

## Supplementary Data

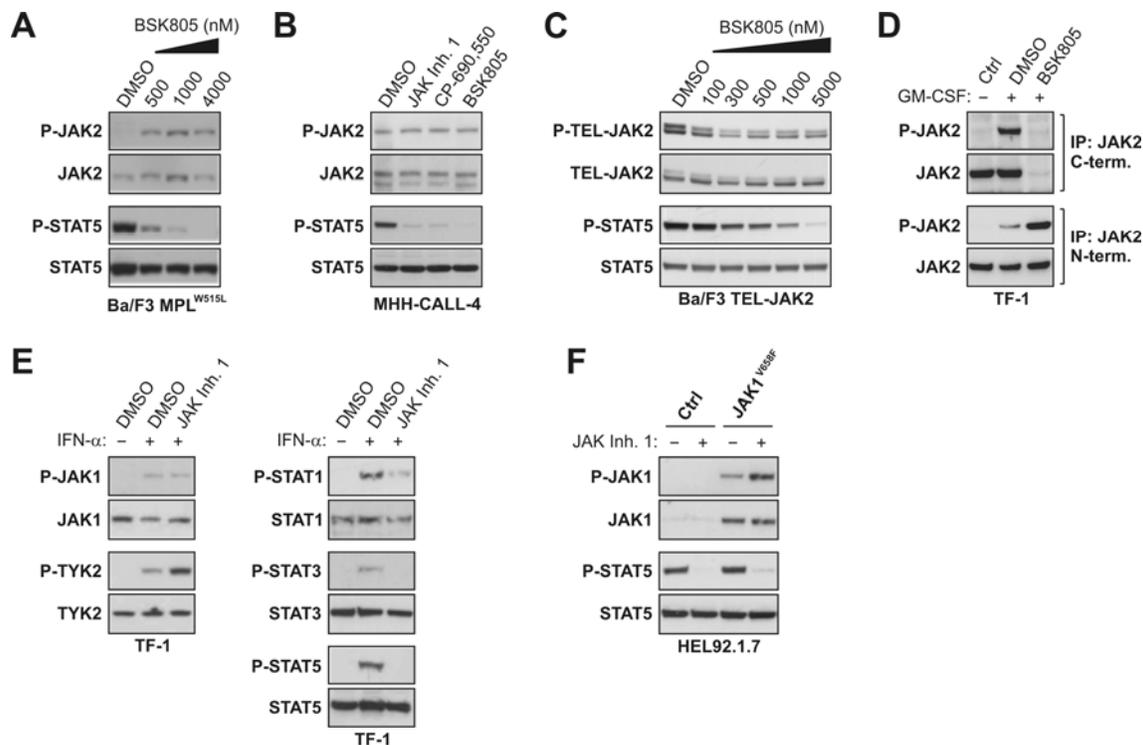
### **Modulation of activation-loop phosphorylation by JAK inhibitors is binding mode dependent**

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## Supplementary Figures

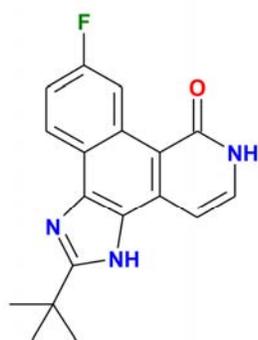


Supplementary Figure S1

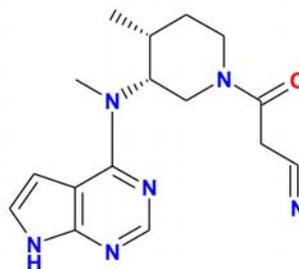
**Supplementary Figure S1. Analysis of JAK inhibitor effects on JAK activation-loop phosphorylation in different cellular models.** **A**, Ba/F3 MPL<sup>W515L</sup> cells were treated for 4 hours with the indicated concentrations of NVP-BSK805 and then extracted for Western blot analysis of JAK2 Y1007/Y1008 phosphorylation and STAT5 Y694 phosphorylation. Total levels of JAK2 and STAT5 were probed for as loading controls. **B**, MHH-CALL-4 cells were treated for 1 hour with 1  $\mu$ M of the indicated compounds and then extracted for Western blot analysis as described above. **C**, Ba/F3 TEL-JAK2 cells were treated with increasing concentrations of NVP-BSK805 for 1 h and then extracted for Western blot detection of TEL-JAK2 phosphorylation (using a JAK2 Y1007/Y1008 phospho-specific antibody), TEL-JAK2 (using a TEL antibody) and STAT5 Y694 phosphorylation. **D**, TF-1 cells were starved in medium without GM-CSF overnight and then either pre-treated with DMSO or NVP-BSK805

