Identification and Characterization of JAK2 Pseudokinase Domain Small Molecule Binders

David E. Puleo[†], Kaury Kucera[†], Henrik M. Hammarén[‡], Daniela Ungureanu[‡], Ana S. Newton[§], Olli Silvennoinen[‡], William L. Jorgensen[§] and Joseph Schlessinger[†*]

[†]Department of Pharmacology, [§]Department of Chemistry, Yale University, New Haven, CT 06520; [‡]Faculty of Medicine and Biosciences, University of Tampere, Tampere, Finland 33014

Supporting Information

Table of Contents

1. General Information

AT9283, BI-D1870, JNJ-7706621, and PRT062607 were purchased from Selleckchem. IKK-2 Inhibitor VI was purchased from Santa Cruz Biotechnology.

2. Fluorescence Polarization Screen

A high-throughput fluorescence polarization (FP) screen was conducted at the Yale Small Molecule Discovery Center using a Tecan Aquarius robot and Freedom EVO Workstation. A V&P Scientific 384-pin tool transferred small molecules (10 mM) from stock library plates into black 384-well assay plates (Invitrogen) containing 7.0 μM JAK2 JH2 and 20 nM BODIPY-ATP (Invitrogen) in assay buffer (20 mM Tris pH 8.0, 150 mM NaCl, 20% glycerol, 10 mM $MgCl_2$ 0.5 mM TCEP and 0.01% Brij-35 and 2.5% DMSO). Assays were performed in a total volume of 20 μl with a final concentration of 10 μM compound. FP measurements were read after 60-minute incubation at room temperature on a Wallac Envision Plate Reader. Each assay plate included negative control (no added compounds) and positive control (ATP). Percent effect was calculated using the average of the negative and positive controls on each plate. Z' analysis[1] was performed in order to ensure assay credibility. The JH2 protein was screened against the Selleckchem (355 compounds) and Enzo (80 compounds) Kinase Inhibitor Libraries in duplicate.

3. JAK2 JH2 Protein Purification

Human JAK2 JH2 (residues 536-812) containing three surface mutations (W659A, W777A, F794H) and a C-terminal thrombin-cleavable hexa-histidine tag was expressed in baculovirus-infected *Sf9* cells. Cell pellets were re-suspended in lysis buffer containing 20 mM Tris, pH 8.0, 0.5 M NaCl, 20% Glycerol, 0.25 mM TCEP supplemented with cOmplete EDTA-free protease-inhibitor cocktail (Roche) and lysed using a cell disruptor (Avestin). Lysates were clarified via centrifugation at 16,500 rpm for 45 minutes. Supernatant was applied to pre-equilibrated Ni-NTA agarose (Qiagen) in batch, washed in lysis buffer containing 10 mM imidazole, and eluted in lysis buffer containing 200 mM imidazole. Elution samples containing JAK2 protein were dialyzed overnight using 10,000 MWCO Slide-A-Lyzer dialysis cassettes (Thermo Scientific) into 20 mM Tris, pH 8.0, 35 mM NaCl, 20% Glycerol, 0.25 mM TCEP. Protein was applied to a Mono Q HR 16/60 (GE Healthcare) equilibrated in dialysis buffer and eluted in a linear gradient from 35-200 mM NaCl.

4. JAK2 JH2 Protein Crystallization and Structure Solution

Mono Q elutions containing JH2 protein were concentrated and loaded onto a Superdex 200 Increase 10/300 (GE Healthcare) pre-equilibrated in 20 mM Tris pH 8.0, 100 mM NaCl, 10% Glycerol, 1.0 mM TCEP. The hexa-histidine tag was not cleaved. Fractions containing JAK2 JH2 were concentrated to ~5-7 mg/ml using a 10,000 MWCO Vivaspin (GE Healthcare). Small molecule was added to the protein at 1.5 molar excess and allowed to incubate on ice for ~1-3 hours before setting up crystallization trials. JAK2 JH2 was crystallized at 4°C in 0.1 M Tris, pH 8.0, 0.2 M Sodium acetate, 12-20% PEG 4,000. Streak seeding was used to obtain optimal crystals, which grew to full size after 2-3 days. Alternatively, unoccupied JAK2 crystals were soaked with small molecule compounds. To do so, JH2 protein was crystallized at 4°C in

0.1 M Tris, pH 8.0, 0.2 M Sodium acetate, 12-20% PEG 4,000. Crystals were transferred to a stabilizing solution of 0.1 M Tris, pH 8.0, 0.2 M Sodium acetate, 22% PEG 4,000 containing 1-2 mM compound and left at 4°C between 24-72 hours. Crystals were cryo-protected stepwise in soaking solution containing 18% glycerol then flash-frozen in liquid nitrogen.

X-ray diffraction data for the JAK2 JH2/JNJ-7706621 and JAK2 JH2/AT9283 crystals were collected at Yale University on a Rigaku Micromax 007 HF+ rotating anode X-ray source and Pilatus 200k detectors. Data were scaled and merged using HKL-2000[2]. Data for the JAK2 JH2/IKK-2 Inhibitor VI crystal were collected at the National Synchrotron Light Source I (NSLSI) at Brookhaven National Laboratory on beam line X25. Data were scaled and merged using HKL-2000. Data for the JAK2 JH2/PRT062607 and JAK2 JH2/BI-D1870 crystals were collected at Argonne National Laboratory beam line 24-ID-E and were scaled and merged using the automated XDS[3] at APS.

Initial phases were calculated via molecular replacement in Phaser[4] using the JAK2 pseudokinase domain (PDB ID 4FVP) lacking waters and buffer as the search model. Maximum-likelihood refinement was performed in PHENIX[5]. Rigid body refinement was used during the first refinement. TLS refinement was used in every subsequent refinement. Waters were placed automatically by PHENIX in early refinements. New water as well as buffer molecules were added manually using Coot[6] during the refinement process. Small molecule coordinate files and restraints were generated using PHENIX eLBOW. Small molecule compounds were placed into electron density using Coot before the final refinement. PyMOL[7] molecular viewer was used to generate figures.

