Supplemental Data

Methods

**Patient Platelet Lysate Collection**

To collect platelet lysate, patient blood was diluted 1:1 in wash buffer (150 mM NaCl, 20 mM HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid]; pH 6.5) and centrifuged at 60g for 7 minutes. Platelet-rich plasma (PRP) was centrifuged at 240g for 10 minutes and platelets resuspended in RIPA buffer as previously described.

**Chemicals and Reagents**

rhTPO was a gift from Don Foster, Zymogenetics. All signaling antibodies were from Cell Signaling Technologies, except N-terminal-specific anti-human c-MPL (Amgen Pharmaceuticals), C-terminal-anti-human c-MPL and biotinylated-anti-phosphotyrosine (both from Millipore), anti-actin (Sigma-Aldrich), anti-IκB-α (BioLegend), anti-polyubiquitin (Biomol) and anti-V5 (Invitrogen). Secondary antibodies were from Santa Cruz Biotechnology, apart from Alexa Fluor 488-anti-rabbit (Invitrogen). Ruxolitinib was purchased from ChemieTek.

**Constructs**

Wild-type human *JAK2* was cloned into the retroviral vector, *pQCXIN* (Clontech). The *JAK2* point mutations were introduced with the QuikChange-II site-directed mutagenesis kit (Agilent) using the following oligonucleotides; for *JAK2*<sub>R564Q</sub>, g1691a

5′-caagagtttttaaggggtacaagagataaggagactacgg-3′ and g1691a<sub>_antisense</sub>

5′-ccgtagttctcttcttttttagcctttaaaaatcatttg-3′; for *JAK2*<sub>V617F</sub>, g1849t 5′

agcattttgttaattatggagtgtgttctgagacgaga-3′ and g1849t<sub>_antisense</sub>

5′-tctggtcagaaacataactcataatgttaaaccacagtgc-3′. Mutations were confirmed by sequencing DNA isolated from individual clones. For transient overexpression of V5-tagged *Socs3*, murine *Socs3* was cloned into *pEF1/V5-HisA*. 
**Cell Line Generation and Maintenance**

The interleukin-3 (IL-3)-dependent prolymphoid cell line Ba/F3 expressing human c-MPL (BaF-MPL) was generated and maintained as previously described\(^1\). The same protocol was used to generate and maintain a BaF/3 cell line expressing human EPOR (BaF-EPOR). Stably expressing WT or mutant JAK2 BaF-MPL or BaF-EPOR cells were generated using the pQCXIN-JAK2 constructs, also as described\(^1\). Selection was performed with 500µg/ml Geneticin (Invitrogen). Total c-MPL and JAK2 protein expression was determined by western blot and c-MPL cell surface expression was confirmed using flow cytometry as previously described\(^2\).

**Real Time PCR**

Human/mouse JAK2 cDNA ratios were calculated for each cell line by real time PCR as described in\(^3\), using Taqman Gene Expression assays Hs01078117_m1 for human JAK2 and Mm01208495_m1 for mouse Jak2 (Applied Biosystems) and standard curves generated from linearized pcDNA3.1 plasmids containing either human JAK2 or mouse Jak2.

**Transient V5-Socs3 Expression**

For transient expression of V5-Socs3, cells were transfected using a Neon nucleoporator (Invitrogen; Pulse voltage: 1700V, Pulse width: 10ms, Pulse number: 1) and incubated for 24hr before starvation.

**Immunoblotting and Immunoprecipitation**

Following starvation and/or TPO-stimulation, cells were lysed and proteins fractionated by SDS-PAGE and transferred to PVDF membranes as previously described\(^1\). For JAK2 ubiquitination assays, cells were treated with 10µM MG132 for 60 mins, prior to lysis. Protein detection, quantification and immunoprecipitation were performed using previously described methods\(^1\).
**Annexin V and BrdU staining**

The Annexin-V-FLUOS staining kit (Roche Diagnostics) and FITC BrdU Flow Kit (BD Biosciences) were both used according to the manufacturers' protocols. Flow cytometric analysis was performed using a BD Accuri C6 flow cytometer (BD Biosciences) and data analyzed using FCS Express software (De Novo Software). Each experiment was performed in triplicate.

**JAK2 Kinase Assay**

An *in vitro* JAK2 kinase assay was performed as described\(^4\). Data shown is from three independent experiments.

**MTT Proliferation Assays**

MTT assays were performed on human JAK2-expressing Ba/F3-MPL or Ba/F3-EPOR clones as previously described\(^1\) following incubation with 0-1\(\mu\)g/ml TPO or 0-2U/ml EPO for 48 hrs under starved conditions. Each data point is expressed as a percentage of proliferation stimulated by a maximal dose of murine IL-3 (4\(\mu\)l/ml murine IL-3 supernatant). Each experiment was performed at least in triplicate.

**Ruxolitinib treatment**

Cells in log-phase growth were grown under starved conditions (RPMI 1640 with 2% FBS) for 48 hrs with 0.1nM-1\(\mu\)M ruxolitinib, or DMSO as control. The number of viable cells was then counted and expressed as a percentage of the DMSO control. Data was collected from 3 independent repeats and the IC\(_{50}\) value was calculated using Prism software (Graphpad).
**Statistical analysis**

Data are mean plus or minus SEM. Statistical analyses were performed using InStat software (Graphpad Software). The statistical tests used were Student \( t \) test, unless otherwise stated.

**References**


Supplemental Figure 1. Effects of JAK2 mutations in Ba/F3-EPOR cell lines. (A) Western blot confirming equal levels of JAK2 and EPOR expression in the mutant JAK2 Ba/F3-hEPOR cell lines. (B) XTT cell viability assay in response to EPO under starved conditions for 48 hours. (C) Western blot of show levels of phosphorylation of signaling proteins in response to EPO stimulation.