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Genetic ablation of Pim1 or pharmacologic inhibition with TP-3654 ameliorates myelofibrosis in murine models

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Abstract

Myelofibrosis (MF) is the deadliest form of myeloproliferative neoplasm (MPN). The JAK inhibitor Ruxolitinib can reduce constitutional symptoms but it does not substantially improve bone marrow fibrosis. Pim1 expression is significantly elevated in MPN/MF hematopoietic progenitors. Here, we show that genetic ablation of Pim1 blocked the development of myelofibrosis induced by Jak2V617F and MPLW515L. Pharmacologic inhibition of Pim1

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AUTHOR CONTRIBUTIONS

A.D. performed research, analyzed data and wrote the manuscript; D.N. performed research and analyzed data; Y.Y performed research; B.T.L. performed data analysis; M.F.R. performed research; P.F. performed research; Z.W., R.H., and W.T. performed RNA-seq data analysis; M.S. performed research; R.E.H. performed histopathologic analysis; J.M.F and S.L.W. provided TP-3654 and suggestions for research design; C.Z. supervised RNA-seq data analysis; G.M. designed the research, analyzed data and wrote the manuscript.

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with a second-generation Pim kinase inhibitor TP-3654 significantly reduced leukocytosis and splenomegaly, and attenuated bone marrow fibrosis in Jak2V617F and MPLW515L mouse models of MF. Combined treatment of TP-3654 and Ruxolitinib resulted in greater reduction of spleen size, normalization of blood leukocyte counts and abrogation of bone marrow fibrosis in murine models of MF. TP-3654 treatment also preferentially inhibited Jak2V617F mutant hematopoietic progenitors in mice. Mechanistically, we show that TP-3654 treatment significantly inhibits mTORC1, MYC and TGF-β signaling in Jak2V617F mutant hematopoietic cells and diminishes the expression of fibrotic markers in the bone marrow. Collectively, our results suggest that Pim1 plays an important role in the pathogenesis of MF, and inhibition of Pim1 with TP-3654 might be useful for treatment of MF.

Keywords

Myelofibrosis; PIM1; JAK2; TP-3654; Ruxolitinib

INTRODUCTION

Myelofibrosis (MF) is the most severe form of myeloproliferative neoplasms (MPN) characterized by bone marrow fibrosis, aberrant hematopoiesis, splenomegaly and frequent transformation to acute myeloid leukemia. Although the overall median survival for patients with MF is ~5 years, those with intermediate and high-risk disease have a median survival of 16 to 35 months [1, 2]. A somatic JAK2V617F mutation was found in 50-60% patients with MF [3-6]. Mutations in thrombopoietin receptor (MPL) and calreticulin (CALR) were also detected in MF [7-11]. Constitutive activation of the JAK/STAT signaling has been observed in MPN/MF hematopoietic cells [11]. Currently approved JAK inhibitors, Ruxolitinib and Fedratinib, can reduce splenomegaly and alleviate constitutional symptoms but they do not offer significant improvement of bone marrow (BM) fibrosis [12-16]. While JAK inhibitor therapy represents a major advancement in the management of MF, treatment failure and transformation to acute myeloid leukemia are observed in many cases [17]. Therefore, there is an unmet need to identify new therapeutic targets and develop novel therapies for MF.

The proviral integration site for Moloney murine leukemia virus (Pim) family of serine/threonine kinases have been implicated in the regulation of cell growth, survival and cell cycle [18]. Pim kinases have been shown to be overexpressed in various human cancers [19]. Furthermore, overexpression of Pim kinases has been linked to drug resistance in cancer [20, 21]. Small molecule Pim kinase inhibitors have been developed and undergoing testing in various malignancies [22-26]. Unlike Jak2 knockout mice, which exhibit severe defects in hematopoietic development [27, 28], mice deficient for all three Pim kinases (Pim1/Pim2/Pim3) are viable and fertile [29], indicating that Pim kinases are not essential for tissue homeostasis and targeting of Pim kinases will have wide therapeutic window.

We found that Pim1 expression is significantly increased in hematopoietic progenitors of Jak2V617F knock-in mice and MF patients. So, we hypothesize that Pim1 might play a role in the pathogenesis of MF. In this study, we investigated the effects of Pim1 deletion in Jak2V617F and MPLW515L-induced myelofibrosis. We also tested the efficacy of TP-3654,

a second-generation PIM1 kinase inhibitor, in murine models of MF. Here, we show that Pim1 plays an important role in the pathogenesis of MF, and inhibition of Pim1 by TP-3654 significantly reduces MPN disease burden and ameliorates bone marrow fibrosis in murine models.

MATERIALS AND METHODS

Mice

Pim1 and Pim2 knock-out [29], conditional Jak2V617F knock-in [30] and Mx1-Cre [31] mice were previously described. UBC-GFP mouse (stock # 004353) was purchased from the Jackson Laboratory. All animal studies were performed in accordance with the guidelines approved by the IACUC of University of Virginia School of Medicine.

Patient samples

Collection of blood and bone marrow (BM) samples from MF patients was approved by the institutional review board of the University of Virginia School of Medicine. Written informed consent was obtained from all subjects in accordance with the Declaration of Helsinki.

Cell cultures

Murine BA/F3-EpoR-JAK2V617F, BA/F3-MPLW515L and BA/F3-MPL-CALRdel52 cells and Human HEL and SET2 cells were cultured in RPMI 1640 plus 10% FBS. BAF/3 (parent) and BA/F3-EpoR cells were cultured in RPMI 1640 plus 10% FBS and 1ng/ml IL-3. UKE-1 cells were cultured in IMDM supplemented with 10% FBS, 10% DHS and 1 μM Hydrocortisone.

Real-time quantitative PCR

Total RNA was extracted using RNeasy Mini kit (Qiagen) and cDNA samples were prepared by using QuantiTect Reverse Transcription kit (Qiagen). Real-time quantitative PCR (RT-qPCR) was performed with a Quantstudio3 machine (Applied Biosystems) using SYBR Green PCR master mix (Quatabio). The data were normalized to *GAPDH,18S*, or *HPRT* and fold changes in gene expression were determined by the ΔΔCt method. Sequences of the primers are available in Supplementary Table 1.

Inhibitors

TP-3654 was provided by Tolero Pharmaceuticals. Ruxolitinib was purchased from Chemietek. TP-3654 was administered by oral gavage at 150mg/kg qd and Ruxolitinib at 60mg/kg qd.

Statistical analysis

Results are expressed as mean ± SEM. Student's t test or analysis of variance (ANOVA) was used to determine the statistical significance (GraphPad, Prism Version 9). $P<0.05$ was considered statistically significant.

Additional Methods are available in the Supplementary Information.

RESULTS

Deletion of Pim1 inhibits the development of myelofibrosis in Jak2V617F mice

Gene expression analysis (GSE79198) revealed that Pim1 expression is significantly elevated in Jak2V617F mice LT-HSC compared with control LT-HSC (Fig. 1A). Analysis of MPN patient gene expression data (GSE54644) also showed significantly increased PIM1 expression in MF granulocytes compared with healthy controls (Fig. 1B). RT-qPCR analysis further validated significantly increased Pim1 expression in Jak2V617F mouse HSC and MF CD34+ cells compared with respective control cells (Fig. 1C-D).

Immunoblotting analysis showed increased PIM1 protein expression in the peripheral blood mononuclear cells (PBMC) and BM of MF patients compared with healthy controls (Supplementary Fig.1A-B). We also observed increased Pim1 protein expression in the BM of mice expressing Jak2V617F and MPLW515L (Supplementary Fig.1C-D). In addition, we found increased Pim1 protein expression in BA/F3 cells expressing JAK2V617F, MPLW515L or CALRdel52 mutant compared with BA/F3 parent cells (Supplementary Fig.1E).

Next, we investigated the effects of PIM1 depletion on JAK2V617F-expressing cells. We observed that lentiviral shRNA-mediated knockdown of PIM1 significantly inhibited the proliferation of JAK2V617F-expressing human HEL and UKE-1 cells and murine BA/F3-EpoR-JAK2V617F cells but not WT JAK2-expressing BA/F3-EpoR cells (Supplementary Fig. 2A-D), suggesting that Pim1 may play an important role in the survival/growth of MPN cells expressing oncogenic JAK2V617F mutant.

To assess the role of Pim1 in myelofibrosis induced by Jak2V617F, we crossed Pim1 knockout [29] mice with conditional Jak2V617F knock-in [30] and Mx1Cre [31] mice. Mice expressing heterozygous Jak2V617F exhibit a polycythemia vera (PV) phenotype, whereas mice expressing homozygous Jak2V617F rapidly develop BM fibrosis [30, 32]. We utilized homozygous Jak2V617F (Mx1Cre; $Jak2^{VF/VF}$) and Pim1-deficient homozygous Jak2V617F (Pim1KO; Mx1Cre; $Jak2^{VF/VF}$) mice to assess the role of Pim1 in Jak2V617F-induced MF. We transplanted BM cells (1 x 10^6 cells/recipient) from control (wild-type), Pim1KO, Mx1Cre; $Jak2^{VF/VF}$ (hereafter $Jak2^{VF/VF}$) and Pim1KO; Mx1Cre; $Jak2^{VF/VF}$ (hereafter Pim1KO; $Jak2^{VF/VF}$) mice into lethally irradiated syngeneic C57BL/6 recipient mice. At 4 weeks after transplantation, the recipient animals were injected with 3 doses of pI-pC to induce Cre-mediated Jak2V617F expression and the mice were analyzed at 16 weeks after pI-pC induction. $Jak2^{VF/VF}$ mice exhibited significant increase in white blood cell (WBC), neutrophil (NE), red blood cell (RBC), hematocrit (HCT) and platelet (PLT) counts compared to WT control mice (Fig. 1E). Deletion of Pim1 in the homozygous Jak2V617F mice significantly reduced the WBC and neutrophil counts to almost wild-type levels (Fig. 1E). Pim1 deletion alone in wild type background caused modest reduction in WBC and neutrophil counts at 16 weeks after transplantation whereas RBC, hematocrit and platelet counts were comparable to control wild type mice (Fig. 1E).

