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Supplementary Materials for

Accumulation of JAK activation loop phosphorylation is linked to type I JAK inhibitor withdrawal syndrome in myelofibrosis

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Table S1. Primary myelofibrosis patient characteristics.

Patient #	Sex/Age	Mutation	Karyotype	Ruxolitinib dose	Hb (g/dL)	Platelets ($x10^6$/mL)	White Cells ($x10^6$/mL)	Spleen size or cm below costal margin
RAH1	M/83	JAK2 V617F (VAF 59%)	46, XY	20 mg bd	136	285	18.4	0 cm BCM
RAH2	M/63	JAK2 V617F (VAF 49%)	46, XY	20 mg bd	122	368	8.7	15 cm BCM
RAH3	M/75	JAK2 V617F (VAF 30%)	46/,XY del20q	10 mg bd	93	78	10.0	9 cm BCM
RAH4	F/61	CALR	46/,XX	15 mg bd	105	454	25.4	13 cm BCM
SU669	M/68	JAK2 V617F (VAF 48%)	+9	10 mg bd	11.5	75	42	Splenectomy
SU467	M/67	JAK2 V617F (VAF 28%)	46,XY	0	8.0	50	5.9	18 x12 cm
SU469	M/63	JAK2 V617F (VAF 97%)	46,XY	0	11.1	88	18.7	19.7 x16.3 cm
SU477	M/73	JAK2 V617F (VAF 87%)	46,XY	15 mg bd	9.3	212	10.0	16.5 cm BCM
SU478	M/78	JAK2 V617F (VAF 45%) Post-PV	46,XY	5 mg bd	10.6	40	34.8	15.5 cm BCM
SU479	M/72	JAK2 V617F	46,XY	20 mg bd	9.6	243	34.8	21.5 cm BCM
SU510	M/74	JAK2 V617F (VAF 22%)	46,XY	0	9.7	778	41.4	Splenectomy
SU482	F/67	JAK2 V617F (VAF 82%)	45,X,-del5 q31q35),t(16;21]	0	8.4	15	33.8	Unknown
SU178	M/58	CALR	46,XY	0	11.5	331	7.4	7 cm BCM
SU239	F/59	CALR	46,XY	0	11.9	209	12.7	5 cm BCM
SU594	F/71	CALR	46,XY	20 mg bd	10.3	105	46.1	5 cm BCM
SU497	M/43	CALR	46,XY	0	11.6	745	6.6	Not palpable
SU533	M/83	CALR	46,XY	0	10.3	83	20.3	11.5 cm BCM
SU280	F/76	CALR	46,XX	0	11.6	471	4.1	Not palpable

Table S2. Clinical characteristics and genotype of reported cases of ruxolitinib withdrawal syndrome.

Sex/Age	Mutation	Cytogenetics	Time to Symptom Onset (Days)	Symptoms	Reference
F/56	JAK V617F (VAF = 39%)	Unknown	2 (after taper initiation)	Pyrexia, cough, mild diarrhea	9
			5	Hypotension, tachycardia, acute respiratory distress syndrome	
F/59	JAK2 V617F	Unknown	<7	Respiratory distress, severe anemia, splenomegaly	17
F/69	JAK2 V617F	Complex t(8;22)(q24.1;q112), t(14;18)(q32;q21)	1	Septic shock-like syndrome, hypotension, pyrexia, confusion	17
M/44	JAK2 V617F	Unknown	1	Respiratory distress, pleural effusion, pericardial effusion	17
M/64	JAK2 V617F	Unknown	3	Pyrexia, splenomegaly, fatigue, pruritis, night sweats	17
F/56	JAK2 V617F	Unknown	14	Digital artery thrombosis, polyarticular arthritis	17
F/70	JAK2 V617F	70% trisomy(9) and del(20q)	28	Abdominal pain, massive splenomegaly, acute renal failure, hyperkalemia, hyperphosphatemia	19
M/76	JAK2 V617F (VAF = 62.6%)	Isolated del(20)	1	Pyrexia, inflammatory syndrome, acute respiratory distress syndrome	20
F/72	Unknown	Unknown	14	Hypotension, tachycardia, tachypnea, hypoxia	21
M/76	JAK2 V617F	Del(17)	7	Pyrexia, hypotension, low-grade disseminated intravascular coagulation, spontaneous tumor lysis syndrome	24

