The vascular bone marrow niche influences outcome in chronic myeloid leukemia via the E-selectin - SCL/TAL1 - CD44 axis

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Supplementary Figure 1

A-D) Time of contact (seconds) of human unsorted CML cells from the peripheral blood or bone marrow of 4 different patients labelled with Cell Tracker Orange CMTMR and injected into vehicle- or GMI-1271 (20 mg/kg/dose) -treated unirradiated Rag-2/- CD47-/ IL-2 receptor γ-/ mice with the calvarial endothelium by in vivo microscopy. The P values are as indicated (t-test). The Rag-2/- CD47-/ IL-2 receptor γ-/ mice had been treated with vehicle or GMI-1271 2 hours before injection of cells.
**Supplementary Figure 2**

A) Treatment schedule for recipients of BCR-ABL1+ bone marrow treated with vehicle, 20 mg/kg GMI-1271, 100 mg/kg imatinib or the combination of GMI-1271 plus imatinib. Vehicle and imatinib were administered daily, while GMI-1271 was given twice daily. B-E) Leukocytes per μl (P = 0.0008, ANOVA; Tukey Test) (B) and percentage of GFP+ (BCR-ABL1+) CD11b+ myeloid cells in the peripheral blood on day 17 after transplantation (P = 0.031, ANOVA; Tukey Test) (C) and spleen weight in mg (P = 0.024, ANOVA; Tukey Test) (D) and percentage of GFP+ (BCR-ABL1+) Lin-cKit+ cells (P = 0.001, ANOVA; Tukey Test) (E) in the bone marrow of Balb/c recipients of BCR-ABL1-transduced BM treated with vehicle (black), GMI-1271 (dark gray), imatinib (light gray) or imatinib plus GMI-1271 (white) (n=20). F-H) Shortest three-dimensional distance to the endothelium (P = 0.002, ANOVA; Tukey Test, n=3) (F) or bone (G-H) in μm of GFP+ (BCR-ABL1+) Lin- (G) or BCR-ABL1+ BaF3 (F and H) cells injected into vehicle (timepoints: 0, 14 and 18 hours)-, imatinib (timepoints: 0, 14 and 18 hours)- or imatinib + GMI-1271 (timepoints for GMI-1271: -2, 0, 14 and 18 hours)-treated unirradiated Tie2GFP mice.
A) Number of adherent BCR-ABL1+ BaF3 cells plated on recombinant E-selectin (1 μg) in the presence of 20 μM GMI-1271 and 0.05 % BSA (black), GMI-1271 and 0.05 μg soluble E-selectin (gray) or GMI-1271 and 0.1 μg soluble E-selectin (white) (P = 0.04 and P = 0.021, respectively, ANOVA; Tukey Test, n=3). B) Relative expression of SCL/TAL1 in K562 cells plated on recombinant E-selectin in the presence of vehicle (black), 20 μM GMI-1271 (dark gray),10 μM imatinib (light gray) or imatinib plus GMI-1271 (white) (P = 0.007, ANOVA; Tukey Test , n=3). C-G) Relative expression of MYC, GATA1, SPI1 (PU.1), KLF1 or RUNX1 in K562 cells plated on recombinant E-selectin in the presence of vehicle (black), GMI-1271 (dark gray), imatinib (light gray) or imatinib plus GMI-1271 (white) (concentrations as above) (ANOVA; Tukey Test, n=3).
Supplementary Figure 4

A) Relative expression of Scl/Tal1 in BCR-ABL1+ BaF3 cells plated on recombinant E-selectin in the presence of GMI-1271 and 0.05% BSA (black), GMI-1271 and 0.05 μg soluble E-selectin (gray) or GMI-1271 and 0.1 μg soluble E-selectin (white) (P = 0.028, ANOVA; Tukey Test, n=4).
Supplementary Figure 5