at 1  $\mu$ M for 30 minutes. Then cells were stimulated or not with 20 ng/mL GM-CSF for 10 minutes, followed by extraction for immuno-precipitation (IP) of JAK2 using an antibody directed either towards the amino- or the carboxyl-terminus. Levels of JAK2 Y1007/Y1008 phosphorylation and of the IPd JAK2 protein were detected by Western blotting. **E**, TF-1 cells were starved in medium without GM-CSF for 4 h and then either pre-treated with DMSO or JAK inhibitor 1 at 1  $\mu$ M for 1 h. Cells were then stimulated or not with 20 ng/mL IFN- $\alpha$  for 10 minutes, followed by extraction for Western blot analysis of JAK1 Y1022/Y1023 phosphorylation and TYK2 Y1054/Y1055 phosphorylation, as well as STAT1 (Y701), STAT3 (Y705) and STAT5 (Y694) phosphorylation. Total levels of JAK1, TYK2, STAT1, STAT3 and STAT5 were probed for as loading controls. **F**, HEL92.1.7 cells were transiently transfected with the empty vector (Ctrl) or the corresponding vector for expression of JAK1<sup>V658F</sup>. After 24 h, cells were either treated with 1  $\mu$ M JAK inhibitor 1 or DMSO vehicle for 1 h, followed by extraction for Western blot detection of P-JAK1 (Y1022/Y1023) and P-STAT5 (Y694) levels. Total levels of STAT5 and JAK1 were probed for as loading controls and to verify expression of exogenous mutant JAK1<sup>V658F</sup>, respectively.

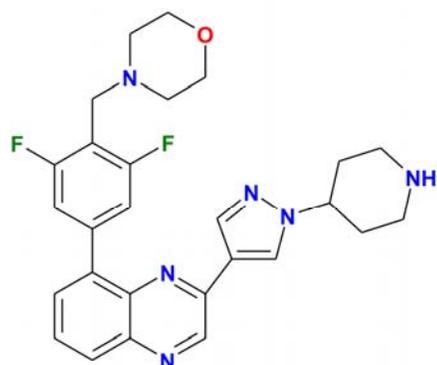
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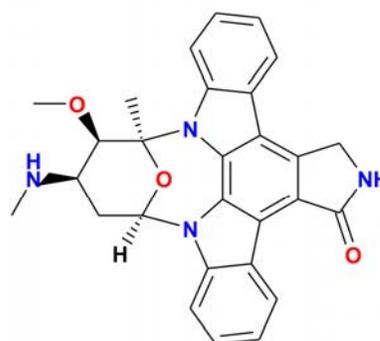
**CP-690,550**