5. JAK2 JH1 Protein Purification

Human JAK2 JH1 (840-1132) containing an N-terminal TEV-cleavable hexa-histidine tag was expressed in baculovirus-infected *Sf9* cells. Cells were re-suspended in lysis buffer containing 20 mM Tris, pH 8.0, 0.5 M NaCl, 20% Glycerol, 0.25 mM TCEP supplemented with cOmplete EDTA-free protease-inhibitor cocktail (Roche) and lysed using a cell disruptor (Avestin). Lysates were clarified via centrifugation and applied to pre-equilibrated Ni-NTA agarose (Qiagen) in batch. Beads were washed with lysis buffer containing 10 mM imidazole and eluted in lysis buffer containing 200 mM imidazole. Elution samples containing JAK2 protein were dialyzed overnight using 10,000 MWCO Slide-A-Lyzer dialysis cassettes (Thermo Scientific) into 20 mM Tris, pH 8.0, 0.25 M NaCl, 20% Glycerol, 0.25 mM TCEP. Protein was concentrated then diluted 10 fold in 20 mM Tris, pH 8.0, 20% Glycerol, 0.25 mM TCEP, then applied to a Mono Q HR 16/60 column pre-equilibrated in 20 mM Tris, pH 8.0, 25 mM NaCl, 20% Glycerol, 0.25 mM TCEP. Protein was eluted in a linear gradient to 500 mM NaCl.

6. JAK2 JH1 Protein Crystallization and Structure Solution

For co-crystallization, small molecule was added at three-fold molar excess after dialysis. After the Mono Q step, fractions containing JAK2 were concentrated and applied to a Superdex 200 Increase 10/300 (GE Healthcare) equilibrated 20 mM Tris, pH 8.0, 250 mM NaCl, 5% Glycerol, 1.0 mM TCEP. The hexa-histidine tag was not cleaved. Protein was concentrated to ~7 mg/ml, to which three-fold molar excess small molecule was added again. Complex was allowed to incubate on ice for ~1-3 hours before setting up crystallization trials. JH1 crystallized in 0.1 Sodium citrate pH 5.6, 0.2 M Ammonium sulfate, 30% PEG 4000 at 20°C. Crystals grew to full size after 1-2 days. Crystals were cryo-protected stepwise in mother liquor containing 10% Glycerol then flash-frozen in liquid nitrogen.

X-ray diffraction data were collected at Yale University on a Rigaku Micromax 007 HF+ rotating anode X-ray source and Pilatus 200k detector. Data were scaled and merged using HKL-2000. Initial phases were calculated using molecular replacement in Phaser. The JAK2 kinase domain (PDB ID 2B7A) lacking waters and ligands was used as the search model. Maximum-likelihood refinement was performed in PHENIX. Rigid body refinement was used during the first refinement. TLS refinement was used in every subsequent refinement. Waters were placed automatically by PHENIX in early refinements. New waters as well as buffer molecules were added manually using Coot during the refinement process. Small molecule coordinate files and restraints were generated using PHENIX eLBOW. Small molecule compounds were placed into electron density using Coot before the final refinement. PyMOL molecular viewer was used to generate figures.

7. Isothermal Titration Calorimetry

Mono Q elutions containing JH1 or JH2 protein were concentrated and loaded onto a Superdex 200 Increase 10/300 pre-equilibrated in 50 mM Hepes, 150 mM NaCl, 10% Glycerol, 1.0 mM TCEP, 4% DMSO, pH 8.0. Elutions containing JH1 or JH2 were pooled, concentrated to 1-10 μM, and loaded into the 1.43 ml cell of a VP-ITC (MicroCal). Small molecules were diluted in size exclusion buffer and brought to a final concentration of 10-100 μM. Small molecules were loaded into a 250 μl VP-ITC syringe and titrated into protein at 20°C with 290 rpm stirring speed. ITC experiments were performed at least in duplicate. Data were analyzed using ORIGIN software.

It is noteworthy that different values were obtained for binding affinities of small molecules to JH2 using FP and ITC measurements (Newton *et. al.*, accompanying manuscript). The variation in K_d values between FP and ITC likely stems from the difference in the assay

format used for determining binding affinities by the two approaches. ITC is a label-free technique where direct binding between small molecule and protein is measured. On the other hand, the FP measurements involve indirect binding determination via a competition assay. Rossi and Taylor[8] describe a detailed comparison of the merits and deficiencies of using FP and ITC measurements for determining the binding affinity of small molecules to proteins and other macromolecular binders. Stevers *et. al.*[9] saw a ~5-fold difference between values obtained in FP and ITC measurements. Specifically, the article refers to CFTR_R6 peptide binding to a 14-3-3β protein. The binding affinities determined via FP and ITC were 15 μM and 75 μM, respectively. In addition, the authors report that they see the "same trend" in FP versus ITC measurement. We have also seen similar rank-order trend for binding of different compounds to JH2 domains using FP and ITC measurements of different compounds.

8. References

(1) Zhang, J. H.; Chung, T. D.; Oldenburg, K. R. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen* **1999,** 4, 67-73.

(2) Otwinowski, Z.; Minor, W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* **1997,** 276, 307-26.

(3) Kabsch, W. Xds. *Acta Crystallogr D Biol Crystallogr* **2010,** 66, 125-32.

(4) McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J. Phaser crystallographic software. *J Appl Crystallogr* **2007,** 40, 658-674.

(5) Adams, P. D.; Afonine, P. V.; Bunkoczi, G.; Chen, V. B.; Davis, I. W.; Echols, N.; Headd, J. J.; Hung, L. W.; Kapral, G. J.; Grosse-Kunstleve, R. W.; McCoy, A. J.; Moriarty, N.