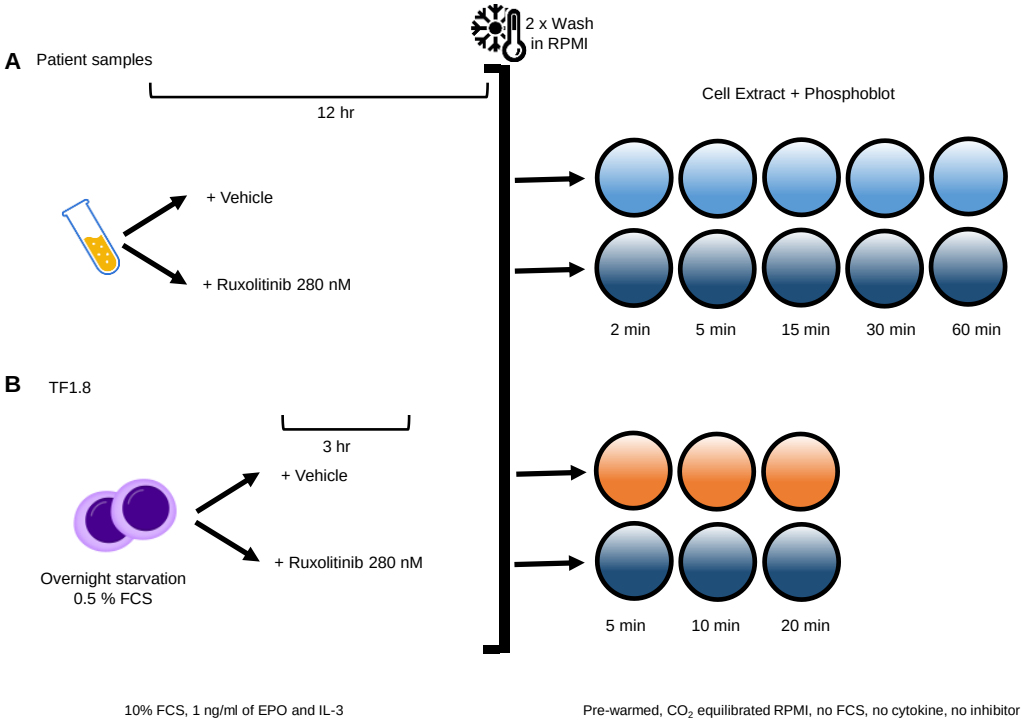


Fig. S1. Graphical representation of experiments used in JAK inhibitor withdrawal studies in patient samples and TF1.8 cells.