Flow cytometric analysis showed significant expansion of myeloid ($Gr\text{-}1^+/Mac\text{-}1^+$) cells in the BM and spleens of $Jak2^{VF/VF}$ mice (Fig. 1F). Deletion of Pim1 significantly

reduced Gr-1^{+}/Mac-1^{+} cells in the BM and spleens of Pim1KO; Jak2$^{VF/VF}$ mice (Fig. 1F). Flow cytometric analysis further revealed that Pim1 deletion significantly reduced the LSK (Lin^{-}Sca1^{+}c-kit^{+}) and myeloid progenitors (LK; Lin^{-}c-kit^{+}) in the BM of Pim1KO; Jak2$^{VF/VF}$ mice compared with Jak2$^{VF/VF}$ mice (Fig. 1G). We also observed a marked decrease in common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP), and megakaryocyte-erythroid progenitors (MEP) in the BM of Pim1KO; Jak2$^{VF/VF}$ mice compared to Jak2$^{VF/VF}$ mice (Fig. 1H). Hematopoietic progenitor colony assays showed significant reduction in CFU-GM colonies in the BM of Pim1KO; Jak2$^{VF/VF}$ mice as compared to Jak2$^{VF/VF}$ mice (Fig. 1I). Mice with Pim1 deletion alone exhibited modest reduction of BM LSK cells and CFU-GM colonies compared with wild type mice (Fig. 1G, I). The Jak2$^{VF/VF}$ mice exhibited enlargement of spleen (Fig. 1J), as expected. Pim1 deletion in Jak2$^{VF/VF}$ mice significantly reduced the spleen size/weight (Fig. 1J). Pim1 deletion in wild type background also caused reduction of spleen size (Fig. 1J). Histopathologic analysis showed extensive fibrosis in the BM of Jak2$^{VF/VF}$ mice (Fig. 1K). Deletion of Pim1 significantly reduced BM fibrosis in Pim1KO; Jak2$^{VF/VF}$ mice (Fig. 1K). Overall, these data suggest an important role of Pim1 in the pathogenesis of MF induced by Jak2V617F.

Deletion of Pim1 inhibits the development of myelofibrosis in MPLW515L mouse model—To determine the requirement of Pim1 in MPLW515L-evoked myelofibrosis, we used the retroviral BM transplantation model of MPLW515L [33]. BM cells from control (WT) and Pim1 knockout mice were transduced with retroviruses expressing MPLW515L and transplanted into lethally irradiated C57BL/6 recipient mice. High titer MPLW515L retrovirus was used and comparable level of transduction was observed in WT and Pim1 knockout mice BM cells (Supplementary Fig. 3A-B). Recipients of Pim1 knockout BM expressing MPLW515L (Pim1KO; MPLW515L) showed significantly reduced WBC, neutrophil and platelet counts compared with recipients of WT BM expressing MPLW515L (WT; MPLW515L) at 8-12 weeks after transplantation (Fig. 2A). RBC count, however, was not affected by Pim1 deletion (Fig. 2A). Deletion of Pim1 significantly increased survival in mice expressing MPLW515L (Fig. 2B). We also observed marked decrease in spleen size/weight in Pim1KO; MPLW515L mice compared with WT; MPLW515L mice (Fig. 2C). Flow cytometric analysis showed significant reduction in myeloid (Gr-1^{+}/Mac-1^{+}) and megakaryocytic (CD41^{+}) precursors in the BM and spleens of Pim1KO; MPLW515L mice compared with WT; MPLW515L mice (Fig. 2D-E). Pim1 deletion significantly reduced number of CFU-GM colonies in the BM of Pim1KO; MPLW515L mice compared with WT; MPLW515L mice (Fig. 2F). More importantly, deletion of Pim1 significantly reduced BM fibrosis in mice expressing MPLW515L (Fig. 2G). However, deletion of Pim2 did not significantly alter the blood counts, spleen size or BM fibrosis in mice expressing MPLW515L (Supplementary Fig. 4A-C).

Pim inhibitor TP-3654 significantly inhibits hematopoietic cells expressing MPN driver mutants

Next, we investigated the effects of Pim kinase inhibition on hematopoietic cells expressing JAK2V617F and MPLW515L. We used TP-3654, a novel second-generation Pim kinase inhibitor [24], which is more potent but less toxic than the first-generation Pim inhibitor SGI-1776 [22]. TP-3654 also has a greater selectivity for Pim1 than Pim2 or Pim3 [24].

We observed that treatment of TP-3654 significantly reduced proliferation of JAK2V617F and MPLW515L mutant expressing BA/F3-EpoR-JAK2V617F and BAF3-MPLW515L cells but not in WT JAK2 expressing BAF/3-EpoR cells (Fig. 3A-B and Supplementary Fig. 5A). TP-3654 treatment also significantly inhibited proliferation of human JAK2V617F-positive HEL, UKE-1 and SET-2 cells (Fig. 3C-E). Combined treatment of TP-3654 and Ruxolitinib resulted in greater inhibition of proliferation in BA/F3-EpoR-JAK2V617F, HEL, UKE-1 and SET-2 cells (Fig. 3F-I).

We also tested the effects of TP-3654 or TP-3654/Ruxolitinib on cell apoptosis. We observed that TP-3654 treatment alone caused significant apoptosis in BA/F3-EpoR-JAK2V617F and HEL cells but not in WT JAK2 expressing BA/F3-EpoR cells (Fig. 3J and Supplementary Fig. 5B-D). Combined treatment of TP-3654 and Ruxolitinib resulted in more apoptosis in BA/F3-EpoR-JAK2V617F and HEL cells (Fig. 3J and Supplementary Fig. 5C-D). Formal drug interaction analysis indicates that TP-3654 acts synergistically with Ruxolitinib to induce apoptosis in BA/F3-EpoR-JAK2V617F cells (Fig. 3K).

We next investigated the effects of TP-3654/Ruxolitinib treatment on MF patient CD34+ cells. TP-3654 alone significantly reduced myeloid colony formation in MF CD34+ cells (Fig. 3L). Combined treatment of TP-3654 and Ruxolitinib resulted in greater inhibition of colony formation in MF CD34+ cells (Fig. 3L). TP-3654/Ruxolitinib treatment also resulted in reduction of colony formation in healthy control CD34+ cells (Supplementary Fig. 6), although their effects were greater against MF CD34+ cells than healthy control CD34+ cells.

To determine whether TP-3654 treatment can overcome resistance to Ruxolitinib in cells expressing JAK2V617F, we generated Ruxolitinib-resistant BA/F3-EpoR-JAK2V617F-Rux^{R} cells. As expected, Ruxolitinib treatment did not inhibit STAT5 phosphorylation or cell proliferation in BA/F3-EpoR-JAK2V617F-Rux^{R} cells (Supplementary Fig. 7A-B). However, TP-3654 treatment resulted in dose-dependent reduction of proliferation in Ruxolitinib-resistant BA/F3-EpoR-JAK2V617F-Rux^{R} cells (Supplementary Fig. 7C). Furthermore, combining low-dose TP-3654 with Ruxolitinib caused significantly enhanced inhibition of BA/F3-EpoR-JAK2V617F-Rux^{R} cells (Supplementary Fig. 7D). Interestingly, we observed upregulated expression of Pim1 mRNA and protein levels in Ruxolitinib-resistant BA/F3-EpoR-JAK2V617F-Rux^{R} cells compared with BA/F3-EpoR or BA/F3-EpoR-JAK2V617F cells (Supplementary Fig. 7E-F). Ectopic overexpression of Pim1 WT significantly reduced the sensitivity of Ruxolitinib in BA/F3-EpoR-JAK2V617F cells (Supplementary Fig. 8A-B), suggesting that increased expression of Pim1 may alter the response to Ruxolitinib treatment.

Pim inhibitor TP-3654 alone or in combination with Ruxolitinib diminishes myelofibrosis in Jak2V617F mouse model of MF

We next evaluated the efficacy of Pim inhibitor TP-3654 in homozygous Jak2V617F knock-in mouse model of MF. The experimental approach is depicted in Fig. 4A. BM cells from the pI-pC induced Mx1Cre; Jak2$^{VF/VF}$ mice were transplanted into lethally irradiated C57BL/6 recipient mice. At six weeks after BMT, mice were randomized into four groups to receive treatment of vehicle, TP-3654 (150 mg/kg), Ruxolitinib (60 mg/kg) or TP-3654

(150 mg/kg) plus Ruxolitinib (60 mg/kg) by oral gavage once daily for 6 weeks. Treatment of TP-3654 alone significantly reduced WBC and neutrophil counts in $Jak2^{VF/VF}$ mice (Fig. 4B). Combined treatment of TP-3654 and Ruxolitinib normalized WBC and neutrophil counts in these animals (Fig. 4B).

Flow cytometric analysis showed significant reduction in myeloid ($Gr\text{-}1^{+}/Mac\text{-}1^{+}$) cells in the BM and spleens of $Jak2^{VF/VF}$ mice treated with TP-3654 compared with vehicle treatment (Fig. 4C-D). Combined treatment of TP-3654/Ruxolitinib resulted in significantly greater reduction of $Gr\text{-}1^{+}/Mac\text{-}1^{+}$ cells in the BM and spleens of these mice (Fig. 4C-D). We also observed a significant reduction in LSK, LK and GMP populations in the BM and spleens of mice treated with TP-3654 or TP-3654 plus Ruxolitinib (Fig. 4E-G). Hematopoietic progenitor colony assays showed significant reduction of CFU-GM colonies in the BM of $Jak2^{VF/VF}$ mice treated with TP-3654 or TP-3654/Ruxolitinib combination compared with vehicle or Ruxolitinib treatment (Fig. 4H). Spleen size/weight was significantly reduced by TP-3654 or Ruxolitinib treatment alone (Fig. 4I). Combined treatment of TP-3654 and Ruxolitinib resulted in significantly greater reduction of spleen size/weight in $Jak2^{VF/VF}$ mice (Fig. 4I). Histopathologic analysis showed significant attenuation of BM fibrosis in $Jak2^{VF/VF}$ mice treated with TP-3654 (Fig. 4J). Combined treatment of TP-3654 and Ruxolitinib resulted in greater reduction of BM fibrosis in $Jak2^{VF/VF}$ mice (Fig. 4j). These data suggest that TP-3654 alone or in combination with Ruxolitinib may be useful against MF.

