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# Pharmacological Inhibition of Insulin Growth Factor-1 Receptor (IGF-1R) Alone or in Combination With Ruxolitinib Shows Therapeutic Efficacy in Preclinical Myeloproliferative Neoplasm Models

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**Abstract**

Even after development of the JAK1/JAK2 inhibitor ruxolitinib, myeloproliferative neoplasm (MPN) patients require novel therapeutic options. While ruxolitinib can considerably improve quality of life and prolong survival, it does not modify the natural disease course in most patients. Moreover, resistance develops with prolonged use. Therefore, various combination treatments are currently being investigated. Published data provide a compelling rationale for the inhibition of insulin growth factor-1 receptor (IGF-1R) signaling in MPN. Here we report that genetic and pharmacological inhibition of IGF-1R selectively reduced Jak2$^{V617F}$-driven cytokine-independent proliferation ex vivo. Two different structurally unrelated IGF-1R inhibitors ameliorated disease phenotype in a murine MPN model and significantly prolonged survival. Moreover, in mice, low-dose ruxolitinib synergized with IGF-1R inhibition to increase survival. Our data demonstrate preclinical efficacy of IGF-1R inhibition in a murine MPN model.

## Introduction

Myeloproliferative neoplasms (MPN) constitute a group of clinically and molecularly overlapping entities that arise clonally from an aberrant stem cell and include essential thrombocythemia, polycythemia vera (PV), and primary myelofibrosis (PMF). In MPN patients, several alterations cause constitutive activation of JAK/STAT signaling, including activating mutations in the JAK2 kinase itself, as well as mutations in thrombopoietin receptor (MPL) and calreticulin (CALR).[1–3] While ruxolitinib, a JAK1/JAK2 inhibitor is US Food and Drug Administration (FDA)-approved for the treatment of both PMF and PV, this drug only marginally affects the number of circulating neoplastic cells.[4,5] Therapeutic benefit is derived from its ability to reduce spleen size and to mitigate an often significant improvement in constitutional symptoms. Through these effects, ruxolitinib increases survival in PMF patients, but with rare exceptions is unable to alter the biological disease course.[6,7]

Several approaches are being taken to improve the efficacy of ruxolitinib treatment or, more broadly, of JAK/STAT inhibition in the treatment of MPN. These include the development of second-generation inhibitors that either target the mutated JAK2 kinase, JAK2$^{V617F}$, more selectively, or exhibit more favorable side effect profiles and pharmacodynamics, allowing more consistent inhibition of both wild type (wt) and mutant JAK2.[8] Alternatively, a variety of combination treatments are being investigated, including combining ruxolitinib with the histone deacetylase (HDAC) inhibitor vorinostat,[9] lenalidomide,[10] or the PI3-kinase (phosphatidylinositol 3-kinase) inhibitor buparlisip.[11]

Several findings suggest a role for the insulin growth factor-1 receptor (IGF-1R) in the pathophysiology of MPN. First, MPN patients display higher plasma levels of IGF-1R[12] and insulin growth factor binding protein-1 (IGFBP-1) than healthy controls.[13] Second, PV cells are hypersensitive to IGFBP-1, showing maximal erythroid colony growth at concentrations 100-fold lower than those required by control cells.[14] In fact, Axelrad and colleagues[14] proposed that it is not Epo-independence, but rather insulin growth factor-1 (IGF-1) hypersensitivity that accounts for the pathognomonic phenomenon termed "endogenous erythroid colonies" (EECs) in PV. This model is supported by the observation that the IGF-1R is constitutively phosphorylated in PV patients.[12]

Data from the lab of Stefan Constantinescu suggests that IGF-1 cooperates with aberrant JAK/STAT signaling to promote proliferation of neoplastic cells.[15] IGF-1 stimulation selectively increased proliferation of cells expressing JAK2$^{V617F}$, but not JAK2$^{wt}$, augmenting phosphorylation of JAK2$^{V617F}$ at tyrosine Y1007/Y1008. Again, phosphorylation of JAK2$^{wt}$ was not affected by IGF-1.

From these data, we hypothesized that inhibition of IGF-1R signaling will impair JAK2$^{V617F}$-driven aberrant cell growth and proliferation. Furthermore, we tested the hypothesis that inhibition of IGF-1 signaling synergizes with JAK2 inhibition to attenuate disease burden and progression in a JAK2$^{V617F}$-driven murine MPN model.

## Methods

### Generation of the Mx1-Cre Jak2$^{V617F}$ knock in and bone marrow transplantation (BMT) MPN mouse models

Conditional floxed Jak2$^{V617F}$ knock-in mice were a kind gift from Jean Luc Villeval.[16] These mice were crossed with

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Mx1-Cre mice (Tg(Mx1-cre)1Cgn: JAX stock No. 003556) to induce expression of Jak2$^{V617F}$.[17] Mice heterozygous for Jak2$^{V617F}$ (henceforth termed "Jak2$^{V617F}$ mice") were used for treatment. Due to the possible in vivo activation of the interferon (IFN) system (leakiness), induction of the Mx1-Cre construct and development of an MPN phenotype did not require polyinosinic polycytidylic acid (pIpC) induction, as previously published.[17] In addition, in this model, Jak2$^{V617F}$ is expressed throughout embryogenesis in tissues where Mx1 is activated. This fact may account for the reduced life span of our mice.[17]

To generate the BMT MPN model, floxed Jak2$^{V617F}$ mice were crossed with a Cre-Del strain to obtain mice heterozygously expressing Jak2$^{V617F}$ in all bone marrow (BM) cells. BM from these Jak2$^{V617F}$-heterozygous mice, which carry the CD45.2 isotype, was mixed at a 1:1 ratio with BM from wt Bl/6 CD45.1 expressing mice (Bl/6 Ptprc Ly5.1; JAX stock No. 002014 ) and transplanted into lethally irradiated Bl/6 CD45.1 recipients (1 million cells transplanted per animal).

Mice were housed under pathogen-free conditions, regularly tested negative for 34 pathogens by sentinel mouse analysis, in accordance with committee approved animal protocols (Environment and consumer protection of Baden Württemberg, Germany, G-17/043).

## Treatment and analysis of JAK2$^{V617F}$ knockin and BMT mice

Jak2$^{V617F}$ mice were treated by oral gavage with the following drugs, alone or in combination as indicated: 15 or 25 mg/kg linsitinib (in 25 mM tartaric acid); 60 mg/kg ruxolitinib (in 25 mM tartaric acid); 20 mg/kg picropodophyllin (PPP; in sodium citrate, pH: 4.0). As no uniform dosing schedule for linsitinib existed in either murine models or clinical trials at the time of study initiation, we chose a continuous dosing for 14 days followed by a 7 day drug holiday. Observations were terminated at the latest at 17 weeks since vehicle control animals rarely survived past this point and statistically significant comparisons would therefore not have been possible had treatment been extended further. Peripheral blood was drawn by retrobulbar puncture and analyzed on an ADVIA 120 (Siemens, Erlangen, Bayern, Germany) or a URIT 5250 VET hematological analyzer.

## Human and murine colony assays

### *Human colonies*

Peripheral blood MNCs were seeded at a density of 2 × 10$^5$ cells/mL in methylcellulose medium with or without erythropoietin (EPO; H4330 and H4230, respectively, STEMCELL Technologies, Vancouver, BC, Canada) and incubated for 14 days at 37°C, 5% $CO_2$. The number of colonies was determined after 14 days.

### *Murine colonies*

Bone marrow was seeded in methylcellulose medium with EPO (H3434, STEMCELL Technologies) and incubated for 8 days at 37°C, 5% $CO_2$. For genotyping, individual colonies were picked, deposited in phosphate buffered saline (PBS), and a fraction subjected to polymerase chain reaction (PCR) analysis for Jak2$^{wt}$ and Jak2$^{V617F}$ as previously described.[16]

## Cell lines and in vitro treatments

Ba/F3 cells expressing Jak2$^{WT}$ or Jak2$^{V617F}$ in the presence or absence of murine IL-3, as indicated, were treated with linsitinib (OSI-906, OSI Pharmaceuticals, Farmingdale, NY, USA), ruxolitinib (JAKAVI) or PPP (72435, DC lab, Shanghai, Pudong District, China) at the doses shown. For the competitive culture experiments, Ba/F3 expressing either Jak2$^{wt}$ or Jak2$^{V617F}$, which carry the green fluorescent protein (GFP) marker from the initial Jak2 retroviral transduction, were lentivirally transduced to express mCherry in addition. Subsequently, GFP$^+$/mCherry$^+$ Jak2$^{wt}$ and Jak2$^{V617F}$ expressing Ba/F3 cells were cultured either separately or mixed at a 1:1 ratio (GFP$^+$-Jak2$^{wt}$ with GFP$^+$/mCherry$^+$-Jak2$^{V617F}$) and treated with increasing concentrations of PPP for 72 hours. Afterward, the percentage of viable, mCherry positive cells was determined.