A) Immunoblot of the expression of SCL/TAL1 (42 kDa) or lamin B1 (72 kDa) in K562 cells transduced with empty vector control or two different sh SCL/TAL1-expressing lentiviruses. The immunoblot is representative of 2 independent experiments. B) Western blot of the expression of SCL/TAL1 (42 kDa) or lamin B1 (72 kDa) in K562 cells transduced with empty vector control- or SCL/TAL1-overexpressing lentivirus. The immunoblot is representative of 2 independent experiments. C) Median fluorescence intensity (MFI) of CD44 on K562 cells transduced with empty vector control-or the SCL/TAL1-overexpressing lentivirus as in B) \( (P = 0.019; \text{t-test}) \). D) Relative expression of CD44 on CD34+ human cells before and after infection with the SCL/TAL1-overexpressing lentivirus \( (P = 0.0001; \text{t-test, } n=3) \). E) Cloning of a 700bp NheI - HindIII fragment from the human CD44 regulatory element containing SCL/TAL1 binding sites (underlined) into the pGL4.23 vector for use in the luciferase assays. SCL/TAL1 binding sites have the sequence CANNTG.
Supplementary Figure 6

A) Design of 4 primers binding to regions within the CD44 regulatory element on human chromosome 11, used in the ChIP assay. B) Non-binding of SCL/TAL1 to an irrelevant region on chromosome 18 in K562 cells treated with vehicle or imatinib as measured by a ChIP assay using an anti-SCL/TAL1 (white) or a control IgG (black) antibody and primers against chromosome 18.
A) Immunoblot of the expression of SCL/TAL1 (42 kDa), CD44 (82 kDa) or lamin B1 (72 kDa) in BCR-ABL1+ BaF3 cells cotransduced with empty vector control- or the Scl/Tal1-overexpressing lentivirus. The immunoblot is representative of 2 independent experiments. B) Southern blot showing distinct proviral integration sites and, consequently, disease clonality in spleens (taken at the time of death as shown in Figure 3F) of Balb/c recipients of bone marrow transduced with BCR-ABL1 alone (lanes 1-3), BCR-ABL1 plus empty vector control- (lanes 4-10) or BCR-ABL1 plus Scl/Tal1-overexpressing lentivirus (lanes 11-17). The difference in disease clonality as a measure of engraftment of leukemia-initiating clones between BCR-ABL1 plus empty vector control- versus BCR-ABL1 plus Scl/Tal1-overexpressing lentivirus is not statistically significant, but shows a trend towards reduced clonality in recipients of bone marrow transduced with BCR-ABL1 and Scl/Tal1-overexpressing lentivirus. C) Time of contact (seconds) of BCR-ABL1+ BaF3 cells labelled with CMTMR and injected into vehicle- or GMI-1271 (20 mg/kg)-treated unirradiated Tie2-GFP mice with the calvarial endothelium by in vivo microscopy ($P = 0.05$, t-test, n=2). The mice were treated 2 hours before injection.
**Supplementary Figure 8**

A) Gating strategy for the analysis of cell cycling with ki-67 and DAPI in GFP+ (BCR-ABL1+) BaF3 and GFP- (BCR-ABL1-) BaF3 cells overexpressing CD44. B-C) Cell cycle analysis by ki-67-staining of serum-starved BaF3 cells coexpressing BCR-ABL1 and CD44 (ANOVA; Tukey Test, n=3) (B) or BaF3 cells expressing BCR-ABL1 (ANOVA; Tukey Test, n=3) (C) plated on 1% bovine serum albumin (BSA)-coated plates and treated with vehicle, 20 μM GMI-1271, 10 μM imatinib or the combination of GMI-1271 plus imatinib. The cells were starved at 37°C. D-E) CDK4 expression (D) and corrected total cell fluorescence (CTCF) for CDK4 (t-test, n=3) (E) by immunofluorescence in GFP+ (BCR-ABL1+) BaF3 cells plated on E-selectin in the adhesion assay, performed in the presence of vehicle or GMI-1271. F-G) Corrected total cell fluorescence (CTCF) for SCL/TAL1 (P = 0.04, t-test, n=2) (F) and p16 (P = 0.002, t-test, n=2) (G) on Lin- cells from Balb/c recipients of BCR-ABL1-transduced BM treated with vehicle (black) or GMI-1271 (white). H) Median fluorescence intensity (MFI) of CD44 on different GFP- (BCR-ABL1-) or GFP+ (BCR-ABL1+) cell populations from FVB mice transplanted with BCR-ABL1-transduced bone marrow on day 17-18 after transplantation. The P values are as indicated (ANOVA; Tukey Test, n=7).
Supplementary Figure 9

