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Supplemental Methods

Immunoblotting

Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors. Protein concentrations of clarified cell lysates were determined by Pierce™ BCA Protein Assay kit (Thermo Scientific) and a Benchmark Plus Microplate Spectrophotometer (Bio-Rad). Following SDS-PAGE, proteins were transferred to PVDF membrane and immunoblots were performed using the following antibodies: P-BAD (S112) (#9296); hu-cl-PARP (#5625); pp70S6K (T389) (#9234); P-S6 (S235/236) (#4858); S6 (#2217); P-4EBP1 (T37/46) (#2855); PIM2 (#4730); PIM3 (#4165); pPRAS40 (T246) (#2997); pAKT (S473) (#9271); p70S6K (#9202); 4EBP1 (#9644); and GAPDH (#5174) (Cell Signaling Technology); vinculin (sc-73614) and PIM1(sc-13513) (Santa Cruz Biotechnology); pSTAT5 (Y694) (611964) (BD Biosciences); and horseradish peroxidase-conjugated secondary antibodies (Thermo Scientific). Blots were developed using chemiluminescence (Thermo Scientific and Amersham).

Calculation of synergy

Synergy was assessed using the Bliss model of independence, where the expected additive % growth inhibition (% GI_{add}) of the combination of drug 1 and drug 2 was determined by the formula: % $GI_{add} = \%GI_1 + \%GI_2 - (\%GI_1)(\%GI_2)/100$, where $\%GI_1$ and $\%GI_2$ are the observed percent growth inhibition of individual drugs alone. If the observed growth inhibition of the combination of drug 1 and drug 2 is greater than % GI_{add} then the combination of drugs at the concentrations used is considered synergistic.[1]

Ex vivo colony forming assays

Blood was treated with HetaSep™ (STEMCELL Technologies, Inc.) to remove the major portion of red blood cells. Peripheral blood mononuclear cells (PBMCs) were obtained by ficoll gradient separation. PBMCs (0.12×10^5 to 4×10^5) were plated in 1.1 mL of methylcellulose medium containing rhSCF, rhIL-3, and rhGM-CSF (MethoCult™ #H4534; STEMCELL Technologies, Inc.). The final DMSO concentration in all plated samples was 0.1%. Cells were incubated at 37°C with 5% CO_2 and colonies were enumerated after 12–14 days. Samples were plated in duplicate for each treatment and the average of these were used to calculate % of control (DMSO). Graphical representation of data and statistical analyses were obtained utilizing Prism (GraphPad Software, Inc., San Diego, CA).

Histology

After sternum decalcification and paraffin embedding, bone marrow slices were prepared and stained with H&E and for reticulin (Chandler's Precision Reticulum Stain, American MasterTech). Reticulin fibrosis scoring was performed by a hematopathologist according to the European consensus grading scale.[2] Images were captured using an Olympus BX51 microscope with 20X or 60X objectives (200X and 600X total magnification) at room temperature using a SPOT Insight camera and SPOT 5.1 imaging software (SPOT Imaging, a Division of Diagnostic Instruments, Inc., Sterling Heights, MI).

Ruxolitinib persistent cells

Ruxolitinib persistent cells were previously described.[3]

Supplemental Methods (continued)

References

1. Foucquier J, Guedj M. Analysis of drug combinations: current methodological landscape. Pharmacol Res Perspect. 2015;3(3):e00149.

2. Thiele J, Kvasnicka HM, Facchetti F, Franco V, van der Walt J, Orazi A. European consensus on grading bone marrow fibrosis and assessment of cellularity. Haematologica. 2005;90(8):1128-1132.

3. Mazzacurati L, Lambert QT, Pradhan A, Griner LN, Huszar D, Reuther GW. The PIM inhibitor AZD1208 synergizes with ruxolitinib to induce apoptosis of ruxolitinib sensitive and resistant JAK2-V617F-driven cells and inhibit colony formation of primary MPN cells. Oncotarget. 2015;6(37):40141-40157.

Supplemental Table 1

MPN patient information of primary samples used in this study.