**NVP-BSK805**

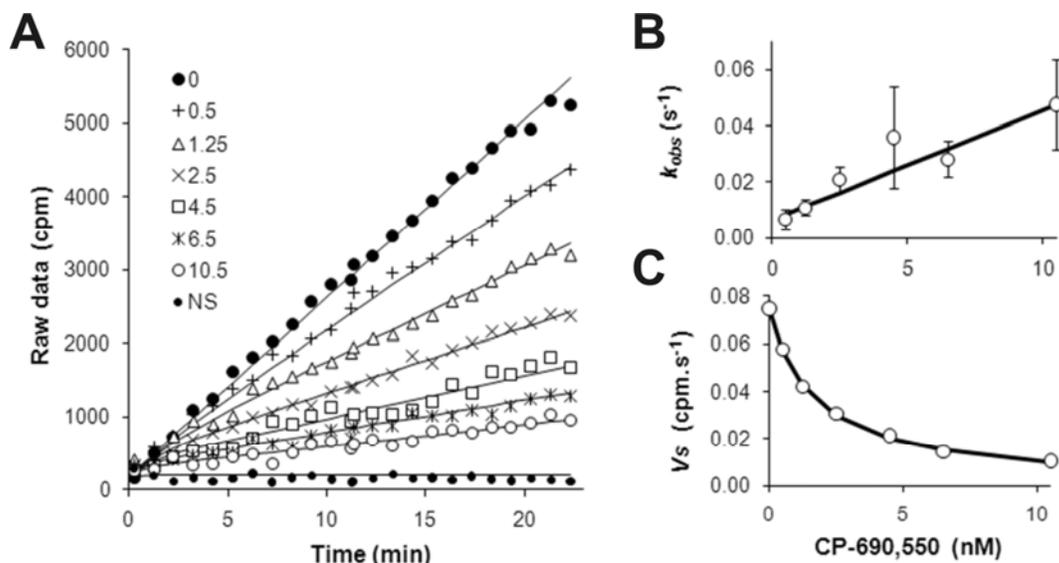


**Staurosporine**



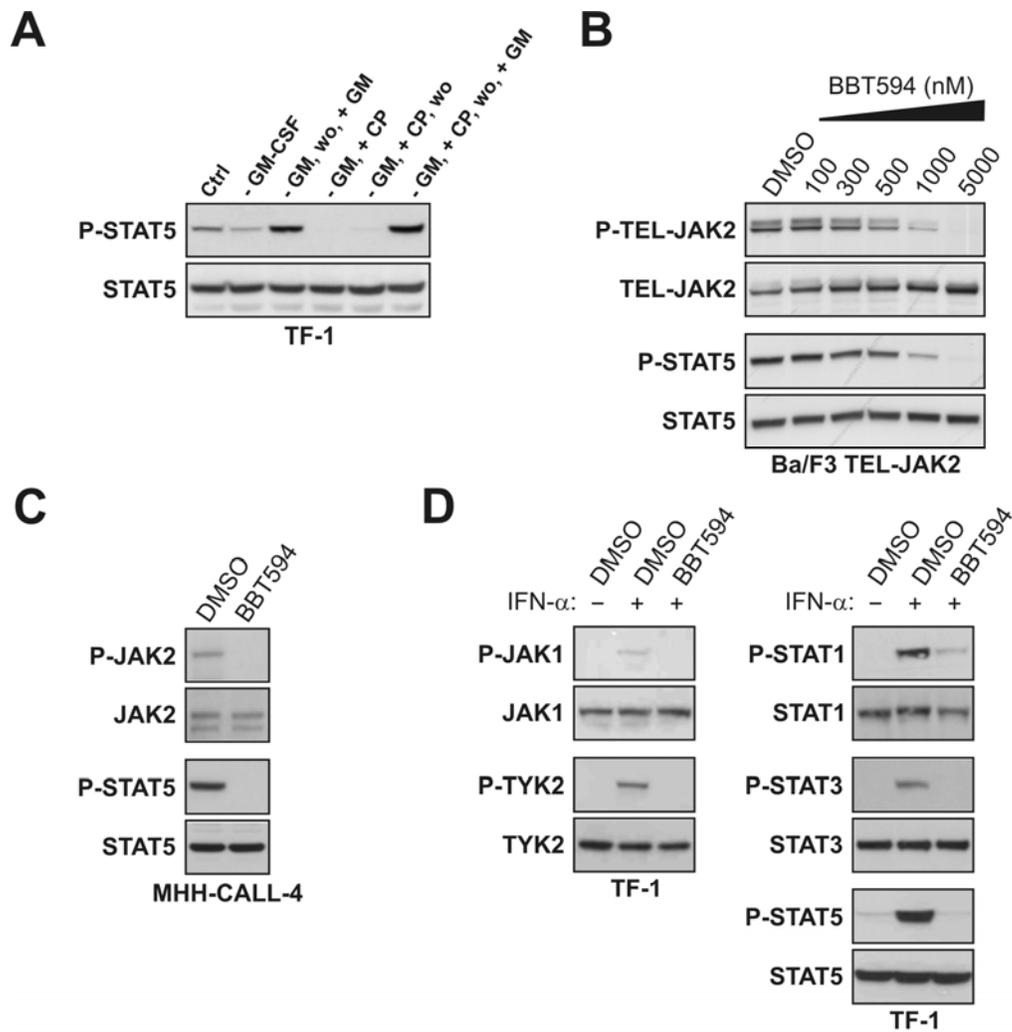
**Supplementary Figure S2**

**Supplementary Figure S2. Overview of ATP-competitive type-I inhibitors used in this study.** The chemical structures of JAK Inhibitor 1 (tetracyclic pyridone scaffold-based compound, also termed “pyridone 6”), CP-690,550 (pyrrolo[2,3-d]pyrimidine-based compound; tofacitinib), NVP-BSK805 (2,8-diaryl-quinoxaline-based compound) and staurosporine (indolo[2,3-a]carbazole alkaloid) are shown.



Supplementary Figure S3

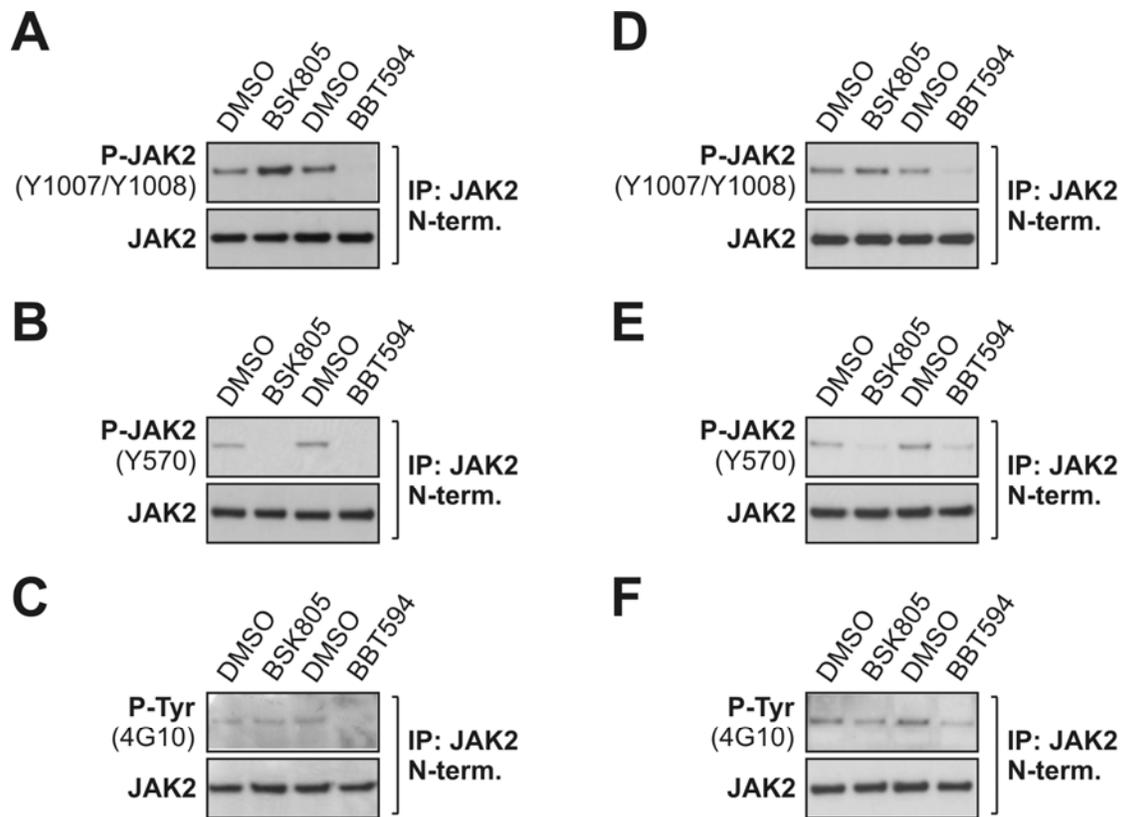
**Supplementary Figure S3. Binding kinetics of CP-690,550, as assessed using the recombinant JAK2 kinase (JH1 domain) *in vitro*.** The JAK2 JH1 enzyme was incubated at room temperature in 0.9 mL medium containing the inhibitor, 1% DMSO, 1 mM  $MnCl_2$ , 5 mM  $MgCl_2$ , 1 mM DTT, 50  $\mu$ g/mL BSA, 0.01% Brij35, 0.1  $\mu$ M  $[\gamma\text{-}^{33}\text{P}]\text{-ATP}$ , 30  $\mu$ M EQEDEPEGDYFEWLE (peptide substrate), and 50 mM Tris-HCl pH 7.5. The kinase reaction was initiated by addition of JAK2 JH1 (0.32 ng/30  $\mu$ L or 20 pM active protein) and stopped after various times by transferring 30  $\mu$ L of this medium to 96-well plates containing 120  $\mu$ L of 200 mM  $H_3PO_4$ . **A**, Bound  $^{33}\text{P}$  as a function of time and inhibitor concentration (nM) as indicated in the graph. Each point is the average of two determinations from a representative experiment. Curves were globally fitted to eq. 6 by non-linear 3D regression. The predicted  $k_{obs}$  (**B**) and  $V_s$  (**C**) are plotted in the right upper and lower panels, respectively. Points in the right panels represent these parameters ( $\pm$  SE) calculated from each curve with eq. 1 by non-linear 2D regression.



