

Supplementary Information

Stat5 is indispensable for *bcr/abl*-positive leukaemia maintenance

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Supplementary Figure 1

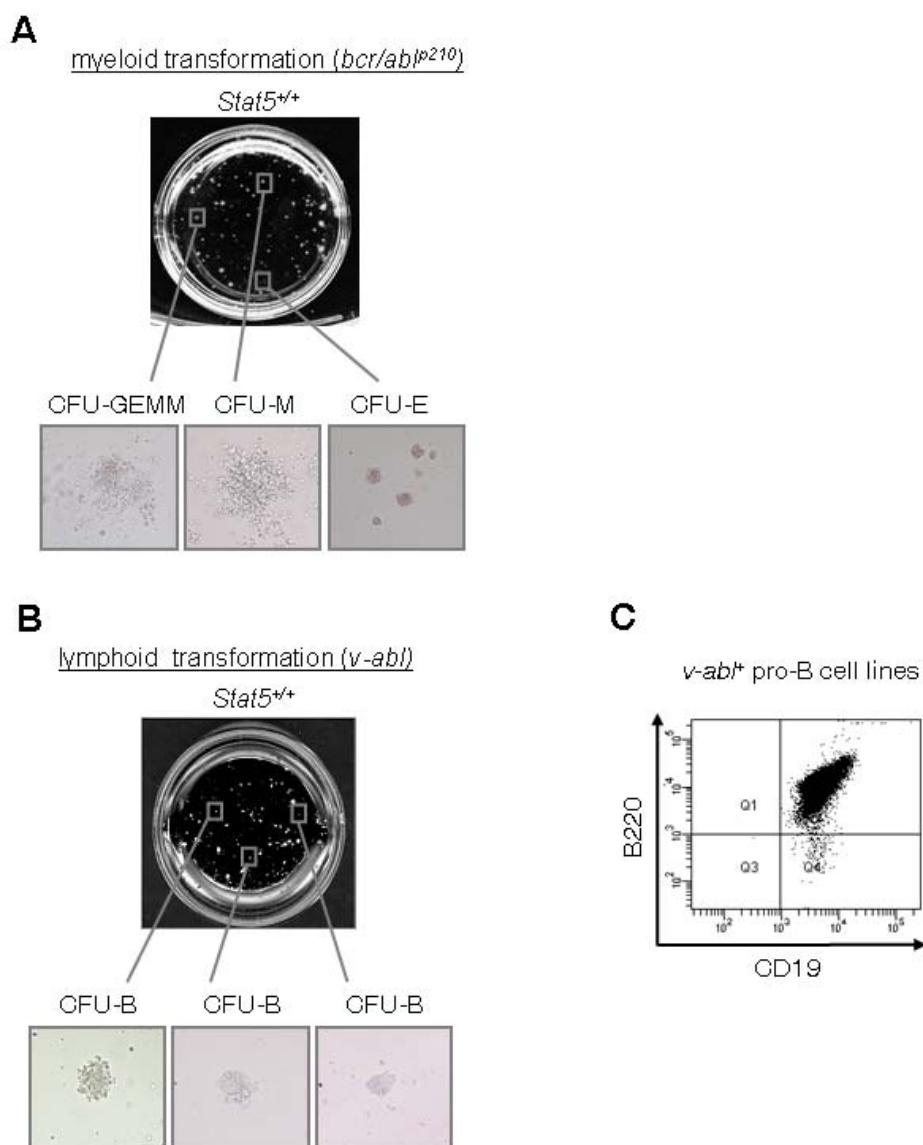


Figure S1. Morphology of colony-forming-units (CFUs) induced by *bcr/abl*^{p210} and *v-abl*.

(A) Transformation by *bcr/abl*^{p210} results in myeloid transformation as indicated by the outgrowth of CFU-GEMM (granulocyte-erythrocyte-macrophage-megakaryocyte), CFU-M (macrophage) and CFU-E (erythroid). No outgrowth of growth-factor-independent cell lines was observed. (B) Transformation by *v-abl* results in lymphoid transformation as indicated by the outgrowth of CFU-B (B-cell). In contrast to myeloid clones, lymphoid clones give rise to stable growth-factor independent pro-B cell lines (CD19⁺ B220⁺) *in vitro*. (C) One representative flow cytometric profile out of 10 picked clones is depicted.