## Lentiviral infections for knockdown experiment

Ba/F3 cells were seeded at a density of 2 × 10$^5$/mL. A total of 2 mL of cells were infected with lentiviral particles expressing a short hairpin ribonucleic acid (shRNA) targeting murine insulin like growth factor-1-receptor (mIGF-1R) (TL320384, Origene Technologies, Rockville, MD, USA, sequence GAAGATCCGCCATTCTCATGCCTTGGTCT) or a control shRNA (TR30021) at a multiplicity of infection (MOI) of 5. Transduced cells were assayed 96 hours after infection.

## Cell proliferation assay

Cell proliferation was determined in duplicate by enumerating viable cells using a trypan blue exclusion assay or an AlamarBlue proliferation assay.

## Apoptosis assay

Apoptosis was determined by fluorescence activated cell sorting (FACS) analysis using an AF647-coupled AnnexinV (640912, BioLegend, San Diego, CA, USA) and PI staining (11348639001, Sigma, St. Louis, MO, USA). Early apoptotic cells (annexinV+/PI–) and late apoptotic cells (annexinV+/PI+) were quantified.

## Semiquantitative reverse transcription polymerase chain reaction (RT-PCR)

RNA was isolated using the RNeasy mini kit (74104, Qiagen, Hilden, Nordrhein-Westfalen, Germany) and complementary deoxyribonucleic acid (cDNA) was synthesized using the Multiscribe reverse transcriptase kit (10121214, Applied Biosystems, Foster City, CA, USA). Semiquantitative PCR was performed using the following primer pairs: IGF-1R-F (5′-GACCTCTGTTACCTCTCCAC-3′), IGF-1R-R (5′-GATGAGACGAAGGTTCTTCAG-3′) and mB2M-F (5′-CTTTCTGGTGCTTGTCTCACTGAC-3′) and mB2M-R (5′-GGTGGCGTGAGTATACTTGAATTTG-3′).

## Western blot

Cells were serum starved for 16 hours in Roswell Park Memorial Institute medium (RPMI) with 0.7% fetal calf serum (FCS) before inhibitor treatment. Cell lysates in RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxylate, 1% nonyl phenoxypolyethoxylethanol [NP40]) were subjected to western blotting with the following antibodies, all from Cell Signaling Technologies (Danvers, MA, USA): pIGF-1R (3021) (phosphorylated insulin like growth factor-1-receptor); pJAK2 (3771) (phosphorylated janus kinase 2); pSTAT5 (9351) (phosphorylated signal transducer and activator of transcription 5); pMEK (9121) (phosphorylated MAPK/ERK kinase); pERK (4370) (phosphorylated extracellular signal regulated kinase); pAKT (9275) (phosphorylated protein kinase B); IGF-1R

(3027); JAK2 (3230); STAT5 (9352); MEK (9122); ERK (9102); AKT (9272). Blots were stripped and reprobed against beta actin (Sigma A5441) to ensure equal loading. Immune complexes were detected using chemiluminescence (K12-045-D20, Advansta, San Jose, CA, USA).

### Densitometry analysis

Band intensities obtained by chemiluminescence detection of western blots were quantified using the Image J software (National Institutes of Health, Bethesda, MD, USA).

### Statistical analysis

Statistical significance was determined by Student *t* tests, paired or unpaired, 1 or 2 tailed, as indicated. Kaplan-Meier survival curves were calculated using the log-rank (Mantel-Cox) test.

## Results

To test the hypothesis that hypersensitivity to IGF-1 contributes to MPN pathophysiology, we reduced expression of the IGF-1R by RNA interference in Ba/F3 cells expressing either Jak2$^{wt}$ or Jak2$^{V617F}$. Cells were lentivirally transduced to express either a scrambled, control shRNA or an shRNA against IGF-1R (shIGF-1R). Since IGF-1 plays a vital role in various aspects of cellular metabolism,[18–20] a drastic reduction in protein levels might prove toxic. We therefore sought a moderate reduction of IGF-1R expression. Both IGF-1R mRNA and protein expression were reduced by 40%–50% (Figure 1A and Supplemental Digital Content, Figure 1A, http://links.lww.com/HS/A153). Diminished IGF-1R expression reduced cell numbers both in Jak2$^{wt}$- and in Jak2$^{V617F}$-positive cells, irrespective of the presence of IL-3 (Figure 1B). Concomitantly, the fraction of cells in early and late apoptosis was significantly increased in IGF-1R shRNA-treated cells (Figure 1C, D and Supplemental Digital Content, Figure 1B, http://links.lww.com/HS/A153). These data suggested a role for IGF-1R signaling in promoting Ba/F3 cell survival that is irrespective of the Jak2 status and is retained in the absence of cytokine.

We subsequently investigated the effect of pharmacological IGF-1R inhibition using linsitinib (OSI-906), a selective, orally bioavailable dual IGF-1R/insulin receptor (IR) inhibitor.[21] While linsitinib did not affect the proliferation of either Jak2$^{wt}$ or Jak2$^{V617F}$ cells in the presence of IL-3, IGF-1R inhibition significantly reduced cytokine independent proliferation of Jak2$^{V617F}$ Ba/F3 cells in a dose-dependent manner (Figure 2A, B). This reduction was explained by a significant increase in apoptosis following IGF-1R inhibition only in the absence of cytokine (Figure 2C and Supplemental Digital Content, Figure 2, http://links.lww.com/HS/A153). Concomitantly, linsitinib treatment selectively reduced phosphorylation of IGF-1R, JAK, and STAT5 as well as MAPK (mitogen activated protein kinase)/ERK (extracellular signal regulated kinase) (MEK) in cytokine-deprived Jak2$^{V617F}$ BA/F3 cells, while protein kinase B (AKT) and extracellular signal regulated kinase (ERK) remained unaffected (Figure 2D, E and Supplemental Digital Content, Figure 3, http://links.lww.com/HS/A153). Conversely, IGF-1R inhibition had no effect on STAT5 phosphorylation in either Jak2$^{wt}$ or Jak2$^{V617F}$ cells grown in the presence of IL-3 while under these conditions, it effectively inhibited ERK phosphorylation in both Jak2$^{wt}$ and Jak2$^{V617F}$ cells (Supplemental Digital Content, Figure 4, http://links.lww.com/HS/A153). These data demonstrate that pharmacological inhibition of IGF1-R signaling counteracts the constitutive activation of JAK-STAT pathways evoked by the Jak2$^{V617F}$ mutation. Inhibition of MEK without subsequent inhibition of ERK phosphorylation may be due to alternative ERK activation, such as the mitogen activated protein kinase kinase kinase 8/cancer Osaka thyroid oncogene isoform 1 (MAP3K8/COT1)-mediated ERK phosphorylation shown by Johannessen et al.[22] Since the AKT and ERK-mediated proliferative pathways were not affected by linsitinib treatment in the absence of cytokine, our data raise the possibility that IGF-1R inhibition may selectively target the Jak2$^{V617F}$-positive MPN clone under these conditions.

We investigated whether the selective effect on Jak2$^{V617F}$-positive cells could also be observed in vivo. To this end, we used our recently published *Mx-Jak2$^{V617F}$* mouse strain.[17] As described, hematopoiesis in *Mx-Jak2$^{V617F}$* mice is derived both from Jak2$^{wt}$ and Jak2$^{V617F}$ stem cells. We treated mice with linsitinib for 14 day cycles, as previously published.[21] However, we reduced the dose by 60% to minimize toxicity. Our mice received a total of 2 treatment cycles, either linsitinib (25 mg/kg; n = 8) or vehicle control (n = 12), interrupted by a 7-day drug holiday (Figure 3A). Linsitinib treatment was well tolerated, as mice did not lose weight (Figure 3B) and showed no other signs of distress. Complete blood counts were assessed on day 0 and on day 35, following 2 treatment cycles.

While the disease progressed in vehicle-treated animals, witnessed by an increase in the white blood cell (WBC), leukocyte counts declined significantly in the linsitinib-treated mice (Figure 3C). Likewise, IGF-1R inhibitor treatment lowered the mean platelet count, but this difference did not reach statistical significance (Figure 3D). At the same time, the hematocrit remained unchanged, possibly due to the extended life span of erythrocytes (Figure 3E). Therefore, spleen weights were not impacted (Figure 3F), as the extramedulary hematopoiesis inducing splenomegaly in this model includes an extensive erythroid component.[17] Most importantly, we observed a highly significant increase in the survival of linsitinib-treated animals compared to vehicle controls (Figure 3G). As previously reported for other murine Jak2$^{V617F}$ models,[23] the main cause of death in our animals were thromboembolic and bleeding complications.

