Suporting Information

Kinase-independent small-molecule inhibition of JAK-STAT signaling

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Experimental Procedures

Cell culture and reagents. INS-1E cells (generously provided by Claes Wollheim and Pierre Maechler, University of Geneva, Switzerland [1]) were maintained in RPMI1640 containing 11 mM glucose, 10% fetal bovine serum, 10 mM HEPES, 50 μM 2-mercaptoethanol, and 1 mM sodium pyruvate, and cultivated at 37ºC with 5% CO_2 in a humidified atmosphere. DLD-1 colon cancer cell line was purchased from ATCC and were maintained in RPMI1640 containing 11 mM glucose, 10% fetal bovine serum, and cultivated at 37ºC with 5% CO_2 in a humidified atmosphere. pRK5-Jak2-FLAG expression vector was generously provided by Dr. Christin Carter-Su (University of Michigan Medical School) and Dr. James Ihle (St. Jude Children's Research Hospital). pLentiCRISPR.v2 vector was from Addgene (Addgene no. 52961). Recombinant human IL-1β, IFN-γ, and TNF-α were purchased from R&D Systems. CellTiter-Glo, Dual-Glo and Caspase-Glo 3/7 assay kits were purchased from Promega. Antibodies were obtained from Abcam (USP9X), Cell Signaling Technology (STAT1, p-STAT1(Y701), JAK2 and secondary horseradish peroxidase-conjugated secondary antibodies), and Sigma (tubulin, actin). GAS reporter assay kit for STAT1 activity and primers for RT-qPCR were from SABiosciences. Chemiluminescence detection system SuperSignal and Monomeric Avidin agarose kit were from Thermo Fisher Scientific. Compounds BRD6283 and WP1130 were synthesized at the Broad Institute and were dissolved in 100% DMSO. VM001 was synthesized at University of Michigan, College of Pharmacy[2]. ABT-737 and ruxolitinib were purchased from Selleck Chem. QuickExtract DNA Extraction solution was from Epicentre. Prime STAR GXL DNA Polymerase was from Takara. PCR Cloning Kit and NEB 10-beta chemically competent cells were from New England Biolabs. Human HEK293 cells overexpressing USP9X-FLAG[3] (generously provided by Dr. Dario Alessi, University of Dundee, Scotland) were maintained in DMEM containing 11 mM glucose, 10% fetal bovine serum, 15 μg/ml blasticidin, 100 μg/ml hygromycin B and cultivated at 37ºC with 5% CO_2 in a humidified atmosphere. pcDNA3-HA-Ub

vector was from Addgene. Lactacystin was from Tocris Bioscience. 4% paraformaldehyde was from Alfa Aesar. AlexaFluor-conjugated secondary antibody, Hoechst 33342 reagent, Lipofectamine2000 transfection reagent, SYPRO Orange and total JAK2 ELISA kit were from Life Technologies. Antibodies for IPO9, and XPO1 were from Abcam. Antibodies for ASK1, β-CATENIN, p-STAT1(Y727), HA, and MCL1 were from Cell Signaling Technology. Thapsigargin, tunicamycin, G418, M2 Monoclonal antibody for FLAG tag and 3XFLAG peptide were from Sigma. Magnetic IP/Co-IP kit was from Thermo Scientific. RT-pPCR primers for rat *Mcm6* and *Blvrb* were from SABiociences. Ub-AMC was from Boston Biochem.

Human islet culture. Human islets from deceased organ donors were obtained through the Integrated Islet Distribution Program (IIDP) and the National Disease Research Interchange (NDRI). The purity and viability of human islets are reported to be 80–85% and 86–95%, respectively. Specific data on individual donors is reported in Supplementary Information. Islets were cultured in 96- and 384-well plates as reported previously [4].

Cytokine treatment. INS-1E cells were treated with 10 ng/mL IL-1β, 50 ng/mL IFN-γ, and 25 ng/mL TNF-α in RPMI1640 media containing 1% fetal bovine serum, in the presence or absence of compounds. Human pancreatic islets were treated with 0.5 ng/mL human IL-1β, 50 ng/mL human IFN-γ and 25 ng/mL human TNF-α in the presence or absence of BRD0476 for 6 days.

Glucose-stimulated insulin secretion. Dissociated human islet cells were incubated for 2 hours in KRBH buffer (135 mM NaCl, 3.6 mM KCl, 5 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4, 0.1% BSA) lacking glucose. Cells were

subsequently incubated with KRBH buffer containing 2 mM or 16 mM glucose for 1 hour. The supernatant was collected for measurement of secreted insulin by ELISA (Alpco).

Viability assays. Measurement of cellular ATP, caspase-3 activation, and mitochondrial membrane potential in the presence or absence of cytokines and BRD0476 was performed as previously described[5,6].

RNA interference. INS-1E cells were seeded at 5,000 cells/well using a Multidrop Combi (Thermo Labsystems) in white optical 384-well plates. 100 nM of scrambled siRNA or siRNAs targeting *Usp9x* were transfected using DharmaFECT reagent (Dharmacon). Transfected cells were cultured for 72 hours, treated with cytokines for an additional 48 hours, and subjected to cell-based assays.

Reporter-gene assay. INS-1E cells were seeded at 10,000 cells/well using a Multidrop Combi (Thermo Labsystems) in white optical 384-well plates. The cells were then transfected with GAS reporter or control plasmids using DharmaFECT reagent (Dharmacon). After overnight incubation, cells were treated with cytokines and compounds for 18hr. Luminescence was measure using Dual-Glo Luciferase (Promega).

Western blotting. INS-1E cells were seeded at the appropriate density, and after overnight incubation, cells were treated with cytokines and compounds for 1 hour, followed by lysis in RIPA buffer. Total protein was separated by 4-12% SDS-PAGE and transferred to a PVDF membrane and probed with the appropriate antibody. Blots were developed using the chemiluminescence detection system SuperSignal and light emission was captured using an Imaging Station 4000MM PRO (CareStream).

Gene-expression profiling. Microarray analysis was performed by the Broad Institute Genetic Analysis Platform on 500 ng of total RNA using Rat 230_2.0 arrays (Affymetrix). All data are MIAME-compliant, with the raw data deposited in Gene Expression Omnibus (GEO), accession number GSE42897.

Quantitative PCR. RNA was isolated using the RNeasy Mini Kit (Qiagen). RNA was reverse-transcribed with random primers using the High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems), followed by quantitative PCR with Power SYBR Green PCR Master Mix (Applied Biosystems) on an Applied Biosystems 7900HT real-time PCR machine.