Supplementary Figure 2

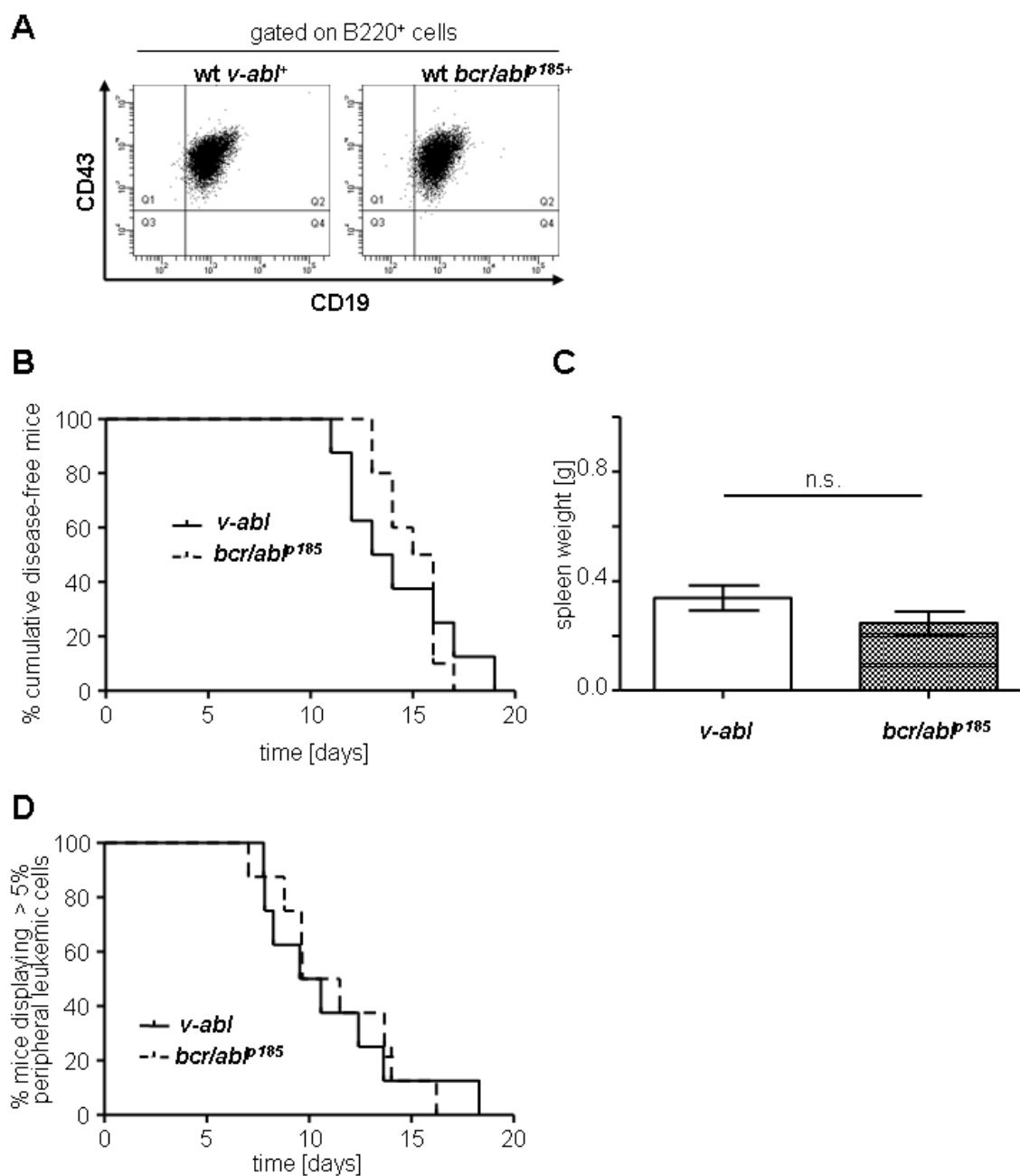


Figure S2. Comparability of $v-abl^+$ and bcr/abl^{p185+} lymphoid leukaemia *in vivo*.