MPN Sample #	Diagnosis	Sex	Age at time of blood draw	JAK2-V617F	Ongoing treatment at time of collection
1	ET	Female	45	positive	hydroxyurea and aspirin
2	PV	Male	78	positive	anagrelide and ruxolitinib
3	PV	Male	55	positive	aspirin
4	PV	Female	66	positive	hydroxyurea and aspirin
5	PMF	Male	82	positive	ruxolitinib
6	PV	Male	56	positive	aspirin
7	PV	Male	36	positive	aspirin
8	PV	Male	64	positive	hydroxyurea and aspirin

Note: sample #3 and #6 are from the same patient but obtained 6 months apart.

Supplemental Figure 1

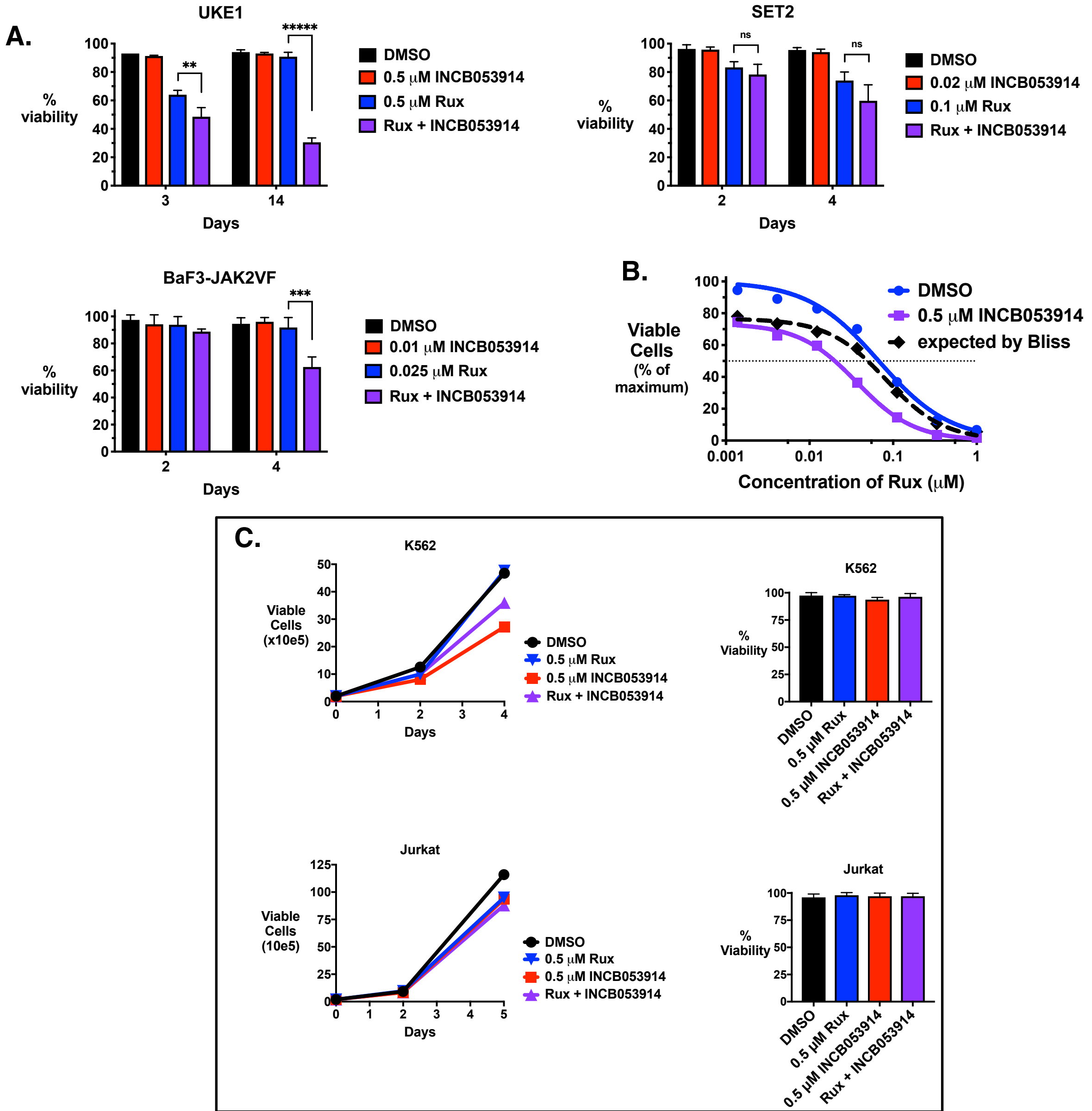

Figure S1. INCB053914 in combination with ruxolitinib reduces cell viability in MPN model cells but does not affect growth or viability of K562 or Jurkat cells. (A) UKE1, SET2, and BaF3-JAK2VF cells were cultured with DMSO (0.1%) or the indicated concentrations of INCB053914 and ruxolitinib, alone and together, and % cell viability (+/- SD) was determined by trypan blue exclusion (** = $P < 0.01$, *** = $P < 0.001$, and ***** = $P < 0.000001$ by unpaired *t* test, ns = not significant)). (B) SET2 cells were incubated with a range of ruxolitinib in the absence (DMSO, blue) or presence of 0.5 μM INCB053914 (purple) and the % of relative viable cells present after 72 hr was determined using CellTiter-Glo®. The expected additive % of relative viable cells at each ruxolitinib concentration in the presence of INCB053914 was determined by the Bliss model of independence and plotted as the black dashed line. % of relative viable cells lower than this dashed line indicates synergy. (C) K562 (top) and Jurkat (bottom) cells were cultured with DMSO (0.1%) (black), 0.5 μM ruxolitinib (blue), 0.5 μM INCB053914 (red), and both drugs together (purple) and viable cell numbers (left) and percent cell viability (+/- SD) (right) (at Day 4 for K562 and at Day 5 for Jurkat) were determined by trypan blue exclusion.