A pathognomonic feature of MPN is the formation of EPO-independent erythroid colonies, so called EECs.[24] In diligent experiments, Axelrad and colleagues[14] showed that, rather than resulting from Epo independence, EEC colony growth actually results from hypersensitivity to IGF-1. Therefore, we determined the consequence of pharmacological IGF-1R inhibition on colony formation using peripheral blood MNCs from PV patients and healthy controls. Although linsitinib did not affect the colony forming potential in the presence of Epo (Figure 4A, B), Epo-independent EEC colony formation was significantly reduced, suggesting that IGF-1R inhibition selectively affects the malignant clone (Figure 4C).

The JAK1/2 inhibitor ruxolitinib is FDA approved for the treatment of PMF and PV patients. While this inhibitor shows efficacy in reducing size of the splenomegaly and normalizing hematological parameters, it does not significantly reduce the size of the malignant clone in most patients.[4,5,25,26] Moreover, a large proportion of patients discontinue ruxolitinib therapy due both to intolerance and to the development of resistance or relapse.[6] We therefore investigated whether low doses of linsitinib and ruxolitinib synergize to reduce proliferation and induce apoptosis in BaF/3 cells. In the presence of cytokines, the low doses used showed no effect either alone or in combination, irrespective of the presence of Jak2$^{V617F}$ (Figure 5). However, in cytokine-independent Jak2$^{V617F}$ cells, while low doses of either drug alone again showed no effect, combined Jak and Igf-1R inhibition synergized both in reducing proliferation and in inducing apoptosis (Figure 5A–C and Supplemental Digital Content, Figure 5, http://links.lww.com/HS/A153). Concomitantly, IGF-1R phosphorylation was decreased significantly by the combination treatment in comparison to ruxolitinib alone (Figure 5D, E). Stat5 phosphorylation was inhibited significantly more by the combination of low doses of both drugs, than by either agent

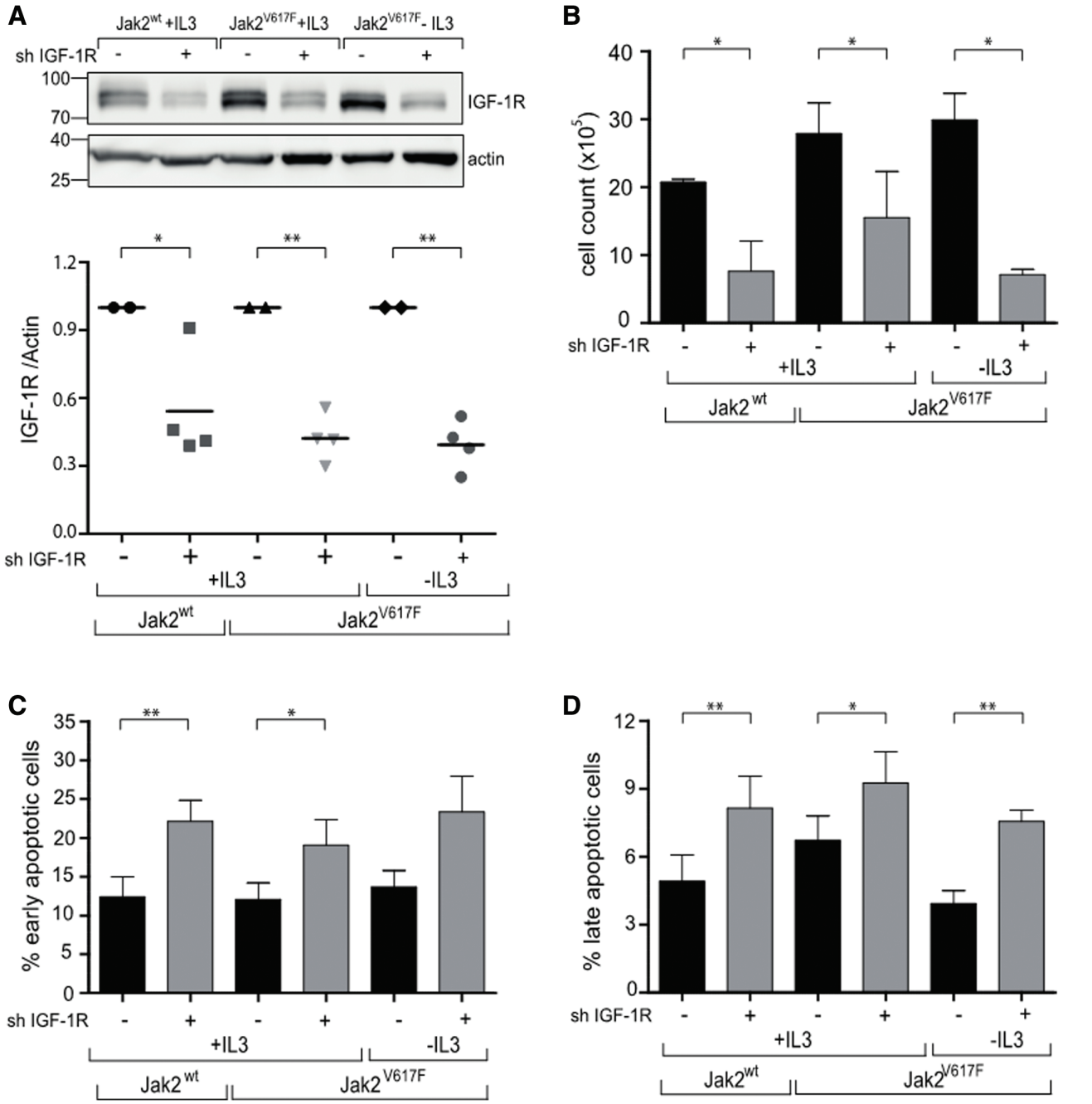


**Figure 1. Knockdown of IGF-1R reduces cell proliferation and increases apoptosis in Ba/F3 cells.** Ba/F3 cells expressing either Jak2$^{wt}$ or Jak2$^{V617F}$ in the presence or absence of IL-3 were infected with a lentivirus containing an shRNA against IGF-1R or a scr control shRNA at an MOI of 5. (A), Protein was isolated 96 h after infection. Top: Representative western blot using an antibody against IGF-1R. Actin was used as a loading control. Bottom: Densitometric analysis of all western blots. Horizontal lines represent mean values. (B), Total cell counts assessed by trypan blue exclusion assay. (C, D), Infected cells were stained with an AF647-coupled annexinV and PI followed by FACS analysis. (C), Percentages of early apoptotic (annexinV positive) and (D) late apoptotic (annexinV and PI double positive) cells are shown. Bars represent mean and SEM of 3 independent experiments conducted in duplicate. Paired 2-tailed Student *t* test was performed to compare IGF-1R depletion to control conditions . $^{*}P < 0.05$, $^{**}P < 0.01$. AF647 = alexa fluor 647; FACS = fluorescence activated cell sorting; IGF-1R = insulin growth factor-1 receptor; IL3 = interleukin 3; MOI = multiplicity of infection; PI = propidium iodide; scr = scrambled; SEM = standard error of mean; shRNA = short hairpin RNA.

alone (Figure 5D, E and Supplemental Digital Content, Figure 6, http://links.lww.com/HS/A153). Again, these effects were not observed in cells expressing either Jak2$^{wt}$ or Jak2$^{V617F}$ grown in the presence of IL-3 (Supplemental Digital Content, Figure 7, http://links.lww.com/HS/A153). These data suggest that low doses of linsitinib and ruxolitinib may selectively and synergistically affect the Jak2-mutated clone. In addition to Jak1 and Jak2, ruxolitinib has been shown to inhibit the MAPK/ERK kinases 2 and 3[27] among others. Therefore, it is possible that the synergistic effect of ruxolitinib and IGF-1R inhibition is due to non Jak-mediated effects on the part of the former. On the other hand, Gupta et al[28] have demonstrated an interaction between IGF-1R and JAK2 in enhancing neurite outgrowth of neuronal cells, providing evidence that both signaling pathways converge

