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ORIGINAL ARTICLE

PAK-dependent STAT5 serine phosphorylation is required for BCR-ABL-induced leukemogenesis

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The transcription factor STAT5 (signal transducer and activator of transcription 5) is frequently activated in hematological malignancies and represents an essential signaling node downstream of the BCR-ABL oncogene. STAT5 can be phosphorylated at three positions, on a tyrosine and on the two serines S725 and S779. We have investigated the importance of STAT5 serine phosphorylation for BCR-ABL-induced leukemogenesis. In cultured bone marrow cells, expression of a STAT5 mutant lacking the S725 and S779 phosphorylation sites ($STAT5^{SASA}$) prohibits transformation and induces apoptosis. Accordingly, $STAT5^{SASA}$ BCR-ABL^+ cells display a strongly reduced leukemic potential *in vivo*, predominantly caused by loss of S779 phosphorylation that prevents the nuclear translocation of STAT5. Three distinct lines of evidence indicate that S779 is phosphorylated by group I p21-activated kinase (PAK). We show further that PAK-dependent serine phosphorylation of STAT5 is unaffected by BCR-ABL tyrosine kinase inhibitor treatment. Interfering with STAT5 phosphorylation could thus be a novel therapeutic approach to target BCR-ABL-induced malignancies.

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Keywords: BCR-ABL; STAT5; serine phosphorylation; nuclear translocation

INTRODUCTION

Janus kinase/signal transducer and activator of transcription (JAK/STAT) molecules are key players in a number of highly conserved signaling pathways involved in cell-fate decisions such as differentiation, proliferation and apoptosis.[1] Mounting evidence pinpoints a role for JAK/STAT signaling in human cancer and STAT proteins are attracting increasing interest as potential molecular targets for cancer therapy.[2] Constitutively active forms of JAK2 have been identified as drivers of myeloid and T lymphoid leukemia.[3–5] Studies in STAT5a/b-deficient mice have revealed that STAT5a/b are essential effectors for JAK2-triggered leukemogenesis.[6,7] In other malignancies, STAT5 signaling is activated downstream of oncogenic tyrosine kinases and contributes to transformation and tumor maintenance. An example of a tyrosine kinase that exerts its oncogenic function via STAT5 is the Abelson (*BCR-ABL*) oncogene, generated by a reciprocal translocation t(9;22) and found in leukemic cells of human chronic myeloid leukemia and acute lymphoid leukemia patients.[8,9] Fusion with the BCR protein turns the Abelson kinase into a constitutively active tyrosine kinase capable of transforming hematopoietic cells. Deletion of STAT5 during induction or maintenance of BCR-ABL^+ leukemia leads to abrogation of the disease.[10,11]

STAT proteins are phosphorylated on tyrosine and serine residues and phosphorylation is generally necessary for full transcriptional activity, although there is mounting evidence that unphosphorylated STAT1 activates a certain subset of target genes.[12] Tyrosine phosphorylation allows dimerization of STAT molecules that is believed to be a prerequisite for nuclear translocation.[13] The importance of phosphorylated STAT5 for hematopoietic malignancies is underlined by observations in lymphoid, myeloid and erythroid leukemias that have constitutive $STAT5^{Y694}$ phosphorylation.[14,15] The introduction of constitutively active STAT5a mutants into murine hematopoietic cells suffices to induce multilineage leukemia in mice.[16]

Although the role of serine phosphorylation in transcriptional control has been intensively investigated, only limited information is available about its importance in STAT5a/b function.[17–20] Serine phosphorylation of STAT1 is required for cytotoxic T-cell responses and/or interferon-γ-mediated innate immunity.[21,22] Phosphorylation of STAT3 on S727 is needed for Ras-mediated tumor formation.[23] Consistently, serine phosphorylation of STAT3 has been linked to the growth of solid tumors such as prostate or skin cancer.[24,25] Moreover, STAT3 and STAT1 are constitutively phosphorylated on serine residues in a subset of acute myeloid leukemia[26] as well as B-cell chronic lymphocytic leukemia, although the significance of the modification is still unclear.

The causal link between serine phosphorylation of STAT5a and leukemogenesis has only recently been established.[27] Using bone marrow (BM) transplantations, we described a critical role for STAT5a serine phosphorylation in STAT5a-driven leukemogenesis (using a constitutively active murine STAT5a as driver oncogene).[27] The importance of this result was underlined by the finding that both serine residues of STAT5a (S726 and S780, corresponding to murine S725 and S779) are phosphorylated in human myeloid malignancies including acute myeloid leukemia and BCR-ABL^{p210+} chronic myeloid leukemia.[27] This study provided the first indication

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that serine phosphorylation of STAT5a might play a part in myeloid leukemia driven by constitutively active STAT5a, indirectly implying that serine phosphorylation of STAT5a might be required in other naturally occurring malignancies that depend on STAT5. We thus investigated whether STAT5 serine phosphorylation is downstream of oncogenic tyrosine kinases, using BCR-ABL-induced disease as a model system. We report here that serine phosphorylation of STAT5a is necessary for nuclear localization of STAT5 in BCR-ABL$^+$ cells. We identify group I p21-activated kinases (PAKs) as upstream regulators and suggest that they might represent an attractive therapeutic point of attack independent of BCR-ABL kinase activity.

MATERIALS AND METHODS

Mouse strains

Mx-1Cre,[28] *Stat5a/b*$^{fl/fl}$,[29] C57Bl/6J and NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ; The Jackson Laboratory, Bar Harbor, ME, USA) were maintained under pathogen-free conditions at the University of Veterinary Medicine Vienna (Vienna, Austria). All animal experiments were approved by the institutional ethics committee and conform to Austrian laws (license BMWF-68.205/0218-II/3b/2012).

Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuikChange site-directed Mutagenesis Kit from Stratagene (La Jolla, CA, USA) according to the manufacturer's instructions (using pMSCV-STAT5a-IRES-GFP as parental vector).

Generation of leukemic cell lines and *in vitro* deletion of endogenous STAT5

The following leukemic cell lines were used: murine BCR-ABL^{p185+}, v-ABL^{p160+}, Ba/F3^{p210+} and Ba/F3^{p185+} pro-B cells as well as the human K562 and KU812 cell lines (both myeloid; BCR-ABL^{p210+}). To generate v-ABL^{p160+} and BCR-ABL^{p185+} cell lines, fetal liver cells of a *Stat5*$^{fl/fl}$ x *Stat5*$^{fl/fl}$*Mx-1Cre* cross were transformed and maintained in RPMI supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol and 100 U/ml penicillin, 100 μg/ml streptomycin (PAA, Pasching, Austria) as previously described.[30] For *Stat5* deletion, stable *Stat5*$^{fl/fl}$*Mx-1Cre* BCR-ABL^{p185+} cell lines were incubated for 48 h in 1000 U/ml recombinant interferon-β (PBL Interferon Source, Piscataway, NJ, USA). After 2 weeks, deletion efficiency was verified by genotyping PCR as described before.[11]

Transfection of leukemic cell lines

Stat5$^{fl/fl}$*Mx-1Cre* BCR-ABL^{p185+} cell lines were transduced with pMSCV-IRES-GFP-based constructs encoding individual STAT5 variants by co-culture with gp + E86 ecotropic retroviral producer cells as described previously.[31] Vector-positive (GFP$^+$) cells were sorted using a fluorescence-activated cell sorting (FACS) Aria III device (BD Biosciences, San Jose, CA, USA).

Transplantation studies in mice

A total of 2500 BCR-ABL^{p185+} cells were injected via the tail vein into nonirradiated NSG mice. Mice were monitored daily. Sick mice were killed and analyzed for spleen weight, white blood cell count and the presence of STAT5-vector-positive leukemic cells (GFP$^+$) in BM, spleen and peripheral blood (PB) by flow cytometry. Differential hemograms were assessed using a VetABC Blood Counter (Scil Animal Care, Viernheim, Germany). The Hemacolor staining kit (Merck Millipore, Billerica, MA, USA) was used for hematoxylin and eosin staining.

Flow cytometry and cell sorting of leukemic cells

A total of 5×10^5 cells were stained and analyzed by a FACS Canto II flow cytometer equipped with 488, 633 and 405 nm lasers using the FACS Diva software (Becton-Dickinson, Franklin Lakes, NJ, USA) as described before.[11] High-purity FACS sorting was performed on a FACS Aria III equipped with a 488 nm laser at 4 °C (Becton-Dickinson).

Transfection and immunofluorescence staining of HEK cells

HEK 293T cells were transfected with a pcDNA 3.1.-based vector expressing BCR-ABL^{P185} using PolyFect (Qiagen, Hilden, Germany). Cells were cultured with Dulbecco's modified Eagle's medium (PAA) high glucose supplemented with 10% fetal calf serum (PAA), 50 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin, 100 μg/ml streptomycin (PAA) and 1000 μg/ml G418 (InvivoGen, San Diego, CA, USA) to select for stable BCR-ABL^{P185}-expressing cells. The localization of yellow fluorescent protein (YFP)-tagged STAT5 protein was examined by immunofluorescent laser scanning microscopy (Olympus IX71, 20-fold magnification) using a 530/550 nm filter (U-MNG2 filter, Olympus, Tokyo, Japan).

Cell extracts and immunoblotting

Whole-cell extracts and cellular fractionations were performed as previously described.[10,32] For immunoblotting, proteins (50–100 μg) were separated on a 7% SDS polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were probed with antibodies from Santa Cruz (Dallas, TX, USA) against STAT5a/b (N-20; C-17), α-tubulin (DM1A), β-actin (C-15), HSC70 (B-6) and pERK^{Y204} (E-4). Lamin-B (ab45848-100) was purchased from Abcam (Cambridge, UK). The pSTAT5^{S725}- and pSTAT5^{S779}-specific antibodies were generated by immunization of rabbits (Eurogentec, Liége, Belgium). The following antibodies were purchased from Cell Signaling (Danvers, MA, USA): PAK1 (2602), PAK2 (2608), pPAK1^{T423}/pPAK2^{T402} (2601), pCrkl^{Y207} (3181) and STAT1 (9172). pSTAT5^{Y694} (611964) and Rac1 (610650) were obtained from BD Transduction Laboratories (San Jose, CA, USA). Immunoreactive bands were visualized by chemoluminescence (20X LumiGLO Reagent and 20X Peroxide, Cell Signaling).

Semiquantitative real-time PCR

RNA was isolated from murine BCR-ABL^{p185+} cells expressing wild-type or mutant STAT5 variants using peqGOLD TriFast reagent (Peqlab, Erlangen, Germany). RNA (1 μg) was transcribed by employing the iSCRIPT cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time PCR was performed on a MyiQ2 cycler (Bio-Rad) with SsoAdvanced SYBR GreenSupermix (Bio-Rad) and Primers for *Bcl2* (forward (fwd) 5′-ACTGAGTACCTGAACCGGC ATC-3′, reverse (rev) 5′-GGAGAAATCAAACAGAGGTCGC-3′), *Cish* (fwd 5′-AGACGTTCTCCTACCTTCGG-3′, rev 5′-TGACCACATCTGGGAAGGC-3′) and *Gapdh* (fwd 5′-TGTGTCCGTCGTGGATCTGA-3′, rev 5′-CCTGCTTCACCACCTT CTTGA-3′). Target gene expression was normalized to *Gapdh*.