Supplemental Figure 2

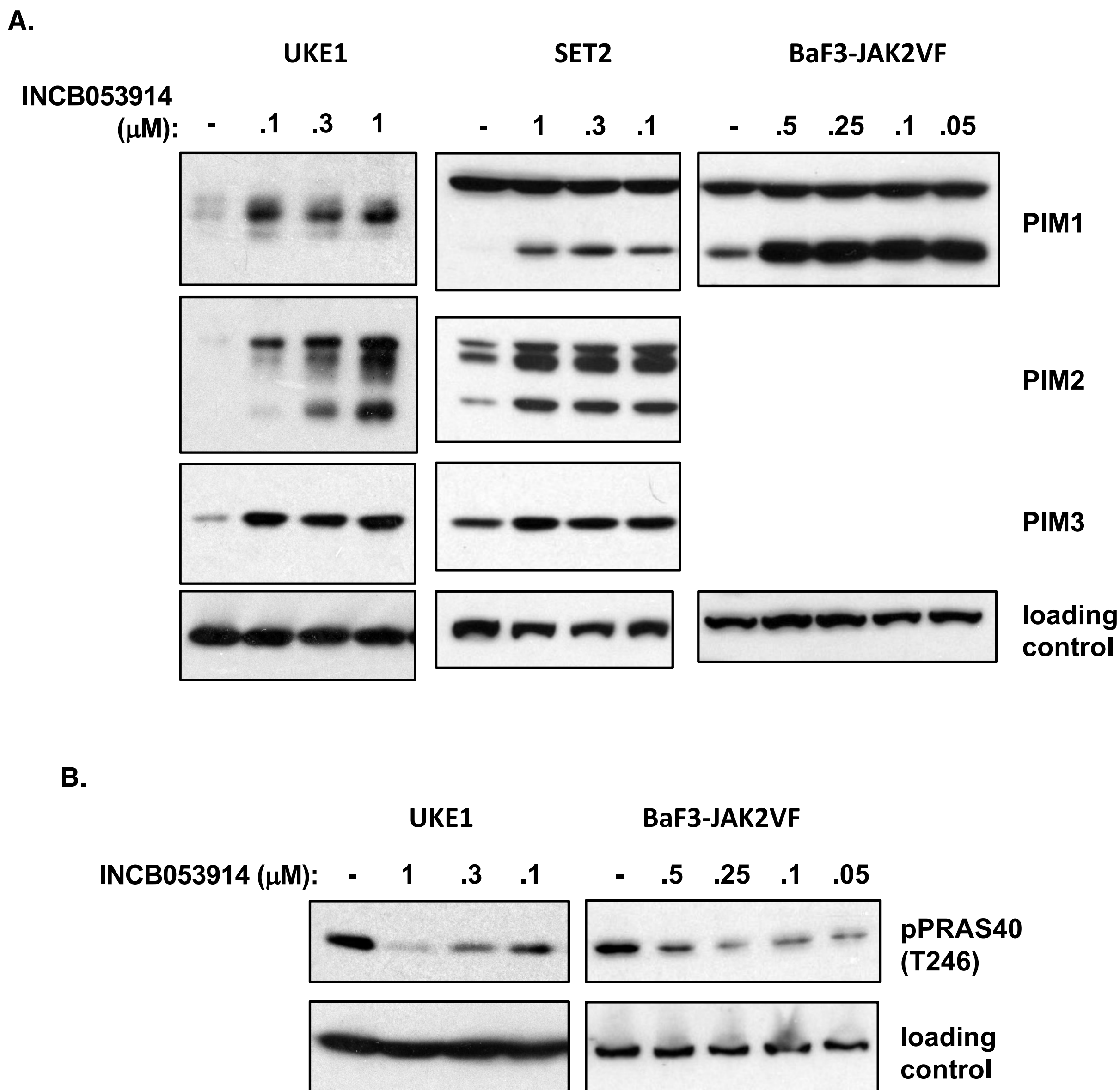

Figure S2. PIM stabilization and inhibition of phospho-PRAS40 indicate on-target inhibition of PIM family members. Cell lysates of UKE1, SET2, and BAF3-JAK2VF cells incubated with the indicated concentrations of INCB053914 for 4 hr were immunoblotted for PIM1, PIM2, and PIM3, as well as GAPDH (for UKE1) and vinculin (for SET2 and BaF3-JAK2VF) as loading controls (A), and pPRAS40-T246 and GAPDH (for UKE1) and vinculin (for BaF3-JAK2VF) as loading controls (B). PIM2 and PIM3 were not detected in BaF3-JAK2VF cells.

