46	CML CP	46,XX,t(9;22)	51	f	93.6	11.5	568	9	4	<5	n.t.	D	n.a.
47	CML CP	46,XY,t(9;22)	62	m	335	9	902	5	5	<5	n.t.	D	n.a.
48	CML CP	46,XY,t(9;22)	55	m	40.9	13.9	889	10	n.t.	n.t.	28.48	D	n.a.
48	CML FU	46,XY	62	m	5.12	13.3	303	0	n.t.	1	n.t.	FU (87 months, IM)	n.t./CCyR

No	diagnosis	karyotype	age (yrs)	gender (m/f)	WBC (G/L)	Hb (g/dL)	Plt (G/L)	basophil PB (%)	blasts PB (%)	blasts BM (%)	BCR/ABL1 (%)	Samples used at D/FU (months after therapy; TKI)	MR/CR
49	CML CP	46,XX,t(9;22)	66	f	127.2	10.1	607	5	9	<5	n.t.	D	n.a.
49	CML FU TKI resistant	n.t.	76	f	4.23	9.4	86	n.t.	1	2	52.29	FU (131 months, IM)	NMR/n.t.
50	CML CP	46,XX,t(9;22)	24	f	77	12.3	363	5	2	1	47,22	D	n.a.
50	CML CP	46,XX,t(9;22)	25	f	506.92	7.9	197	3	9	n.t.	n.t.	FU	NMR/NR
51	CML CP	n.t.	46	f	570.5	7.5	342	3	1	2	57,54	D	n.a.
52	CML CP	n.t.	68	f	97,83	10,9	11,2	6	1	1	51,63	D	n.a.
53	CML AP	46,XX,t(9;22)	27	f	178.3	10.2	626	15	7	5	71.765	D	n.a.
54	CML AP	46,XX,t(9;22)	86	f	230	8.9	783	4	11	3	100	D	n.a.
55	CML AP	46,XY,t(9;22),t(8;17)	39	m	190.9	11	347	15	3	2	56.38	D	n.a.
56	CML BP ly	46,XY,t(9;22)	72	m	20.35	10	12	0	69	80	86	D	n.a.
57	CML BP ly	45,XX,-7,t(9;22)	35	f	32.97	9.7	172	2	30	50	97.758	D	n.a.
58	CML BP ly	46,XX,t(9;22)	66	f	16.6	13.3	48	n.t.	80	100	62	D	n.a.
59	CML BP ly	46,XY,t(9;22)	72	m	104.43	9.8	73	0	46	36	87.593	D	n.a.
60	CML BP ly	46,XY,t(9;22)	38	m	137.35	7.3	29	n.t.	50	n.t.	n.t.	D	n.a.
61	CML BP my	complex, t(9;22)	84	f	149.8	5.5	62	1	63	83	61.699	D	n.a.
62	CML BP my	46,XX,t(9;22)	60	f	94.4	10.9	45	1	35	75	38.806	D	n.a.
63	CML BP my	46,XY,t(9;22)	43	m	5.5	12.4	158	n.t.	n.t.	25	48.16	D	n.a.

No, number; yrs, years; m, male; f, female; WBC, white blood count; Hb, hemoglobin; Plt, platelet count; PB, peripheral blood; BM, bone marrow; G/L, 10^9 cells per liter; g/dL, gram per deciliter; CP, chronic phase; AP, accelerated phase; BP, blast phase; my, myeloid; ly, lympathic; D, diagnosis, FU, follow-up; IM, imatinib; NIL, nilotinib; DAS, dasatinib; MR/CR, molecular response/ cytogenetic response; CHR, complete hematologic response; CMR, complete molecular response; MMR, major molecular response; NMR, no molecular response; NR, no response; CCyR, complete cytogenetic response; n.t.; not tested; n.a., not applicable. *In these patients a cryptic translocation of BCR/ABL1 was detectable by FISH in leukemic cells.

Supplementary Table S3A

Patients´ characteristics – normal/reactive BM

No	diagnosis	age (yrs)	gender (m/f)	WBC (G/L)	Hb (g/dL)	Plt (G/L)	blasts PB (%)	blasts BM (%)
1	NHL	70	m	3.6	10.2	189	0	2
2	NHL	23	m	11.7	14.1	210	0	2
3	NHL	71	m	13	14.7	108	0	1
4	solid tumor	52	m	8	15	203	0	1
5	suspected SM	23	m	6	14.4	165	0	1
6	NHL	72	m	13.8	14.3	286	0	1
7	NHL	40	m	5.5	14.6	247	0	1
8	NHL	77	f	3.9	10.2	110	0	1
9	NHL	28	m	5.2	16.9	198	0	2
10	NHL	61	m	8.1	7.6	530	0	1
11	solid tumor	27	f	9.1	13.8	354	0	1
12	NHL	63	f	6.8	14.6	208	0	1
13	Hodgkin	38	m	14.8	13.1	234	n.t.	2
14	NHL	66	f	5.9	14.9	307	0	1
15	Hodgkin	23	m	11.9	12.1	427	0	2
16	NHL	58	f	5.1	12	230	0	2
17	MGUS	52	m	7.2	15.3	219	0	1
18	NHL	45	m	5.1	14.5	245	0	1
19	suspected SM	32	m	4.3	15.2	278	0	1
20	suspected SM	31	f	4.7	13.7	229	0	<5
21	suspected SM	54	m	9.5	16	356	0	<5
22	suspected SM	44	f	7.1	14.1	267	0	<5
23	suspected SM	50	m	7.4	15.3	242	n.t.	1
24	suspected SM	35	m	7.3	14.2	241	0	1

No, number; yrs, years; m, male; f, female; WBC, white blood count; Hb, hemoglobin; Plt, platelet count; PB, peripheral blood; BM, bone marrow; G/L, 10^9 cells per liter; g/dL, gram per deciliter; NHL, Non Hodgkin's lymphoma, MGUS, monoclonal gammopathy of undetermined significance; SM, systemic mastocytosis; n.t.; not tested.

Supplementary Table S3B

Patients´ characteristics – idiopathic cytopenia of undetermined significance (ICUS)

No	diagnosis	age (yrs)	gender (m/f)	WBC (G/L)	Hb (g/dL)	Plt (G/L)	basophils PB (%)	blasts PB (%)	blasts BM (%)
1	ICUS	82	f	7.4	11.8	82	0	0	1
2	ICUS	37	f	2.7	11.6	196	2	0	0
3	ICUS	56	f	1.6	11.6	25	0	0	1
4	ICUS	69	m	2.4	10.6	1	2	0	1
5	ICUS	79	m	3.4	13.2	89	1	0	1
6	ICUS	44	f	4	10.8	189	2	0	1
7	ICUS	64	m	3.7	9.8	67	1	0	1
8	ICUS	62	m	5	8.4	94	0	0	2
9	ICUS	34	f	2.5	13.7	159	1	0	1
10	ICUS	68	f	2.5	13.8	189	1	0	2
11	Haemolysis	42	m	15.7	7.8	259	2	1	1
12	Agranulocytosis	47	m	1.7	10.6	399	2	0	2
13	ICUS	60	m	1.5	10.9	45	0	0	2
14	ICUS	53	f	3.2	11.3	173	0	0	1

No, number; yrs, years; m, male; f, female; WBC, white blood count; Hb, hemoglobin; Plt, platelet count; PB, peripheral blood; BM, bone marrow; G/L, 10^9 cells per liter; g/dL, gram per deciliter; ICUS; idiopathic cytopenia of undetermined significance.

Supplementary Table S3C

Patients´ characteristics – systemic mastocytosis

No	diagnosis	age (yrs)	gender (m/f)	mutations	karyotype	WBC (G/L)	Hb (g/dL)	Plt (G/L)	basophils PB (%)	blasts PB (%)	blasts BM (%)
1	ISM	53	f	KIT D816V	46,XX	9.7	13.6	263	0	0	1
2	ISM	36	f	KIT D816V	46,XX	5.1	13.6	193	0	0	1
3	ISM	25	m	KIT D816V	46,XY	7.5	15.1	223	1	0	2
4	ISM	69	f	none	46,XX	5.1	13.3	236	0	0	1
5	ISM	63	f	KIT D816V	46,XX	6	12.7	285	1	0	2
6	ISM	25	f	none	n.t.	6.3	12.8	212	0	0	1
7	ISM	56	m	KIT D816V	46,XY	6.8	13.8	223	0	0	2

No, number; yrs, years; m, male; f, female; WBC, white blood count; Hb, hemoglobin; Plt, platelet count; PB, peripheral blood; BM, bone marrow; G/L, 10^9 cells per liter; g/dL, gram per deciliter; ISM, indolent systemic mastocytosis; n.t., not tested.

Supplementary Table S3D

Patients´ characteristics – myeloproliferative neoplasm

No	diagnosis	age (yrs)	gender (m/f)	mutations	karyotype	WHO	WBC (G/L)	Hb (g/dL)	Plt (G/L)	basophils PB (%)	blasts PB (%)	blasts BM (%)
1	MPN	70	m	JAK2 V617F	n.t.	PMF	8.1	13.4	375	0	0	1
2	MPN	67	f	JAK2 V617F	46,XX	ET	8.9	13.2	660	2	0	2
3	MPN	48	m	JAK2 V617F	46,XY	ET	9	14.5	974	5	0	1
4	MPN	61	m	JAK2 V617F	46,XY	PMF	8.7	8.7	138	1	0	1
5	MPN	45	f	JAK2 V617F	46,XX	PMF	11.1	14.6	966	1	0	1
6	MPN	21	f	JAK2 V617F	n.t.	ET	8.7	13.5	605	2	0	2

No, number; yrs, years; m, male; f, female; WHO, world health organisation; WBC, white blood count; Hb, hemoglobin; Plt, platelet count; PB, peripheral blood; BM, bone marrow; G/L, 10^9 cells per liter; g/dL, gram per deciliter; MPN; myeloproliferative neoplasm; PMF, primary myelofibrosis; ET, essential thrombocythemia; JAK, Janus kinase.