Proliferation assays and focused compound screen

K562 and murine BCR-ABL^{p185+} cells expressing wild-type or mutant STAT5 variants were seeded in 384-well plates (Corning, Corning, NY, USA) at a concentration of 10 000 cells per well in 50 μl medium. Kinase inhibitor libraries (Tocris Kinase Inhibitor Toolbox from Tocris (Bristol, UK), Merck Kinase Inhibitor Library I and Merck Kinase Inhibitor Library III both from Merck-Millipore) were added at a screening concentration of 10 μM. Dasatinib at 1 μM was used as a positive control and all wells contained a final dimethyl sulfoxide concentration of 0.1%. After 24 h of incubation, CellTiter-Glo (Promega, Fitchburg, WI, USA) was added and luminescence measured with an Envision plate reader (Perkin Elmer, Waltham, MA, USA). Data were normalized to internal controls by linear regression to the mean of the 32 dimethyl sulfoxide wells (set to 100% of control) and the mean of the 32 positive control wells (set to 0% of control) using Pipeline Pilot (Accelrys, San Diego, CA, USA). Screening data were visualized with Spotfire (Spotfire Inc., Cambridge, MA, USA) software. Screening was performed in duplicate.

The methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was conducted in 96-well plates with 20 000 cells per well in 100 μl medium. Kinase inhibitors (Kinase Inhibitor Toolbox, Tocris) were added at 10 μM concentration followed by 24 h of incubation. Positive and negative controls were included as above. Cells were incubated for 3 h with 10 μl MTT (5 mg/ml MTT; Sigma-Aldrich). Upon addition of 100 μl acidified isopropanol (4 mM HCl, 0.1% Nonidet P-40; Sigma-Aldrich), absorbance was measured at 590 nm on an EnSpire multimode plate reader (Perkin Elmer).

Kinase inhibitor studies

K562, KU812, BCR-ABL^{p185+} and v-ABL^{p160+} leukemic cell lines were seeded in a six-well dish at a concentration of 10^6 cells per ml. Kinase inhibitors were added and after incubation at 37 °C and 5% CO_2, cells were harvested, washed twice with ice-cold phosphate-buffered saline and subjected to immediate lysis as described previously.[33] The following inhibitors were purchased from Calbiochem (Billerica, MA, USA): KN-93, H-89, PD98059, TDZD-8, roscovitine and olomoucine. PIM1 kinase inhibitor,

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BIO and IPA-3 were purchased from Tocris. Flavopiridol, CAL-101 and foretinib were purchased from Selleck Chemicals (Houston, TX, USA). Anisomycin was purchased from Sigma-Aldrich; olomoucine II from Alexis Biochemical (San Diego, CA, USA) and SB 203580 from Jena-Bioscience (Jena, Germany). All inhibitors were dissolved in dimethyl sulfoxide. As negative control 0.1% dimethyl sulfoxide was used.

Immunoprecipitation studies

STAT5- or PAK1-specific antibodies were incubated with lysates for 1.5 h at room temperature, and then for 1 h with magnetic beads conjugated to proteins A and G (Bio-Adembeads PAG, Ademtech, Pessac, France). Beads were pelleted with a magnet and washed 3 times. Protein complexes were eluted by incubation with PAG elution buffer (Ademtech) and analyzed by immunoblotting.

In vitro kinase assays

Recombinant mouse TAT-STAT5a and TAT-STAT5b were produced and purified as described previously.[34] Recombinant mouse PAK1 and Cdc42/PAK1 were purchased from SignalChem (Richmond, BC, Canada) and recombinant Rac1 from Cytoskeleton (Denver, CO, USA). Recombinant TAT-STAT5 proteins (20 ng) were incubated with PAK1 kinase (100 ng) in buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$, 2 mM $MnCl_2$ and 100 μM ATP for 30 min at 37 °C. Kinase reactions were stopped by adding Laemmli buffer and STAT5 phosphorylation was detected by immunoblotting with an anti-pSTAT5^{S779} antibody (Affinity Bioreagents, Golden, CO, USA).

^{3}H thymidine incorporation assays

A total of 50 000 cells per well were seeded in 96-well plates, inhibitors were added and cells were pulsed with 1 μCi of ^{3}H thymidine (Perkin Elmer) per well. Cells were harvested 18 h later using a Filtermate Harvester (Perkin Elmer). ^{3}H thymidine uptake was determined using a Top Count 4.00 Scintillation Counter (Perkin Elmer). All experiments were performed in triplicate.

shRNA knockdown experiments

For short hairpin RNA (shRNA)-mediated knockdown of PAK2 in murine BCR-ABL^{p185+} cells, TRC clone TRCN0000025213 (Open Biosystems, Huntsville, AL, USA) was used and a nonsilencing shRNA (RHS4080) served as control. Lentivirus production and infection were performed as previously described.[12]

Statistical analysis

Analysis was performed by means of an unpaired *t*-test or a one-way analysis of variance followed by Tukey's test. Data are presented as averages ± s.d. and were analyzed by GraphPad (GraphPad Software, San Diego, CA, USA). Differences in Kaplan–Meier plots were assessed for statistical significance using the logrank test.

RESULTS

Expression of STAT5^{SASA} hampers leukemic cell viability *in vitro*

To test the role of STAT5 serine phosphorylation in cellular transformation, we infected murine wild-type (wt) fetal liver cells with v-ABL^{p160+} in combination with STAT5^{wt}, STAT5^{SASA} or an empty vector control (pMSCV-IRES-GFP). The STAT5^{SASA} construct contains two serine sites mutated to alanine: STAT5^{S725A} and STAT5^{S779A} (a diagram of the STAT5 mutants is provided in Supplementary Figure 1). Whereas the expression of STAT5^{wt} conferred a slight survival advantage, the percentage of cells expressing STAT5^{SASA} declined within 8 days till no cells were detectable (Figure 1a). Similar results were obtained when GFP$^+$ cells were sorted 2 days after infection; expression of STAT5^{SASA} provoked a rapid decrease in GFP$^+$ cell numbers (Figure 1b). Cell cycle analysis (propidium iodide) and apoptosis staining (Annexin V) were performed 2, 3 and 6 days after sorting. STAT5^{SASA} cells accumulated in the sub G_0/G_1 phase after 2 days (Figure 1c) and at day 3 the number of Annexin V-positive cells increased and reached 100% on day 6 (Figure 1d). This indicates that the expression of STAT5^{SASA} induces apoptosis.

We next investigated whether established cell lines tolerate the expression of STAT5^{SASA} mutants (Figure 1e). We infected v-ABL^{p160+} and BCR-ABL^{p185+} cells with STAT5^{SASA} or STAT5^{wt}. As controls two dominant negative versions of STAT5 were used—STAT5$^{\Delta 749}$ and STAT5^{Y694F}—lacking the C-terminal transactivation domain or the critical tyrosine phosphorylation site required for dimerization. As expected, the proportion of STAT5$^{\Delta 749}$- and STAT5^{Y694F}-expressing cells steadily declined and on day 3 no viable cells were detectable. Similarly, we failed to obtain viable cells after expression of STAT5^{SASA} (Figure 1e and Supplementary Table 1). Consistent with the idea that STAT5 is essential in v-ABL^{p160+} cells, the expression of transcriptionally inactive STAT5 mutants significantly impairs the outgrowth of cell lines (STAT5$^{\Delta 749}$ and STAT5^{Y694F}). We succeeded in establishing a single STAT5^{SASA}-positive cell line (BCR-ABL^{p185+}). The experiments indicate that the expression of a STAT5 mutant lacking the critical serine sites S725 and S779 does not support transformation and severely impairs viability of v-ABL^{p160+} and BCR-ABL^{p185+} cells.

Expression of STAT5^{SASA} in leukemic cells enhances disease latency

It is conceivable that the strong inhibitory effect that we observed *in vitro* is overcome by cytokines and growth factors *in vivo* that act in synergy with BCR-ABL. The role of STAT5 serine phosphorylation in *in vivo* leukemogenesis was tested using the BCR-ABL^{p185+} leukemic cell line expressing STAT5^{SASA} (Supplementary Figure 1). The maternal cell line harbors *Stat5$^{fl/fl}$Mx-1Cre* alleles and therefore allows the deletion of endogenous *Stat5* by a single interferon-β treatment. The resulting cell lines express only the retroviral *Stat5* construct and are either STAT5^{SASA} or STAT5^{wt} positive.

The cells were transplanted into nonirradiated NSG mice (scheme in Figure 2a). We observed a significant increase in disease latency upon transplantation of STAT5^{SASA}-expressing leukemic cells compared with the cohort that had received STAT5^{wt} cells (Figure 2a). In a subsequent experiment, all animals were killed on day 18 (scheme in Figure 2b). We noticed a significant attenuation of the severity of the disease upon transplantation of STAT5^{SASA} leukemic cells as shown by significantly reduced spleen weight (Figure 2c) and white blood cell count (Figure 2d). Blood smears revealed lower numbers of tumor cells in the PB (Figure 2e). Histological spleen sections substantiated these findings and showed less infiltration of leukemic cells (Figure 2f). Differences in disease severity were also obvious when we monitored leukemic cells in PB (Figure 2g) and spleens (Figure 2h) by FACS analysis. Although we observed no differences in leukemic infiltration in the BM on day 18 (12.4 ± 8.2 vs 12.3 ± 5.3 for the STAT5^{wt} and STAT5^{SASA} groups, $P =$ nonsignificant, data not shown), there were profound differences in the PB and in the spleen. In summary, the leukemic cell load was significantly reduced upon transplantation of BCR-ABL^{p185+} STAT5^{SASA} cells.

Single mutation of STAT5^{S779} prolongs disease latency

To investigate whether leukemia progression *in vivo* is modulated by phosphorylation of STAT5 on S725 or on S779 or on both positions, we transduced murine *Stat5$^{fl/fl}$Mx-1Cre* BCR-ABL^{p185+} cell lines with a STAT5^{S725A} or a STAT5^{S779A} construct and deleted endogenous *Stat5* by means of interferon-β. Whereas we rapidly succeeded in obtaining cell lines expressing STAT5^{S725A}, the generation of cells expressing STAT5^{S779A} required six rounds of infection and selection before a stable cell line could be established. The cells were transplanted into NSG mice, with experimental animal groups subsequently termed STAT5^{wt}, STAT5^{S725A}, STAT5^{S779A} and STAT5^{SASA}. Animals of the STAT5^{S725A} group differed in overall survival from those in the STAT5^{wt} group.