Supplemental Figure 3

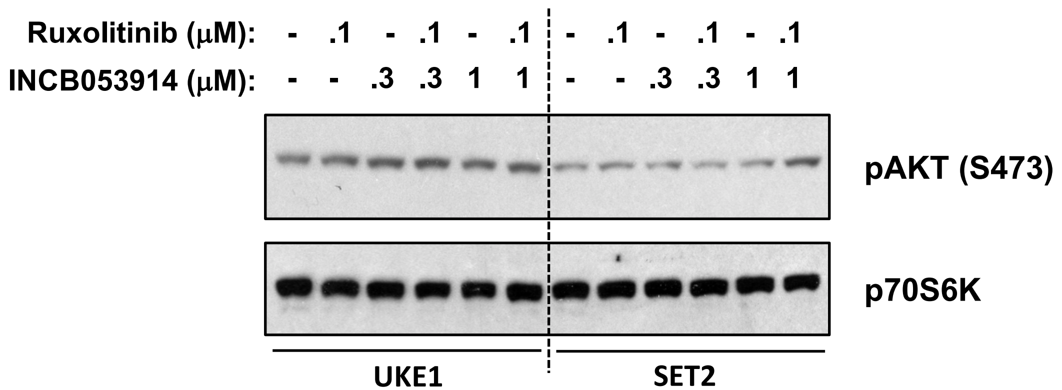

Figure S3. INCB053914 and ruxolitinib do not affect phospho-AKT levels at the same concentrations that demonstrate suppression of markers of mTORC1 signaling. Cell lysates of UKE1 and SET2 cells incubated with the indicated concentrations of INCB053914 and ruxolitinib, alone and in combination, for 4 hr were immunoblotted to see effects on pAKT (S473) levels. A p70S6K immunoblot is shown as a loading control.

