Stat5 is indispensable for the maintenance of bcr/abl-positive leukaemia

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INTRODUCTION

Tumourigenesis caused by the Bcr/Abl oncoprotein is a multi-step process proceeding from initial to tumour-maintaining events and finally results in a complex tumour-supporting network. A key to successful cancer therapy is the identification of critical functional nodes in an oncogenic network required for disease maintenance. So far, the transcription factors Stat3 and Stat5a/b have been implicated in bcr/abl-induced initial transformation. However, to qualify as a potential drug target, a signalling pathway must be required for the maintenance of the leukaemic state. Data on the roles of Stat3 or Stat5a/b in leukaemia maintenance are elusive. Here, we show that both, Stat3 and Stat5 are necessary for initial transformation. However, Stat5- but not Stat3-deletion induces G0/G1 cell cycle arrest and apoptosis of imatinib-sensitive and imatinib-resistant stable leukaemic cells in vitro. Accordingly, Stat5-abrogation led to effective elimination of myeloid and lymphoid leukaemia maintenance in vivo. Hence, we identified Stat5 as a vulnerable point in the oncogenic network downstream of Bcr/Abl representing a case of non-oncogene addiction (NOA).

Tumourigenesis is a multi-step process that requires the expression of oncogenes or the downregulation of tumour suppressor genes (Hanahan & Weinberg, 2000). The tumour initiation process is driven by continuous oncogene expression which frequently develops into oncogene addiction during tumour maintenance (e.g. in the case of Bcr/Abl, EGFR, Flt3-ITD, Jak2V617F, c-Kit) (Weinstein, 2002). The process of tumour maintenance is associated with rewiring of signalling pathways and by acquiring additional genetic mutations (e.g. mutations or deletions of Trp53 or Bcl-2 over-expression) (Letai et al, 2004; Sherr, 2004; Ventura et al, 2007). Such additional mutations may complicate the success of therapies directed against the transforming oncogene as has been shown for bcr/abl-driven disease (Moon et al, 2009; Wendel et al, 2006).

Bcr/abl-induced leukaemia is characterized by a t(9;22) (q34;q11) translocation leading to the expression of a chimeric fusion gene product (Bcr/Abl) representing a constitutively active tyrosine kinase. This translocation is mainly linked to two distinct haematopoietic disorders: acute lymphoid leukaemia (ALL) and chronic myelogenous leukaemia (CML) (Deininger et al, 2000). Targeting the Bcr/Abl oncoprotein by the kinase inhibitor imatinib-mesylate and related substances has been a major breakthrough. This success is only compromised by treatment-insensitive mutations within the transforming oncogene itself (Chu et al, 2005; Griswold et al, 2006; Shah et al, 2002). Thus, despite the success story of Bcr/Abl kinase inhibitors, additional therapeutic strategies are required. Among the signalling pathways that may allow therapeutic interference is the Jak-Stat signalling pathway that has been implicated in tumourigenesis (Bromberg, 2002; Ho et al, 1999; Levine et al,
RESULTS

Initial myeloid and lymphoid transformation require Stat5 and Stat3

We have previously shown that initial lymphoid transformation by bcr/ablP185 and v-abl oncogenes critically depends on Stat5 in vitro and in vivo ([Hoelbl et al, 2006] and Fig 1A). Here, we investigated whether initial myeloid transformation induced by the Bcr/AblP210 oncogene, also depends on Stat5. Hence, bcr/ablP210-induced colony formation in growth-factor free methylcellulose was investigated using Stat5null-null (foetal livers (FLs) (ED 14). Importantly, the frequency of HSC numbers in Stat5null-null FLs is relatively normal (Hoelbl et al, 2006; Li et al, 2007; Yao et al, 2006) resulting in a comparable target population for transformation. We found that myeloid transformation critically depends on Stat5 in a gene-dosage dependent manner (Fig 1B). Next, we investigated the role of Stat3 in initial myeloid and lymphoid transformation. Since Stat3-deficiency results in early embryonic lethality (ED 6.5) (Takeda et al, 1997) we relied on BM cells derived from Stat3null/fl/Mx1Cre mice that were treated with polyinosinic: polycytidyllic acid (p(I:C)) to induce Stat3 deletion in vivo. Similarly, significant reductions of v-abl+ and bcr/ablP210+ colony numbers were observed for Stat3-/- that on transduction of v-abl- and bcr/ablP210- respectively (Fig 1C,D). Numbers of target cells were comparable within each experimental group as determined by flow cytometric analysis of HSC-enriched Lin-c-kit-Sca-1+/+ population for transformation. We found that myeloid trans- formed cells are phenotypically identical and share comparable disease kinetics (ED 14). Importantly, the frequency of HSC numbers in Stat5null-null FLs is relatively normal (Hoelbl et al, 2006; Li et al, 2007; Yao et al, 2006) resulting in a comparable target population for transformation. We found that myeloid transformation critically depends on Stat5 in a gene-dosage dependent manner (Fig 1B). Next, we investigated the role of Stat3 in initial myeloid and lymphoid transformation. Since Stat3-deficiency results in early embryonic lethality (ED 6.5) (Takeda et al, 1997) we relied on BM cells derived from Stat3null/fl/Mx1Cre mice that were treated with polyinosinic: polycytidyllic acid (p(I:C)) to induce Stat3 deletion in vivo. Similarly, significant reductions of v-abl+ and bcr/ablP210+ colony numbers were observed for Stat3-/- that on transduction of v-abl- and bcr/ablP210- respectively (Fig 1C,D). Numbers of target cells were comparable within each experimental group as determined by flow cytometric analysis of HSC-enriched Lin-c-kit-Sca-1+/+ population for transformation. We found that myeloid trans- formed cells are phenotypically identical and share comparable disease kinetics (Supporting Information Fig 1).

