

MYELOID NEOPLASIA

Improved targeting of JAK2 leads to increased therapeutic efficacy in myeloproliferative neoplasms

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Key Points

- Genetic deletion of JAK2 in vivo shows that MPN cells remain fully dependent on JAK2 signaling for survival.
- Dual JAK2 targeting with JAK and HSP90 inhibitors offers increased efficacy in murine models and primary samples.

The discovery of *JAK2/MPL* mutations in patients with myeloproliferative neoplasms (MPN) led to clinical development of Janus kinase (JAK) inhibitors for treatment of MPN. These inhibitors improve constitutional symptoms and splenomegaly but do not significantly reduce mutant allele burden in patients. We recently showed that chronic exposure to JAK inhibitors results in inhibitor persistence via JAK2 transactivation and persistent JAK–signal transducer and activator of transcription signaling. We performed genetic and pharmacologic studies to determine whether improved JAK2 inhibition would show increased efficacy in MPN models and primary samples. *Jak2* deletion in vivo led to profound reduction in disease burden not seen with JAK inhibitors, and deletion of *Jak2* following chronic ruxolitinib therapy markedly reduced mutant allele burden. This demonstrates that JAK2 remains an essential target in MPN cells that survive in the setting of chronic JAK inhibition. Combination therapy with the heat shock protein 90 (HSP90) inhibitor PU-H71 and ruxolitinib reduced total and phospho-JAK2 and achieved more potent inhibition of downstream signaling than ruxolitinib monotherapy. Combination treatment improved blood counts, spleen weights, and reduced bone marrow fibrosis compared with ruxolitinib alone. These data suggest alternate approaches that increase JAK2 targeting, including combination JAK/HSP90 inhibitor therapy, are warranted in the clinical setting. (*Blood*. 2014;123(13):2075-2083)

Introduction

Myeloproliferative neoplasms (MPNs) are chronic myeloid malignancies characterized by the clonal expansion of myeloid lineage cells. The classical MPN include chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). The majority of patients with PV, ET, and PMF harbor a highly conserved somatic mutation in the tyrosine kinase *JAK2* (*JAK2V617F*).¹⁻⁴ In addition, *JAK2* exon 12 mutations are observed in *JAK2V617F*-negative PV⁵ and mutations in the thrombopoietin receptor (*MPLW515L/K/A*) are observed in *JAK2V617F*-negative ET/PMF.⁶ These mutations result in constitutive activation of the Janus kinase–signal transducer and activator of transcription (JAK-STAT) signaling pathway and their identification led to the clinical development of JAK kinase inhibitors for MPN patients.

In 2011, the dual JAK1/JAK2 inhibitor INCB18424 (ruxolitinib; Jakafi) was approved for the treatment of myelofibrosis (MF) based on the ability of ruxolitinib to markedly reduce splenomegaly and ameliorate MF-associated symptoms. Despite the important clinical benefits of ruxolitinib therapy, JAK inhibition with ruxolitinib and

with other JAK inhibitors does not substantively reduce mutant allele burden as is observed with BCR-ABL kinase inhibition in CML.⁷ Similar results have been observed in murine models of MPN driven by activated *Jak2/Mpl* mutations, in which JAK inhibition leads to improved blood counts and splenomegaly but does not reduce mutant allele burden.^{8,9} The limited efficacy of JAK inhibitors in vivo might be due to incomplete pathway inhibition at clinically tolerable doses, the presence of other disease alleles, or incomplete dependence on JAK2 by the MPN clone.

We recently demonstrated that chronic exposure of MPN cells to ruxolitinib leads to the development of disease persistence and reduced sensitivity to JAK inhibition.¹⁰ We observed JAK inhibitor persistence (JAK^{Per}) in MPN cell lines, mouse models, and primary samples from patients treated with ruxolitinib. Of note, MPN cells that are resistant to ruxolitinib were also insensitive to other JAK inhibitors including JAK inhibitor I and TG101348, and persistence was not associated with acquisition of secondary mutations in *JAK2*. In JAK^{Per} cells, we found that JAK2 is stabilized by type I JAK inhibitors, which facilitates heterodimeric association of JAK2 with

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JAK1 and TYK2, JAK2 transactivation, and reactivation of JAK-STAT signaling. Although in vitro mutagenesis screens have identified a small set of JAK inhibitor resistance alleles in *JAK2*,^{11,12} to date, second-site mutations in *JAK2* have not been identified in patients, consistent with incomplete inhibition of JAK-STAT signaling with existing JAK inhibitors.

These data have led investigators to question whether JAK2 represents an essential therapeutic target in MPN, and has led to diminished expectations of JAK-targeted therapy in MPN patients. We therefore sought to investigate whether JAK2 is a critical target in MPN in vivo using genetic studies, and sought to develop a therapeutic approach that improves JAK2 inhibition in vivo and increases therapeutic efficacy. Here we show that JAK2 is critically required for disease pathogenesis, both for initiation and maintenance of disease. Furthermore, we show that genetic deletion of *Jak2* can overcome JAK^{Per} in vivo. We have previously demonstrated that JAK2 is a heat shock protein 90 (HSP90) client protein and JAK^{Per} cell lines remain sensitive to PU-H71, an HSP90 inhibitor.^{10,13} Based on these genetic and pharmacologic data, we investigated the efficacy of combined JAK2 and HSP90 inhibitors in preclinical MPN models such that we can inform the clinical development of improved treatment regimens for MPN patients.

Methods

Murine models and analysis of mice

Jak2^{fl/fl} mice were a kind gift from Kay-Uwe Wagner (University of Nebraska Medical Center, Omaha, NE).¹⁴ They were backcrossed into C57BL/6 for 7 generations and then crossed to C57BL/6 Mx1-Cre mice. For *Jak2* deletion studies, bone marrow (BM) cells from CD45.2 *JAK2^{fl/fl}* Mx1-Cre positive and negative mice were enriched using CD117 microbeads from Miltenyi Biotec and transduced with viral supernatants containing MSCV-*hMPLW515L*-green fluorescent protein (GFP). One million transduced cells along with 500 000 CD45.1 CD117-positive BM cells were tail vein injected into CD45.1 recipients and bled 14 to 21 days after transplantation to assess engraftment and chimerism. For initiation experiments, mice received 4 intraperitoneal (IP) doses of 100 μ L of polyI:polyC (pI:pC; 1 mg/mL) every other day starting at 14 days post-tail vein injection. For maintenance experiments, mice received pI:pC injections starting 19 days following tail vein injection. All mice were sacrificed 3 months after tail vein injection for histologic analysis and flow cytometry. For ruxolitinib experiments, mice were randomized to receive vehicle (20% Captisol in 58 mM citrate buffer, weight/volume), 60 mg/kg ruxolitinib twice daily by oral gavage or pI:pC by IP injection at day 18 after tail vein injection. All mice were euthanized 6 weeks later for further analysis.