Supplemental Figure 4

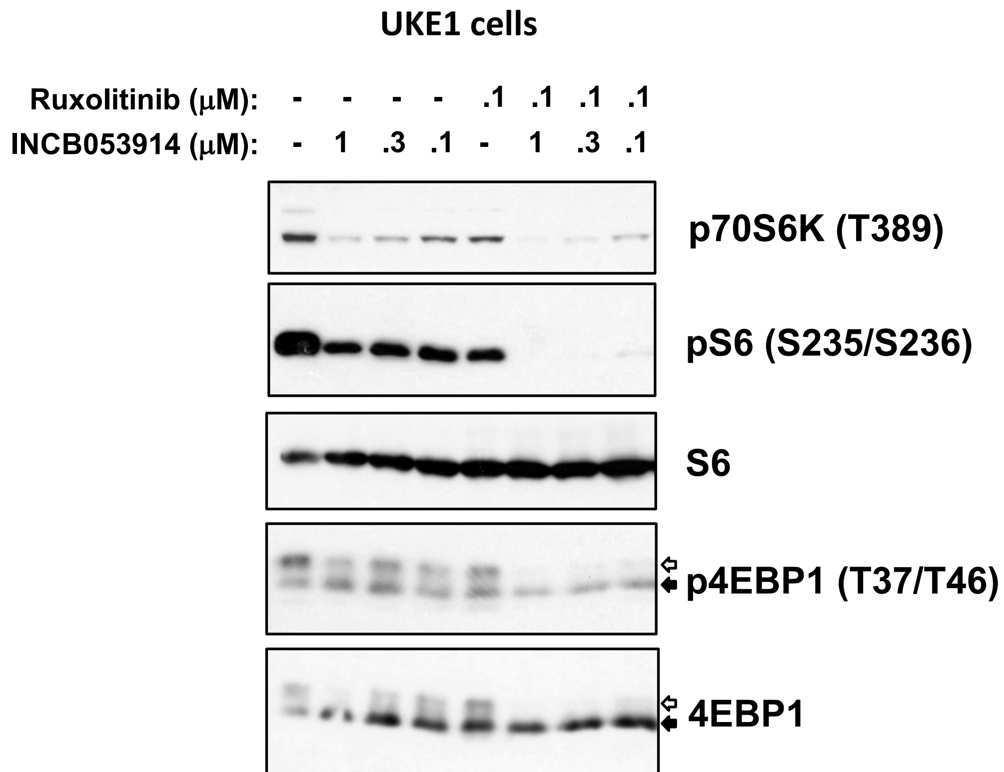

Figure S4. INCB053914 and ruxolitinib in combination synergistically suppress markers of mTORC1 signaling in UKE1 cells. Cell lysates of UKE1 cells incubated with INCB053914 and ruxolitinib, alone and in combination, at the indicated concentrations for 4 hr were immunoblotted for pS6 (S235/S236), S6, p4EBP1 (T37/T46), and 4EBP1. The open and closed arrows indicate the migration of hyper-phosphorylated and hypo-phosphorylated 4EBP1 protein, respectively.