Supplementary Table S4

Detection of BCR/ABL1 in sorted CML cells by FISH

Cell Population	% BCR/ABL1+ cells
Patient #11*	
$CD34^+/CD38^-/CD25^+$ cells	100%
$CD34^+/CD38^-/CD25^-$ cells	52%
Patient #34*	
$CD34^+/CD38^-/CD25^+$ cells	100%
$CD34^+/CD38^-/CD25^-$ cells	49%
Patient #50*	
$CD34^+/CD38^-/CD25^+$ cells	100%
$CD34^+/CD38^-/CD25^-$ cells	30%

Fluorescence in situ hybridization (FISH) was performed on sorted stem cell fractions. FISH was performed as described in the methods-section in this appendix. A total number of at least 20 interphases were examined in each cell sample.
*Patient numbers (#) refer to the list of patients provided in Supplementary Table S2.

Supplementary Table S5A

Major genes up-regulated by gene array analysis on KU812 cells transduced with a CD25 shRNA (KU812 CD25 shRNA, clone #2) compared to KU812 cells transduced with a random control shRNA (KU812 RDM shRNA)

up-regulated gene products (mRNA)		Gene Array Data KU812 CD25 shRNA vs KU812 RDM shRNA
Gene Symbol	Name, Synonym(s), Abbreviation(s) Gene Description	fold up-regulation
DAZ2	deleted in azoospermia 2	25.6
DAZ4	deleted in azoospermia 4	23.2
DAZ3	deleted in azoospermia 3	22.7
DAZ1	deleted in azoospermia 1	19.5
USP26	ubiquitin specific peptidase 26	15.6
---	TCONS_00014766	
	linc-ZFHX4-2 chr8:+:76851941-76864003	8.9
RAB27B	RAB27B, member RAS oncogene family	8.7
IGFBP7	insulin-like growth factor binding protein 7	8.4
SSX2	synovial sarcoma, X breakpoint 2	8.1
---	ENST00000524132	
	cdna:known chromosome:GRCh37:8:36394909:36636682:-1 gene:ENSG00000253363	7.2
---	TCONS_l2_00021973	
	linc-SCLT1-4 chr4:-:132851185-132897107	6.8
RASGEF1A	RasGEF domain family, member 1A	6.5
---	TCONS_00008716	
	linc-TRIML2-5 chr4:-:190200224-190201335	6.5
---	TCONS_00014696	
	linc-SNTG1-2 chr8:+:50080641-50105812	6.4
SSX1	synovial sarcoma, X breakpoint 1	5.8
IGF1	insulin-like growth factor 1 (somatomedin C)	5.8
---	TCONS_00025434	
	linc-KIF2B-1 chr17:+:50995287-51004254	5.7
ZFP42	zinc finger protein 42 homolog (mouse)	5.5
HBE1	hemoglobin, epsilon 1	5.1
DSCR8	Down syndrome critical region gene 8	5.1
MAGEA6	melanoma antigen family A, 6	4.7
---	TCONS_00026582	
	linc-MBP-7 chr18:-:75701644-75702115	4.6
ARHGAP32	Rho GTPase activating protein 32	4.6
---	TCONS_l2_00025436	
	linc-MAP3K7-4 chr6:-:93432720-93433547	4.4
FSTL5	follistatin-like 5	4.4
INHBA	inhibin, beta A	4.3
DSCR4	Down syndrome critical region gene 4	4.3
---	TCONS_00026222	
	linc-MBP-1 chr18:-:75683254-75692482	4.2
---	TCONS_00015680	
	linc-ANKRD20A1-14 chr9:+:45484571-45488153	4.1

up-regulated gene products (mRNA) Gene Symbol	Name, Synonym(s), Abbreviation(s) Gene Description	Gene Array Data KU812 CD25 shRNA vs KU812 RDM shRNA fold up-regulation
SNORD62A	small nucleolar RNA, C/D box 62A	4.1
GRID2	glutamate receptor, ionotropic, delta 2	4.1
HEPH	hephaestin	4.0
CXADRP3	coxsackie virus and adenovirus receptor pseudogene 3	4.0
SNORD85	small nucleolar RNA, C/D box 85	4.0
RRAGD	Ras-related GTP binding D	4.0
MIR4266	microRNA 4266	3.9
ZNF385D-AS1	ZNF385D antisense RNA 1 (non-protein coding)	3.8
CTSE	cathepsin E	3.7
ENPP6	ectonucleotide pyrophosphatase/phosphodiesterase 6	3.7
MAGEA12	melanoma antigen family A, 12	3.7
ENPP5	ectonucleotide pyrophosphatase/phosphodiesterase 5	3.6
---	TCONS_00017351 linc-CHM-1 chrX:-:86979060-86983512	3.5
---	TCONS_l2_00027522 linc-XRCC2-3 chr7:-:153097004-153109319	3.5
SAMD5	sterile alpha motif domain containing 5	3.5
CSAG2	CSAG family, member 2	3.4
NPC1L1	NPC1 (Niemann-Pick disease, type C1, gene)-like 1	3.4
CRYBA4	crystallin, beta A4	3.3

After calculating the differences in mRNA expression levels from paired samples, top up-regulated genes were selected and expressed as fold up-regulation in KU812 CD25 shRNA cells compared to KU812 RDM shRNA cells. Genes are listed in the order of up-regulation.

Supplementary Table S5B

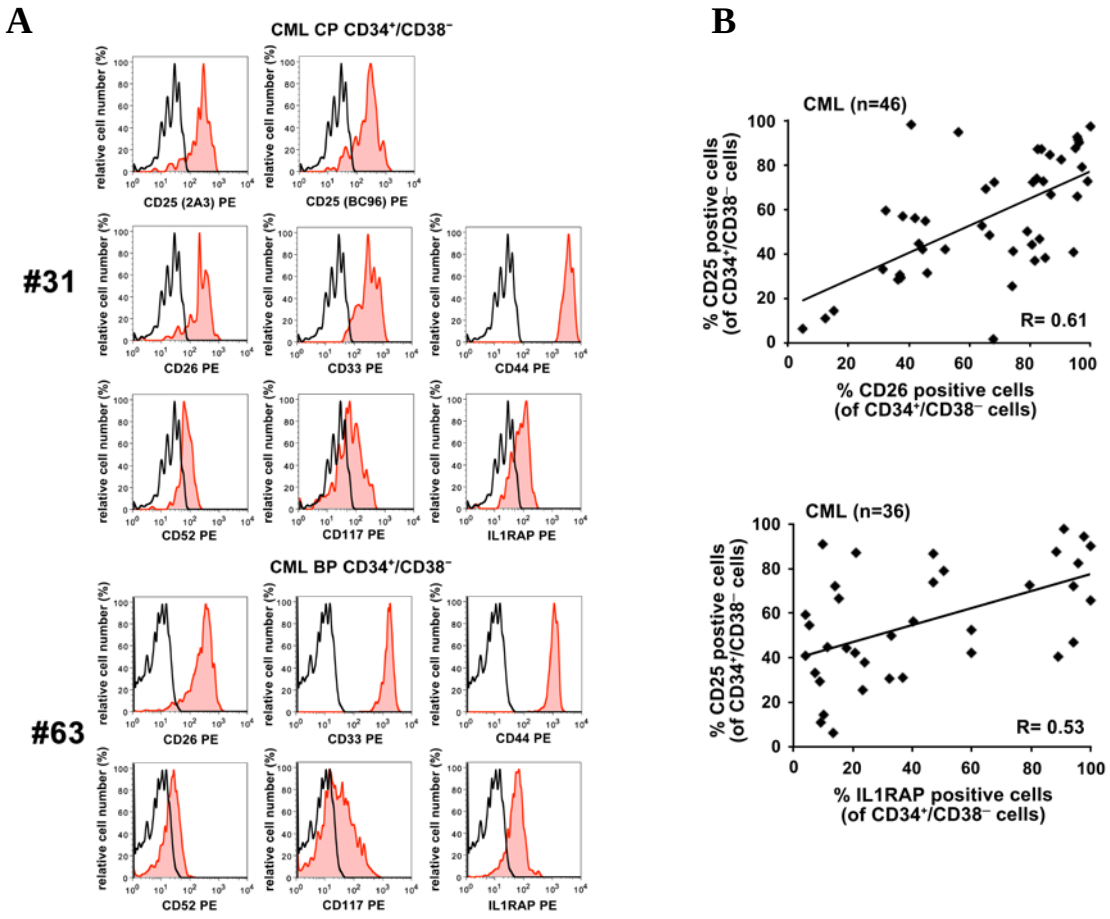
Major genes down-regulated by gene array analysis on KU812 cells transduced with a CD25 shRNA (KU812 CD25 shRNA, clone #2) compared to KU812 cells transduced with a random control shRNA (KU812 RDM shRNA)

