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BCR-ABL uncouples canonical JAK2-STAT5 signaling in chronic myeloid leukemia

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Constitutive activation of STAT5 is critical for the maintenance of chronic myeloid leukemia (CML) characterized by the BCR-ABL oncoprotein. Tyrosine kinase inhibitors (TKIs) for the STAT5-activating kinase JAK2 have been discussed as a treatment option for CML patients. Using murine leukemia models combined with inducible ablation of JAK2, we show JAK2 dependence for initial lymphoid transformation, which is lost once leukemia is established. In contrast, initial myeloid transformation and leukemia maintenance were independent of JAK2. Nevertheless, several JAK2 TKIs induced apoptosis in BCR-ABL$^+$ cells irrespective of the presence of JAK2. This is caused by the previously unknown direct 'off-target' inhibition of BCR-ABL. Cellular and enzymatic analyses suggest that BCR-ABL phosphorylates STAT5 directly. Our findings suggest uncoupling of the canonical JAK2-STAT5 module upon BCR-ABL expression, thereby making JAK2 targeting dispensable. Thus, attempts to pharmacologically target STAT5 in BCR-ABL$^+$ diseases need to focus on STAT5 itself.

The BCR-ABL fusion oncoprotein is the product of the Philadelphia chromosome translocation and the defining molecular event of CML[1,2]. BCR-ABL has constitutive tyrosine kinase activity[3] that can be inhibited effectively by small molecule TKIs such as imatinib (Gleevec) and others[4,5]. The majority of patients treated with imatinib in the early chronic phase of CML enter durable remission[6,7]. Conversely, some patients do not respond (primary resistance) or lose previously achieved responses (secondary resistance). The best understood mechanisms of secondary resistance are point mutations in the BCR-ABL kinase domain that can render the kinase insensitive to imatinib inhibition[8,9]. Imatinib resistance can lead to disease progression to the accelerated and terminal blast crisis phase of CML, with poor response to BCR-ABL TKI therapy and short median survival times[10]. Therefore, targeting strategies must be combined to further increase therapeutic success.

Aside from BCR-ABL expression, activation of the transcription factor STAT5 is a signaling hallmark of CML[11–13]. Of the several dozen intensively characterized mediators of BCR-ABL action, STAT5 is among the few critical for leukemia initiation and the very few critical for leukemia maintenance, therefore qualifying as an attractive drug target[14–18]. Additionally, STAT5 contributes to CML progression and imatinib resistance[19]. STAT5, however, is a very challenging direct drug target as it is a transcription factor lacking an enzymatic domain that can be targeted readily with a small molecule inhibitor. Conversely, STAT5 activation is achieved by phosphorylation of a single tyrosine residue (Tyr694) that leads to SH2 domain–dependent dimerization and transcriptional activation. Therefore, STAT5 signaling could be inhibited by targeting the upstream kinase that phosphorylates Tyr694 (ref. 20). JAK kinases, in particular JAK2, are the predominant activators of STAT5 (refs. 21,22). JAK2 is constitutively activated by point mutations and chromosomal translocations in different myeloproliferative neoplasms that lead to constitutive STAT5 activation, including polycythemia vera, essential thrombocythemia, primary myelofibrosis, myelodysplastic syndrome and primary acute myeloid leukemia[23]. Even in the context of solid tumors with high STAT activation, JAK2 TKIs may represent a valuable strategy as several human tumor cell lines respond with growth inhibition and apoptosis to JAK2 TKI treatment[24]. Because STAT5 activation is necessary in Ph$^+$ cells, several reports suggest the use of JAK2 TKIs for the treatment of Ph$^+$ cells[25–27]. Pharmacological inhibition or knockdown of JAK2 leads to lower viability of imatinib-sensitive and imatinib-resistant CML cells[28]. These studies prompted us to undertake a study using JAK2-deficient mice in CML mouse models and to investigate the mechanism of action of newly available JAK2 TKIs in BCR-ABL–induced STAT5 activation and leukemogenesis[29–31]. Here we describe how the JAK2-STAT5 module is differentially required in initial leukemic transformation by BCR-ABL. JAK2 is involved in lymphoid but not myeloid transformation. In contrast, disease maintenance was unaltered after ablation of JAK2 but continuously required STAT5. Biochemical and pharmacological studies indicated that rewiring of signaling in CML cells uncouples the JAK2-STAT5 module and puts STAT5 under direct control of BCR-ABL.

RESULTS

Treatment of *BCR-ABL1*$^+$ cells with imatinib and JAK2 TKIs

We first investigated whether simultaneous inhibition of JAK2 and BCR-ABL exerted additive or synergistic growth inhibitory effects. The human cell lines K562 and Ku812 (both expressing p210$^{BCR-ABL}$) and Sup-B15 (expressing p185$^{BCR-ABL}$), as well as freshly prepared murine cells expressing p185$^{BCR-ABL}$, were exposed to increasing concentrations of imatinib with and without JAK2 TKIs. An overview of all TKIs used in this manuscript including information regarding potency and stage of clinical development is in **Supplementary Results**, **Supplementary Table 1**. We were

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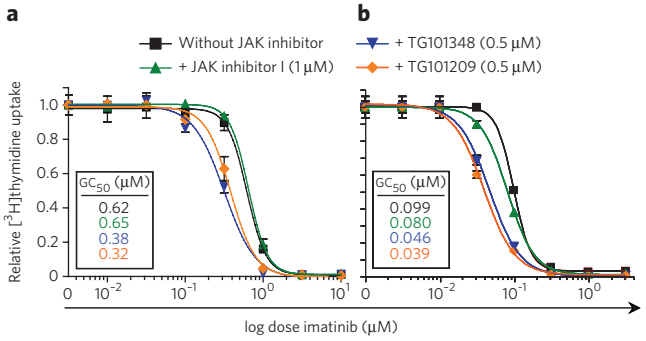

Figure 1 | Combination treatment of JAK2 TKIs and imatinib in BCR-ABL$^+$ human cell lines. (**a**,**b**) Dose-response curves for imatinib alone and combined with JAK inhibitor I (1 μM), TG101348 (0.5 μM) and TG101209 (0.5 μM) were analyzed in p210$^{BCR\text{-}ABL+}$ human CML cell lines K562 (**a**) and KU812 (**b**). Data are mean ± s.d. from a representative experiment done in triplicate.

interested in the compounds JAK inhibitor I and TG101209, as well as TG101348, which is currently used in clinical trials[32]. Dose-response curves for imatinib were monitored to compare the half-maximum growth inhibitory concentrations (GC_{50}) with and without different JAK2 TKIs at concentrations that completely inhibited JAK2 kinase activity (**Supplementary Fig. 1a**). We observed a reduction of more than 50% in GC_{50} values for imatinib in K562 and Ku812 cells upon simultaneous treatment with either TG compound but not with JAK inhibitor I (**Fig. 1a**,**b**). We obtained comparable results in murine p185$^{BCR\text{-}ABL}$-expressing cells (**Supplementary Fig. 1b**). The p185$^{BCR\text{-}ABL}$-expressing human cell line Sup-B15 showed low imatinib sensitivity, but addition of either of the two TG compounds led to greater inhibition of cell proliferation by imatinib (**Supplementary Fig. 1c**). Apoptosis assays using annexin V and propidium iodide staining confirmed these observations (**Supplementary Fig. 1d**). We found no correlation between drug sensitivity and expression of JAK2 and STAT5 (**Supplementary Fig. 1e**). These findings suggest that TG101348 and TG101209 led to greater imatinib sensitivity of all BCR-ABL–dependent cell lines under investigation.