Supplemental Figure 5

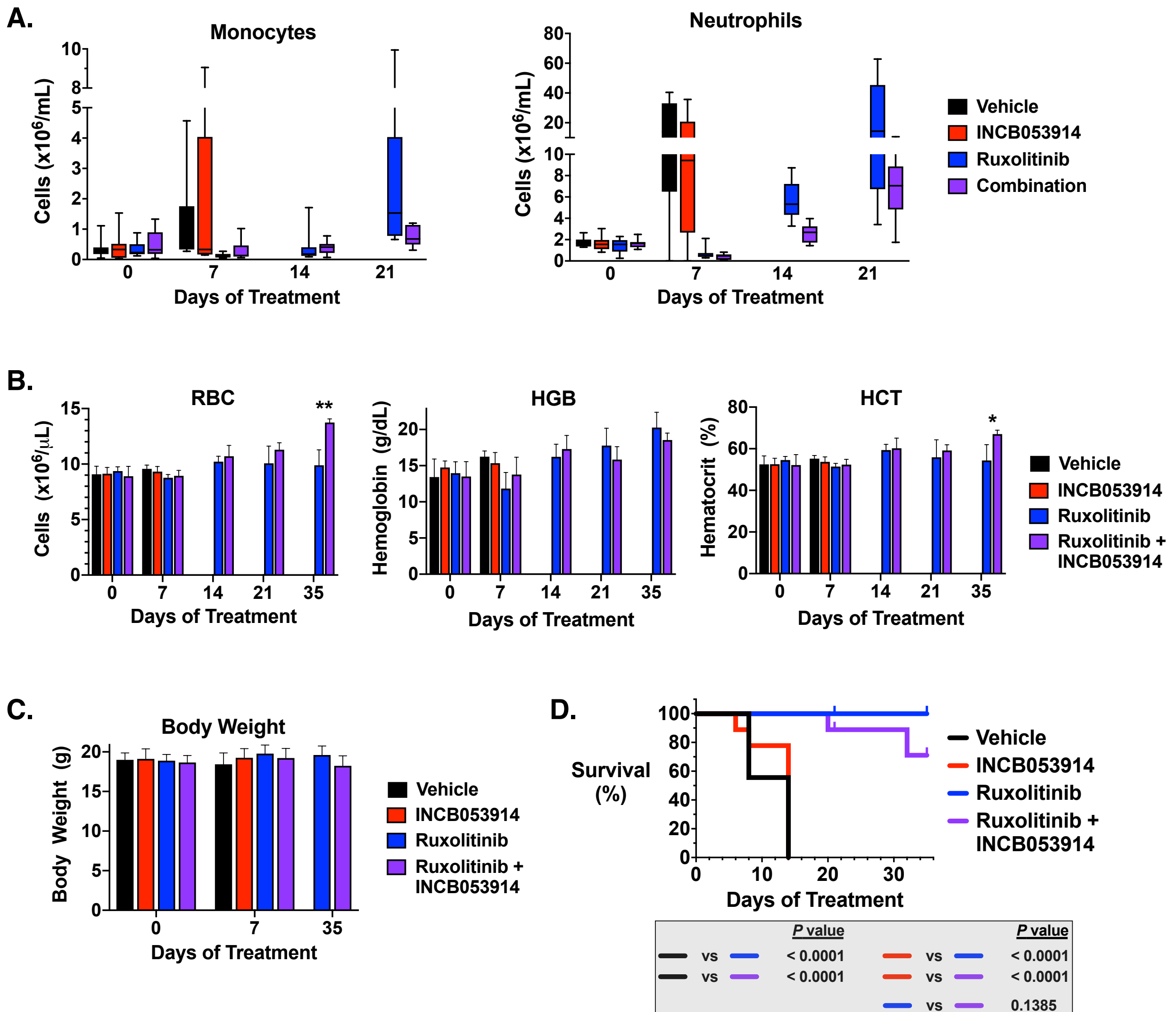

Figure S5. INCB053914 antagonizes the development of MPN that persists during ruxolitinib treatment of a murine MPN model. This figure provides additional data, obtained following drug treatment of mice bearing an MPN induced by MPL-W515L, that supplements Figure 6. Shown are: (A) monocyte and neutrophil counts, (B) red blood cell (RBC), hemoglobin (HGB), and hematocrit (HCT) levels, and (C) body weights of mice following the indicated days of treatment with vehicle (black), INCB053914 (100 mg/kg) (red), ruxolitinib (60 mg/kg) (blue), or the combination of INCB053914 and ruxolitinib (purple) twice daily orally. In the box and whisker plots, boxes indicate 25th to 75th percentile, whiskers indicate the range, and the horizontal line indicates the median. In the bar graphs error bars indicate SD. (* = $P < 0.05$, ** = $P < 0.01$, by unpaired *t* test) (D) A Kaplan-Meier plot of survival is shown. Three healthy appearing animals each from the ruxolitinib and combination groups were sacrificed for analysis on day 21 of treatment. The experiment was stopped after 35 days when all remaining animals were euthanized. Log-rank (Mantel Cox) test indicated no statistical difference between the ruxolitinib and combination groups while each of these groups were significantly different than vehicle and INCB053914 mono-therapy groups.