For ruxolitinib and PU-H71 combination studies, BM cells were isolated from fluorouracil (5-FU)-treated Balb/C donor mice, transduced with *hMPLW515L-IRES-GFP* retrovirus and injected into lethally irradiated Balb/C recipients. Fourteen days following injection, mice were randomized to receive vehicle, 30 mg/kg ruxolitinib twice daily by oral gavage, 90 mg/kg ruxolitinib twice daily by oral gavage, and 30 mg/kg ruxolitinib with 75 mg/kg PU-H71 thrice weekly by IP injection. All mice were bled at day 14 following start of treatment. Two mice from each arm were euthanized for further analysis. At the 2-week time point, a subset of mice receiving 30 mg/kg ruxolitinib alone also started receiving 75 mg/kg PU-H71. Also, the ruxolitinib dose was increased to 90 mg/kg in a subset of mice receiving combination treatment. At 4 weeks from the start of drug treatment, all mice were euthanized for further analysis. Ruxolitinib was synthesized by the Bradner laboratory at the Dana-Farber Cancer Institute and PU-H71 was synthesized by the Chiosis laboratory at Memorial Sloan-Kettering Cancer Center as previously reported.^{15,16}

Animal care was in strict compliance with institutional guidelines established by the Memorial Sloan-Kettering Cancer Center, the Guide for the

Care and Use of Laboratory Animals, and the Association for Assessment and Accreditation of Laboratory Animal Care International. For histopathology, tissues were fixed in 4% paraformaldehyde and then embedded in paraffin for analysis. Tissue samples were stained using hematoxylin and eosin as well as a Gordon and Sweet stain for reticulin fibers (ammoniacal silver procedure).

Flow cytometry analysis

BM and spleen cells were filtered, and red blood cells were lysed and washed in phosphate-buffered saline (PBS). Cells were incubated with the following antibodies for 30 minutes on ice in PBS plus 2% bovine serum albumin (BSA). For staining of myeloid progenitors, the antibodies used were CD11b, Gr-1, Ter119, CD3, CD4, NK1.1, B220, CD19 conjugated to allophycocyanin Cy7 (APCCy7) (Biolegend), c-kit-phycoerythrin (PE) (Biolegend), Scal-PECy7 (BD Pharmingen), CD16/32-eFluor 450 (eBioscience), CD34-Alexa Fluor 660 (eBioscience). For chimerism and mature leukocyte staining, the antibodies used were CD45.1-eFluor 450 (eBioscience), CD45.2-APC, CD11b-PECy7, Gr1-PE (Biolegend). Data were collected on LSRFortessa (BD Biosciences) and analysis was performed on FlowJo.

Immunoblotting for patient samples

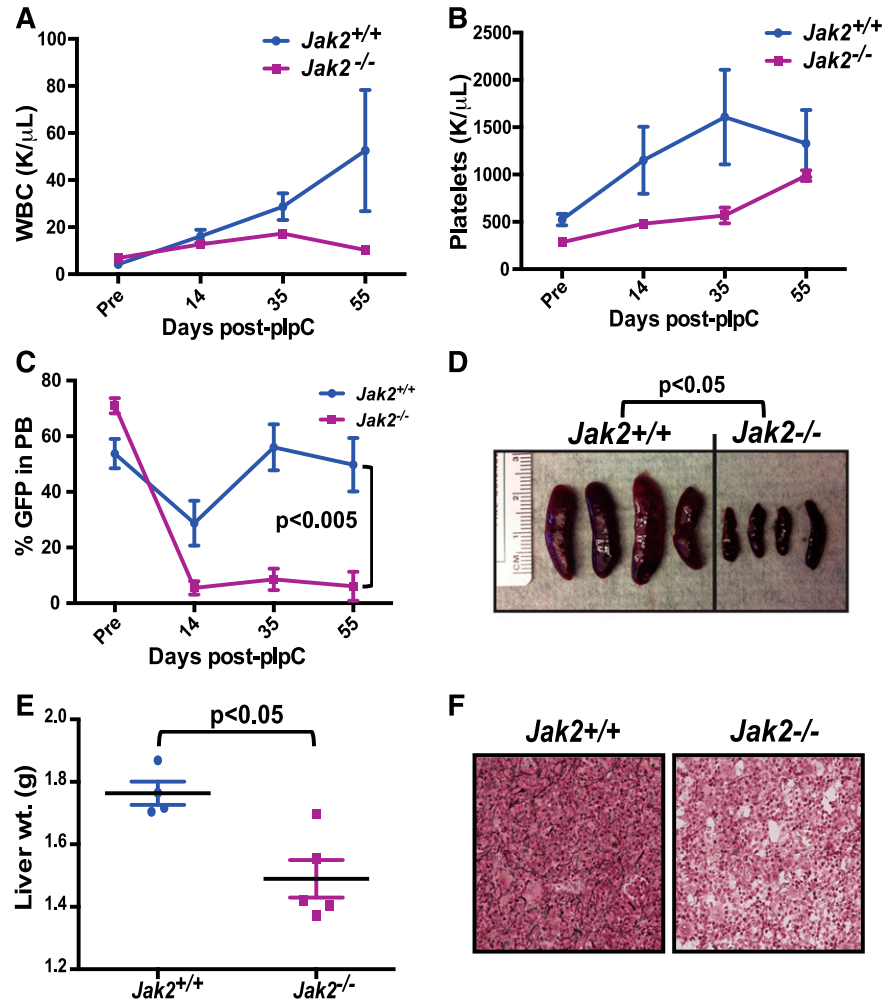
The institutional review boards of Memorial Sloan-Kettering Cancer Center approved sample collection and all experiments. Informed consent was obtained from human subjects before study. The study was conducted in accordance with the Declaration of Helsinki. Mononuclear cells were freshly extracted using Ficoll separation from peripheral blood (PB) and used for studies. Cells were treated with 500 nM PU-H71 for 16 hours, washed in ice-cold PBS, and collected in lysis buffer (150 mM NaCl, 20 mM Tris [pH 7.4-7.5], 5 mM ethylenediaminetetraacetic acid, 1% Triton-X, 10% glycerol) containing Protease Arrest (G-Biosciences), Phosphatase Inhibitor Cocktail II (EMD Chemicals). Protein was quantified using the Bio-Rad Bradford protein estimation and 30 to 50 μ g was loaded per well in 4% to 12% Bis-Tris electrophoresis gels (Invitrogen). Protein was transferred on to 0.45-micron nitrocellulose membranes. Antibodies used for western blotting included pSTAT5 and phosphorylated and total JAK2, pSTAT3, and pMAPK (all from Cell Signaling Technologies) and Actin (EMD Chemicals).