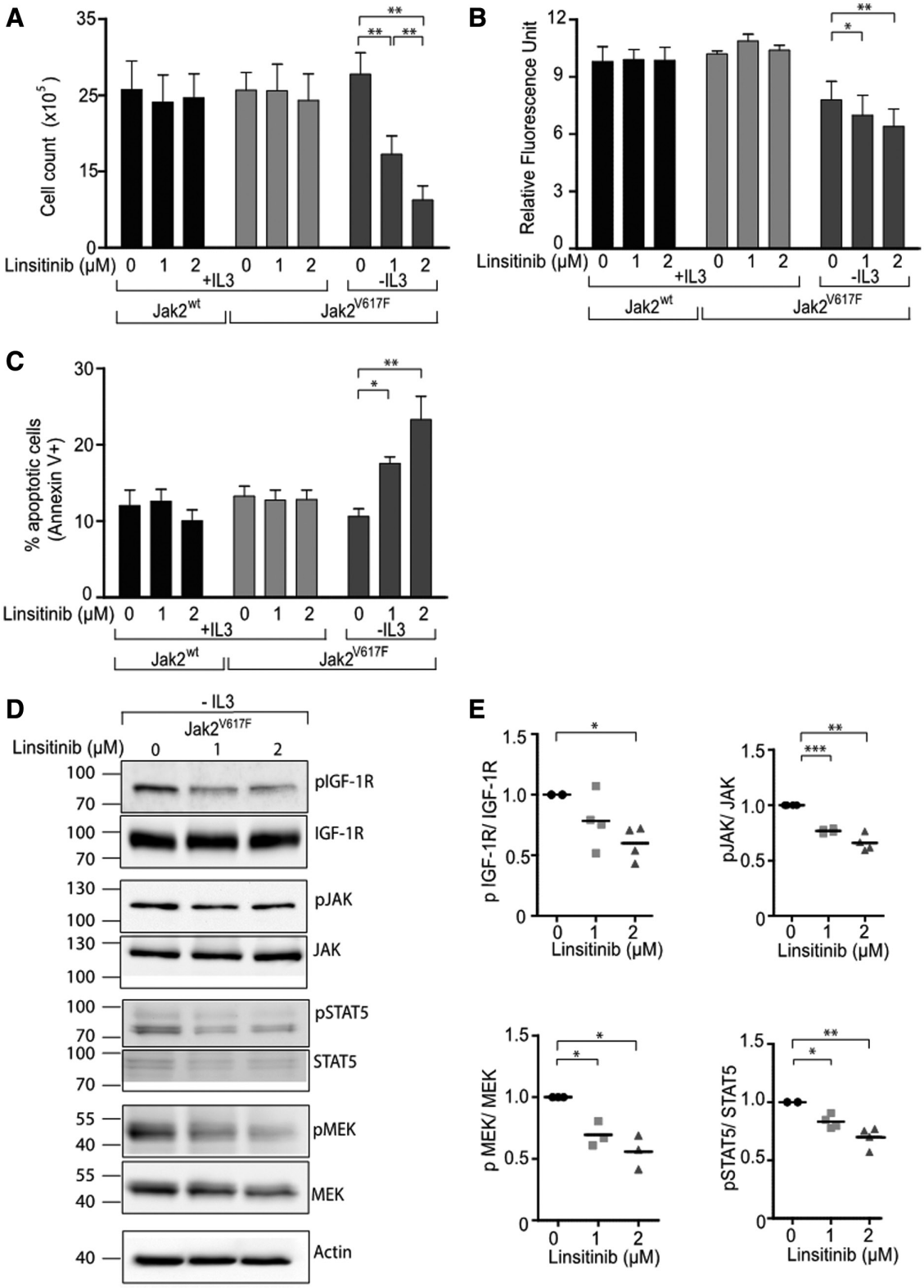


**Figure 2. The IGF-1R inhibitor linsitinib selectively reduces cell proliferation and increases apoptosis of cytokine-independent Jak2V617F-positive Ba/F3 cells.** Ba/F3 cells expressing Jak2$^{WT}$ or Jak2$^{V617F}$, in the presence or absence of IL-3, were treated with DMSO, 1 or 2 μM linsitinib. (A), Total cell counts assessed by trypan blue exclusion assay 96 h after treatment are shown. (B), Alamar blue cell proliferation assay 96 h after treatment is depicted. (C), Treated Ba/F3 cells were stained with an AF647-coupled annexinV and PI, followed by FACS analysis. Percentages of apoptotic cells (annexinV and PI positive) are shown. Bars in (A–C) represent mean and SEM of 3 independent experiments conducted in duplicate. (D), Representative western blots determining phosphorylation of indicated signal transducers in serum-starved IL-3–independent Jak2$^{V617F}$ Ba/F3 cells treated with linsitinib as indicated for 6 h. Actin was used as a loading control. (E), Densitometric analysis of western blots from 4 independent experiments. Data and mean are shown. Paired 2-tailed Student *t* test was used for pairwise comparison. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. AF647 = alexa fluor 647; DMSO = dimethyl sulfoxide; FACS = fluorescence activated cell sorting; IGF-1R = insulin growth factor-1 receptor; ; IL-3 = interleukin 3; Jak = janus kinase; MEK = MAPK/ERK kinase PI = propidium iodide; pIGF-R = phosphorylated insulin like growth factor-1-receptor; pJak = phosphorylated Janus kinase; pMEK = phosphorylated MAPK/ERK kinase; pSTAT5 = phosphorylated signal transducer and activator of transcription 5; SEM = standard error of mean; STAT5 = signal transducer and activator of transcription 5.

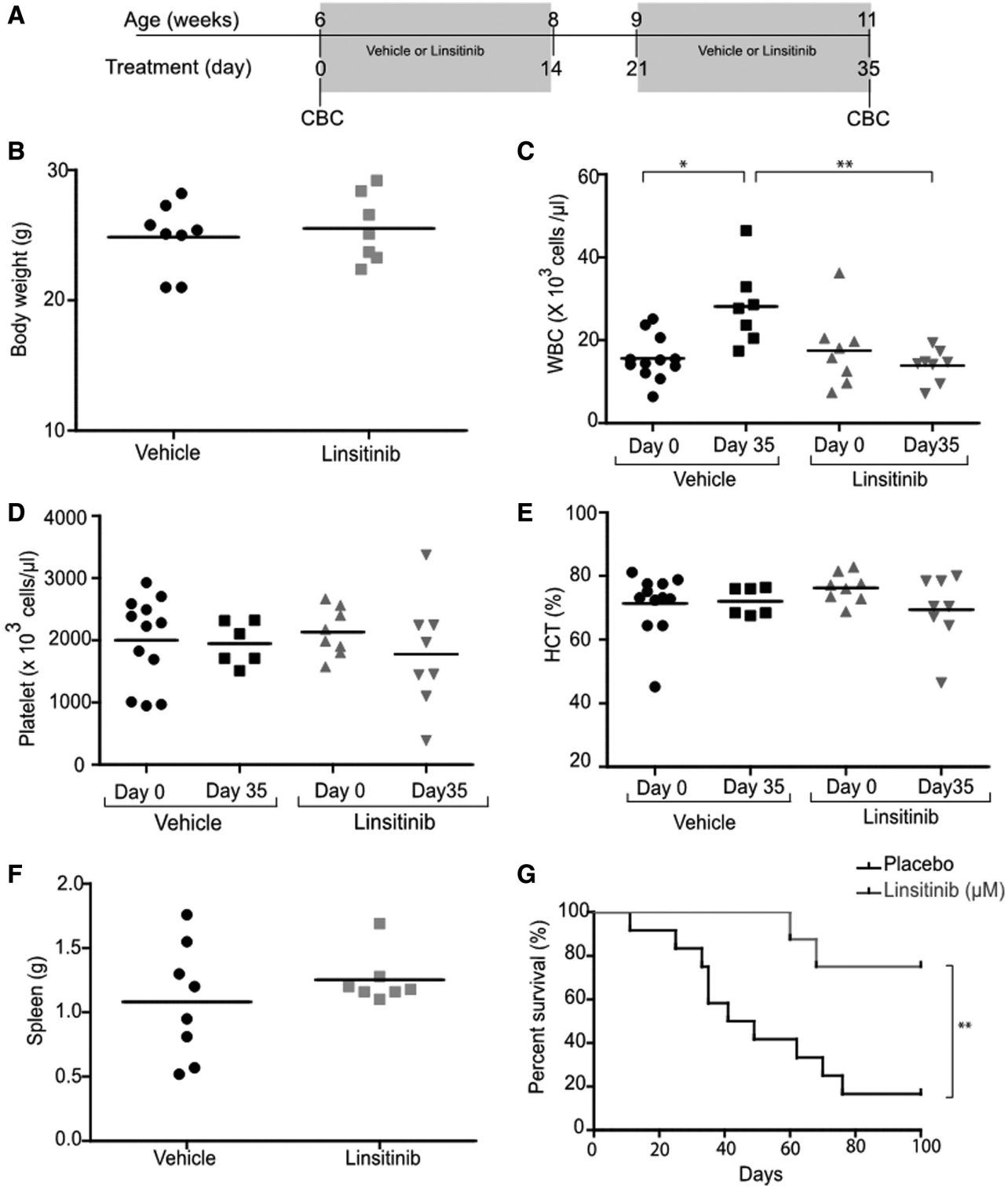


**Figure 3. Linsitinib reduces disease progression and increases survival of Jak2V617F knock-in mice.** (A), Schematic of the treatment plan. Six week old mice were treated with either vehicle (25 mM tartaric acid; n = 12) or 25 mg/kg linsitinib (n = 8) for 14 d followed by a drug holiday of 7 d and a second cycle treatment of 14 d. Blood was drawn on day 0 and 35. (B) Body weight and mean on day 35 is shown. (C, D), Hematological parameters at the indicated time points. (C), WBC counts. (D), Platelet numbers. (E), Hematocrit. Unpaired 2-tailed Student *t* tests were used for pre-post comparisons and for the assessment of linsitinib effects. $^{*}P < 0.05$, $^{**}P < 0.01$. (F), Spleen weight. (G), Kaplan-Meier survival curve of vehicle and linsitinib-treated mice. For statistical analysis, the log rank (Mantel-Cox) test was used. $^{**}P < 0.01$. CBC = complete blood counts; HCT = hematocrit; WBC = white blood cell counts.

and suggesting a mechanism for synergy albeit in a different cell type.