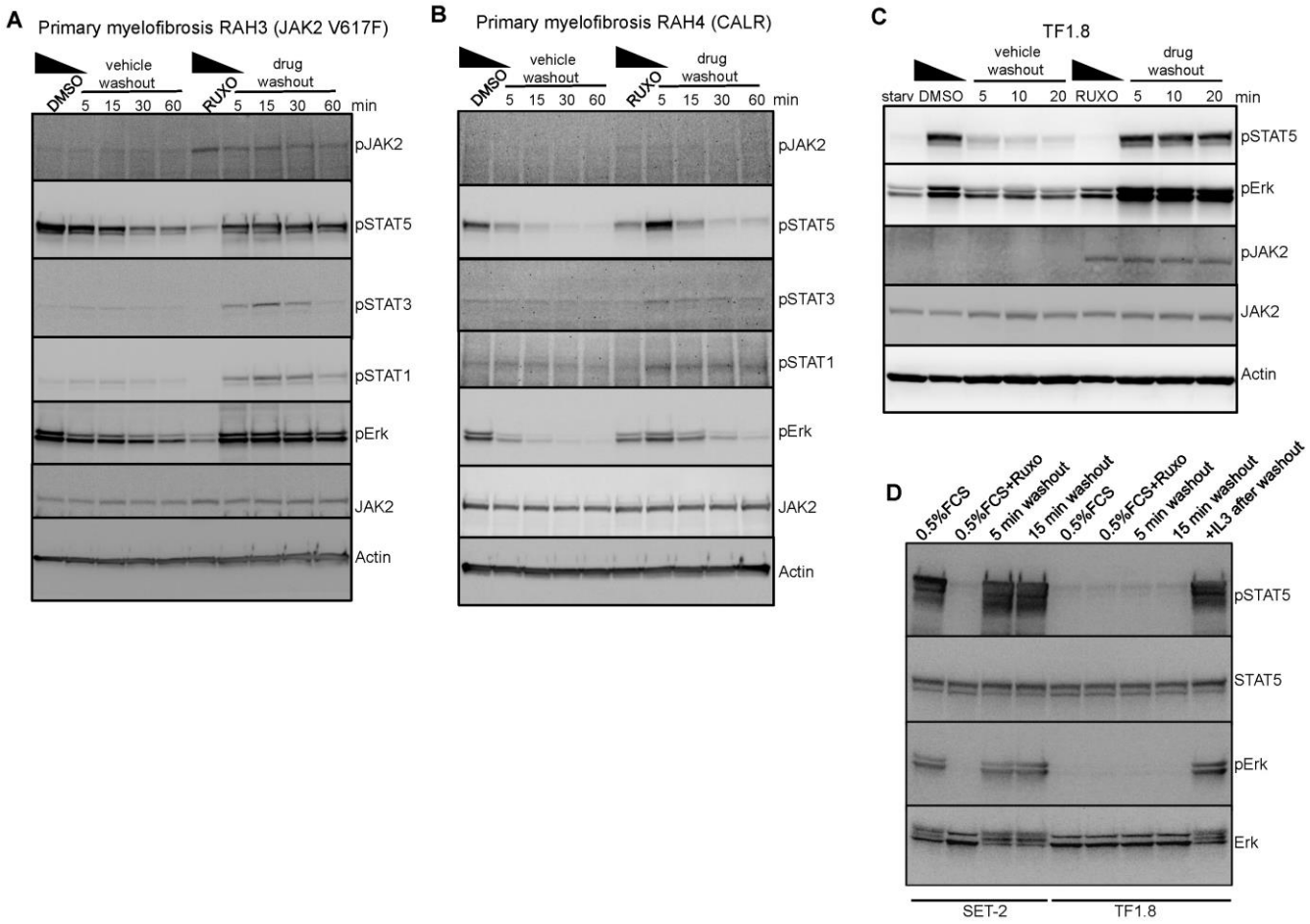


Fig. S2. Ruxolitinib washout triggers intracellular signaling. Primary cells from MPN patient RAH3 carrying the JAK2^{V617F} mutation (**A**) or patient RAH4 carrying a calreticulin mutation (**B**), were analysed for p-JAK2, p-STAT5, p-STAT3, p-STAT1 and p-Erk following withdrawal of ruxolitinib as described in Figure 1. (**C**) After overnight incubation in 0.5% FCS, TF1.8 cells were cultured in 10% FCS with 1 ng/ml EPO and IL-3 and 280 nM ruxolitinib or DMSO for 3 hours. Cells were extensively washed in cold media and cultured without additives for the indicated periods of time, cell lysates were prepared and immunoblotted for the indicated proteins. (**D**) SET-2 or TF1.8 cells were incubated in 0.5% FCS for 6 hours before addition of 280nM ruxolitinib for 90 minutes. Cells were washed and cultured in RPMI media for 5 and 15 minutes. Whole cell lysates were prepared and immunoblotted with p-STAT5, p-Erk, STAT5 and Erk antibodies. To confirm that TF1.8 cells were able to activate JAK dependent signalling after 15 minutes of ruxolitinib washout, cells were stimulated with 50 ng/ml IL-3 for 5 minutes.