W.; Oeffner, R.; Read, R. J.; Richardson, D. C.; Richardson, J. S.; Terwilliger, T. C.; Zwart, P. H. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* **2010,** 66, 213-21.

(6) Emsley, P.; Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* **2004,** 60, 2126-32.

(7) DeLano, W. L. The PyMOL Molecular Graphics System; DeLano Scientific: San Carlos, CA, 2002; http://www.pymol.org.

(8) Rossi, A. M.; Taylor, C. W. Analysis of protein-ligand interactions by fluorescence polarization. *Nat Protoc* **2011,** 6, 365-87.

(9) Stevers, L. M.; Lam, C. V.; Leysen, S. F.; Meijer, F. A.; van Scheppingen, D. S.; de Vries, R. M.; Carlile, G. W.; Milroy, L. G.; Thomas, D. Y.; Brunsveld, L.; Ottmann, C. Characterization and small-molecule stabilization of the multisite tandem binding between 14-3-3 and the R domain of CFTR. *Proc Natl Acad Sci U S A* **2016,** 113, E1152-61.

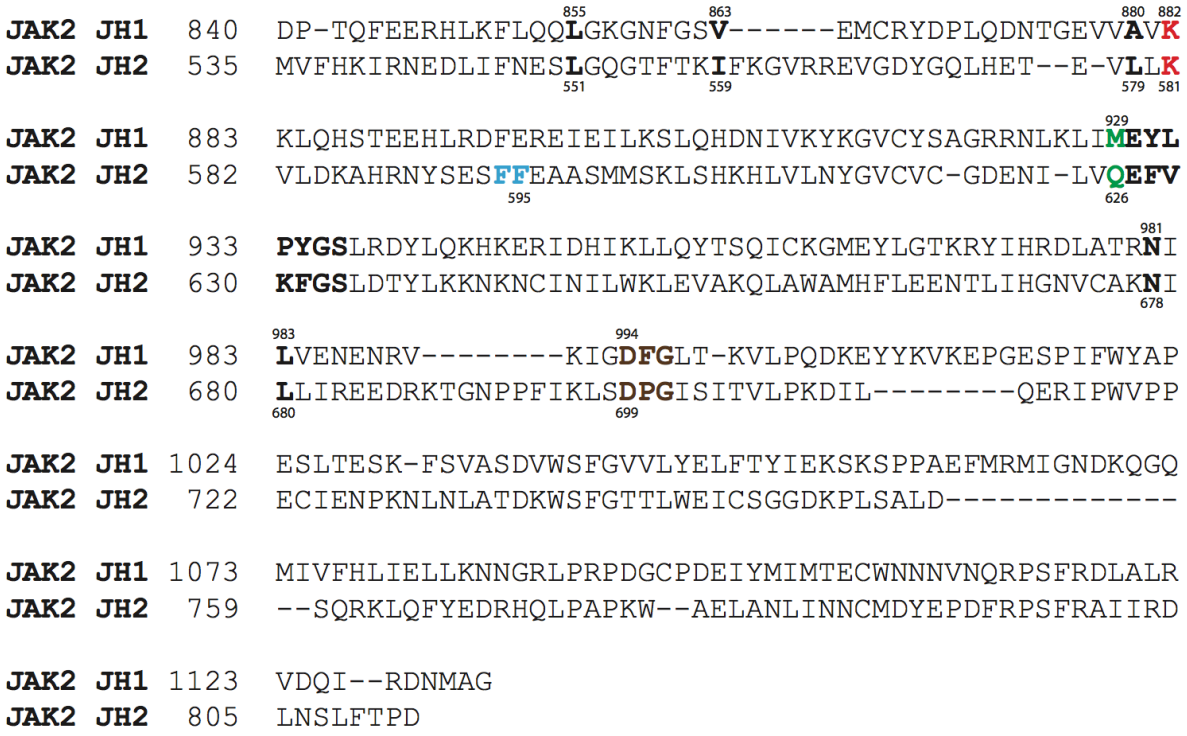

Figure S1. Sequence alignment of JAK2 JH1 and JH2 domains. Residues surrounding the small molecules in the ATP-binding pocket are highlighted in black. The conserved β3 lysine is highlighted in red. The gatekeeper residues of JH1 and JH2 are highlighted in green. F594 and F595 in JH2 are highlighted in blue. The DFG motif in JH1 and DPG motif in JH2 are highlighted in brown.