A) Histogram showing the expression of CD44 on GFP⁺ (BCR-ABL⁺) BaF3 cells treated with 10μM imatinib for 6 hours. The data is representative of 3 independent experiments. B) Median fluorescence intensity (MFI) of CD44 on GFP⁺ (BCR-ABL⁺) BaF3 treated with imatinib at increasing concentrations for 4 hours ($P = 0.015$, ANOVA; Tukey Test, n=3). C) Immunoblot showing the expression of SCL/TAL1, pSCL/TAL1 T90 or vinculin in the non-adherent versus adherent fractions of BCR-ABL⁺ BaF3 cells plated on recombinant E-selectin in the presence of vehicle, GMI-1271, imatinib or imatinib + GMI-1271. The cells had been allowed to adhere for 6 hours at 37°C.
Supplementary Figure 10

A) Histogram showing the expression of CD44 on BaF3 cells transduced with BCR-ABL1- or BCR-ABL1 T315I-expressing retrovirus. The data are representative of 3 independent experiments. B) Median fluorescence intensity (MFI) of CD44 on BaF3 cells transduced with BCR-ABL1-, BCR-ABL1 T315I-, BCR-ABL1 E255K- or BCR-ABL1 M351T-expressing retrovirus ($P = 0.034$, ANOVA; Tukey Test, $n=3$). C) Number of adherent BCR-ABL1+ or BCR-ABL1 T315I+ BaF3 cells 6 hours after plating on recombinant E-selectin ($P = 0.011$, t-test, $n=3$). 70,000 cells had been plated and allowed to adhere at 37°C. D) Cell cycle analysis by ki-67-staining of serum-starved, non-adherent versus adherent BaF3 cells expressing BCR-ABL1 (black) or BCR-ABL1 T315I (white) plated on recombinant E-selectin. A greater number of adherent BCR-ABL1 T315I+ cells were found in the G0 phase of the cell cycle ($P = 0.042$, ANOVA; Tukey Test, $n=3$). The data were normalized to BCR-ABL1+ BaF3 adherent cells in the G0 phase. The cells were starved and 70,000 cells had been plated and allowed to adhere for 6 hours at 37°C.
Supplementary Figure 11

A) Annotated MS/MS spectrum of the pS122-bearing peptide "oxMVQLpSPPALAAPAAPGR" on SCL/TAL1. Matched band y-ions are color-coded with blue and red, respectively. Loss of water and ammonia are in orange. Fragments with neutral loss of a phosphate group are marked with stars.
Supplementary Figure 12

Schematic demonstrating the effects of inhibition of E-selectin in the bone marrow microenvironment on chronic myeloid leukemia stem cells via modulation of the SCL/TAL1 – CD44 axis.
**Supplementary Table 1: List of primers used**

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Scl = Stem cell leukemia gene  
Tal1 = T-cell acute lymphocytic leukemia protein 1  
Runx = Runt-related transcription factor  
KLF = Krueppel-like factor  
CDK = Cyclin-dependent kinase  
GAPDH = glyceraldehyde phosphate dehydrogenase  
GUS = beta-glucuronidase
Supplementary movie

Representative movie recorded by 2-photon in vivo microscopy of the bone marrow calvarium of a non-irradiated Tie2-GFP mouse injected with $10^6$ BCR-ABL1+ BaF3 cells, labelled with CMTMR (orange). Bones are visualized in blue due to second harmonic generation. The movie was taken for 10 minutes at 10 frames/second.
**Supplementary methods**

*Bone marrow transduction and transplantation*

Generation of MSCV-IRES GFP BCR-ABL1 retrovirus and transplantation were performed as described (1). Briefly, donor BM cells were harvested from 5-fluorouracil (5-FU)-treated Balb/c (or FVB in figures 1E and S8H) mice, transduced twice with cryopreserved retrovirus expressing MSCV-IRES GFP BCR-ABL1 and transplanted into sublethally irradiated recipient Balb/c (or FVB) mice (750 cGy) at a dose of 3 x 10⁵ cells i.v.