Results

JAK2 is required for initiation of *MPLW515L*-mediated disease

Retroviral expression of mutant *MPLW515L* in hematopoietic cells in vivo results in the development of a highly penetrant, lethal MPN with features of ET/PMF, including leukocytosis, thrombocytosis, extramedullary hematopoiesis, and extensive BM fibrosis.⁶ Given that this model depends on expression of a mutant cytokine receptor, we evaluated the effect of *Jak2* deletion on disease development in vivo. Germline deletion of *Jak2* results in embryonic lethality.^{17,18} We therefore used a conditional knockout model of *Jak2*¹⁴ in which *Jak2* is deleted in an inducible and hematopoietic-specific manner by Cre-recombinase expressed under the control of the Mx1 promoter. BM cells from *Jak2* floxed (*Jak2^{fl/fl}*) *Mx1-Cre⁺* and *Mx1-Cre⁻* mice expressing the CD45.2 congenic marker were transduced with the GFP-tagged *MPLW515L* retrovirus and transplanted into irradiated CD45.1 recipients along with an equal number of CD45.1 support BM cells. Two weeks following transplantation, we documented engraftment by the presence of GFP-positive cells in PB of transplanted mice. Before mice developed overt disease, *Jak2* was deleted by injection of pI:pC and we followed the proportion of GFP-positive cells and CD45.1:CD45.2 ratios in hematopoietic cells (supplemental Figure 1, available on the *Blood* Web site).

Figure 1. JAK2 is required for initiation of *MPLW515L*-mediated disease. Deletion of *Jak2* following engraftment of *MPLW515L*-transduced BM leads to significant reduction in (A) WBC counts, (B) platelets, (C) mutant allele burden in terms of GFP-positive cells in PB ($P < .005$), and (D) spleen ($P < .05$) and (E) liver size ($P < .05$) as compared with controls. (F) BM fibrosis assessed by reticulin staining is absent in *Jak2*-deleted BM. (Data are represented at mean \pm standard error of the mean [SEM]; *Jak2*^{+/+}, $n = 4$; *Jak2*^{-/-}, $n = 5$).



These data revealed marked differences in disease onset and progression in mice transplanted with *Jak2*^{fl/fl} *Mx1-Cre*⁺ and *Mx1-Cre*⁻ cells. All mice receiving *Jak2*^{fl/fl} *Mx1-Cre*⁻ cells developed severe, lethal MPN characterized by leukocytosis, thrombocytosis, splenomegaly, and MF. By contrast, we did not observe significant disease in mice transplanted with *Jak2*^{fl/fl} *Mx1-Cre*⁺ cells; white blood cell (WBC) (52.52 K/ μ L vs 10.27 K/ μ L) and platelet counts (Plt) (1328 K/ μ L vs 988 K/ μ L) remained normal consistent with an absence of myeloproliferation (Figure 1A-B). More importantly, *MPL* mutant allele burden as measured by percentage of GFP-positive cells was significantly reduced compared with controls (4.9% vs 44.3%, $P < .005$) (Figure 1C). Spleen and liver sizes were also significantly reduced in mice transplanted with *Jak2*^{fl/fl} *Mx1-Cre*⁺ cells compared with mice transplanted with *Jak2*^{fl/fl} *Mx1-Cre*⁻ cells (spleen 0.176 g vs 0.577 g, $P < .05$; liver 1.48 g vs 1.76 g, $P < .05$) (Figure 1D-E). Notably, BM fibrosis, a hallmark feature of this PMF model, was completely absent in mice transplanted with *Jak2*^{fl/fl} *Mx1-Cre*⁺ compared with marked reticulin fibrosis in mice engrafted with *Jak2*^{fl/fl} *Mx1-Cre*⁻ cells (Figure 1F).

With extended observation, 1 mouse in the *Mx1-Cre*⁺ cohort manifested a persistent GFP-positive population in the PB, and subsequently developed elevated blood counts and an enlarged spleen (supplemental Figure 2A) consistent with disease progression. Polymerase chain reaction (PCR) analysis documented incomplete excision and expansion of unexcised GFP-positive cells over time, as can be seen by presence of the floxed allele in PB (supplemental

Figure 2B). These data strongly indicate an absolute requirement for JAK2 in disease initiation in the *MPLW515L* model of MF.

JAK2 plays a critical role in survival of the MPN mutant clone

We then asked whether JAK2 was required in the maintenance of the disease clone in the *MPLW515L* model. *Jak2* was excised by administration of pI:pC 4 to 6 weeks after BM transplantation, at which time all mice had evidence of myeloid expansion and overt disease. Similar to the results with *Jak2* deletion at disease initiation, loss of JAK2 when mice had overt MF resulted in a rapid, potent reduction in leukocytosis and Plt compared with mice transplanted with *Jak2*^{fl/fl} *Mx1-Cre*⁻ cells (WBC 18.71 K/ μ L vs 95.78 K/ μ L, $P < .005$; Plt 456 K/ μ L vs 1171 K/ μ L, $P < .005$) (Figure 2A-B). *Jak2* deletion caused marked reduction in spleen sizes compared with mice transplanted with *Jak2*^{fl/fl} *Mx1-Cre*⁻ cells (0.16 g vs 0.63 g, $P < .005$) (Figure 2C and supplemental Figure 3A). Most importantly, we observed a rapid, significant reduction in mutant allele burden (GFP-positive cells) in the PB and BM (PB 34.04% vs 77.12%, $P < .005$; BM 20.92% vs 84.56%, $P < .005$) (supplemental Figure 3B and Figure 2D). Of note, the marked reduction in mutant allele burden seen with *Jak2* deletion is not observed with maximal kinase inhibition in this same model.^{9,10}

Deletion of *Jak2* led to reduced pathologic myeloproliferation, including a decrease in extramedullary hematopoiesis, restoration of normal splenic architecture, and abrogation of BM fibrosis