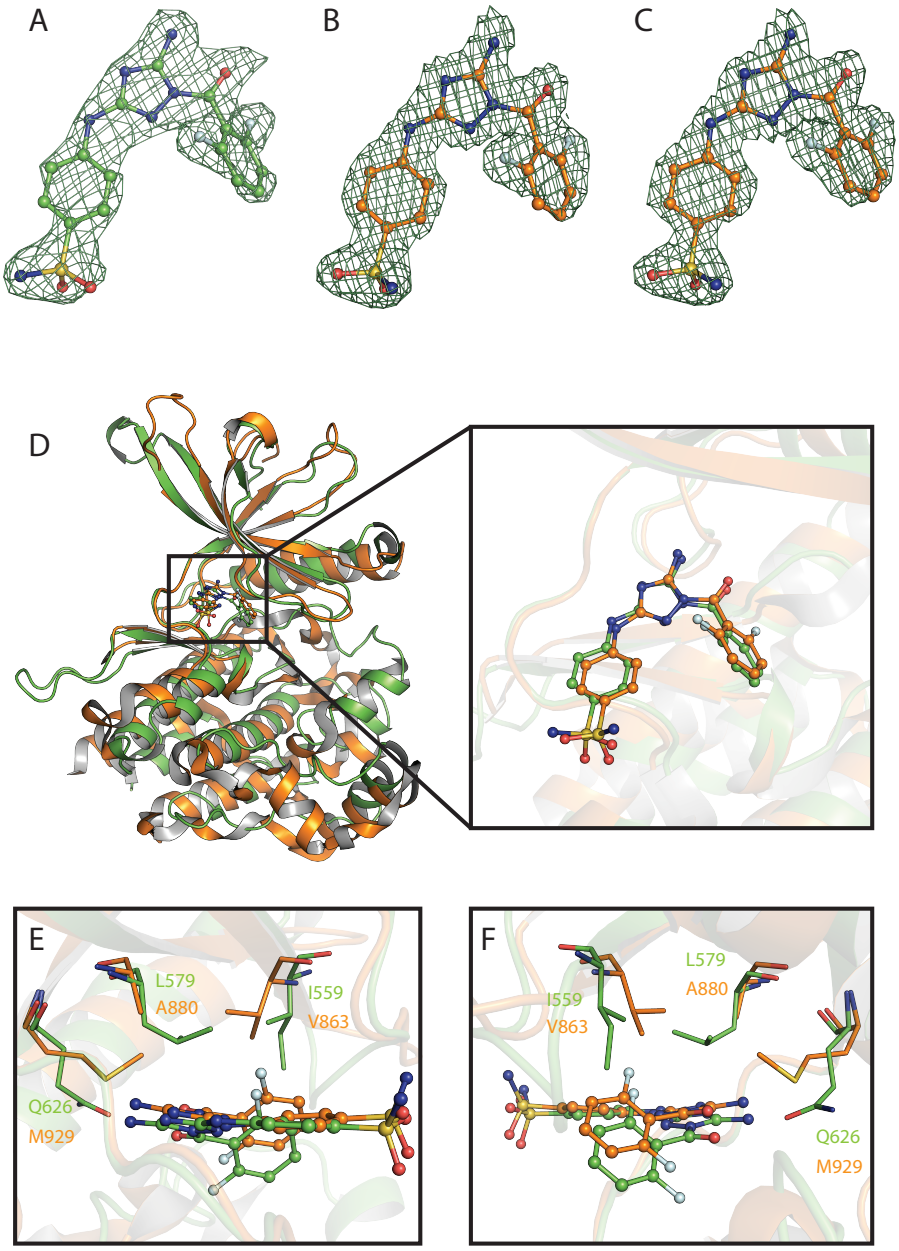

Figure S2. (A-C) Electron density map (F_o-F_c contoured at 3σ) of JNJ-7706621 at the (A) JH2 as well as JH1 (B, chain A; C, chain B) ATP-binding sites. The map was calculated without JNJ-7706621 in the model but present during refinements. (D) Structural alignment of JAK2 JH1 (orange) and JH2 (green) domains in complex with JNJ-7706621. The hinge regions of JH1 domain (chain A, residues 929-935) and JH2 domain (residues 626-632) were used for the alignment. (E and F) Magnified views of the ATP-binding pocket.