Taken together, these data reveal the absolute requirement of the transcription factors Stat3 and Stat5 for the initial transformation event downstream of v-Abl and Bcr/AblP210. Stat5, but not Stat3, is essential for cell cycle progression and survival of lymphoid leukaemic cells in vitro

Transformed cells rewire pathways for growth and survival. Only signalling pathways essential for the maintenance of the oncogenic state qualify as useful therapeutic targets. Hence, we next investigated the consequences of Stat3- or Stat5-deletion on already established leukaemic cells. Since bcr/ablP210+ myeloid cells do not give rise to stable, growth-factor free cell lines in vitro, we used v-abl+ lymphoid cell lines for the following in vitro studies. V-abl+ transformed cells readily become growth-factor independent in vitro. Thus, we retrovirally transduced Stat3null/fl/Mx1Cre and Stat5null/fl/Mx1Cre derived BM and control cells with v-abl, a murine variant of bcr/ablP185. V-abl- and bcr/ablP185 transformed cells are phenotypically identical and share comparable disease kinetics in vivo (Supporting Information Fig 2). Stable cell lines of all genotypes were generated (CD19+, B220+, CD43+) analysed for proliferation rate, growth factor independent colony formation and homing to haematopoietic organs in vivo with comparable results (data not shown). We used recombinant IFN-β to activate Cre-recombainase in Stat3null/fl/Mx1Cre and Stat5null/fl/Mx1Cre cells in vitro (Fig 2A and B).

As depicted in Fig. 2, IFN-β treatment had no effect on cell proliferation in Stat3null/fl/Mx1Cre cells. In contrast, we observed changes in cell cultures of Stat5null/fl/Mx1Cre cells (Fig 2C and D). Cell cycle profiles obtained 48h after the initiation of IFN-β treatment revealed a profound G0/G1 cell cycle arrest in Stat5null/fl/Mx1Cre cells (67.7±1.7% compared to 45.4±5.2%...
of untreated cells within $G_0/G_1$ phase) whereas the cell cycle profiles of $Stat3^{+/+}/Mx1Cre$ cells remained unaltered ($44.9 \pm 4.8\%$ compared to $38.5 \pm 3.9\%$ of untreated cells within $G_0/G_1$ phase) (Fig 2E and F). The cell cycle arrest in IFN-$\beta$-treated $Stat5^{+/+}/Mx1Cre$ cells was followed by apoptosis analysed by Annexin V/PI stains 9 days post deletion. $58.3 \pm 6.2\%$ and $32 \pm 1.3\%$ of IFN-$\beta$ treated $Stat5^{+/+}/Mx1Cre$ cells were double-positive for Annexin V/PI and single-positive for PI, respectively. The time-span between IFN-$\beta$ treatment and cell death results from the long half life of Stat5 in these cells (data not shown). No changes in the viability of IFN-$\beta$ treated $Stat3^{+/+}/Mx1Cre$ cells were detectable (Fig 2G and H) even after 30 days. After 10 days in the presence of IFN-$\beta$ no viable cells were detected in IFN-$\beta$ treated $Stat5^{+/+}/Mx1Cre$ cell cultures. Our attempts to rescue Stat5 deficiency by re-expression of Stat5 target genes such as D-type cyclins, c-myc, bcl-xL or bcl2 failed (Fig 2I). Only re-expression of wild-type (wt) Stat5, but not of transcriptionally inactive mutants (Stat5$^{Delta49}$ and Stat5$^{Y694F}$), was able to protect cells from proliferation arrest and apoptosis upon deletion of endogenous Stat5 (Supporting Information Fig 2D). We therefore chose this time point to initiate p(I:C) treatment which was repeated every 4 days in order to efficiently target this highly proliferating ALL-like disease (scheme in Fig 3B). Hence, we concluded that Stat5, but not Stat3, is required for the maintenance of the malignant state of transformed lymphoid leukaemic cells in vivo.

Stat5 is required for lymphoid leukaemia maintenance in vivo

To study the role of Stat5 in lymphoid leukaemia maintenance in vivo, we transplanted $Stat5^{+/+}/Mx1Cre$ v-abl$^+$ cells into Rag2$^{-/-}$, c-myc$^{-/-}$ mice ($1 \times 10^6$ cells/mouse). $Rag2^{-/-}/c-myc^{-/-}$ mice lack lymphoid cells and are therefore particularly suited to monitor lymphoid leukaemia. Recipient mice were subsequently divided into two groups (Fig 3A) with one group receiving p(I:C) to induce type I IFN responses and to delete Stat5 within the leukaemic cells. The second group was mock-injected with PBS. Preliminary experiments had revealed that 7 days post-transplantation mice display first signs of sickness with elevated numbers of leukaemic cells in the peripheral blood (Supporting Information Fig 2D). We therefore chose this time point to initiate p(I:C) treatment which was repeated every 4 days in order to efficiently target this highly proliferating ALL-like disease (scheme in Fig 3B).