(A) $V-abl^+$ and bcr/abl^{p185+} cell lines share comparable pro-B cell surface marker expression (CD43, CD19, B220). (B) 1×10^6 cells of either $v-abl^+$ or bcr/abl^{p185+} lymphoid leukaemic cells were injected i.v. into syngenic $Rag2^{-/-}$ mice (n=8 and n=10, respectively). No differences in survival time were observed (mean survival time of 13.5 days and 15.5 days,

respectively). **(C)** Spleen weights of terminally diseased mice revealed no significant differences. Data represent means \pm SD. **(D)** Diagram depicts time points of appearance of more than 5% leukaemic cells in peripheral blood as determined by flow cytometric analysis (mean time-to-event for “*v-abl* group”: 10 days, for “*bcr/abl*^{p185} group” 10.5 days).

Supplementary Figure 3

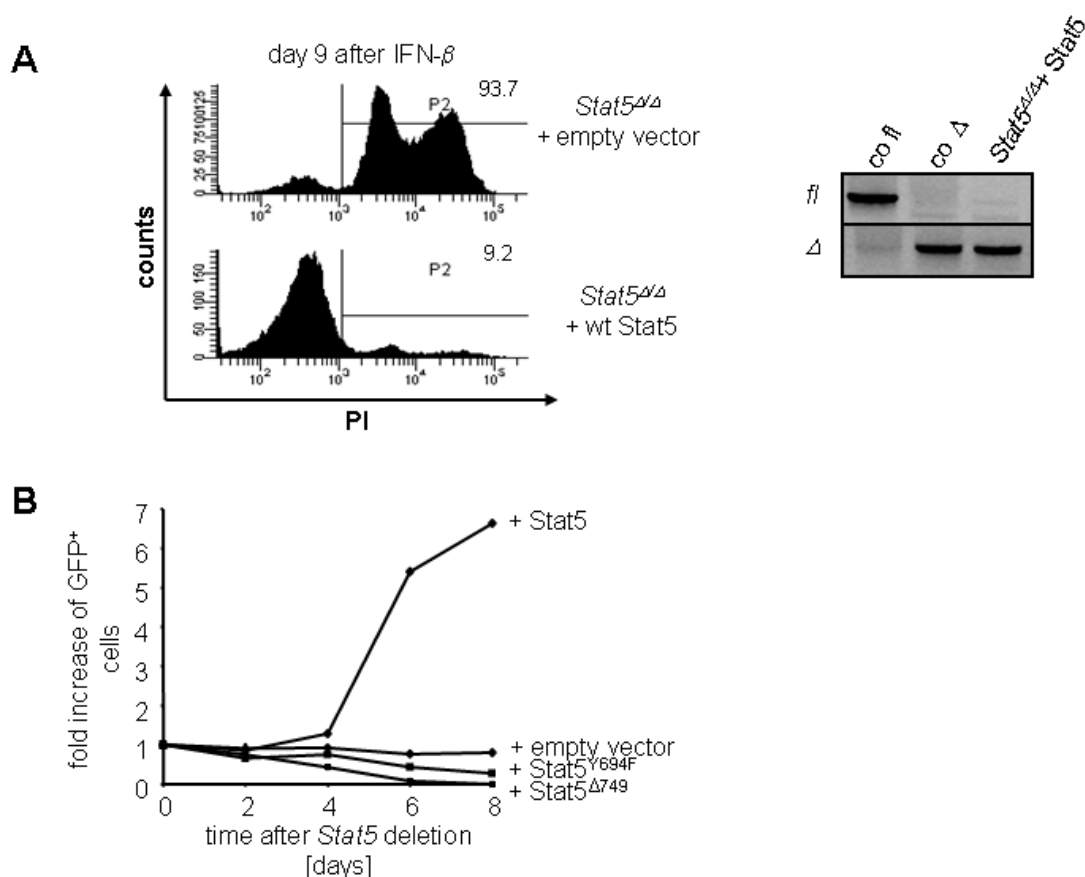


Figure S3. *Stat5* re-expression rescues effects of *Stat5*-deficiency

(A) *Stat5^{fl/fl}Mx1Cre* cell lines were transduced with retroviruses encoding either empty vector or wt *Stat5*. Apoptotic cells were determined by PI staining in a non-hypotonic buffer and FACS analysis after *Stat5* deletion via IFN- β (left panel). PCR analysis revealed complete deletion of wt *Stat5*-re-expressing cells (right panel).