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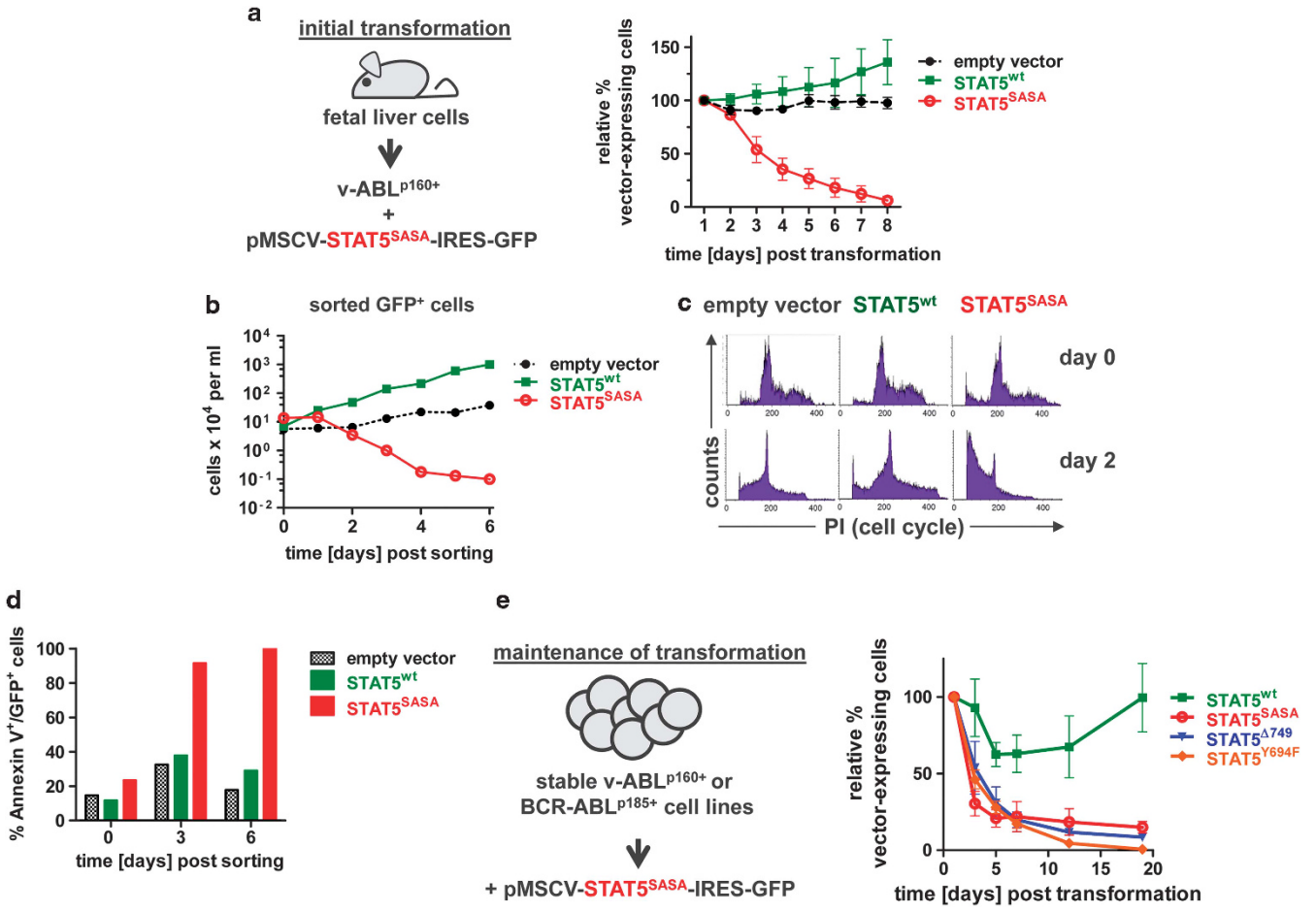


Figure 1. STAT5^{SASA} expression does not support transformation *in vitro*. (**a**) The wt fetal liver cells were simultaneously infected with v-ABL^{p160+} and STAT5^{wt}, STAT5^{SASA} or the empty vector (pMSCV-IRES-GFP; $n=3$, $n=3$ and $n=6$, respectively). GFP$^+$ cells were monitored via FACS analysis. (**b**) Growth curve of GFP$^+$ cells. Three days after co-infection, vector-positive cells were sorted. The experiment was performed in triplicate; one representative experiment is depicted. Post sorting, propidium iodide (PI) cell cycle (**c**) and Annexin V (**d**) stainings were performed at indicated time points. (**e**) Stably transformed wt leukemic cells were infected with STAT5^{wt}, STAT5^{SASA}, STAT5$^{\Delta749}$ or the empty vector ($n=8$, $n=3$, $n=12$ and $n=13$, respectively). Outgrowth of GFP$^+$ cells was monitored via FACS analysis.

Survival of mice in the STAT5^{S779A} and STAT5^{SASA} groups was further enhanced. Remarkably, the combined loss of both serine phosphorylation sites further delayed disease latency compared with single loss of STAT5^{S779} phosphorylation (Figure 3a). FACS analysis of leukemic cell infiltrates in the BM (Figure 3b) revealed significant levels of leukemic cells expressing STAT5^{wt}, STAT5^{S725A} or STAT5^{S779A}, whereas only low numbers of STAT5^{SASA} cells were detected. A comparable picture was found in PB (Figure 3c). Numbers of STAT5^{SASA} cells were significantly reduced compared with other experimental groups. In contrast, FACS analysis of spleens (Figure 3d) showed decreased numbers of GFP$^+$ cells in mice of both the STAT5^{S779A} and STAT5^{SASA} groups when compared with the STAT5^{S725A} and the STAT5^{wt}. These data indicate that STAT5^{S779} phosphorylation is dominating leukemogenesis *in vivo*.

STAT5^{S779} phosphorylation is required for nuclear localization

To investigate how serine phosphorylation modulates leukemogenesis, we analyzed the subcellular localization of the modified proteins. Mouse STAT5 serine mutants were introduced into HEK 293T cells stably expressing BCR-ABL^{p185}. The mutants were C-terminally tagged with YFP to monitor subcellular localization. As expected, BCR-ABL^{p185} activates STAT5 and causes nuclear accumulation of STAT5^{wt}-YFP (Figure 4a). Similarly, STAT5^{S725A}-YFP was primarily found in the nucleus. In contrast, the STAT5^{S779A} and STAT5^{SASA} proteins failed to accumulate in the nucleus, instead being evenly distributed throughout the cells (Figure 4b, upper panel). To investigate whether STAT5^{S779A} and STAT5^{SASA} proteins regain the ability to move to the nucleus upon dimerization with a STAT5^{wt} partner, we additionally transfected untagged STAT5^{wt} proteins (Figure 4b, lower panel) into the cells. The co-transfection caused increased nuclear accumulation of the STAT5^{S779A}-YFP and STAT5^{SASA}-YFP proteins, suggesting that serine phosphorylation of one partner of the STAT5 heterodimer suffices for nuclear localization. The phospho-mimetic variants (S→D) STAT5^{S725D}, STAT5^{S779D}, STAT5^{SDSD} served as controls and efficiently accumulated in the nucleus (Figure 4c and Supplementary Table 2).

To examine whether the findings are relevant to the situation in leukemic pro-B cells, we prepared cytoplasmic and nuclear fractions of BCR-ABL^{p185+} cells expressing STAT5^{wt}, STAT5^{S725A}, STAT5^{S779A}, STAT5^{SASA} or the corresponding phospho-mimetic mutants and performed immunoblotting with anti-STAT5a/b antibodies (Figure 4d), using Lamin B and α-tubulin to control for the quality of our nuclear and cytoplasmic fractions. Consistent with the findings in HEK 293T cells, there were barely detectable amounts of STAT5 proteins in the nuclei of BCR-ABL^{p185+} cells expressing STAT5^{S779A} or STAT5^{SASA}. This indicates that STAT5^{S779} phosphorylation of at least one STAT5 molecule within a homodimer is required for translocation to the nucleus. In line with this observation, the STAT5 target genes *Cish* and

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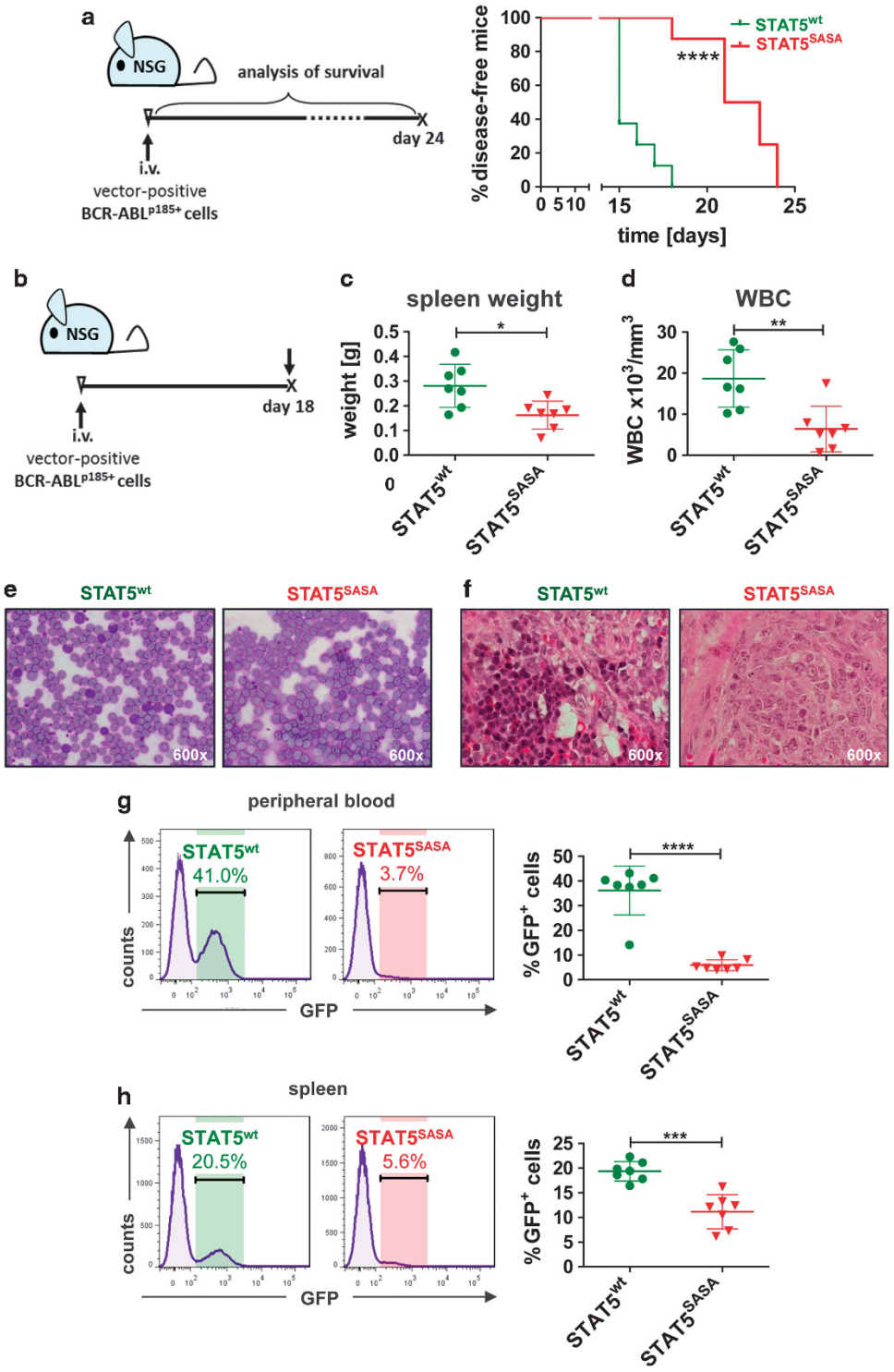
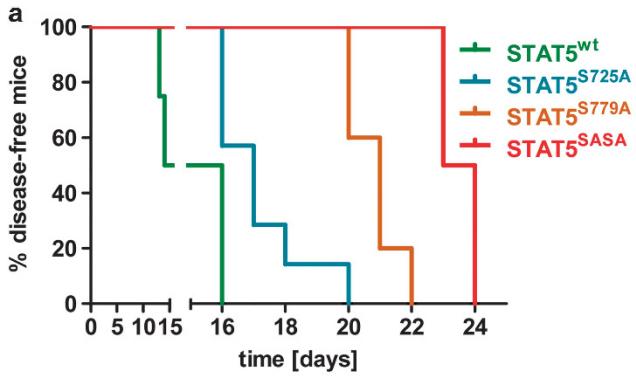