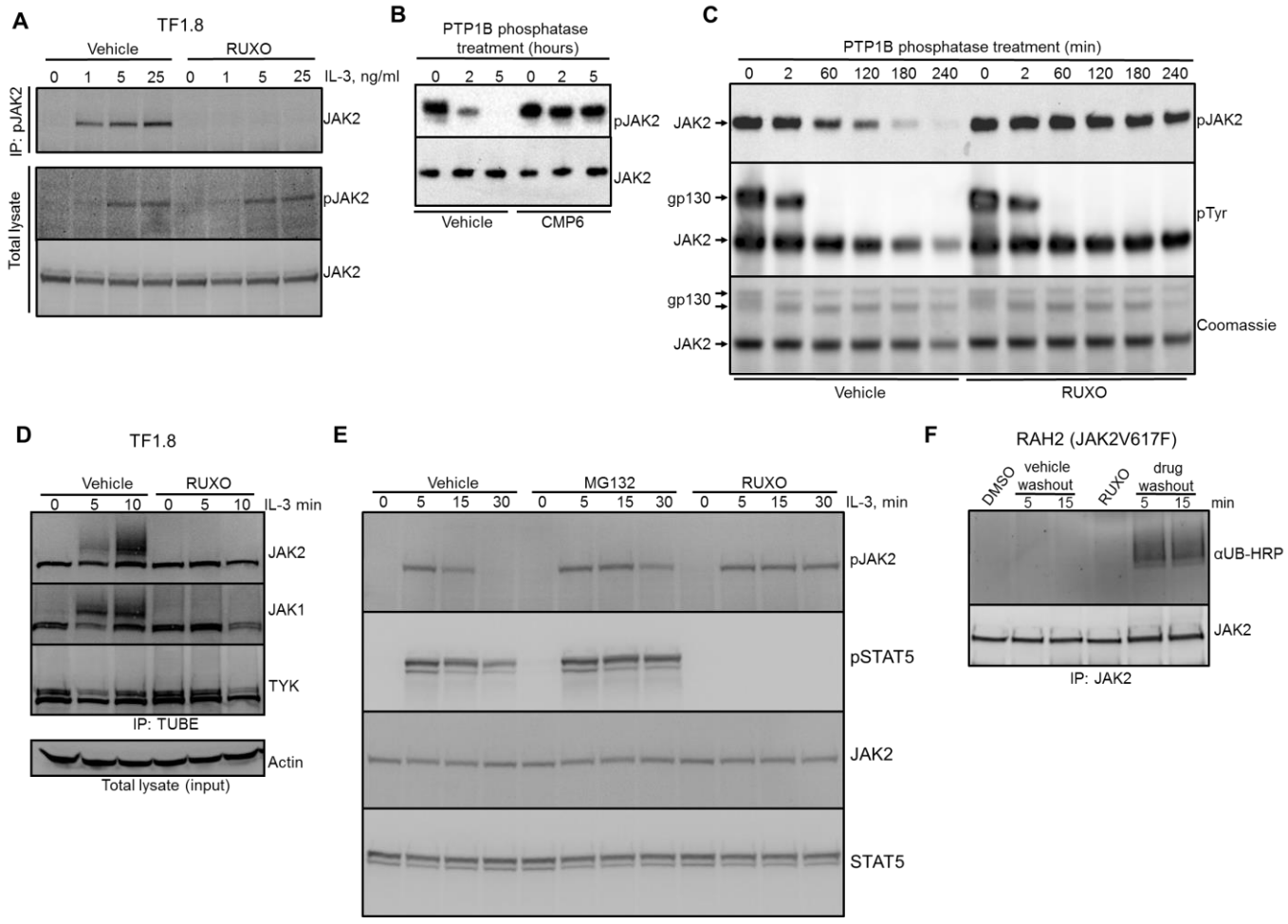
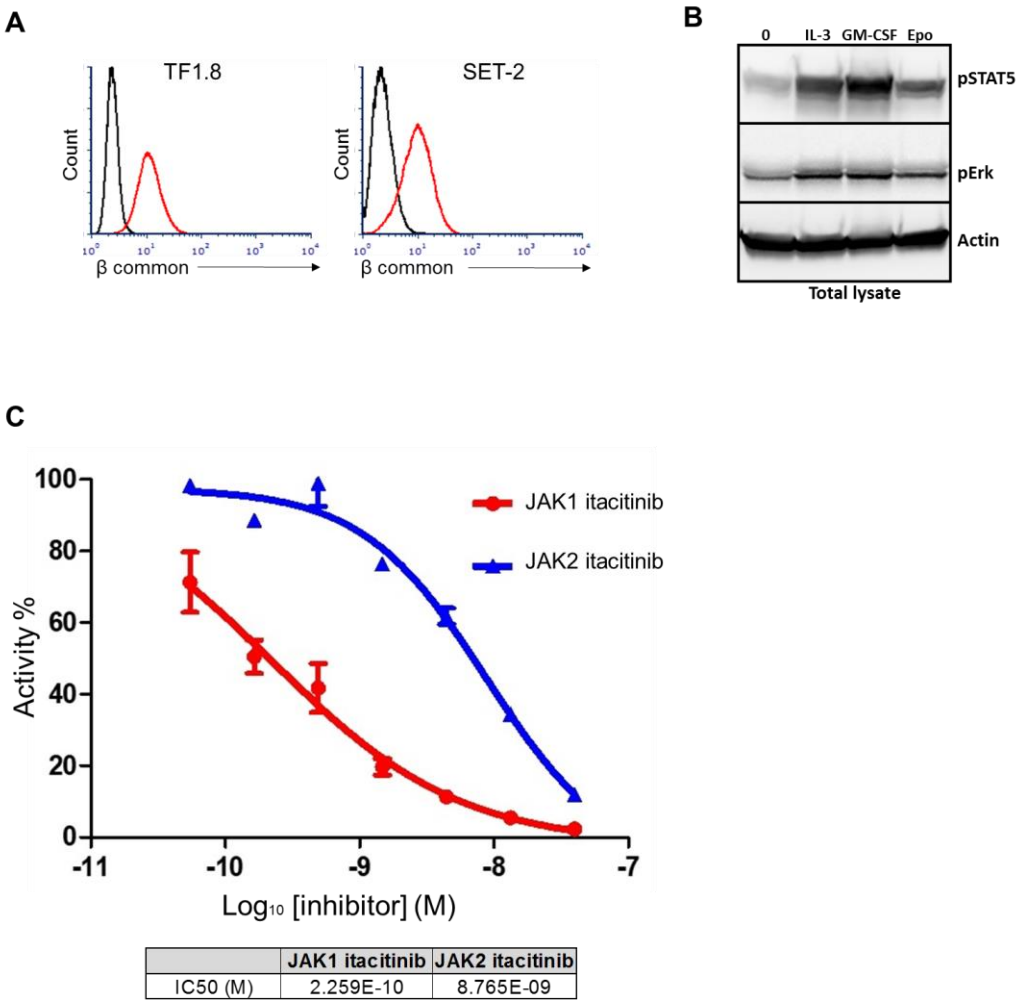