(B) *Stat5^{fl/fl}Mx1Cre* cell lines were transduced with retroviruses encoding either empty vector, wt *Stat5*, *Stat5^{Y694F}* (preventing nuclear translocation (Gouilleux et al, 1994; Stoecklin et al, 1997; Yamashita et al, 1998) and *Stat5^{Δ749}* (dominantly negative *Stat5* (Moriggi et al, 1996). Endogenous *Stat5* was deleted via IFN- β and outgrowth of GFP⁺ cells was monitored by flow cytometry.

Supplementary Figure 4

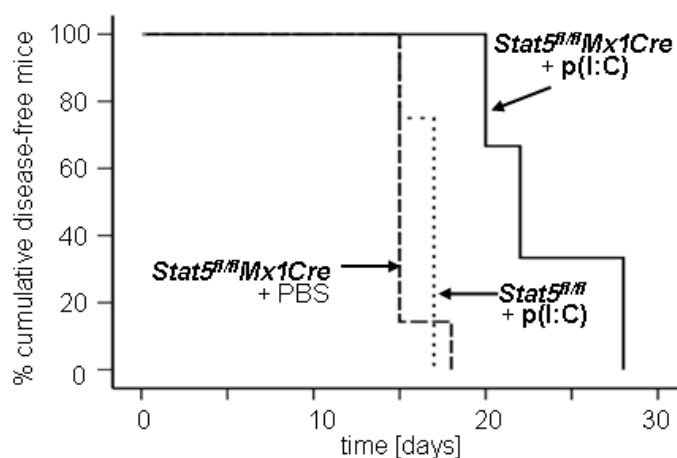


Figure S4. Transplantation of *Stat5^{fl/fl} Mx1Cre* and *Stat5^{fl/fl} v-abl⁺* cell lines into wt *C57BL/6J* mice.

1×10^6 cells of *Stat5^{fl/fl} Mx1Cre* or *Stat5^{fl/fl}* cell lines (n=3 each) were injected via tail vein. From day 7 on, 400 μ g p(I:C) was injected i.p. every four days until mice diseased. Kaplan-Meier analysis revealed a significant difference in survival time of mice after *Stat5*-deletion compared to control group (“*Stat5^{fl/fl} Mx1Cre + p(I:C)*” n= 6 vs. “*Stat5^{fl/fl} Mx1Cre* untreated” n=7 $p < 0.001$; “*Stat5^{fl/fl} Mx1Cre* untreated” vs. *Stat5^{fl/fl} + p(I:C)*” n=4 n.s.). Mice of the “*Stat5^{fl/fl} Mx1Cre + p(I:C)*” group displayed a mean survival of 23.3 days, whereas mice of the “*Stat5^{fl/fl} Mx1Cre* untreated” and “*Stat5^{fl/fl} + p(I:C)*” groups survived on average 15.4 and 16.5 days, respectively.

Supplementary Figure 5

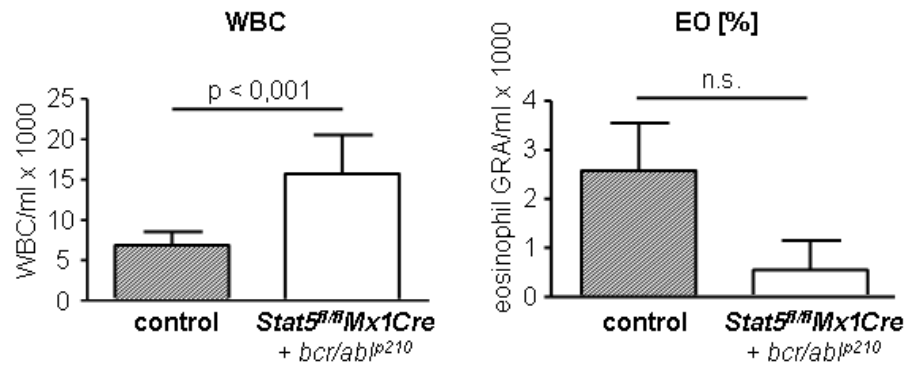


Figure S5. WBC and eosinophil counts from mice that received *bcr/abl^{p210}*-transduced *Stat5^{fl/fl}Mx1Cre* BM cells at the time point of p(I:C) injection.