In the homing assay, 2.5×10⁶ 5-FU–treated BM cells, transduced with BCR-ABL1 were intravenously injected into Balb/c mice treated with vehicle or GMI-1271. 18 h later BM and spleen of the recipient mice were analysed for GFP⁺ LKS cells.

For the Scl/Tal1 overexpression experiment, 2.5×10⁵ 5-FU–treated BM cells, transduced with MSCV IRES RFP BCR-ABL1 retrovirus alone or double transduced with MSCV IRES RFP BCR-ABL1 retrovirus and SiEW empty vector or Scl/Tal1 overexpressing lentivirus (SiEW vector), were transplanted into sublethally irradiated recipient Balb/c mice (750 cGy). The SCL/Tal1-overexpressing SiEW vector was a kind gift from Joern Lausen (2), and lentivirus was generated via transfection of 293T cells with the SCL/Tal1-overexpressing SiEW vector, Delta 8.9 and VSV, as described (1).

**Drug treatment**

Mice were treated twice a day with vehicle (saline) or GMI-1271 (GlycoMimetics Inc., Maryland, USA) by intraperitoneal injection at 20 mg/kg/dose from day 10 to 30 after transplantation. Concomitantly, imatinib was administered via oral gavage once a day at 100 mg/kg (Enzo Life Sciences, ALX-270-492, Farmingdale, NY) from day 10 to 30 after transplantation.

For *in vitro* assays BCR-ABL1⁺ BaF3 or K562 cells were treated for 8 hours with different compounds that inhibit signaling pathways downstream of BCR-ABL1 at the following
concentrations: Imatinib (Enzo Life Sciences, ALX-270-492, Farmingdale, NY) at 10μM (3, 4), wortmannin (PI3K inhibitor, Selleckchem, S2758) at 20μM (5) and MK-2206 (inhibitor of AKT1/2/3, Selleckchem, S1078) at 20μM (6). *In vitro* GMI-1271 was used at 20μM (7).

Analysis of diseased mice and tumor burden

The leukemic burden in diseased animals was assessed by weekly analysis of peripheral blood by a Scil Vet animal blood counter (Scil Animal Care Company, Viernheim, Germany) and by staining leukocytes from peripheral blood with a phycoerythrin (PE)-conjugated antibody to CD11b (myeloid cells) (clone M1/70, Biolegend, San Diego, CA) and analysis by GFP expression (leukemic cells are CD11b+ GFP+). Spleens were weighed at death. Leukemia-initiating cells were analyzed by staining for GFP, c-Kit and lineage (B220, CD5, Gr-1 and Ter119 cocktail) (BD Biosciences, Franklin lakes, NJ).

Cell lines

K562 and BaF3 cells were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures and later also authenticated there. BaF3 cells were transduced with MSCV IRES GFP (empty vector)-, MSCV IRES GFP BCR-ABL1-, MSCV IRES GFP BCR-ABL1T315I-, MSCV IRES GFP BCR-ABL1E255K- or MSCV IRES GFP BCR-ABL1M351T-expressing retrovirus. All constructs were kind gifts from Richard A. Van Etten.

Southern blotting

Genomic DNA from spleen was digested with the restriction enzyme BglIII, run on an agarose gel and transferred onto a nylon membrane. The blot was then hybridized with a radioactively labeled probe targeting the GFP gene, in order to detect distinct proviral integration sites in each sample, as described (1, 8). The blot hybridized with an RFP probe was generated by the
non-radioactive Digoxigenin11dUTP (DIG) method according to the manufacturer’s instructions (Roche, Mannheim, Germany).