We observed a prolonged survival of mice that had received $Stat5^{+/+}/Mx1Cre$ leukaemic cells and p(I:C) treatment compared to mice that were mock-injected with PBS. To control for effects of p(I:C) per se, mice that had received $Stat5^{+/+}$ cells were also p(I:C) treated (Fig 3C). Whereas mice from the ‘$Stat5^{+/+}/Mx1Cre + PBS$’ and ‘$Stat5^{+/+} + p(I:C)$’ groups displayed obvious severe signs of sickness from day 16 on, animals...
harbouring Stats5\(^{+/−}\) leukaemic cells appeared healthy with normal mobility, fur and weight. Mice where Stats5 had been deleted in the leukaemic cells survived significantly longer (mean survival of 49 days compared to 20 and 16 days in the ‘Stats5\(^{+/−}\) + p(I:C)’ and ‘Stats5\(^{+/−}\)Mx1Cre + PBS’ groups, respectively). We compared diseased animals sacrificed on days 16 and 20 (‘Stats5\(^{+/−}\)Mx1Cre + PBS’ group) to healthy appearing mice of the ‘Stats5\(^{+/−}\)Mx1Cre + p(I:C)’ group. Whereas BMs and spleens of the ‘Stats5\(^{+/−}\)Mx1Cre + PBS’ group were densely infiltrated with B220\(^{+}\)CD19\(^{+}\) cells, we hardly detected leukaemic cells in mice of the ‘Stats5\(^{+/−}\)Mx1Cre + p(I:C)’ group (Fig 3D). Similar results were obtained when immunocompetent mice were used as recipient animals (Supporting Information Fig 4). However, finally all mice succumbed to leukaemia. Examination of the leukaemic cells revealed lack of genomically Stats5 deleted cells and Stats5 protein expression (Fig 3E and data not shown). We reasoned that p(I:C)-induced deletion was incomplete in vivo and that some cells escaped deletion. This scenario is supported by the fact that we still could induce cell cycle arrest and apoptosis in the ex vivo derived leukaemic cells by IFN-\(\beta\) treatment (Fig 3F). This rules out that the cells have acquired secondary mutations overcoming the Stats5-requirement.

Stat5 is required for bcr/abl\(^{210}\)-induced myeloid leukaemia maintenance in vivo

Transplantation of bcr/abl\(^{210}\) BM cells is a reliable method to develop a fatal rapidly progressing myeloproliferative illness in mice which is commonly determined ‘CML-like’ disease (Pear et al, 1998; Van Etten, 2001). We retrovirally transduced BM cells from 5-fluorouracil (FU) pretreated Stats5\(^{+/−}\) mice with bcr/abl\(^{210}\) IRES GFP and injected them i.v. into lethally irradiated wt mice (1 \(\times\) 10\(^6\) cells). In order to determine the optimal time point to initiate p(I:C) injection preliminary experiments were performed (\(n = 6\)). Under our experimental conditions 6 weeks after the initial transplantation the animals displayed first signs of disease indicated by 12.3 \(\pm\) 3.7% bcr/abl\(^{+/−}\)/GFP\(^{+}\) cells in the BM accompanied by the doubling of peripheral white blood cell counts (WBCs) and a decrease in eosinophil cell numbers (Fig 4A and Supporting Information Fig 5). A single p(I:C) injection at that time eradicated bcr/abl\(^{+/−}\)/GFP\(^{+}\) cells in the BM when analysed 10 days thereafter, whereas increasing numbers were detected in p(I:C)-treated control
animals (Fig 4A). This indicates that p(I:C) treatment and thus Stat5 deletion was capable to eradicate the disease at that given time point. Our attempts to wait with the p(I:C) application till the animals displayed more severe signs of leukaemia such as weight loss or reduced mobility failed. After displaying severe signs of leukaemia the animals die rapidly within less than 5 days. This time frame is insufficient to deplete the leukaemic cells of Stat5 protein despite the successful genomic deletion. The long half life of the Stat5 protein is also comprehensible from the fact that it takes 9 days to eradicate v-abl transformed cells in vitro after IFN-β treatment (Fig 2 and data not shown). Accordingly, the following experimental protocol was set up (Fig 4B). Peripheral WBCs were monitored weekly as internal control. P(I:C) treatment and Stat5 reduction resulted in a significantly prolonged survival of the Stat5fl/fl Mx1Cre mice compared to all other groups (p < 0.001 compared to every other group, Fig 4C). Untreated mice succumbed to disease from week 7 on. Any time when a group of control mice severely diseased and had to be sacrificed, one animal of the ‘Stat5fl/fl Mx1Cre + p(I:C)’ group was analysed in parallel to allow a direct comparison. Sixteen weeks post-transplantation, the number of leukaemic cells in the peripheral blood was clearly reduced in the ‘Stat5fl/fl Mx1Cre + p(I:C)’ compared to the ‘Stat5fl/fl Mx1Cre + PBS’ group. Reduction of Stat5 in the Stat5fl/fl Mx1Cre + p(I:C) group was verified by immunoblotting of peripheral blood leukocytes 13, 14, 16 and 18 weeks post-treatment (indicated censored events in Fig 4C and D). Similarly, histological stainings revealed a reduced infiltration of BM and spleen with leukaemic cells paralleled by a diminished pStat5 staining (Fig 4E). These findings indicated a reduction of the leukaemic cell load after p(I:C) treatment and Stat5 deletion. Measuring spleen sizes underlined the effect of p(I:C) treatment (Fig 4F). Whereas the diseased mice showed significantly enlarged spleens, the spleens of the p(I:C) treated Stat5fl/fl Mx1Cre mice were of regular size and comparable to age-matched control mice. The BMs of all animals were cultured in a medium supplemented with SCF, Flt3-ligand (Flt3-L), IgF-1, IL-3, IL-6, GM-SCF and dexamethasone as described previously (Kieslinger et al, 2000). As listed in Supporting Information Table 1, no bcr/abl+/GFP+ cells grew out from BMs of p(I:C) treated-, Stat5fl/fl Mx1Cre-transplanted mice. In contrast, after 4 weeks significant numbers of bcr/abl+/GFP+ cells were
detected in control cultures, transplantation of these cells in lethally irradiated mice re-initiated leukaemia (Supporting Information Fig 6).