Role of JAK2 in initial transformation by BCR-ABL

To delineate the role of JAK2 in BCR-ABL–driven leukemia, we crossed *Jak2$^{fl/fl}$* mice with *Mx1-Cre* mice. This enabled efficient, inducible deletion of *Jak2* in hematopoietic cells by poly(I:C) or tamoxifen treatment, respectively[18,33]. We investigated BCR-ABL–dependent transformation using colony-forming assays. Bone marrow derived from *Jak2$^{fl/fl}$Mx1-Cre*, *Jak2$^{fl/+}$Mx1-Cre* and *Jak2$^{fl/+}$* mice treated with poly(I:C) (resulting genotypes, *Jak2$^{\Delta/\Delta}$*, *Jak2$^{\Delta/+}$* and *Jak2$^{fl/+}$*, respectively; **Supplementary Fig. 2a**) were infected with retroviruses encoding different oncogenic ABL variants. p210$^{BCR\text{-}ABL}$ causes myeloid cell transformation, whereas p185$^{BCR\text{-}ABL}$ and the murine viral oncoprotein p160$^{v\text{-}ABL}$ support the transformation and outgrowth of lymphoid pro-B cell lines. *Jak2$^{\Delta/\Delta}$* cells could form colonies only upon infection with *p210$^{BCR\text{-}ABL1}$* (**Fig. 2a**), whereas infection with *p160$^{v\text{-}Abl}$* did not allow the outgrowth of growth factor–independent colonies (**Fig. 2b**). Deletion of one allele of *Jak2* led to a strong decrease in colony numbers compared with wild type, indicating a gene-dosage effect. Notably, the degree of lymphoid transformation of *Jak2$^{\Delta/\Delta}$* cells varied with genetic background. JAK2 dependence of lymphoid transformation was strongest in mixed sw129-Bl6-C57 bone marrow and was lower in a pure C57/Bl6 background. To further investigate these findings we used fetal liver cells derived from *Jak2*-deficient embryonic day 12.5 (E12.5) embryos on a sw129-Bl6-C57 background. This procedure was necessary because of the embryonic lethality of conventional *Jak2*-deficient mice[34]. Again, hematopoietic cells devoid of *Jak2* cannot be transformed by the oncogene *p160$^{v\text{-}Abl}$* or by *p185$^{BCR\text{-}ABL1}$*, whereas the outgrowth of p210$^{BCR\text{-}ABL}$-expressing colonies was not affected (**Fig. 2c**). These data show that JAK2 is involved in the initial transformation of lymphoid cells by *BCR-ABL1* oncogenes but is dispensable for myeloid transformation. To investigate possible signaling differences between p210$^{BCR\text{-}ABL}$ and p185$^{BCR\text{-}ABL}$, we used Ba/F3 cells. STAT5 phosphorylation was greater in Ba/F3 *p210$^{BCR\text{-}ABL1}$* cells compared with Ba/F3 *p185$^{BCR\text{-}ABL1}$* cells (**Fig. 2d** and

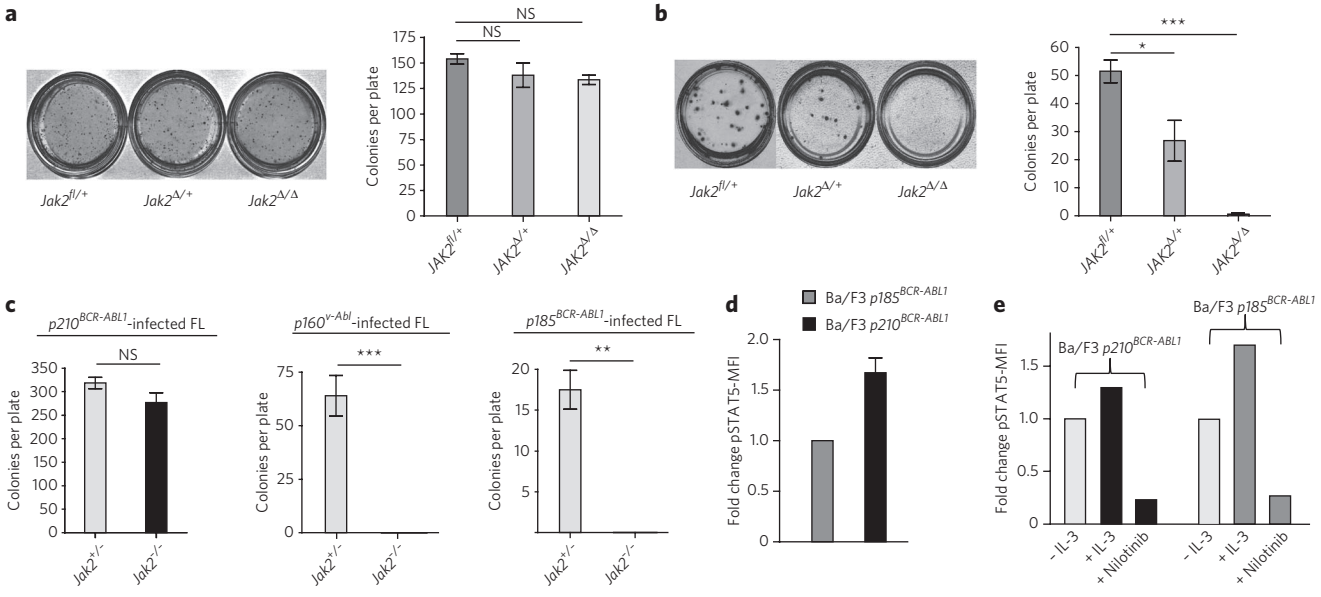

Figure 2 | JAK2 is essential for initial lymphoid but not myeloid transformation. (**a**,**b**) Colony-formation assay of *Jak2$^{fl/+}$*, *Jak2$^{fl/\Delta}$* and *Jak2$^{\Delta/\Delta}$* BM infected with *p210$^{BCR\text{-}ABL1}$* (**a**) or *p160$^{v\text{-}Abl}$* (**b**). (**c**) Colony-formation assay of fetal liver (FL) cells derived from *Jak$^{+/-}$* and *Jak2$^{-/-}$* embryos infected with oncogenes. (**d**) Intracellular pSTAT5 FACS of Ba/F3 cells expressing p185$^{BCR\text{-}ABL}$ or p210$^{BCR\text{-}ABL}$. Fold difference in mean fluorescence intensity (MFI) of pSTAT5. (**e**) Ba/F3 *p210$^{BCR\text{-}ABL1}$* and Ba/F3 *p185$^{BCR\text{-}ABL1}$* cells were treated with IL-3 or 1,000 nM nilotinib. Fold change in pSTAT5 MFI compared with untreated cells (-IL-3). Data are mean ± s.d. NS, not significant; *P < 0.05, **P < 0.01, ***P < 0.001.

Supplementary Fig. 2b). In agreement with this observation, we found that IL-3 stimulation led to a stronger additive STAT5 phosphorylation in Ba/F3 cells expressing p185$^{BCR-ABL}$ (**Fig. 2e**). This led us to conclude that p210$^{BCR-ABL}$ has a greater ability to induce STAT5 phosphorylation in Ba/F3 cells, a finding that may explain the different potency of p210$^{BCR-ABL}$ and p185$^{BCR-ABL}$ in transforming *Jak2*-deficient cells.

Viability of Ph$^+$ lymphoid cells does not depend on JAK2

We next investigated whether the importance of JAK2 for lymphoid leukemia initiation by p160^{v-ABL} or p185$^{BCR-ABL}$ is maintained after the initial transformation event. We generated p160^{v-ABL}- or p185$^{BCR-ABL}$-transformed polyclonal cell populations from the bone marrow of *Jak2$^{fl/fl}$Mx1-Cre* and *Jak2$^{fl/fl}$* mice. At this time point the bone marrow cells still expressed JAK2, as no CRE expression had been induced by poly(I:C) or interferon treatment. These cells did not differ in expression of pro-B cell surface markers or in their proliferation capacity and represent a polyclonal mixture of p160^{v-ABL}- or p185$^{BCR-ABL}$-transformed cells (**Supplementary Fig. 3a–c**). After establishment, CRE-mediated deletion of *Jak2* was induced by interferon-β (IFN-β) *in vitro*, and efficient *Jak2* deletion was verified by immunoblotting and PCR (**Fig. 3a** and **Supplementary Fig. 3d**). Because proliferation and viability of *p160^{v-Abl+}* cell lines depends on STAT5, we included *p160^{v-Abl} Stat5a$^{fl/fl}$Mx1-Cre* cells as control[17,18]. Whereas Cre-mediated *Stat5a* deletion readily induced growth arrest and apoptosis, we observed no changes upon deletion of *Jak2* in p160^{v-ABL}- or p185$^{BCR-ABL}$-transformed cells (**Fig. 3b–d**). We further characterized *Jak2*-deleted cells and found no differences compared-with maternal cells. Neither the expression of pro-B cell surface markers nor cell proliferation, as measured via carboxyfluorescein succinimidyl ester (CFSE) staining and [^{3}H]thymidine incorporation assays, was altered (**Fig. 3e** and **Supplementary Fig. 3e**,**f**). Thus, in contrast to STAT5, JAK2 seems dispensable for cell viability and growth of Ph$^+$ lymphoid cells after the initial transformation event. This suggests uncoupling of the JAK2-STAT5 signaling module in lymphoid cells driven by p160^{v-ABL} or p185$^{BCR-ABL}$.