**Adhesion assay**

48-well plates were coated with recombinant murine E-selectin–IgG1 Fc fusion protein (575-ES, R&D Systems, Minneapolis) (1µg in 100 µl) or 1% BSA as control, as described (9), for 16 hours at 4°C in 20 mM TBS (pH 7.6) with 1 mM CaCl₂. Unbound selectin was removed, wells were blocked for 1 hour at room temperature with 1% BSA and washed 4 times with Iscove modified Dulbecco medium with 1% bovine serum albumin (BSA). 20,000 murine Lin⁻ c-Kit⁺ BCR-ABL1⁺ cells from diseased mice in triplicate or 50,000 human CML cells (4 from peripheral blood, 1 from bone marrow) were added to control or E-selectin-coated wells and treated with vehicle, imatinib (10 µM) (3, 4), GMI-1271 (20 µM) (7) or imatinib plus GMI-1271. After incubation at 4°C for 6 hours the plates were washed 4 times and adherent cells were counted. For the adhesion experiments with BaF3 cells BCR-ABL1⁺ BaF3, BaF3 transduced with empty vector, BCR-ABL1³¹⁵⁺ or BCR-ABL1⁺ BaF3 cells overexpressing CD44 were serum-starved for 12 hours at 37°C and added in duplicate to E-selectin-coated or control plates. The four drugs were then added to the cells, before the plates were carefully centrifuged and left at 37°C for 6 hours. After incubation, non-adherent versus adherent cells were harvested and stained for CD44 (103011, Biolegend, San Diego). Non-adherent cells were removed by pipetting off the supernatant, whereas adherent cells were removed after three wash steps.

Cell cycle analysis was performed or protein lysates generated after allowing BCR-ABL1⁺ BaF3 to adhere to E-selectin-coated wells (as above) for 6 hours at 37°C in the presence of vehicle, imatinib (10 µM), GMI-1271 (20 µM) or imatinib plus GMI-1271.
In order to investigate the expression of genes involved in the cell cycle status of BM cells, all cells from each well were harvested after the adhesion assay and RNA was prepared. Genes and primers used are listed in supplementary table 1.

For the “soluble E-selectin experiment”, BCR-ABL1+ BaF3 were added to E-selectin-coated 48-well plates, before the plates were centrifuged at low speed and kept in an incubator at 37 °C for one hour. Thereafter, 0.05% BSA, 0.05 or 0.1 μg E-selectin plus GMI-1271 (20 μM) were added to the cells. Consequently, the plate was incubated at 37 °C for 6 hours and then washed 4 times. Adherent cells were counted, while non-adherent cells were removed for RNA preparation.

**Immunofluorescence studies**

Non-adherent cells from the adhesion assay, as described above, were harvested and cytopspun onto poly-L-lysine-coated slides. The cells were fixed with 4 % paraformaldehyde for 20 minutes at room temperature and permeabilized with 0.25 % Triton X-100 for 10 minutes. The cells were then blocked with 2% BSA and incubated with a primary antibody to CDK4 (sc-23896, Santa Cruz Biotechnology, Dallas), CDK6 (sc-796, Santa Cruz Biotechnology, Dallas), SCL/TAL1 (ab236520, Abcam, Berlin) or p16 (10883-1-AP, Proteintech UK) at a 1:50 dilution overnight at 4 °C. Cells were washed and incubated with the secondary antibody Alexa Fluor 488/ Alexa Fluor 555 (Invitrogen, Grand Island, NY) at a 1 : 500 dilution for one hour at room temperature. The slides were mounted using mounting media with DAPCO (290734, Sigma, Taufkirchen, Germany) and images were taken on a Confocal Laser Scanning microscope (CSLM). Analyses were performed using ImageJ. The corrected total cell fluorescence (CTCF) was calculated by measuring the integrated density of the nucleus for SCL/TAL1, p16, CDK4 and CDK6 by using the formula CTCF = Integrated Density – (Area of selected cell x mean fluorescence of background readings).
Flow cytometric analysis of cell cycle status

Analysis of cell cycle status was performed by Ki67 staining (652403, Biolegend, San Diego) according to the manufacturer’s instructions. For Ki67 staining non-adherent versus adherent cells from the adhesion assay, as described above, or bone marrow cells of primary mice (FVB) with CML were harvested, fixed and stained overnight with Ki67 staining (652403, Biolegend, San Diego) according to the manufacturer’s instructions and DAPI was incorporated for 15 mins. The stained cells were acquired on a BD LSR Fortessa, BD Biosciences, Franklin Lakes, NJ).