Supplementary Figure S4

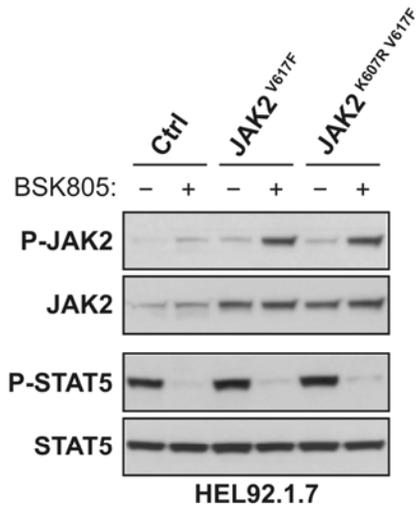
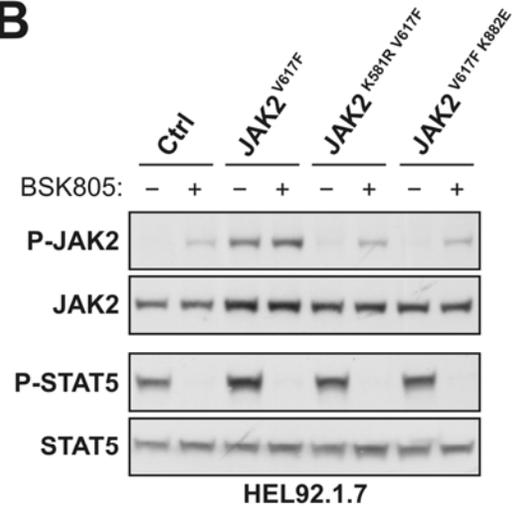
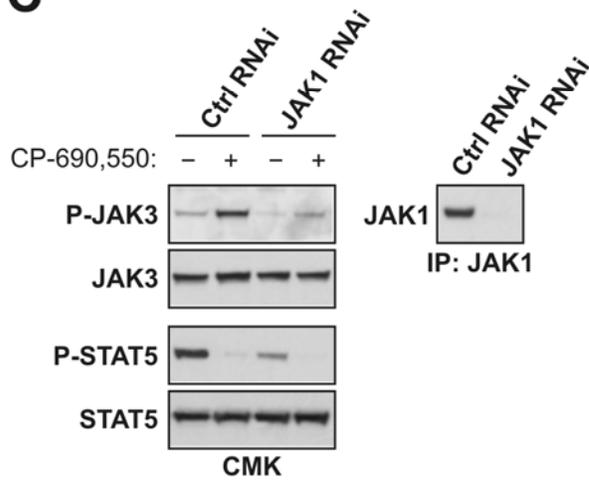
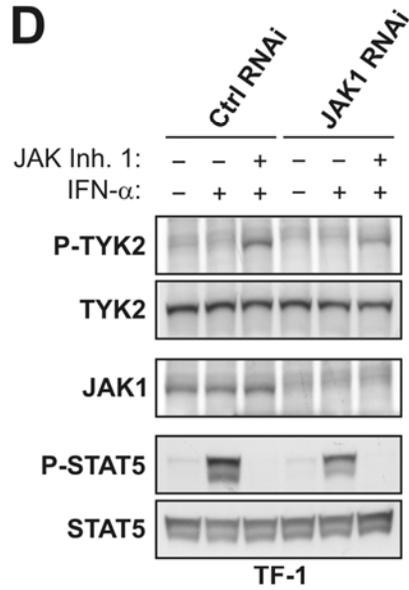
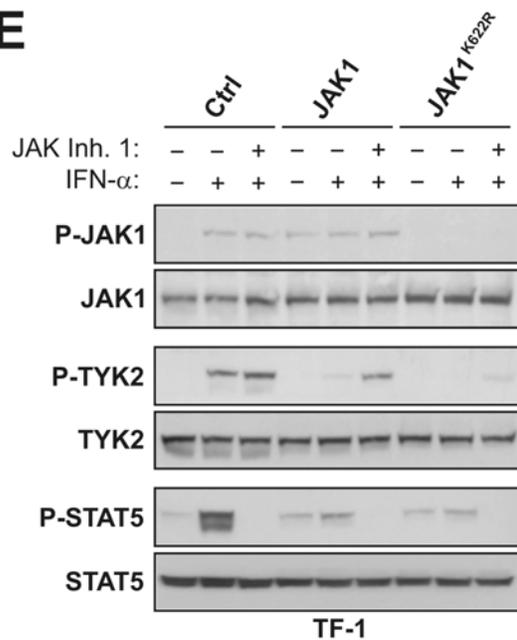
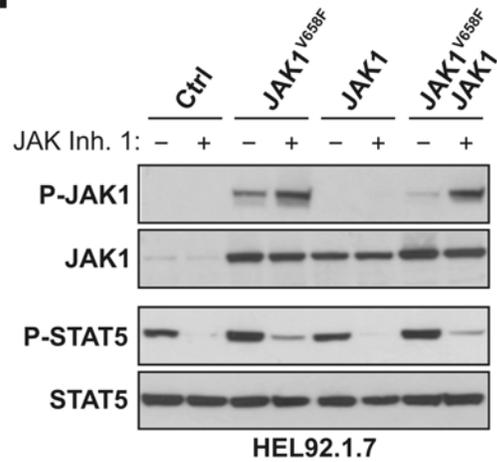
**Supplementary Figure S4. JAK type-I inhibitor treatment and washout in factor-starved TF-1 cells, and suppression of both JAK activation-loop phosphorylation and STAT phosphorylation upon JAK type-II inhibitor treatment.** **A**, treatment of overnight GM-CSF-starved (-GM) TF-1 cells with CP-690,550 (+CP) at 1  $\mu$ M for 2 h, followed by inhibitor washout (wo) for 2 h, did not lead to increased phosphorylated STAT5 (Y694) levels in the absence of GM-CSF. Levels of JAK2 Y1007/Y1008 phosphorylation were below detection limits in TF-1 cells. Levels of STAT5 phosphorylation in untreated control (Ctrl) cells are shown for sake of reference. **B**, Ba/F3 TEL-JAK2 cells were treated with increasing concentrations of NVP-BBT594 for 1 h and then extracted for Western blot detection of TEL-

JAK2 phosphorylation (using a JAK2 Y1007/Y1008 phospho-specific antibody), TEL-JAK2 (using a TEL antibody) and STAT5 Y694 phosphorylation. **C**, MHH-CALL-4 cells were treated for 1 h either with DMSO or with 1  $\mu$ M NVP-BBT594 and then extracted for Western blot detection of JAK2 Y1007/Y1008 phosphorylation and STAT5 Y694 phosphorylation. Total levels of JAK2 and STAT5 were probed for as loading controls. **D**, TF-1 cells were starved in medium without GM-CSF for 4 h and then either pre-treated with DMSO or 1  $\mu$ M NVP-BBT594 for 1 h. Cells were then stimulated or not with 20 ng/ml IFN- $\alpha$  for 10 minutes, followed by extraction for Western blot analysis of JAK1 Y1022/Y1023 phosphorylation, TYK2 Y1054/Y1055 phosphorylation, as well as STAT1 (Y701), STAT3 (Y705) and STAT5 (Y694) phosphorylation. Total levels of JAK1, TYK2, STAT1, STAT3 and STAT5 were probed for as loading controls.



Supplementary Figure S5

**Supplementary Figure S5. Analysis of JAK2 tyrosine phosphorylation following treatment of JAK2<sup>V617F</sup> mutant cell lines with JAK type-I or type-II inhibitors. A-C,** JAK2<sup>V617F</sup> mutant SET-2 cells were either treated with DMSO, 1  $\mu$ M NVP-BSK805 or 1  $\mu$ M NVP-BBT594 for 30 minutes, followed by extraction for immuno-precipitation (IP) of JAK2 using an antibody directed towards the amino-terminus. Levels of JAK2 Y1007/Y1008 phosphorylation, of JAK2 Y570 phosphorylation and of overall tyrosine phosphorylation of the IPd JAK2 protein were detected by Western blotting. **D-F,** HEL92.1.7 cells were treated as described above for the analysis of JAK2 tyrosine phosphorylation.