Next, we asked whether TP-3654/Ruxolitinib treatment can reduce already established BM fibrosis. The experimental approach is depicted in Fig. 5A. At 14 weeks after transplantation of BM cells from Mx1Cre; $Jak2^{VF/VF}$ mice, we randomly sacrificed a few mice from the cohort and confirmed BM fibrosis by reticulin staining. Remaining mice were then randomized to receive treatment with vehicle, TP-3654, Ruxolitinib or TP-3654 plus Ruxolitinib for 6 weeks. Treatment of TP-3654 or TP-3654/Ruxolitinib combination significantly reduced the WBC and neutrophil counts in these animals (Fig. 5B). Frequency of myeloid precursors ($Gr\text{-}1^{+}/Mac\text{-}1^{+}$) in the BM and spleens was significantly reduced by treatment of TP-3654 or TP-3654/Ruxolitinib combination (Fig. 5C). Spleen size/weight was significantly reduced by TP-3654 or Ruxolitinib treatment alone (Fig. 5D). Combining TP-3654 with Ruxolitinib resulted in significantly greater reduction of spleen size/weight in these animals (Fig. 5D). Most importantly, TP-3654 treatment significantly attenuated BM fibrosis in these animals (Fig. 5E). Combined treatment of TP-3654 and Ruxolitinib resulted in greater reduction of BM fibrosis in this mouse model (Fig. 5E).

Treatment of TP-3654 preferentially inhibits Jak2V617F mutant hematopoietic progenitors

To investigate whether TP-3654 can inhibit disease causing Jak2V617F mutant stem/progenitor cells, BM cells from Mx1Cre; $Jak2^{VF/VF}$ GFP+ mice (6 weeks after pI-pC induction) were mixed with WT C57BL/6 mice BM cells at a ratio of 1:1 and then transplanted into lethally irradiated C57BL/6 mice (outlined in Fig. 6A). At 6 weeks after BMT, mice were randomized to receive treatment with vehicle, TP-3654 (150 mg/kg), Ruxolitinib (60 mg/kg) or TP-3654 (150 mg/kg) plus Ruxolitinib (60 mg/kg) once daily for 12 weeks. As expected, vehicle-treated chimeric mice exhibited high WBC, neutrophil

and platelet counts in their peripheral blood (Fig. 6B). TP-3654 treatment significantly reduced WBC, neutrophil and platelet counts (Fig. 6B). Combined treatment of TP-3654 and Ruxolitinib resulted in greater reduction of WBC, neutrophil and platelet counts (Fig. 6B). Furthermore, treatment of TP-3654 or TP-3654/Ruxolitinib combination caused significant reduction in the percentage of Jak2V617F mutant GFP+ LSK, LK, myeloid ($Gr\text{-}1^+$), and megakaryocytic ($CD41^+$) cells in the BM of the chimeric mice (Fig. 6C-F). Together, these results suggest that treatment of TP-3654 or TP-3654/Ruxolitinib combination preferentially inhibits Jak2V617F mutant hematopoietic progenitors.

Treatment of TP-3654 alone or in combination with Ruxolitinib ameliorates myelofibrosis in MPLW515L mouse model

We next assessed the efficacy of TP-3654 and TP-3654/Ruxolitinib combination in MPLW515L mouse model of MF [33]. BM cells from WT BALB/c mice were transduced with retroviruses expressing MPLW515L and transplanted into lethally irradiated syngeneic recipient mice. At three weeks after transplantation, mice were randomized to receive treatment with vehicle, TP-3654, Ruxolitinib or TP-3654 plus Ruxolitinib (outlined in Fig. 7A.) As expected, vehicle-treated mice exhibited markedly increased WBC and neutrophil counts in their peripheral blood (Fig. 7B). Treatment of TP-3654 alone for 3 weeks significantly reduced WBC and neutrophil counts in the MPLW515L mice (Fig. 7B). Combined treatment of TP-3654 and Ruxolitinib caused greater reduction of WBC and neutrophil counts in these animals (Fig. 7B). Treatment of TP-3654 or TP-3654/Ruxolitinib also resulted in significant reduction of myeloid precursors ($Gr\text{-}1^+/Mac\text{-}1^+$) in the BM and spleens of these mice (Fig. 7C). Treatment of TP-3654 alone or in combination with Ruxolitinib significantly increased survival in mice expressing MPLW515L compared with vehicle treatment (Fig. 7D). Treatment of TP-3654 or Ruxolitinib significantly reduced the splenomegaly (Fig. 7E). However, combined treatment of TP-3654 and Ruxolitinib resulted in greater reduction of spleen size in MPLW515L mice (Fig. 7E). Histopathologic analysis revealed significantly reduced BM fibrosis in MPLW515L mice treated with TP-3654 (Fig. 7F). Combined treatment of TP-3654 and Ruxolitinib exhibited significantly greater reduction of BM fibrosis in MPLW515L mice (Fig. 7F) suggesting that TP-3654 alone or in combination with Ruxolitinib may be useful in the treatment of MF.

We also assessed the effects of TP-3654/Ruxolitinib treatment on wild type C57BL/6 mice. We observed that treatment of TP-3654 or TP-3654/Ruxolitinib combination for 3 weeks did not significantly alter the body weight in wild type mice (Supplementary Fig. 9A). TP-3654 treatment caused modest reduction of platelet counts whereas Ruxolitinib treatment slightly reduced RBC, hemoglobin and hematocrit levels in wild type mice (Supplementary Fig. 9B). All the blood counts were within normal range in wild type mice treated with TP-3654/Ruxolitinib, indicating that TP-3654 or TP-3654/Ruxolitinib combination treatment is well-tolerated and has modest effect on normal hematopoiesis.

Effects of Pim1 inhibition on gene expression and cell signaling in hematopoietic cells expressing Jak2V617F

To gain insights into the mechanism(s) by which inhibition of Pim1 reduces disease burden and ameliorates myelofibrosis, we performed RNA-sequencing on sorted LSK

cells from Mx1Cre; Jak2$^{VF/VF}$ mice treated with vehicle or TP-3654. Heat maps showed significantly down-regulated genes in TP-3654-treated LSK cells compared with vehicle-treated LSK cells (Fig. 8A). Analysis of RNA sequencing data revealed that 261 genes were significantly down-regulated (p-adj <0.05, fold change <−1.5 fold) in TP-3654-treated LSK cells compared with vehicle-treated LSK cells. Gene Set Enrichment Analysis (GSEA) [34] revealed significant downregulation of genes related to cell proliferation, inflammatory response, MYC targets and TGF-β response in TP-3654-treated LSK cells compared with vehicle-treated LSK cells (Fig. 8B).

We next compared genes that were significantly downregulated by Pim1 inhibition with genes that were upregulated in gene expression data derived from MF patients (GSE54644). There was an overlap of 359 genes, which were upregulated in MF patients and significantly downregulated in TP-3654-treated Jak2$^{VF/VF}$ mice LSK cells (Fig. 8C). Gene Ontology analysis of overlapping genes showed enrichment for cell cycle, inflammatory response, myeloid cell development, mTORC1 signaling, cytokine response and MYC targets (Fig. 8C), indicating that these pathways were upregulated in MF and downregulated by Pim1 inhibition.

We next performed cell signaling studies in MF patient PBMC and Jak2$^{VF/VF}$ mice BM following treatment with TP-3654 alone or in combination with Ruxolitinib. We observed reduced phosphorylation of EIF4B, S6RP, 4E-BP1, BAD and SMAD2 and decreased expression of c-MYC in MF PBMC and Jak2$^{VF/VF}$ BM cells upon TP-3654 or TP-3654/Ruxolitinib treatment (Fig. 8D-E and Supplementary Fig. 10A-B). We also assessed the effects of Pim1 deletion on Jak2V617F-induced cell signaling using Jak2$^{VF/VF}$ and Pim1KO; Jak2$^{VF/VF}$ mice BM cells. We observed increased phosphorylation of EIF4B, S6RP, 4E-BP1, BAD and SMAD2 and upregulated expression of c-MYC in Jak2$^{VF/VF}$ BM cells compared with control WT mice BM (Fig. 8F). Deletion of Pim1 markedly reduced phosphorylation of EIF4B, S6RP, 4E-BP1, BAD and SMAD2 and reduced the expression of c-MYC in Pim1KO; Jak2$^{VF/VF}$ BM (Fig. 8F). Lentiviral shRNA-mediated knock-down of PIM1 also resulted in similar decrease in phosphorylation of S6RP, 4E-BP1 and BAD and downregulation of c-MYC expression in JAK2V617F-positive HEL cells (Supplementary Fig. 11). TP-3654 treatment, however, did not cause any reduction of PIM1, PIM2 or PIM3 expression in HEL cells (Supplementary Fig. 12). Instead, there was a modest increase in PIM1 and PIM2 expression upon TP-3654 treatment, possibly due to a compensatory mechanism.

Immunohistochemistry analysis on BM sections from Jak2$^{VF/VF}$ mice treated with TP-3654 or TP-3654 plus Ruxolitinib also showed marked reduction in S6RP and BAD phosphorylation compared with vehicle or Ruxolitinib treatment (Supplementary Fig. 13A-B), suggesting that cell growth/survival signaling are affected by TP-3654 treatment.

Inhibition of Pim1 reduces TGF-β1 level and diminishes expression of fibrotic markers

Increased levels of TGF-β1 have been found in patients with MF [35], and TGF-β1 has been suggested to play a role in the pathogenesis of MF [36, 37]. We assessed the serum TGF-β1 level in Jak2$^{VF/VF}$ mice treated with TP-3654/Ruxolitinib. We observed that TP-3654 or

TP-3654 plus Ruxolitinib treatment significantly reduced serum TGF-β1 level in $Jak2^{VF/VF}$ mice (Fig. 8G).

Next, we assessed the expression of fibrotic markers in the BM following TP-3654 treatment. Collagen deposition in the BM mesenchymal stromal cells (MSC) has been linked to myelofibrosis [35]. We previously showed that TGF-β1 stimulation significantly increased Collagen I and III expression in the BM MSC [38, 39]. Since we observed that TP-3654 treatment inhibits the TGF-β signaling pathway, we asked if TP-3654 could inhibit TGF-β1-induced Collagen expression in BM MSC. Indeed, we observed that TP-3654 significantly inhibited TGF-β1-induced Collagen I and III mRNA expression in BM MSC (Supplementary Fig. 14A-B). Immunofluorescence staining also showed that TP-3654 treatment markedly inhibited TGF-β1-induced Collagen I and III expression in BM MSC (Supplementary Fig. 15A-B). TGF-β1 can induce Snail and α-smooth muscle actin (αSMA) expression and promote epithelial-mesenchymal transition (EMT) and extracellular matrix (ECM) production in various tissue fibrosis [40, 41]. Immunohistochemistry analysis on the BM sections showed that treatment of TP-3654 or TP-3654/Ruxolitinib combination markedly reduced Snail and αSMA expression in $Jak2^{VF/VF}$ mice BM (Fig. 8H). These data suggest that inhibition of Pim1 by TP-3654 blocks the expression of fibrotic markers and prevents the development/progression of myelofibrosis.