down-regulated gene products (mRNA) Gene Symbol	Name, Synonym(s), Abbreviation(s) Gene Description	Gene Array Data KU812 CD25 shRNA vs KU812 RDM shRNA fold down-regulation
MS4A2	membrane-spanning 4-domains	0.1
CYTL1	cytokine-like 1	0.2
SIGLEC17P	sialic acid binding Ig-like lectin 17	0.2
CPA3	carboxypeptidase A3 (mast cell)	0.2
IL-2RA	interleukin 2 receptor, alpha	0.2
---	TCONS_l2_00021793 linc-ZFP42-7 chr4:+:188334717-188336851	0.2
CDK15	cyclin-dependent kinase 15	0.2
SIGLEC6	sialic acid binding Ig-like lectin	0.3
CRISP3	cysteine-rich secretory protein 3	0.3
PAH	phenylalanine hydroxylase	0.3
HEY1	hairy/enhancer-of-split related, YRPW motif 1	0.3
SLAMF6	SLAM family member 6	0.3
GPR65	G protein-coupled receptor 65	0.3
---	ENST00000522354 havana:lincRNA chromosome:GRCh37:8:69760977:69764998:-1 gene:ENSG00000254337	0.3
CXCL10	chemokine (C-X-C motif) ligand 10	0.3
RN5S126	RNA, 5S ribosomal 126	0.3
---	TCONS_00017655 linc-RBMY1B chrY:+:23300673-23332061	0.3
---	TCONS_00011730 linc-HDGFL1-1 chr6:+:22349446-22517940	0.3
IL-1RL1	interleukin 1 receptor-like 1	0.3
IGLV2-18	immunoglobulin lambda variable 2-18	0.3
SLC18A2	solute carrier family 18, member 2	0.4
SNAI2	snail homolog 2 (Drosophila)	0.4
CA2	carbonic anhydrase II	0.4
SGK1	serum/glucocorticoid regulated kinase 1	0.4
---	ENST00000408076 ncrna:miRNA chromosome:GRCh37:8:29786121:29786205:1 gene:ENSG00000221003 gene_biotype:miRNA	0.4
TMPRSS11F	transmembrane protease, serine 11F	0.4
MIR181B1	microRNA 181b-1	0.4
LUST	RBM5 antisense RNA	0.4
PPAP2A	phosphatidic acid phosphatase type 2A	0.4
---	TCONS_00001394 linc-LRRC38-3 chr1:-:14717544-14746458	0.4
RAB37	RAB37, member RAS oncogene family	0.4

down-regulated gene products (mRNA)		Gene Array Data KU812 CD25 shRNA vs KU812 RDM shRNA fold down-regulation
Gene Symbol	Name, Synonym(s), Abbreviation(s) Gene Description	
---	ENST00000364556 ncrna:misc_RNA chromosome:GRCh37:3:110445868:110445968:1 gene:ENSG00000201426	0.4
RNU7-27P	RNA, U7 small nuclear 27 pseudogene	0.4
---	ENST00000362601 ncrna:misc_RNA chromosome:GRCh37:1:202884008:202884108:-1 gene:ENSG00000199471	0.4
---	BC016361 Homo sapiens, clone IMAGE:4105785, mRNA	0.4
---	ENST00000458954 ncrna:miRNA chromosome:GRCh37:8:12435514:12435661:1 gene:ENSG00000238460 gene_biotype:miRNA transcript_biotype:miRNA	0.4
TRAF5	Homo sapiens TNF receptor-associated factor 5	0.4
---	ENST00000408265 ncrna:miRNA chromosome:GRCh37:7:96920826:96920908:-1 gene:ENSG00000221192	0.4
BCL2	B-cell CLL/lymphoma 2	0.4
---	ENST00000459100 ncrna:miRNA chromosome:GRCh37:8:12493613:12493761:1 gene:ENSG00000238323 gene_biotype:miRNA	0.4
41XAF1	XIAP associated factor 1	0.4
PRR9	proline rich 9	0.4
IKZF2	IKAROS family zinc finger 2 (Helios)	0.4
MIR548I3	microRNA 548i-3	0.4
---	TCONS_00014864 linc-KHDRBS3-5 chr8:+:135732687-135736134	0.4
RN5S82	RNA, 5S ribosomal 82	0.4
LIMD2	LIM domain containing 2	0.4
GPR174	G protein-coupled receptor 174	0.4
MAOB	monoamine oxidase B	0.4
---	TCONS_00005769 linc-ANO10-2 chr3:-:44462618-44465499	0.4

After calculating the differences in mRNA expression levels from paired samples, top down-regulated genes were selected and expressed as fold down-regulation in KU812 CD25 shRNA cells compared to KU812 RDM shRNA cells. Genes are listed in the order of down-regulation.

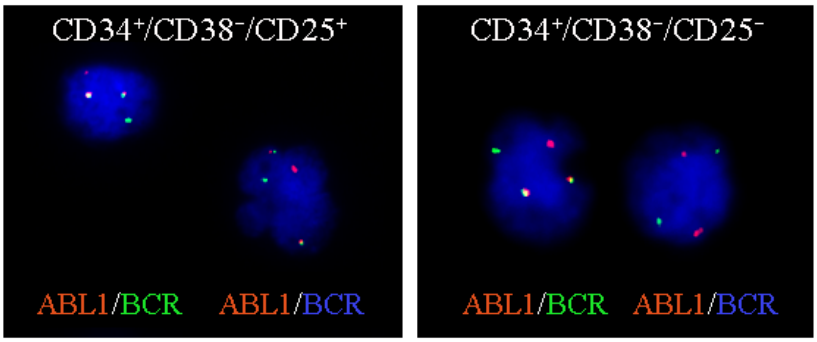
Supplementary Figure S1



CML LSCs co-express CD25 together with other stem cell markers

A: Histograms show the expression of CD26, CD33, CD44, CD52, CD117 and IL-1RAP on $CD34^+/CD38^-$ LSCs from one patient with CML chronic phase (CP, n=1, upper panels) and one patient with CML blast phase (BP, n=1, lower panels). The reactivity of $CD34^+/CD38^-$ LSCs with 2 different CD25 mAb (2A3 and BC96) was also tested in the patient with CML CP (upper histograms). Black open histograms represent the matched isotype control, red histograms represent expression of investigated surface antigens. The patient-numbers (#) are also indicated and refer to the list of patients shown in Supplementary Table S2. B: Correlations between the percentages of $CD25^+$ stem cells, SCs (of $CD34^+/CD38^-$ SC) and $CD26^+$ SCs (n=46, upper panel) and between $CD25^+$ SCs and IL-1RAP$^+$ SCs (n=36, lower panel) in the bone marrow or blood of patients with BCR/ABL1+ CML.

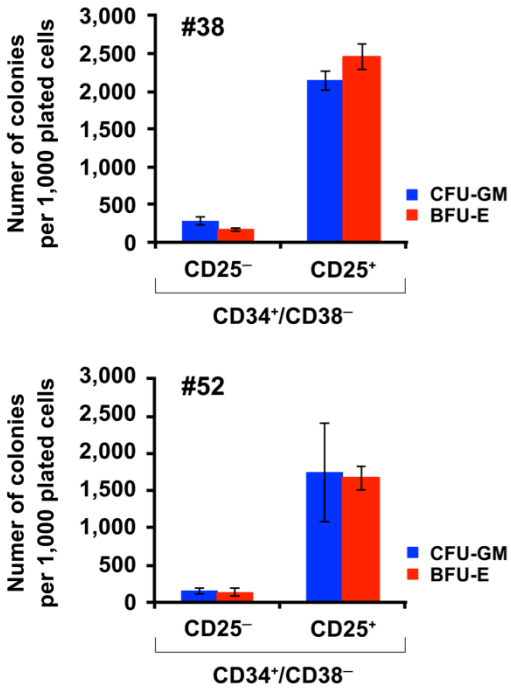
Supplementary Figure S2



Expression of BCR/ABL1 in $CD34^+/CD38^-/CD25^+$ stem cells obtained from a patient with CML as determined by fluorescence in situ hybridization (FISH)

FISH analysis was performed using purified $CD34^+/CD38^-/CD25^+$ CML stem cells (left panel) and $CD34^+/CD38^-/CD25^-$ stem cells (right panel) obtained from one (the same) patient with CML (#34, see Supplementary Table S2). FISH was performed on cytospin slides using a triple-color staining approach, with one probe specific for ABL1 (red) and two specific for BCR (green and blue). Red-green and red-blue fusion-spots indicate BCR/ABL1. Quantitative FISH data are shown in Supplementary Table S4.

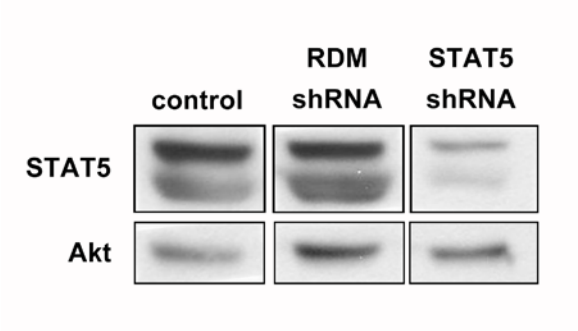
Supplementary Figure S3



Clonogenic growth of CML LSCs and normal stem cells obtained from two patients with Ph+ CML

$CD34^+/CD38^-/CD25^-$ (presumably normal) stem cells and $CD34^+/CD38^-/CD25^+$ LSCs were highly enriched (purity >95%) from bone marrow mononuclear cells in two patients with chronic phase CML (patient #38 and #52 in Supplementary Table S2) by flow cytometry. CD25− (presumably normal) stem cells and LSCs were cultured on irradiated M2-10B4 feeder-cells in LTC-IC (Myelocult) medium for 3 weeks. Then, cells were harvested and cultured in a CFU-GM culture assay (Methocult containing colony-stimulating cytokines) for 2 weeks. CFU-GM (blue bars) and BFU-E colonies (red bars) were counted under an inverted microscope. Results show the numbers of colonies per 1,000 plated cells and represent the mean±S.D. of triplicates. After counting, colonies were picked and pooled (10 colonies per plate) and the isolated RNA subjected to qPCR analysis to measure BCR/ABL1 levels. In colonies grown from $CD34^+/CD38^-/CD25^+$ LSCs, almost all cells were found to be BCR/ABL1+ (>80%), whereas in the $CD34^+/CD38^-/CD25^-$ (presumably normal) stem cells, BCR/ABL1 mRNA levels were low or undetectable (0-5.2%).