Figure 2. Expression of STAT5^{SASA} impairs leukemogenesis *in vivo*. (**a**) STAT5^{SASA}- or STAT5^{wt}–expressing BCR-ABL^{p185+} cells were injected intravenously (i.v.) into NSG mice (2500 cells/mouse; $n=8$ each). Survival curves of recipients are depicted. The median survival was 15 and 22 days for STAT5^{wt} and STAT5^{SASA} group. (**b**) Scheme depicting experimental setup of data shown in (**c**–**h**). Transplantation was performed as described in (**a**) ($n=7$ each). All animals were killed on day 18. (**c**) Spleen weights of the STAT5^{SASA} group were 1.6-fold reduced (0.28 ± 0.09 and 0.16 ± 0.06 g for the STAT5^{wt} and STAT5^{SASA} groups, respectively, $P<0.05$). Data represent mean ± s.d. (**d**) White blood cell counts (WBCs) were 2.9-fold reduced in mice of the STAT5^{SASA} group (18.7 ± 7 × 10^3/mm^3 and 6.4 ± 5.5 × 10^3/mm^3, for the STAT5^{wt} and STAT5^{SASA} groups, $P<0.01$). Data represent mean ± s.d. (**e**) Blood smears show reduced lymphocyte load in mice of the STAT5^{SASA} group (4.3 ± 0.8% vs 0.7 ± 0.8% of blasts relative to red blood cells for the STAT5^{wt} and STAT5^{SASA} groups, respectively, $P<0.001$). One representative example per group is depicted. (**f**) Hematoxylin and eosin (H&E)-stained spleen sections. One representative example per group is depicted. (**g**, **h**, left panels) Representative FACS plots showing infiltration of GFP$^+$ cells in (**g**) PB (36.1 ± 9.9% vs 5.9 ± 2.2% GFP$^+$ cells in STAT5^{wt} and STAT5^{SASA} groups, $P<0.0001$) and (**h**) spleen (19.3 ± 2 vs 11.1 ± 3.5 for STAT5^{wt} and STAT5^{SASA} groups, $P<0.001$). Data obtained from entire cohorts are summarized. Reduced numbers of GFP$^+$ cells in PB and spleens of the STAT5^{SASA} group (PB: 6.1-fold; spleen: 1.7-fold). Data represent mean ± s.d. Asterisks denote statistical significance (*$P\leqslant0.05$; **$P\leqslant0.01$; ***$P\leqslant0.001$; ****$P\leqslant0.0001$).

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	statistical significance
$STAT5^{wt}$ vs. $STAT5^{S725A}$	p<0,05 (*)
$STAT5^{wt}$ vs. $STAT5^{S779A}$	p<0,01 (**)
$STAT5^{wt}$ vs. $STAT5^{SASA}$	p<0,01 (**)
$STAT5^{S725A}$ vs. $STAT5^{S779A}$	p<0,01 (**)
$STAT5^{S725A}$ vs. $STAT5^{SASA}$	p<0,01 (**)
$STAT5^{S779A}$ vs. $STAT5^{SASA}$	p<0,01 (**)

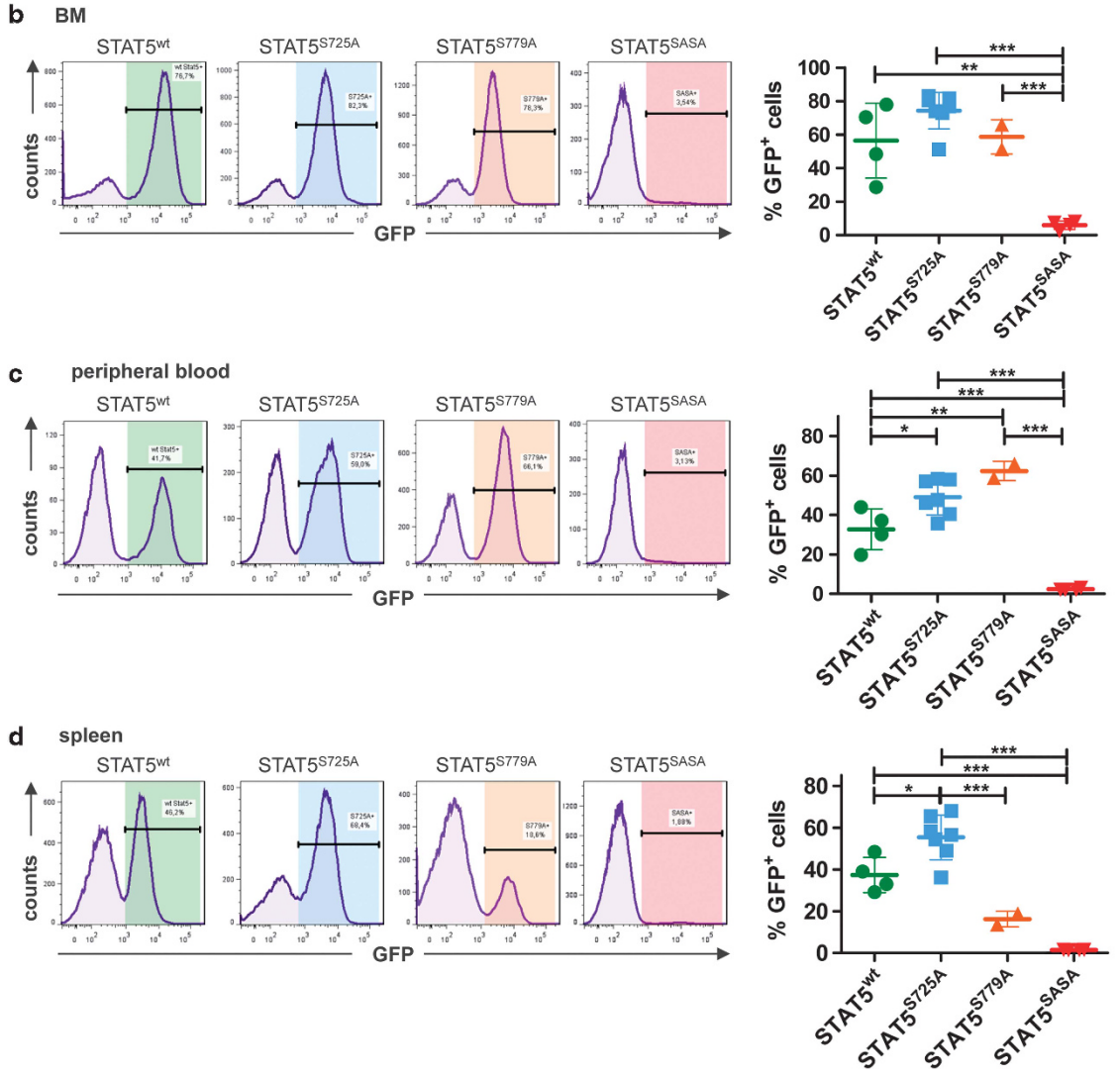


Figure 3. Expression of $STAT5^{S779A}$, $STAT5^{S725A}$ or $STAT5^{SASA}$ suppresses the leukemic potential of BCR-ABL^{p185+} cells *in vivo*. (**a**) Kaplan–Meier plot of NSG mice upon transplantation of $STAT5^{wt}$-, $STAT5^{S725A}$-, $STAT5^{S779A}$- or $STAT5^{SASA}$-expressing leukemic cells ($n=4$, $n=7$, $n=5$ and $n=4$, respectively). The median survival for the $STAT5^{wt}$, $STAT5^{S725A}$, $STAT5^{S779A}$ and $STAT5^{SASA}$ groups was 15, 17, 21, 23.5 days, respectively. Table summarizes statistical significances between indicated experimental groups. (**b**) FACS analysis of BMs for infiltration of GFP^+ cells (56.4 ± 22.3%, 74.3 ± 10.9%, 58.7 ± 10.3% and 6 ± 2.5% GFP^+ cells for groups $STAT5^{wt}$, $STAT5^{S725A}$,$STAT5^{S779A}$ and $STAT5^{SASA,}$ respectively). (**c**) In PB, 32.8 ± 10.4%, 49.1 ± 9.1%, 62.3 ± 5% and 2.4 ± 0.5% GFP^+ cells in the $STAT5^{wt}$, $STAT5^{S725A}$, $STAT5^{S779A}$ and $STAT5^{SASA}$ groups were detected. (**d**) Analysis of spleens resulted in 37.4 ± 8.4%, 55.4 ± 10.7%, 16.3 ± 3.7% and 1.5 ± 0.1% GFP^+ cells in the $STAT5^{wt}$, $STAT5^{S725A}$, $STAT5^{S779A}$ and $STAT5^{SASA}$ groups. One representative FACS plot per experimental group is depicted. Data in right panels represent mean ± s.d. Three mice of the $STAT5^{S779A}$ display censored events and were excluded from FACS analysis. Asterisks denote statistical significances as determined by a (**a**) logrank test or (**b**, **c**, **d**) a one-way analysis of variance (ANOVA) followed by Tukey's test (*$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.001$).