Deletion of *Jak2* does not affect CML disease progression

Although JAK2 is dispensable for initial myeloid transformation *in vitro*, an essential position of JAK2 may be envisioned *in vivo*. Conversely, the observed dependence on JAK2 of initial lymphoid transformation *in vitro* could also indicate that JAK2 is required for lymphoid leukemia maintenance *in vivo*. To test these possibilities, we injected three individually derived p160^{v-ABL}- and p185$^{BCR-ABL}$-transformed *Jak2$^{fl/fl}$* cell pools and their Jak2-deleted counterparts (*Jak2$^{\Delta/\Delta}$*) into RAG2$^{-/-}$ γc$^{-/-}$ mice and monitored leukemia development. Leukemia evolved with no differences in disease latency between genotypes (**Fig. 4a**,**b**). To study the impact of *Jak2* deletion on myeloid leukemia formation, we used bone marrow derived from *Jak2$^{fl/fl}$* and *Jak2$^{fl/fl}$CreERT2* mice. Tamoxifen treatment led to efficient deletion of *Jak2* in the bone marrow, which was subsequently infected with a retrovirus encoding *p210$^{BCR-ABL1}$* (**Fig. 4c**). The infected bone marrow was transplanted into NOG mice, and disease onset was monitored (**Fig. 4d**). *Jak2* deletion did not influence disease latency or expression of surface markers Gr1, Mac1, c-Kit and Sca1 (**Fig. 4d** and **Supplementary Fig. 4a**). We confirmed deletion of *Jak2* by PCR of leukemic cells derived from spleens from the diseased mice, verifying the contribution to the leukemic population (**Fig. 4e**). Collectively, these findings rule out the possibility that JAK2 has a substantial role in BCR-ABL–driven lymphoid and myeloid leukemia maintenance *in vivo*.

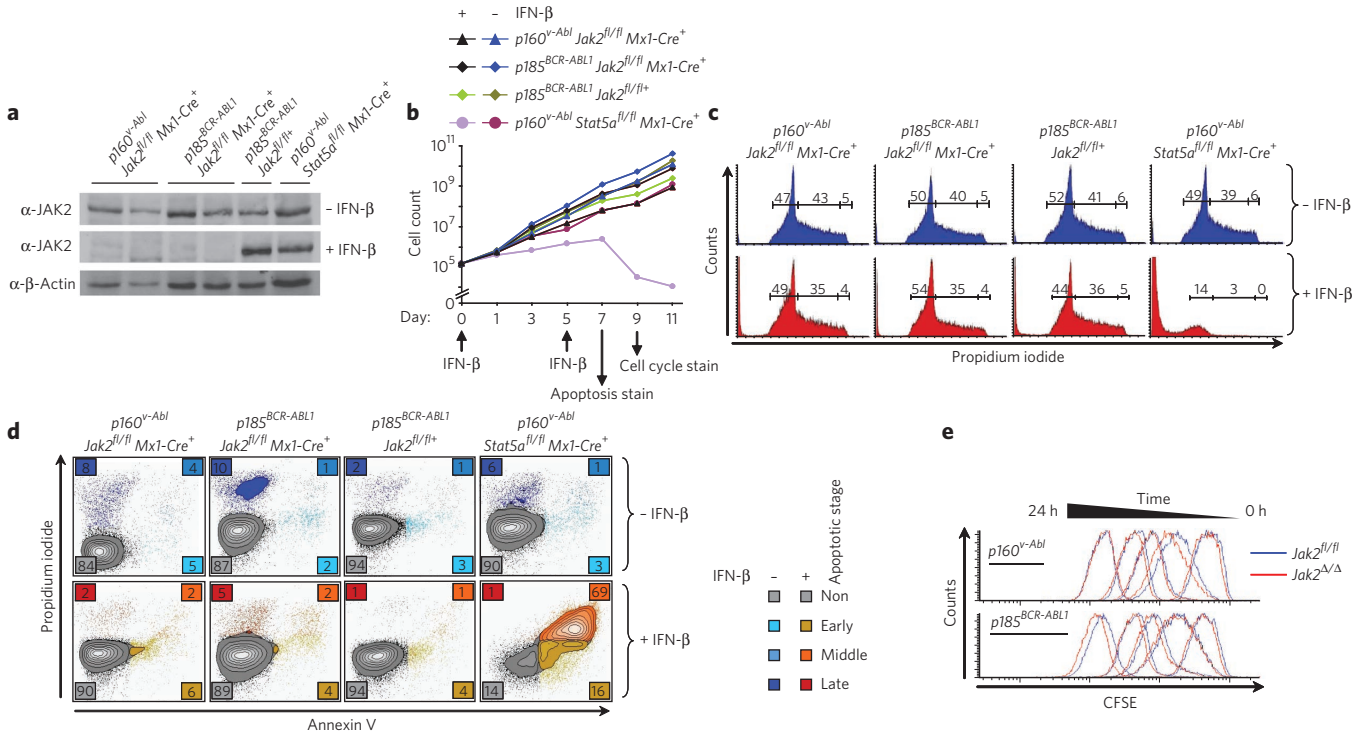

Figure 3 | JAK2 is not required for cell viability of transformed cells *in vitro*. (**a**) Immunoblot verifying IFN-β-induced deletion of *Jak2* in *p160^{v-Abl+}* and *p185$^{BCR-ABL1+}$* cells. Raw data is in **Supplementary Figure 8**. (**b**) Growth curves of *p160^{v-Abl+}* and *p185$^{BCR-ABL1+}$Jak2$^{fl/fl}$Mx1-Cre* cells after *in vitro* deletion of *Jak2*. *p185$^{BCR-ABL1+}$Jak2$^{fl/fl}$* and *p160^{v-Abl+}Stat5a$^{fl/fl}$Mx-1 Cre* cell lines were negative and positive controls, respectively. (**c**) Cell cycle profiles of cell lines 9 d after start of IFN-β treatment and initiation of *Jak2* deletion. Numbers, percentage of cells in G_0/G_1, S or M phase. (**d**) Apoptosis stain of cell lines 7 d after initiation of IFN-β treatment. Propidium iodide$^-$–annexin V$^+$ cells, early apoptosis; propidium iodide$^+$–annexin V$^+$ cells, middle apoptosis; propidium iodide$^+$–annexin V$^-$ cells, late apoptosis. Percentages of each apoptotic stage are in corner of each blot. (**e**) Proliferation assay of *p160$^{v-Abl1+}$* and *p185$^{BCR-ABL1+}$Jak2$^{fl/fl}$* and *Jak2$^{\Delta/\Delta}$* cells via CFSE dilution staining over 24 h.