To test whether the synergism observed in the cell culture model is also effective in vivo, we treated *Mx*-Jak2$^{V617F}$ mice[17] displaying a full MPN phenotype with a vehicle control or with either low dose ruxolitinib or linsitinib single agent or with the combination of both drugs (Figure 6A). Six week old mice were treated on a 2 weeks on, 1 week off schedule for a total of 17

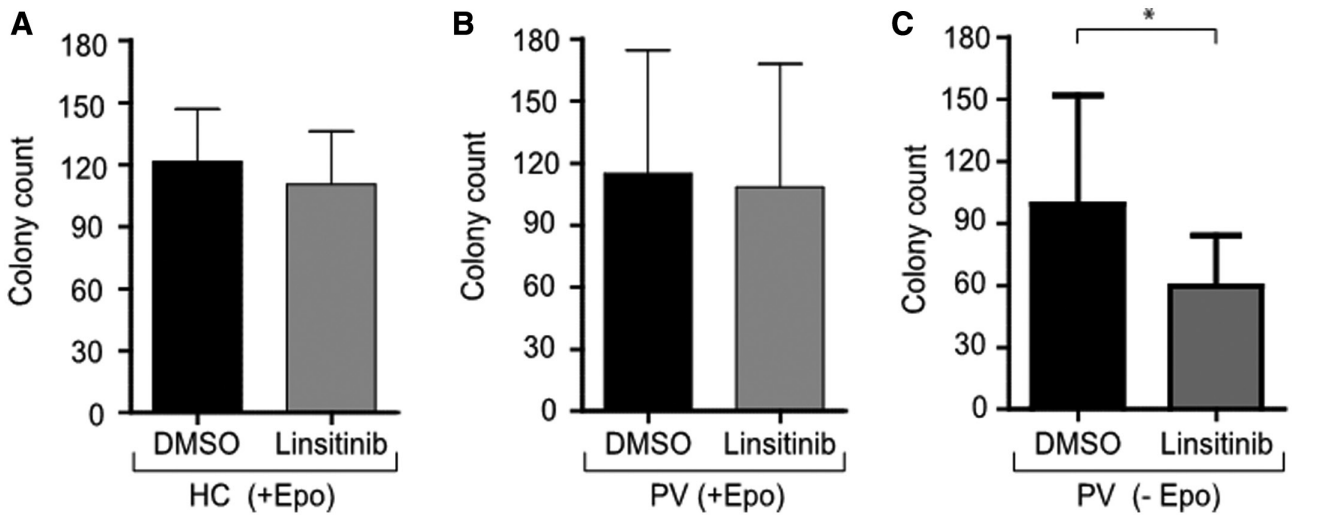


**Figure 4. Linsitinib targets the erythropoietin-independent clone in PV patients.** (A–C), Enumeration of colonies grown from MNCs of (A) HC and (B, C) PV patients in the presence (A, B) or absence (C) of EPO with or without linsitinib treatment. Colonies were scored on day 14. Bars show colony count per milliliter as mean and SEM of n = 5 healthy controls and n = 5 PV patients. Wilcoxon matched-pairs signed rank test was used for pairwise comparison. *$P < 0.05$. DMSO = dimethyl sulfoxide; EPO = erythropoietin; HC = healthy controls; MNC = mononuclear cell; PV = polycythemia vera; SEM = standard error of mean.

weeks. The combination regiment caused minimal toxicity as the mice maintained the same weight as the vehicle control–treated mice throughout the 17-week period (Figure 6B). As we have previously shown for this MPN model,[17] vehicle-treated mice died rapidly, before the end of the 77-day treatment. Neither low dose ruxolitinib nor linsitinib monotherapy prolonged survival. In contrast, the combination of both drugs significantly increased survival compared to vehicle or ruxolitinib alone (Figure 6C). While red blood cells and platelets were unaffected (Figure 6D–F) both WBC counts and spleen weights were significantly reduced by the combination treatment (Figure 6G, H). However, substantial splenomegaly persists in animals treated with both ruxolitinib and linsitinib. Nonetheless, combined Jak2 and IGF-1R inhibition ameliorated the MPN phenotype and significantly prolonged survival in this murine MPN model.

Because clinical development of linsitinib was paused, we examined the effect of a second IGF-1R inhibitor, PPP (PPP/AXL1717) currently undergoing phase I/II investigation for non-small cell lung carcinoma (NSCLC), squamous cell carcinomas and adenocarcinomas of the lung, as well as for malignant astrocytoma.[29] Similar to linsitinib (Figure 2A), PPP did not affect IL-3–stimulated proliferation of either $Jak2^{wt}$ or $Jak2^{V617F}$ cells but significantly reduced cytokine independent growth of $Jak2^{V617F}$ expressing Ba/F3 cells (Figure 7A). Concomitantly, PPP increased apoptosis selectively in the absence of cytokine (Figure 7B and Supplemental Digital Content, Figure 8, http://links.lww.com/HS/A153).

To further investigate the selectivity of PPP against $Jak2^{V617F}$ cells, we lentivirally transduced the $Jak2^{wt}$ or $Jak2^{V617F}$ expressing Ba/F3 cells, that express GFP, to express mCherry in addition. mCherry-positive Ba/F3 $Jak2^{wt}$ or $Jak2^{V617F}$ cells were cultured alone with increasing concentrations of PPP in a lower dose of IL-3 (0.1 ng/mL) which still allows the growth of $Jak2^{wt}$ cells. Again, PPP treatment decreased the viability and cell numbers of $Jak2^{V617F}$ but not of $Jak2^{wt}$ cells (Figure 7C, D, left and middle histograms). Hence the presence of mCherry did not alter our previous observations of selective inhibition of $Jak2^{V617F}$ cell growth (Figure 7A, B).

Subsequently, we mixed $Jak2^{wt}$ cells, expressing only GFP with $Jak2^{V617F}$ cells that express both GFP and mCherry at a 1:1 ratio and determined the percentage of viable and mCherry-positive cells following 72 hours of culture with increasing doses of PPP (Figure 7C, D, right histograms). In this coculture, the number of mCherry-positive $Jak2^{V617F}$ cells declined significantly with PPP treatment. Furthermore, we conducted the identical set of experiments detailed earlier, this time treating the cells either with lower doses of PPP or ruxolitinib alone or with a combination of both drugs. Again, $Jak2^{V617F}$-expressing Ba/F3 cells were selectively targeted by either PPP alone or by the drug combination (Figure 7E, F). At the level of protein phosphorylation, Jak2/Stat5 inhibition selectively occurs in cytokine-independent cells, grown in the absence of growth factor as these effects were not observed in cells expressing either $Jak2^{wt}$ or $Jak2^{V617F}$ grown in the presence of IL-3 (Figure 7G, H as well as Supplemental Digital Content, Figure 9, http://links.lww.com/HS/A153 and Figure 10, http://links.lww.com/HS/A153).

We probed the efficacy of PPP in our MPN mouse model, employing the same schedule of four 14-day treatment cycles with intermittent 7-day drug holidays (Figure 8A). Six week old mice were treated with PPP (20 mg/kg; n = 9) or vehicle alone (sodium citrate; n = 11). PPP treatment was well tolerated as mice maintained their body weight (Figure 8B). IGF-1R inhibition again significantly enhanced overall survival (Figure 8C) and, while it had no effect on the hematocrit (Figure 8D), it limited the expansion of leukocytes (Figure 8E), especially the expansion of neutrophils (Figure 8F) and significantly lowered platelet numbers (Figure 8G). The decrease in neutrophils was confirmed by FACS analysis of both peripheral blood and spleen at autopsy, which revealed a significant decrease in the percentage of $Gr\text{-}1^{+}/Mac\text{-}1^{+}$ cells in both compartments (Figure 8H, I). Splenomegaly was also significantly reduced by PPP treatment (Figure 8J).

Because animals in our *Mx-Jak2*$^{V617F}$ mouse strain carry both $Jak2^{wt}$ and $Jak2^{V617F}$ cells, we genotyped erythroid colonies from animals treated with vehicle or with PPP to determine whether IGF-1R inhibition selectively reduced $Jak2^{V617F}$-positive cells. As the $JAK2^{V617F}$ clone has an augmented erythroid drive in mice,[16] an average of 96% of colonies treated with vehicle are $JAK2^{V617F}$ by genotype (Figure 8K). Treatment with PPP reduced the percentage of $JAK2^{V617F}$ cells to an average of 72%, allowing the growth of 28% wt cells. These data suggest that IGF-1R inhibition could allow re-emergence of wt hematopoiesis, known to remain in MPN patients often even after long disease durations.