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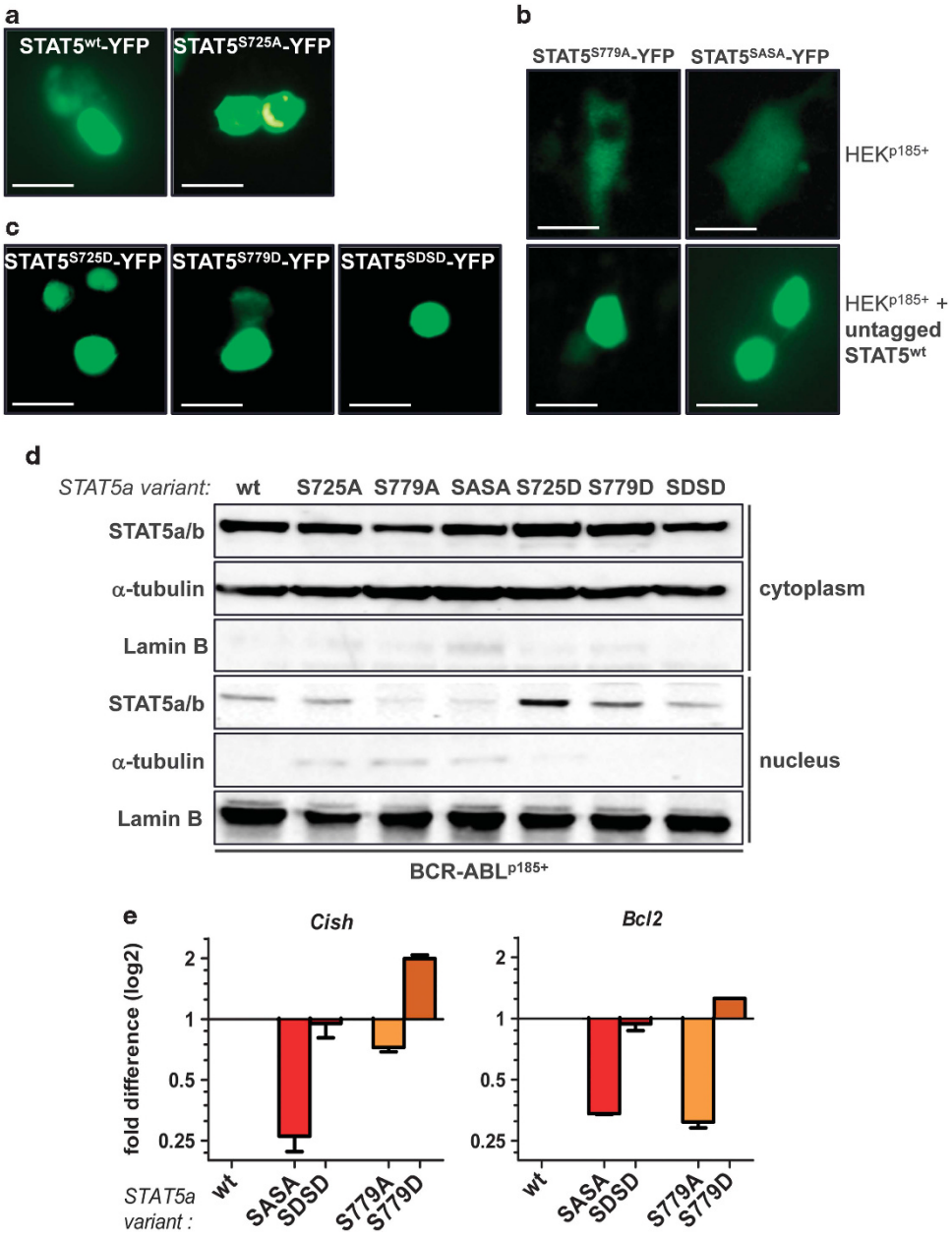


Figure 4. STAT5^{S779} phosphorylation is a prerequisite for nuclear translocation in BCR-ABL^{p185+} cells. (**a**, **b**) Immunofluorescence of HEK 293T cells stably expressing BCR-ABL^{p185}-transfected with YFP-tagged STAT5 variants. Scale bars 10 μm. (**a**) STAT5^{wt} and STAT5^{S725A} translocate to the nucleus. (**b**) STAT5^{S779A} and STAT5^{SASA} fail to translocate to the nucleus (upper panel). The concomitant expression of untagged STAT5^{wt} alters the nuclear localization of STAT5^{S779A} and STAT5^{SASA} proteins (lower panel). (**c**) Immunofluorescence of HEK 293T^{p185+} cells transfected with phospho-mimetic mutants (STAT5^{S725D}, STAT5^{S779D} or STAT5^{SDSD}, all YFP-tagged). Scale bars 10 μm. (**d**) Immunoblotting for STAT5a/b of nuclear and cytoplasmic fractions of BCR-ABL^{p185+} cells. Lamin B and α-tubulin served as controls of nuclear and cytoplasmic fractions. (**e**) mRNA expression of *Cish* and *Bcl2* normalized to *Gapdh* in murine BCR-ABL^{p185+} cells expressing exclusively STAT5a mutant variants was determined by real-time PCR.

Bcl2 were not transcribed in cells expressing STAT5^{SASA} and STAT5^{S779A} but within normal range in cells expressing STAT5^{SDSD} and STAT5^{S779D} (Figure 4e).

Group I PAK kinases as upstream regulators of STAT5^{S779} in BCR-ABL^{p185+} cells

Blocking STAT5^{S779} phosphorylation and thereby nuclear translocation of STAT5 might represent a way to inhibit the transcriptional activity of STAT5, which is essential for maintenance of BCR-ABL-driven disease. To identify the kinase(s) upstream of STAT5^{S779} we performed *in silico* screens employing group-based phosphorylation scoring, KinasePhos, NetPhosK, prediction of protein kinase-specific phosphorylation site, PredPhospho, Scansite and PhosphoMotif finder. Results for potential candidates are summarized in Figure 5a. As both murine STAT5^{S779} and human STAT5^{S780} are flanked by prolines, we focused on hits that represent proline-directed serine/threonine kinases. Mitogen-activated protein kinases (MAPKs) and cyclin-dependent kinases (CDKs) were consistently identified. Protein kinases A, B and C (PKA, PKB and PKC), glycogen synthase kinase-3β, Ca^{2+}/calmodulin-dependent protein kinases, PAKs and mammalian target of

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a

Kinase group	Predicted kinases	In silico prediction*	Screened hit compounds
CMGC	MAPK	7/7	5-Iodotubercidin, JNK Inhibitor V, MEK Inhibitor I, p38 MAP Kinase Inhibitor, JX401, PD 198306, SB 220025, SC-68376
	CDK	7/7	BIO, bohemine, Cdc7/Cdk9 Inhibitor, Cdk1 Inhibitor, Cdk2 Inhibitor II, CR8 (R)-Isomer, CR8 (S)-Isomer, olomoucine II, roscovitine, SU9516
	GSK3-β	3/7	BIO, GSK-3b Inhibitor VIII
AGC	PKA, PKB, PKC	5/7	KT5720
CAMK	CAMK	1/7	--
	PIM	0/7	PIM1 Kinase Inhibitor IV
STE	PAK	1/7	p21-activated Kinase Inhibitor III (IPA-3)
	Tpl2	0/7	Tpl2 Kinase Inhibitor, Tpl2 Kinase Inhibitor II
Atypical protein kinases	mTOR	1/7	Compound 401
	PI3-K, ATM, DNA-PK	0/7	ATM kinase Inhibitor, DNA-PK Inhibitor III, TGX-221, PI3-Kγ Inhibitor

* number of positive predictions / screened databases

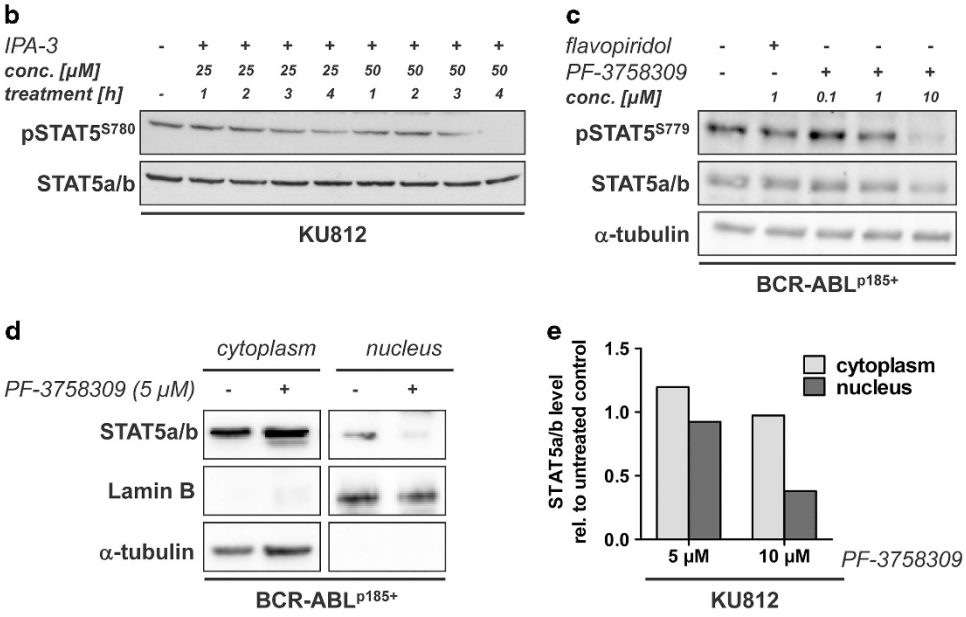


Figure 5. Inhibition of group I PAK kinases diminishes STAT5^{S779} phosphorylation and nuclear localization of STAT5. (**a**) Summary of candidate kinases obtained from *in silico* predictions and screening hit compounds targeting serine/threonine (Ser/Thr) kinases. For screening, K562 and BCR-ABL^{p185+} cell lines were incubated with kinase inhibitor libraries. Reduction of cell viability was assessed via CellTiter-Glo assay. (**b**) Immunoblotting of KU812 cells treated with IPA-3 (group I PAK kinase inhibitor) at 25 or 50 μM for up to 4 h. (**c**) Immunoblotting of BCR-ABL^{p185+} cells incubated for 5 h either with flavopiridol or PF-3758309 at indicated concentrations. (**d**) Immunoblotting of nuclear and cytoplasmic fractions of BCR-ABL^{p185+} cells treated with PF-3758309 (5 μM; 5 h). Lamin B and α-tubulin served as controls of nuclear and cytoplasmic fractions. (**e**) Densitometric analysis of immunoblotting of nuclear and cytoplasmic fractions of KU812 cells treated with PF-3758309 (5 and 10 μM; 5 h). As negative controls in (**a**–**e**), cells were treated with 0.1% dimethyl sulfoxide (DMSO).

rapamycin were predicted with low frequency. In parallel, we initiated a drug screen in the human cell line K562 and in a murine BCR-ABL^{p185+} cell line. To control for hits that lead to apoptosis without interfering with STAT5 serine phosphorylation, we included cells expressing a phospho-mimetic variant of STAT5^{S779} (STAT5^{S779D}) and excluded positive hits obtained in these cells. Compounds that induced loss of cell viability in >50% of the cells were further tested. Figure 5a summarizes two individual rounds of experiments. The target profiles of hit compounds were largely consistent with the *in silico* predictions. In a next step, we used western blot analysis for validation and found that two independently acting group I PAK kinase inhibitors (IPA-3 and PF-3758309) suppress STAT5^{S779} phosphorylation in both human and murine BCR-ABL^{+} cells (Figures 5b and c).

CDK inhibitors were also analyzed in more depth as CDK8 has previously been defined as upstream kinase for STAT proteins.[12,35] We treated BCR-ABL^{p185+} cells with CDK inhibitors including flavopiridol and analyzed levels of pSTAT5^{S779} by immunoblotting. As depicted in Figure 5c and Supplementary Figure 2, we failed to detect any reduction of pSTAT5^{S779} upon inhibition of CDKs.

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Similarly, inhibitors of MAPK, PKA, PKB, PKC, Ca^{2+}/calmodulin-dependent protein kinases, mammalian target of rapamycin, glycogen synthase kinase-3β and PIM1—that were predicted as potential upstream kinases—failed to exert any effect (Supplementary Figures 3 and 4).

These data lead to a testable prediction: if PAK kinases phosphorylate $STAT5^{S779}$, PAK kinase inhibition should prevent the accumulation of nuclear STAT5. In line with the results in $STAT5^{S779A}$ mutant cells, we indeed observed a reduction of nuclear STAT5 upon treating murine BCR-ABL^{p185+} and KU812 cells with the PAK inhibitor PF-3758309 (Figures 5d and e). This led us to conclude that group I PAK kinases are direct or indirect upstream regulators of $STAT5^{S779}$ phosphorylation, controlling the nuclear localization of STAT5.

