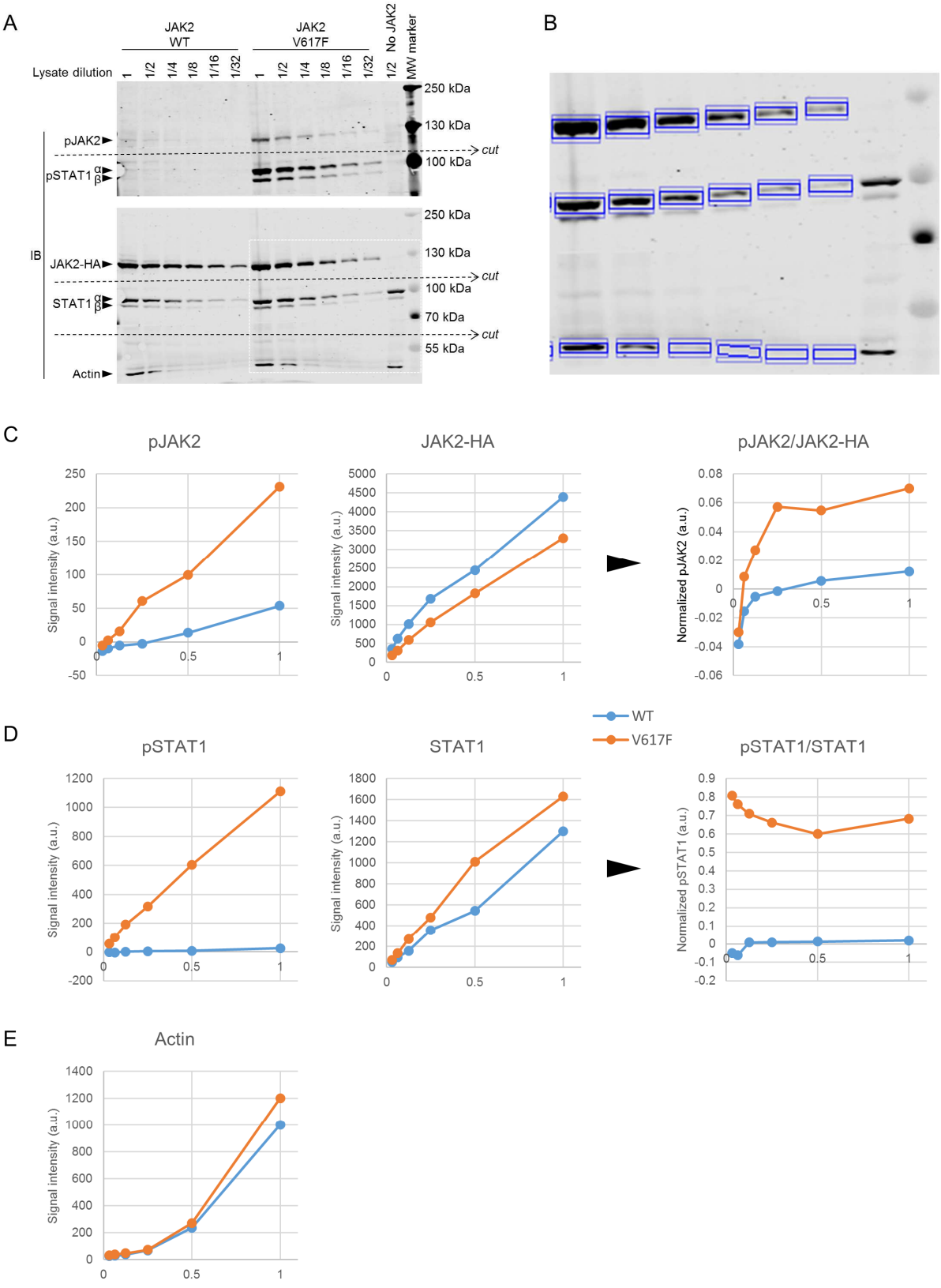
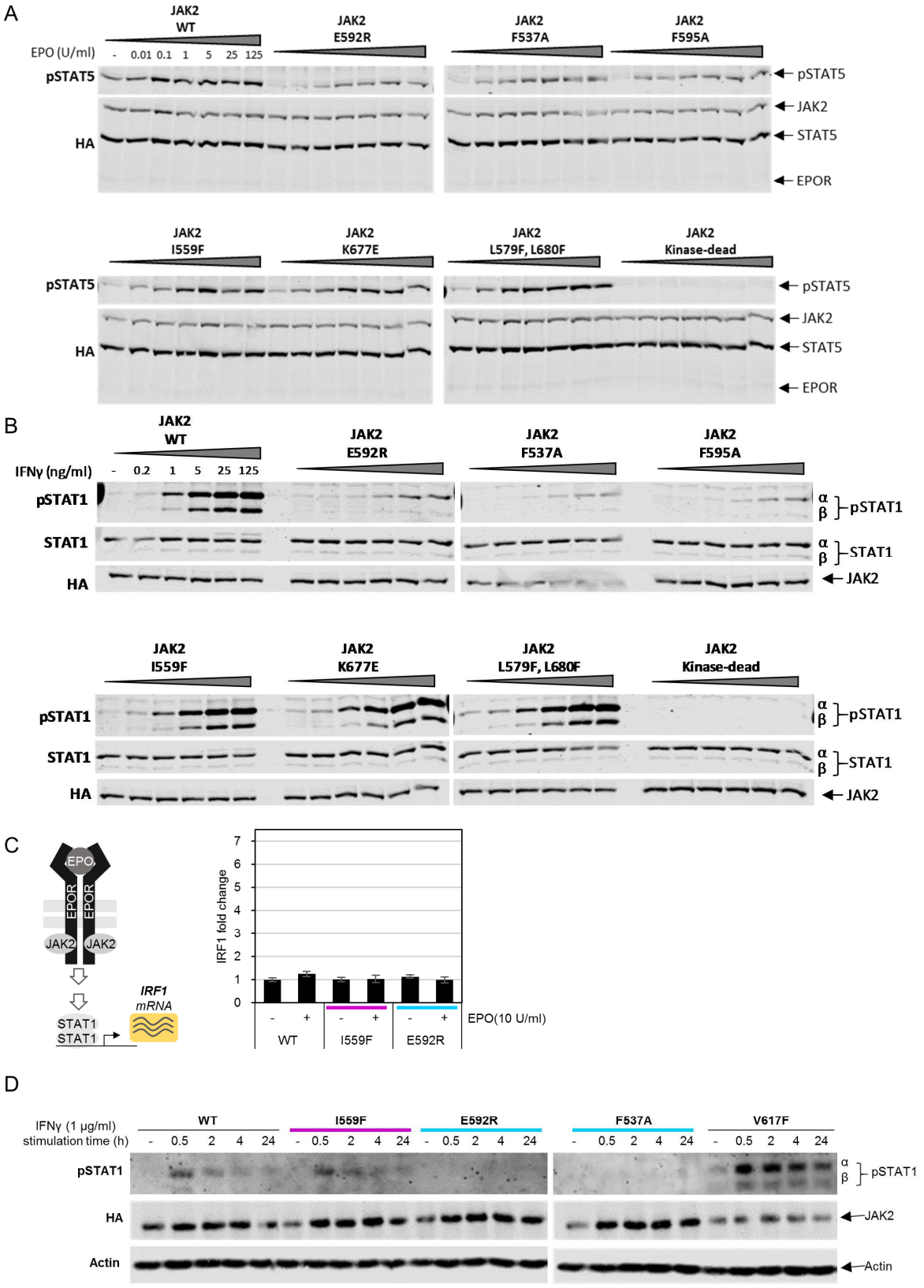
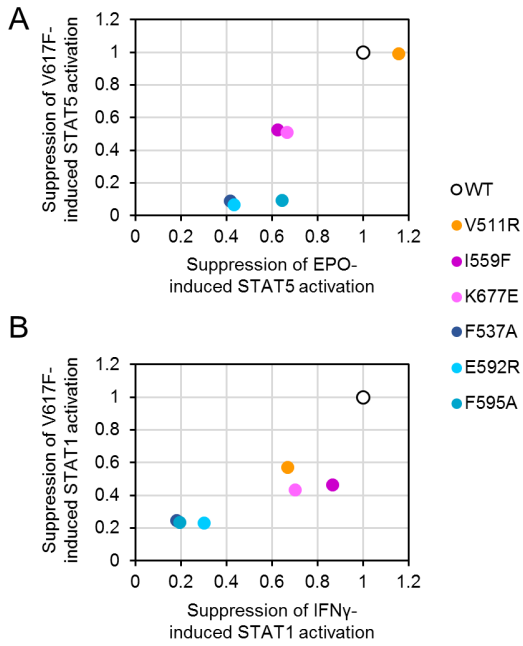