Fig. S3. Type I JAK2 inhibitor protects JAK2 from degradation and down-regulation. (**A**) TF1.8 cells were starved overnight in the presence of 0.5% FCS, pre-incubated with either vehicle or 280 nM of ruxolitinib for 10 minutes and stimulated with different doses of IL-3 for 5 minutes. Cells were lysed and subjected to immunoprecipitation with p-JAK2 antibody followed by immunoblotting with JAK2 antibody. As a control, total lysates from the same experiment were immunoblotted with p-JAK2 and JAK2 antibodies. (**B**) Recombinant JAK2 kinase domain was mixed with recombinant PTP1B and either no inhibitor or Type I JAK inhibitor CMP6 in phosphatase assay buffer. Phosphatase reactions were incubated at room temperature for 0, 2 and 5 hours, fractionated by SDS-PAGE and immunoblotted with p-JAK2 or JAK2 antibodies. (**C**) Recombinant JAK2 kinase domain and gp130 were mixed with recombinant PTP1B and either no inhibitor or ruxolitinib in phosphatase assay buffer. Phosphatase reactions were incubated at room temperature for 0, 2, 60, 120, 180 and 240 minutes, fractionated by SDS-PAGE and immunoblotted with p-JAK2 or anti-phospho-tyrosine (4G10) antibodies. Coomassie staining was used as a loading control. (**D**) TF1.8 cells were starved overnight in the presence of 0.5% FCS, pre-incubated for 10 minutes with MG132 plus either vehicle or ruxolitinib and stimulated with 25 ng/ml of IL-3 for 0, 5 and 10 minutes. Cells were lysed and subjected to precipitation with TUBE-agarose followed by immunoblotting with JAK2, JAK1 and TYK antibodies.