Supplemental Figure 6

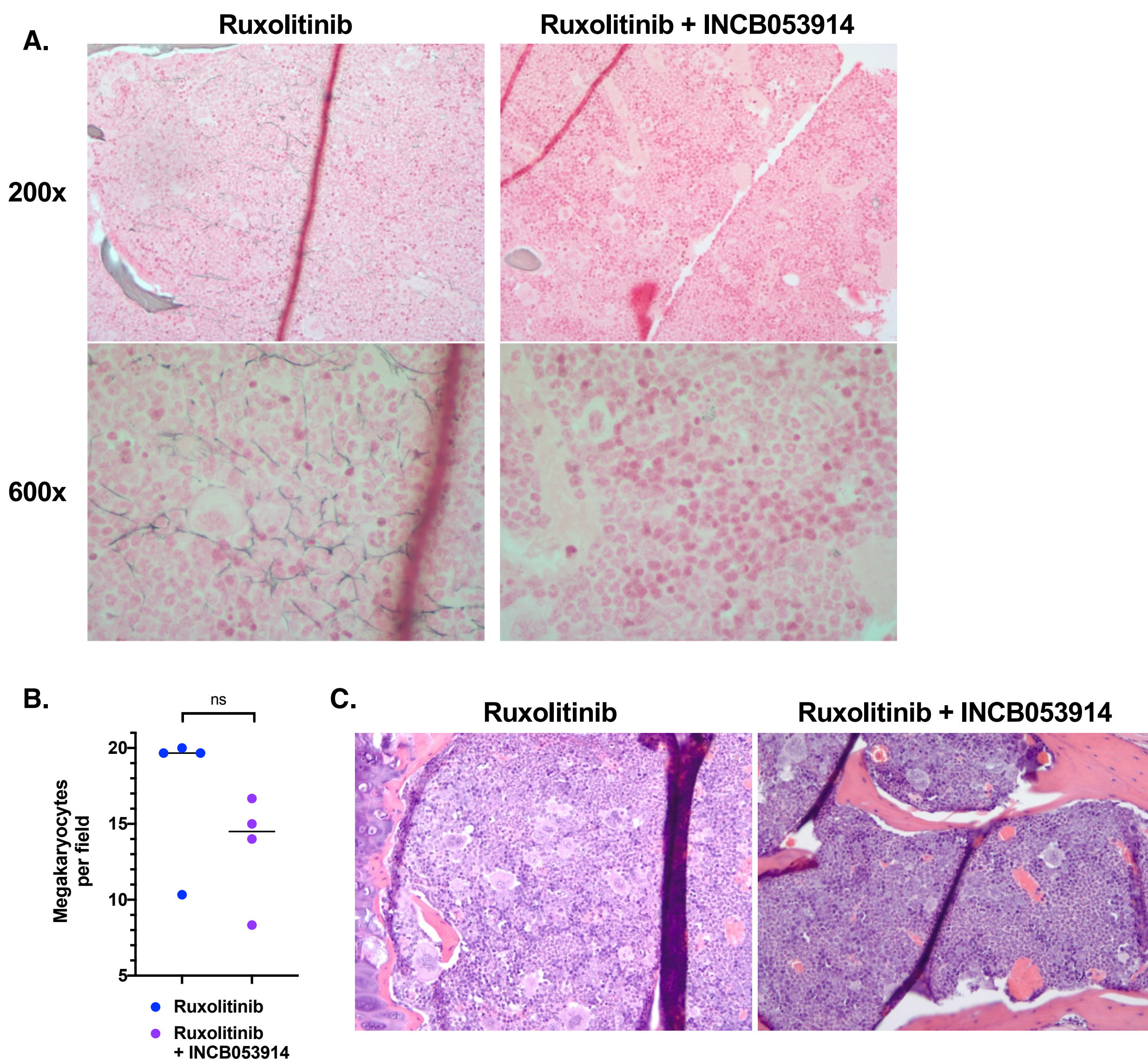

Figure S6. Addition of INCB053914 to ruxolitinib therapy reduces reticulin staining and slightly reduces megakaryocyte numbers compared to ruxolitinib-treated animals in a murine MPN model. This figure provides additional data, obtained following drug treatment of mice bearing an MPN induced by MPL-W515L, that supplements Figure 6. (A) Representative fields of reticulin staining of bone marrow obtained from ruxolitinib (overall MF score = 0.56) and ruxolitinib plus INCB053914 combination therapy (overall MF score = 0.04) treated mice are shown. (B) Megakaryocyte counts from H&E stains of bone marrow obtained from ruxolitinib and ruxolitinib plus INCB053914 combination therapy treated mice are shown. Dots represent averages of three fields (n=4 mice per group), the bar indicates the median. (C) Representative fields of bone marrow (H&E, 200x) obtained from ruxolitinib and ruxolitinib plus INCB053914 combination therapy treated mice are shown. Images were captured using an Olympus BX51 microscope with 20X or 60X objectives (200X and 600X total magnification) at room temperature using a SPOT Insight camera and SPOT 5.1 imaging software (SPOT Imaging, a Division of Diagnostic Instruments, Inc., Sterling Heights, MI).