DISCUSSION

Myelofibrosis is a deadly blood neoplasia with limited treatment options that are mostly palliative. Patients with MF are in a dire need for new effective treatment. In this study, we demonstrate that Pim1 plays an important role in myelofibrosis evoked by Jak2V617F and MPLW515L. Further, we show that inhibition of Pim1 with TP-3654 significantly inhibits MPN hematopoietic cells and progenitors and ameliorates bone marrow fibrosis in murine models.

We observed elevated Pim1 expression in hematopoietic progenitors of Jak2V617F knock-in mice and MF patients. So, we assessed the role of Pim1 in MF. We found that deletion of Pim1 significantly reduced WBC and neutrophil counts, normalized spleen size and abrogated BM fibrosis without significantly affecting RBC and hematocrit levels in homozygous Jak2V617F mice. We further tested the effects of Pim1 deletion in MPLW515L-induced MF. Similar to Jak2V617F model, we observed significant reduction of leukocytosis and spleen size and abrogation of BM fibrosis in Pim1-deleted MPLW515L mice. These results establish an important role of Pim1 in the development of MF. Since Pim1 deletion does not inhibit RBC and hematocrit levels in Jak2V617F mice, targeting of Pim1 alone may not be suitable for PV.

Data from our Pim1 genetic deletion study prompted us to investigate the effects of Pim1 inhibition in animal models of MF. Similar to the effects of Pim1 gene deletion, inhibition of Pim1 by TP-3654 alone significantly reduced leukocytosis and splenomegaly and improved BM fibrosis in both Jak2V617F and MPLW515L murine models of MF. Notably, TP-3654 treatment alone exhibited greater inhibition of BM fibrosis than Ruxolitinib treatment. We further show that TP-3654 preferentially inhibits Jak2V617F mutant hematopoietic

progenitors in mice. Thus, treatment of TP-3654 alone or in combination with Ruxolitinib may have therapeutic potential for treatment of MF. Recent studies [42, 43] have suggested combinatorial effect of pan-PIM inhibitors (INCB053914, PIM447) with Ruxolitinib in MPN pre-clinical models. However, these studies did not find significant in vivo efficacy of PIM inhibitors (INCB053914, PIM447) alone against myelofibrosis [42, 43]. In contrast, our studies show that TP-3654 alone significantly inhibited BM fibrosis in murine models.

We also provided some mechanistic insights into the inhibition of MPN/MF by Pim1 inhibition. We observed increased phosphorylation of mTORC1 signaling proteins, S6RP, 4E-BP1 and EIF4B, in Jak2$^{VF/VF}$ BM cells, and deletion of Pim1 markedly inhibited the phosphorylation of these mTORC1 signaling proteins in Pim1KO; Jak2$^{VF/VF}$ BM. S6RP, 4E-BP1 and EIF4B are known to regulate mRNA translation and cell growth [44]. Treatment of TP-3654 or TP-3654/Ruxolitinib inhibited the phosphorylation of these mTORC1 signaling proteins in MF PBMC and Jak2$^{VF/VF}$ BM cells. We also observed phosphorylation of BAD (Ser112) in Jak2$^{VF/VF}$ BM cells. Pim1 promotes BAD phosphorylation, which results in increased cell survival [45]. Pim1 deletion or inhibition with TP-3654 reduced the phosphorylation of BAD in Jak2$^{VF/VF}$ BM cells and MF PBMC and thus decreased survival of these cells. Increased expression of c-MYC has been observed in MF [46]. We also observed elevated c-MYC expression in Jak2$^{VF/VF}$ BM cells. Pim1 inhibition or deletion reduced the expression of c-MYC in MF PBMC and Jak2$^{VF/VF}$ BM. Thus, suppression of Pim1 activity may inhibit MPN cell growth/survival by altering the activation/expression of these signaling proteins.

Previous studies have suggested an important role for TGF-β signaling in various tissue fibrosis [40, 47]. Elevated levels of TGF-β1 also have been observed in patients with MF as well as in mouse models of MF [35-37, 48]. Interestingly, we observed that treatment of TP-3654 or TP-3654/Ruxolitinib significantly reduced the TGF-β1 level in Jak2$^{VF/VF}$ mice. It remains to be identified how inhibition of Pim1 reduces the TGF-β1 level. It is plausible myeloid cells in the BM that secrete TGF-β1 are reduced upon treatment of TP-3654 or TP-3654/Ruxolitinib. We also observed reduced phosphorylation of SMAD2 upon treatment of TP-3654 or deletion of Pim1 in Jak2$^{VF/VF}$ mice BM, suggesting that Pim1 may regulate the TGF-β1 signaling pathway. This is consistent with a recent study suggesting that PIM1 interacts with SMAD2 and SMAD3 and phosphorylates SMADs to induce gene expression in renal-cell carcinoma cells [49]. TGF-β1 can also induce expression of Collagen, Snail and αSMA, which have been implicated in tissue fibrosis and cancer metastasis [40, 41]. We observed that treatment of TP-3654 significantly reduced Collagen I and III expression in BM MSC. Treatment of TP-3654 also significantly reduced Snail and αSMA expression in the BM of Jak2$^{VF/VF}$ mice. Thus, suppression of Pim1 activity may prevent the development/progression of BM fibrosis by inhibiting the TGF-β signaling and thereby reducing the expression of fibrotic markers.

In conclusion, we demonstrate that Pim1 plays an important role in the pathogenesis of myelofibrosis. Both genetic deletion and pharmacologic inhibition of Pim1 significantly reduce MPN disease burden and ameliorate BM fibrosis in multiple murine models of MF. Combined treatment of TP-3654 and Ruxolitinib results in greater inhibition of MPN cells and abrogation of BM fibrosis in mice. Initial data from a dose-escalation trial of TP-3654

in advanced cancers (NCT03715504) show that TP-3654 is well-tolerated in humans without exhibiting significant toxicity [50]. Based on the results from these studies, a Phase I clinical trial of TP-3654 in patients with MF has been initiated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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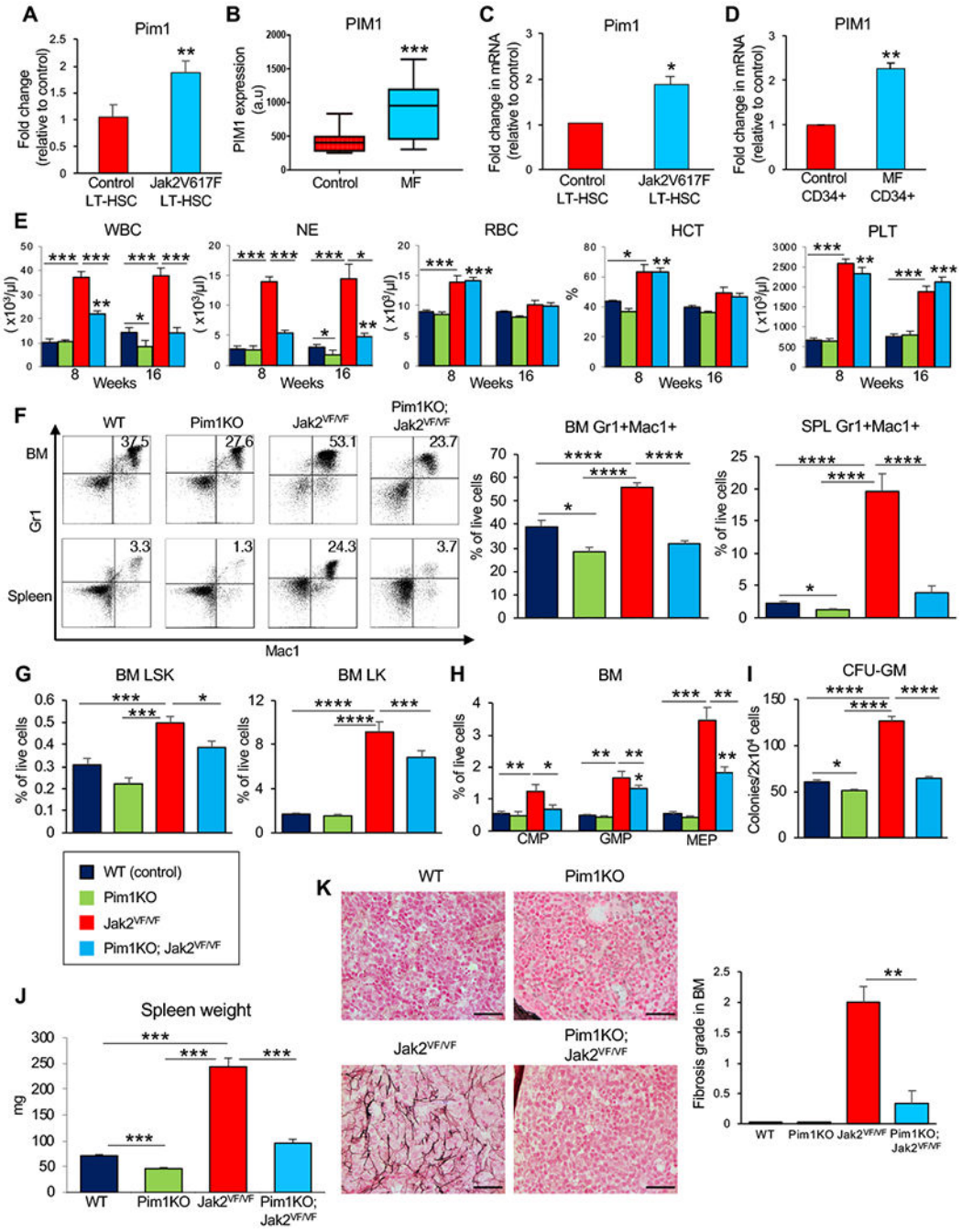

Fig. 1. Deletion of Pim1 blocks the development of myelofibrosis in Jak2V617F mouse model.