Additionally, whole cell lysates were immunoblotted with actin antibody. (**E**) TF1.8 cells were starved overnight in the presence of 0.5% FCS, pre-incubated with either vehicle, 10 uM MG132 or 280 nM ruxolitinib for 10 minutes and stimulated with 25 ng/ml of IL-3 for 0, 5, 15 and 30 minutes. Whole cell lysates were prepared and immunoblotted with p-JAK2, p-STAT5, JAK2 and STAT5 antibodies. (**F**) Mononuclear cells from myelofibrosis patient RAH2 were cultured in 10% FCS with 1 ng/ml EPO and IL-3 and 280 nM of ruxolitinib or DMSO for 12 hours. Cells were then washed in cold RPMI and cultured in MG132 without additives for 5 or 15 minutes. Cells were lysed and subjected to immunoprecipitation with JAK2 antibody followed by immunoblotting with Ub-HRP or JAK2 antibody



	JAK1 itacitinib	JAK2 itacitinib
IC50 (M)	2.259E-10	8.765E-09

Fig. S4. Selective targeting of JAK1 kinase by itacitinib. (**A**) Flow cytometric analysis of TF1.8 and SET-2 cells with βc antibody (red line) and an isotype control (black line). (**B**) SET-2 cells were starved overnight in 0.5% FCS and stimulated for 5 minutes with nil, 250 ng/ml IL-3, 25 ng/ml GM-CSF or 25 ng/ml Epo. Whole cell lysates were prepared and immunoblotted with p-STAT5, p-Erk and Actin antibodies. (**C**) *In vitro* kinase assay to determine the JAK1 IC_{50} for itacitinib. JAK2 was included to determine specificity of Itacitinib towards JAK1