PAK kinases directly phosphorylate $STAT5^{S779}$

Whereas PAK1 and PAK2 are expressed in human BCR-ABL^{+} cells, it appears that only PAK2 is found in murine leukemic cells: we failed to detect any PAK1 protein in murine BCR-ABL^{p185+} cells (Figure 6a). Co-immunoprecipitation experiments revealed complexes of STAT5, PAK1, PAK2 and Rac1 in KU812 cells (Figure 6b). STAT5 was consistently associated with PAK1, PAK2 in K562 (Supplementary Figure 5) and in murine BCR-ABL^{+} cells (Supplementary Figure 6). The interaction was specific for STAT5 as no complexes were detectable upon immunoprecipitation of STAT1 in K562 or murine BCR-ABL^{+} cells (Supplementary Figures 5 and 6). Thus, group I PAK kinases directly interact with STAT5.

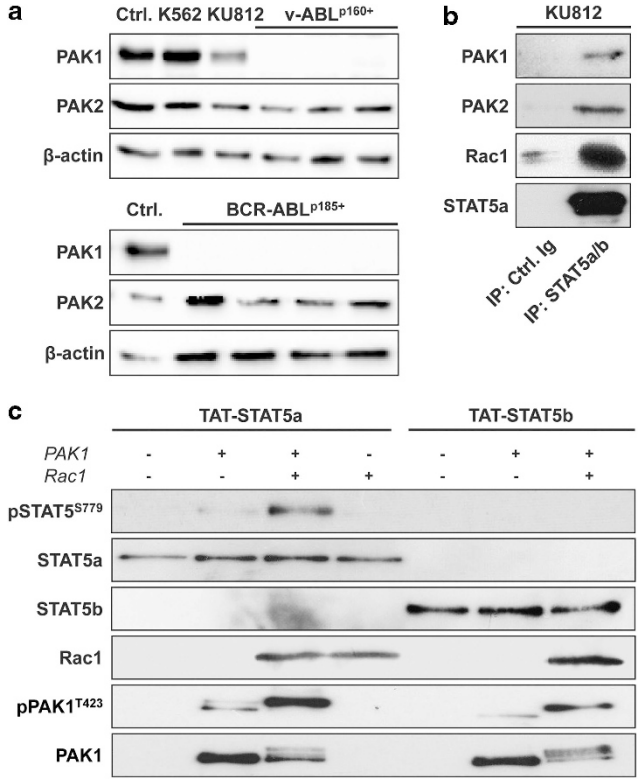


Figure 6. PAK1 directly phosphorylates $STAT5^{S779}$. (**a**) Protein expression levels of PAK1 and PAK2 in K562, KU812, v-ABL^{p160+} (upper panel) and BCR-ABL^{p185+} (lower panel) cells. (**b**) Co-immunoprecipitation of STAT5 in KU812 cells. PAK1, PAK2 and Rac1 co-immunoprecipitate with STAT5a. (**c**) *In vitro* kinase assays using recombinant TAT-STAT5a, PAK1 and Rac1 proteins. As a negative control, TAT-STAT5b was included. Activated PAK1 was detected by an antibody directed against $pPAK1^{T423}$. PAK1 phosphorylates $STAT5^{S779}$ in the presence of Rac1.

To investigate whether PAK kinases phosphorylate STAT5, we performed *in vitro* kinase assays, incubating recombinant PAK1 kinase with recombinant TAT-STAT5a protein. Only in the presence of Rac1—which is required to activate PAK kinases—did phosphorylation of $STAT5^{S779}$ become apparent (Figure 6c). As negative controls we used recombinant mouse STAT5b protein that is highly homologous to STAT5a as well as a truncated version of STAT5a ($STAT5^{\Delta 749}$) (Supplementary Figure 7a). Stat5b does not harbor a serine at position 779 but at position 778. However, $STAT5b^{778}$ is not flanked by prolines. We failed to detect any signal using these constructs. Identical results were obtained when we used recombinant PAK2 (Supplementary Figure 7b). The data support the conclusion that group I PAK kinases directly phosphorylate $STAT5^{S779}$. To further substantiate the link between PAK1/2 and $STAT5^{S779}$ phosphorylation, we performed knockdown experiments against PAK2 in murine BCR-ABL^{p185+}-transformed cells (Supplementary Figures 8a and b). We focused on PAK2 here as murine BCR-ABL^{p185+} cells do not express PAK1 (see Figure 6a). Despite the successful knockdown of PAK2, the remaining PAK2 proteins displayed increased kinase activity. Accordingly, $STAT5^{S779}$ phosphorylation was enhanced indicating a so far unrecognized pronounced feedback loop that was independent of mitogen-activated protein kinase kinase activity as the mitogen-activated protein kinase kinase inhibitor U0126 remained without effect (Supplementary Figure 8c).

$STAT5^{S779}$ phosphorylation is independent of BCR-ABL kinase activity

As $STAT5^{Y694}$ phosphorylation depends on BCR-ABL kinase activity, we investigated whether phosphorylation of $STAT5^{S725}$ and $STAT5^{S779}$ requires BCR-ABL kinase activity. BCR-ABL^{p185+} cells were treated with imatinib (2 μM) and the kinetics of $STAT5^{S725}$ and $STAT5^{S779}$ phosphorylation monitored. As expected, $STAT5^{Y694}$ phosphorylation decreased within 15 min and was hardly detectable after 60 min. In contrast, the level of $STAT5^{S725}$ and $STAT5^{S779}$ phosphorylation remained unaffected for 6 h (Figure 7a). This indicates that STAT5 serine phosphorylation is independent of BCR-ABL kinase activity and does not require concomitant $STAT5^{Y694}$ phosphorylation. We reasoned that—if independent of BCR-ABL kinase activity—activation of PAK kinases should not be impaired by imatinib treatment. We used antibodies that recognize phosphorylated $STAT5^{S780}$, PAK1 and PAK2 ($pPAK1^{T423}$ and $pPAK2^{T402}$), corresponding to the activated forms of the proteins, after incubation of KU812 cells with 2 μM imatinib. As expected, $STAT5^{Y694}$ phosphorylation rapidly declined (Figure 7b). However, there was no change to the levels of activated PAK1 and PAK2 and the extent of $STAT5^{S780}$ phosphorylation also remained unaltered upon imatinib treatment, suggesting that $STAT5^{S780}$ is phosphorylated independently of BCR-ABL.

Support came from an experiment with cells overexpressing a Tyr-phosphorylation mutant of STAT5 ($STAT5^{Y694F}$), $STAT5^{SASA}$ or $STAT5^{wt}$. STAT5 is phosphorylated on residues S725 and S779 even in the absence of tyrosine phosphorylation (Figure 7c). We reasoned that BCR-ABL^{p185+} cells with reduced PAK protein have an enhanced susceptibility if treated with PAK inhibitors. This was indeed the case; the half-maximal inhibitory concentration (IC_{50}) for PF-3758309 was reduced from 8.79 to 0.63 nM (random vs PAK2 shRNA) whereas the IC_{50} for imatinib remained unchanged (Figure 7d).

In summary, the data indicate that there are two distinct and independent pathways that control the phosphorylation and thus the intracellular localization of STAT5: phosphorylation of $STAT5^{Y694}$ controls the protein's dimerization, whereas $STAT5^{S779}$ phosphorylation directly regulates its intracellular localization (Figure 7e).

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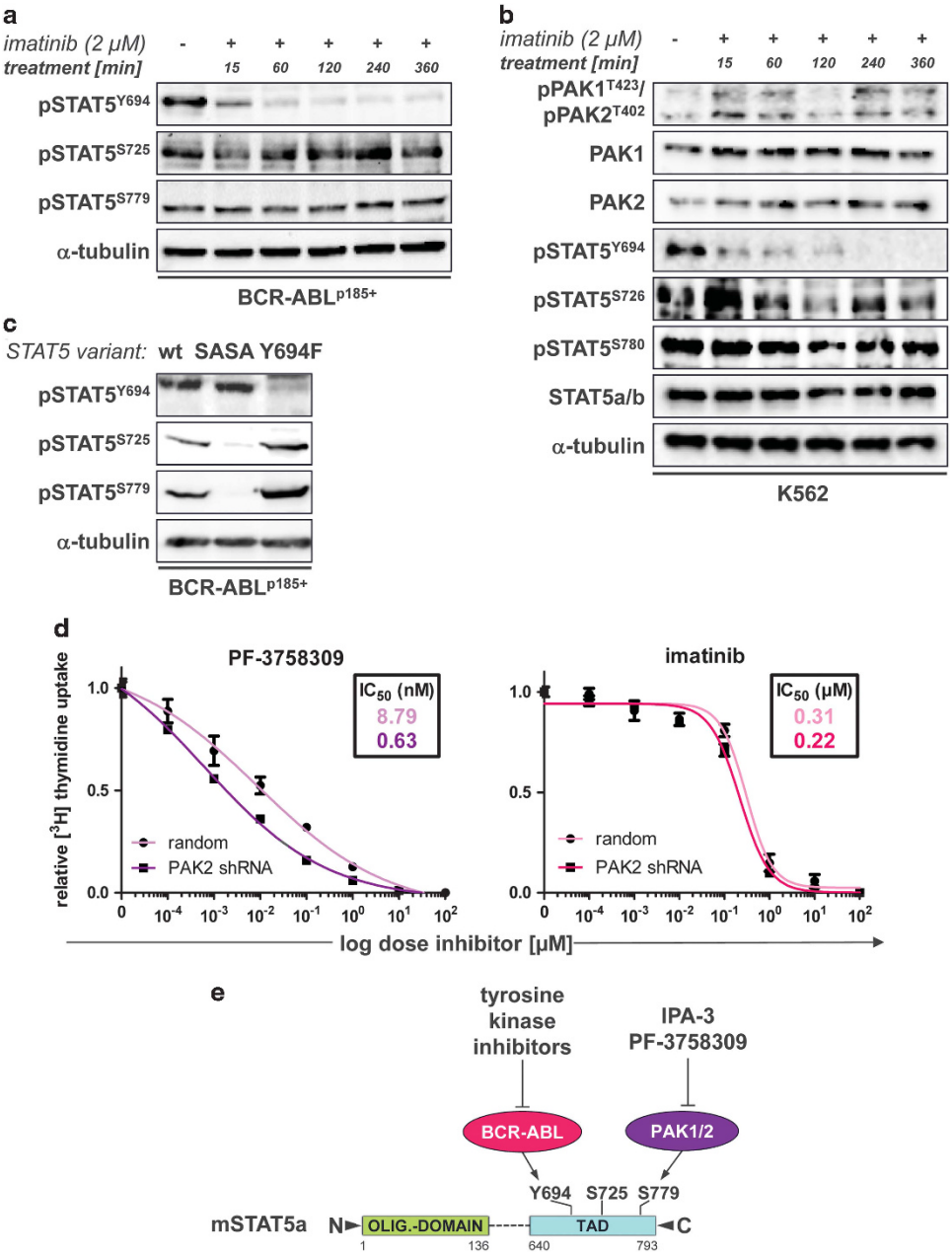


Figure 7. STAT5^{S725} and STAT5^{S779} phosphorylations are independent of BCR-ABL^{P185} kinase activity and STAT5^{Y694} phosphorylation. (**a**) BCR-ABL^{P185+} cells were treated with imatinib (2 μM) and STAT5 phosphorylation on residues Y694, S725 and S779 monitored by immunoblotting. (**b**) Immunoblotting of K562 cells after treatment with 2 μM imatinib for indicated time points. Levels of active versions of PAK1 and PAK2 (pPAK1^{T423}; pPAK2^{T402}) do not alter upon inhibition of BCR-ABL kinase activity. (**c**) Immunoblotting of *Stat5$^{fl/fl}$ Mx-1Cre^{+}* BCR-ABL^{P185+} cells expressing indicated STAT5 mutants. Endogenous *Stat5* was deleted via interferon-β (IFN-β) treatment 4 weeks before use. STAT5 proteins harboring a Y694 mutation maintain phosphorylations on S725 and S779. (**d**) Dose–response curves of PAK2 shRNA- or random shRNA-expressing BCR-ABL^{P185+} cells toward PF-3758309 (left panel) or imatinib (right panel). (**e**) Scheme summarizing activation of STAT5 via PAK1/2 kinases independently of BCR-ABL kinase activity.