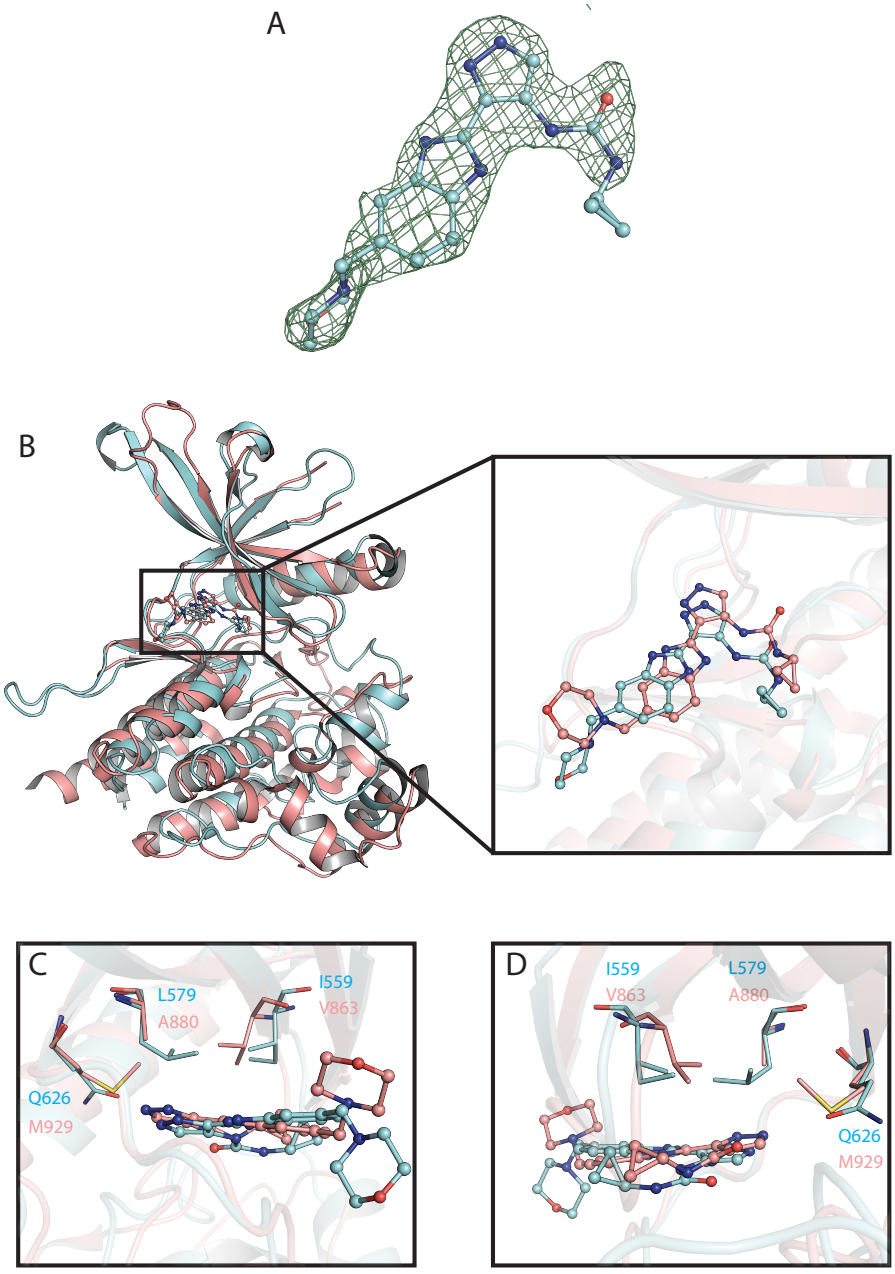

Figure S3. (A) Electron density map (F_o-F_c contoured at 3σ) of AT9283 at the JH2 domain ATP-binding site. The map was calculated without AT9283 in the model but present during refinements. (B) Structural alignment of JAK2 JH1 (pink) and JH2 (blue) domains in complex with AT9283. The hinge regions of the JH1 domain (chain A, residues 929-935) and JH2 domain (residues 626-632) were used for the alignment. (C and D) Magnified views of the ATP-binding pocket.

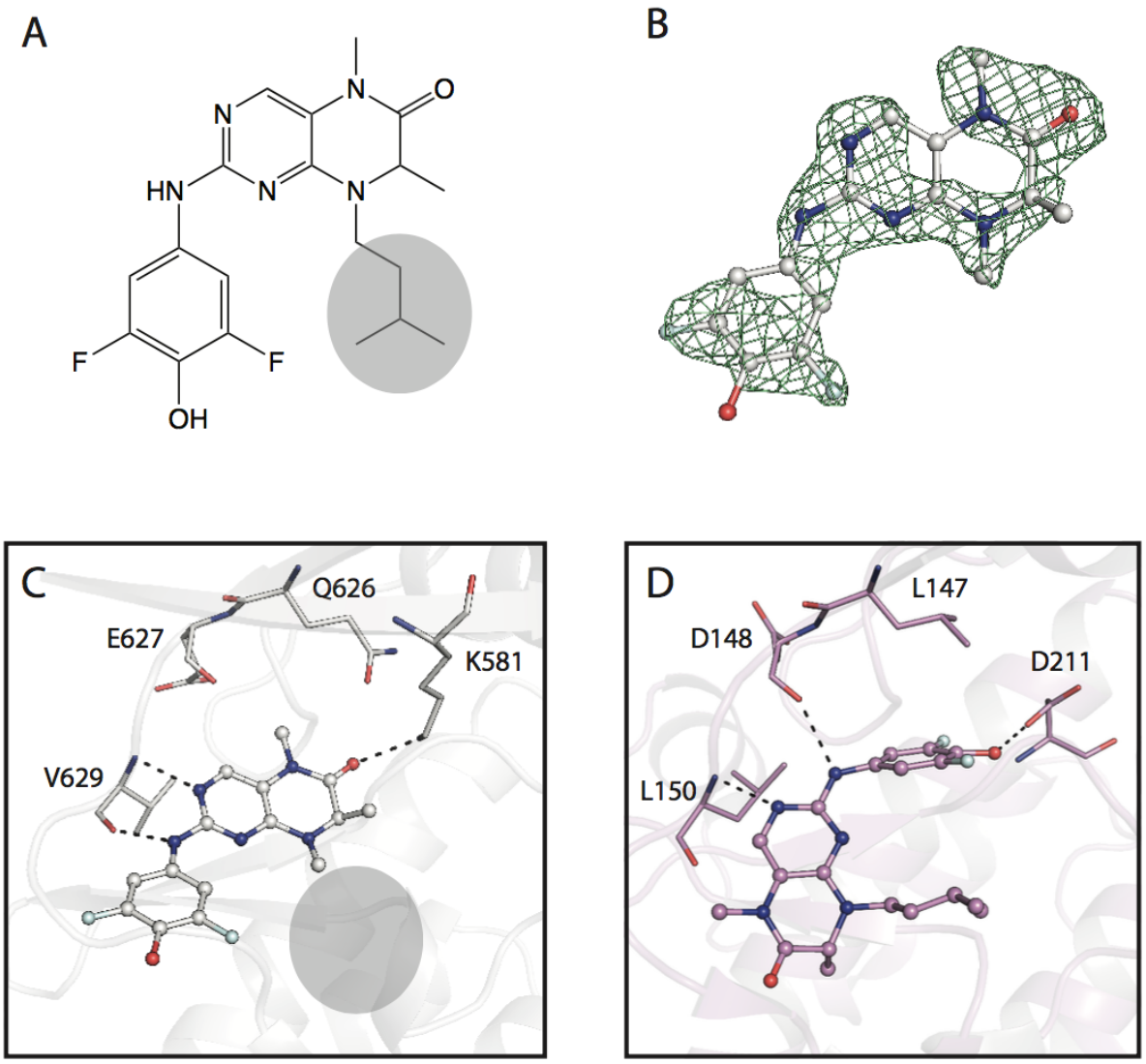

Figure S4. (A) Chemical structure of BI-D1870, a ribosomal S6 kinase inhibitor and hit from the screen. The isopentane group (circled in grey) lacks electron density and, thus, is omitted from structure. (B) Electron density map (F_o-F_c contoured at 3σ) of BI-D1870 at the JH2 active site. The map was calculated without BI-D1870 in the model but present during refinements. (C) Magnified view of the ATP-binding pocket of JH2 domain in complex with BI-D1870. The missing isopentane group is circled in grey. (D) Magnified view of the ATP-binding pocket of ribosomal S6 kinase 2 (RSK2) in complex with BI-D1870 (PDB ID 5D9K). The BI-D1870 binding mode differs between the JAK2 JH2 domain and RSK2 complexes.