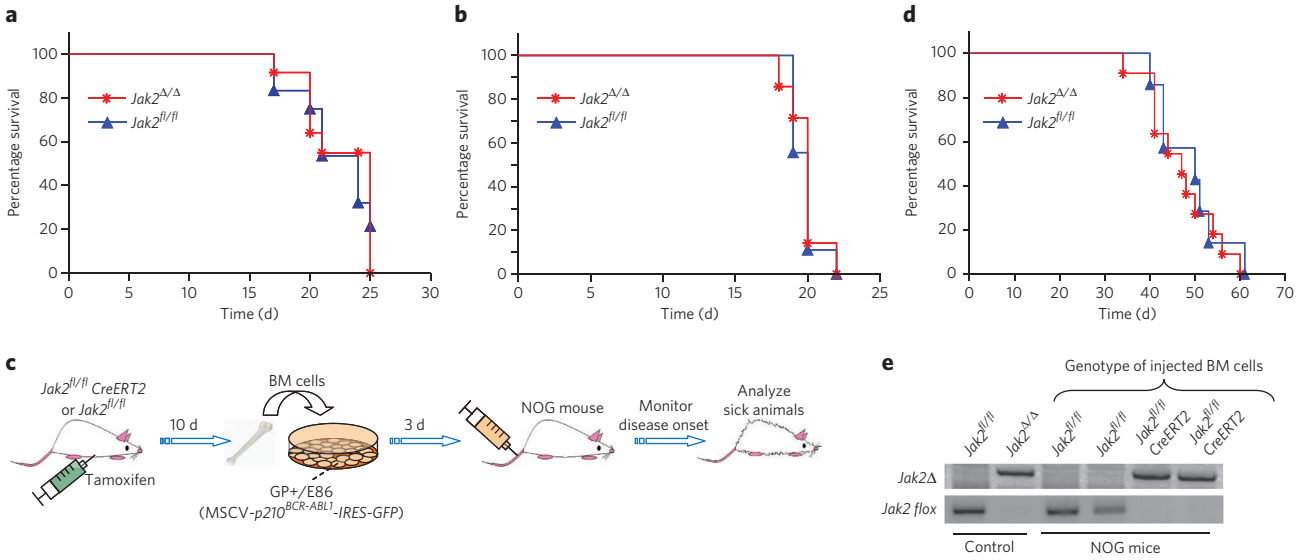

Figure 4 | Deletion of *Jak2* had no effect on lymphoid and myeloid leukemia development *in vivo*. (**a**,**b**) Kaplan Meier blot of Rag2$^{-/-}$ γc$^{-/-}$ mice transplanted with p160$^{v\text{-ABL}}$-transformed (n = 8 per genotype; **a**) and p185$^{BCR\text{-}ABL}$-transformed *Jak2*$^{fl/fl}$ (n = 9) or *Jak2*$^{Δ/Δ}$ (n = 7) cells (**b**). (**c**) Procedure used to infect *Jak2*$^{Δ/Δ}$ BM cells with *p210*$^{BCR\text{-}ABL1}$ and re-injection into NOG mice to monitor disease onset. (**d**) Kaplan Meier blot of NOG mice injected with 1.7 × 10^7 *Jak2*$^{fl/fl}$ (n = 7) or *Jak2*-deficient (n = 11) BM cells infected with MSCV-p210$^{BCR\text{-}ABL}$-IRES-GFP. (**e**) PCR analysis of *Jak2* and *Jak2 flox* allele in leukemic cells derived from diseased NOG mice engrafted with *p210*$^{BCR\text{-}ABL1+}$*Jak2*$^{fl/fl}$ or *Jak2*$^{Δ/Δ}$ BM. Raw data is in **Supplementary Figure 8**.

BCR-ABL is an off target of some JAK2 TKIs

Our results indicated that JAK2 is not essential for BCR-ABL–dependent leukemia maintenance. In contrast, we and others have shown growth inhibition of *BCR-ABL1*$^+$ cells by JAK2 kinase inhibitors (**Fig. 1**)[28,35]. We thus used our conditional *Jak2* knockout cell lines to test whether JAK2 is required for growth inhibition and measured dose response curves for imatinib and JAK2 TKIs in *p160*$^{v\text{-}Abl+}$ and *p185*$^{BCR\text{-}ABL1+}$*Jak2*$^{fl/fl}$ and *Jak2*$^{Δ/Δ}$ cells. The GC_{50} values for all TKIs were unchanged irrespective of whether JAK2 was expressed (**Fig. 5a** and **Supplementary Fig. 5a**). Inhibition of proliferation by JAK2 TKIs without JAK2 must therefore represent an off-target effect. Although TG101348, TG101209 and JAK inhibitor I have similar IC_{50} values for JAK2 *in vitro*, the GC_{50} of JAK inhibitor I was much higher (**Fig. 5a**). This indicated that the suspected 'off-target' is inhibited with different efficiencies by the JAK2 TKIs. To define whether the off-target effects induced by JAK2 TKIs represent nonspecific cytotoxic effects or impinge on inhibition of STAT5 tyrosine phosphorylation, we used intracellular fluorescence-activated cell sorting (FACS) staining for phospho-STAT5 (pSTAT5) after treatment with different concentrations of JAK2 TKIs. Irrespective of the concentration applied, pSTAT5 expression did not differ between *Jak2*$^{fl/fl}$ and *Jak2*$^{Δ/Δ}$ cells (**Fig. 5b**,**c**, **Supplementary Fig. 5b** and **Supplementary Table 2**). We observed that STAT5 phosphorylation was lower for concentrations of the TG compounds and AG490 greater than their GC_{50} values (**Fig. 5c**). In contrast, even with 100 μM JAK inhibitor I, pSTAT5 expression remained unaffected. In addition, deletion of JAK2 did not change imatinib sensitivity (**Fig. 5c** and **Supplementary Fig. 5a**). As the off-target effects of JAK2 TKIs in *BCR-ABL1*$^+$ cell lines caused inhibition of STAT5 phosphorylation, obvious candidates are the other members of the JAK kinase family and BCR-ABL. When testing the latter possibility, we observed that cellular BCR-ABL activity and BCR-ABL autophosphorylation were inhibited with IC_{50} values of 1.3 μM and 8.7 μM, respectively, for TG101209 and 2.5 μM and 10.6 μM, respectively, for TG101348 (**Fig. 5d**). Furthermore, both TG101209 and TG101348 inhibited BCR-ABL kinase activity *in vitro* with IC_{50} values of 1.0 μM and 2.3 μM, respectively (**Fig. 5e**). We also included the JAK1 and JAK2 inhibitor INCB-018424 (ruxolitinib) in our analysis, as this compound is now in phase III clinical trials for myelofibrisis and may be the first JAK inhibitor to obtain regulatory approval[29]. In contrast to the TG compounds, INCB-018424 and JAK inhibitor I did not inhibit BCR-ABL activity at concentrations up to 20 μM (**Fig. 5e**). This lack of an inhibitory effect on BCR-ABL activity is in agreement with our results showing no decrease in pSTAT5 after treatment with JAK inhibitor I (**Fig. 5c**). It also explains the lack of changes in GC_{50} values upon combination with imatinib (**Fig. 1b–e**). Finally, both TG101209 and TG101348 strongly induced apoptosis in five different BCR-ABL–expressing cell lines at 2.0 μM, whereas JAK inhibitor I or INCB-018424 had no effect at 5.0 μM (**Fig. 5f** and **Supplementary Fig. 5c**). Collectively, these data show that growth inhibition and induction of apoptosis of BCR-ABL$^+$ cells by certain JAK2 TKIs at low micromolar concentrations are correlated with their ability to inhibit BCR-ABL kinase activity.

STAT5 activation depends only on BCR-ABL kinase activity

Next, we tested whether JAK2 signaling is required for STAT5 activation in Ba/F3 cells. Treatment of Ba/F3 cells expressing either p210$^{BCR\text{-}ABL}$ or p185$^{BCR\text{-}ABL}$ with different JAK2 TKIs did not affect STAT5 activation, whereas the parental Ba/F3 cell line showed strong inhibition of STAT5 phosphorylation upon JAK2 inhibition (**Fig. 6a**). Thus, expression of BCR-ABL made Ba/F3 cells refractory to JAK2 inhibition, suggesting that STAT5 is under dominant control of a different activator and that the JAK2-STAT5 axis is largely uncoupled.