**A****B****C****D****E****F**

**Supplementary Figure S6. Analysis of the role of JH1 and JH2 domains in JAK type-I inhibitor-induced increase of JAK activation-loop phosphorylation, and impact of JAK depletion at oligomeric cytokine receptor complexes.** **A**, HEL92.1.7 cells were transiently transfected with the indicated JAK2 constructs. Control cells were transfected with the empty vector (Ctrl). After 24 h, cells were either treated with 1  $\mu$ M NVP-BSK805 or DMSO vehicle for 30 minutes, followed by extraction for Western blot detection of JAK2 Y1007/Y1008 and STAT5 Y694 phosphorylation. **B**, HEL92.1.7 cells were transiently transfected with the indicated JAK2 constructs. Control cells were transfected with the empty vector (Ctrl). After 24 h, cells were either treated with 1  $\mu$ M NVP-BSK805 or DMSO vehicle for 30 minutes, followed by extraction for Western blot detection of P-JAK2 (Y1007/Y1008) and P-STAT5 (Y694) levels. **C**, non-targeting siRNA oligos or oligos directed towards JAK1 were transfected into CMK cells. After 24 h cells were either treated with 1  $\mu$ M CP-690,550 or DMSO vehicle for 1 h, followed by extraction for Western blot detection of P-JAK3 (Y980) and P-STAT5 (Y694) levels. The degree of RNAi-mediated JAK1 depletion was verified by immunoprecipitation of JAK1 and Western blotting. **D**, non-targeting siRNA oligos or oligos directed towards JAK1 were transfected into TF-1 cells. After 48 h cells were and then starved for GM-CSF overnight. The following day cells were either treated with 1  $\mu$ M JAK inhibitor 1 or DMSO vehicle for 30 minutes. Cells were then stimulated or not with 10 ng/mL IFN- $\alpha$  for 10 minutes, followed by extraction for Western blot analysis of TYK2 Y1054/Y1055 phosphorylation and STAT5 Y694 phosphorylation. Total levels of TYK2 and STAT5 were probed for as loading controls. The degree of RNAi-mediated JAK1 depletion was verified by Western blotting. **E**, TF-1 cells were transiently transfected with the indicated JAK1 constructs. Control cells were transfected with the empty vector (Ctrl). After 24 h, cells were starved in medium without GM-CSF for 4 hours and then either pre-treated with DMSO or 1  $\mu$ M JAK inhibitor 1 for 1 h. Cells were then stimulated or not with 20 ng/ml IFN- $\alpha$  for

10 minutes, followed by extraction for Western blot analysis of JAK1 Y1022/Y1023 phosphorylation, TYK2 Y1054/Y1055 phosphorylation and STAT5 Y694 phosphorylation. Total levels of JAK1, TYK2 and STAT5 were probed for as loading controls. F, HEL92.1.7 cells were transiently transfected with the indicated JAK1 constructs. Control cells were transfected with the empty vectors (Ctrl). Where single JAK1 mutant constructs were transfected, the respective empty vector of the other JAK1 mutant construct was co-transfected to deliver comparable amounts of plasmid DNA into cells. After 24 h, cells were either treated with 1  $\mu$ M JAK Inhibitor 1 or DMSO vehicle for 1 hour, followed by extraction for Western blot detection of levels of JAK1 Y1022/Y1023 and STAT5 Y694 phosphorylation.

## **Supplementary Table Legends**

### **Supplementary Table S1. Activity of NVP-BBT594 in biochemical and cell-based assays.**

The IC<sub>50</sub> (mean of two experiments) of NVP-BBT594 on the JAK2 kinase was determined in a biochemical kinase assay with the active enzyme (1). Half-maximal growth inhibition (GI<sub>50</sub>) of NVP-BBT594 was determined in SET-2, CMK and K-562 cell lines (mean ± SD, n = 4).

### **Supplementary Table S2. Overview of phosphopeptides detected by mass-spectrometry following treatment of SET-2 cells with different JAK inhibitors.**

For treatment details see Table 1. Changes in phosphopeptide abundance following JAK inhibitor treatment versus DMSO vehicle control treatment in the different experiments are displayed as log<sub>2</sub> ratios calculated from duplicate runs. No value = not detected.

### **Supplementary Table S3. X-ray crystallographic data collection and refinement**

**statistics.** Diffraction data were collected at the Swiss Light Source (beamline X10SA). Raw diffraction data were processed and scaled with the XDS/XSCALE software package. The program BUSTER was used for structure refinement using all diffraction data between 20 and 1.34 Å resolution, excluding 5% of the data for cross-validation.

## Supplementary Methods

### *RNA interference*

JAKs were depleted using the following stealth™ RNAi oligonucleotides (Invitrogen, Carlsbad, CA, USA): JAK1: duplex 1 5'-GCACAGAAGACGGAGGAAAUGGUAU-3' and duplex 2 5'-GCCUUAAGGAAUAUCUCCAAAGAA-3'; JAK2: duplex 1 5'-GCAACAGAGCCUAUCGGCAUGGAAU-3' and duplex 2 5'-GCAGCAAGUAUGAUGAGCAAGCUUU-3'; JAK3: duplex 1 5'-GCCAUGGGUCCUUCACCAAGAUUUA-3' and duplex 2 5'-GGGCCAUAGACAUGUAUCUGCGAAA-3'; TYK2: duplex 1 5'-CCCAGAGAUGCAAGCCUGAUGCUAU-3' and duplex 2 5'-CCAUCUGAAGACAGUCCAUGAGAA-3'. Non-targeting control stealth™ RNAi oligo: Catalogue #45-2001. The stealth™ RNAi oligonucleotide to deplete BTK was 5'-GCUCCAUGUCUGAAGAUGAAUUCAU-3', and 5'-AUAGGUGACAAUUUCGUUAUAGGAGG-3' for depletion of LYN. Typically, oligos were diluted in water at 20 μM and stored at -20°C. Cells were transfected with RNAi oligos using Nucleofactor™ Solution V (Amaxa GmbH, Cologne, Germany) and the Amaxa system (Amaxa, Nucleofactor™ II) according to the instructions of the manufacturer.