A Microarray gene expression analysis shows significant increase in Pim1 mRNA expression in mice Jak2V617F LT-HSCs compared with control LT-HSCs (n=3) (GSE79198). **B** Analysis of microarray gene expression data (GSE54644) shows that PIM1 expression is significantly increased in granulocytes of MF patients compared with healthy controls (Control=11, MF=18). **C** RT-qPCR validates significant increase in Pim1 mRNA expression in Jak2V617F LT-HSC compared with control LT-HSC. The mRNA expression was normalized by *18S* (n=4). **D** Expression of PIM1 mRNA was assessed in PB CD34+

cells from MF patients and healthy controls (n=4) by RT-qPCR and normalized with *18S* expression. **E** Peripheral blood WBC, neutrophil (NE), RBC, hematocrit (HCT) and platelet (PLT) counts were assessed at 8 and 16 weeks after pI-pC injection (n=7-10). **F** Flow cytometric analysis of granulocyte/monocyte ($Gr\text{-}1^+/Mac\text{-}1^+$) precursors in the BM and spleens of control, Pim1KO, $Jak2^{VF/VF}$ and Pim1KO; $Jak2^{VF/VF}$ mice are shown in representative dot plots and bar graphs as mean ± SEM. **G-H** Flow cytometric analysis of LSK, LK, CMP, GMP and MEP in the BM are shown in bar graphs as mean ± SEM. **I** BM cells (2×10^4) from WT (control), Pim1KO, $Jak2^{VF/VF}$ and Pim1KO; $Jak2^{VF/VF}$ mice were plated in methylcellulose medium with cytokines. CFU-GM colonies are presented in bar graphs as mean ± SEM (n= 7-8). **J** Spleen size/weight in WT (control), Pim1KO, $Jak2^{VF/VF}$ and Pim1KO; $Jak2^{VF/VF}$ mice are shown in bar graphs as mean ± SEM (n=7-8). **K** Representative images of reticulin stained BM sections are shown at 16 weeks after transplantation. Scale bars, 15μm. Histological gradings of BM fibrosis are illustrated in the bar graphs as mean ± SEM (n=4-6 per group). One-way ANOVA was used for comparisons of all 4 groups of mice, and the Student's t test (unpaired two-tailed) was used to compare between 2 groups of mice (*p<0.05; **p<0.005, ***p<0.0005, ****p<0.00005).

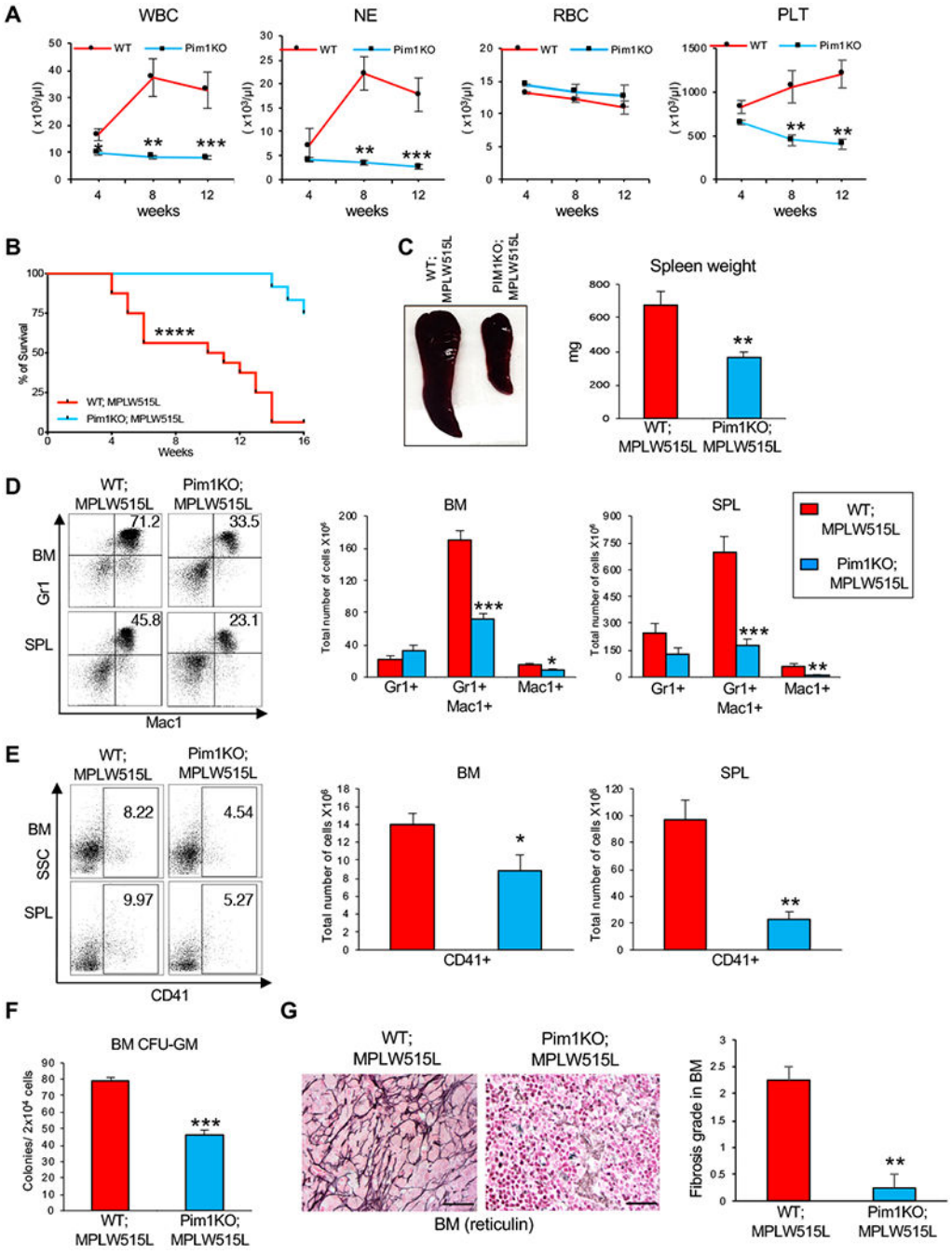

Fig. 2. Genetic deletion of Pim1 inhibits myelofibrosis in MPLW515L mouse model of MF.

A Peripheral blood WBC, NE, RBC and PLT counts were assessed at 4, 8 and 12 weeks after transplantation (n=10-12). **B** Kaplan-Meier analysis showed significant increased survival in Pim1KO; MPLW515L mice compared to WT; MPLW515L mice (n=12-15 in each group; ****p<0.0001; log-rank test). **C** Spleen size/weight in WT; MPLW515L and Pim1KO; MPLW515L mice (n=10-12). **D-E** Representative dot plots of Gr-1$^+$/Mac-1$^+$ and CD41$^+$ precursor cells in the BM and spleens of WT; MPLW515L and Pim1KO; MPLW515L mice. Total numbers of Gr-1$^+$/Mac-1$^+$ and CD41$^+$ cells in the BM and spleens

are shown in bar graphs as mean ± SEM. **F** CFU-GM colony formation was assessed in the BM of WT; MPLW515L and Pim1KO; MPLW515L mice. Data are presented in bar graphs as mean ± SEM (n= 8). **G** Representative images of reticulin stained BM sections are shown at 12 weeks after transplantation. Scale bars, 15μm. Histological gradings of BM fibrosis are illustrated in the bar graphs as mean ± SEM (n=4). (*p<0.05; **p<0.005, ***p<0.0005; Student's t test).

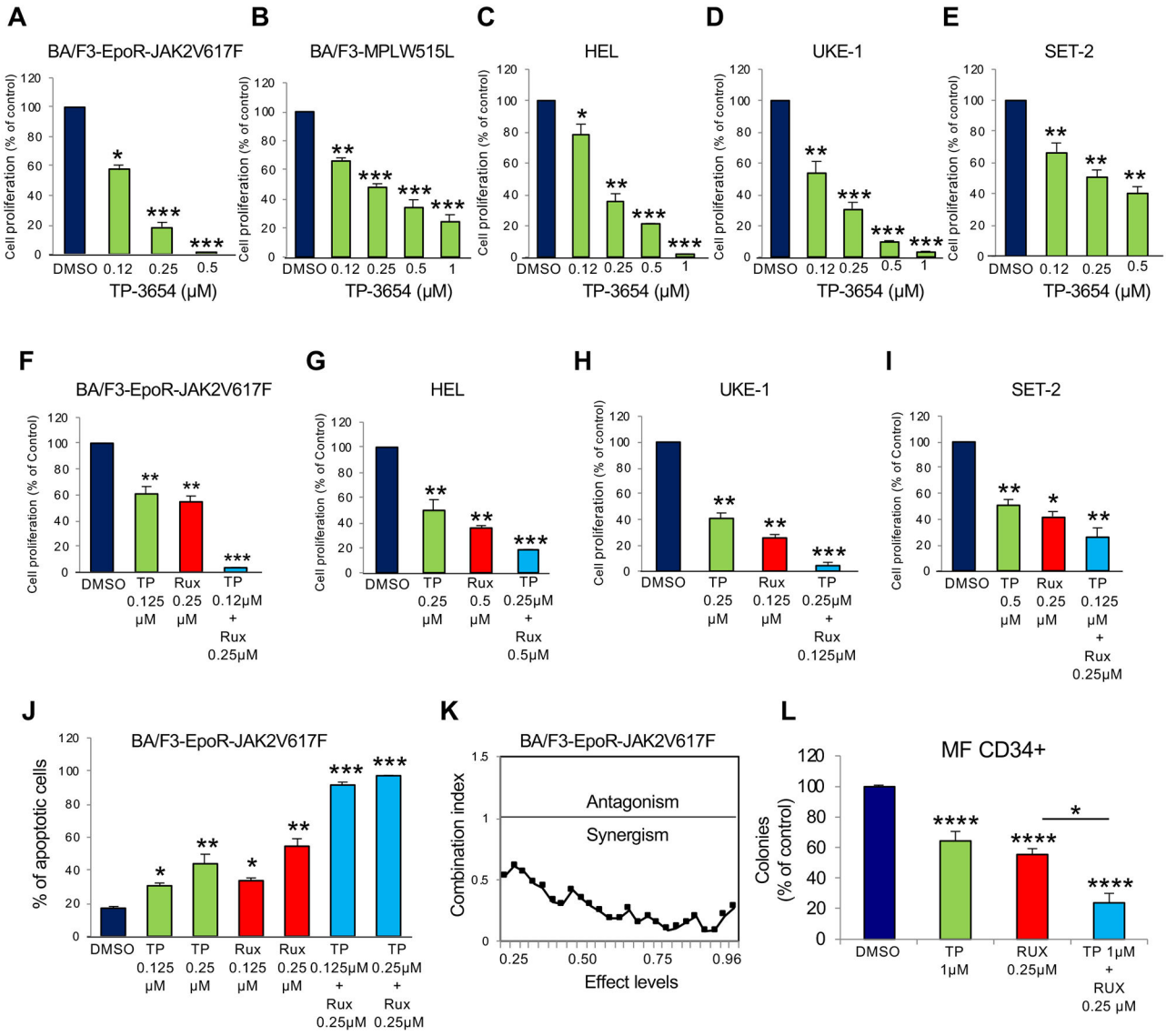

Fig. 3. Pim inhibitor TP-3654 alone or in combination with Ruxolitinib significantly inhibits hematopoietic cells expressing MPN-associated mutants.