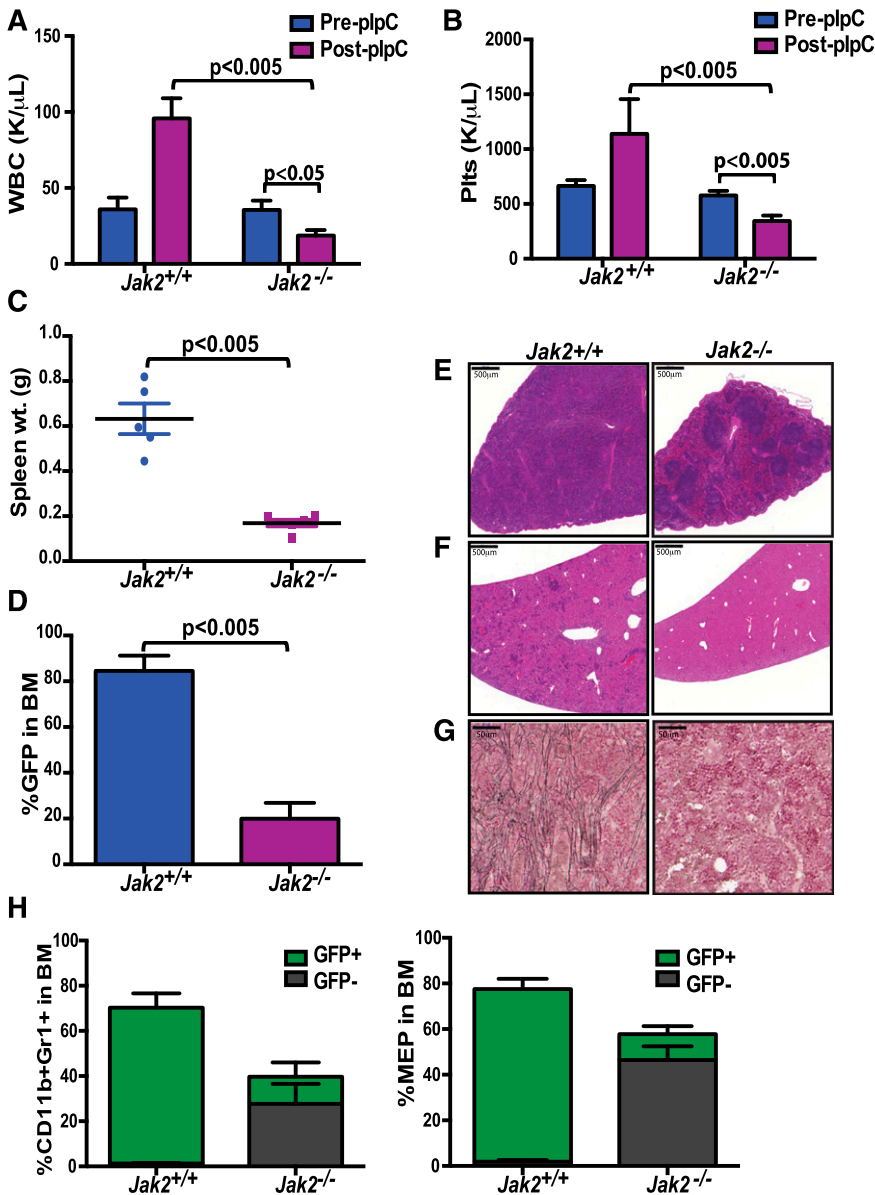


Figure 2. JAK2 plays a critical role in the survival of MPN clone. Excision of *Jak2* following disease establishment led to reduction in (A) WBC, (B) platelets, (C) spleen sizes, and (D) GFP-positive cells in BM ($P < .005$). Loss of JAK2 results in significant improvement in disease features including (E) restoration of splenic architecture, (F) decrease in myeloid infiltration in liver, and (G) reduction in BM fibrosis. (H) There was also a reduction in CD11b⁺Gr1⁺ cells and MEP population in *MPLW515L*-transduced mice lacking *Jak2*. (Data are represented at mean \pm SEM; *Jak2*^{+/+}, n = 5; *Jak2*^{-/-}, n = 6).

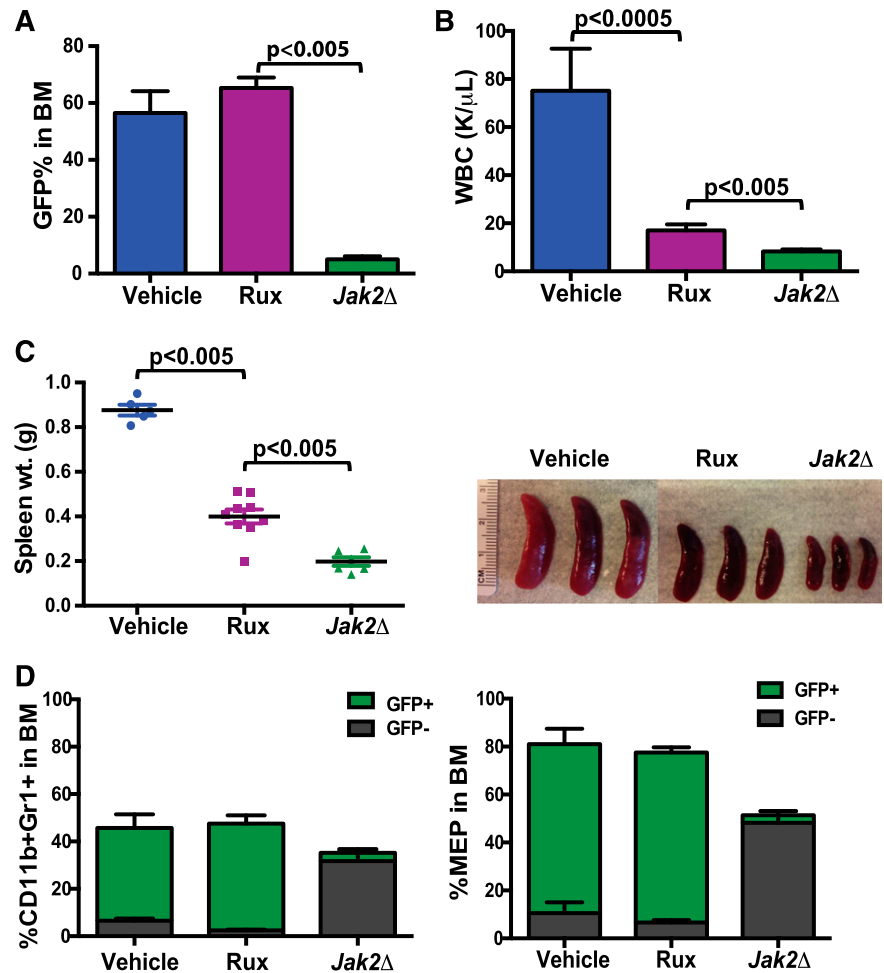
(Figure 2E-G and supplemental Figure 4). These data indicate that elimination of JAK2 in mutant MPN cells can reverse BM fibrosis, contrary to what has been observed with JAK inhibition in the preclinical and clinical setting. *Jak2* deletion normalized the expansion of the CD11b⁺Gr1⁺ myeloid lineages and megakaryocytic-erythroid progenitor (MEP) compartment observed in this MF model (Figure 2H and supplemental Figure 5). As in the studies done at disease initiation, we documented that the remaining GFP-positive mutant cells in the BM had incomplete excision of *Jak2*, based on genotypic analysis of sorted GFP-positive BM cells (supplemental Figure 6). These data demonstrate that JAK2 is required for maintenance of the mutant MPN clone.