**Stat5 is required for engraftment and repopulation of bcr/abl^{p210+} leukaemia in secondary recipients**

These data suggested that Stat5 is required for the maintenance of leukaemia initiating cells driving bcr/abl^{p210} induced myeloid disease. CML-like disease in mice is driven by a bcr/abl+c-kit-Leukaemia initiating or leukaemic stem cell (LSC) defined by the ability to carry on disease to a secondary recipient and to replenish the leukaemic cell pool (Krause et al, 2006; Wang & Dick, 2005). A definite cure of CML can only be achieved when LSCs are successfully eliminated. Therefore, we tested the effects of Stat5-deletion in LSCs by investigating their ability to engraft and repopulate leukaemia in a secondary recipient. We used bcr/abl^{p210} infected Stat5^{fl/fl}Mx1Cre bcr/abl^{+/GFP} cells to transplant lethally irradiated wt recipient mice. When first signs of disease evolved, indicated by elevated WBCs, the BM of the affected animals was prepared (data not shown). At that time, 13.4±3.3% of the BM cells were bcr/abl^{+}/GFP^{+} (Fig 5, upper panel) which mainly consisted of Mac1^{+}Gr1^{+}.

**Figure 4. Myeloid leukaemia maintenance depends on Stat5.**

A. FACS analysis of BM cells 10 days after a single p(I:C) treatment. Six weeks post-transplantation of Stat5^{fl/fl} or Stat5^{fl/fl}Mx1Cre bcr/abl^{+/GFP} cells, mice received p(I:C) i.p.

B. Experimental setup for the deletion of Stat5 in myeloid leukaemia.

C. Kaplan–Meier plot displaying overall survival of p(I:C)-treated and control recipient mice harbouring Stat5^{fl/fl}Mx1Cre and Stat5^{fl/fl} myeloid leukaemia. One single p(I:C) treatment was performed 6 weeks after transplantation in order to delete Stat5 in leukaemic cells. At the time point of analysis (18 weeks post-injection), 100% (10/10) of Stat5^{fl/fl}Mx1Cre p(I:C)-treated mice are alive, while recipients from all other groups succumb to leukaemia (n = 8 for Stat5^{fl/fl}Mx1Cre + PBS, n = 3 for Stat5^{fl/fl} + p(I:C) and n = 3 for Stat5^{fl/fl} + PBS; p < 0.001; mean survival time of 12.5, 11.3 and 12 weeks, respectively).

D. Immunoblot analysis of Stat5 expression in peripheral blood cells 7 (#1), 8 (#2), 10 (#3) and 12 (#4) weeks after p(I:C) treatment. These censored events are indicated in (C).

E. Blood smears and histological sections of spleens and BM. Treatment with p(I:C) leads to a massive reduction of WBCs (left panel) and phospho-Stat5 expression (right panels) in Stat5^{fl/fl}Mx1Cre-bcr/ abl^{p210+} transplanted mice compared to controls (PBS).

F. Macroscopic anatomy of spleens from Stat5^{fl/fl}Mx1Cre mice treated with p(I:C) and control mice. A spleen of one age-matched healthy wt mouse is depicted as control.
Secondary leukaemia formation.

Mac1

2.1/C6

(C6)

(28.5 ± 8.9%) cells being mainly stem/progenitor cells (Lin−c-kit+Sca-1−; 86.7 ± 20.5%). Deletion of Stat5 was induced by treating the BM cells ex vivo with recombinant IFN-β (1000 U/ml). We had to omit our initial plan to transplant a pure Stat5−/− population into secondary recipients since we never completely deleted Stat5 despite intense efforts using various concentrations of IFN-β (Fig 5, middle panel). We reasoned that Stat5−/+ cells might have a severe disadvantage in vitro and decided to transplant an IFN-β treated ‘mixed’ population of Stat5−/−Mx1Cre and Stat5+/+Mx1Cre cells. This attempt has the advantage that the co-transplanted non-deleted LSCs served as internal control for successful transplantation when investigating whether the Stat5−/+ LSCs contribute to CML in vivo.

Two weeks after the secondary transplant, recipient mice displayed clear signs of disease including decreased mobility and weight loss. All mice had developed leukaemia with enlarged tumour cells (lower middle panel). Two weeks after the secondary transplant, recipient mice displayed clear signs of disease including decreased mobility and weight loss. All mice had developed leukaemia with enlarged tumour cells (lower middle panel).