### *Generation of Ba/F3 cell lines stably expressing MPL<sup>W515L</sup>, EpoR and TEL-JAK2*

Ba/F3 cells stably expressing MPL<sup>W515L</sup> were described previously (2). Ba/F3 EpoR cells were generated by transfecting human EpoR using the Amaxa nucleofection system. Subsequently, while gradually withdrawing mouse IL-3, the concentration of hygromycin was reduced from 200 to 100 μg/mL and medium was supplemented with 4 U/mL recombinant human erythropoietin (rhEpo) (#CRE600B, Cell Sciences, Canton, MA, USA). Cells were

serially diluted to obtain stable clones. The Ba/F3 EpoR cell line (clone 9) was grown in RPMI medium supplemented with 10% FCS, 2 mM L-glutamine, 1% v/v penicillin/streptomycin, 100 µg/mL hygromycin B and 4 U/mL rhEpo. Ba/F3 cells rendered cytokine independent by expression of TEL-JAK2, a cytoplasmic fusion protein found in acute lymphoblastic leukemia (3), were cultured in RPMI medium supplemented with 10% FCS, 2 mM L-glutamine, 1% penicillin/streptomycin and 800 µg/mL geneticin.

#### *Site-directed mutagenesis and transient transfection*

FERM (Y114A), JH2 (K581R, K607R) and JH1 (K822E) mutations were introduced by site-directed mutagenesis using a human JAK2<sup>V617F</sup> cDNA construct in pDEST<sup>TM</sup>12.2 (Invitrogen, Carlsbad, CA, USA). Point mutations K622R and K648R were introduced in the JH2 domain of human JAK1 using a cDNA construct in pDEST. The JAK1<sup>V658F</sup> mutation was generated by site-directed mutagenesis using a human JAK1 cDNA construct in pcDNA3.1. The integrity of the *JAK* mutants was verified by sequencing (Solvias AG, Basel, Switzerland) the entire open reading frames. For transient transfections, the corresponding pDEST JAK constructs (approximately 2 µg of the respective plasmids per 1 x 10<sup>6</sup> cells transfected) were transfected into HEL92.1.7, Ba/F3 EpoR or TF-1 cells in 100 µL of Nucleofector solution V (Amaxa GmbH, Cologne, Germany) according to the manufacturer's instructions using the Amaxa system. Cells were then grown in their respective medium for 24 h. In co-transfection experiments with 2 different JAK1 constructs, the respective empty vector of the other JAK1 mutant construct was co-transfected to deliver comparable amounts of plasmid DNA into cells, when the effect of the single JAK1 mutant construct transfected alone was assessed.

#### *Inhibition of cellular ATP production*

ATP levels were depleted by treating cells with 20  $\mu$ M oligomycin A (Enzo Life Sciences, Butler Pike, USA) and 10 mM 2-deoxy-D-glucose (Sigma, Switzerland) for 30 minutes (4). Cells were extracted in protein lysis buffer (50 mM HEPES, 150 mM NaCl, 25 mM  $\beta$ -glycerophosphate, 25 mM NaF, 5 mM EGTA, 1 mM EDTA, 15 mM PPI, 1% NP-40, 0.2 mM sodium vanadate, 1 mM PMSF and 1 mM DTT) and ATP levels were measured on a Wallac 1420 multilabel VICTOR<sup>2</sup> counter (PerkinElmer, Waltham, MA, USA) using the Kinase-Glo<sup>®</sup> Max kit (Promega, Madison, WI, USA). Values for ATP were normalized to sample protein content and expressed as % of vehicle control.

#### *Proliferation assays*

Anti-proliferative activity of JAK inhibitors was determined essentially as described (1).

#### *Enzymatic assays with JAK2 JH1*

Kinetic parameters of JAK inhibitors with the JAK2 JH1 enzyme were carried out by a radiometric filter-binding assay using [ $\gamma$ -<sup>33</sup>P]-ATP essentially as described (1). Rate constants were determined from progress curves of the JAK2 kinase reaction. The inhibited reaction was described by pseudo first-order kinetics according to the equation

$$(eq. 1) \quad P = v^S * t + \frac{(v^0 - v^S) * (1 - e^{-k_{obs} * t})}{k_{obs}} + NS$$

where  $P$  is the phosphorylated product,  $v^S$  is the rate of the enzymatic reaction at steady-state,  $v^0$  the initial velocity of the reaction,  $t$  the time, and  $k_{obs}$  the pseudo first-order rate constant characterizing the establishment of the steady-state velocity (5).  $P$  is represented by bound