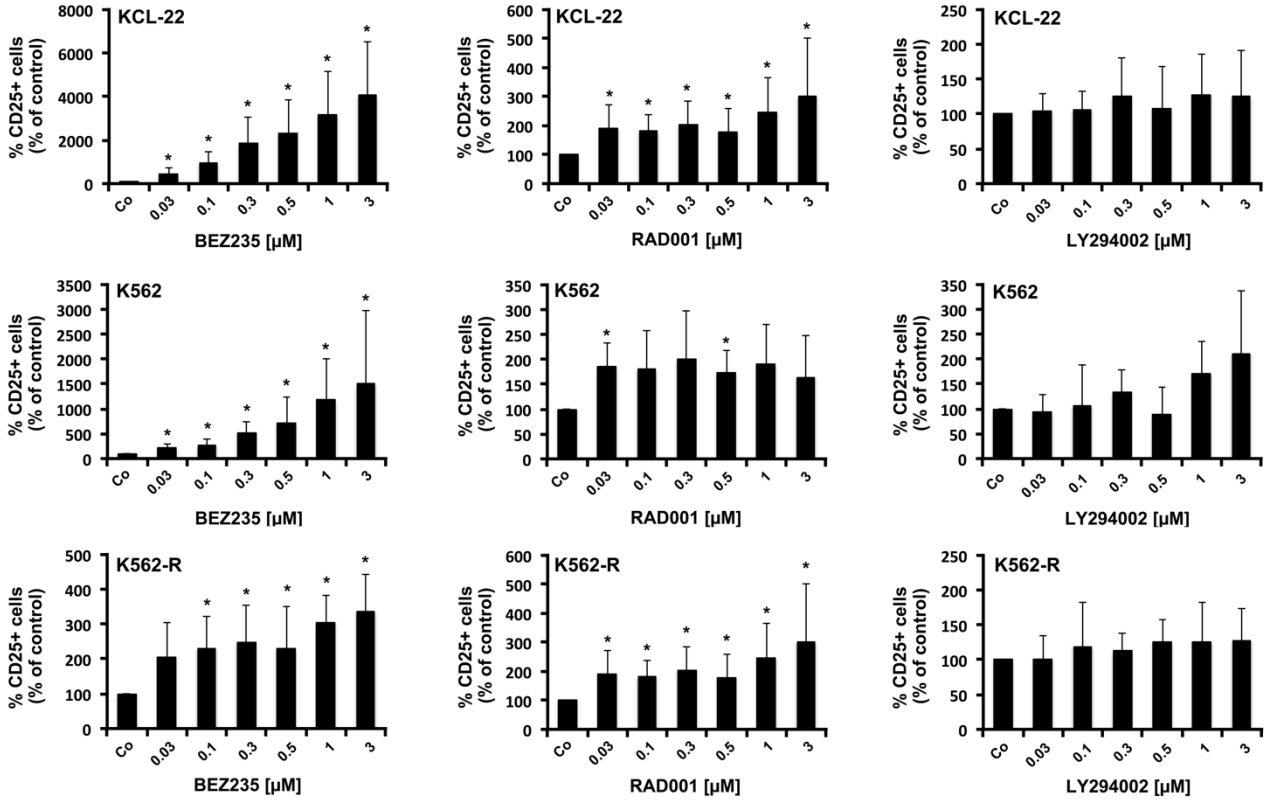
Supplementary Figure S4



Western blotting confirmed the knock-down of STAT5 in KU812 cells

Western blotting was performed with untransduced KU812 cells (control) and KU812 cells transduced with a random control shRNA (RDM shRNA) or a shRNA against STAT5 (STAT5 shRNA) as described in the Material and Methods part in the main document, using an antibody against STAT5. Akt served as a loading control.

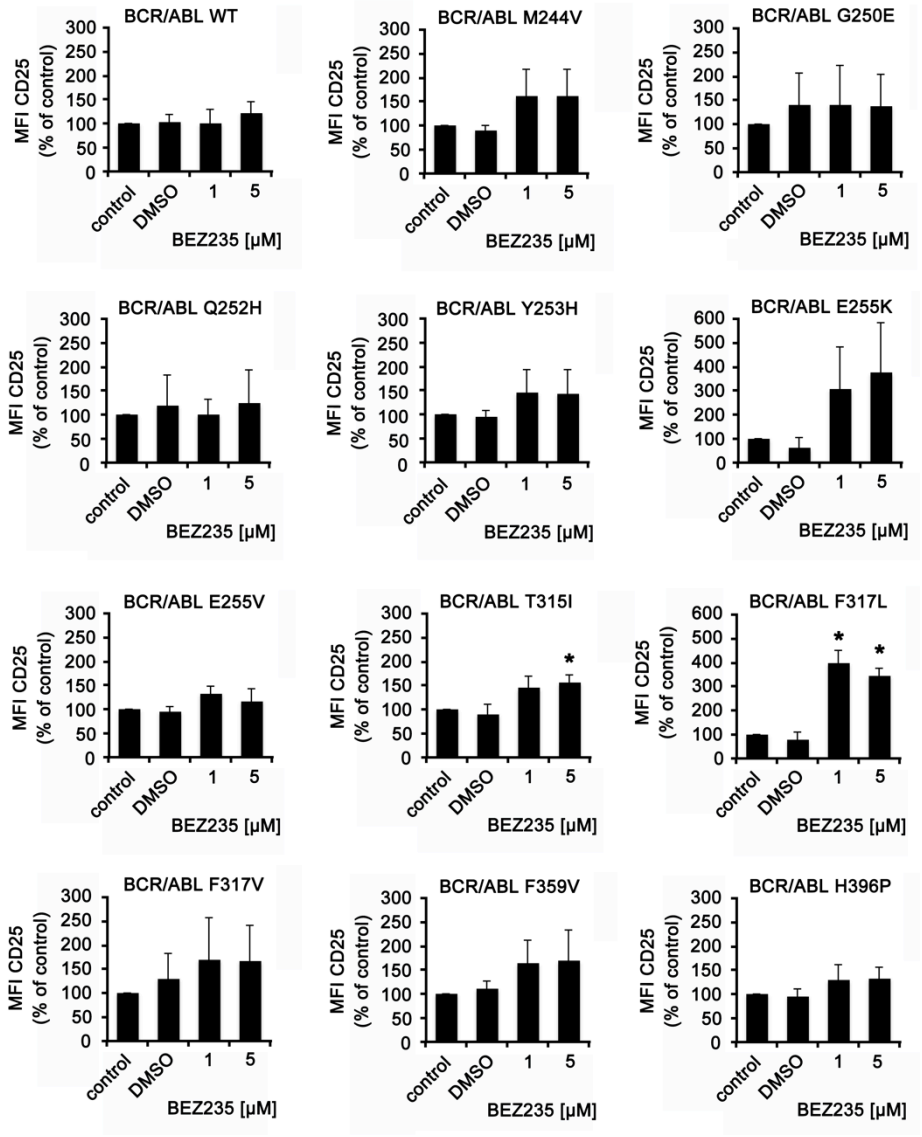
Supplementary Figure S5A



Drug-induced upregulation of CD25 in CML cell lines

KCL-22 cells (upper panels), K562 cells (middle panels), and K562-R cells (lower panels) were incubated in control medium (Co) or various concentrations of BEZ235 (left panels), RAD001 (middle panels), or LY294002 (right panels) (each 0.03-3 µM) at 37°C for 24 hours. Thereafter, expression of CD25 on these cell lines (percent of CD25-positive cells) was assessed by flow cytometry. Results are expressed as percent of control (percent $CD25^+$ cells in Co = 100%) and represent the mean±S.D. from at least 5 independent experiments. Asterisk (*): $p<0.05$ compared to untreated cells (Co).

Supplementary Figure S5B



Effects of BEZ235 on CD25 surface expression in Ba/F3 cells expressing diverse BCR/ABL1 mutant forms

Ba/F3 cells expressing several BCR/ABL1 mutants (as indicated) were incubated in control medium (control) or in various concentrations of BEZ235 (1 and 5 µM) or DMSO control at 37°C for 24 hours. After incubation, cells were analyzed for their CD25 surface expression using flow cytometry. Results are expressed as mean fluorescence intensities (MFI) of CD25 (percent of medium control) and represent the mean±SD from 3 independent experiments. Asterisk (*) indicates $p<0.05$ compared to untreated cells.