A
JAK2 WT
JAK2 V617F
No JAK2
MW marker
Lysate dilution
1
1/2
1/4
1/8
1/16
1/32
250 kDa
130 kDa
100 kDa
70 kDa
55 kDa
cut
pJAK2
pSTAT1 α
pSTAT1 β
IB
JAK2-HA
STAT1 α
STAT1 β
Actin
B
C
pJAK2
JAK2-HA
pJAK2/JAK2-HA
Signal intensity (a.u.)
Normalized pJAK2 (a.u.)
D
pSTAT1
STAT1
pSTAT1/STAT1
WT
V617F
Normalized pSTAT1 (a.u.)
E
Actin


A
JAK2 WT
JAK2 E592R
JAK2 F537A
JAK2 F595A
EPO (U/ml)
- 0.01 0.1 1 5 25 125
pSTAT5
HA
pSTAT5
JAK2
STAT5
EPOR
JAK2 I559F
JAK2 K677E
JAK2 L579F, L680F
JAK2 Kinase-dead
B
IFNγ (ng/ml)
- 0.2 1 5 25 125
pSTAT1
STAT1
HA
α
β
pSTAT1
STAT1
JAK2
C
EPO
EPOR
EPOR
JAK2
JAK2
STAT1
STAT1
IRF1
mRNA
IRF1 fold change
0 1 2 3 4 5 6 7
- + - + - +
EPO(10 U/ml)
WT
I559F
E592R
D
IFNγ (1 μg/ml)
stimulation time (h)
WT
I559F
E592R
F537A
V617F
- 0.5 2 4 24
pSTAT1
HA
Actin
α
β
pSTAT1
JAK2
Actin


A
Suppression of V617F-induced STAT5 activation
1.2
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
1.2
Suppression of EPO-induced STAT5 activation
WT
V511R
I559F
K677E
F537A
E592R
F595A

B
Suppression of V617F-induced STAT1 activation
1.2
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
1.2
Suppression of IFNγ-induced STAT1 activation

Supplementary

Materials and Methods

Plasmid constructs and mutagenesis. Full-length human JAK2 and erythropoietin receptor (EPOR) were cloned into pCIneo expression vector using SalI-NotI restriction sites. Full-length human STAT5A was in pXM vector. JAK2 and STAT5A were C-terminally hemagglutinin (HA)-tagged. EPOR was HA-tagged N-terminally after the signal peptide (between residues 30 and 31). Site-directed mutagenesis was performed using QuikChange (Agilent) according to manufacturer's instructions, and verified by Sanger sequencing. For luciferase reporter assays, Firefly luciferase reporter constructs for STAT5 (Spi-Luc [1]) or STAT1 (IRF-GAS [2]) was used together with a constitutively expressing *Renilla* luciferase plasmid. For analysis of subcellular localization of JAK2 mutants, JAK2-YFP fusion constructs were made by cloning JAK2 without stop codon to pEGFP vector using SalI-XmaI restriction sites. The YFP variant (mCitrine – gift from Robert Campbell, Michael Davidson, Oliver Griesbeck, Roger Tsien; Addgene plasmid #54594) was cloned to XmaI-NotI restriction sites resulting in the JAK2-YFP fusion construct. A short flexible linker (amino acids RSIAT) was also inserted between JAK2 and YFP during cloning. EPOR FRET reporter constructs were created by fusing CFP or YFP to the N-terminus of EPOR truncated after residue 340 by overlap extension PCR. The fused EPOR-YFP and EPOR-CFP fragments were cloned to the bidirectional pBOF-vector [3] to ensure equal expression of the EPOR FRET pair. Cotransfection with pTetOn vector and doxycycline (0.1 µg/ml) was used during transfection to induce expression of FRET reporter constructs.