Supplemental Figure 7

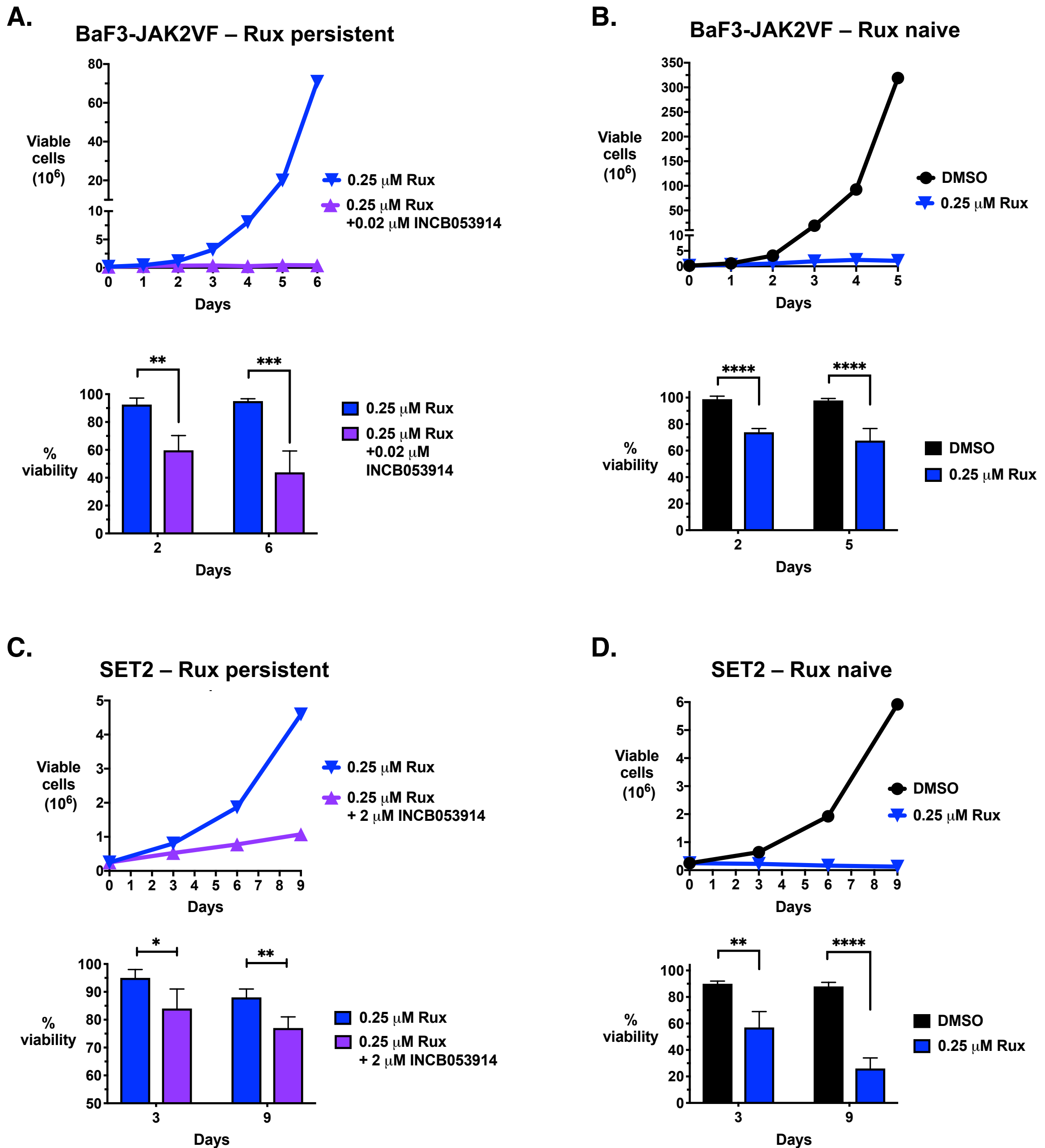

Figure S7. INCB053914 antagonizes the growth of ruxolitinib persistent MPN model cell lines. The effect of INCB053914 on the growth and viability of ruxolitinib-persistent BaF3-JAK2VF (A) and SET2 (C) cells are shown. The effect of the same concentrations of ruxolitinib (Rux) which the ruxolitinib-persistent cells proliferate in on ruxolitinib naïve BaF3-JAK2VF (B) and SET2 (D) cells are also shown, demonstrating the ruxolitinib persistent nature of the cells in (A) and (C). BaF3-JAK2VF cells and SET2 cells were initially plated at $2x10^5$ and $2.5x10^5$ per mL, respectively. Cells were counted by trypan blue exclusion and passed into fresh medium and drug as needed. (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, and **** = $P < 0.0001$ by unpaired t test).