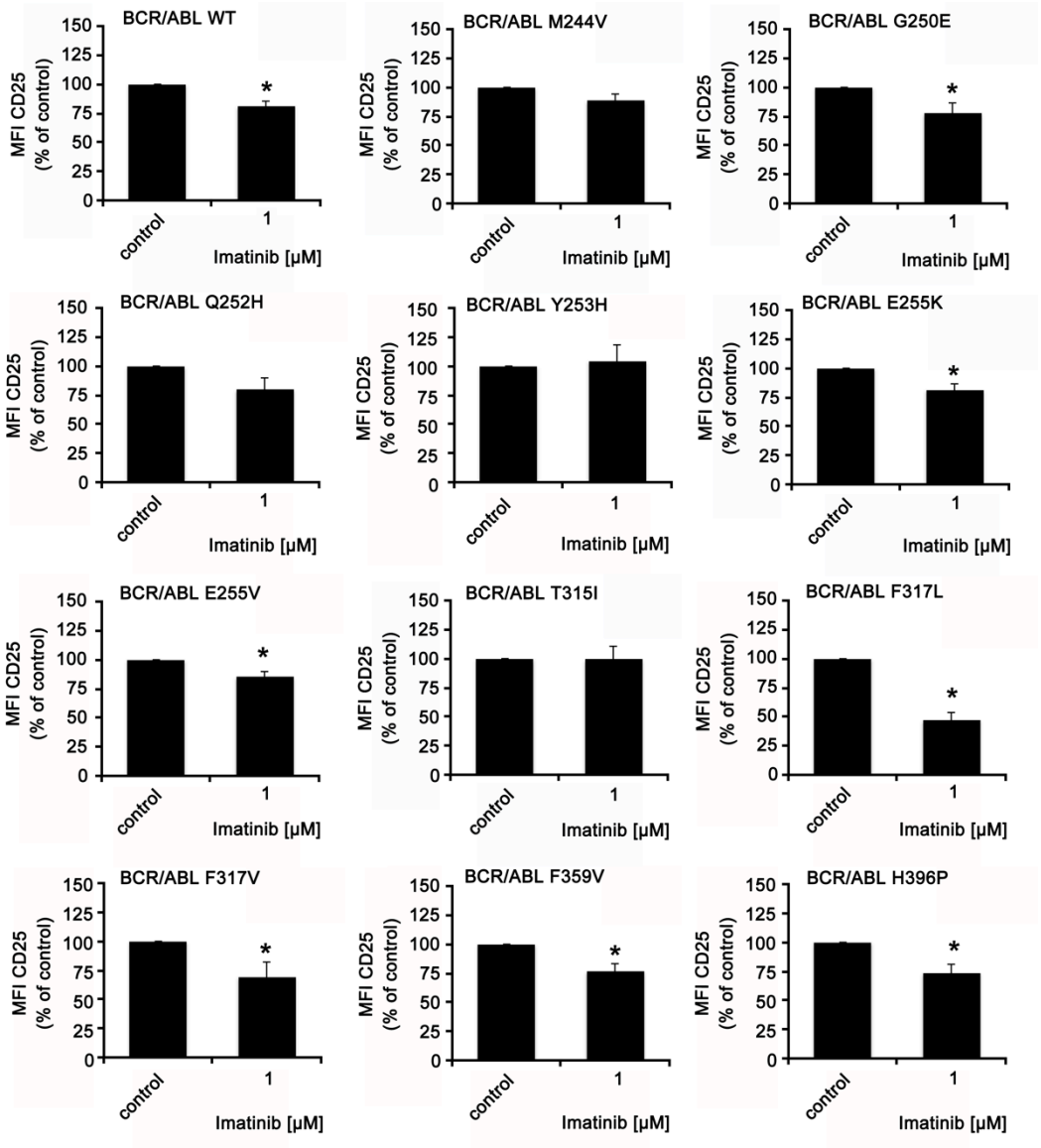
Supplementary Figure S5C

Effects of the dual PI3K/mTOR blocker BEZ235 on proliferation of Ba/F3 cells expressing diverse BCR/ABL1 mutants

BCR/ABL1 mutant	IC_{50} [μM]
wild type (WT)	0.5-1
M244V	0.1-0.5
G250E	0.1-0.5
Q252H	0.1-0.5
Y253H	0.5-1
E255K	0.1-0.5
E255V	0.1-0.5
T315I	0.1-0.5
F317L	0.1-0.5
F317V	0.1-0.5
F359V	0.5-1
H396P	0.5-1

Ba/F3 cells were incubated with various concentrations of BEZ235 at 37°C for 48 hours. Proliferation was measured by analyzing ^{3}H-thymidine uptake.
IC_{50}, inhibitory concentration (50%).

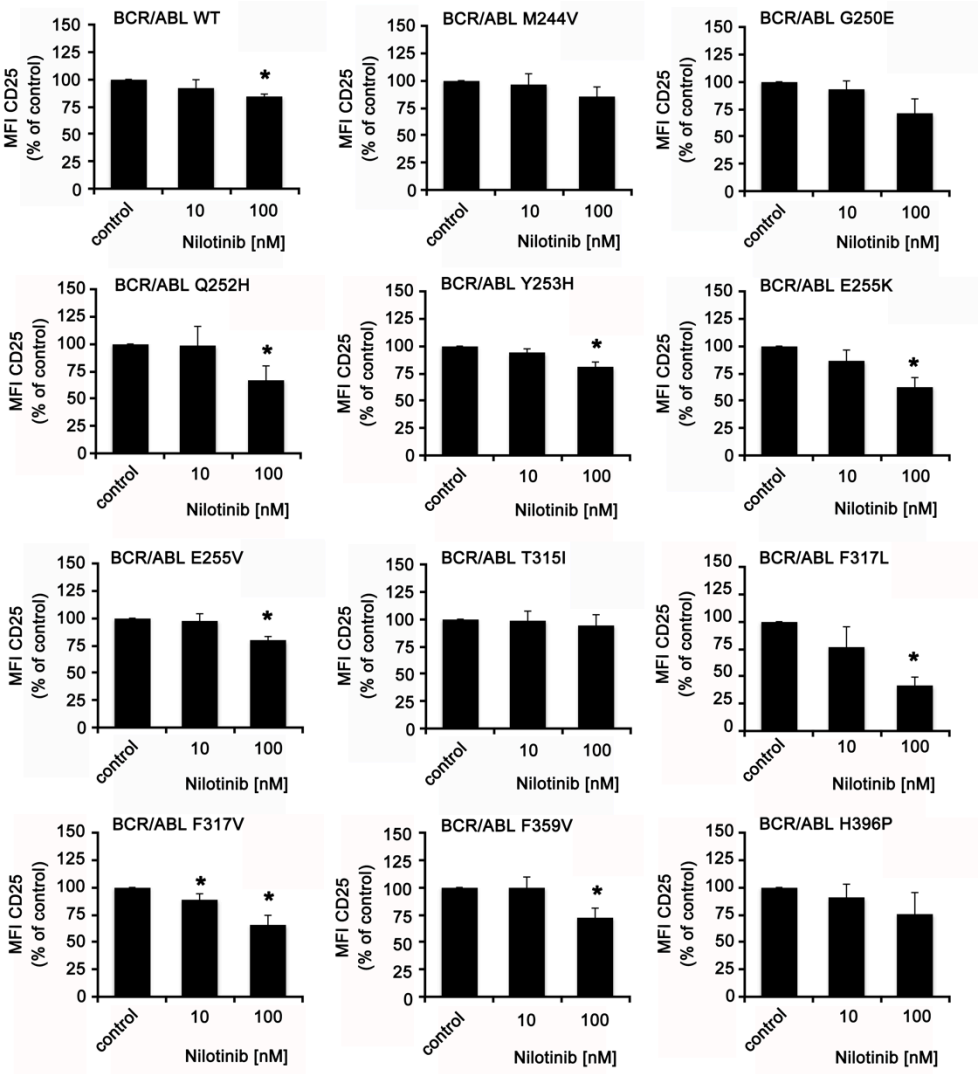
Supplementary Figure S6A



Effects of imatinib on CD25 surface expression in Ba/F3 cells expressing diverse BCR/ABL1 mutants

Ba/F3 cells expressing several BCR/ABL1 mutants were incubated in control medium (control) or in various concentrations of imatinib (1 µM) at 37°C for 24 hours. After incubation, cells were analyzed for their CD25 surface expression using flow cytometry. Results are expressed as MFI of CD25 (percent of control) and represent the mean±SD from 3 independent experiments. Asterisk (*) indicates $p<0.05$ compared to untreated cells.

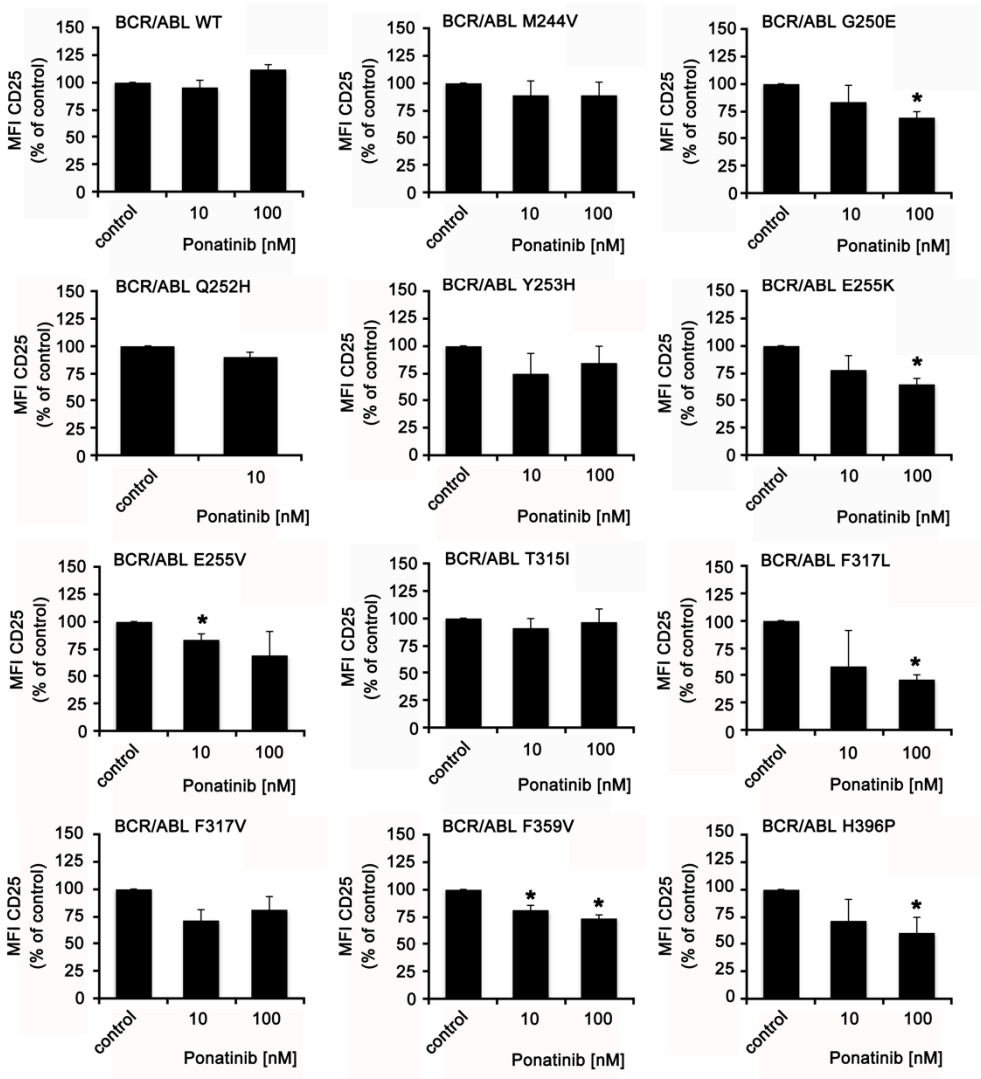
Supplementary Figure S6B



Effects of nilotinib on CD25 surface expression in Ba/F3 cells expressing various imatinib-resistant BCR/ABL1 mutants

Ba/F3 cells expressing BCR/ABL1 mutants were incubated in control medium (control) or in medium containing nilotinib (10 nM or 100 nM) at 37°C for 24 hours. After incubation, cells were analyzed for CD25 expression by flow cytometry. Results are expressed as MFI of CD25 (percent of medium control) and represent the mean±SD from 3 independent experiments. Asterisk (*) indicates $p<0.05$ compared to untreated cells (control).