Mammalian cell culture. JAK2-deficient γ2A human fibrosarcoma cells [4] were cultured using standard culturing conditions in DMEM (Lonza) supplemented with 10% fetal bovine serum (FBS, Sigma), 2 mM L-glutamine (Lonza), and antibiotics (0.5% Pen/Strep, Lonza).

For transfection, cells were seeded on 6-, 12-, or 24-well tissue culture plates and transfected using FuGENE HD (Promega) according to manufacturer's instructions. Cells were transfected for 24–48 h and, where needed, cytokine stimulated in starvation medium without FBS for 30 min (for immunoblotting) or 5 h (for reporter assays) unless otherwise specified, with human recombinant EPO (NeoRecormon, Roche), or human recombinant IFNγ (Peprotech).

Immunoblotting. After transfection/stimulation, cells were washed with ice-cold PBS, and lysates collected in cold lysis buffer (50 mM Tris-Cl pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mM NaF, 2 mM vanadate, 8.3 µg/ml aprotinin, 4.2 µg/ml pepstatin, and 1 mM phenylmethanesulfonyl fluoride). Lysates were centrifuged and used directly for SDS-PAGE/immunoblotting or stored at -20 ℃. Immunoblots were blocked with bovine serum albumin (BSA) and double-stained with the following diluted primary antibodies: HA Tag (1:2000, Aviva Systems Biology OAEA00009), phospho-JAK2 (1:1000, Tyr1007/1008, Millipore 07-606), phospho-STAT5 (1:1000, Tyr694, Cell Signaling 9351), phospho-STAT1 (1:1000, Tyr701 (D4A7), Cell Signaling 7649), STAT1 (1:1000, BD Biosciences 610116), or actin (1:1000, Millipore MAB1501R). Signals were detected using a mixture of goat-anti-rabbit (DyLight 680) and goant-anti-mouse (DyLight 800, Thermo Scientific) secondary antibodies (both at 1:5000 dilution) and read using an Odyssey CLx (LI-COR). Quantification of immunoblot signals was done using Image Studio software (LI-COR) by manually assigning bands to be quantified. For STAT1, only the larger isoform (STAT1α) was assessed. Control experiments were carried out to ensure that signals were within the quasi-linear range of the detection method (See Figure S1).

Luciferase reporter assays. For reporter assays, cells were seeded on 6 or 12-well plates, transfected overnight, transferred onto 96-well plates, let attach overnight, and starved/stimulated for 5 hours, after which signals were detected using the DualGlo

reporter assay kit (Promega) according to manufacturer's instructions. Luminescence was read on an EnVision multiplate reader (Perkin Elmer), and results calculated as Firefly luciferase luminescence divided by *Renilla* luciferase luminescence and normalized to readings from wells of unstimulated cells transfected with JAK2 WT.

RNA isolation and qPCR. For quantitative PCR (qPCR) analysis, γ2A cells were transfected as described above for 28 h, starved for 16 h, stimulated for 2 h with 10 U/ml EPO or 10 ng/ml IFNγ, and then RNA extracted using TRI Reagent (Molecular Research Center) according to manufacturer's instructions. Total RNA (0.2 µg) was reverse transcribed using M-MuLV reverse transcriptase (Thermo Scientific) according to manufacturer's instructions. qPCR reactions with the cDNAs were made using HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne) and primers specific for IRF1 which is an IFNγ responsive gene (Primers- 5'-GCATGAGACCCTGGCTAGAG-3' and 5'-CTCCGGAACAAACAGGCATC-3'). The qPCR reaction was performed using Bio-Rad CFX-384 Real-Time PCR detection system and the gene expression was quantified using comparative C(T) method by normalizing to the expression of TATA-box binding protein (TBP).