WBCs and percentages of eosinophil cells in the peripheral blood of mice that received *bcr/abl^{p210}*-transduced *Stat5^{fl/fl}Mx1Cre* BM (n=18) and age-matched controls (n=10) at the time point of p(I:C) injection (6 weeks post transplantation). Data represent means \pm SD.

Supplementary Table 1

in vitro (SCF, Flt3-L, IgF-1, IL-3, IL-6, GM-SCF, Dex):

genotype of transplanted cells	mouse #	injected i.p.	outgrowth of <i>bcr/abl</i> ⁺ / <i>GFP</i> ⁺ cells
<i>Stat5</i> ^{fl/fl} <i>Mx1Cre</i>	#1	PBS	+
	#2	PBS	+
	#3	PBS	+
	#4	PBS	+
	#5	PBS	+
	#6	PBS	+
	#7	PBS	+
	#8	PBS	+
<i>Stat5</i> ^{fl/fl} <i>Mx1Cre</i>	#1	p(I:C)	-
	#2	p(I:C)	-
	#3	p(I:C)	-
	#4	p(I:C)	-
	#5	p(I:C)	-
	#6	p(I:C)	-
	#7	p(I:C)	-
	#8	p(I:C)	-
	#9	p(I:C)	-
	#10	p(I:C)	-
<i>Stat5</i> ^{fl/fl}	#1	PBS	+
	#2	PBS	+
	#3	PBS	+
<i>Stat5</i> ^{fl/fl}	#1	p(I:C)	+
	#2	p(I:C)	+
	#3	p(I:C)	+

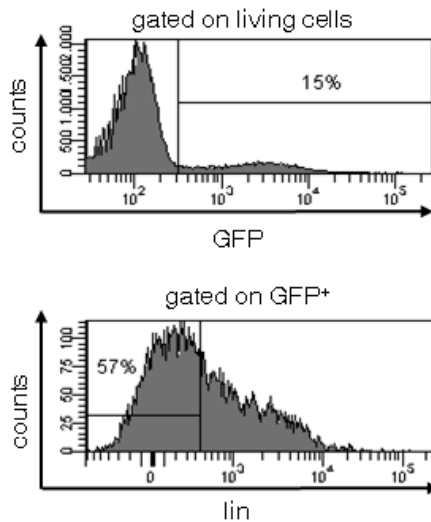
Table S1. Outgrowth of *ex vivo*-derived *bcr/abl*⁺/*GFP*⁺ BM cells *in vitro*.

BM cells were cultured in a medium supplemented with SCF, Flt-3L, IgF-1, IL-3, IL-6; GM-SCF and dexamethasone for a period of four weeks.

Supplementary Figure 6

A

FACS analysis after 4 weeks *in vitro*:



B

Leukemia upon re-injection into lethally irr. mice:

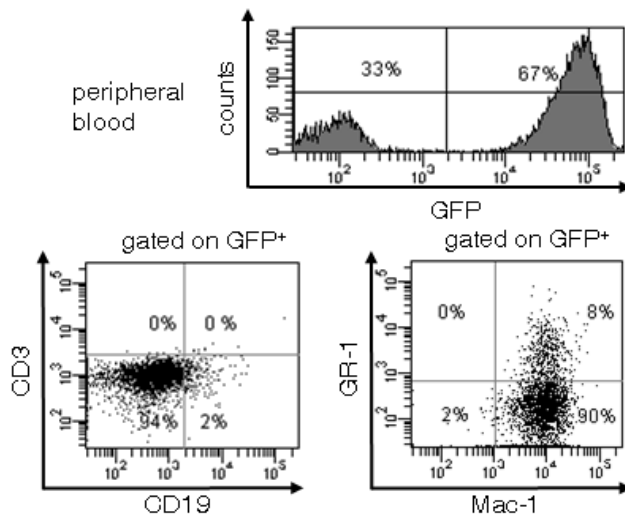


Figure S6. *In vitro* cultivation and re-transplantation of *ex vivo*-derived *bcr/abl*⁺/*GFP*⁺ BM cells.