Western blotting

After treatment of cell lines in vitro (concentrations as found under ‘Drug treatment’), the cells were harvested and washed 2 times with cold PBS. The pellet was then resuspended in buffer A (10mM HEPES, 1.5 mM MgCl₂, 10mM KCl, 0.05% NP40, 0.5 mM DTT, protease and phosphatase inhibitors) and incubated on ice for 10 min. After fractionation the cells were centrifuged at 4°C at 14000 rpm for 10 min. The collected supernatant represents the cytosolic fraction. The pellet was resuspended in buffer B (5mM HEPES, 1.5 mM MgCl₂, 0.2 mM EDTA, 26% Glycerol, 0.5mM DTT, 300mM NaCl, protease and phosphatase inhibitors), sonicated and incubated on ice for 30 min. After centrifugation, the collected supernatant corresponded to the nuclear fraction and was used for further analysis.

Lysates were prepared in RIPA buffer (50 mM Tris HCL, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% NaDOC, 0.1% SDS and 1 mM EDTA, 50 mM NaF, 1 mM Na3VO4, aprotinin and PMSF). Protein concentrations of RIPA lysates or nuclear fractions were measured using the Bradford protein assay (Bio-Rad, Hercules, CA). Equal concentrations of samples were mixed with the SDS running buffer Roti-load 4X (K929.1, Carl Roth, Karlsruhe, Germany), boiled at 95°C for 5 minutes and loaded. The proteins were then blotted onto a nitrocellulose membrane (88018, Thermo Fisher Scientific, Darmstadt) or PVDF membrane (88518, Thermo Fisher
Scientific, Darmstadt) and probed with antibodies to SCL/TAL1 (ab236520, Abcam), SCL/TAL1 (sc12984, Santa Cruz Biotechnology, Dallas), p-TAL1 S122 (ab138646, Abcam, Cambridge, UK), pSCL/TAL1 T90 (SA0807221, Aviva Systems Biology, San Diego), AKT (9272, Cell Signaling, Danvers), pAKT Ser473 (9271, Cell Signaling, Danvers), CD44 (PA5-21419, Invitrogen, Darmstadt), Vinculin (VLN01, Thermo Fisher Scientific, Darmstadt) or the nuclear membrane marker Lamin B1 (ab16048, Abcam). 5% milk powder in TBST or 1X Roti block (A151, Carl Roth, Karlsruhe, Germany) was used for blocking. Anti-Rabbit HRP (70748, Cell Signaling, Danvers) and anti-goat HRP (ab97110, Abcam) were used as secondary antibodies.

**ChIP Assay**

After culture cells were cross-linked with formaldehyde and the reaction was quenched using glycine. After washing in cold PBS, the pellet was resuspended in ChIP lysis buffer. After 1 hour of lysis at 4°C lysates were sonicated to shear DNA to an average fragment length of 500-1000 bp. The cell debris was pelleted and the supernatant was harvested, aliquoted in new tubes, snap-frozen in liquid nitrogen and finally stored at -80°C for further analysis. For immunoprecipitation blocked protein G beads, ChIP lysates and an SCL/TAL1 antibody (Origene, TA590662, Rockville) (7.5 μg) were incubated overnight at 4°C. A rabbit IgG antibody was used as a control (sc-2027, Santa Cruz Biotechnology, Dallas). After several washing steps, DNA was eluted and proteinase K added. Finally, the ChIP DNA was purified using the ChIP DNA Clean and Concentrator kit (Zymo Research, USA, Irvine). The DNA was eluted with 40 μl and qPCR analysis was performed using 2 μl of the chromatin-containing solution with four different primer sets specific for four different regions where SCL/TAL1 binds on the CD44 regulatory element (primers are listed in supplementary table 1). DNA recovery was calculated as percent of the input.
**Luciferase assay**