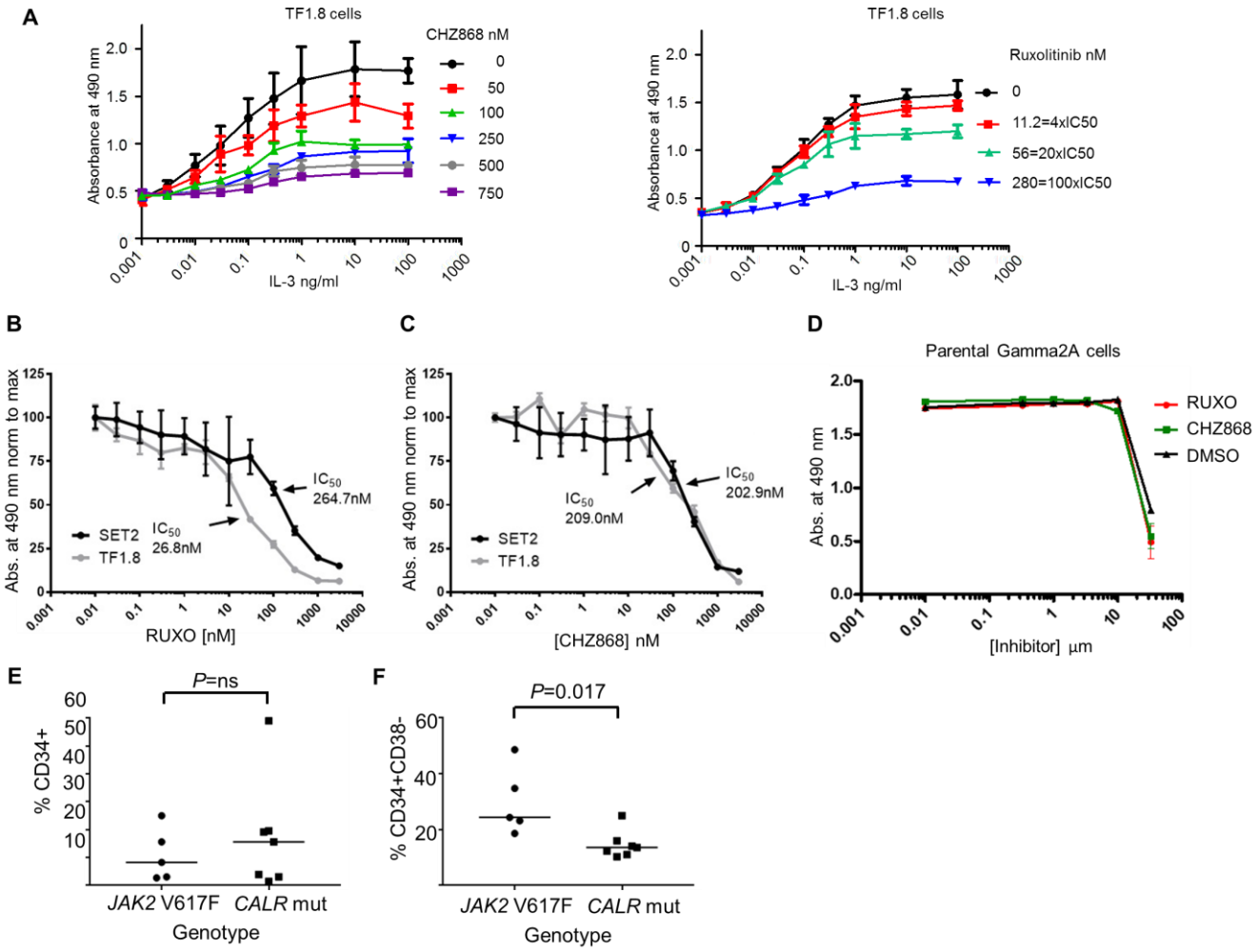


Fig. S5. Type II JAK2 inhibitor CHZ868 blocks proliferation of cells expressing wild-type JAK2 and JAK2^{V617F}. (**A**) TF1.8 cells starved of GM-CSF overnight, were incubated in 10% FCS with titrations of IL-3 in the presence of different doses of ruxolitinib or CHZ868. After 48 hours, cell proliferation was assessed using CellTiter 96 reagent. Data shows the mean of triplicate measurements +/- SD from a representative experiment. (n=3). (**B, C**) Starved TF1.8 cells and SET-2 cells were incubated in 50 ng/ml IL3 and 10% FCS with titrations of ruxolitinib (**B**) or CHZ868 (**C**) in triplicate. After 48 hours, cell proliferation was assessed using CellTiter 96 reagent. Data is expressed as absorbance normalized to maximum proliferation in the presence of DMSO alone and shows the mean of triplicate measurements +/- SD from a representative experiment. Two additional biological replicates were generated in order to obtain mean +/- SEM IC_{50} values (n=3) as depicted in Fig 4C. IC_{50} values were determined using Graph Pad Prism. (**D**) Parental γ2A cells were plated in DMEM media containing 10% FCS and different doses of DMSO, ruxolitinib or CHZ868. After 48 hours, cell proliferation was assessed using CellTiter 96 reagent. Data shows the mean of triplicate measurements +/- SD from a representative experiment from three biological replicates. (**E**) The percentage of CD34+ cells detectable in the mononuclear population of peripheral blood samples