As mentioned above, in our conditional *Jak2* knockout model, another JAK kinase family member (JAK1, JAK3 or TYK2) may compensate for the lack of JAK2 and activate STAT5 and thereby account for the off-target effect of JAK2 TKIs. We carried out short interfering RNA (siRNA)-mediated knockdown of all four JAK kinases alone or in combination in CML cell lines K562 and Ku812. Whereas K562 cells mainly express JAK2 and TYK2, Ku812 cells express all four JAK kinases. Both knockdown of individual and combinations of JAK kinases led to efficient downregulation

of target proteins (**Fig. 6b** and **Supplementary Fig. 6a**). Neither individual knockdown of single JAK kinases nor combined knockdown of all four JAK kinases led to lower STAT5 phosphorylation compared with wild type (**Fig. 6b** and **Supplementary Fig. 6a**). In an independent line of investigation, we treated K562 cells with JAK2 TKIs. We used TG101348 at different concentrations to monitor JAK2-selective (25 nM), JAK2-JAK1-TYK2–selective (250 nM) and pan-JAK (1,000 nM) effects. We further used JAK inhibitor I at concentrations that inhibited all four JAK kinases. We used both JAK2 TKIs at concentrations at which BCR-ABL kinase activity was not inhibited (**Fig. 5e**). Tyrosine kinases of the Src family have been implicated in STAT5 phosphorylation (reviewed in ref. 36). To inhibit SRC kinases, we used the pan-SRC inhibitor SU6656 at 500 nM (ref. 37). At this concentration, all SRC

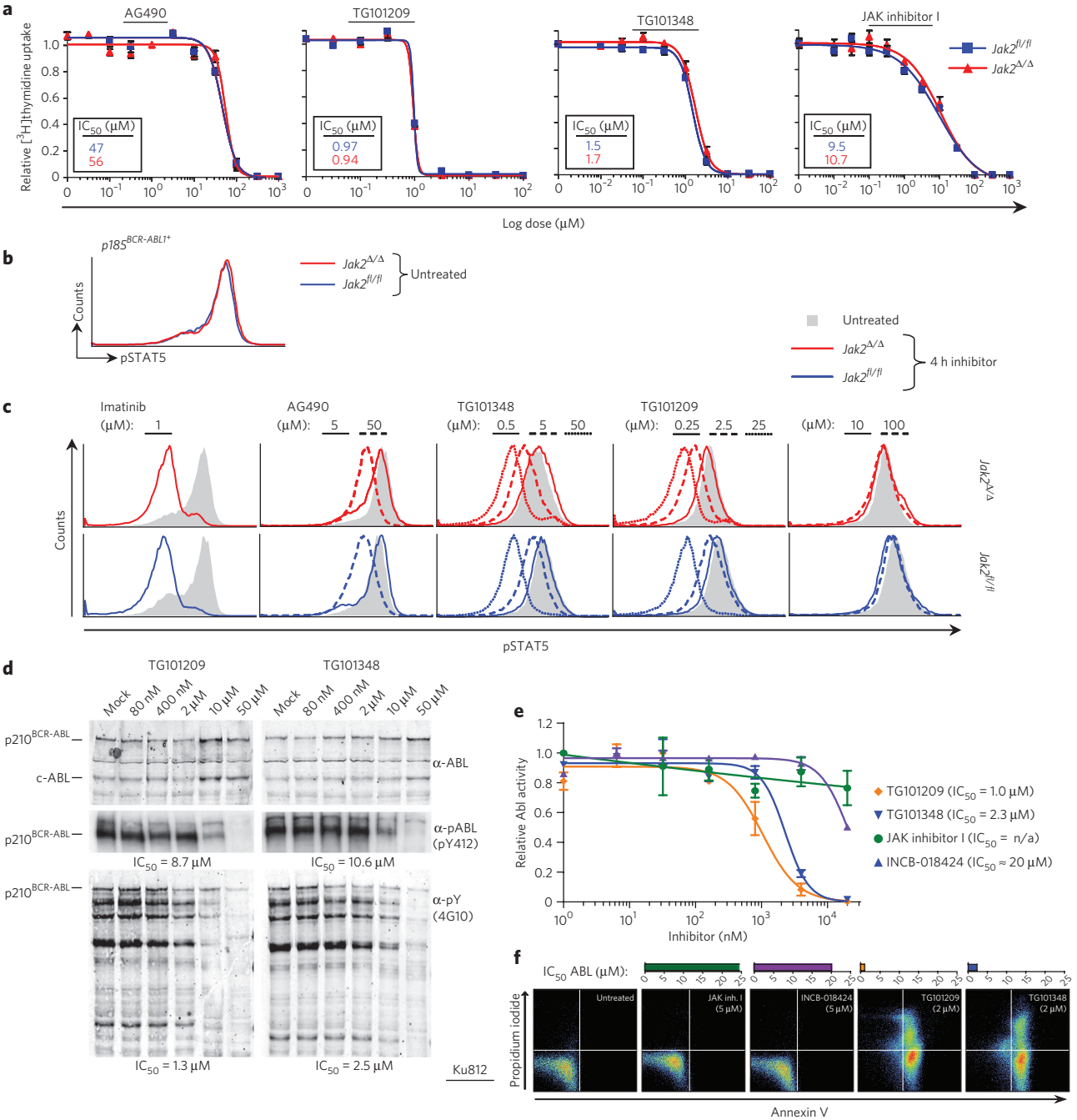

Figure 5 | Deletion of *Jak2* did not influence growth inhibition induced by JAK2 TKI treatment of BCR-ABL1⁺ cells. (**a**) Dose-response curves of *Jak2*$^{fl/fl}$ and *Jak2*$^{\Delta/\Delta}$ cell lines transformed by *p160*$^{v\text{-ABL}}$ (n = 2 per genotype) or *p185*$^{BCR\text{-}ABL^+}$ (n = 2 per genotype) for TKIs AG490, TG101209, TG101348 and JAK inhibitor I. Data are mean ± s.d. of all four cell lines. (**b**,**c**) Intracellular FACS staining for pSTAT5 in *p185*$^{BCR\text{-}ABL^+}$ *Jak2*$^{fl/fl}$ and *Jak2*$^{\Delta/\Delta}$ cells. Histograms, pSTAT5 concentration of untreated cells (**b**) and changes after 4-h treatment with TKIs (**c**). (**d**) Immunoblot for ABL, pABL and phosphotyrosine (pY) (4G10) of *p210*$^{BCR\text{-}ABL^+}$ cell line K562 after treatment with the JAK2 inhibitors TG101209 and TG101348. pABL and pY amounts were quantified, and IC_{50} values from sigmoidal dose-response curves (not shown) were calculated. Raw data is in **Supplementary Figure 8**. (**e**) *In vitro* kinase assay of inhibition of ABL kinase by TKIs INCB-018424, JAK inhibitor I, TG101348 and TG101209. (**f**) Ku812 cells were treated for 72 h with indicated JAK2 TKIs. FACS blots, apoptotic stages of cells measured via annexin V–propidium iodide staining. Top, IC_{50} values of applied TKIs for target BCR-ABL.