[<sup>33</sup>P] (in cpm) at time  $t$ .  $NS$  represents non-specific binding determined in the absence of enzyme. The rate constant  $k_{obs}$  is related to  $k_{on}$  and  $k_{off}$  by the equation

$$(eq. 2) \quad k_{obs} = k_{off} + (k_{on} * [I])$$

where  $[I]$  represents the inhibitor concentration. Since  $K_i = k_{off}/k_{on}$ , eq. 2 can be rewritten to relate  $k_{obs}$  to  $K_i$  and  $k_{on}$

$$(eq. 3) \quad k_{obs} = k_{on} * ([I] + K_i)$$

or to relate  $k_{obs}$  to  $K_i$  and  $k_{off}$

$$(eq. 4) \quad k_{obs} = \frac{k_{off}}{K_i} * ([I] + K_i)$$

The rate of the inhibited reaction at steady-state is related to  $K_i$  by the equation (6)

$$(eq. 5) \quad v^S = \frac{K_i * v^0}{K_i + [I]}$$

By replacing  $k_{obs}$  (eq. 3 or eq. 4) and  $v^S$  (eq. 5) in eq. 1 the following equations are obtained:

$$(eq. 6) \quad P = \frac{K_i * v^0 * t}{K_i + [I]} + \frac{\left( v^0 - \frac{K_i * v^0}{K_i + [I]} \right) * \left( 1 - e^{-k_{on} * ([I] + K_i) * t} \right)}{k_{on} * ([I] + K_i)} + NS$$

$$(eq. 7) \quad P = \frac{K_i * v^0 * t}{K_i + [I]} + \frac{\left( v^0 - \frac{K_i * v^0}{K_i + [I]} \right) * \left( 1 - e^{-k_{off} * ([I] + K_i) * t / K_i} \right)}{k_{off} * ([I] + K_i) / K_i} + NS$$

Progress curves obtained in the presence of different concentrations of inhibitor in at least two independent experiments were combined and fitted as a whole by using eq. 6 and eq. 7 in a 3D fit with  $t$  and  $[I]$  as the independent variables. Nonlinear regression was performed with

XLfit<sup>®</sup> (ID Business Solutions, Guildford, UK). Identical fits were obtained with these equations; eq. 6 gives  $k_{on}$ , eq. 7 gives  $k_{off}$ , and both equations give  $K_i$ . The ratio  $k_{off} / k_{on}$  gives a value which is exactly identical to the  $K_i$  value. With these equations, SE can be obtained for each parameter. A representative experiment is shown in (Supplementary Fig. S3).

#### *Purification of tyrosine-phosphorylated peptides*

SET-2 cells were expanded to  $200 \times 10^6$  cells in T-162 cm<sup>2</sup> flasks and treated with inhibitors at a cell density of approximately  $0.8 \times 10^6$  cells/mL. Following treatments (30 minutes with NVP-BSK805 or CP-690,550 (5  $\mu$ M), or NVP-BBT594 (1  $\mu$ M)), cells were harvested in lysis buffer (20 mM HEPES pH 8.0, 9 M Urea, 1 mM Sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM sodium beta-glycerophosphate) reduced, alkylated and digested with trypsin after dilution to 2 M urea (7). The peptides were acidified to 1% TFA, desalted on SepPak C18 cartridges and eluted with 5 ml of 60% acetonitrile and 0.1% TFA. Lyophilized peptides were dissolved in IP buffer (50 mM MOPS pH 7.2, 10 mM sodium phosphate, 50 mM NaCl) and incubated with 40  $\mu$ L of protein G plus-agarose beads and 100  $\mu$ L of anti-phosphotyrosine antibodies for 16 h at 4°C (7). After elution of the beads with 100 mM glycine pH 2.6, peptides were desalted on Poros R3 and further purified on TiO<sub>2</sub> microcolumns (8).

#### *Mass spectrometry*

Purified phosphopeptides were resuspended in 10% formic acid and injected onto a 15 cm x 75  $\mu$ m ProteoPep 2 PicoFrit column (New Objectives), connected to an LTQ-Orbitrap XL mass spectrometer (Thermo). Buffer A consisted of H<sub>2</sub>O with 0.1% formic acid and Buffer B of 80% acetonitrile with 0.1% formic acid. Peptides were separated using a 120 minutes

gradient from 2% B to 50% B. Data acquisition was done using a 'Top 5 method', where every full MS scan was followed by 5 data-dependent scans on the 5 most intense ions from the parent scan. Full scans were performed in the Orbitrap at 60'000 resolution with target values of 5 E5 ions and 500 ms injection time, while MSMS scans were done in the ion trap with 1E4 ions and 200 ms. Database searches were performed with Mascot Server using the human IPI database (version 3.55). Mass tolerances were set at 10 ppm for the full MS scans and at 0.8 Da for MSMS. Search results were validated using Scaffold (Proteome Software) and peptide identifications accepted which exceeded the 95% confidence level. In case of ambiguous assignments, spectra were manually interpreted for confirmation of identity and localization of the phosphorylation site. Label free quantification was performed on duplicate LC-MS runs for each sample using Progenesis LC-MS (Nonlinear Dynamics Software).

## **Supplementary References**

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