Recombinant protein production, purification, and *in vitro* kinase assay. Recombinant human JAK2 JH2-JH1 (513–1132-6xHis) WT, I559F, and E592R proteins were expressed in High Five insect cells (Thermo Fisher Scientific) using the Bac-to-Bac baculovirus expression system (Invitrogen) according to manufacturer's instructions. After protein expression (10% P3 virus, 48 h, 27 ^{o}C), the cells were collected by centrifugation, resuspended in lysis buffer containing 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 20 mM imidazole supplemented with phosphatase and protease inhibitors (100 mM sodium orthovanadate, 100 mM PMSF, 10 µg/ml pepstatin A), and lysed by applying two freeze-thaw cycles. The lysates were clarified by centrifugation and recombinant

proteins were purified using Ni-NTA agarose (Qiagen) and size-exclusion chromatography in HiLoad 16/600 Superdex 75 pg column (GE Healthcare) equilibrated in 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 0.5 mM TCEP. Protein concentrations were determined by Bradford assay (Bio-Rad) according to manufacturer's instructions. Enzymatic activity of JAK2 JH2-JH1 WT and mutant proteins was determined by time-resolved (TR-)FRET-based Lance Ultra kinase assay (PerkinElmer) under conditions recommended by the manufacturer. Kinase reactions: 100 nM tyrosine kinase substrate ULight™-poly GT, recombinant JAK2 JH2-JH1 WT (60 pM), I559F (150 pM) or E592R (60 pM), 2 nM Eu-labeled anti-phospho antibody, and ATP concentration range of 0–1000 µM, were set up in kinase buffer (50 mM Tris-HCl pH 8.0, 10 mM MgCl2, 1 mM EGTA, 0.05% BSA, 0.01% Brij-35, and 0.5 mM TCEP) on 384-plates (AlphaPlate-384 SW, PerkinElmer). Substrate phosphorylation was detected by measuring TR-FRET (ex. 320 nm, em. 665 nm) in 5 min intervals for 2 h at room temperature using EnVision Multilabel plate reader (PerkinElmer). Activity parameters k_{cat} and K_m were calculated by fitting reaction velocity vs. ATP concentration in GraphPad Prism (GraphPad Software). Kinase reactions were performed in triplicate and results shown are representative data from 2–3 individual experiments.

Microscopy and FRET assay to quantify JAK2 dimerization. For microscopy, cells were seeded on 35 mm glass bottom dish (MatTek), transfected overnight as described above and starved for 8 h. Cells were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde for 15 minutes in room temperature, washed and kept in PBS at 4 ℃ before imaging. The cells were imaged using a Zeiss LSM 780 laser scanning confocal microscope using a Plan Apochromat 63x/1.4 oil immersion objective and images were acquired with 458 nm and 514 nm excitation laser for CFP and YFP, respectively. Fluorescence was monitored between 465–500 nm for CFP and 525–640 nm for YFP with

32-channel QUASAR GaAsP PMT array detector. FRET was monitored by acceptor photobleaching [5] using 514 nm laser and the images were processed with ImageJ. Cell images were sorted based on the expression levels by measuring acceptor fluorescence before photobleaching (independent of FRET) and only cells with approximately equal levels of expression for different constructs were used for the analysis. The cell membrane region was manually segmented for each cell, and FRET efficiency was calculated only from the cell membrane.

Supplementary References

(1) Sliva D, Wood TJ, Schindler C, Lobie PE, Norstedt G. Growth hormone specifically regulates serine protease inhibitor gene transcription via gamma-activated sequence-like DNA elements. J Biol Chem 1994 Oct 21;269(42):26208-26214.

(2) Pine R, Canova A, Schindler C. Tyrosine phosphorylated p91 binds to a single element in the ISGF2/IRF-1 promoter to mediate induction by IFN alpha and IFN gamma, and is likely to autoregulate the p91 gene. EMBO J 1994 Jan 1;13(1):158-167.