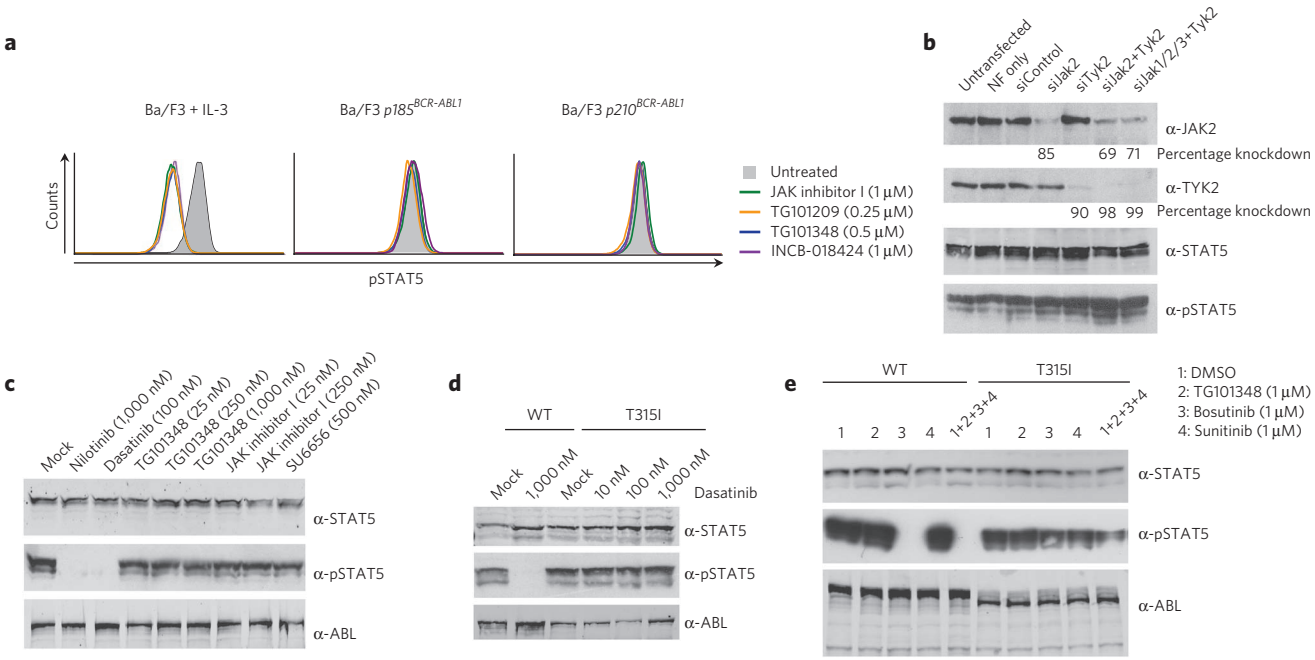

Figure 6 | Evidence that BCR-ABL phosphorylates STAT5. (**a**) Intracellular FACS staining for pSTAT5 of indicated Ba/F3 cell lines with different JAK2 TKIs. (**b**) Immunoblot of JAK2, TYK2, STAT5 and pSTAT5 in K562 cells after siRNA-mediated knockdown of respective kinases. Knockdown relative to controls are indicated. (**c**) Immunoblot of STAT5, pSTAT5 and ABL in K562 cells after treatment with TKI nilotinib, dasatinib, TG101348, JAK inhibitor I or SU6656. (**d**,**e**) Immunoblot of Ba/F3 cells expressing either wild-type $p210^{BCR-ABL}$ or BCR-ABL TKI-resistant $p210^{BCR-ABL}$ T315I mutant. Expression of STAT5, pSTAT5 and ABL after treatment with 10 nM, 100 nM or 1,000 nM dasatinib (**d**) or DMSO, TG101348, bosutinib or sunitinib (**e**, 1 μM each). Raw immunoblot data of **b**–**e** is in **Supplementary Figures 8** and **9**.

kinases but LCK (which is not expressed in myeloid cells) are completely inhibited, whereas BCR-ABL activity is markedly inhibited only at concentrations >1 μM (**Supplementary Fig. 6b**)[37]. As positive controls we used the BCR-ABL TKIs nilotinib and dasatinib, which abrogated STAT5 phosphorylation and led to much lower total tyrosine phosphorylation, indicating efficient inhibition of BCR-ABL. In contrast, TG101348, JAK inhibitor I and SU6656 did not inhibit STAT5 phosphorylation (**Fig. 6c** and **Supplementary Fig. 6c**). These experiments show that STAT5 phosphorylation is independent of JAK or SRC kinase activity. To exclude possible contributions of other tyrosine kinases, we used Ba/F3 cells expressing BCR-ABL T315I. The T315I mutation renders BCR-ABL resistant to all available BCR-ABL TKIs. Dasatinib has a very broad specificity and inhibits the activity of 51 tyrosine kinases of a panel of 80 tyrosine kinases at 1 μM to >90% (ref. 38). We observed no effect on STAT5 phosphorylation in BCR-ABL T315I cells with 1 μM dasatinib (**Fig. 6d** and **Supplementary Fig. 6d**). In contrast, STAT5 phosphorylation was abrogated by 1 μM dasatinib in Ba/F3 cells expressing wild-type BCR-ABL (**Fig. 6d**). The combination of bosutinib (with even broader specificity than dasatinib)[39], the broad-specificity receptor tyrosine kinase inhibitor sunitinib and TG101348 did not block STAT5 activation in BCR-ABL T315I cells (**Fig. 6e** and **Supplementary Fig. 6e**). These data provide evidence that phosphorylation of STAT5 is maintained even under conditions in which most if not all tyrosine kinases other than BCR-ABL T315I are inhibited. Conversely, only BCR-ABL inhibition itself efficiently abrogated STAT5 activation. Although our experiments do not provide evidence for the involvement of additional kinases, it is impossible to completely exclude this possibility.

BCR-ABL phosphorylates STAT5 as efficiently as CRKL

As the data presented above provided evidence for direct phosphorylation of BCR-ABL by STAT5, we coexpressed BCR-ABL and STAT5 or different known BCR-ABL substrates of similar size (SHIP2, CBL and STS-1) in cell lines to evaluate the efficiency of STAT5 phosphorylation by BCR-ABL in comparison to other BCR-ABL substrates. STAT5 was phosphorylated by BCR-ABL with comparable efficiency to the known BCR-ABL substrates SHIP2, CBL and STS-1 (**Fig. 7a**). We next investigated the inhibition of tyrosine phosphorylation of STAT5 and CRKL in cells at different concentrations of the BCR-ABL TKIs nilotinib or dasatinib. Inhibition of CRKL phosphorylation required ~50-fold and ~20-fold higher concentrations of nilotinib and dasatinib, respectively, than inhibition of STAT5 phosphorylation (**Fig. 7b** and **Supplementary Fig. 7a**,**b**). This hypersensitivity underscores the notion of a key position of STAT5 in the BCR-ABL signaling network and may further support the evidence that STAT5 can be phosphorylated directly by BCR-ABL.

We next carried out a series of *in vitro* kinase assay experiments to determine the enzymatic parameters for the putative BCR-ABL-STAT5 kinase-substrate pair. We synthesized peptides encompassing the sequence upstream and downstream of Tyr694 of STAT5A (ref. 40), and of Tyr207 of CRKL[41], as a control for a *bona fide* BCR-ABL substrate (**Supplementary Fig. 7c**). We inspected the sequences and found that both the STAT5 and CRKL phosphorylation sites adhered well to the optimal ABL phosphorylation consensus determined *in vitro*[42]. We carried out *in vitro* kinase assays with these two peptides at different concentrations. We fit the results to the Michaelis-Menten equation, determined K_m and V_{max} and calculated the turnover number (k_{cat}) and catalytic fidelity (k_{cat}/K_m; **Fig. 7c** and **Supplementary Fig. 7d**). The K_m for both peptides was ~100 μM, which is in a range frequently observed for other kinase-substrate pairs and only four- to five-fold higher than for an *in vitro*–selected optimal ABL substrate peptide (**Supplementary Fig. 7e**). The observed catalytic fidelity of BCR-ABL was only ~16-fold higher for the CRKL peptide than for the STAT5 peptide. These results are compatible with direct phosphorylation of STAT5 by BCR-ABL and indicate that STAT5 may be phosphorylated with similar efficiency as the canonical BCR-ABL substrate CRKL.

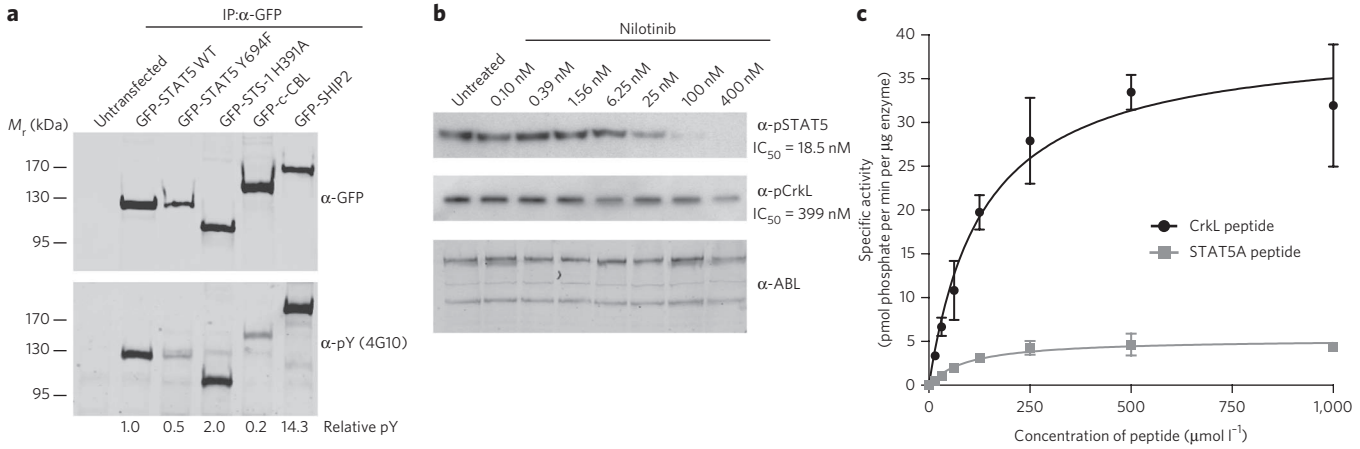

Figure 7 | BCR-ABL phosphorylated STAT5 with comparable efficiency to known BCR-ABL substrates. (**a**) GFP fusion proteins of wild-type (WT) and STAT5 Y694F, STS-1 H391A (phosphatase-dead), c-CBL and SHIP2 were expressed in HEK293 cells stably expressing BCR-ABL. GFP immunoprecipitates were immunoblotted for GFP (top) and phosphotyrosine (bottom). Relative pY amounts were calculated by normalizing phosphotyrosine bands with relative expression. Relative pY for wild-type STAT5 was arbitrarily set to 1.0. (**b**) K562 cells were treated with various concentrations of nilotinib for 4 h. pSTAT5, pCrkL and ABL concentrations were quantified, and IC_{50} values from sigmoidal dose-response curves (not shown) were calculated. (**c**) Recombinant ABL kinase domain was used to carry out kinase assays with 100 μM ATP and increasing concentrations of STAT5 or CrkL peptide. Specific activity (picomoles of incorporated phosphate per minute per microgram of enzyme) was calculated and plotted versus substrate concentration (Michaelis-Menten graph). Values are mean ± s.d. of two experiments done in triplicate. Raw immunoblot data for **a** and **b** is in **Supplementary Figure 10**.