collected from patients with myelofibrosis comparing *JAK2* mutant positive samples with *CALR* mutant samples. Bars represent the median, *P*=0.6 was determined using the Mann-Whitney U test. (**F**) Percent of CD34+CD38- stem/progenitor cells within the CD34+ population in peripheral blood from myelofibrosis patients comparing patients with *JAK2* vs *CALR* mutations. Bars represent the median, *P*=0.017 was determined using the Mann-Whitney U test.

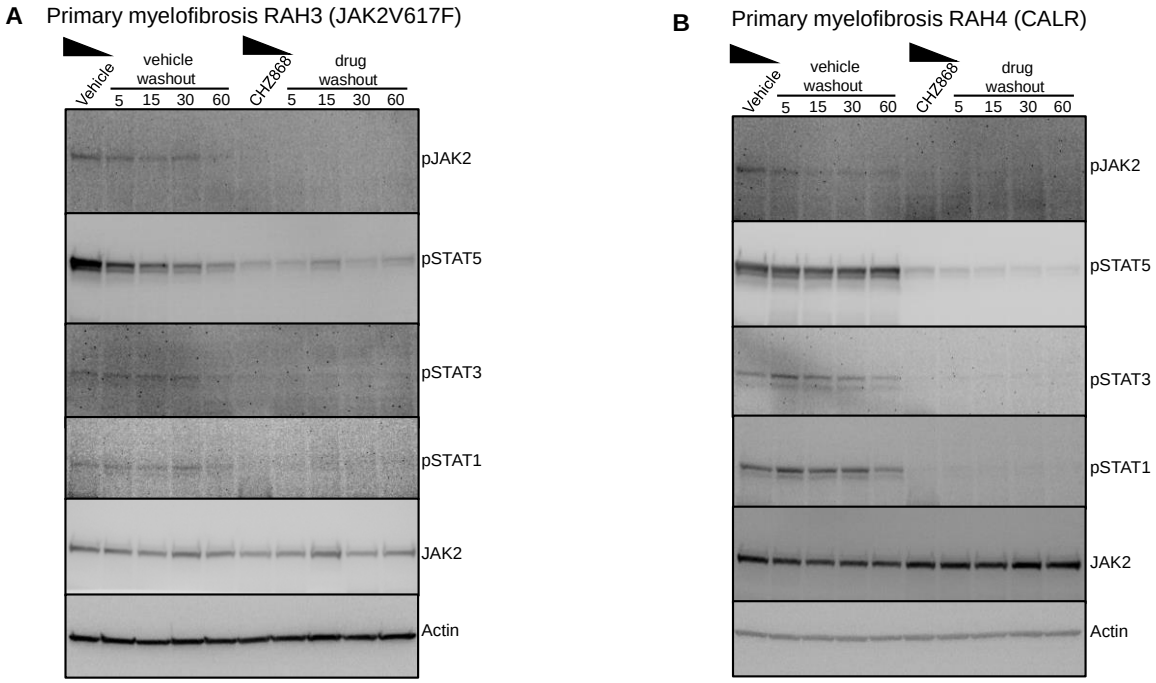


Fig. S6. Type II inhibitor withdrawal does not trigger intracellular signaling. Whole cell lysate from myelofibrosis patient RAH3 carrying a JAK2^{V617F} mutation (**A**) or patient RAH4 carrying a calreticulin mutation (**B**) were immunobloted for p-JAK2, p-STAT5, p-STAT3 and p-STAT1 following withdrawal of CHZ868 as described in Figure 5A.