Affinity pull-down experiments. INS-1E cells were seeded at $5x10^5$ cells/well in 6-well plates. After overnight incubation, cells were treated with the cytokine cocktail for 1 hour in the absence of compound, followed by lysis in RIPA buffer. 100 μg total protein were diluted to 600 μL with PBS and treated for 12 hours with 10 μM BRD0476 biotin-derivative at 4°C. After incubation, 30 μL monomeric avidin agarose, previously equilibrated according to manufacturer's instructions, were added, and incubated 2 hours at room temperature. After centrifugation at 10,000x*g* for 1 minute, the unbound proteins were recovered (flow-through) and the resin was washed twice with 1 mL PBS. The bound protein(s) were eluted with 100 μL 2 mM biotin in PBS (pull-down). 20 μL flow-through and 30 μL pull-down fractions were subjected to Western blot analysis as described above.

Co-immunoprecipitation. $10x10^6$ INS-1E cells were seeded in 10-cm plates. Cells were transfected with 25 μg pRK5-Jak2-FLAG or empty vector using Lipofectamine2000 (Life Technologies). Cells were cultured for 72 hours, lysed with 1 mL IP/Lysis buffer, and centrifuged

at 26,000x*g* for 20 min. 500 μg total protein was immunoprecipitated with 5 μg anti-FLAG M2 antibody for 12 hours at 4°C. Lysate were then incubated with protein A/G magnetic beads for 2 hours, followed by wash steps. Bound proteins were eluted with 100 μL 3XFLAG peptide (150 ng/μL) in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). Total cell lysate and immunoprecipitated protein complexes were subjected to Western blot analysis as described above.

Procedures for generating sgRNA vectors and CRISPR/Cas9-mediated genome engineering. Guide strands targeting the catalytic domain of the *Usp9x* gene were designed and analyzed for potential off-target recognition sites using an online CRISPR design tool (http://www.genome-engineering.org/). The guide strand we selected (5'-AGAATACCGTTCCTGATAGA-3') was constructed from a pair of annealed oligonucleotides and cloned into the expression vector, pLentiCRISPR.v2), according to published protocols[7,8]. These constructs were then transfected into INS-1E cells using Lipofectamine 2000 (Invitrogen), per the manufacturer's instructions. Cells were selected for 7 days with 0.25 μg/ml puromycin, and then seeded as single colonies (2500 cells) in a 10-centimeter plate. Individual clones were selected after 3 weeks in culture, with media changes performed every three days.

Sequence verification of endogenous genes. Genomic DNA was extracted from cell lines arising from single clones with the QuickExtract DNA Extraction solution, and CRISPR target regions amplified using appropriate locus-specific primers (FW: 5'-GGACCAGGAAGCTTTTGGGAGGG-3'; REV: 5'- TGGGGTTAGTGGGAGTTGGCA-3'). Standard PCR conditions were used with Prime STAR GXL DNA Polymerase and 250 ng of genomic DNA according to the manufacturer's instruction for 35 cycles. Correct size of PCR products (803 bp) was checked by agarose gel electrophoresis. PCR products were then cloned into the pMiniT vector using the PCR Cloning Kit according to the manufacturer's instructions

and transformed into NEB 10-beta chemically competent cells. Plasmid DNA was isolated from multiple colonies of each transformation and Sanger sequenced.

Site-directed mutagenesis. The Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific) was used to generate the $JAK2^{K999R}$ and $JAK2^{K850R}$ mutants. The presence of the desired mutation was confirmed by DNA sequencing. To evaluate the effect of mutation on JAK2 signaling, INS-1E cells were seeded at $5x10^5$ cells/well in 6-well plates. Cells were transfected with 4 μg pRK5-Jak2-FLAG, pRK5-Jak2-K999R-FLAG, or pRK5-Jak2-K850R-FLAG vectors using Lipofectamine2000. Transfected cells were cultured for 72 hours, lysed with 300 μL RIPA buffer, and centrifuged at 16,000x*g* for 20 minutes. Total cell lysates were subjected to Western blot analysis as described above.

JAK2 signaling. $10x10^6$ INS-1E cells were seeded in 10-cm cell culture dishes. After overnight incubation, medium was removed and cells were treated with cytokines and compounds for 1 hour, followed by lysis in RIPA buffer. pJAK2(Y1007/1008) levels were determined by ELISA (Life Technologies). The inhibition of JAK family members by BRD0476, WP1130, or ruxolitinib were determined by EMD Millipore (KinaseProfiler).

Network analysis. The protein-protein association network STRING (version 9.05) was used to gain additional insight of potential functional relationship between USP9X and JAK2.

Synthesis of CR-6. To a solution of BRD0476 (8.8 mg, 0.013 mmol) in tetrahydrofuran (THF) (500 μL) was added carbonyldiimidazole (CDI) (7.40 mg, 0.046 mmol) at rt. The reaction mixture was stirred at rt for 2h, diluted with water, and extracted into ethyl acetate. The combined organic layers were concentrated *in vacuo* to provide an imidazole carbamate

intermediate. This crude intermediate (10 mg, 0.013 mmol) was dissolved in THF (500 µL) and Boc-PEG-NH_2 (9.3 µL, 0.039 mmol) and *N*,*N*-diisopropylethylamine (*i*-Pr_2NEt) (2.3 µL, 0.013 mmol) were added to the resulting solution at rt. The reaction mixture was further stirred at rt for 3.5 h, concentrated *in vacuo*, and redissolved in CH_2Cl_2 (400 µL). This solution was washed with water, dried over Na_2SO_4, filtered, and concentrated *in vacuo* to provide a Boc-protected CR-6 intermediate. This crude intermediate was dissolved in dichloromethane (DCM) and trifluoroacetic acid (TFA) (4:1, 500uL), stirred for 20 minutes, concentrated *in vacuo*, and directly purified by HPLC to provide 2.54 mg of CR-6 (0.0030 mmol, 23% yield).

Synthesis of immobilized BRD0476. Following the general procedure of Ong et al.[9], CR-6 was activated with *N*,*N*'-disuccinimidyl carbonate (DSC) in dimethylformamide (DMF) and then immobilized onto Affi-Gel102 solid-phase beads (Bio-Rad) in the presence of triethylamine in dimethylsulfoxide (DMSO), with 12.5% loading.

Synthesis of biotinylated BRD0476. To a solution of crude CR-6 (14 mg, 0.016 mmol) in DMF (0.230 mL) was added *N*-hydroxybenzotriazole hydrate (HOBT) (3.68 mg, 0.024 mmol), *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU) (0.024 mmol, 9.1 mg), *i*-Pr_2NEt (8.38 µl, 0.048 mmol), and biotin (7.8 mg, 0.032 mmol) at rt. The reaction mixture was further stirred for 2.5 h at rt and concentrated *in vacuo*. The residue was diluted with water and extracted into ethyl acetate. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. Purification on silica gel (0-50% methanol in DCM) provided 5.7 mg of biotinylated BRD0476 (0.0053 mmol, 33% yield).