As illustrated in Fig 1B, already Stat5-heterozygosity profoundly affected bcr/abl210-induced myeloid colony formation. To test the effect of Stat5 heterozygosity on bcr/abl210-induced disease in vivo, we repeated the experiments shown in Fig 5 using Stat5−/+Mx1Cre mice as donors of the primary transplant. In this setting, a complete deletion of the floxed Stat5 allele of primary transplant-derived cells in vitro was achieved (Supporting Information Fig 7, middle panel). Transplantation of the resulting heterozygous Stat5−/+ population—comprised of leukaemic and non-leukaemic cells—failed to induce a bcr/abl210-leukaemia. No bcr/abl+/GFPP+ cells were detectable in BMs and all other organs investigated including lymph nodes, spleen and liver of secondary recipients (Supporting Information Fig 7 and data not shown). However, the presence of the Stat5−− allele was confirmed in the BM of the recipient animals proving successful transplantation and reconstitution by non-leukaemic Stat5+/− cells (Supporting Information Fig 7, lower panel). To substantiate this finding we also cultivated ex vivo derived BM cells of the Stat5−/+ transplanted animals under stem-cell-supporting conditions (Zhang & Lodish, 2005). Whereas LSCs from the Stat5−/+Mx1Cre control BMs readily grew out we failed to detect any outgrowth of leukaemic cells from BM of the Stat5−/− group even after 6 weeks (data not shown). Thus, we concluded that Stat5−/− cells contribute to haematopoietic reconstitution of lethally irradiated mice but do not allow the outgrowth of bcr/abl210-leukaemic cells.

Normal haematopoiesis is not significantly affected by Stat5 loss

Therapeutic agents must display a sufficiently large therapeutic window that allows killing tumour cells while sparing normal cells. Since Stat5 has been implicated in particularly in foetal
haematopoietic development (Burchill et al, 2003; Grebien et al, 2008; Hoelbl et al, 2006; Yao et al, 2006), we were interested in the impact of Stat5 deletion on normal adult haematopoiesis.

Stat5-deletion was induced in adult (4 weeks old) Stat5^fl/flMx1Cre, Stat5^fl/+Mx1Cre and wt Mx1Cre mice (n = 7 each) by p(I:C) treatment (Fig 6A). Seven days after the initial p(I:C) treatment the deletion of Stat5 in sorted HSC-enriched Lin^−c-kit^+Sca-1^+ and Lin^−c-kit^+Sca-1^− (including myeloid and lymphoid progenitors) cells was verified by PCR (Fig 6B). In line with published data, despite the successful deletion of Stat5 the total numbers of these cellular fractions was unaltered (Fig 6C) (Wang et al, 2009). Similarly, the frequency of common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs) were unaffected (data not shown). We then collected blood once a week for a total observation period of 8 weeks. The analysis of body weight, WBCs, red blood cell counts (RBCs), haemoglobin (HGB) and haematocrit (HCT) did not reveal any significant effects (Fig 6D and E and data not shown). Minor but not significant alterations were detected in the absolute number of platelets (PLTs) and a slight decrease of monocytes (MO) and granulocytes (GRA) (Fig 6E and F). In addition, analysis of several clinical parameters (triglycerides, glucose, cholesterol, bilirubin, creatine, α-amylase, γ-GT, ALT, AST) did not show any noticeable changes after Stat5-deletion (data not shown). Whereas percentages of CD3^+ cells remained unaltered, ‘Stat5^fl/flMx1Cre + p(I:C)’ mice displayed significantly lower levels of CD19^+ cells (Fig 6F). Moreover, 8 weeks post-Stat5 deletion we did not observe changes in frequencies of LT-HSCs (Lin^−c-kit^+Sca-1^+/Flt-3^−Thy1^+), ST-HSCs (Lin^−c-kit^+Sca-1^+/Flt-3^−Thy1^+ ) or MPPs (Lin^−c-kit^+Sca-1^+/Flt-3^−Thy1^+ ) (Passegue et al, 2004, 2005). However, we observed slight decreases in

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**Figure 6.** Lack of detrimental long-term effects of Stat5-deletion on haematopoiesis.

A. Time course of p(I:C) injections and analysis of mice during 8 weeks (n = 7/group).

B. Lin^−c-kit^+Sca-1^+ (HSC-enriched population) and Lin^−c-kit^+Sca-1^− (include myeloid and lymphoid progenitors) cells were purified by FACS-sorting and the deletion efficiency was determined by PCR. Numbers indicate samples from individual mice.

C. Bar graphs summarize the quantifications of Lin^−c-kit^+Sca-1^+ (left panel) and Lin^−c-kit^+Sca-1^− cells (right panel) as a percentage of total BM cells. Data are means ± SD.

D. Variation of body mass during 8 weeks after p(I:C) treatment. Data are means ± SD.

E. Blood count analysis during 8 weeks after p(I:C) treatment (WBC, white blood cells; RBC, red blood cells; PLT, platelets; HGB, haemoglobin). Data are means ± SD.

F. FACS-analysis of blood cell populations (differential) from indicated mouse genotypes (MO, monocytes; GRA, granulocytes; CD19^+, CD19 positive B-cells; CD3^+ , CD3 positive T-cells). Data are means ± SD.

G. FACS analysis of HSC-subpopulations (LT-HSCs, ST-HSCs and MPPs) in the BM of p(I:C)-treated mice. Percentages are depicted in gates (n = 4).

H. FACS analysis of erythroid development in the BM of mice with indicated Stat5-status after p(I:C)-treatment. Numbers are relative percentages of cells in gates (n = 4).
numbers of cells undergoing erythroid differentiation (Fig 6H). In summary, the deletion of Stat5 for 8 weeks was generally well
taken by the adult animals.