The human CD44 regulatory element, a 700 bp fragment containing SCL/TAL1 binding sites, was cloned into the pGL4.23 vector via NheI and HindIII restriction sites. K562 cells or K562 cells transduced with lentivirus overexpressing SCL/TAL1 or expressing shSCL/TAL1 were transfected with the reporter plasmids and a β-galactosidase expressing vector using Lipofectamine 2000 (Thermo Fisher Scientific, Darmstadt). The transfected cells were incubated at 37°C and 48 h after transfection the luciferase and β-galactosidase activities were analysed by using a luciferase assay system (E1500, Promega, Mannheim) and a β-galactosidase assay kit (75707, Thermo Fisher Scientific, Darmstadt, Germany). To control for transfection efficiency the firefly luciferase activity was normalized to β-galactosidase.

**Immunoprecipitation**

The K562 cells treated with imatinib or the AKT inhibitor were resuspended in RIPA buffer (50mM Tris-HCL (pH-8.0), 150 mM NaCl, 2 mM EDTA (pH-8.0), 1% NP-40, 0.5% sodium deoxycholate) and incubated on ice for 1 hour followed by centrifugation at 14,000 x g at 4°C for 20 minutes. 10 μl of the supernatant was used as control input. The supernatant was probed with Protein G Dynabeads (10003D, Thermo Fisher Scientific, Darmstadt, Germany) and an anti-SCL/TAL1 antibody (TA590662, Origene, Rockville, MD) and incubated at 4°C overnight. The beads were then washed with RIPA buffer and PBST on a magnetic stand. The final lysate was probed with 4x SDS loading dye and incubated for 5 minutes at 95°C. The input and final lysate were analyzed by Western blotting.

**Mass spectrometry**

Quantitative mass spectrometry was performed by using triple SILAC-labeling (SILAC medium, Thermo Scientific, Rockford, USA). Light-, medium- or heavy-labeled K562 cells were used as a DMSO-treated control or after a 2-hour or 4-hour inhibition with 10μM imatinib
or 20µM MK-2206, respectively. The cells were then harvested, immunoprecipitation was performed with an anti-SCL/TAL1 antibody and the lysates were analyzed by mass spectrometry, as described (10).

**In vivo microscopy**

Unirradiated Tie2-GFP reporter mice (GFP expression is under the control of the Tie2 promoter labeling endothelial cells) treated with vehicle or GMI-1271 as above were injected with 200,000 BaF3 cells transduced with MSCV IRES GFP BCR-ABL1- or MSCV IRES CD44 BCR-ABL1- expressing retrovirus and labelled with the Cell Tracker orange CMTMR dye (C2927, Thermo Fisher Scientific, Darmstadt) for 30 minutes at 37° C. The mice were anesthetized by xylene/ketamine and the scalp removed, as described (11). The calvarium was visualized under a 2-photon microscope (LaVision Biotech, Bielefeld) using a 40x objective in a 37° C chamber. Movies were recorded at 1 frame / second using the Inspector Pro software. Each video was taken for a minimum of 15 minutes. The videos were analyzed frame by frame by measuring the time of contact of cells with the GFP⁺ endothelial cells. The shortest three-dimensional distance of BaF3 cells transduced with MSCV IRES GFP BCR-ABL1 or of Lin-GFP⁺ (BCR-ABL1⁺) cells from FVB mice with established CML (days 15-17 after transplantation) to Tie2-GFP⁺ endothelial cells in Tie2-GFP mice (on an FVB background) or the endosteum was measured by ImageJ, as described (11). Recipient Tie2-GFP mice were treated with 20mg/kg GMI-1271 i.p. 2 hours before transplantation and at time points 0, 14 and 18 hours after transplantation, while imatinib was administered at time points 0 (100 mg/kg), 14 (100 mg/kg) und 18 (50 mg/kg) hours after transplantation of leukemia cells. The endosteum can be imaged via second harmonic signal derived from collagen in bone (11).