Establishment of INS-1E cell line stably expressing HA-Ub (INS-1E-Ub). $10x10^6$ INS-1E cells were seeded in 10-cm plates. Cells were transfected with 25 µg pcDNA3-HA-Ub[10] using

Lipofectamine2000 (Life Technologies). Cells were cultured for 72 hours, selected for two weeks with 200 μg/mL G418 and then maintained with 100 μg/mL G418.

JAK2 ubiquitination. $10x10^6$ INS-1E-Ub cells were seeded in 10-cm plates. Cells were treated with cytokines and compounds for 1 hour, followed by lysis with 1 mL IP/Lysis buffer, and centrifuged at 26,000x*g* for 20 min. 500 μg total protein was immunoprecipitated with 5 μg anti-HA antibody for 12 hours at 4°C. Lysate was incubated with protein A/G magnetic beads for 2 hours, followed by wash steps. Bound proteins were eluted with 100 μL SDS Loading buffer. Immunoprecipitated protein complexes were subjected to Western blot analysis as described above.

Purification of recombinant USP9X. For each purification, ten 25-cm dishes of confluent HEK-293 cell lines stably expressing wild-type human FLAG-tagged USP9X were treated for 24 hours with 10 μg/mL tetracycline, washed twice with ice-cold PBS, and lysed in 1 mL of ice-cold IP/Lysis buffer. The combined lysates were centrifuged at 26,000x*g* for 30 minutes at 4°C and the supernatant incubated with 0.2 ml of anti-FLAG M2 affinity gel previously equilibrated with five volumes of TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) overnight at 4°C. The anti-FLAG M2 affinity gel was washed extensively with ten volumes of TBS buffer and then eluted with five volumes of a solution containing 150 ng/mL 3XFLAG peptide in TBS.

STAT1 translocation. INS-1E cells were seeded at 20,000 cells/well using a Multidrop Combi (Thermo Labsystems) in a 96-well black plate. After overnight attachment, cells were treated for 30 minutes with 10 ng/mL IFN-γ in RPMI 1640 media containing 1% fetal bovine serum in the presence or absence of compounds, and fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature. Fixation was stopped by aspirating the PFA and wells were washed with

PBS twice. Fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 20 minutes and blocked with 2% BSA for 2 hours at room temperature. Primary anti-STAT1 antibody was added to the cells and incubated at 1:500 dilution overnight at 4°C, washed three times with 2% BSA, and incubated with AlexaFluor-conjugated secondary antibody at 1:2000 dilution for 1 hour at room temperature. Nuclei were stained with Hoechst 33342. Wells were washed with PBS, and then imaged with an ImageXpress Micro automated microscope (Molecular Devices, Sunnyvale, CA).

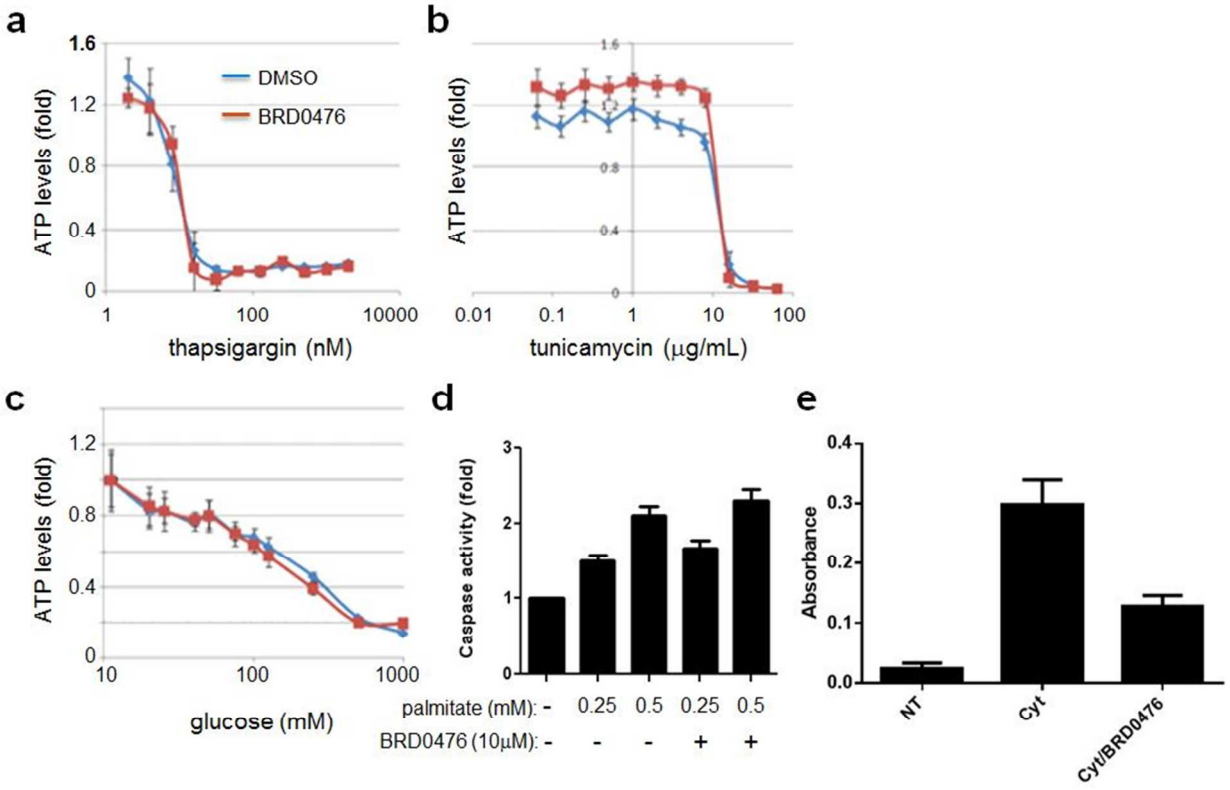
USP9X enzymatic activity. In a 384-well black plate was added 5 μL 10X Reaction Buffer (500 mM Tris-HCl, pH 7.5, 5% NP-40, 50 mM $MgCl_2$ and 1.5 M NaCl), 2 μL of a 0.1 μg/μl solution of recombinant full-length USP9X-FLAG or USP9X catalytic domain, 0.5 μL of 500 mM DTT, and the appropriate amount of compounds when necessary for a total volume of 50 μL. After a 2-hour preincubation at 37°C, 0.5 μL of a 50 μM solution of Ub-AMC was added and incubated at 37°C. The fluorescence was then measured at excitation/emission 380 nm/460 nm. For kinetic experiments, the fluorescence was measured over 50 min. For end-point experiments, the fluorescence was measured after 2 hours.