To assess the effect of PPP in a second, independent murine model, we used a bone marrow transplant model, in which $Jak2^{wt}$ cells, carrying the CD45.1 isotype and $Jak2^{V617F}$ cells, carrying

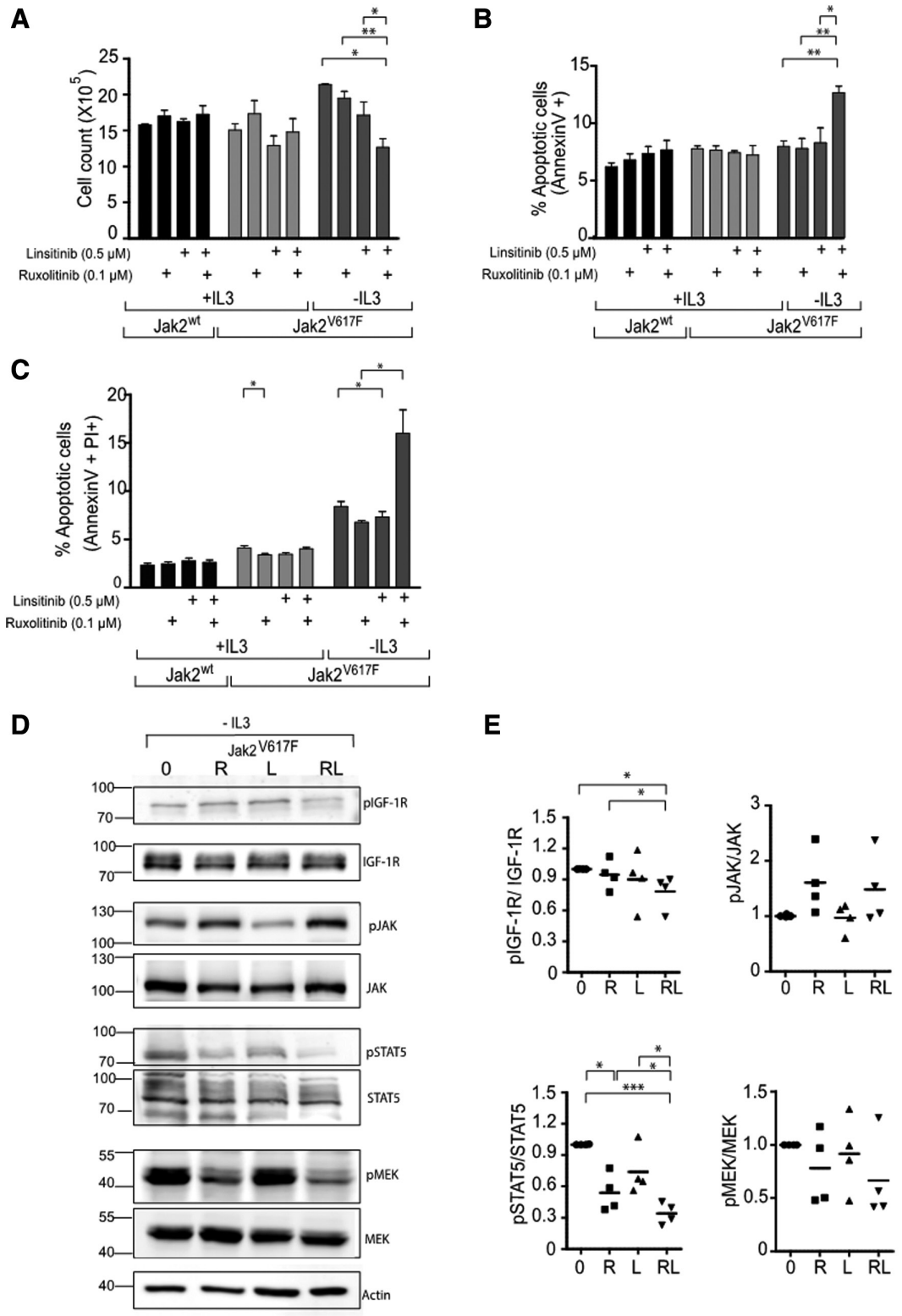


**Figure 5. Ruxolitinib and linsitinib synergistically target cell proliferation and increase apoptosis of cytokine-independent Jak2V617F Ba/F3 cells.** (A–D), Ba/F3 cells expressing either $Jak2^{WT}$ or $Jak2^{V617F}$ in the presence or absence of IL-3 were treated with V, 0.1 μM R, 0.5 μM L or both RL. (A), Total cell counts measured by trypan blue exclusion assay after 48 h are shown. (B, C), Treated Ba/F3 cells were stained with AF647-coupled annexinV and PI and analyzed by FACS. Percentages of (B) early apoptotic cells (annexinV positive) and (C) late apoptotic cells (annexinV and PI double positive) are shown. Bars represents mean and SEM of 4 independent experiments conducted in duplicate. (D), Representative western blots determining phosphorylation of indicated signal transducers in serum-starved IL-3–independent $Jak2^{V617F}$ Ba/F3 cells treated with V, R, L, or RL for 6 h are shown. Actin was used as a loading control. (E), Densitometric analysis of all western blots is shown. Paired 2-tailed Student *t* test was performed for pairwise comparison. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.
AF647 = alexa fluor 647; DMSO = dimethyl sulfoxide; FACS = fluorescence activated cell sorting; IGF-1R = insulin growth factor-1 receptor; IL-3 = interleukin 3; JAK = janus kinase; L = linsitinib; MEK = MAPK/ERK kinase; PI = propidium iodide; pIGF-1R = phosphorylated insulin like growth factor -1-receptor; pJak = phosphorylated janus kinase; pMEK = phosphorylated MAPK/ERK kinase; pSTAT5 = phosphorylated signal transducer and activator of transcription 5; R = ruxolitinib; SEM = standard error of mean; STAT5 = signal transducer and activator of transcription 5; V = DMSO.