DISCUSSION

The JAK/STAT pathway has been shown to be among the most important signaling pathways in the development and maintenance of tumors.[36] Inhibitors of individual JAKs and STATs are currently in development and are thought to hold promise for treating a wide variety of tumors. A number of JAK inhibitors are undergoing clinical trials and first compounds have been approved by the Food and Drug Administration (FDA).[37] Nevertheless, there have not yet been any convincing demonstrations of STAT inhibitors that are both safe and sufficiently specific to be appropriate for use in humans. The critical STAT molecules are STAT3 and STAT5, both of which are constitutively activated in a broad range of solid and hematopoietic tumors. Targeting these molecules directly via their dimerization domains has proven especially difficult. We present a different approach to inhibiting the activity of STAT5 in tumor maintenance, based on blocking an upstream serine kinase and consequently preventing nuclear translocation. It is likely that the mechanism we describe will turn out to be relevant to other types of tumor.

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Although a number of signaling pathways cooperate to support cell viability, the role of STAT5 in BCR-ABL-induced disease is key.[38,39] The deletion of STAT5a/b is incompatible with cell survival[10,11] and STAT5 is essential for initial transformation as well as for leukemia maintenance. Loss of STAT5 signaling causes leukemic cell death even in imatinib-resistant cells. Furthermore, deletion of STAT5 is well tolerated by the adult host organism—at least in the murine system.[10,11] STAT5 thus fulfills all the criteria of a therapeutic target.

STAT proteins lack a catalytic domain but can be targeted by inhibiting critical post-translational modifications. Most attention has been paid to Y694, the phosphorylation of which permits dimerization and nuclear translocation. In nontransformed cells, JAK kinases are responsible for tyrosine phosphorylation, whereas in BCR-ABL$^+$ cells the fusion kinase itself phosphorylates STAT5^{Y694}.[40] Treatment of BCR-ABL$^+$ cells with imatinib or any of the other tyrosine kinase inhibitors abolishes STAT5^{Y694} phosphorylation. All BCR-ABL kinase inhibitors thus indirectly target STAT5^{Y694} phosphorylation, and hence all of them essentially represent a single therapeutic avenue against STAT5.

STAT5 is additionally phosphorylated on highly conserved serine residues in the transactivation domain.[20] We show here that this phosphorylation is independent of STAT5^{Y694} phosphorylation: treatment of BCR-ABL^{p185+} cells with imatinib reduces phosphorylation of STAT5^{Y694} but not of STAT5^{S725} or STAT5^{S779}. Confirmation comes from experiments using a STAT5^{Y694} phosphorylation mutant that retains phosphorylation on STAT5^{S725} and STAT5^{S779}. STAT5a serine phosphorylation has recently been implicated in leukemogenesis.[27] BM transplantation studies using a constitutively active version of STAT5a (cS5a) caused leukemia with STAT5a itself as the driving oncogene but mutation of serine residues (STAT5^{S725A} and STAT5^{S779A}) abrogated disease. This indicated the importance of STAT5 serine phosphorylation but did not address its relevance in forms of leukemia driven by human transforming tyrosine kinases. We provide initial evidence that serine phosphorylation is important for kinase-driven STAT5 hyperactivation. Fetal liver cells could not be transformed with BCR-ABL^{p185} on co-infection with STAT5^{SASA}, whereas transfection of stable leukemic cell lines with STAT5 variants led to only an initial reduction of STAT5^{wt}-expressing cells that may stem from the ability of STAT5 to induce senescence[41] or to increase reactive oxygen species levels in BCR-ABL^{p185+} cells:[42,43] enforced STAT5^{wt} expression may enhance reactive oxygen species levels immediately, whereas STAT5-dependent transcription of anti-apoptotic proteins counteracting reactive oxygen species is delayed. The generation of a cell line that tolerates STAT5^{SASA} allowed us to show that leukemogenesis *in vivo* was significantly delayed. It is noteworthy that out of 21 attempts with individual cell lines and multiple round of retroviral transduction, we finally ended up with one single cell line tolerating STAT5^{SASA} expression—reflecting its detrimental effect on leukemic cell survival. We assume that interference with the transcriptional activity of STAT5 (as mediated by STAT5^{Y694} or STAT5^{S779} mutations) accounts for this problem. Most probably, this cell line evaded Stat5^{SASA}- induced cell death via the acquisition of secondary mutations such as alterations in tumor-suppressor responses. The intracellular location of STAT1 has recently been investigated. The protein has an unconventional nuclear localization signal[44] and mutation of STAT1^{L407A} hinders importin-α5 binding and thus causes cytoplasmic localization.[45,46] Similarly, phosphorylation of STAT5 controls the protein's intracellular localization and Src-dependent tyrosine phosphorylation in STAT5 Src homology 2 domain favors the cytoplasmic accumulation of the protein.[47] We propose that STAT5^{S779} phosphorylation is critically involved in nuclear translocation. The requirement for STAT5^{S779} phosphorylation has previously been overlooked, probably because it has been masked by the presence of endogenous STAT5.[19,20,27,48] Our conclusions are based on observations in BCR-ABL^{p185+} HEK cells as well as in leukemic cells. Tracking of YFP-tagged STAT5 variants showed that STAT5^{S779A} and STAT5^{SASA} proteins fail to accumulate in the nucleus, whereas nuclear accumulation is only achieved by concomitant expression of STAT5^{wt}. This indicates that the presence of one STAT5 partner with an intact S779 within the dimer suffices for nuclear transport.

We provide several lines of evidence that group I PAK kinases are upstream regulators of STAT5^{S779} phosphorylation in BCR-ABL$^+$ cells. First, PAK kinases were identified in drug screens based on viability of the human cell line K562 and of murine BCR-ABL^{p185+} cells. IPA-3, which allosterically blocks the group I PAK kinases at concentrations that make it unsuitable for use in human patients, and the inhibitor PF-3758309, which prevents ATP binding, significantly reduce the viability of both cell lines, accompanied by decreases in the levels of STAT5^{S779} phosphorylation and of nuclear STAT5. The findings are in line with observations in STAT5^{S779A} mutant cells, in which nuclear accumulation of the protein and transcription of target genes is significantly impaired. Second, PAK kinases are able to phosphorylate STAT5^{S779} *in vitro*. Co-immunoprecipitation experiments identified complexes containing both STAT5 and group I PAK kinases. Finally, knockdown experiments revealed the tight connection between STAT5^{S779} phosphorylation and PAK kinases, although in an unexpected manner. Reduced expression of PAK2 in murine BCR-ABL^{p185+} cells is associated with an enhanced activity of the remaining protein and paralleled by increased STAT5^{S779} phosphorylation. This observation might point at a so far unknown feedback loop that tightly adjusts protein expression and activation status. As PAK kinases regulate mitogen-activated protein kinase kinase 1 activation via phosphorylation on S298,[49] this represents an obvious candidate. However, no effects were observed if we blocked mitogen-activated protein kinase kinase 1 in our cellular system. Nevertheless, reduced PAK protein levels render the cells more susceptible to treatment with PAK inhibitors but not to BCR-ABL tyrosine kinase inhibitors, providing further support for our concept.

Group I PAK kinases 1–3 are known to possess auto-inhibitory phosphotyrosine interaction domains and to require activation by a p21 GTPase, either Rac or Cdc42.[50] There is convincing evidence that Rac GTPases are key regulators of BCR-ABL-induced malignancies,[51,52] but the underlying mechanism has remained obscure. Our results suggest that the effect is at least partially mediated by inhibition of nuclear accumulation of STAT5. It is currently unknown which signaling pathways activate PAK kinases in nonsolid tumors, although integrin signaling was recently shown to be crucial for acute myeloid leukemia.[53] The constitutive activation of PAK kinases may also result from the accelerated cell cycle progression in leukemic cells: PAK kinases are active when cells initiate mitosis and rapidly dividing cells might not have sufficient time to deactivate them. PAK kinases have been reported to be overexpressed in human cancers and are considered promising therapeutic targets.[54] They have a wide variety of downstream targets, such as c-Raf and MAPK signaling that contribute to a tumorigenic state, and hence the therapeutic potential of inhibiting PAK kinases is considerable.[54] To the best of our knowledge, there have been no previous studies of PAK kinases and STAT5 in transformed cells, although there is a report that PAK1 regulates lobuloalveolar development in a STAT5^{S779}-dependent manner.[55] Using BCR-ABL$^+$ disease we now show that PAK kinases act via STAT5 in an oncogenic setting, thereby adding STAT5 to the list of signaling mediators downstream of PAK kinases. STAT5 may be one of the key downstream targets of PAK kinases in certain types of tumors, mediating their protooncogenic effects.