(3) Haan C, Rolvering C, Raulf F, Kapp M, Drückes P, Thoma G, et al. Jak1 Has a Dominant Role over Jak3 in Signal Transduction through [gamma] c-Containing Cytokine Receptors. Chem Biol 2011;18(3):314-323.

(4) Kohlhuber F, Rogers NC, Watling D, Feng J, Guschin D, Briscoe J, et al. A JAK1/JAK2 chimera can sustain alpha and gamma interferon responses. Mol Cell Biol 1997 Feb;17(2):695-706.

(5) Bastiaens P, Majoul IV, Verveer PJ, Söling H, Jovin TM. Imaging the intracellular trafficking and state of the AB5 quaternary structure of cholera toxin. EMBO J 1996;15(16):4246-4253.

Supplementary Figure Legends

Figure S1: Validation of immunoblotting quantification method. A: Control immunoblot from whole-cell lysate of γ2A cells transiently transfected with JAK2-HA WT or JAK2-HA V617F as indicated. Lysates were run at different dilutions in lysis buffer to gauge linearity of immunoblotting signal. For detection, immunoblot is cut into three pieces (indicated with dashed lines) and the pieces double stained with Anti-pJAK2 + Anti-HA or Anti-pSTAT1 + STAT1, or single-stained with Anti-Actin. B: Example of quantification procedure using ImageStudio software (LICOR Biosciences). Shown is an example area from the immunoblot shown in (A) and indicated by a white dotted line. Bold blue boxes are the manually assigned areas of interest, each encompassing a single band (note, e.g., that for STAT1, only STAT1α is quantified). The light blue boxes on top and below each band are used to calculate the local background from the lane. From the background, the median signal intensity is used as a background value, which is deducted from the total signal from the area of interest. C: pJAK2 and total JAK2-HA quantifications showing the approximate linearity of the signal over the measured intensity range. Last panel shows the ratio of pJAK2 and JAK2-HA signal intensities, which is used as a measure of phosphorylation status (“Normalized pJAK2”). The point of deviation from linearity on the normalized pJAK2 panel shows the limits of pJAK2+JAK2-HA quantifiability (down to pJAK2 signal intensities of ~50), thus limiting the direct comparison of pJAK2 values to samples with relatively strong pJAK2 signals. D: pSTAT1 and total STAT1 quantifications as delineated above for (C). Note the exceptional linearity of STAT1 signals (and the ensuing stability of the normalized pSTAT1 value in the last panel), thus rendering pSTAT1 better suited for comparison of samples over a wide range of pSTAT1 signal intensities. E: Quantification of Actin signals showing poor linearity over the tested range. Thus Actin was not used for normalization in quantification of immunoblot data.

Figure S2: Characterization of effects of suppressing mutation on cytokine signaling in JAK2 WT background. Related to Figure 4. A and B: Immunoblots related to Figure 4 A and B, respectively. Immunoblots of whole-cell lysates from γ2A cells transiently transfected with full-length JAK2-HA (and mutants thereof), STAT5-HA, and EPOR-HA (A) or full-length JAK2-HA mutants only (B), and stimulated with the indicated amount of cytokine for 30 min. Quantification of immunoblots was done as shown in Figure S1. C: Related to Figure 4 E, qPCR of *IRF1* from RNA extracted from γ2A cells transiently transfected with JAK2-HA mutants and stimulated with EPO for 2 h showing the specificity of *IRF1* induction. D: Immunoblots of whole-cell lysates from γ2A cells transiently transfected with full-length JAK2-HA mutants and stimulated with 1 µg/ml IFNγ for the indicated times before lysis. JAK2-HA E592R and F537A show no induction of pSTAT1 even at longer stimulation times.

Figure S3: JAK2 activation by V617F relies on the same interfaces to activate as JAK2 activation by cytokine. Suppression calculated as STAT5 or STAT1 reporter activity relative to basal activity of JAK2 V617F (y-axes) and stimulated wild-type JAK2 (10 U/ml EPO or 5 ng/ml IFNγ; x-axes). Data for figure is from the same experiments as for Figures 2 and 3.