Diffferential scanning fluorimetry (DSF). DSF was performed on a Roche LightCycler system. All reactions were prepared individually in 384-well PCR plates at a final volume of 6.4 μL. The reactions consisted of 0.1 μg/μL full-length recombinant USP9X-His in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4), incubated with 10 μM of BRD0476 or WP1130. SYPRO Orange was then added to ratio 2:1 protein:SYPRO orange. The final DMSO concentration was 1%. A temperature melt was carried out between 25-95°C with 0.06°C/sec increments and the gain was set to 2. The HRM module was used with an excitation filter of 468 nm and emission filter of

575 nm. Each condition was performed in triplicate. The first derivative of the raw data was analyzed for peaks, which corresponded to the melting temperature (T_m) of USP9X.

Compound characterization. For chemical characterization of BRD0476, please see ref. 11 and 12. BRD0476 (10 μM) was incubated for 30 minutes at 37°C in RPMI media (1% FBS) in the absence or presence of INS-1E beta cells (with no cytokines) in a 384-well plate. A 40 μL aliquot of compound-containing media was removed, and 40 μL cold acetonitrile was added, containing 10 μM spautin-1 as internal standard. We centrifuged at 3000 rcf at 4 °C, and analyzed the supernatant by LC/MS, detecting compounds by diode array (200-500 nm). The diode array peaks were integrated to give a ratio of BRD0476 to spautin-1.

Figure S1



BRD0476 does not protect beta cells from treatments, other than inflammatory cytokines, that cause cell death. Cellular ATP levels were measured after two-day treatment with (**a**) thapsigargin, (**b**) tunicamycin, or (**c**) elevated glucose in the absence (blue) or presence (red) of 10 μM BRD0476. (**d**) Caspase-3 activity after two-day treatment with the indicated concentration of palmitate in the absence or presence of 10 μM BRD0476. (e) Cell-death measurement of DNA-histone complexes released into the cytoplasm by ELISA.

Figure S2

Donor	Source	Islet Purity	Islet Viability	Age	Gender	Height (cm)	Weight (kg)	BMI	Ethnicity
1	U Pitt	85%	95%	36	M	190.5	104	28.7	W
2	Prodo lab	80%	90%	43	N/A	N/A	N/A	27.0	N/A
3	Prodo lab	85%	95%	58	N/A	N/A	N/A	30.4	N/A
4	U Penn	85%	86%	47	M	177.0	89.5	28.6	B

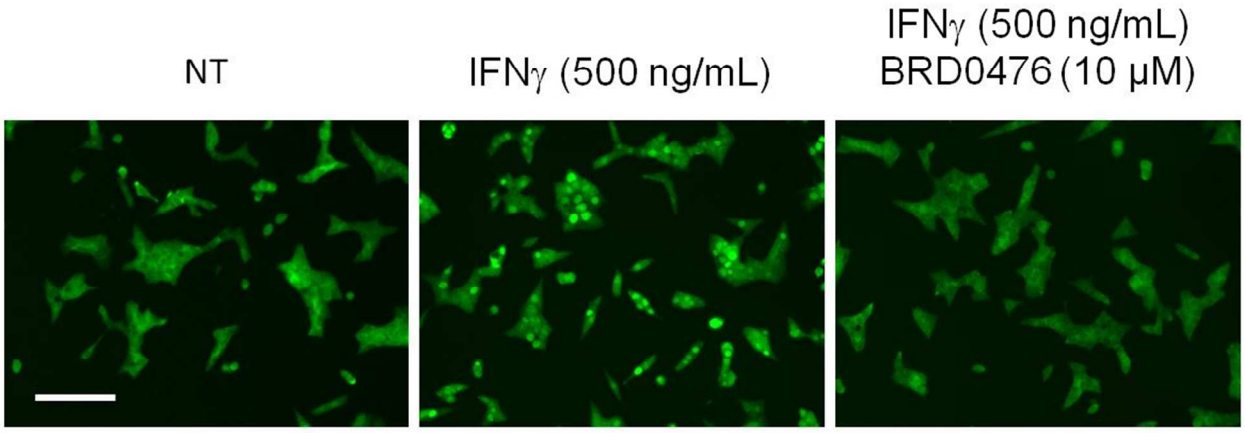
BRD0476 protects human beta cells from cytokine-induced apoptosis. Islet donor information for islet batches used for this study provided. M, male; N/A, information not available; W, white; B, black; BMI, body-mass index.

Figure S3

SYMBOL	Gene	6h	Function
Lrrc39	leucine rich repeat containing 39	1.76	component of the sarcomeric M-band
Nr4a1	nuclear receptor subfamily 4, group A, member 1	1.67	steroid-thyroid hormone-retinoid receptor superfamily
Chac1_predicted	ChaC, cation transport regulator-like 1 (predicted)	1.59	putative pro-apoptotic component of the UPR
Trib3	tribbles homolog 3 (Drosophila)	1.18	putative protein kinase induced by NF-kappaB
Areg	amphiregulin	1.14	member of the epidermal growth factor family
Pcp4l1	Purkinje cell protein 4-like 1	1.06	unknown function
Slc7a11_predicted	solute carrier family 7, member 11 (predicted)	1.01	anionic amino acid transporter (cysteine, glutamate)
Arntl	aryl hydrocarbon receptor nuclear translocator-like	-1.71	basic HLH protein that heterodimerizes with CLOCK
Fam84a	family with sequence similarity 84, member A	-1.12	neurologic sensory protein 1
Adm	adrenomedullin	-1.05	hypotensive peptide

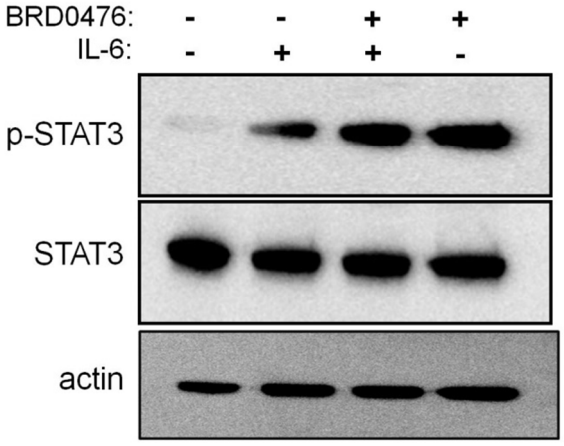
BRD0476 modulates relatively few gene-expression changes in INS-1E after 6 hours. Log_2 fold changes are shown for each gene at indicated times. All genes changed greater than two-fold are shown.

Figure S4



BRD0476 inhibits STAT1 nuclear localization in INS-1E cells after 30-minute treatment with IFNγ. Scale bar, 100μm.