Deletion of *Jak2* is more effective than JAK inhibitor treatment alone

We have previously shown that although MPN cells can rapidly become insensitive to JAK inhibition, knockdown of JAK2 by RNA interference in JAK^{Per} cells leads to growth suppression and inhibition

of downstream signaling.¹⁰ By deleting *Jak2* in vivo, we tested whether we could rescue the persistent phenotype in the *MPLW515L* model of ET/MF. BM from *Jak2*^{fl/fl} *Mx1-Cre*⁺ mice was retrovirally transduced with *MPLW515L-IRES-GFP* and transplanted into lethally irradiated recipients. After disease establishment, mice were randomized to receive vehicle, ruxolitinib, or pl:pC to delete *Jak2*. As reported previously,⁹ although drug treatment improved PB counts, ruxolitinib did not reduce the mutant allele burden in vivo. By contrast, deletion of *Jak2* markedly reduced percentage of GFP-positive cells in the BM (5.03% vs 65.27%, $P < .005$) (Figure 3A). Deletion of *Jak2* reduced blood counts (WBC 8.32 K/ μ L vs 17.06 K/ μ L, $P < .005$) (Figure 3B) and spleen size (0.19 g vs 0.40 g, $P < .005$) (Figure 3C) to a greater extent than observed with chronic JAK inhibitor treatment. Analysis of myeloid and progenitor populations revealed loss of JAK2 led to a significant reduction in MEP and CD11b⁺Gr1⁺ proportions with a dramatic decrease in the contribution of mutant cells to myeloid progenitors and differentiated myeloid compartments (Figure 3D and supplemental Figure 7), which was not observed with ruxolitinib

Figure 3. Deletion of *Jak2* is more effective than JAK inhibitor therapy at reducing disease burden. *MPLW515L*-transduced mice were treated with vehicle, 60 mg/kg ruxolitinib or pl:pC to excise *Jak2*. (A) Deletion of *Jak2* led to significant reduction in mutant allele burden in BM as compared with inhibitor treatment ($P < .005$). It also resulted in further decrease in (B) blood counts and (C) spleen size in *Jak2*-deleted mice compared with mice that received ruxolitinib alone ($P < .005$). (D) Loss of JAK2 also leads to reduction in MEP and CD11b⁺Gr⁺ proportions with a dramatic decrease in the contribution of mutant (GFP⁺) cells. (Data are represented at mean \pm SEM; vehicle, n = 5; Rux, n = 9; *Jak2* deleted, n = 6).



therapy. These results indicate that deletion of *Jak2* is superior to JAK2 inhibitor therapy at reducing disease burden consistent with a dependence on JAK2, which is not amenable to inhibition with clinically available JAK inhibitors.

Combined JAK and HSP90 inhibition is more efficacious than JAK inhibitor monotherapy

The results so far suggest that inhibiting the kinase activity of JAK2 with existing, ATP competitive inhibitors might not sufficiently inhibit JAK2 to obtain adequate efficacy. However, since MPN cells remain dependent on JAK2 expression for their growth and survival, we hypothesized that combination approaches which increase JAK2 inhibition would lead to increased efficacy in vivo. We previously reported that JAK2 is an HSP90 client protein and that treatment of MPN cells with HSP90 inhibitors results in degradation of total and phosphorylated JAK2 and concomitant inhibition of downstream signaling.¹³ Of note, JAK^{Per} cells remain sensitive to PU-H71, an HSP90 inhibitor that is efficacious in preclinical MPN models.¹⁰ We therefore decided to test the efficacy of combined ruxolitinib and PU-H71 in the *MPLW515L* model of ET/MF. The dosing regimens tested included vehicle, 2 different doses of ruxolitinib monotherapy, combined JAK/HSP90 inhibition from the onset and sequential therapy with ruxolitinib followed by combined ruxolitinib/PU-H71 therapy (supplemental Figure 8). We did not observe any hematopoietic or nonhematopoietic toxicity with combined JAK/HSP90 inhibition, and

escalated both agents to their maximally tolerated monotherapy doses in combination studies.

Combination therapy resulted in a potent reduction in total and phosphorylated JAK2 expression, whereas JAK inhibitor therapy alone resulted in increased phospho-JAK2 and JAK2 expression as previously reported.^{10,19} More importantly, we observed more potent inhibition of downstream signaling effectors including phospho- and phospho-MAPK (Figure 4A). Immunohistochemistry studies documented that combination therapy resulted in more potent inhibition of downstream signaling, concomitant with induction of HSP70 expression (supplemental Figure 9A-B). These data indicated that combination therapy results in greater inhibition of the JAK-STAT pathway than maximally tolerated JAK kinase inhibitor monotherapy.