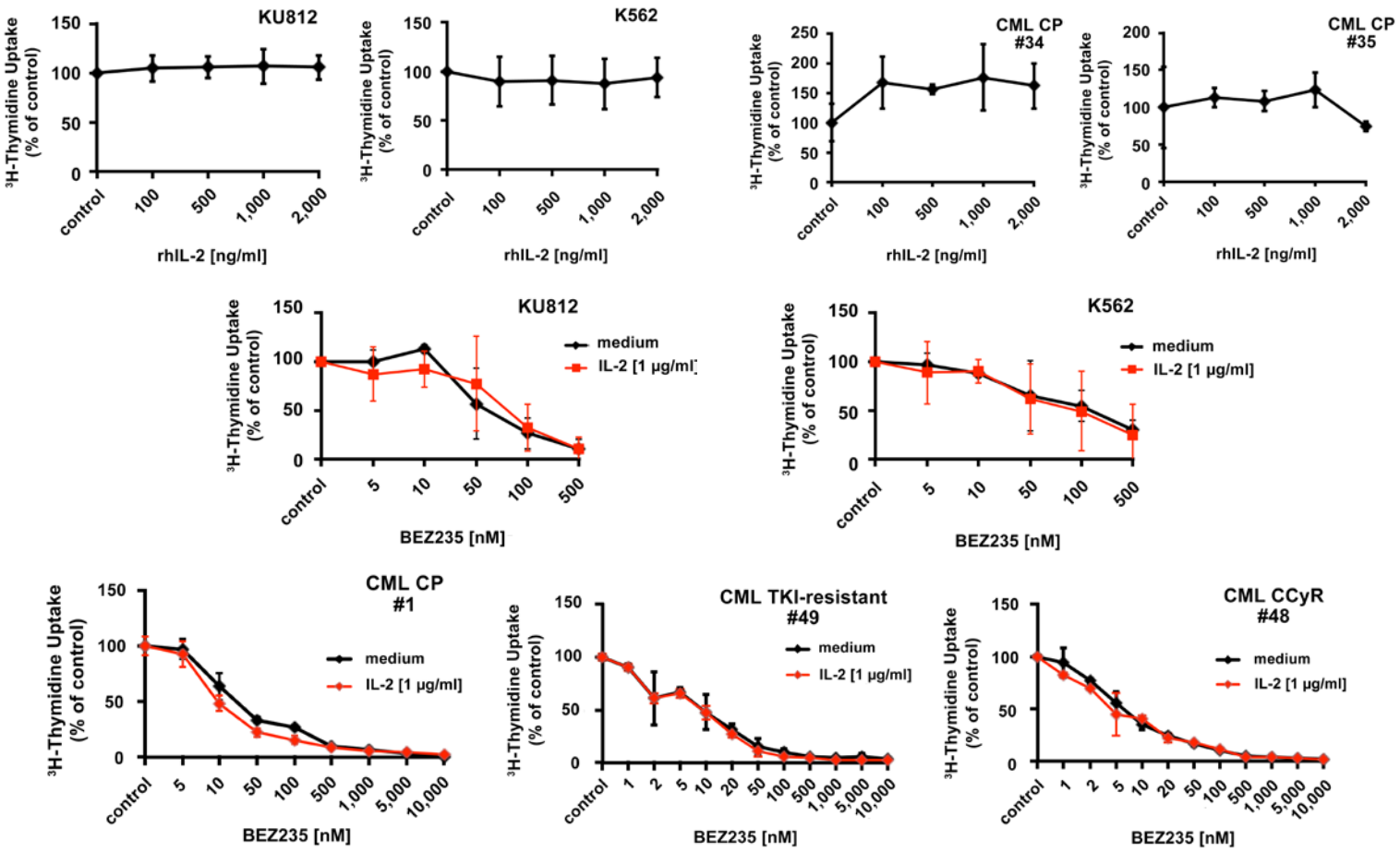
Supplementary Figure S6C



Effects of ponatinib on CD25 expression in Ba/F3 cells expressing various BCR/ABL1 mutants

Ba/F3 cells expressing various imatinib-resistant BCR/ABL1 mutants were incubated in control medium (control) or in medium containing ponatinib (10 nM or 100 nM) at 37°C for 24 hours. After incubation, cells were analyzed for CD25 expression by flow cytometry. Results are expressed as MFI of CD25 (percent of medium control) and represent the mean±SD from 3 independent experiments. Asterisk (*) indicates $p<0.05$ compared to untreated cells (control).

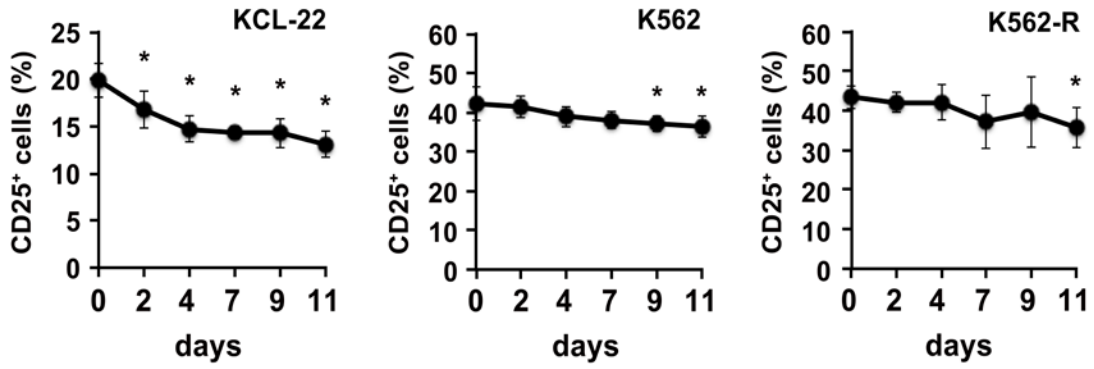
Supplementary Figure S7



Effects of interleukin-2 (IL-2) on growth of untreated and BEZ235-treated CML cells

Upper panels: KU812, K562 or mononuclear cells (MNCs) from two patients with chronic phase (CP) CML were incubated in control medium or in various concentrations of IL-2 (100-2,000 ng/ml) at 37°C for 48 hours. After incubation, ^{3}H-thymidine uptake was measured. Middle and lower panels: KU812 and K562 cells (middle panels) or MNCs from a patient with CP CML, from a TKI-resistant CML patient and from a CML patient with complete cytogenetic response (CCyR) (lower panels) were pre-incubated in control medium (black line) or medium supplemented with 1 µg/ml rhIL-2 (red line) at 37°C for 1 hour. After incubation, cells were washed and incubated in control medium or in various concentrations of BEZ235 at 37°C for 48 hours. Thereafter, ^{3}H-thymidine uptake was measured. Results are expressed as percent of control and represent the mean±SD from at least 3 independent experiments (KU812 and K562 cells) or represent the mean±SD from triplicates (CML MNCs). The patient-numbers (#) refer to Supplementary Table S2.