High Bcl-2 levels or deletion of Trp53 do not relieve Stat5-dependence

Tumour cells frequently acquire additional mutations after long-
term maintenance in culture. We therefore analysed our Stat5fl/fl
Mx1Cre v-abl++ cell lines after 14 months of continuous culture
whether any spontaneously acquired mutation could release the
necessity for Stat5. When we analysed the cell lines for
expression of Trp53, BclXL and Bcl-2, we found that two cell
lines (#1 and #3) had completely lost the Trp53 protein (Fig 7A).
Loss of Trp53 was described to result in decreased sensitivity
towards imatinib (Wendel et al, 2006). Accordingly, these cells
displayed a 5.7-fold reduced sensitivity towards imatinib (IC50
of 0.98 \( \mu \text{M} \) compared to IC50 = 0.17 \( \mu \text{M} \), data not shown). Cell
line #1 additionally displayed a significant up-regulation of the
Bcl-2 protein and a significant decrease in Stat5 protein
expression. An overexpression of the anti-apoptotic protein
Bcl-2 is found in many cancers contributing to tumourigenesis
and resistance to therapies (Letal et al, 2004; Moon et al, 2009;
Oltersdorf et al, 2005). However, when the residual Stat5 protein
was removed by activation of Cre-recombinase, all cell lines still
underwent a G0/G1 cell cycle arrest followed by cell death (Fig 7B and C). Hence, even in the presence of elevated Bcl-2
protein levels, Stat5 was indispensable for proliferation and
survival of leukaemic cells.

Expression of an imatinib-resistant bcr/abl mutant
(bcr/abl\(^{p210T315I}\)) does not relieve Stat5 dependence

Treatment of bcr/abl-induced CML has been significantly
improved by the availability of imatinib (Druker et al, 2001a,
b). However, some patients acquire mutations in the Bcr/Abl
oncoprotein which render them insensitive to imatinib (Chu
et al, 2005; Griswold et al, 2006). The bcr/abl\(^{p210T315I}\) mutation

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**Figure 7. Leukaemic cells harbouring second hits are still sensitive to Stat5-loss.**

A. Immunoblot analysis of long-term cultured (14 months) Stat5fl/flMx1Cre v-abl++ cell lines for Stat5, Trp53, Bcl-2 and BclXL protein expressions. Acquisition of secondary mutations leading to defective Trp53- and/or Bcl-2-expression in two cell lines is depicted.

B. Cell cycle analysis of two cell lines from (A) which were subjected to Stat5-deletion via IFN-\( \beta \)-treatment (#1, #3). Cell cycle and protein expressions were determined by PI staining in a non-hypotonic buffer.

C. Stat5-deletion by IFN-\( \beta \)-administration suffices to induce apoptosis in secondarily mutated cell lines (#1 and #3). Percentages of apoptotic cells are determined by PI staining in a non-hypotonic buffer.

D. Stat5-deletion via IFN-\( \beta \)-induced cell-death of imatinib-resistant bcr/abl\(^{p210T315I}\) BM-derived cells. Five days after treatment, IFN-\( \beta \)-induced apoptosis of Stat5fl/flMx1Cre bcr/abl\(^{p210T315I}\) leukaemic cells. As a control, 100 nM imatinib is sufficient to eradicate wt
Mx1Cre bcr/abl\(^{p210T315I}\) BM-derived cells (upper middle panel). Numbers show percentages of cells in indicated cell cycle phases.
is among the biggest therapeutic challenges in CML therapy, since it mediates complete resistance to imatinib and all of the next generation Abl kinase inhibitors (Quintas-Cardama et al., 2007; Shah et al., 2002). We therefore decided to test whether cells expressing bcr/abl<sup>p210T315I</sup> require Stat5. Stat5<sup>F0/0</sup>Mx1Cre and Stat5<sup>F0/0</sup>Mx1Cre BM cells were infected with retrovirus encoding bcr/abl<sup>p210T315I</sup> and treated either with imatinib or IFN-β-mediated Stat5-deletion. To ensure survival and proliferation of immature progenitors, cells were maintained in a medium supplemented with SCF, Flt3-ligand (Flt3-L), IgF-1, IL-3, IL-6, GM-SCF and dexamethasone as described previously (Kieslinger et al., 2000). Under this condition the IC<sub>50</sub> for imatinib was 83.4 nM when tested in <i>wt</i> Mx1Cre cells transduced with non-mutated bcr/abl<sup>p210</sup> (Supporting Information Fig 8). As expected <i>wt</i> Mx1Cre and Stat5<sup>F0/0</sup>Mx1Cre cells expressing bcr/abl<sup>p210T315I</sup> did not undergo apoptosis upon imatinib treatment (100 nM) Fig 7D, middle panels). In contrast, bcr/abl<sup>p210T315I</sup>-expressing Stat5<sup>F0/0</sup>Mx1Cre cells showed substantial cell death upon loss of Stat5 (Fig 7D, right panels).

**DISCUSSION**

A key to successful new therapeutic strategies is to identify critical functional nodes in the signalling network downstream of an oncogene. Cancer cells undergo extensive adaptations in their signalling and metabolic pathways. Thereby, they may become dependent on certain genes that are not per se canonical oncogenes. In fact, the activity of these genes may become rate limiting for a cancer cell. The term ‘non-oncogene addiction’ (NOA) has been coined recently to describe this phenomenon. The inhibition of these critical players within the signalling network is predicted to induce system failure and thus the cessation of the malignant state (Luo et al., 2009).