(A) FACS analysis of BM cells of diseased mice that were cultured in a medium supplemented with SCF, Flt-3L, IgF-1, IL-3, IL-6; GM-SCF and dexamethasone (one representative example out of 14 is depicted). (B) After four weeks cultured cells were re-

injected into lethally irradiated mice and led to leukaemia within two weeks (n=4). One representative flow cytometric profile of peripheral blood FACS analysis is shown.

Supplementary Figure 7

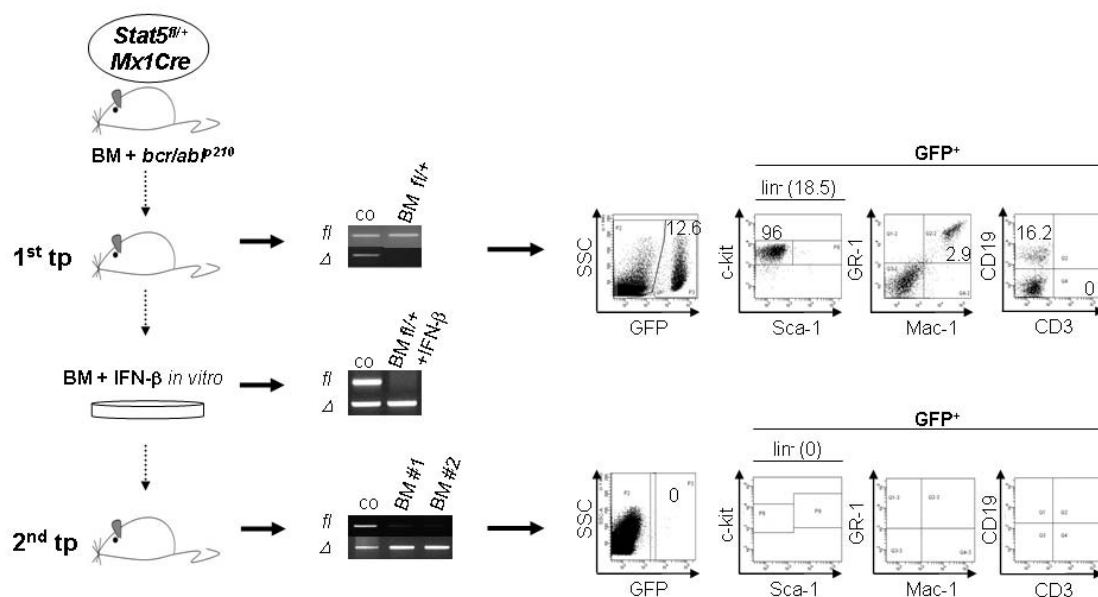


Figure S7. *Stat5*-heterozygosity leads to an abrogation of *bcr/abl^{p210}*-induced disease maintenance.

Stat5^{fl/+} Mx1Cre mice were used as donor mice. As depicted in a scheme (left panel) BM cells were infected with *bcr/abl^{p210}* and transplanted into lethally irradiated recipient mice (“1st tp”). After 12 weeks BM cells were isolated, pooled (n=3), treated with IFN- β for 48h *in vitro* to induce deletion of the *Stat5* alleles and injected into lethally irradiated recipients (“2nd tp”; n=9). Middle panels show PCR analysis of BMs at indicated steps of the experiments. Right panels depict flow cytometric analysis of BM cells after 1st and 2nd tp as indicated. No *bcr/abl⁺/GFP⁺* cells were detected in BM cells of secondary recipients. PCR analysis of the non-leukaemic BM verified successful transplantation (two representative samples are shown). One representative flow cytometric profile of BM cells is depicted. Numbers indicate percentages of cells belonging to individual sub-populations.