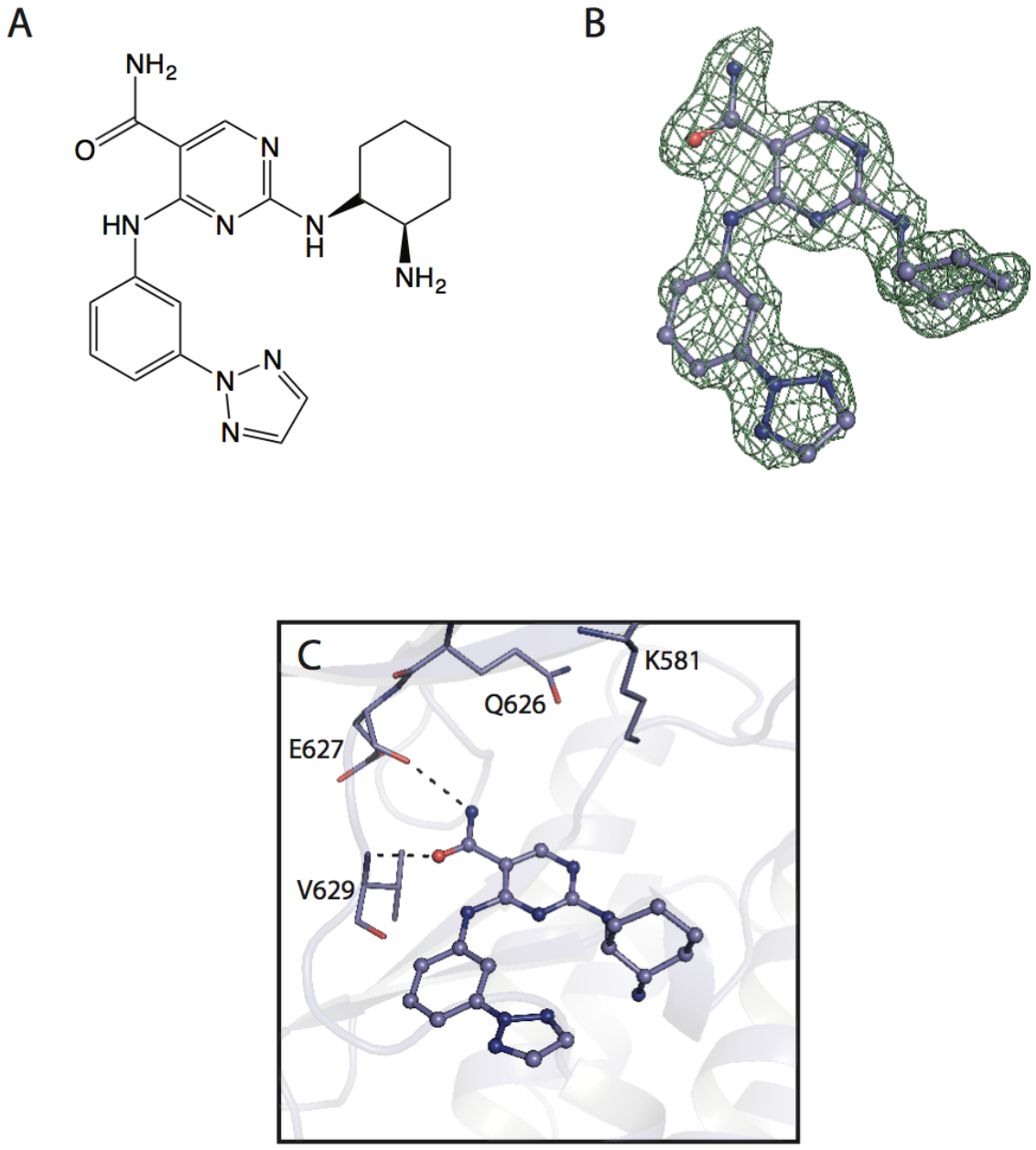

Figure S5. (A) Chemical structure of PRT062607, a Syk inhibitor and hit from the screen. (B) Electron density map (F_o-F_c contoured at 3σ) of PRT062607 at the JH2 domain ATP-binding site. The map was calculated without PRT062607 in the model but present during refinements. (C) Magnified view of the ATP-binding pocket of JH2 domain in complex with PRT062607.