Figure S5



BRD0476 does not inhibit STAT3 signaling. HepG2 cells were treated with IL-6 or BRD0476 for 1 hour, followed by cell lysis and Western blotting for phosphorylated and total STAT3.

Figure S6

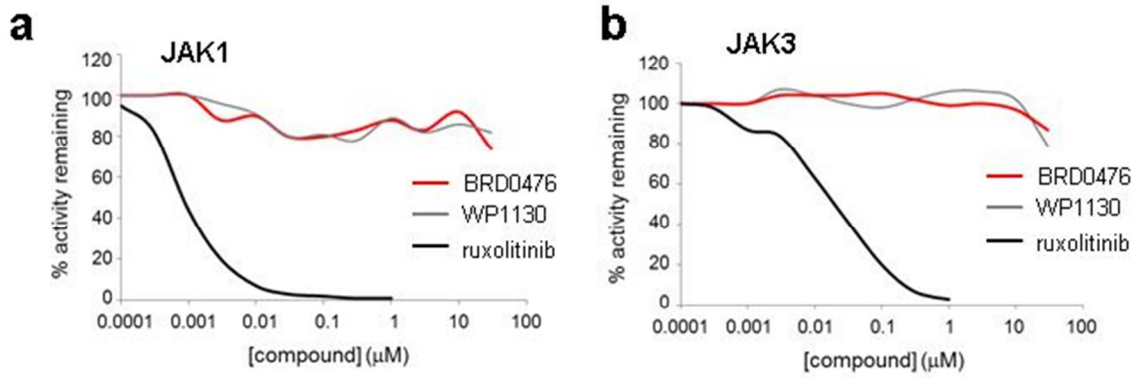
Kinase	Activity
Abl	99
ALK	65
AMPKα1	83
ASK1	99
Aurora-A	104
Aurora-B	77
Axl	89
BTK	109
CaMKI	97
CDK1/cyclinB	104
CDK2/cyclinA	105
CDK5/p25	101
CDK6/cyclinD3	106
CDK7/cyclinH/MAT1	104
CDK9/cyclin T1	112
CHK1	104
CK1γ1	92
CK1(γ)	101
CK2α2	113
c-RAF	101
DRAK1	107
eEF-2K	128
EGFR	103
EphA5	108
EphB4	101
Flt3	109
Fyn	90
GCK	100
GSK3α	137
GSK3β	111
IGF-1R	86
IKKα	130

Kinase	Activity
IKKβ	97
IR	99
IRR	100
IRAK1	96
IRAK4	103
JAK2	108
JAK3	88
JNK1α1	107
JNK2α2	102
JNK3	109
KDR	101
Lck	70
LKB1	85
LOK	103
Lyn	102
MAPK1	109
MAPK2	111
MAPKAP-K2	93
MAPKAP-K3	92
MEK1	110
MKK4(m)	112
MKK6	103
MKK7β	103
MLK1	96
Mnk2	97
MSK1	89
MSK2	86
MST1	100
mTOR	94
NEK2	102
p70S6K	123
PAK2	97

Kinase	Activity
PDGFRβ	102
PDK1	102
Pim-1	98
PKA	139
PKBα	98
PKBβ	92
PKBγ	99
PKCα	94
PKCβII	101
PKCγ	99
PKCδ	111
PKCε	110
PKCη	112
PKCι	108
PKCμ	98
PKCθ	115
PKCζ	108
PKG1α	73
Plk3	103
PRAK	60
ROCK-I	107
ROCK-II	97
Ros	103
Rse	100
Rsk1	102
SAPK2a	96
SAPK2b	94
SAPK3	115
SAPK4	107
Src(1-530)	107
SRPK1	94
TAK1	101

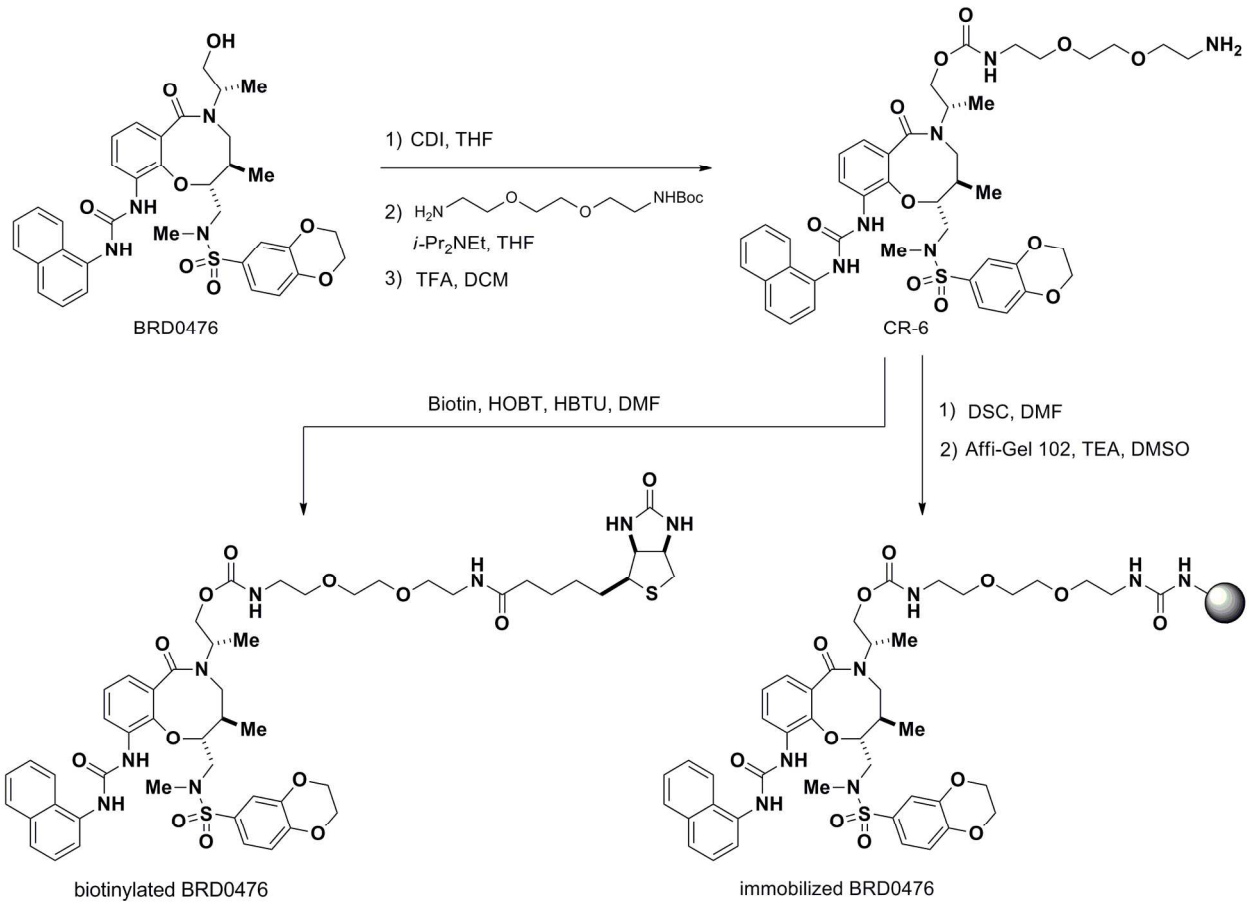
Kinase profiling results with 10 μM BRD0476. Data shown as % activity remaining. Services provided by EMD Millipore.