We assessed inhibitor efficacy 2 weeks following the start of treatment; at that time point, combination therapy significantly reduced WBC and Plt compared with low-dose (30 mg/kg) or high-dose (90 mg/kg) ruxolitinib monotherapy ($P < .0001$) (Figure 4B-C). Combination treatment also led to a further reduction in spleen size compared with ruxolitinib monotherapy (Figure 4D) and increased histopathological improvement, with reduced BM and spleen cellularity, a reduction in megakaryocyte expansion, and a reduction in BM fibrosis (Figure 4E and supplemental Figure 9B). Of note, we observed a greater difference in efficacy with 4 weeks of combination therapy, as combined ruxolitinib/PU-H71 markedly reduced blood counts and organomegaly compared with ruxolitinib alone. Although we first used a suboptimal dose of ruxolitinib therapy

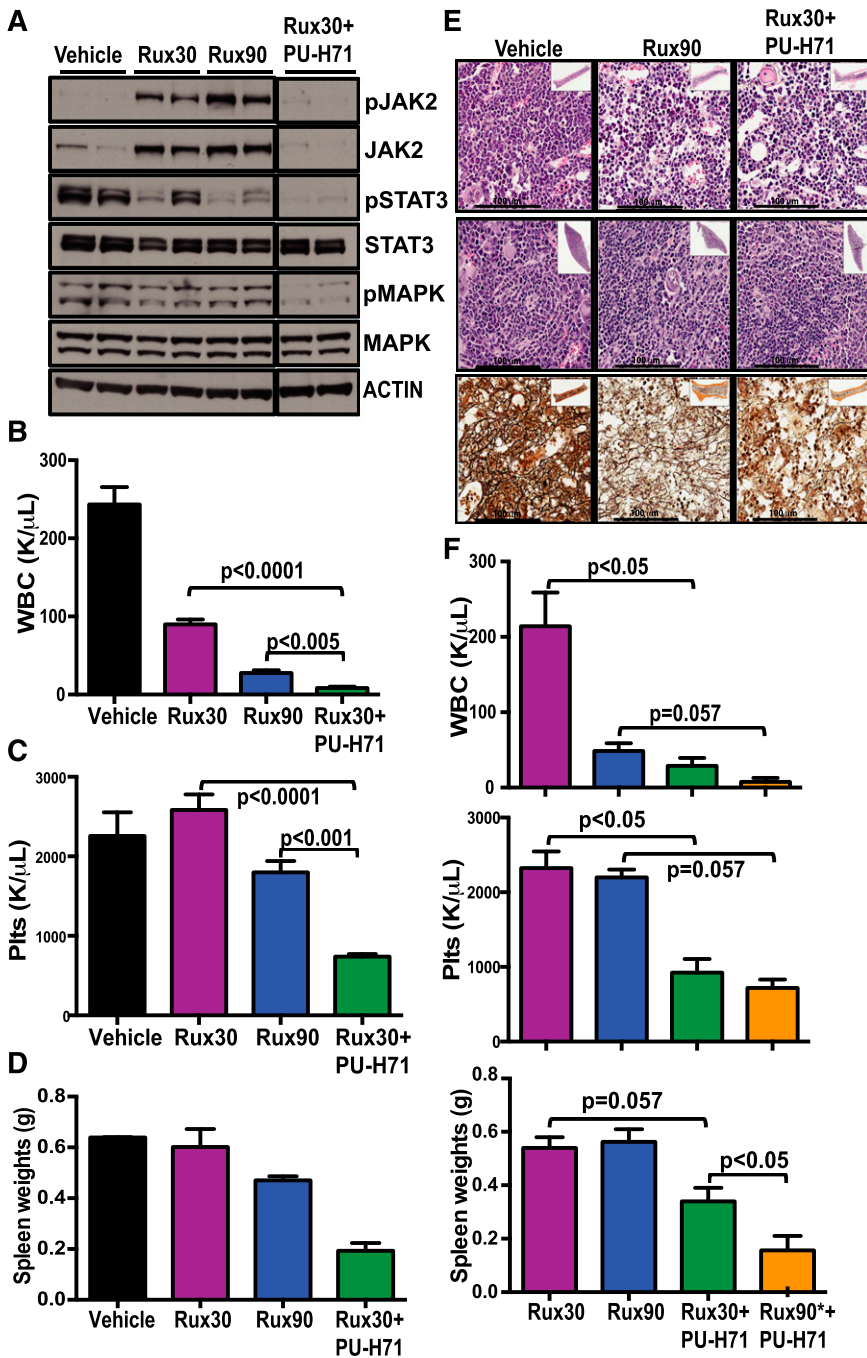


Figure 4. Combination of JAK2 and HSP90 inhibition is more efficacious than JAK inhibitor monotherapy. (A) Combination of ruxolitinib and PU-H71 resulted in JAK2 degradation and inhibition of downstream signaling in splenocytes of *MPLW515L*-transduced mice (all lanes were run on the same gel). After 2 weeks of drug treatment, the ruxolitinib and PU-H71 combination arm had significantly lower (B) WBC and (C) Plt compared with ruxolitinib alone ($P < .0001$, $n = 10$). (D) Spleen size in the combination arm was also lower. (E) Improved histopathology and reduction in BM fibrosis in mice treated with combination of JAK and HSP90 inhibitors. (F) After 4 weeks of drug treatment, the combination group that had ruxolitinib dose increased to 90 mg/kg for 2 weeks (Rux90+PU-H71) had lowest blood counts and spleen sizes compared with ruxolitinib alone. (Data are represented at mean \pm SEM; $n = 4$).

in our combination studies, we found that combining the highest dose of ruxolitinib (90 mg/kg) with PU-H71 was the most efficacious strategy without any increased toxicity (Figure 4F).

Given that the data above were obtained in a *MPLW515L*-driven model of MPN, we investigated whether combined JAK/HSP90 inhibition would demonstrate increased efficacy in a *Jak2V617F*-driven model of MPN. We transplanted BM cells from *Jak2V617F* knock-in mice⁸ into secondary recipients, and following disease engraftment, treated mice with ruxolitinib or with combined ruxolitinib/PU-H71. Combination therapy resulted in a further reduction in blood counts and spleen size in vivo compared with ruxolitinib monotherapy, consistent with increased therapeutic efficacy (supplemental Figure 10).