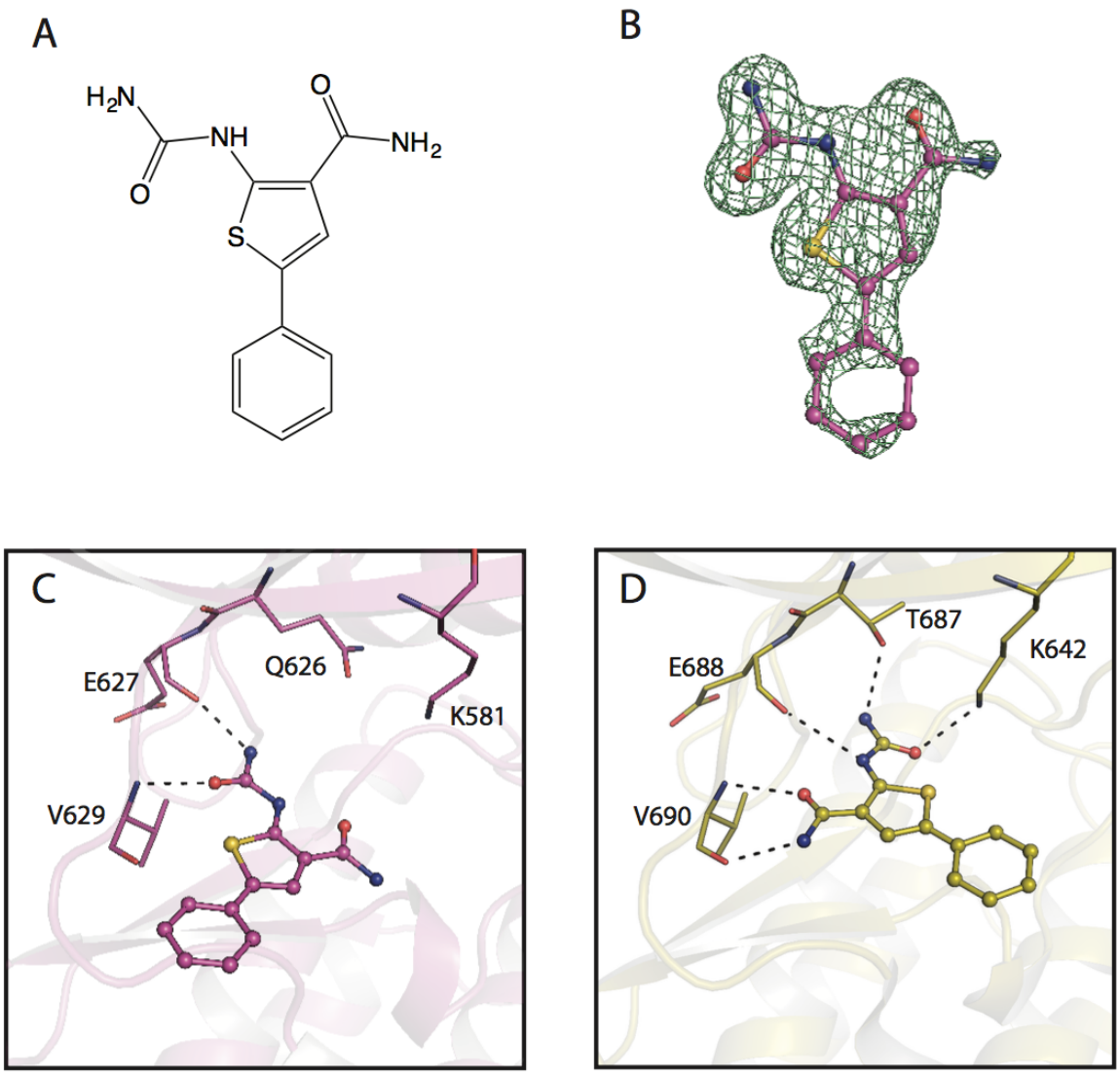

Figure S6. (A) Chemical structure of IKK-2 Inhibitor VI, an analog of the screen hit TPCA-1. (B) Electron density map (F_o-F_c contoured at 3σ) of IKK-2 Inhibitor VI at the JH2 domain ATP-binding site. The map was calculated without IKK-2 Inhibitor VI in the model but present during refinements. (C) Magnified view of the ATP-binding pocket of JAK2 JH2 domain in complex with IKK-2 Inhibitor VI. (D) Magnified view of the ATP-binding pocket of TYK2 JH2 domain in complex with IKK-2 Inhibitor VI (PDB ID 3ZON). The IKK-2 Inhibitor VI binding mode differs between the JAK2 JH2 and TYK2 JH2 domain complexes.

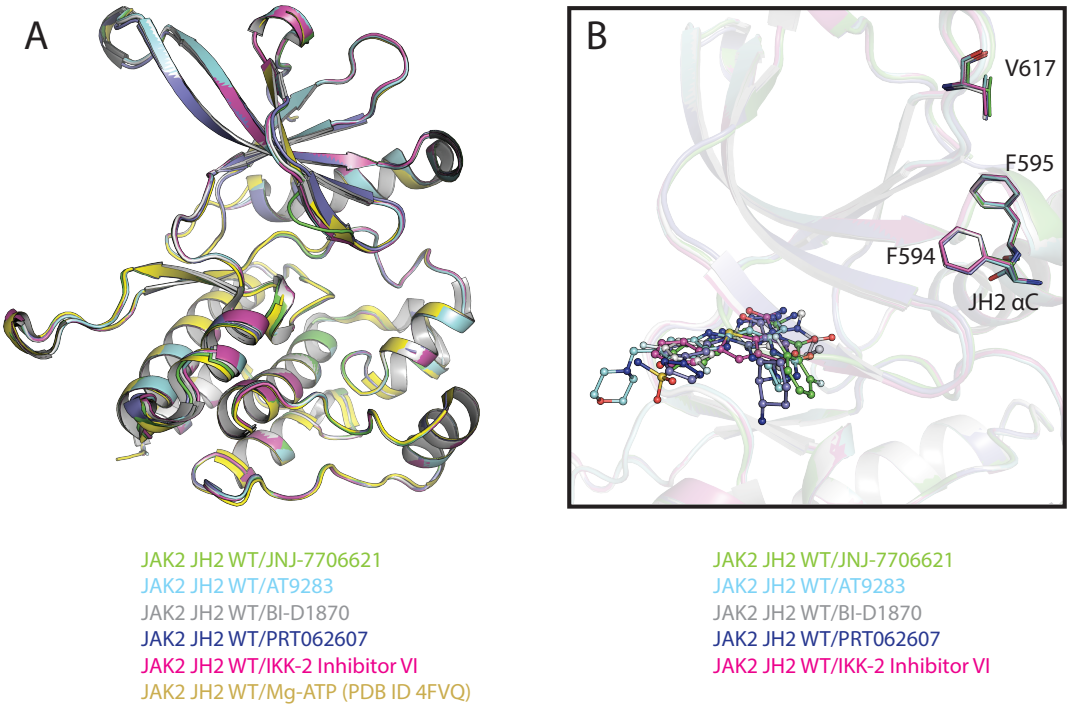

Figure S7: (A) Overlay of the 5 JAK2 JH2 complexes presented in this study, along with the JAK2 JH2 domain/ATP complex (PDB ID 4FVQ). (B) Magnified view of the JH2 domain ATP-binding pocket with all 5 compounds. Small molecules bind to the hinge region, distant from the JH2 αC-helix.