Figure S7



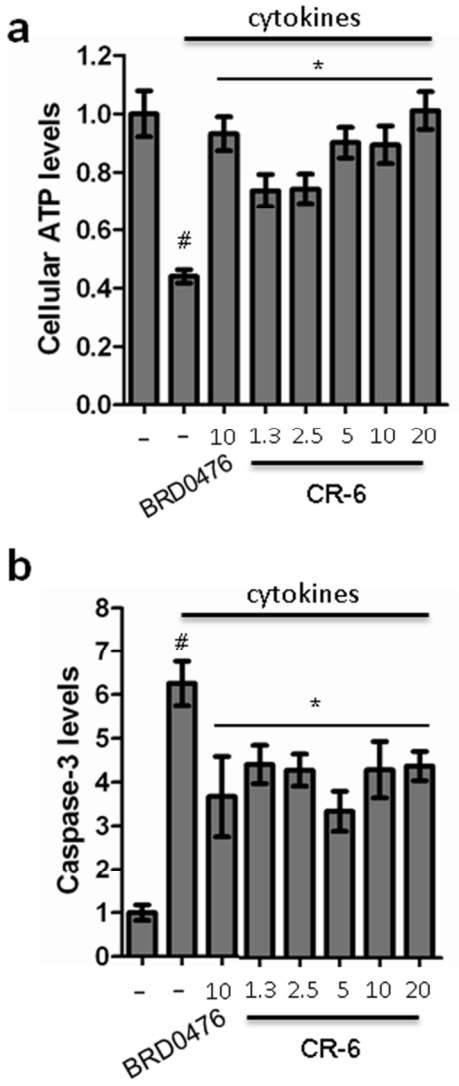
Assessment of biochemical kinase activities of (a) JAK1 and (b) JAK3 and modulation by BRD0476, WP1130, or ruxolitinib. Services provided by EMD Millipore.

Figure S8



Synthetic scheme for preparation of CR-6, PEG-immobilized version of BRD0476, and a biotinylated version of BRD0476.

Figure S9



CR-6 retains activity in INS-1E cells. (**a**) Cellular ATP levels and (**b**) caspase-3 activity after two-day treatment with cytokines and the indicated concentrations of compound. # $p < 0.001$ compared to no treatment, * $p < 0.001$ compared to cytokine treatment, Student's t-test.

Figure S10

Symbol	Exp1,Ratio1	Exp1,Ratio2	Exp2,Ratio1	Exp2,Ratio2	AvgRatio	*p*-value	Protein Description
Mcm6	1.59	1.76	1.63	1.56	1.63	2.29E-26	DNA replication licensing factor MCM6
Blvrb	1.44	0.96	1.55	2.14	1.52	7.19E-23	biliverdin reductase B
Usp9x	0.96	0.74	1.12	1.11	0.98	2.07E-09	ubiquitin specific peptidase 9, X-linked isoform
Ap3b2	0.93	0.31	1.27	0.84	0.84	1.01E-06	adaptor-related protein complex 3, beta 2 subunit
Cand1	0.52	0.54	1.12	0.96	0.78	7.49E-06	cullin-associated NEDD8-dissociated protein 1
n/a	0.61	0.43	0.87	0.97	0.72	6.97E-05	similar to TBC1 domain family member 15
Gapdh	0.79	0.32	0.94	0.83	0.72	7.85E-05	glyceraldehyde-3-phosphate dehydrogenase
Ahcy	0.68	0.39	0.89	0.91	0.72	8.65E-05	adenosylhomocysteinase
Kpna4	0.64	0.5	1.01	0.64	0.70	1.64E-04	importin 4
n/a	0.69	0.64	0.59	0.75	0.67	4.18E-04	similar to CG5937-PA
Lrpprc	0.43	0.53	0.72	0.88	0.64	1.02E-03	leucine-rich PPR motif-containing protein
Ipo9	0.37	0.46	0.74	0.77	0.58	5.14E-03	importin 9
Retsat	0.51	0.41	0.58	0.83	0.58	5.38E-03	all-trans-retinol 13,14-reductase
Xpo1	0.37	0.32	0.91	0.63	0.56	1.09E-02	exportin-1
Timm44	0.37	0.49	0.49	0.74	0.52	2.66E-02	mitochondrial import inner membrane translocase subunit TIM44
Cand2	0.31	0.56	0.50	0.62	0.50	5.11E-02	cullin-associated NEDD8-dissociated protein 2

Quantitative proteomics results, with 16 candidate binders of BRD0476 at $p<0.05$.

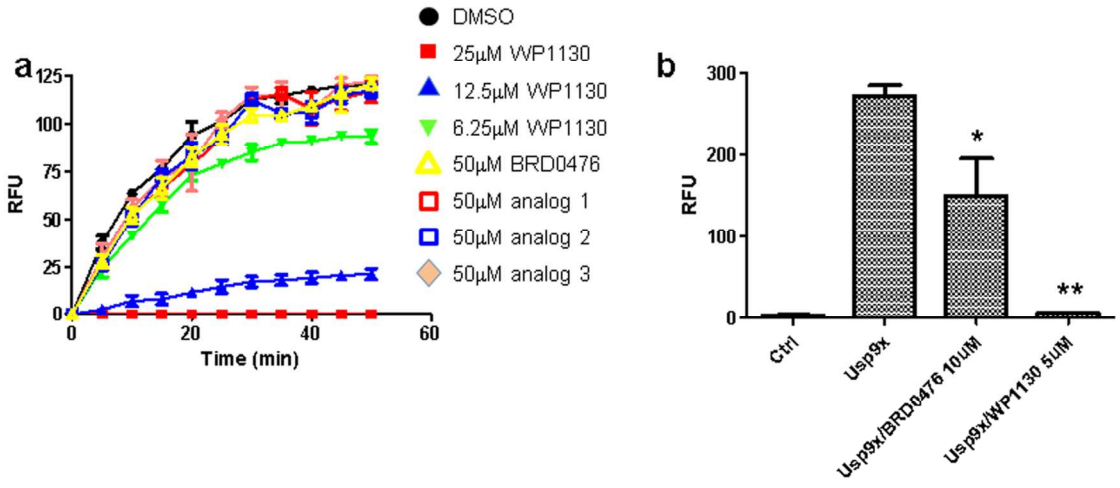
Enrichment ratios for each experiment are in $\log_2$ scale.