A-E BA/F3-EpoR-JAK2V617F, BA/F3-MPLW515L, HEL, UKE-1 and SET-2 cells were treated with vehicle (DMSO) or TP-3654 and cell proliferation was assessed by viable cell counts over 5 days. **F-I** BA/F3-EpoR-JAK2V617F, HEL, UKE-1 and SET-2 cells were treated with vehicle (DMSO), TP-3654, Ruxolitinib and TP-3654 plus Ruxolitinib. Cell proliferation was assessed by viable cell counts over 5 days. TP-3654 alone or in combination with Ruxolitinib showed significant reduction in cell proliferation. Combined treatment of TP-3654 (TP) and Ruxolitinib (Rux) resulted in greater inhibition of cell proliferation. Data from three independent experiments are shown in bar graphs as mean ± SEM. **J** Bar graphs of the flow cytometric analysis of the percentage of apoptotic cells in BA/F3-EpoR-JAK2V617F cells treated with TP-3654, Ruxolitinib and TP-3654/Ruxolitinib combination. Combined treatment of TP-3654 and Ruxolitinib resulted in significant apoptosis in BA/F3-EpoR-JAK2V617F cells. **K** Combined treatment of TP-3654 and

Ruxolitinib exhibit synergistic effects against BA/F3-EpoR-JAK2V617F cells. Combination index <0.5 indicates strong synergism. **L** MF CD34+ cells were plated in methylcellulose medium containing cytokines in the presence of vehicle (DMSO), TP-3654, Ruxolitinib and TP-3654 plus Ruxolitinib. Treatment of TP-3654 alone or in combination with Ruxolitinib significantly inhibits hematopoietic progenitor colonies in MF CD34+. Data are represented in bar graphs as mean ± SEM (n= 8). One-way ANOVA was used for comparisons of all 4 groups of treatment, and the Student's t test (unpaired two-tailed) was used to compare between 2 groups of drug treatment (*p<0.05; **p<0.005, ***p<0.0005, ****p<0.00005).

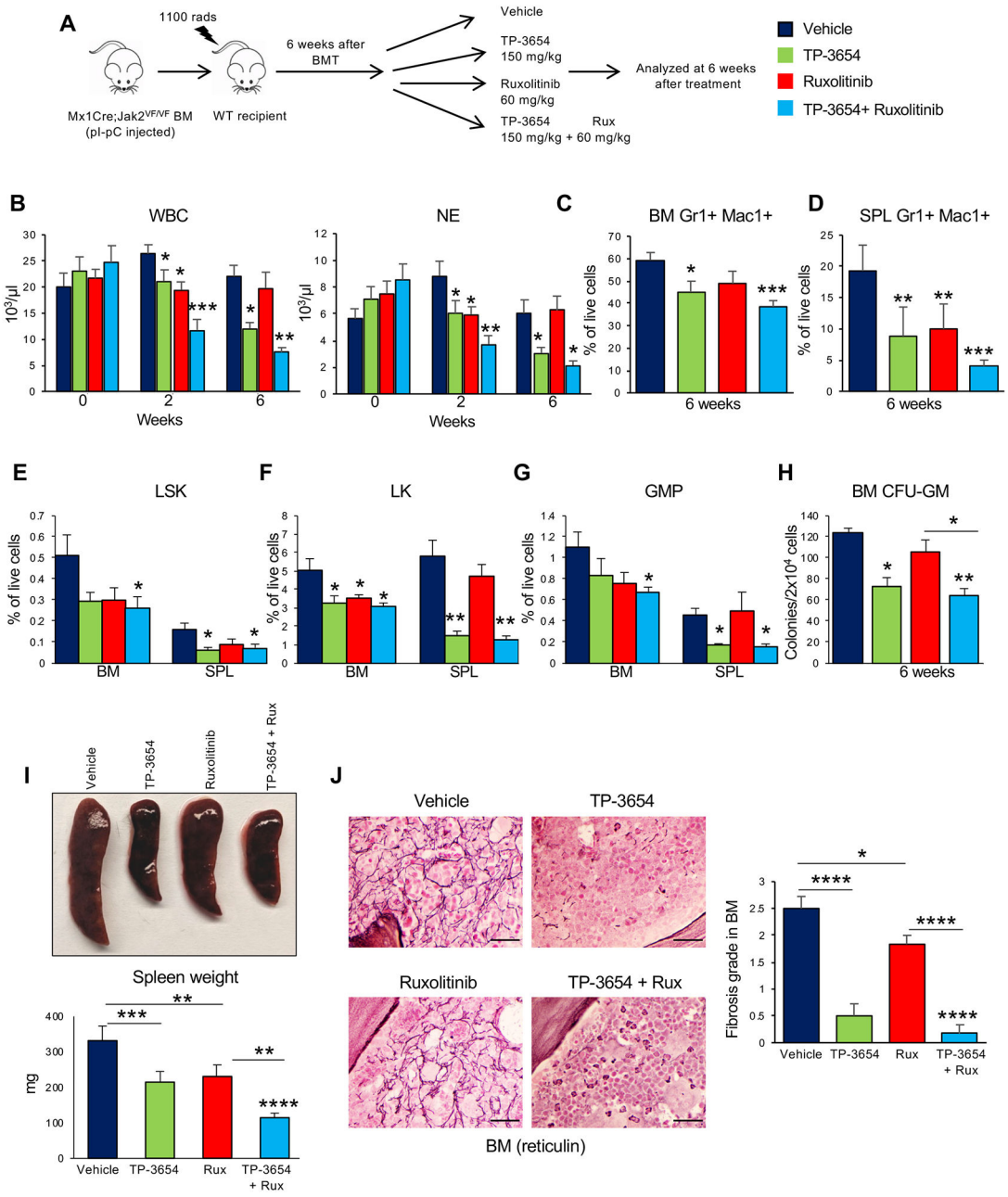

Fig. 4. In vivo administration of TP-3654 alone or in combination with Ruxolitinib ameliorates myelofibrosis in Jak2V617F murine model of MF.

A Schematic of the experimental design to assess the in vivo efficacy of TP-3654/Ruxolitinib in Jak2V617F model of MF. **B** Peripheral blood WBC and NE counts were assessed at 2 and 6 weeks after treatment (n=10-12). **C-D** Frequency of Gr-1^{+}/Mac-1^{+} cells in the BM and spleens (SPL) of drug treated mice is shown in bar graphs as mean ± SEM. **E-G** Flow cytometric analyses of LSK (Lin^{-}Sca-1^{+}c-kit^{+}), LK (Lin^{-}c-kit^{+}), and GMP (Lin^{-}Sca-1^{-}c-kit^{+}CD34^{+}FcγRII/II^{high}) cells in the BM and SPL are shown in bar graphs as mean ± SEM. **H** Jak2$^{VF/VF}$ mice treated with vehicle, TP-3654, Ruxolitinib or TP-3654/Ruxolitinib combination were plated in methylcellulose medium containing cytokines. CFU-

GM colonies were scored 7 days after plating. Data are represented in bar graphs as mean ± SEM (n= 5). **I** Spleen size/weight in $Jak2^{VF/VF}$ mice treated with vehicle, TP-3654, Ruxolitinib or TP-3654 plus Ruxolitinib (n=10-12). **J** Representative images of reticulin stained BM sections from $Jak2^{VF/VF}$ mice treated with vehicle, TP-3654, Ruxolitinib or TP-3654 plus Ruxolitinib for 6 weeks are shown. Scale bars, 15μm. Histological gradings of BM fibrosis are illustrated in the bar graphs as mean ± SEM (n=6). One-way ANOVA was used for comparisons of all 4 groups of treated mice, and the Student's t test (unpaired two-tailed) was used to compare between 2 groups of mice (*p<0.05; **p<0.005, ***p<0.0005, ****p<0.00005).