DISCUSSION

Several JAK2 TKIs are currently in clinical trials and are an emerging class of compounds that has received attention from the pharmaceutical industry and the medical research community[30,31,43,44]. The discovery of the JAK2 V617F mutation in different myeloproliferative neoplasms and the success of BCR-ABL TKIs in treatment of CML have driven this development. Our earlier detailed studies on the necessity of STAT5 signaling in *BCR-ABL1*$^+$ cells did not determine the mechanism of STAT5 activation by BCR-ABL. To study this, we used INCB-018424, TG101349 and TG101209, which potently inhibit JAK2, have considerably greater specificity than the other three JAK kinases and have defined target specificities for other kinases. We potently inhibited JAK2 with relative selectivity at certain concentrations and revisited several experiments from earlier studies done with the frequently used low-potency 'JAK2' inhibitor AG490, which is highly pleiotropic and has an undefined target space.

In addition to pharmacological tools, genetic tools to reliably test the role of JAK2 in BCR-ABL–dependent leukemogenesis have become available only recently[33]. The conditional *Jak2* knockout mouse model used here enabled for the first time several experiments hampered by the embryonic lethality of the conventional *Jak2* knockout. Our genetic data showed that, once a transformed phenotype is established by expression of different ABL oncoproteins, the growth and phenotypic characteristics of the cells and the latency and phenotype of the diseases (lymphoid or myeloid) *in vivo* do not differ regardless of expression of JAK2. In contrast, STAT5 expression is required for leukemia maintenance *in vivo*[18]. Our results suggest decoupling of the cytokine-activated JAK2 pathway from STAT5, which is rewired to signal by ABL oncoproteins upon transformation. Our data further exclude the requirement of other tyrosine kinases in CML cells other than BCR-ABL for STAT5 activation and suggest direct phosphorylation of STAT5 by BCR-ABL. The molecular mechanism by which STAT5 can be directly activated by BCR-ABL is still unclear and is discussed further below.

The differential JAK2 dependence of myeloid and lymphoid transformation could be caused by a tighter regulation of STAT5 activity in lymphoid (versus myeloid) cells that initially require active JAK2, in addition to $p185^{BCR-ABL}$ or $p160^{v-ABL}$, to fully activate STAT5. We speculate that STAT5 activation triggered by $p185^{BCR-ABL}$, in contrast to $p210^{BCR-ABL}$, does not allow sufficient induction of antiapoptotic target genes that may counterbalance the oncogenic stress inflicted by transformation. In agreement with this hypothesis, we observed higher STAT5 activation in Ba/F3 *p210*$^{BCR-ABL1}$ compared with Ba/F3 *p185*$^{BCR-ABL1}$ cells. Alternatively, oncogenic tyrosine kinase activity elicited by $p185^{BCR-ABL}$ or $p160^{v-ABL}$ could be more limiting in lymphoid progenitors than that activity elicited by $p210^{BCR-ABL}$ in myeloid progenitors owing to lower overall expression, lower intrinsic kinase activity, higher activity of negative regulatory pathways, insufficient assembly of the oncogenic signaling complex and other reasons. However, these effects seem to be overcome once transformation is established.

Despite our data excluding a requirement of JAK2 for BCR-ABL–dependent leukemia maintenance *in vivo*, we and others have observed that certain JAK2 TKIs show inhibitory activity in *BCR-ABL1*$^+$ cell lines *in vitro* (**Fig. 1**)[25,28]. We also observed that this effect of TG compounds and AG490 without JAK2. This seemingly paradoxical finding showed that an off-target (other than JAK2) must be responsible for the growth-inhibitory effect of the TG compounds and AG490 on *BCR-ABL1*$^+$ cells. For TG101348, TG101209 and AG490, cellular responses in *BCR-ABL1*$^+$ cells were much weaker compared with cell lines that depend on oncogenic JAK2 (for example, HEL or Ba/F3-JAK2 V617F). GC_{50} is almost two orders of magnitude higher than IC_{50} for 'cognate target' JAK2 *in vitro* (**Fig. 5a** and **Supplementary Fig. 1a–c**)[30,45]. In contrast, imatinib inhibits cell proliferation in the high nanomolar range, in agreement with its IC_{50} for BCR-ABL *in vitro*. The ability of the TG compounds to inhibit BCR-ABL directly in the low micromolar concentration range is compatible with the observed growth-inhibitory effect on *BCR-ABL1*$^+$ cells. Notably, INCB-018424 and JAK inhibitor I had no effect on *BCR-ABL1*$^+$ cells, although they are equally potent JAK2 inhibitors, and, in contrast to the TG compounds, they did not inhibit BCR-ABL kinase activity, even at high micromolar concentrations. We conclude and recommend that for *in vitro* studies of the TG compounds, concentrations ≤500 nM should be used to prevent the 'off-target' inhibition of BCR-ABL. Therefore, we cannot conclude that there is a 'JAK2-specific effect' on the basis of experiments done with TG101348 or TG101209 at concentrations >1 μM,

as BCR-ABL and other kinases are also inhibited. Even more caution should be used with AG490, which, owing to its low potency, is used at high concentrations in many studies. Because of its low potency and broad specificity, AG490 should not be used in future studies as more potent and more specific TKIs are now available, and past results should be carefully interpreted in the light of our new insights.

Another question regards the molecular mechanism of STAT5 activation by BCR-ABL. Although it is not necessary for a kinase substrate *per se* and is the exception for Ser-Thr-kinase substrates, many substrates of tyrosine kinases also interact with their cognate kinase[46]. For BCR-ABL, we and others have characterized such interactions and the resulting oncogenic signaling complexes, but we have not identified a direct interaction between BCR-ABL and STAT5 (refs. 47,48). This is not unusual for kinase substrates and does not challenge our model of direct activation of STAT5 by BCR-ABL, but it may explain some other possibly biologically meaningful observations. We observed that inhibition of STAT5 phosphorylation by BCR-ABL TKIs is more sensitive than inhibition of the phosphorylation of other BCR-ABL substrates or interacting proteins such as CRKL. This may indicate a stronger negative feedback by cellular phosphatases and may enable switch-like behavior of STAT5 activation in response to graded BCR-ABL activation. This sensitive dependence of STAT5 activation on BCR-ABL kinase activity may also explain the role of STAT5 in leukemia initiation and maintenance and the differential requirement of JAK2 kinases for initial transformation in lymphoid versus myeloid cells. The consequences of the lower phosphorylation efficiency we observed for the STAT5 peptide compared with the CRKL peptides are difficult to predict *in vivo*. In a cellular environment and with full-length proteins, docking interactions and relative cellular concentrations will influence the efficiency of phosphorylation of STAT5 by BCR-ABL.

Collectively, our data show that JAK2 is not a drug target in CML and that there is no biological rationale for using JAK2 TKIs in CML patients. Future attempts to improve treatment of BCR-ABL TKI-resistant patients should focus on inhibition of STAT5 itself, as has been recently demonstrated[49]; this is the logical next step in the cascade downstream of BCR-ABL.

METHODS

Cell lines. HEK293, K562, Ba/F3, UT-7 and U937 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen. Cells were retrovirally transduced with wild-type or mutant BCR-ABL using pMSCV-IRES-GFP-based retroviral vectors.