Table S1: Thermodynamic parameters determined using ITC

JAK2 Domain	Compound	ΔH (kcal/mol)	ΔS (cal/mol/deg)	K_d (nM)
JH1	**JNJ-7706621**	-9.5	1.6	31
	AT-9283	-17.1	-22.0	11
JH2	**JNJ-7706621**	-10.4	-3.6	106
	AT-9283	-9.0	-3.8	1323

Table S2: Data Collection and Refinement Statistics

Protein/Small Molecule	**JAK2 JH1/JNJ-7706621**	**JAK2 JH2/JNJ-7706621**
PDB ID	**5USY**	**5USZ**
Data Collection		
X-ray Source	Rigaku 007 HF+	Rigaku 007 HF+
Wavelength (Å)	1.54	1.54
Space Group	C2	$P2_1$
Unit Cell Dimensions		
a, b, c (Å)	137.0, 68.9, 100.5	44.7, 57.5, 60.7
α, β, γ (°)	90.0, 127.1, 90.0	90.0, 110.4, 90.0
Resolution (Å)*	50.0 – 2.0 (2.07 – 2.00)	50.0 – 2.1 (2.18 – 2.10)
No. Total Reflections	150,646	52,368
No. Unique Reflections	50,325	16,371
R_{sym} or R_{meas} (%)*	9.1 (25.1)	14.4 (49.9)
$I/\sigma I$*	9.6 (2.5)	9.6 (2.3)
Completeness (%)*	99.3 (96.4)	96.7 (88.1)
Redundancy*	3.0 (2.8)	3.2 (2.6)
Refinement		
Molecules/ASU	2	1
Resolution (Å)	37.6 – 2.0	41.9 – 2.1
R_{work}/R_{free}	17.6/21.6	17.2/22.4
Number of Atoms		
Protein, Solvent, Ion/Small Molecule	4684, 665, 97	2092, 214, 43
B Factors ($Å^2$)		
Protein, Solvent, Ion/Small Molecule	19.6, 32.6, 34.7	22.0, 30.8, 29.5
RMS Deviations		
Bond (Å)	0.013	0.002
Angle (°)	1.2	0.58

*Values in parentheses refer to the outer resolution shell.
Each data set was collected from a single crystal.

Table S3: Data Collection and Refinement Statistics

Protein/Small Molecule	**JAK2 JH2/AT9283**	**JAK2 JH2/BI-D1870**
PDB ID	**5UT0**	**5UT1**
Data Collection		
X-ray Source	Rigaku 007 HF+	APS 24-ID-E
Wavelength (Å)	1.54	0.98
Space Group	$P2_1$	$P2_1$
Unit Cell Dimensions		
a, b, c (Å)	44.1, 57.6, 60.4	44.4, 57.5, 60.6
α, β, γ (°)	90.0, 109.9, 90.0	90.0, 110.3, 90.0
Resolution (Å)*	50.0 – 2.1 (2.18 – 2.10)	56.9 – 1.9 (2.02 – 1.95)
No. Total Reflections	60,191	79,465
No. Unique Reflections	16,717	21,107
R_{sym} or R_{meas} (%)*	11.0 (32.3)	13.9 (93.0)
$I/\sigma I$*	8.9 (1.9)	12.4 (1.9)
Completeness (%)*	99.9 (99.7)	99.7 (99.8)
Redundancy*	3.6 (2.4)	3.8 (3.8)
Refinement		
Molecules/ASU	1	1
Resolution (Å)	41.4 – 2.1	56.9 – 1.9
R_{work}/R_{free}	16.7/22.5	16.4/20.1
Number of Atoms		
Protein, Solvent, Ion/Small Molecule	2104, 250, 48	2097, 220, 48
B Factors (Å^2)		
Protein, Solvent, Ion/Small Molecule	21.6, 32.1, 32.9	24.5, 35.7, 43.2
RMS Deviations		
Bond (Å)	0.003	0.012
Angle (°)	0.60	1.17

*Values in parentheses refer to the outer resolution shell.
Each data set was collected from a single crystal.

Table S4: Data Collection and Refinement Statistics

Protein/Small Molecule	**JAK2 JH2/PRT062607**	**JAK2 JH2/IKK-2 Inhibitor VI**
PDB ID	**5UT2**	**5UT3**
Data Collection		
X-ray Source	APS 24-ID-E	NSLS X25
Wavelength (Å)	0.98	1.10
Space Group	$P2_1$	$P2_1$
Unit Cell Dimensions		
a, b, c (Å)	44.2, 57.2, 61.2	44.3, 57.6, 60.4
α, β, γ (°)	90.0, 110.6, 90.0	90.0, 109.9, 90.0
Resolution (Å)*	57.2 – 1.7 (1.81 – 1.75)	50.0 – 1.5 (1.55 – 1.50)
No. Total Reflections	107,445	148,193
No. Unique Reflections	28,976	45,642
R_{sym} or R_{meas} (%)*	8.3 (92.7)	3.9 (18.0)
$I/\sigma I$*	16.3 (1.7)	27.0 (5.1)
Completeness (%)*	99.8 (99.9)	99.5 (97.2)
Redundancy*	3.7 (3.3)	3.2 (2.7)
Refinement		
Molecules/ASU	1	1
Resolution (Å)	57.2 – 1.7	40.9 – 1.5
R_{work}/R_{free}	16.1/20.1	15.5/16.9
Number of Atoms		
Protein, Solvent, Ion/Small Molecule	2122, 285, 45	2113, 327, 40
B Factors (Å^2)		
Protein, Solvent, Ion/Small Molecule	26.6, 38.8, 30.7	19.9, 34.6, 35.5
RMS Deviations		
Bond (Å)	0.009	0.008
Angle (°)	0.97	0.97

*Values in parentheses refer to the outer resolution shell.
Each data set was collected from a single crystal.