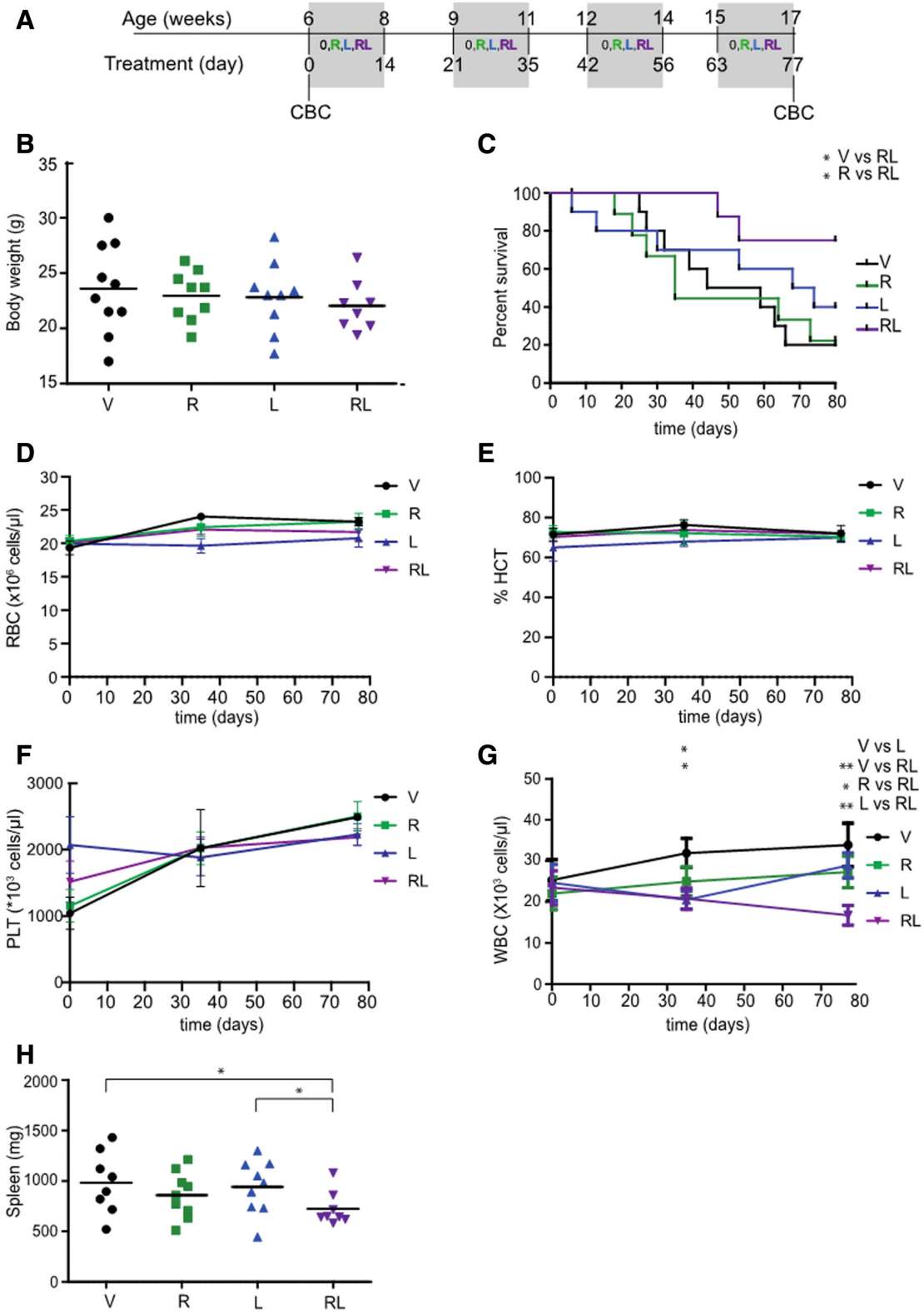


**Figure 6. Combination treatment of ruxolitinib and linsitinib reduces disease progression and increases survival of Jak2V617 knock-in mice.** (A), Schematic of the treatment plan. Six week old mice at the age of 6 wk were treated with either vehicle as 25 mM V (n = 10), 60 mg/kg R (n = 10), 15 mg/kg L (n = 10) or both (RL; n = 8) for 14 d in 4 cycles with 7-d drug holiday in between each cycle. Blood was drawn on days 0, 35, and 77 for analysis of hematological parameters. (B), Body weights of the treated mice at the end of treatment are shown. (C), Kaplan-Meier survival curve of V-, R-, L-, or RL-treated mice is shown. For statistical analysis, the log rank (Mantel-Cox) test was performed. *$P < 0.05$. (D–G), CBCs on days 0, 35, and 77 are shown: (D), RBC; (E), HCT; (F), PLT; (G), WBC counts; (H), spleen weight of treated mice is depicted. (B, D–H), Data along with mean value are represented. Unpaired 1-tailed Student *t* test was performed to compare between treated groups. *$P < 0.05$, **$P < 0.01$. CBC = complete blood counts; HCT = hematocrit; L = linsitinib; PLT = platelets; R = ruxolitinib; RBCs = red blood cells; V = tartaric acid; WBC = white blood cells.

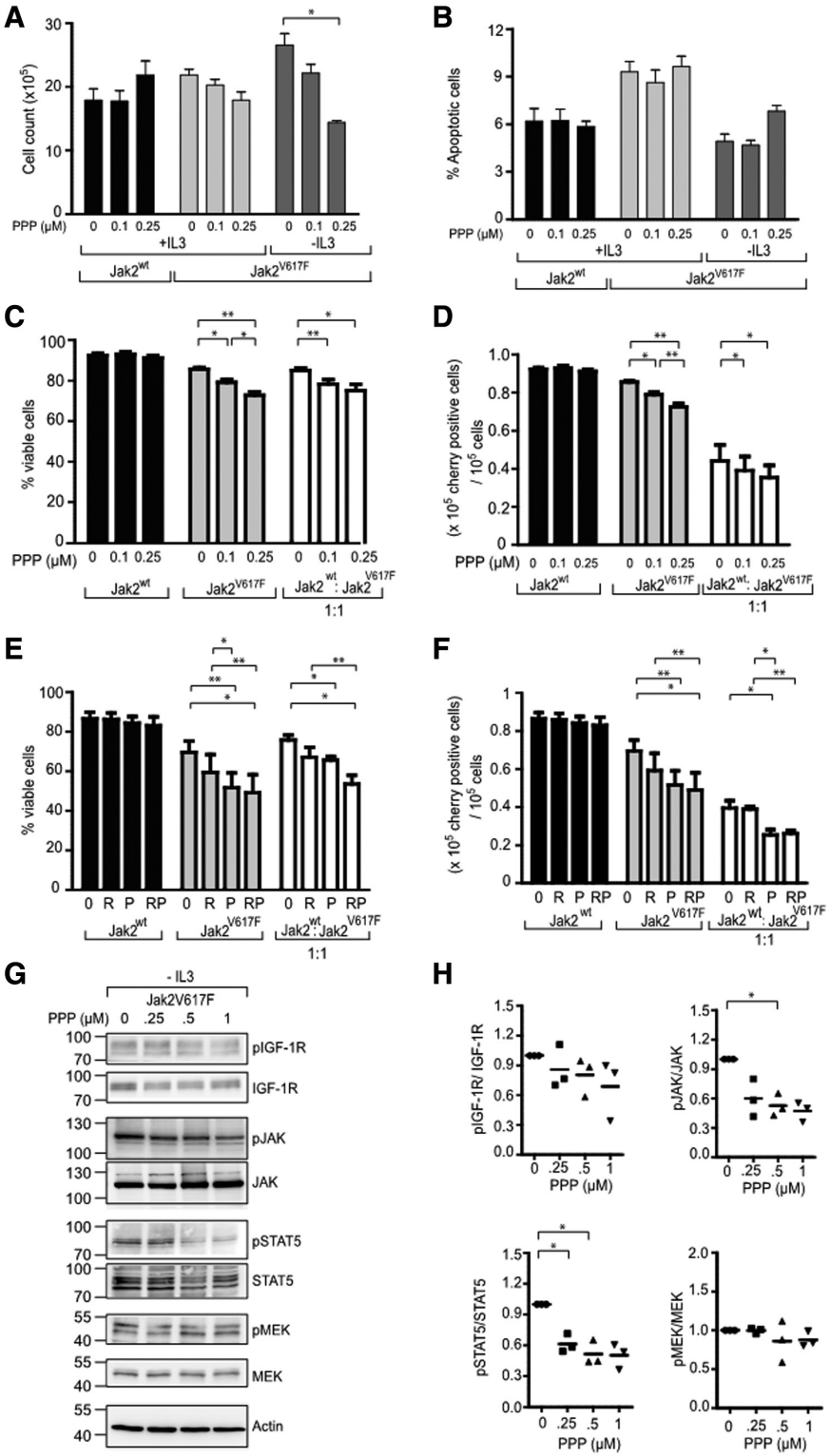


**Figure 7. The IGF-1R inhibitor PPP selectively reduces cell proliferation and increases apoptosis of cytokine-independent Jak2V617F Ba/F3 cells.** (A–D), Ba/F3 cells expressing Jak2$^{WT}$ or Jak2$^{V617F}$ in the presence or absence of IL-3 were treated with DMSO, 0.1 or 0.25 μM PPP. (A), Total cell counts assessed by trypan blue exclusion assay 96 h after treatment are shown. (B), Treated Ba/F3 cells were stained with an AF647-coupled annexinV and PI and analyzed by FACS. Percentages of apoptotic cells (annexinV/PI positive cells) are depicted. Bars represent mean and SEM of 4 independent experiments performed in duplicate. (C–F), Ba/F3 expressing either Jak2$^{wt}$ or Jak2$^{V617F}$, as depicted were lentivirally transduced to express mCherry in addition and cultured either separately (left and middle sets of histograms) or mixed at a 1:1 ratio (Jak2$^{wt}$-GFP and Jak2$^{V617F}$-GFP + mCherry, left sets of histograms) in increasing concentrations of PPP for 72 h (C and D) or treated with O or P or R alone or with a RP (E and F), and the percentage of viable cells (C and E) and mCherry-positive cells (D and F) determined. Bars represent mean and SEM of 4 independent experiments performed in duplicate. (G), Representative western blots determining phosphorylation of the indicated signal transducers in serum-starved IL-3–independent Jak2$^{V617F}$ Ba/F3 cells treated with PPP for 6 h are shown. Actin was used as a loading control. (H), Densitometric analysis of all western blots is shown. Data along with mean of 3 independent experiments. Paired 2-tailed Student *t* test was used for pairwise comparison. *$P < 0.05$. **$P < 0.01$. AF647 = alexa fluor 647; DMSO = dimethyl sulfoxide; FACS = fluorescence activated cell sorting; GFP = green fluroescent protein; IGF-1R = insulin growth factor-1 receptor; IL-3, PI = propidium iodide; O = DMSO; P = PPP; PPP = picropodophyllin; R = ruxolitinib; RP = combination of R and PPP; SEM = standard error of mean.

