Supporting Information

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SI Materials and Methods

Cell Culture and Transfection. JAK2-deficient γ2A fibrosarcoma cells and COS7 cells were cultured in DMEM supplemented with 10% (vol/vol) FBS (Life Technologies), 4 mM L-Glutamine, and 1% PenStrep (Sigma) at 37 °C in 5% (vol/vol) CO_2. Cells were transfected at 60% confluency using FuGENE6 (Promega) or Xtreme-GENE9 (Roche) according to the manufacturers' instructions using 300 ng of plasmid DNA per six-well plate. Amount of DNA was varied for each construct to obtain similar expression levels. After 10 h cells were starved in serum-free DMEM overnight. Stimulation was done for 30 min with human EPO (NeoRecormon, Roche) at 200 U/mL or with hIFNγ (Peprotech) at 0.5 µg/mL.

Immunoblotting. Cells were lysed by scraping into Triton-X cell lysis buffer (50 mM Tris•Cl pH 7.5, 10% (vol/vol) glycerol, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mM NaF). Lysates were centrifuged and the supernatant was used directly for SDS/PAGE and immunoblotting. Blots were blocked using 1% BSA (Sigma) and double-stained with phosphospecific antibodies [pJAK2(Y1007–Y1008) at 1:2,000 (Cell Signaling, cat. no. 3771), pSTAT1(Y701) 1:1,000 (Cell Signaling, cat. no. 9171), pSTAT5A(Y694) 1:1,000 (Cell Signaling, cat. no. 9351), pJAK1(Y1022–Y1023) 1:1,000 (Millipore, cat. no. 07–849), and HA 1:2,000 (Covance, cat. no. MMS-101P)]. Secondary antibody incubation was done in a mix of IRDye-labeled anti-rabbit 1:5,000 (LI-COR, cat. no. 926–68021) and anti-mouse 1:10,000 (Thermo Scientific, cat. no. 35521), after which signals were detected and quantified using a LI-COR Odyssey CLx. A minimum of three independent experiments were performed for each condition.

Plasmid Constructs. For mammalian cell culture and recombinant plasmid production, mutations were introduced by QuikChange site-directed mutagenesis (Agilent Technologies) into full-length human JAK2-HA in pCIneo and pFastBac1 JAK2(513–827-6xHis for wild type or V617F) or JAK2(536–812-6xHis, W659A, W777A, and F794H for "wild type," or W659A, W777A, and V617F for "V617F") pFastBac1 plasmids (1), respectively. Human EPOR-HA and STAT5A-HA used for mammalian cell culture were in pCIneo and pXM eukaryotic expression vectors, respectively. For in vivo bone marrow transplant experiments, human JAK2 mutants were generated using QuikChange site-directed mutagenesis in pMSCV-IRES GFP plasmids. pMSCV-IRES GFP JAK2 and pMSCV-IRES GFP JAK2 V617F were used as templates. pMSCV-IRES GFP plasmid integrity was checked by Hind III cleavage. All mutations in all plasmids were verified by sequencing.

Retroviral Transduction and Bone Marrow Transplantation. Total bone marrow cells from 5-FU–treated (single i.p. injection, 150 mg/kg, 7 d before harvesting) C57BL/6 donor mice (Harlan Laboratories) were transduced and transplanted into C57BL/6 female recipients lethally irradiated with 12 Gy (2). Mice were kept under specified pathogen-free conditions with free access to food and water. Blood was collected into EDTA-coated microtainers (BD Biosciences) by tail vein sampling 12 wk posttransplantation. Complete blood counts were determined on the ADVIA120 hematology analyzer using the Multispecies software (Bayer).

Statistical Analysis of in Vivo Results. Results are presented as means ± SEM. To assess statistical significance among individual cohorts, one-way ANOVA with subsequent Bonferroni posttest was conducted (Graph Pad Prism, vs. 4.00, 2003), and P values less than 0.05 were considered significant.

Fluorometric TSA. Thermal-shift experiments were carried out in 96-well PCR plates in a final volume of 25 µL with the following reagent concentrations: 8x Sypro Orange (Molecular Probes, cat. no. S6551), 5.5 µM protein Ni-NTA eluate, 20 mM $MgCl_2$, 20 mM Tris·Cl (pH 8.0), 500 mM NaCl, 20% (vol/vol) glycerol, and differing ATP concentrations. Reactions were heated in a real-time CFX96 PCR cycler (Bio-Rad) at 1 °C per min from 4 °C to 95 °C with a fluorescence read every 1 °C. Fluorescence data were then normalized to represent unfolding curves, which were fitted to a Boltzmann sigmoidal equation with GraphPad Prism to obtain Tm values.