Infections, *in vitro* transformation assays and establishment of cell lines. For the preparation of fetal liver cells, *Jak2*$^{+/-}$ mice (mixed background, sw129/Bl6/C57) were set up for breeding. Fetal livers were prepared at E14 and single-cell suspensions were infected with viral supernatant derived either from A010 cells (Ab-MuLV producer cell line) or from GP^{+}/E86 p185$^{BCR\text{-}ABL}$-IRES-eGFP or GP^{+}/E86 p210$^{BCR\text{-}ABL}$-IRES-eGFP producer cell lines with 4 μg ml^{-1} polybrene. Similarly, bone marrow cells of tibiae and femora of *Jak2*$^{fl/fl}$, *Jak2*$^{fl/}$ and *Jak2*$^{\Delta/\Delta}$ mice were infected. Cells were then maintained in RPMI medium enriched with 5% (v/v) FCS to establish cell lines or were immediately plated in cytokine-free methylcellulose at a density of 2.5 × 10^{5} cells ml^{-1}. Colonies were counted after 10 d by light microscopy. All experiments were done in triplicate; mock-infected cells were used as negative controls and did not allow the outgrowth of growth factor–independent colonies.

***In vitro* deletion of *Jak2* and analysis of cell lines.** For deletion of *Jak2* in *Jak2*$^{fl/fl}$*Mx1-Cre p160*$^{v\text{-}Abl+}$ *or p185*$^{BCR\text{-}ABL1+}$ cell lines, cells were treated with 1,000 U ml^{-1} recombinant IFN-β (Serotech). *Jak2*$^{fl/fl}$ and *Stat5a*$^{fl/fl}$*Mx1-Cre* cells treated with INF-β were included as negative and positive controls, respectively. For dose-response curves, 2 × 10^{4} cells were plated in 96-well dishes in triplicate. At 16 h after addition of the TKIs, 0.1 μCi [^{3}H]thymidine per well (PerkinElmer) was added and cells were incubated for another 8 h. Dose-response curves were evaluated using Graph Pad software. To evaluate the onset of apoptosis, 5 × 10^{6} cells were stained with propidium iodide and an APC-conjugated antibody to annexin V (BD Bioscience) and analyzed using a FACS device. For evaluation of proliferation rate, 5 × 10^{6} cells were stained with CFSE using the CellTrace CFSE Proliferation Kit (Invitrogen) and CFSE-MFI was measured over 24 h. Cell cycle profiles were obtained by staining 5 × 10^{6} cells with propidium iodide (50 μg ml^{-1}) in hypotonic lysis solution (0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100, 100 μg ml^{-1} RNAse) and incubated at 37 °C for 30 min before measurement via FACS.

Maintenance of mice, *in vivo* deletion of *Jak2* and bone marrow transplants of cells. *Jak2*$^{fl/fl}$ (mixed, Sv129 × C57Bl/6), *Mx1-Cre, CreERT2, NOD/Shi-scid/IL-2Rγ*^{null} (NOG), and *Rag2*$^{-/-}$*cγ*$^{-/-}$ mice were maintained at Biomedical Research Institute (Medical University of Vienna). All animal experiments were carried out in accordance with protocols approved by Austrian law. For *in vivo* deletion of *Jak2*, two different approaches were used: (i) *Jak2*$^{fl/fl}$*Mx1-Cre* mice were injected intraperetoneally with 400 μg p(I:C) (Sigma) at days 2, 4 and 6 before BM extraction and (ii) *Jak2*$^{fl/fl}$*CreERT2* mice received 1 mg Tamoxifen (Sigma) daily over 5 d by intraperitoneal injection, starting at day 7 before BM extraction. For transplantation studies, BM cells from 6-week-old donor mice were cocultivated on *p210*$^{BCR\text{-}ABL1}$ retroviral producer cells for 48 h with IL-3 (25 ng ml^{-1}), IL-6 (50 ng ml^{-1}), SCF (50 ng ml^{-1}) and 4 μg ml^{-1} polybrene and injected via tail vein into NOG mice. For stable cell line transplantation, 1 × 10^{5} cells were injected via tail vein into *Rag2*$^{-/-}$*cγ*$^{-/-}$ mice.

FACS analysis. Cells were analyzed by a BD FACS-Canto II FACS device and BD FACS Diva software (Beckton Dickinson). The following antibodies were used: CD19-FITC, CD43-PE, B220-PerCP, AnnexinV-APC, PY-STAT5-Alexa Fluor 647 (all BD Bioscience Pharmingen). For intracellular pSTAT5 staining, 5 × 10^{6} cells were fixed by 2% (v/v) paraformaldehyde (Aldrich) with PBS at room temperature for 10 min. Cells were permeabilized with 99% (v/v) ice-cold methanol for 20 min at −20 °C and incubated with αCD16/CD32 (FCγIII/II receptor; BD Bioscience Pharmingen) and 5 μl phospho-tyrosine-STAT5 antibody at room temperature for 90 min before FACS analysis.

Immunoblotting. Samples were analyzed by standard procedures using antibodies to ABL (Ab-3, Oncogene Science), phosphotyrosine (4G10, Millipore), phospho-ABL (Tyr-245, Cell Signaling Technology), phospho-ABL (Tyr-412, Cell Signaling Technology), phospho-STAT5 (pY696, Cell Signaling Technology), STAT5 (Cell Signaling Technology) and HA (HA11, Covance), JAK1, JAK2, JAK3 and TYK2 (all Cell Signaling Technology). Secondary antibodies were labeled with either AlexaFluor 680-labeled goat mouse IgG–specific (Molecular Probes) or IRDye800 goat rabbit IgG–specific (Rockland) and detected using the Li-Cor Odyssey system. Alternatively, peroxidase-labeled mouse-specific or rabbit HRP–specific antibodies (AP Biotech) were used.

Kinase inhibitors. TKIs used were TG101348, TG101209 and INCB-018424 (Symansis), JAK inhibitor I (Millipore), AG490 (Merck), SU6656 (Sigma Aldrich) and imatinib, nilotinib, dasatinib and bosutinib (WuXi PharmaTech). Purity (>95%) of all TKIs was confirmed using HPLC and mass spectrometry analysis. Stock solutions (10 mM) of TKIs were prepared in DMSO and diluted in kinase assay buffer for *in vitro* kinase assays or cell culture medium to indicated final concentrations.

Peptides. Peptides were custom synthesized by Eurogentec. Purity (all >95%) and identity of the peptides were confirmed using HPLC and mass spectrometry analysis. Stock solutions of peptides (5 mM) were prepared in water and diluted in kinase assay buffer to indicated final concentrations. Peptides used for kinase assays had the following sequences: STAT5A (Y694) peptide, biotin-KAVDGYVK-PQIK-amide (calculated relative molecular mass (M_r), 1570.62 Da; observed M_r, 1,570.0 Da); CrkL (Y207) peptide, biotin-EPAHAYAQPQTT-amide (calculated M_r, 1,538.40 Da; observed M_r, 1,537.6 Da); optimal Abl peptide, biotin-GGEAIYAAP-FKK-amide (calculated M_r, 1,477.46 Da; observed M_r, 1,477.1 Da).

***In vitro* kinase assays.** Recombinant JAK2 was purchased from Carna Biosciences. Recombinant Abl kinase domain was overexpressed and purified from *Escherichia coli*. Immunoprecipitation of BCR-ABL or c-ABL protein, and ABL *in vitro* kinase assays were carried out as described[50].

Statistics. Two-tailed Student's *t*-tests were used for statistical analysis. Difference was considered significant when $P < 0.05$. Data are mean ± s.d. of number of determinations and were analyzed by Graph Pad software.

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Author contributions

O.H. did the experiments in **Figures 5d,e**, **6b–f** and **7**. W.W. did the experiments in **Figures 1**, **2d,e**, **3b–e**, **4b–e**, **5a–c,g** and **6a**. E.E. did the experiments in **Figures 2a,b**, **3a** and **4a**. I.K. provided technical assistance and vital tools for experiments in **Figures 5d,e**, **6b–f** and **7**. F.G. did the experiments in **Figure 2c**. K.-U.W. provided the conditional Jak2 knockout mice. G.S.-F. and V.S. designed the experiments and interpreted the data. O.H., W.W., G.S.-F. and V.S. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

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