Genetic or pharmacologic loss of JAK2 can overcome inhibitor persistence in vivo and in patient samples

The approval of ruxolitinib for MF has led to its use as first-line treatment of MF patients with significant splenomegaly and symptoms. As such, we investigated whether improved JAK2 targeting following initial ruxolitinib monotherapy might lead to significant therapeutic benefit. We first assessed whether genetic deletion of *Jak2* after ruxolitinib treatment could reduce myeloproliferation and disease burden in vivo. BM from *Jak2^{fl/fl} Mx1-Cre⁺* mice was retrovirally transduced with *MPLW515L-IRES-GFP* and transplanted into lethally irradiated recipients. Following disease establishment, we initiated treatment with ruxolitinib (supplemental Figure 11). Although

ruxolitinib treatment improved blood counts and reduced spleen size, we did not observe a reduction in mutant allele burden with 5 weeks of ruxolitinib therapy (supplemental Figure 12). By contrast, deletion of *Jak2* following ruxolitinib treatment led to a significant reduction in mutant allele burden in BM (23.28% vs 65.27%, $P < .005$) (Figure 5A), blood counts (WBC 4.99 K/ μ L vs 17.06 K/ μ L, $P < .001$) (Figure 5B), and spleen sizes (0.149 g vs 0.40 g, $P < .005$) (Figure 5C) as compared with mice that continued to receive drug. Furthermore, deletion of *Jak2* after long-term ruxolitinib treatment (5 weeks) prevented disease relapse, which is observed with cessation of treatment even after longer-term ruxolitinib therapy (Figure 5D). The percentage of GFP-positive cells, which persisted at a high-level despite 5 weeks of ruxolitinib treatment, was also decreased by deletion of *Jak2* (Figure 5E). These results indicate that deletion of *Jak2* in vivo can successfully eliminate mutant MPN cells that survive in the setting of chronic JAK2 kinase inhibition.

We then asked whether we could leverage this finding for therapeutic purposes. We initiated combination JAK2/HSP90 therapy following ruxolitinib monotherapy. After 2 weeks of ruxolitinib treatment, we added 75 mg/kg PU-H71 treatment to a subset of mice that were receiving monotherapy (supplemental Figure 8). Two weeks of combination treatment resulted in a significant reduction in leukocytosis and thrombocytosis compared with ruxolitinib monotherapy (WBC 35.22 K/ μ L vs 214 K/ μ L; $P < .05$; Plt 613 K/ μ L vs 2323 K/ μ L, $P < .05$) (Figure 5F). Addition of PU-H71 to ruxolitinib monotherapy also reduced splenomegaly to a greater extent than observed with ruxolitinib monotherapy (0.33 g vs 0.53 g, $P < .05$) (Figure 5G).

We have previously demonstrated that hematopoietic cells from MPN patients treated with ruxolitinib are insensitive to JAK2 kinase inhibition ex vivo and exhibit persistent downstream signaling with ex vivo JAK inhibitor exposure.¹⁰ We therefore asked whether treatment with an HSP90 inhibitor increased JAK-STAT pathway inhibition in samples from MF patients receiving ruxolitinib therapy. As postulated, PU-H71 treatment led to degradation of total and activated JAK2 and abrogation of downstream STAT and MAPK signaling (Figure 5H). Thus, PU-H71 is effective in inhibiting JAK-STAT signaling in primary samples that are insensitive to JAK inhibition.

Discussion

The discovery of mutations in the JAK-STAT pathway in a majority of MPN patients led to the hypothesis that this pathway activation is a central feature of MPN pathogenesis. Expression of mutant *JAK2/MPL* alleles transforms hematopoietic cells to cytokine-independent growth, and in vivo expression of these alleles recapitulates many features of MPNs. These experiments are consistent with the notion that JAK-STAT pathway mutations are indeed drivers of MPN pathogenesis. However, kinase inhibition of JAK2 has not resulted in the molecular responses that have been observed with ABL kinase inhibitors in CML. Although MPN patients experience an improvement in splenomegaly, and constitutional symptoms, treatment with JAK inhibitors is not curative and does not significantly decrease mutant allele burden. These studies therefore led investigators to question the role of activated JAK2 signaling in MPN pathogenesis and the ultimate role of JAK-targeted therapies as a long-term therapeutic strategy for MPN patients.

The identification of secondary resistance mutations in response to chronic inhibition is often considered a hallmark of effective targeted therapy consistent with sufficient target inhibition to select for genetic resistance. This has been observed with ABL kinase inhibitors in CML,²⁰ EGFR inhibitors in lung cancer,²¹ and FLT3 inhibitors in acute myeloid leukemia.²² However, this has not been the case with JAK2 inhibitors to date, suggesting either incomplete dependence on JAK2 or insufficient inhibition of the target in the clinical setting. Our previous in vitro studies suggested a putative mechanism for insufficient JAK-STAT pathway inhibition based on activation of JAK2 by other JAK kinases. The underlying mechanism is based on the stabilization of activated JAK2 by the binding of type I inhibitors, including ruxolitinib.¹⁹ These data suggest that MPN cell lines require JAK2, at least in part, as a scaffold to maintain downstream signaling even in the context of inhibition of its catalytic activity. This can at least partially be overcome by type II JAK kinase inhibitors; however, type II JAK inhibitors have not been developed for in vivo studies to date. As such, there is a need to determine whether JAK2 remains a critical therapeutic target in MPN cells, and whether there are pharmacologic approaches that can be used to improve JAK2 targeting and therapeutic response.

In this study, we demonstrate that *Jak2* is essential in vivo by deleting *Jak2* in an inducible, tissue-specific manner at different stages of disease and following JAK inhibitor therapy. *Jak2* deletion led to significant improvements in blood counts, organomegaly, and reduced mutant allele burden, which has not been shown using JAK inhibitors as monotherapy. We also showed that genetic loss of *Jak2* was more efficacious than kinase inhibition alone in vivo. Furthermore, deletion of *Jak2* was curative in this model, as disease did not recur with the exception of mice with incomplete deletion of *Jak2*, consistent with a genetic requirement for JAK2 signaling in MPN. Taken together, these data suggest that JAK2 remains an exceptional therapeutic target in MPN and that we have not seen the impact of maximal JAK kinase inhibition in MPN patients with existing agents to date.

We previously demonstrated that JAK2 is an HSP90 client protein and treatment with HSP90 inhibitors leads to degradation of total and phosphorylated JAK2 along with inhibition of downstream signaling.¹³ JAK inhibitors and HSP90 inhibitors likely have pleiotropic effects. It is possible that the increased therapeutic efficacy seen with combination therapy is due to HSP90-mediated effects on different client proteins, and JAK inhibitors may in part exert their effects through amelioration of inflammatory signaling. In these studies, we show that combination ruxolitinib/PU-H71 therapy was more efficacious than JAK inhibitor monotherapy at inhibiting JAK-STAT signaling and reducing myeloproliferation without attendant increases in toxicity. Preliminary observations from monotherapy trials with the HSP90 inhibitors AUY922 and PU-H71 for refractory MPN patients suggest significant clinical efficacy of HSP90 inhibition in MPN patients (R.R. and R.L.L., unpublished data). Combination studies in which HSP90 inhibition is added to JAK kinase inhibitor therapy are being planned subsequent to these trials.