MANT-ATP Binding Assay. Binding of MANT-ATP (Jena Biosciences) to protein was measured with a Quantamaster spectrofluorometer (Photon Technology International) by measuring FRET between the protein and MANT at a final volume of 140–150 µL. Reaction mixtures (20 mM Tris•HCl pH 8.0, 200 mM NaCl, 10% (vol/vol) glycerol, 2 mM DTT, 1.5 µM or 2 µM for recombinant JAK2 and JAK1 JH2 protein, respectively, with 10 mM of either $MgCl_2$, $MnCl_2$, $CaCl_2$, or no cation at all) were excited at 280 nm and fluorescence measured as a 300–500-nm emission scan. MANT-ATP stocks were titrated in concentrated stocks (10 µM, 100 µM, and 1,000 µM). Binding was measured from emitted fluorescence at 440–450 nm corrected for the primary inner filter effect. The K_d value was calculated from at least triplicate measurements using GraphPad Prism essentially as described in ref. 3, taking into account protein dilution and ligand depletion.

1. Bandaranayake RM, et al. (2012) Crystal structures of the JAK2 pseudokinase domain and the pathogenic mutant V617F. *Nat Struct Mol Biol* 19(8):754–759.
2. Tiedt R, et al. (2008) Ratio of mutant JAK2-V617F to wild-type Jak2 determines the MPD phenotypes in transgenic mice. *Blood* 111(8):3931–3940.
3. Zeqiraj E, et al. (2009) ATP and MO25α regulate the conformational state of the STRADalpha pseudokinase and activation of the LKB1 tumour suppressor. *PLoS Biol* 7(6):e1000126.
4. Heinig M, Frishman D (2004) STRIDE: A web server for secondary structure assignment from known atomic coordinates of proteins. *Nucleic Acids Res* 32(Web Server issue): W500–W502.

Fig. S1. The V617F mutation in JAK2 JH2 causes a slight change in the shape of the ATP binding pocket. Shown are the crystal structures of ATP-bound wild-type JAK2 JH2 (PDB ID code 4FVQ) and JAK2 JH2 V617F (PDB ID code 4FVR). The solvent-accessible surface (estimated using a water sphere model of 1.4 Å radius) of the domain is shown as a mesh structure that is viewed from inside the protein domain. Key residues and ATP are highlighted as stick structures. Coloring is as described in Fig. 1*A*.

Fig. S2. Suppression of the V617F phenotype is not caused by loss of Ser523 or Tyr570 phosphorylation. JAK2(Y1007–Y1008) phosphorylation in γ2A cells transfected with the JAK2 constructs is shown. Bar graph shows quantification of phosphorylation from immunoblots, as described for Fig. 2. Error bars are SDs from three independent experiments.

Fig. S3. Mutation of the ATP binding pocket in JAK2 JH2 V617F suppresses inducibility by cytokine. Show is the fold change over basal JAK2(Y1007–Y1008) phosphorylation upon EPO stimulation (200 U/mL) in γ2A cells cotransfected with full-length JAK2-HA and EPOR. Phosphorylation levels were normalized to JAK2-HA levels. The data shown are from the same experiments as in Fig. 4*A*. Error bars are SDs from three independent experiments.

Fig. S4. Blood counts from murine bone marrow transplantation experiment. *A–F* show blood counts from mice transplanted with retrovirally transduced bone marrow. Mice were analyzed 12 wk posttransplantation, when mice transduced with JAK2 V617F had developed an MPN-like phenotype. No signs of systemic disease were observed in any of the mice (e.g., splenomegaly) during the period analyzed. All values shown were measured from the same mice as data shown in Fig. 4*B*. Results show mean ± SEM. *n* = 8 for each group. *$P < 0.05$. MCV, mean corpuscular volume; RBC, red blood cells.

Fig. S5. Characterization of JAK1 JH2 ATP binding. Shown is the MANT-ATP binding assay with recombinant JAK1 JH2. (*A*) Experiment looking for cation preference in ATP binding of JAK1 JH2. Measurements were done in singlet only. (*B*) Quantification of MANT-ATP binding to JAK1 JH2 in the presence of Mg^{2+}. Error bars in *B* are SDs from three experiments.

Fig. S6. Molecular dynamic simulations show a stabilizing effect for ATP binding to JAK2 JH2. (*A* and *B*) Root-mean-square fluctuation calculated for each residue in JAK2 JH2 over the course of the simulations. The secondary structure of JAK2 JH2 is shown schematically on the *x* axis. (*C*) Root-mean-square deviation values (in Å) over the whole domain of JAK2 JH2 wild type and V617F with and without ATP. (*D*) α-helicity of the C helix during the course of the simulation as predicted by the VMD plugin Timeline using the secondary structure prediction algorithm STRIDE (4). The α-helical nature is shown in blue; all other conformations are shown in yellow.