Figure S11

target	confirmation of knock-down	restores ATP levels	blocks effect of BRD0476
Mcm6	qPCR	no	no
Blvrb	qPCR	no	no
Usp9x	Western	**yes**	no
Gapdh	Western	no	no
Kpna4	Western	no	no
Ipo9	Western	no	no
Xpo1	Western	no	no

Summary of siRNA-mediated knock-down of candidate binders, and their effects on cytokine-induced cell death in the absence (column 3) or presence (column 4) of BRD0476.

Figure S12



Effects of BRD0476, analogs, and WP1130 on (a) biochemical activity of catalytic domain of USP9X and (b) full-length USP9X. See Supplementary Experimental Procedures. * $p <$ 0.001, ** $p < 10^{-7}$, Student's t-test.

Figure S13

Enzyme	BRD0476	Ub-ald
$USP2_{CD}$	104	1.3
USP5	104	1.3
ataxin 3	94	3.6
$USP7_{FL}$	100	1.0
$USP8_{FL}$	101	0.8
USP25	93	0.6
UCH-L1	100	0.6
UCH-L3	99	0.6
BAP1	98	1.0
$A20_{CD}$	90	74
Otubain 1	69	4.5

Biochemical profiling of BRD0476 against eleven deubiquitinases. Activity shown as percent activity remaining. Ubiquitin-aldehyde used as positive control. Services provided by Boston Biochem.

Figure S14

Round 1:

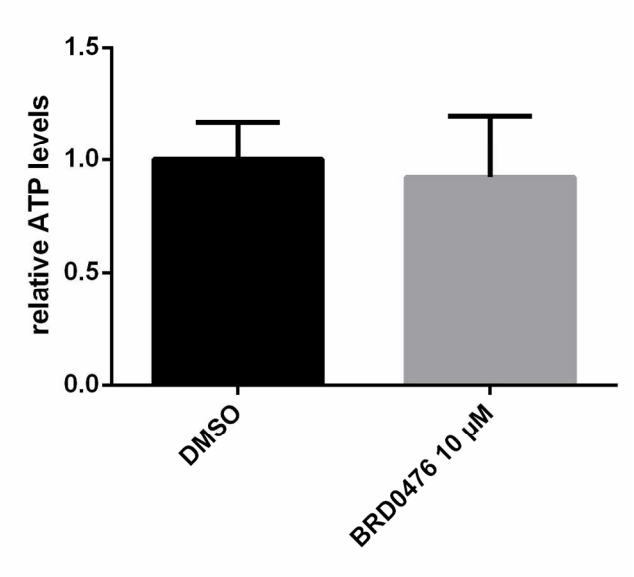
	Tm (°C)	ΔTm (°C)
1% DMSO	52.23 ± 0.04	
BRD0476	51.71 ± 0.14	-0.52
WP1130	50.00 ± 0.13	-2.23

Round 2:

	T_m (°C)	ΔT_m (°C)
1% DMSO	48.19 ± 0.17	
BRD0476	48.00 ± 0.36	-0.19
WP1130	47.77 ± 0.05	-0.42
both	46.66 ± 0.25	-1.53

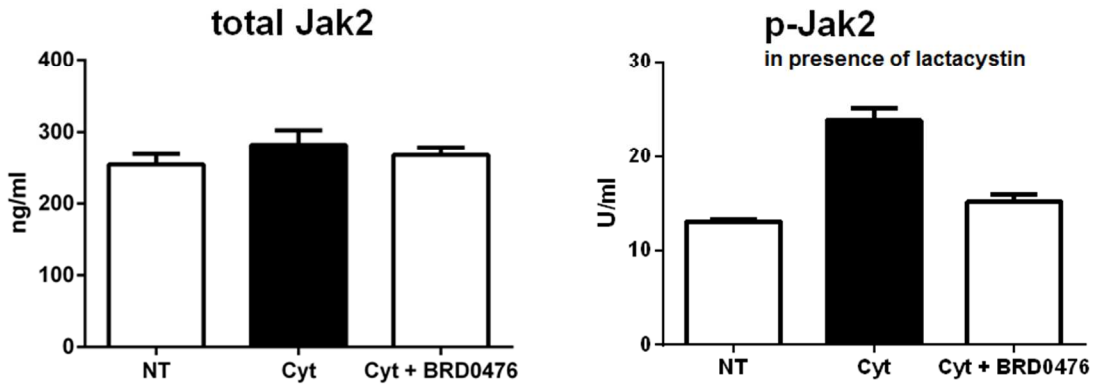
Differential scanning fluorimetry on FLAG-tagged USP9X isolated from HEK cells. Melting temperature after compound or vehicle treatment, and the change in melting temperature induced by compounds. Negative values indicate a destabilization of protein. Round 2 indicates an experiment to test the effects of incubating USP9X with both compounds at the same time. Efforts to detect USP9X binding by surface plamon resonance, either by immobilizing the protein or the small molecule, were unsuccessful.

Figure S15



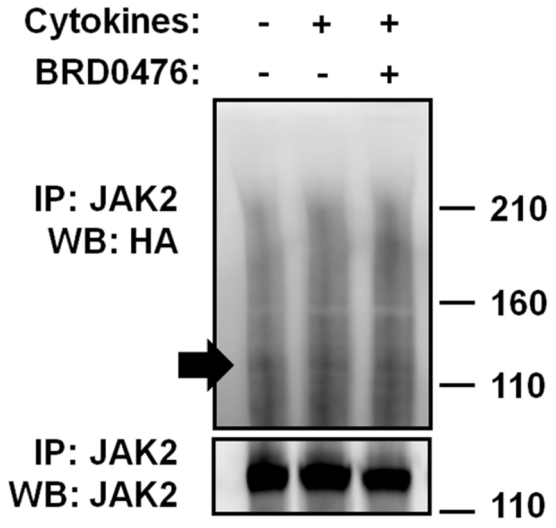
BRD0476 alone has no effect on DLD-1 colon cancer cell death. Cells were treated for 48 hours with DMSO or 10 μM BRD0476 before measuring cellular ATP levels. Final DMSO concentration was 0.2%.