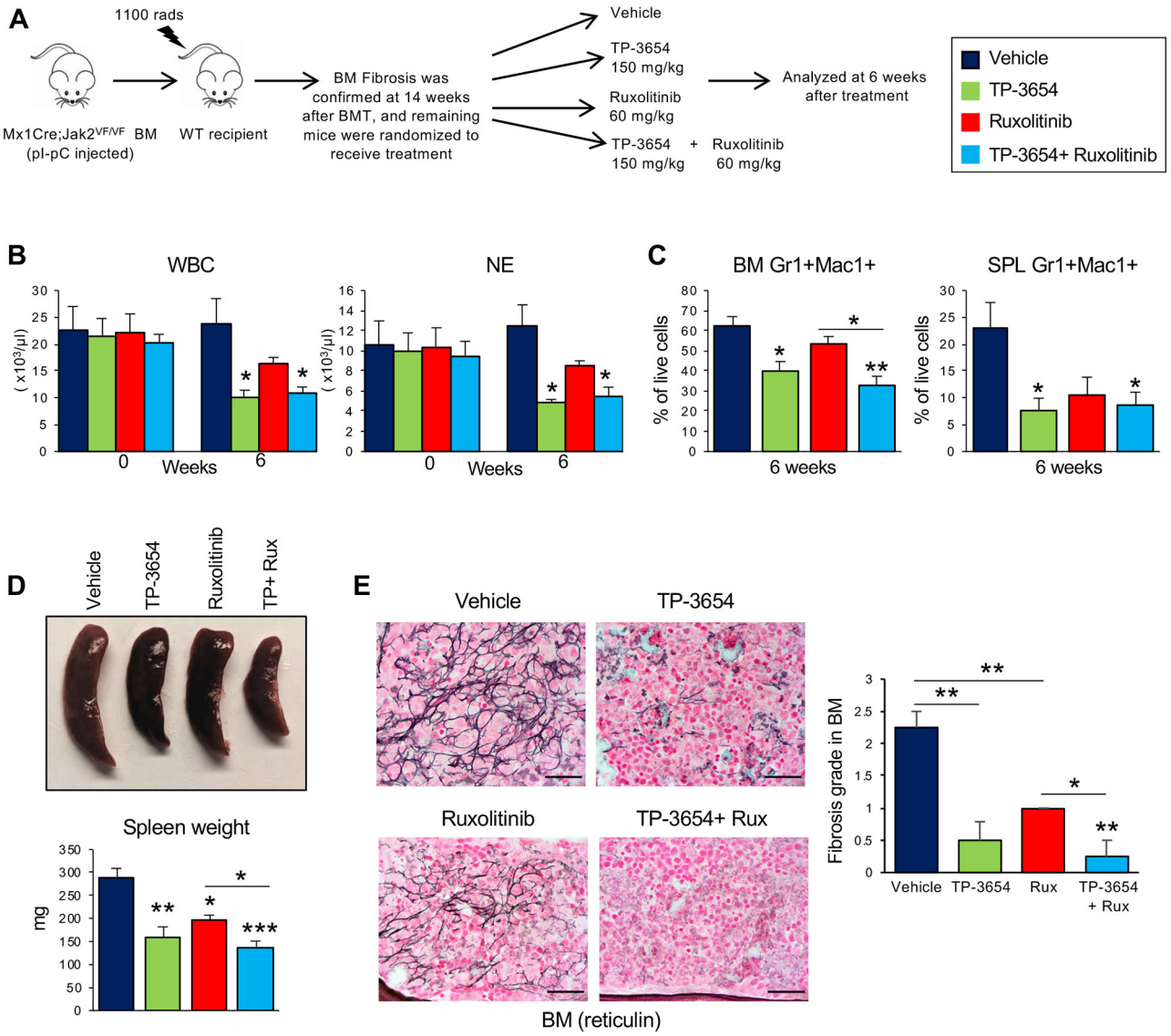

Fig. 5. In vivo administration of TP-3654 alone or in combination with Ruxolitinib improves established BM fibrosis in Jak2V617F mouse model.

A Schematic of the experimental design to test the effects of TP-3654 or TP-3654/ Ruxolitinib combination on established BM fibrosis. **B** Peripheral blood WBC and NE counts were assessed before and after treatment (n=5). **C** Frequency of Gr-1$^+$/Mac-1$^+$ cells in the BM and SPL of drug treated mice is shown in bar graphs as mean ± SEM. **D** Spleen size/weight was significantly reduced in all treatment groups compared with vehicle treated mice while combined treatment of TP-3654 and Ruxolitinib resulted in greater reduction of splenomegaly (n=5). **E** Representative images of BM reticulin fibrosis in mice treated with vehicle, TP-3654, Ruxolitinib or TP-3654 plus Ruxolitinib for 6 weeks are depicted. Scale bars, 15µm. Histological gradings of BM fibrosis are illustrated in the bar graphs as mean ± SEM (n=4). One-way ANOVA was used for comparisons of all 4 groups of treated mice, and the Student's t test (unpaired two-tailed) was used to compare between 2 groups of mice (*p<0.05, **p<0.005; and ***p<0.0005).

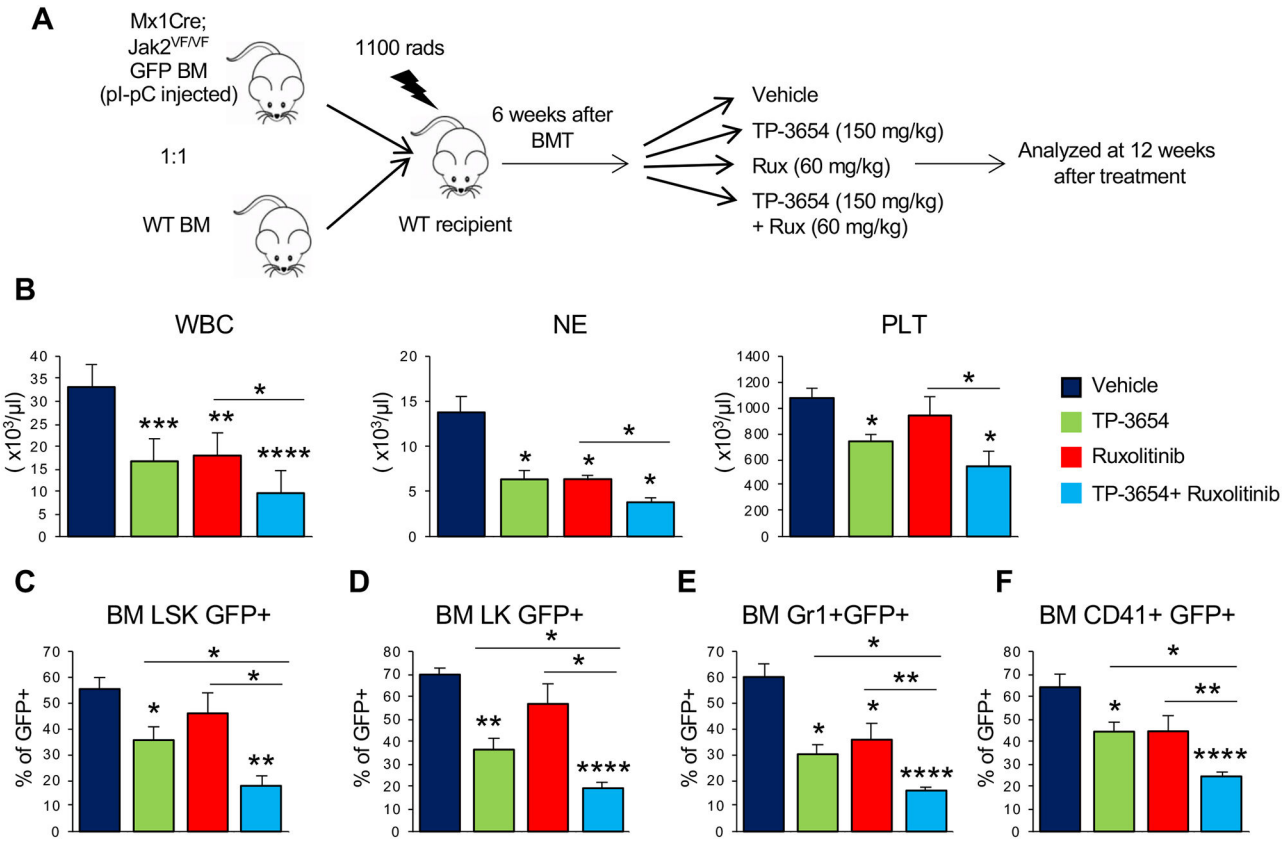

Fig. 6. TP-3654 alone or in combination with Ruxolitinib treatment preferentially inhibits Jak2V617F mutant hematopoietic progenitors.

A Schematic on competitive BM transplantation approach to assess the effects of TP-3654/ Ruxolitinib on Jak2V617F mutant hematopoietic progenitors. BM cells ($5x10^5$) from Mx1Cre; $Jak2^{VF/VF}$; GFP mice (6 weeks after pI-pC injection) was mixed with WT C57BL/6 mice BM ($5x10^5$) at a 1:1 ratio and transplanted into lethally irradiated WT C57BL/6 recipient mice. Six weeks after BMT, recipient mice were randomized to receive treatment with vehicle, TP-3654 (150mg/kg), Ruxolitinib (60mg/kg) or TP-3654 (150mg/kg) plus Ruxolitinib (60mg/kg). Drugs were administered orally once daily for 12 weeks. **B** Peripheral blood WBC, NE and PLT counts were assessed at 12 weeks after treatment (n=5). **C-F** Percentages of $Jak2^{V617F}$ mutant GFP+ LSK ($Lin^-Sca\text{-}1^+c\text{-}kit^+$), GFP+ LK ($Lin^-c\text{-}kit^+$), GFP+ Gr1+ and GFP+CD41+ cells in the BM of chimeric animals at 12 weeks after treatment are shown; bar graphs represent mean ± SEM (n=5). One-way ANOVA was used for comparisons of all 4 groups of treated mice, and the Student's t test (unpaired two-tailed) was used to compare between 2 groups of mice (*p<0.05; **p<0.005, ***p<0.0005; **** p<0.00005).