We show here that bcr/abl-transformed leukaemic cells are addicted to Stat5 for maintaining the leukaemic state. Thus, Stat5 fulfills the criteria of an indispensable functional node within the signalling networks downstream of Bcr/Abl. Accordingly, Stat5 represents a potential drug target. The deletion of Stat5 in leukaemic cells resulted in G<sub>s</sub>/G<sub>1</sub> cell cycle arrest followed by apoptosis. Several signalling pathways are activated downstream of Bcr/Abl and contribute to leukaemia development; the long list includes PI3K-isofoms and Ras-dependent pathways. More recent insights highlight the significance of Hedgehog signalling (Dierks et al., 2008; Zhao et al., 2009). In spite of the complexity of the Bcr/Abl-controlled signalling network, Stat5 appears to have a privileged position that is conserved even in the absence of intact Trp53 signalling, as well as in imatinib-resistant cells. This is underscored by the finding that the mere expression of a single Stat5 target genes such as <i>c-myc</i>, <i>bel-xl</i>, <i>bel-2</i>, <i>cyclin D2</i>, <i>cyclin D3</i> or <i>CIS</i> could not replace Stat5 expression. Importantly, the addiction to Stat5 extends to the LSC compartment. LSCs have been characterized in myeloid bcr/abl<sup>p210</sup>-induced leukaemia by their ability to allow for serial transplantation of the disease (Krause et al., 2007; Wang & Dick, 2005). One of the big current therapeutic challenges is to find strategies how to target and eradicate such LSCs. The most frequently used drug in CML therapy—imatinib—induces apoptosis in bcr/abl<sup>L</sup> cells but fails to eradicate LSCs in vivo (Krause & Van Etten, 2007; Neering et al., 2007). In this context, it is worth pointing out that even one of the most dreaded imatinib-resistant mutants of Bcr/Abl—Bcr/ Abl<sup>p210T315I</sup>—remains strictly dependent on Stat5. Hence neither a mutated Bcr/Abl nor the genetic instability associated with the abrogation of Trp53 allowed for the emergence of Stat5-independent leukaemic clones—even in combination with an upregulation of the anti-apoptotic protein Bcl-2.

These observations support the concept that targeting Stat5 provides new therapeutic opportunities. However, a potential therapeutic target is only useful, if upon blockage normal cells are spared at the expense of tumour cells. This issue has recently been addressed: deletion of Stat5 was reasonably well-tolerated. After 4 months WBCs and HCTs were reduced. This was paralleled by increased numbers of actively cycling HSCs (Wang et al., 2009). Our own observation covered a period over 8 weeks and confirmed the overall tolerability of Stat5 deletion, normal haematopoiesis was not compromised to an appreciable extent. We note that there is an apparent discrepancy between these observations and the strong effects associated with the non-conditional ablation of Stat5 (Greben et al., 2008; Hoelbl et al., 2006; Yao et al., 2006). It is, however, obvious that substantial differences can exist between the phenotypic manifestations of a gene defect acting during embryonic and foetal development and the consequences of eliminating a gene in an adult animal. Foetal and adult HSCs, in particular, differ in important features (Kim et al., 2007; Mikkola & Orkin, 2006). Another observation favours the use of Stat5 as potential drug target. We observed that the mere lowering of Stat5 levels in bcr/ abl<sup>p210T315I</sup>-Stat5<sup>F0/0</sup>Mx1Cre cells by IFN-β treatment was sufficient to prevent leukaemia engraftment in secondary recipient animals. This observation again provides evidence for a role of Stat5 in LSCs. It also further supports the concept of Stat5 as a potential drug target. The data suggest that a partial blockage of Stat5—which is well tolerated in normal tissue—may already be deleterious for the bcr/abl<sup>L</sup> cell population. While it is difficult to extrapolate these experiments in mice to patients, at the very least these observations justify the assumption that potential side effects of Stat5 blockage will not a priori preclude their use in clinics. This conjecture is further supported by the development of an inhibitor targeting Jak2. Jak2 is essential for erythropoiesis—but nevertheless, Jak2 inhibitors have successfully entered clinical trials (Hexner et al., 2008; Wernig et al., 2008). For the past decade, the development of signalceptor-based therapies has concentrated on the ‘druggable’ genome which represents proteins with enzymatic functions. There has been a paradigm shift more recently. Many proteins previously considered difficult or impossible to target are thought to be accessible to small molecules, because these can be designed to bind ‘hot-spots’ on contact surfaces and to disrupt protein–protein interaction. The feasibility of this approach is exemplified by binders of the Bcl-2 family such as ABT-737 (Wells & McClendon, 2007). Thus, it is conceivable that Stat proteins may also be targeted by low molecular weight compounds that target the dimeric interphase or interaction
The paper explained

**PROBLEM:**

Acute lymphoid leukaemia (ALL) and chronic myelogenous leukaemia (CML) can be induced by the chimeric fusion gene product Bcr/Abl, a constitutively active tyrosine kinase. A complex signalling network downstream of Bcr/Abl supports proliferation and survival of the leukaemic cells. Bcr/Abl kinase inhibitors (e.g. Imatinib) can hamper these signals and induce cell death but several mutations were described that confer resistance to these inhibitors. Here we tested whether the transcription factors Stat3 and Stat5, acting downstream of Bcr/Abl are critical for leukaemia maintenance and are alternative pharmaceutical targets.