The importance of STAT5 serine phosphorylation for transformation and its independence from the BCR-ABL-STAT5^{Y694} axis offers a therapeutic opportunity that is distinct from that afforded by current tyrosine kinase inhibitors. Inhibiting PAK1 and/or PAK2 may prevent nuclear localization of STAT5 and as a consequence its oncogenic activity. Targeting PAK kinases may represent a

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feasible way to circumvent the difficulties in developing effective direct inhibitors of STAT5 and might provide a promising strategy for treating cancers with hyperactivated STAT5.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Bromberg J. Stat proteins and oncogenesis. *J Clin Invest* 2002; **109**: 1139–1142.
2 Turkson J, Jove R. STAT proteins: novel molecular targets for cancer drug discovery. *Oncogene* 2000; **19**: 6613–6626.
3 Lacronique V, Boureux A, Valle VD, Poirel H, Quang CT, Mauchauffé M *et al.* A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. *Science* 1997; **278**: 1309–1312.
4 James C, Ugo V, Le Couédic J-P, Staerk J, Delhommeau F, Lacout C *et al.* A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature* 2005; **434**: 1144–1148.
5 Kralovics R, Passamonti F, Buser AS, Teo S-S, Tiedt R, Passweg JR *et al.* A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med* 2005; **352**: 1779–1790.
6 Yan D, Hutchison RE, Mohi G. Critical requirement for Stat5 in a mouse model of polycythemia vera. *Blood* 2012; **119**: 3539–3549.
7 Schwaller J, Parganas E, Wang D, Cain D, Aster JC, Williams IR *et al.* Stat5 is essential for the myelo- and lymphoproliferative disease induced by TEL/JAK2. *Mol Cell* 2000; **6**: 693–704.
8 Ilaria Jr RL, Van Etten RA. P210 and P190(BCR/ABL) induce the tyrosine phosphorylation and DNA binding activity of multiple specific STAT family members. *J Biol Chem* 1996; **271**: 31704–31710.
9 Sillaber C, Gesbert F, Frank DA, Sattler M, Griffin JD. STAT5 activation contributes to growth and viability in Bcr/Abl-transformed cells. *Blood* 2000; **95**: 2118–2125.
10 Hoelbl A, Kovacic B, Kerenyi MA, Simma O, Warsch W, Cui Y *et al.* Clarifying the role of Stat5 in lymphoid development and Abelson-induced transformation. *Blood* 2006; **107**: 4898–4906.
11 Hoelbl A, Schuster C, Kovacic B, Zhu B, Wickre M, Hoelzl MA *et al.* Stat5 is indispensable for the maintenance of bcr/abl-positive leukaemia. *EMBO Mol Med* 2010; **2**: 98–110.
12 Putz EM, Gotthardt D, Hoermann G, Csiszar A, Wirth S, Berger A *et al.* CDK8-mediated STAT1-S727 phosphorylation restrains NK cell cytotoxicity and tumor surveillance. *Cell Rep* 2013; **4**: 437–444.
13 Leonard WJ, O'Shea JJ. Jaks and STATs: biological implications. *Ann Rev Immunol* 1998; **16**: 293–322.
14 Carlesso N, Frank DA, Griffin JD. Tyrosyl phosphorylation and DNA binding activity of signal transducers and activators of transcription (STAT) proteins in hematopoietic cell lines transformed by Bcr/Abl. *J Exp Med* 1996; **183**: 811–820.
15 Chai SK, Nichols GL, Rothman P. Constitutive activation of JAKs and STATs in BCR-Abl-expressing cell lines and peripheral blood cells derived from leukemic patients. *J Immunol* 1997; **159**: 4720–4728.
16 Moriggl R, Sexl V, Kenner L, Duntsch C, Stangl K, Gingras S *et al.* Stat5 tetramer formation is associated with leukemogenesis. *Cancer Cell* 2005; **7**: 87–99.
17 Decker T, Kovarik P. Serine phosphorylation of STATs. *Oncogene* 2000; **19**: 2628–2637.
18 Wen Z, Zhong Z, Darnell Jr JE. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell* 1995; **82**: 241–250.
19 Yamashita H, Nevalainen MT, Xu J, LeBaron MJ, Wagner KU, Erwin RA *et al.* Role of serine phosphorylation of Stat5a in prolactin-stimulated beta-casein gene expression. *Mol Cell Endocrinol* 2001; **183**: 151–163.
20 Beuvink I, Hess D, Flotow H, Hofsteenge J, Groner B, Hynes NE. Stat5a serine phosphorylation. Serine 779 is constitutively phosphorylated in the mammary gland, and serine 725 phosphorylation influences prolactin-stimulated in vitro DNA binding activity. *J Biol Chem* 2000; **275**: 10247–10255.
21 Pilz A, Kratky W, Stockinger S, Simma O, Kalinke U, Lingnau K *et al.* Dendritic cells require STAT-1 phosphorylated at its transactivating domain for the induction of peptide-specific CTL. *J Immunol* 2009; **183**: 2286–2293.
22 Varinou L, Ramsauer K, Karaghiosoff M, Kolbe T, Pfeffer K, Muller M *et al.* Phosphorylation of the Stat1 transactivation domain is required for full-fledged IFN-gamma-dependent innate immunity. *Immunity* 2003; **19**: 793–802.
23 Gough DJ, Corlett A, Schlessinger K, Wegrzyn J, Larner AC, Levy DE. Mitochondrial STAT3 supports Ras-dependent oncogenic transformation. *Science* 2009; **324**: 1713–1716.
24 Aziz MH, Manoharan HT, Sand JM, Verma AK. Protein kinase Cepsilon interacts with Stat3 and regulates its activation that is essential for the development of skin cancer. *Mol Carcinog* 2007; **46**: 646–653.
25 Qin HR, Kim H-J, Kim J-Y, Hurt EM, Klarmann GJ, Kawasaki BT *et al.* Activation of signal transducer and activator of transcription 3 through a phosphomimetic serine 727 promotes prostate tumorigenesis independent of tyrosine 705 phosphorylation. *Cancer Res* 2008; **68**: 7736–7741.
26 Schuringa JJ, Wierenga AT, Kruijer W, Vellenga E. Constitutive Stat3, Tyr705, and Ser727 phosphorylation in acute myeloid leukemia cells caused by the autocrine secretion of interleukin-6. *Blood* 2000; **95**: 3765–3770.
27 Friedbichler K, Kerenyi MA, Kovacic B, Li G, Hoelbl A, Yahiaoui S *et al.* Stat5a serine 725 and 779 phosphorylation is a prerequisite for hematopoietic transformation. *Blood* 2010; **116**: 1548–1558.
28 Kuhn R, Schwenk F, Aguet M, Rajewsky K. Inducible gene targeting in mice. *Science* 1995; **269**: 1427–1429.
29 Cui Y, Riedlinger G, Miyoshi K, Tang W, Li C, Deng CX *et al.* Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. *Mol Cell Biol* 2004; **24**: 8037–8047.
30 Kovacic B, Stoiber D, Moriggl R, Weisz E, Ott RG, Kreibich R *et al.* STAT1 acts as a tumor promoter for leukemia development. *Cancer Cell* 2006; **10**: 77–87.
31 Sexl V, Piekorz R, Moriggl R, Rohrer J, Brown MP, Bunting KD *et al.* Stat5a/b contribute to interleukin 7-induced B-cell precursor expansion, but abl- and bcr/abl-induced transformation are independent of stat5. *Blood* 2000; **96**: 2277–2283.
32 Schreiber E, Matthias P, Müller MM, Schaffner W. Rapid detection of octamer binding proteins with ''mini-extracts'', prepared from a small number of cells. *Nucleic Acids Res* 1989; **17**: 6419.
33 Schuster B, Hendry L, Byers H, Lynham SF, Ward MA, John S. Purification and identification of the STAT5 protease in myeloid cells. *Biochem J* 2007; **404**: 81–87.
34 Harir N, Pecquet C, Kerenyi M, Sonneck K, Kovacic B, Nyga R *et al.* Constitutive activation of Stat5 promotes its cytoplasmic localization and association with PI3-kinase in myeloid leukemias. *Blood* 2007; **109**: 1678–1686.
35 Bancerek J, Poss ZC, Steinparzer I, Sedlyarov V, Pfaffenwimmer T, Mikulic I *et al.* CDK8 kinase phosphorylates transcription factor STAT1 to selectively regulate the interferon response. *Immunity* 2013; **38**: 250–262.
36 Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med* 2004; **10**: 789–799.
37 Menet CJ, Rompaey L, Van, Geney R. Advances in the discovery of selective JAK inhibitors. *Prog Med Chem* 2013; **52**: 153–223.
38 Krause DS, Van Etten RA. Right on target: eradicating leukemic stem cells. *Trends Mol Medi* 2007; **13**: 470–481.
39 Steelman LS, Franklin RA, Abrams SL, Chappell W, Kempf CR, Bäsecke J *et al.* Roles of the Ras/Raf/MEK/ERK pathway in leukemia therapy. *Leukemia* 2011; **25**: 1080–1094.
40 Hantschel O, Warsch W, Eckelhart E, Kaupe I, Grebien F, Wagner K-U *et al.* BCR-ABL uncouples canonical JAK2-STAT5 signaling in chronic myeloid leukemia. *Nature chemical biology* 2012; **8**: 285–293.
41 Mallette FA, Gaumont-Leclerc M-F, Huot G, Ferbeyre G. Myc down-regulation as a mechanism to activate the Rb pathway in STAT5A-induced senescence. *J Biol Chem* 2007; **282**: 34938–34944.
42 Warsch W, Grundschober E, Berger A, Gille L, Cerny-Reiterer S, Tigan A-S *et al.* STAT5 triggers BCR-ABL1 mutation by mediating ROS production in chronic myeloid leukaemia. *Oncotarget* 2012; **3**: 1669–1687.
43 Warsch W, Grundschober E, Sexl V. Adding a new facet to STAT5 in CML: multitasking for leukemic cells. *Cell cycle* 2013; **12**: 1813–1814.
44 McBride KM, Banninger G, McDonald C, Reich NC. Regulated nuclear import of the STAT1 transcription factor by direct binding of importin-alpha. *EMBO J* 2002; **21**: 1754–1763.
45 Melen K, Kinnunen L, Julkunen I. Arginine/lysine-rich structural element is involved in interferon-induced nuclear import of STATs. *J Biol Chem* 2001; **276**: 16447–16455.
46 Meyer T, Begitt A, Lödige I, Van Rossum M, Vinkemeier U. Constitutive and IFN-gamma-induced nuclear import of STAT1 proceed through independent pathways. *EMBO J* 2002; **21**: 344–354.
47 Chatain N, Ziegler P, Fahrenkamp D, Jost E, Moriggl R, Schmitz-Van de Leur H *et al.* Src family kinases mediate cytoplasmic retention of activated STAT5 in BCR-ABL-positive cells. *Oncogene* 2013; **32**: 3587–3597.
48 Clark DE, Williams CC, Duplessis TT, Moring KL, Notwick AR, Long W *et al.* ERBB4/HER4 potentiates STAT5A transcriptional activity by regulating novel STAT5A serine phosphorylation events. *J Biol Chem* 2005; **280**: 24175–24180.

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49 Slack-Davis JK, Eblen ST, Zecevic M, Boerner SA, Tarcsafalvi A, Diaz HB *et al.* PAK1 phosphorylation of MEK1 regulates fibronectin-stimulated MAPK activation. *J Cell Biol* 2003; **162**: 281–291.
50 Lim L, Manser E, Leung T, Hall C. Regulation of phosphorylation pathways by p21 GTPases. The p21 Ras-related Rho subfamily and its role in phosphorylation signalling pathways. *Eur J Biochem* 1996; **242**: 171–185.
51 Dummler B, Ohshiro K, Kumar R, Field J. Pak protein kinases and their role in cancer. *Cancer Metastasis Rev* 2009; **28**: 51–63.
52 Thomas EK, Cancelas JA, Zheng Y, Williams DA. Rac GTPases as key regulators of p210-BCR-ABL-dependent leukemogenesis. *Leukemia* 2008; **22**: 898–904.
53 Miller PG, Al-Shahrour F, Hartwell KA, Chu LP, Järås M, Puram RV *et al.* In vivo RNAi screening identifies a leukemia-specific dependence on integrin beta 3 signaling. *Cancer Cell* 2013; **24**: 45–58.
54 Kumar R, Gururaj AE, Barnes CJ. p21-activated kinases in cancer. *Nat Rev Cancer* 2006; **6**: 459–471.
55 Wang R-A, Zhang H, Balasenthil S, Medina D, Kumar R. PAK1 hyperactivation is sufficient for mammary gland tumor formation. *Oncogene* 2006; **25**: 2931–2936.

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