Supplementary Figure 8

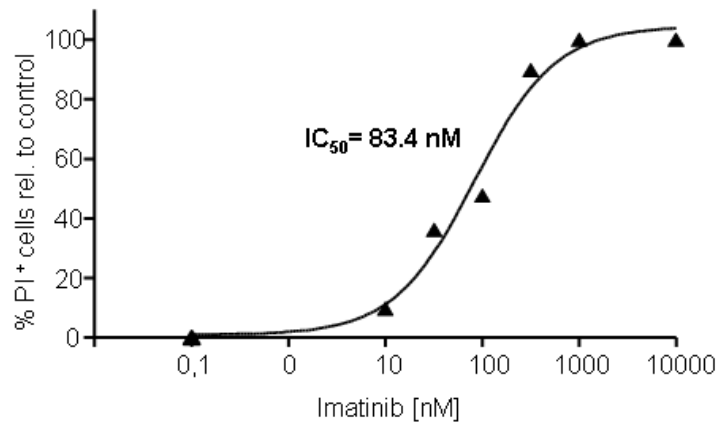


Figure S8. Determination of IC₅₀ value of imatinib on *in vitro* cultivated *bcr/abl*^{p210}-expressing wt *Mx1Cre* BM cells.

Infected BM was treated with variable concentrations of imatinib for 7 days. Thereafter percentages of PI⁺ cells were determined by FACS analysis. IC₅₀ value was computed using Hill equation and OLS regression.

Supplemental Experimental Procedures

Retroviral constructs

Bcr/abl^{p210} was cloned into a pMSCV-IRES-GFP, *bcr/abl*^{p210T315I} into a pMSCV-IRES-dsRed backbone.

Tissue culture conditions and virus preparation

Transformed FL cells and tumor-derived cell lines were maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS), 100 U/ml penicillin/streptomycin, 50 μM β-mercaptoethanol and 2 mM L-glutamine (“RPMI complete”). A010 and gp+E86 cells were maintained in DMEM medium containing 10% fetal calf serum, 100 U/ml penicillin/streptomycin, 50 μM β-mercaptoethanol and 2 mM L-glutamine (“DMEM complete”). Cell culture media were purchased from Sigma. Standard supplements were purchased from Gibco.

A010 cells produce an ecotropic replication-deficient form of the Abelson murine leukaemia virus (A-MuLV) encoding the *v-abl* oncogene and were a generous gift of Dr. Naomi Rosenberg. Gp+E86 retroviral packaging cell lines for Stat5 target genes, *bcr/abl*^{p210} as well as mutated version thereof (*bcr/abl*^{p210T315I}) were established by transfection (Lipofectamine Invitrogen®). Cells were sorted for expression of fluorescent proteins using BD FACS-Vantage device.

Bcr/abl^{p210} and *bcr/abl*^{p210T315I}-infected BM cells were maintained in Stem Cell Pro medium supplemented with mSCF (50 ng/ml), murine IL-3 (2 ng/ml), Flt3-L (10 ng/ml), murine GM-CSF (3 ng/ml), IGF-1 (40 ng/ml), dexamethasone (1 μg/ml) and mouse IL-6 (0.5 ng/ml) (all purchased from R&D Systems) (“stem cell medium”) as described before (Kieslinger et al, 2000).

Infections and establishment of cell lines

For the preparation of *v-abl*⁺ lymphoid cell lines, animals were set up for breeding. Fourteen days after conception pregnant animals were sacrificed and FLs prepared. Single cell

suspensions from FLs were infected for one hour with viral supernatant derived from A010 cells in the presence of 7 µg/ml polybrene as described previously (Sexl et al, 2000). The cells were then maintained in RPMI complete medium and observed for the outgrowth of stable transformed cell lines.