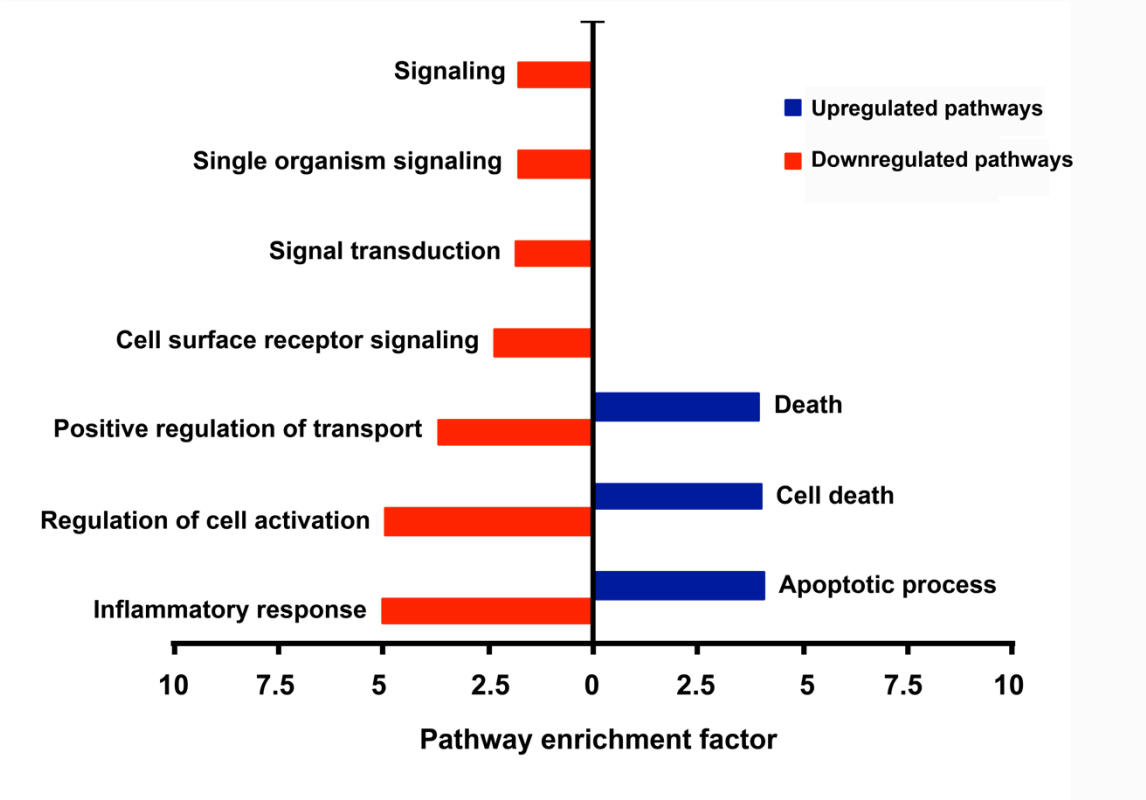
Supplementary Figure S8



Effect of transduced CD25 on growth of CML cells lines

KCL-22 cells (left panel), K562 cells (middle panel), or K562-R cells (right panel) were transduced with a LentiORF IL2RA (CD25) gene construct, and purified by cell sorting (GFP^+ cells). Then, CD25-transduced cells were mixed 1:1 with KU812 cells transduced with a control construct (empty vector) and cultured at 37°C. The percentage of $CD25^+$ cells in these cultures was analyzed 3 times a week by flow cytometry using an APC-conjugated mAb (BC96) directed against CD25. Results show the percent of $CD25^+$ cells and represent the mean±S.D. from 6 independent experiments. Asterisk (*): $p<0.05$ compared to percent $CD25^+$ cells measured on day 0.

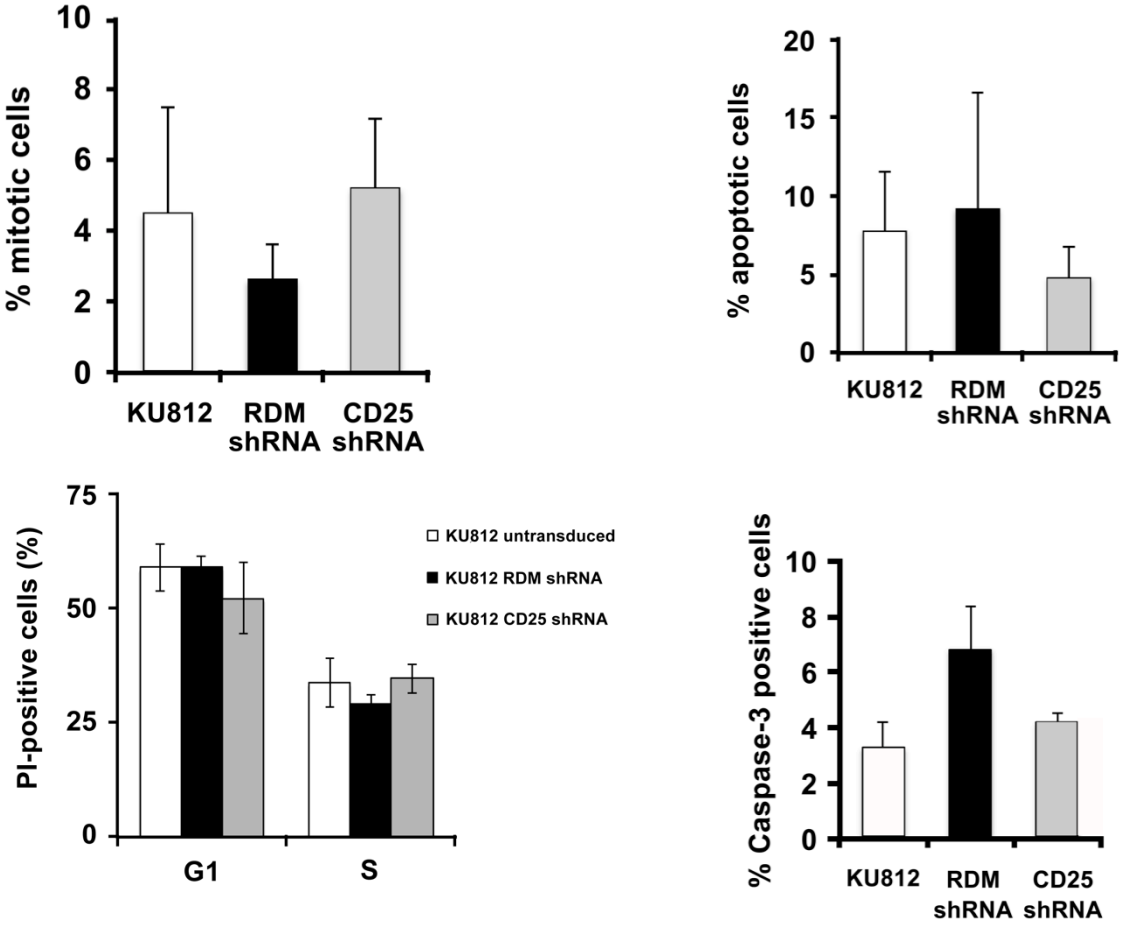
Supplementary Figure S9



Pathway analysis of KU812 cells transduced with a CD25 shRNA

KU812 cells were transduced with a shRNA against CD25 (clone #2) or a RDM control shRNA as described in the text of the main document. Transduced cells were subjected to RNA isolation and gene array analysis using Affymetrix technology to determine CD25 shRNA-induced upregulation and downregulation of genes. All genes that were at least two-fold (up- or down-) regulated by CD25 shRNA-transduction were applied to pathway enrichment analyses using the panther program (www.pantherdb.org). As visible, these analyses revealed a significant enrichment of upregulated genes (blue bars) and downregulated genes (red bars) in certain pathways related to growth, signal transduction, or cell death. The figure shows the pathway enrichment factor of significantly influenced pathways ($p<0.05$) determined by the PANTHER program and statistical evaluation after Bonferroni correction as described (15).

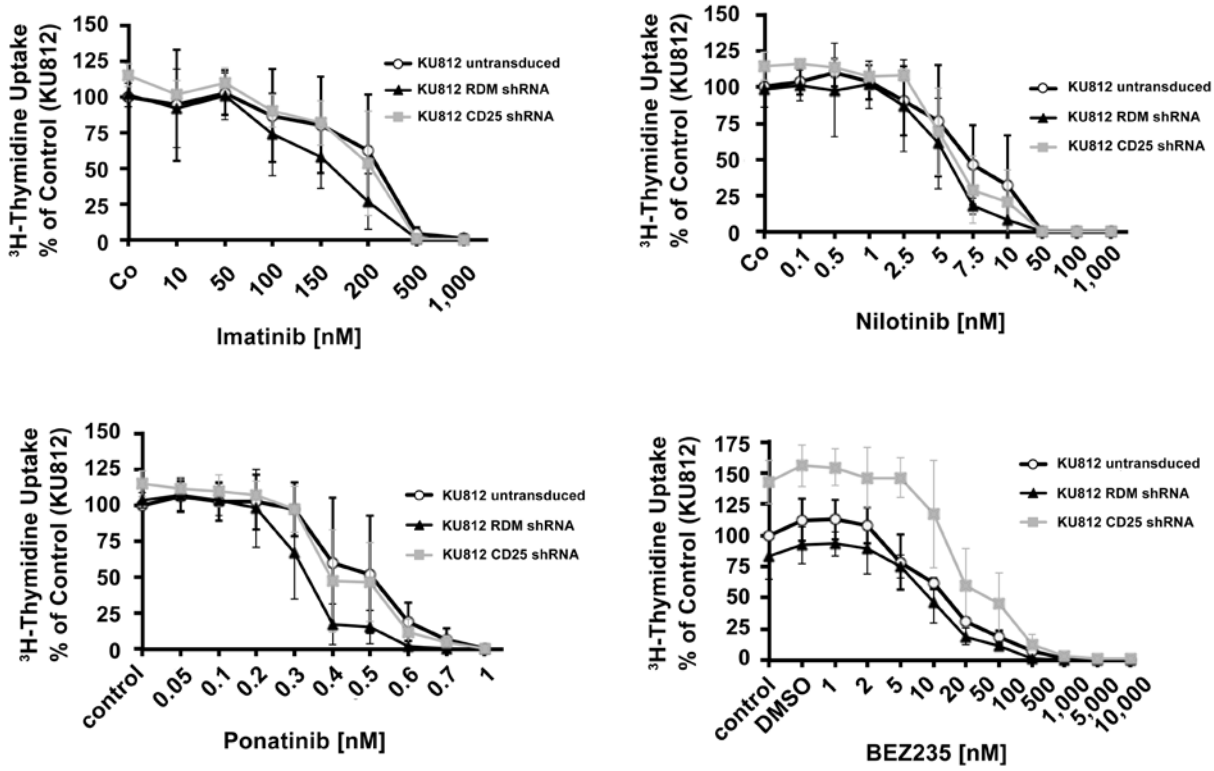
Supplementary Figure S10



Effects of shRNA-induced knock-down of CD25 on cell cycle progression and apoptosis in KU812 cells

Untransduced KU812 cells (open bars) and KU812 cells transduced with a random (RDM) shRNA (black bars) or CD25 shRNA (clone #2, gray bars) were spun on cytospin-slides and stained with Wright-Giemsa. Mitotic cells (upper left panel) and apoptotic cells (upper right panel) were counted on Wright-Giemsa-stained slides under an inverted microscope (Olympus, Tokyo, Japan). Results are expressed as percent of mitotic/apoptotic cells (of all cells) and represent the mean±SD from 3 independent experiments. Flow cytometric cell cycle anaylsis did not reveal a significant difference in the percentage of cells in G1 or S phase in untransduced KU812 cells and KU812 cells transduced with RDM shRNA or CD25 shRNA. Results are expressed as percent PI-positive cells and represent the mean±SD from 6 independent experiments (lower left panel). Apoptosis was also determined by flow cytometry, using an antibody against active caspase-3 (lower right panel). Results are expressed as percent caspase-3 positive cells and represent the mean±SD from 5 independent experiments.