Taken together, these data indicate that MPN cells are dependent on JAK2 expression for their growth and survival, and therapies that lead to increased inhibition of JAK-STAT signaling should be arduously investigated in preclinical and clinical settings. These may include combination JAK/HSP90 inhibitor therapy, type II JAK kinase inhibitors, and other combination approaches with additive inhibitory effects on the JAK-STAT pathway. Given the limited ability of JAK kinase inhibition to achieve disease-modifying activity in MPN patients, we predict that novel approaches which

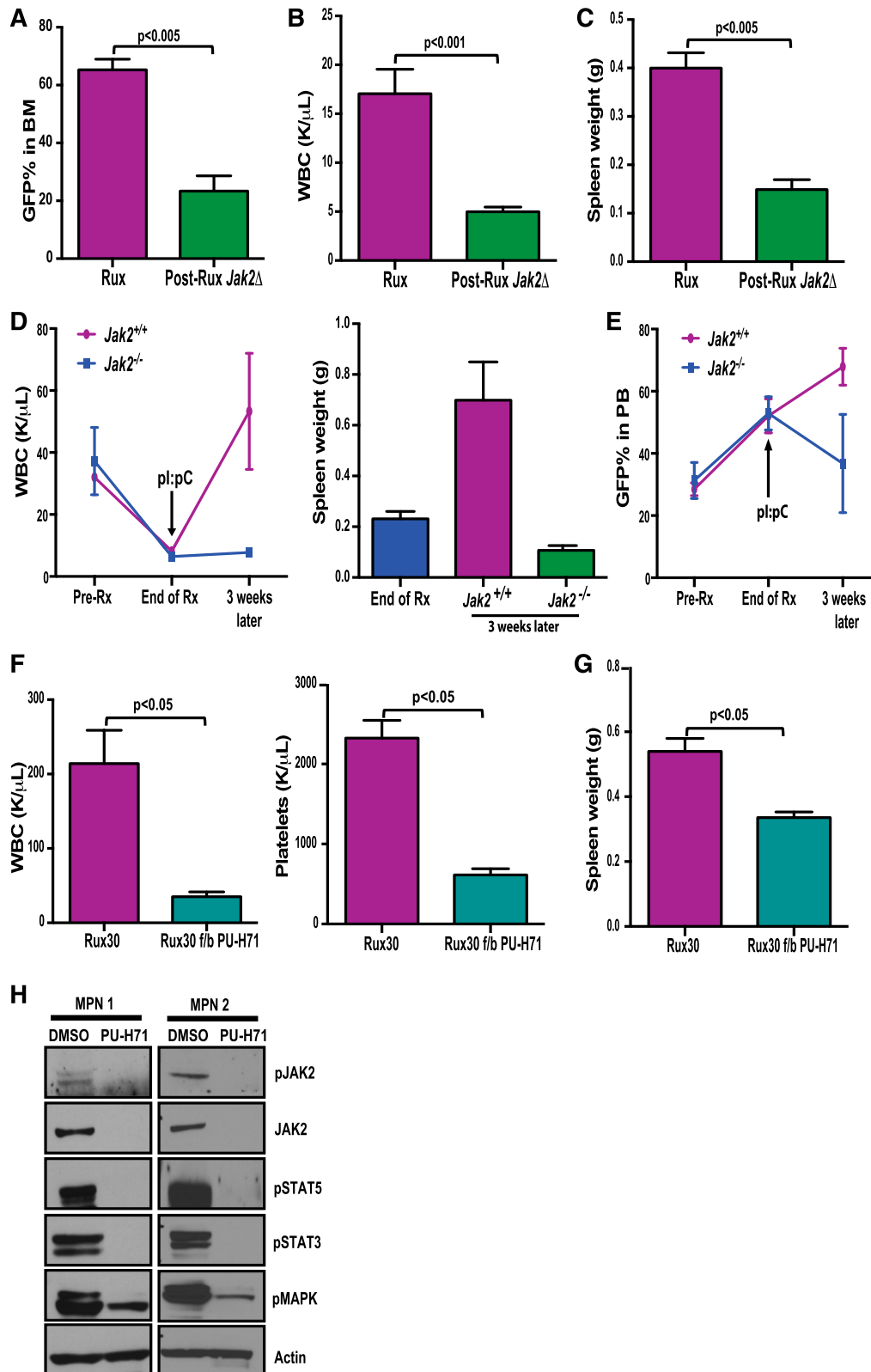


Figure 5. Genetic loss or pharmacologic degradation of JAK2 subsequent to JAK inhibitor monotherapy can overcome persistence. Deletion of *Jak2* following 2 weeks of ruxolitinib treatment resulted in reduction of (A) mutant allele burden ($P < .005$), (B) blood counts ($P < .001$), and (C) spleen weights ($P < .005$) compared with mice that continued to receive ruxolitinib (Rux, $n = 9$; *Jak2* deleted, $n = 6$). Deletion of JAK2 after long-term (5 weeks) of ruxolitinib treatment prevents disease relapse following cessation of treatment by reducing (D) WBC and spleen weights as well as (E) mutant allele burden in *Jak2*-deleted mice ($n = 3$). (F) Addition of PU-H71 to ruxolitinib monotherapy resulted in significant reduction in blood counts. (G) Spleen sizes were further reduced by PU-H71 therapy ($P < .05$, $n = 4$). (Data are represented at mean \pm SEM). (H) Mononuclear cells from ruxolitinib-treated MPN patients were isolated and treated with 0.5 μ M PU-H71 for 16 hours, which led to inhibition of JAK2 and downstream STAT-MAPK signaling.

lead to increased JAK2 inhibition in the clinical setting will improve outcomes for patients with JAK-dependent malignancies.

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Authorship

Contribution: N.B., P.K., and R.L.L. conceived the project; N.B., P.K., S.M., and R.L.L. designed experiments; N.B., P.K., S.M., M. Keller, and K.S. performed experiments; N.B., P.K., S.M., and R.L.L. analyzed data; M. Kleppe and R.R. provided reagents and samples; H.J.P., S.K.S., T.T., and G.C. provided PU-H71 and assisted with design and performance of in vivo studies; J.Q. and J.E.B. provided ruxolitinib; and N.B., P.K., and R.L.L. wrote the manuscript with input from all other authors.

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