***In vitro* colony formation assay**

For *bcr/abl^{p210}*-induced colony formation assays, single cell suspensions from FLs (ED 14) **or BMs** were co-cultivated on *bcr/abl^{p210}* producer cells in the presence of IL-3 (25 ng/mL), IL-6 (50 ng/mL), SCF (50 ng/mL) and 7 µg/ml polybrene for 48 hours as described previously (Sexl et al, 2000). After infection, 1×10^4 GFP⁺ cells were resuspended in 3 ml cytokine-free methylcellulose (Stem Cell Technologies) and plated in 35 mm dishes (1.5 ml each). *V-abl*-induced colony assays were performed as described previously (Hoelbl et al, 2006; Kovacic et al, 2006). After eight days colonies were counted by light microscopy (Leica Fluovert microscope, 4 x magnification). Images of cell culture dishes were scanned using a standard on-desk scanner.

Flow cytometry and differential haemograms

Single cell suspensions were pre-incubated with αCD16/CD32 antibodies to prevent non-specific Fc-receptor-mediated binding. Subsequently, 5×10^5 cells were stained with monoclonal antibodies conjugated with fluorescent markers and analyzed by a FACSCantoII flow cytometer using FACSDiva software (Becton-Dickinson). The following antibodies, all purchased from BD Biosciences, were used: B220 (RA3-6B2), CD19 (1D3), CD3ε (145-2C11), Mac-1 (M1/70), GR-1 (RB6-8C5), Thy1.2 (53-2.1), Flt-3 (A2F10.1), CD71 (M-A712), Ter119, mouse-lineage panel and Sca-1 (D7). C-kit (2B8) was purchased from eBioscience.

Cell cycle and apoptosis analysis: 1×10^6 cells were stained with PI (50 µg/ml) in a hypotonic lysis solution (0.1% sodium citrate, 0.1% triton X-100, 100 µg/ml RNase) and incubated at 37°C for 30 minutes. Analysis of cells undergoing apoptosis was performed by

staining with Annexin V and PI (1 µg/ml) according to the manufacturer's protocol (BD Biosciences).

Differential hemograms from peripheral blood were assessed using a VetABC Blood Counter (Scil Animal Care, Viernheim, Germany).

Cell extracts and Immunoblotting

Cells were lysed in a buffer containing protease and phosphatase inhibitors (50 mM Hepes, pH 7.5, 0.1% Tween-20, 150 mM NaCl, 1 mM EDTA, 20 mM β-glycero-phosphate, 0.1 mM sodium vanadate, 1 mM sodium fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM PMSF, respectively). Protein concentrations were determined using a BCA-kit (Pierce, Rockford, IL).

Proteins (50-100 µg) were separated on an 8% SDS polyacrylamide gel and transferred onto Immobilon membranes. Membranes were probed with antibodies directed against Stat5 (C-17), Trp53 (C-11), Bcl-2 (C-21), Bcl_{XL}(H-5), β-actin (C-4) and holo-ERK (C-16) (all purchased from Santa Cruz Biotechnologies). Immunoreactive bands were visualized by chemoluminescent detection (ECL detection kit; Amersham, Arlington Heights, UK) using protein A-conjugated horseradish peroxidase (Amersham, Arlington Heights, UK).

Immunohistochemistry

Paraffin-embedded sections of spleens and BM were deparaffinized and pretreated by boiling in 10 mM citrate buffer (pH 6.0). Tissue peroxidase activity was quenched by incubation in 3% H₂O₂ in PBS for 10 min, followed by blocking in 1% BSA. Immunohistochemistry was performed with a pSTAT5 antibody (Tyr 694) (Santa Cruz Biotechnology). Peroxidase reaction was done using ImmunoCruz™ Staining Systems according to manufacturer's protocol. Nuclei were stained with haematoxylin.

***In vitro* imatinib sensibility assay of *bcr/abl*^{p210T315I} - transformed cells**

BM cells from 6 weeks old *Stat5^{fl/fl}Mx1Cre* and *Stat5^{+/+}Mx1Cre* mice were co-cultivated with retroviral producer cells encoding *bcr/abl*^{p210} or an imatinib-resistant version (*bcr/abl*^{p210T315I})

as described for colony formation assays. After 48 hours of infection cells were maintained in stem cell medium as described above and treated either with 100 nM imatinib or 1000 U/ml recombinant IFN- β . Cells were analyzed by flow cytometry for cell cycle distribution daily.

Supplemental References

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