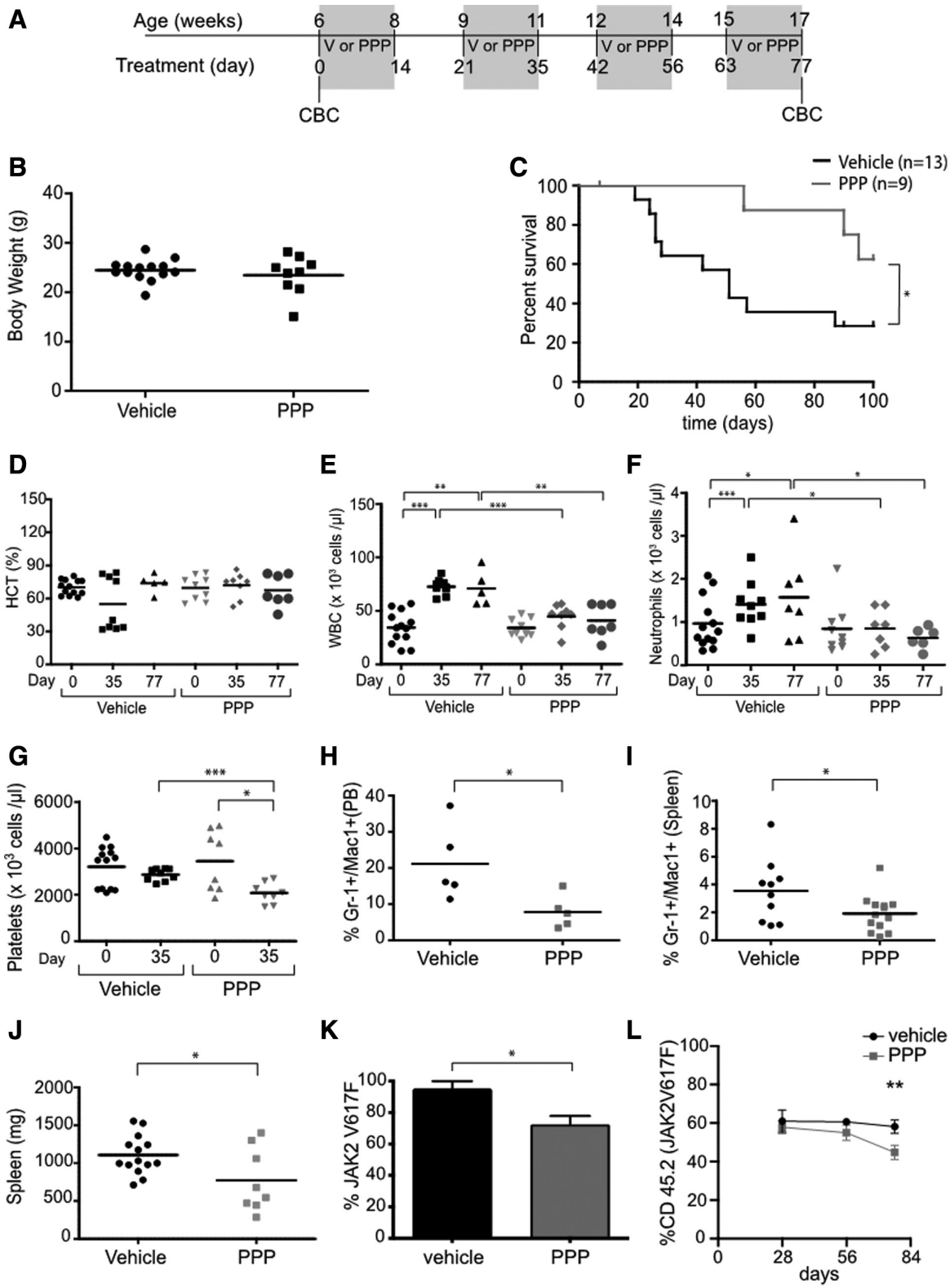


**Figure 8. PPP reduces disease progression and improves survival of Jak2V617F knock-in mice.** (A), Schematic of the treatment plan. Six week old mice were treated with vehicle (sodium citrate) (n = 14) or 20 mg/kg PPP (n = 9) for 14 d in 4 cycles with 7-d drug holiday in between each cycle. Blood was drawn on days 0, 35, and 77 for analysis of hematological parameters. (B), Body weight of treated mice at the end of the analysis is shown. (C), Kaplan-Meier survival curve of 12 vehicle and 8 PPP treated *Mx*-Jak2$^{V617F}$ mice is shown. For statistical analysis, the log rank (Mantel-Cox) test was performed. $P < 0.05$. (D), HCT. (E), WBC counts. (F), Neutrophil numbers; (G), platelet counts. (H and I), Percentage of Gr-1$^+$/Mac-1$^+$ cells determined by FASC analysis of peripheral blood (H) and spleens (I) at autopsy. (J), Spleen weights at the end of analysis are shown. Data along with mean is represented. Paired Student *t* test was performed for pre-post treatment comparison and unpaired Student *t* test was performed for assessment of PPP effect. (K), Genotype of erythroid colonies grown from mice treated either with vehicle (n = 27) or PPP (20 mg/kg; n = 22) as indicated. (L), Percentage of CD45.2/Jak2$^{V617F}$-positive cells in mice transplanted with a 1:1 ratio of CD45.1/Jak2$^{wt}$ and CD45.2/Jak2$^{V617F}$ cells following PPP (20 mg/kg) or vehicle treatment for the indicated number of days (n = 4 each). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. CBC = complete blood counts; HCT = hematocrit; PB = peripheral blood; PPP = picropodophyllin; V = vehicle; WBC = white blood cell.

the CD45.2 isotype, were mixed at a 1:1 ratio and transplanted into lethally irradiated mice. Beginning 2 weeks after transplantation, mice were treated with either vehicle or PPP (20 mg/kg), with the dosing schedule described above. Peripheral blood was analyzed for CD45 isotype on days 28, 56, and 84 of treatment. As shown in Figure 8L, PPP treatment led to a continuous depletion of Jak2$^{V617F}$ cells, while vehicle treatment had no effect on the percentage of Jak2$^{V617F}$ cells.

Our data demonstrate preclinical efficacy of IGF-1R inhibition in 2 different MPN models.

## Discussion

Because of the persistent need for more efficacious therapies for MPN patients, we investigated the effect of genetic or pharmacological inhibition of IGF-1R signaling. We show that 2 structurally unrelated IGF-1R inhibitors have similar effects both on Jak2$^{V617F}$-dependent cell lines and in an MPN murine model. The 2 inhibitors used, linsitinib and PPP, differ in their substrate specificity and in their mechanism of action. Linsitinib, an adenosine triphosphate (ATP) competitive inhibitor, prevents both IR and IGF-1R signaling, while PPP is a IGF-1R–specific substrate competitive inhibitor.[30,31] While we acknowledge that all inhibitors carry the risk of off-target effects, the 2 structurally dissimilar compounds have very similar effects in our models. We therefore propose that the observed effects result from inhibition of IGF-1R autophosphorylation, which both drugs effect.

Many preclinical and clinical studies are currently investigating combination therapies to improve the efficacy of ruxolitinib therapy and to postpone the development of drug resistance. These include combination with the hypomethylating agents decitabine or azacytidine (NCT02076191 and NCT01787487), with the PI3Kinase delta inhibitors TGR-1202 or Parsaclisib (NCT02493530 and NCT02718300), the JAK1 inhibitor Itacitinib (NCT03144687), the BET inhibitor CPI-0610 (NCT02158858), the HDAC inhibitor Pacrinostat (NCT02267278), and the ubiquitin ligase modulator lenalidomide (NCT01375140) to name just a few. Given this plethora of possible combinations, what are the distinct advantages of adding yet another candidate? We believe that the pathophysiological rationale for IGF-1R inhibition in MPN is compelling. The argument arises from the detailed and meticulous work by Dr Axelrad and colleagues, recently continued by Dr Constantinescu's laboratory.[12–15] The data consistently show hypersensitive and aberrant IGF-1R signaling in MPN. The pathognomonic aberrant erythroid colony growth in MPN is due to IGF-1 hypersensitivity. Moreover, IGF-1 stimulation selectively increases proliferation of JAK2$^{V617F}$, but not JAK2$^{wt}$ expressing cells[15] offering a discriminatory feature between the neoplastic clone and the residual healthy hematopoiesis that persists in most patients.

Our data provide evidence in a murine model that IGF-1R inhibition, alone or in low-dose combination with ruxolitinib can curtail disease progression and prolong survival. While erythrocytosis and splenomegaly are only marginally affected by IGF-1R inhibition, the time-dependent increase in WBC is significantly curtailed both by monotherapy and in combination (Figures 3C and 6D). We propose that reduced leukocytosis and delay of the accompanying organ failure contributes to increased survival in our model.

Devising novel therapeutic regiments for MPN patients is demanding for a delightful reason: despite the considerable burden these diagnoses place on patients, life expectancy is often measured in decades and quality of life can be enjoyably high for extended times. Therefore, new drug candidates must possess very favorable toxicity, safety and tolerability profiles to be considered for MPN patients, especially when early intervention with the intention of preventing progression is contemplated. The IGF-1 inhibitor PPP/AXL1717 showed limited toxicity in a trial of NSCLC patients.[29] Moreover, several antibodies against IGF-1R have been developed including cixutumumab, which has already completed several clinical trials in patients with sarcoma, liver cancer, NSCLC, and prostate cancer. Anti-IGF-1R antibody therapy could provide a particularly nontoxic approach for MPN patients.

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### Disclosures

The authors have no conflicts of interest to disclose.

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