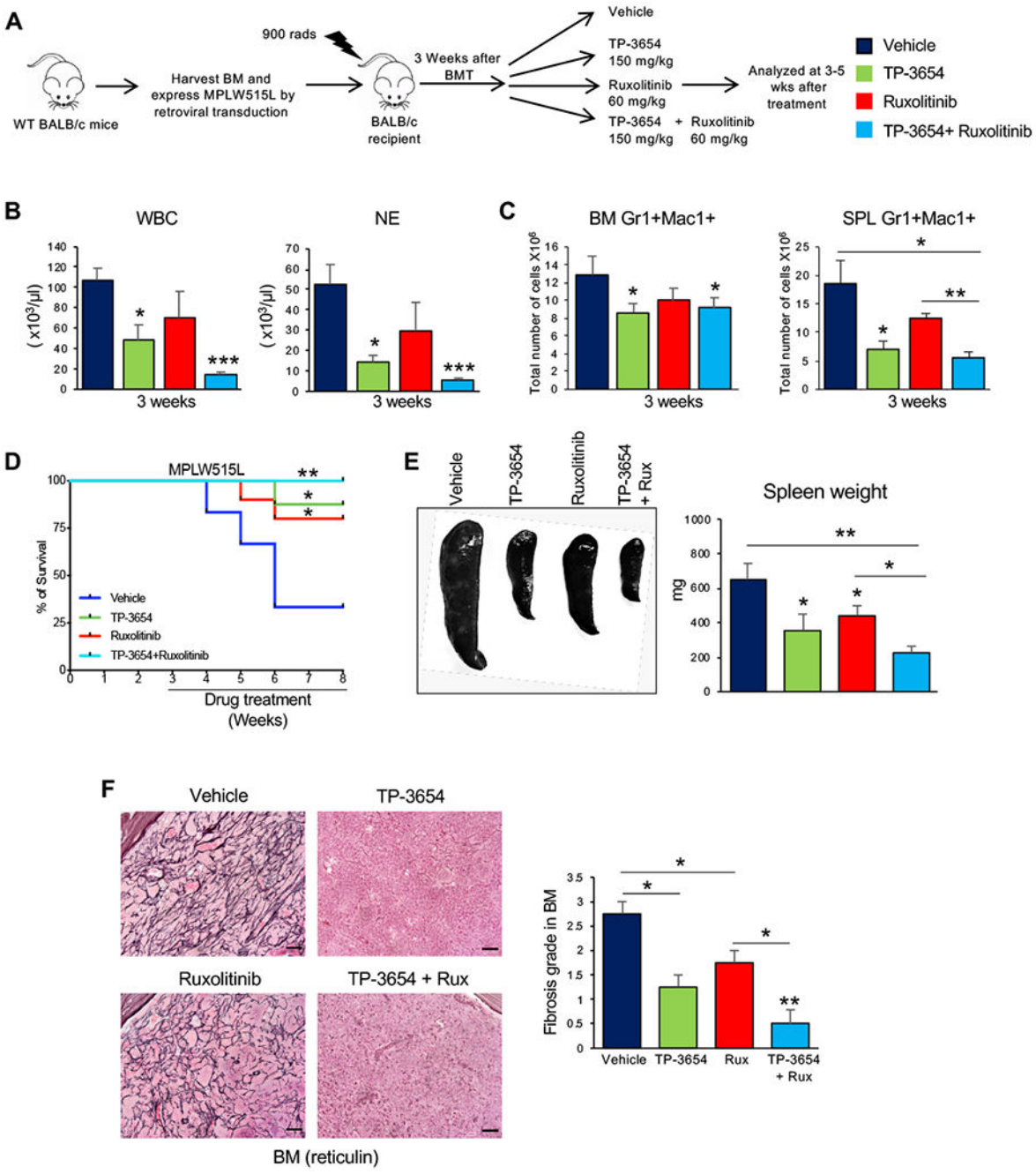

Fig. 7. Inhibition of Pim1 by TP-3654 diminishes myelofibrosis in MPLW515L mouse model of MF.

A Schematic of the experimental design to assess the efficacy of Pim1 inhibition in MPLW515L mouse model of MF. **B** Peripheral blood WBC and NE counts were assessed at 3 weeks after treatment (n=5-9). **C** Total numbers of Gr-1$^+$/Mac-1$^+$ cells in the BM and spleens of drug treated mice are shown in bar graphs as mean ± SEM (n=5). **D** Kaplan-Meier analysis showing significant increase in the survival of MPLW515L mice treated with TP-3654 compared with vehicle-treated MPLW515L mice. Combined treatment of TP-3654 and Ruxolitinib significantly improved survival in MPLW515L mice compared

to untreated or single drug treatment groups (n=8-12 per group; *p<0.05; **p<0.005, log-rank test). **E** Spleen size/weight was significantly reduced in all treatment groups compared with vehicle-treated mice while combined treatment of TP-3654 and Ruxolitinib exhibited greater reduction of spleen size (n= 5-9). **F** Representative images of BM reticulin fibrosis in MPLW515L mice treated with vehicle, TP-3654, Ruxolitinib or TP-3654 plus Ruxolitinib for 3 weeks are depicted. Scale bars, 15μm. Histological gradings of BM fibrosis are illustrated in the bar graphs as mean ± SEM (n=4). One-way ANOVA was used for comparisons of all 4 groups of treated mice, and the Student's t test (unpaired two-tailed) was used to compare between 2 groups of mice (*p<0.05; **p<0.005, ***p<0.0005).

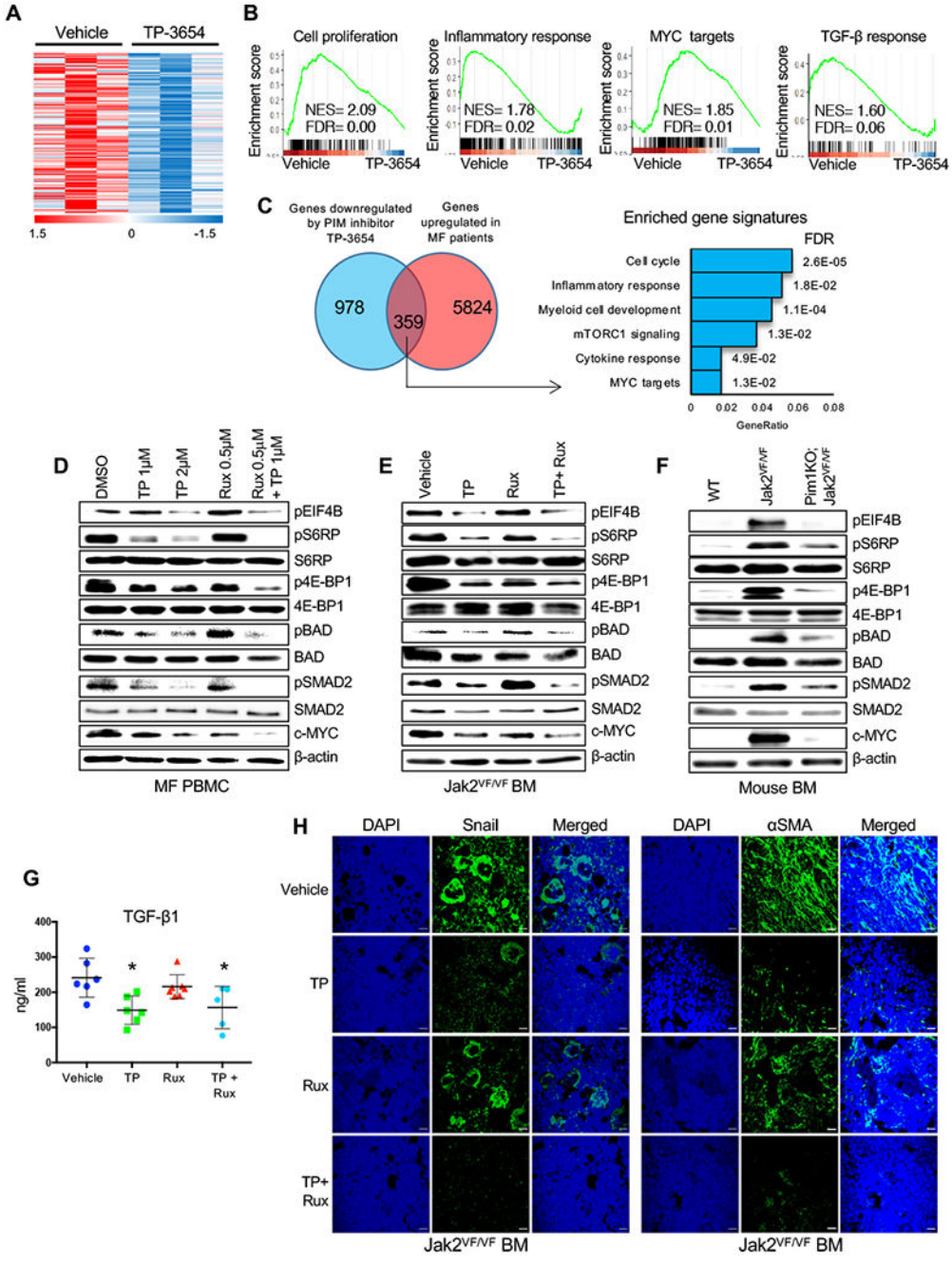

Fig. 8. Suppression of Pim1 activity alters gene expression and cell signaling in MPN cells and attenuates the expression of fibrotic markers.

A Heat map showing top 150 significantly downregulated genes (p<0.05, −1.5-fold) in TP-3654 treated Jak2$^{VF/VF}$ mice LSK cells compared with vehicle treated Jak2$^{VF/VF}$ mice LSK cells. **B** Gene-set enrichment analyses (GSEA) of the RNA-sequencing data from TP-3654-treated Jak2$^{VF/VF}$ mice LSK cells compared with vehicle treated Jak2$^{VF/VF}$ mice LSK cells. Enrichment plots of selected gene sets/pathways with normalized enrichment score (NES) and false discovery rate (FDR) are shown. **C** Venn diagram showing the overlap between upregulated genes in MF patient granulocytes and genes downregulated in

TP-3654 treated Jak2$^{VF/VF}$ mice LSK cells. The cutoffs were FDR-adjusted p < 0.05. **D** PBMC obtained from MF patients were treated with DMSO or TP-3654 (TP), Ruxolitinib (Rux) or TP plus Rux at indicated concentrations for 6 hours. Immunoblotting was performed using phospho-specific or total antibodies as indicated. Treatment of TP-3654 alone significantly reduced phosphorylation of EIF4B, S6RP, 4E-BP1, BAD and SMAD2 and reduced the expression of c-MYC in MF PBMC. Combined treatment of TP-3654 with Ruxolitinib caused greater reduction of phosphorylation or expression of these signaling proteins. **E** BM cells obtained from Jak2$^{VF/VF}$ mice following in vivo treatment with Vehicle, TP-3654, Ruxolitinib or TP-3654/Ruxolitinib combination were subjected to immunoblotting using phospho-specific or total antibodies as indicated. **F** Immunoblotting showed markedly reduced phosphorylation of EIF4B, S6RP, 4E-BP1, BAD and SMAD2 and decreased expression of c-MYC in Pim1-deleted Jak2$^{VF/VF}$ mice BM. β-actin was used as a loading control. **G** Serum TGF-β1 levels in Jak2$^{VF/VF}$ mice treated with vehicle, TP-3654, Ruxolitinib and TP-3654/Ruxolitinib combination were assessed by ELISA (n= 5-7). One-way ANOVA was used for comparisons of all 4 groups of treated mice, and the Student's t test (unpaired two-tailed) was used to compare between 2 groups of mice (*p<0.05). **H** Representative immunofluorescence images showing decreased expression of Snail and αSMA in the BM sections of Jak2$^{VF/VF}$ mice treated with TP-3654 alone or in combination with Ruxolitinib. Snail, αSMA (green) and DAPI (blue). Scale bars, 15μm.