**RESULTS:**

We developed a tumour-specific gene-deletion approach to dissect the roles of Stat3 and Stat5 in Bcr/Abl-induced leukaemia maintenance. We found that both are required for the initial transformation by Bcr/Abl. Once established, only Stat5 is crucial for viability and proliferation of leukaemic myeloid and lymphoid cells. The absolute necessity for Stat5 is conserved in Imatinib-resistant cells and is also maintained when Trp53 signalling is disrupted or Bcl-2 over-expressed.

**IMPACT:**

In many leukaemia patients, effective treatment with Imatinib is hampered by the occurrence of mutations in Bcr/Abl. Our study identified Stat5 as an Achilles’ heel in the signalling network downstream of Bcr/Abl. Thus, inhibition of Stat5—alone or in combination with Bcr/Abl—may provide a novel therapeutic approach for treatment of leukaemia.

MATERIALS AND METHODS

Mice and genotyping

Stat5<sup>fl/fl</sup>Mx1Cre (mixed C57BL/6J/×Sv129), Stat3<sup>fl/fl</sup>Mx1Cre (mixed C57BL/6J/×Sv129), C57BL/6J and Rag2<sup>−/−</sup>/γc<sup>−/−</sup> (C57BL/6J) mice were maintained at the Biomedical Research Institute (Medical University of Vienna) and at the NIH (Bethesda, Maryland), C57BL/6J/×Sv129 F1 (here referred to as B6129F1) at the Institute of Molecular Pathology (IMP, Vienna) under specifically pathogen-free sterile conditions. Genotyping of mice and cells was performed as described previously (Cui et al, 2004). The sensitivities of the Stat5<sup>fl</sup> and Δ PCRs were determined by limited dilution and we are able to detect 100 Stat5<sup>fl</sup> or Stat5<sup>Δ/Δ</sup> cells within the starting material (data not shown). All animal experiments were carried out in accordance with protocols approved by Austrian law.

BM transplants of bcr/abl<sup>210</sup>-infected cells and deletion of Stat5

For BM transplantation studies two different approaches were used. (i) Donor mice (6 weeks of age) were injected i.p. with 5-FU (150 mg/kg body weight). BM cells were co-cultivated on bcr/abl<sup>210</sup> retroviral producer cells for 48 h in the presence of IL-3 (25 ng/ml), IL-6 (50 ng/ml), SCF (50 ng/ml) and 7 μg/ml polybrene and injected via tail vein into lethally irradiated (10 Gy) wt recipients (1 × 10<sup>6</sup> cells/mouse). For

in vivo deletion of Stat5, mice received 400 μg p(1-C) (Sigma) at a single dose 6 weeks post-transplantation. (ii) BM cells from 6 weeks old donor mice were co-cultivated on bcr/abl<sup>210</sup> retroviral producer cells as described above. BM cells (1 × 10<sup>6</sup>) were injected into lethally irradiated B6129F1 recipient mice. BM cells of three diseased animals were pooled and treated with recombinant IFN-β (1000 U/ml; Serotech) for 48 h to delete Stat5. Thereafter cells were transplanted into lethally irradiated secondary recipients (B6129F1).

Deletion of Stat5 and Stat3 in lymphoid leukaemic cell lines

For in vivo deletion of Stat5 or Stat3 in lymphoid leukaemia studies, 1 × 10<sup>6</sup> or 1 × 10<sup>5</sup> u-abl<sup>+</sup> cells were injected via tail vein into Rag2<sup>−/−</sup>/γc<sup>−/−</sup> or C57BL/6J mice, respectively. From day 7 on, mice received 400 μg p(1-C) i.p. every 4 days to induce Stat5-deletion in the transplanted leukaemic cells. Mice injected with PBS served as controls. Upon signs of sickness (decreased mobility, weight loss and scruffy fur), mice were sacrificed and lymphatic organs were analysed for leukaemic cell (CD19<sup>+</sup>, B220<sup>+</sup>) infiltrations by flow cytometry.

For in vitro deletion of Stat5, Stat5<sup>fl/fl</sup>Mx1Cre u-abl<sup>+</sup> cells were seeded at a density of 3 × 10<sup>5</sup> cells/ml and incubated for 48 h in 1000 U/ml recombinant IFN-β (Serotech) in complete RPMI. Stat5<sup>fl/fl</sup> u-abl<sup>+</sup> cells treated with IFN-β and untreated Stat5<sup>fl/fl</sup>Mx1Cre u-abl<sup>+</sup> served as controls. Cells were analysed by flow cytometry for cell cycle progression and apoptosis every day.

Statistical analysis

Statistics were carried out using Student’s t-test or Mann-Whitney U-test as appropriate. Transplant experiments were analysed for statistical significance using log-rank test. Data are presented as averages ± SD and were analysed by GraphPad<sup>®</sup> and SPSS<sup>®</sup> software. Additional information regarding analysis tissue culture conditions, immunoblotting, transformation and imatinib sensibility assays is available within the Supporting Information.
Author contributions
V.S. conceived the project and V.S. and A.H. wrote the paper. A.H., C.S., B.K., S.F., F.G., W.W. and G.S. performed the experiments on Stat5 in vitro and on lymphoid leukaemia in vivo. A.H. and C.S. evaluated data and performed statistics. A.H., B.K., B.Z., M.K. executed in vivo studies on Stat5 deletion in myeloid leukaemia. M.A.H. conducted the experiments on Stat3. Stat5<sup>−/−</sup> mice were contributed by L.H., Stat5<sup>−/−</sup> mice by V.P. H.B. and R.M. significantly contributed material and valuable scientific input.

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Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

For more information
Jak-Stat Signalling—from Basics to Disease: www.jak-stat.at

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