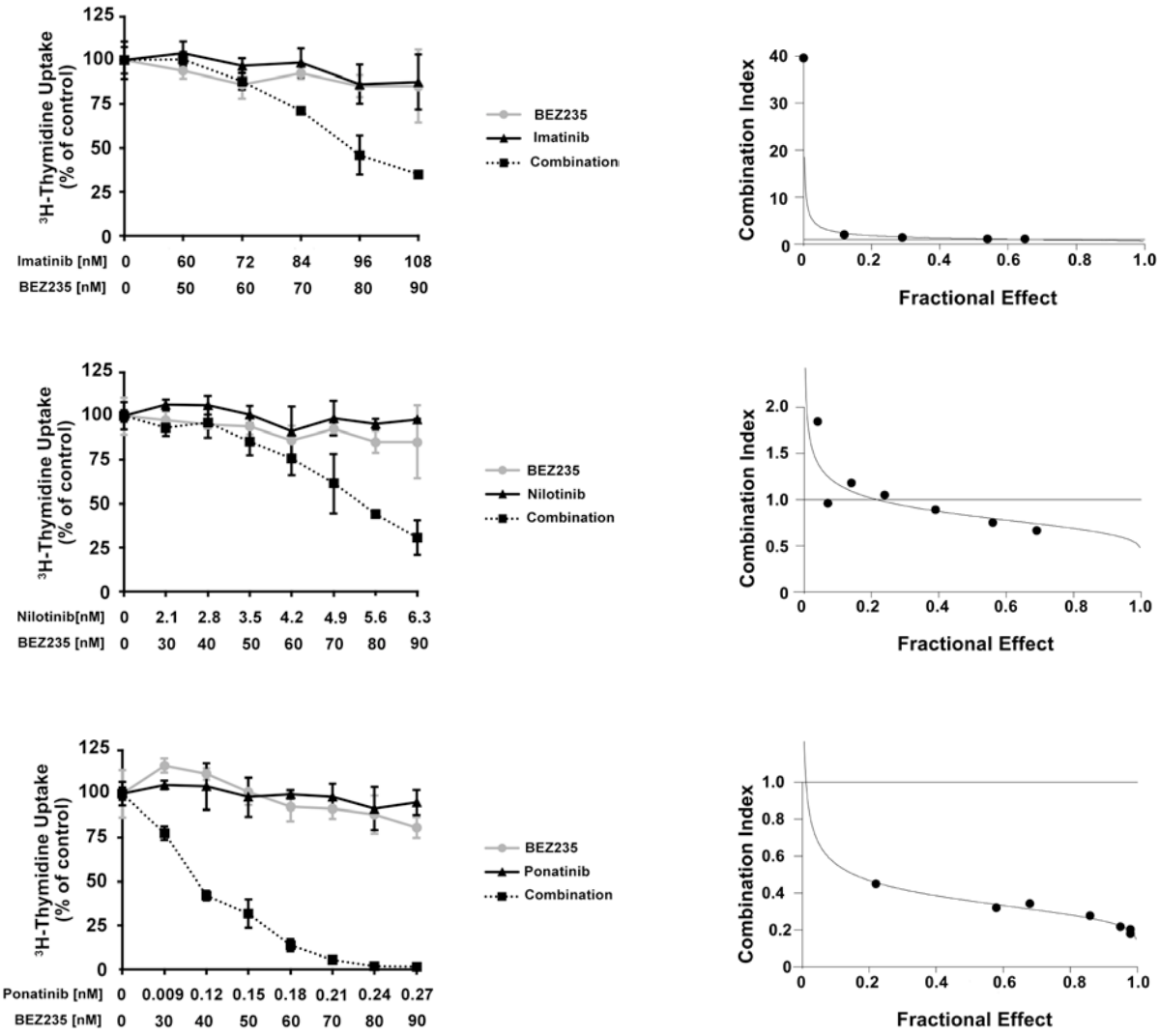
Supplementary Figure S11A



Effects of shRNA-induced knock-down of CD25 on the responsiveness of KU812 cells to imatinib, nilotinib, ponatinib and BEZ235

Untransduced KU812 cells (○-○) and KU812 cells transduced with a random control (RDM) shRNA (▲-▲) or a CD25 shRNA (clone #2, ■-■) were incubated in control medium (control) or in various concentrations of imatinib, nilotinib, ponatinib or BEZ235 at 37°C for 48 hours. In the lower right panel, the DMSO solvent control is also shown. After incubation, ^{3}H-thymidine uptake was measured. Results are expressed as percent of control (untransduced KU812 cells) and represent the mean±SD from 3 independent experiments.

Supplementary Figure S11B



The PI3K/mTOR blocker BEZ235 synergize with imatinib, nilotinib and ponatinib in inducing growth inhibition in KU812 cells

KU812 cells were incubated in control medium (control) or in various concentrations of BEZ235 alone or in combination with the BCR/ABL1 tyrosine kinase inhibitors imatinib (upper panel), nilotinib (middle panel) or ponatinib (lower panel) at fixed ratio of drug concentrations at 37°C for 48 hours. After incubation, ^{3}H-thymidine uptake was measured. Results are expressed as percent of control (0) and represent the mean±SD from triplicates. In each case, the combination index value (CI) was calculated by Calcusyn software (right panels). A CI of 1 indicates an additive effect, a CI below 1 a synergistic drug-interaction, and a CI greater than 1 an antagonistic effect.

References

1. Vardiman JW, Melo J, Baccarani M, Thiele J. Chronic myelogenous leukemia, BCR/ABL1 positive. In: Swerdlow SH, Campo E, Harris NL, et al, editors. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, 4th Edition. Lyon, France: IARC Press; 2008 p.32-7.

2. Sokal JE, Cox EB, Baccarani M, Tura S, Gomez GA, Robertson JE, et al. Prognostic discrimination in "good-risk" chronic granulocytic leukemia. Blood 1984;63:789-99.

3. Hasford J, Pfirrmann M, Hehlmann R, Allan NC, Baccarani M, Kluin-Nelemans JC, et al. A new prognostic score for survival of patients with chronic myeloid leukemia treated with interferon alfa. Writing Committee for the Collaborative CML Prognostic Factors Project Group. J Natl Cancer Inst 1998;90:850-8.

4. Hasford J, Baccarani M, Hoffmann V, Guilhot J, Saussele S, Rosti G, et al. Predicting complete cytogenetic response and subsequent progression-free survival in 2060 patients with CML on imatinib treatment: the EUTOS score. Blood 2011;118:686-92.

5. Baccarani M, Saglio G, Goldman J, Hochhaus A, Simonsson B, Appelbaum F, et al; European LeukemiaNet. Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. Blood 2006;108:1809-20.

6. Baccarani M, Cortes J, Pane F, Niederwieser D, Saglio G, Apperley J, et al; European LeukemiaNet. Chronic myeloid leukemia: an update of concepts and management recommendations of European LeukemiaNet. J Clin Oncol 2009;27:6041-51.

7. Martinelli G, Iacobucci I, Soverini S, Cilloni D, Saglio G, Pane F, et al. Monitoring minimal residual disease and controlling drug resistance in chronic myeloid leukaemia patients in treatment with imatinib as a guide to clinical management. Hematol Oncol 2006;24:196-204.

8. Hughes T, Deininger M, Hochhaus A, Branford S, Radich J, Kaeda J, et al. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. Blood 2006;108:28-37.

9. Müller MC, Cross NC, Erben P, Schenk T, Hanfstein B, Ernst T, et al. Harmonization of molecular monitoring of CML therapy in Europe. Leukemia 2009;23:1957-63.

10. Florian S, Sonneck K, Hauswirth AW, Krauth MT, Schernthaner GH, Sperr WR, et al. Detection of molecular targets on the surface of CD34+/CD38- stem cells in various myeloid malignancies. Leuk Lymphoma 2006;47:207-22.

11. Herrmann H, Cerny-Reiterer S, Gleixner KV, Blatt K, Herndlhofer S, Rabitsch W, et al. CD34(+)/CD38(-) stem cells in chronic myeloid leukemia express Siglec-3 (CD33) and are responsive to the CD33-targeting drug gemtuzumab/ozogamicin. Haematologica 2012;97:219-26.

12. Herrmann H, Sadovnik I, Cerny-Reiterer S, Rülicke T, Stefanzl G, Willmann M, et al. Dipeptidylpeptidase IV (CD26) defines leukemic stem cells (LSC) in chronic myeloid leukemia. Blood 2014;123:3951-62.

13. Sadovnik I, Lierman E, Peter B, Herrmann H, Suppan V, Stefanzl G, et al. Identification of Ponatinib as a potent inhibitor of growth, migration, and activation of neoplastic eosinophils carrying FIP1L1-PDGFRA. Exp Hematol 2014;42:282-93.e4.

14. Peter B, Cerny-Reiterer S, Hadzijusufovic E, Schuch K, Stefanzl G, Eisenwort G, et al. The pan-Bcl-2 blocker obatoclax promotes the expression of Puma, Noxa, and Bim mRNA and induces apoptosis in neoplastic mast cells. J Leukoc Biol 2014;9:95-104.

15. Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, Daverman R, et al. PANTHER: a library of protein families and subfamilies indexed by function. Genome Res 2003;13:2129-41.

16. Brothman AR, Persons DL, Shaffer LG. Nomenclature evolution: Changes in the ISCN from the 2005 to the 2009 edition. Cytogenet Genome Res 2009;127:1-4.