200,000-500,000 unsorted peripheral blood or bone marrow cells (4 from peripheral blood, 1 from bone marrow) from untreated CML patients were labeled with CMTMR dye and injected into non-irradiated Rag-2 -/- interleukin-2 receptor γ gamma -/- CD47 -/- knockout mice (cat.
no. 025730, The Jackson Laboratory, Bar Harbor, Maine; C57/Bl6 background) treated with GMI-1271 or vehicle. Dextran FITC (46945, Sigma, Taufkirchen, Germany) was injected at 1 mg per mouse. After 2 hours of injection, the injected cells were visualized by 2-photon microscopy and recordings were performed as described above. The videos were analyzed frame by frame by measuring the time of contact of cells with the endothelium marked by Dextran FITC. The included supplementary movie has been compressed to 10 frames/second.

Human cells

Human CD34+ cells were isolated from healthy, allogeneic bone marrow donors by bone marrow aspiration (Permit #329-10 from the local ethics committee). The cells were immunomagnetically enriched according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) and expanded using Stem Span (SFemi, Stemcell Technologies, Cologne, Germany).

Human, unsorted CML cells from untreated patients for the in vivo microscopy experiments and the adhesion assay were obtained from peripheral blood (4 patients) or bone marrow (1 patient). These samples were provided by the University Clinic Mannheim and their use was approved by the ethical committee II of Heidelberg University (Mannheim Faculty) (protocol approval number: 0214.6/2002). The human CML samples (from PB) for the experiments in figure 7B originated from the "Centre de Ressources Biologiques" of the Hospices Civils de Lyon, Pierre Bénite, France. Each patient had signed an informed consent, generated and approved by the legal local ethical committee of the hospices civils de Lyon, Lyon, France.

Gene expression profiling data and statistical analysis of CML patients

To analyze expression of SCL/TAL1 and CD44 in CML patient samples we examined two published data sets (12, 13). The first data set (13) included 90 samples from patients in various
phases of CML (42 chronic phase, 17 accelerated phase and 31 blast crisis samples), and also included CD34+ sorted samples from 5 healthy individuals (Rosetta Inpharmatics DNA microarray platform). The log10 ratio of expression of each individual patient sample is shown relative to expression in a pool of CP samples. Among 42 CP CML patient samples, 37 were drawn prior to allogeneic transplantation and had available outcomes to assess the impact of SCL/TAL1 expression on outcomes after allogeneic transplantation. Patients had not been treated with tyrosine kinase inhibitor therapy prior to transplantation. For the analysis of relapse and mortality data, three outcomes were examined; death (n = 13), relapse (n = 10), and a combination of death or relapse to assess relapse free survival (n = 20), calculating time in study by subtracting date of transplant from date of death, date of relapse, or date of relapse or death, respectively. For patients without event, time was calculated as the number of days between transplant and last contact. We estimated hazard ratios (HRs) and 95% confidence intervals (CIs) associated with each outcome using Cox proportional hazards regression models. We modeled SCL/TAL1 expression levels by splitting into high and low groups at the third quartile, and also as a continuous measurement. HRs and CIs reported for continuous SCL/TAL1 represent a 0.25 increase in SCL/TAL1 expression levels. We created Kaplan-Meier curves and used the Log-rank test to detect a difference between high and low SCL/TAL1 expression groups, splitting at the third quartile.

The second data set (12) included 59 available CD34+ sorted samples from CP CML patients prior to treatment with imatinib mesylate (HG-U133 Plus 2.0 GeneChip arrays, Affymetrix). For these analyses the average expression of multiple probe sets for SCL/TAL1 (1561651_s_at, 206283_s_at, 216925_s_at) and CD44 (1557905_s_at, 1565868_at, 204489_s_at, 204490_s_at, 209835_x_at, 210916_s_at, 212014_x_at, 212063_at, 217523_at, 229221_at) were used.
RNA was extracted from leukocytes from peripheral blood of patients with CML and healthy individuals and qPCR for SCL/TAL1 and CD44 was performed. Consequently, delta Ct values for SCL/TAL1 and CD44 were compared.

Chronic and blast crisis CML samples were obtained from the Fred Hutchinson Cancer Research Center from Institutional Review Board approved protocols with written informed consent in accordance with the Declaration of Helsinki (IR files: 3910, 4874, 5309, 5152).

References