Figure S16



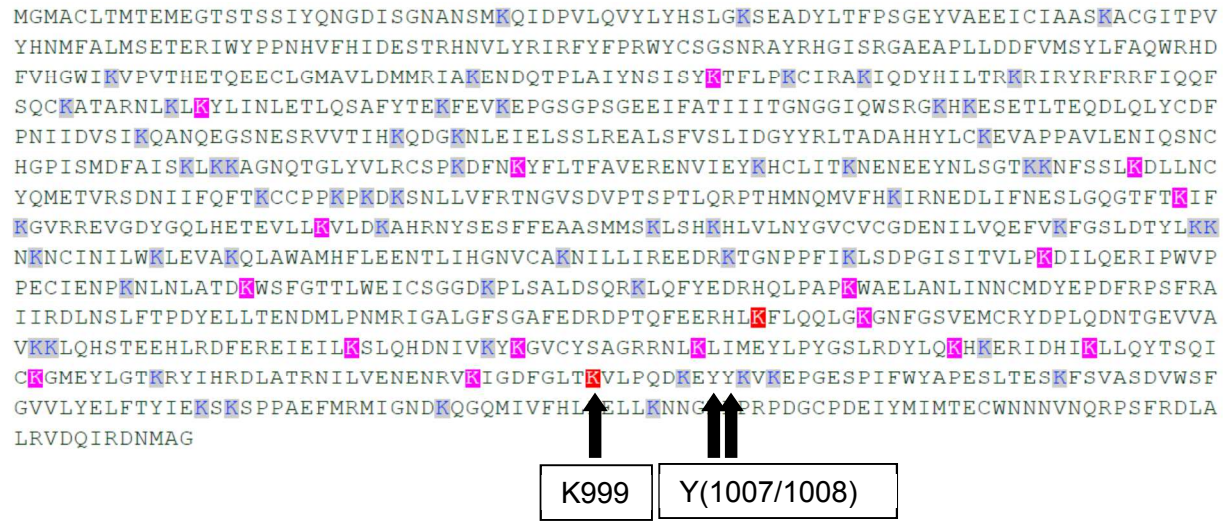
BRD0476 has no effect on total JAK2 levels, and reduces phosphorylation of JAK2 in the presence of 300 nM lactacystin. INS-1E cells were treated with cytokines in absence or presence of 10 μM BRD0476. Protein levels were determined by ELISA.

Figure S17



Ubiquitination status of JAK2. INS-1E-Ub cells were treated as described in the Supplementary Methods. JAK2 immunoprecipitations were evaluated for ubiquitination and total JAK2 levels.

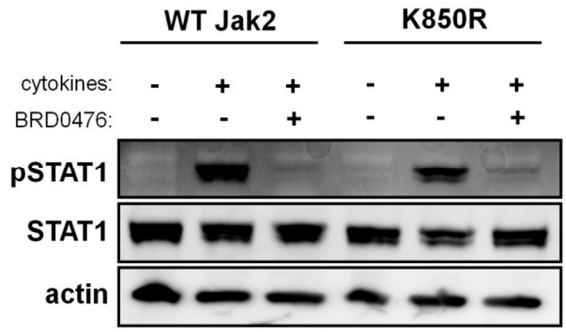
Figure S18



Label	Score Range	Sensitivity	Specificity
Low	[0.725 ~ 0.851]	35.4%	90%
High	(0.851 ~)	11.1%	98%

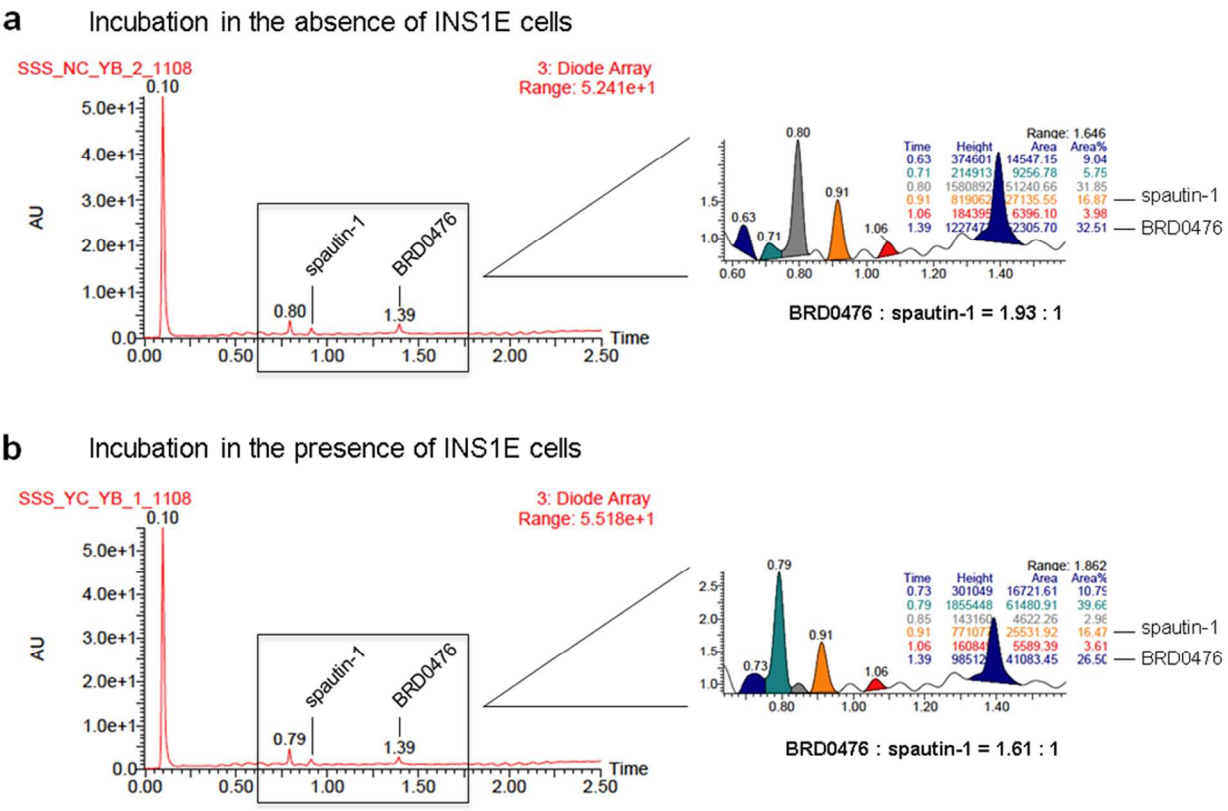
Prediction of ubiquitination sites in JAK2. Lysines in red are high-confidence predicted sites.

Figure S19



Mutation of lysine 850 to arginine has no effect on BRD0476 activity on STAT1 phosphorylation.

Figure S20



Detection of BRD0476 in cell-culture media. LC/MS traces are expanded to show finer detail of BRD0476 peaks (dark blue) and spautin-1 internal standard peaks (orange) after 30-minute incubation in the (**a**) absence or (**b**) presence of INS-1E cells. The ratio of BRD0476:spautin is 1.93:1 to in the absence of cells, and 1.61:1 in the presence of cells.

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