JAK2 IS A THERAPEUTIC TARGET IN MYELOPROLIFERATIVE NEOPLASMS

by

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ABSTRACT

Myeloproliferative neoplasms (MPN) are clonal hematological disorders characterized by dysregulated proliferation of one or more mature myeloid lineages. The identification of activating somatic mutations in tyrosine kinase *JAK2* and in the thrombopoietin receptor gene (*MPL*) in a majority of patients with MPN led to clinical development and FDA approval of JAK kinase inhibitors such as ruxolitinib for the treatment of these diseases. JAK2 inhibitor therapy improves MPN-associated splenomegaly and systemic symptoms but does not significantly decrease or eliminate the MPN clone in most patients. We therefore sought to characterize mechanisms by which MPN cells persist despite chronic inhibition of JAK2. Our studies showed that MPN cells could survive in the context of chronic JAK inhibitor exposure by reactivating the JAK-STAT pathway via the formation of heterodimers between JAK2 and other JAK kinases. This finding was recapitulated in murine models as well as in samples from MPN patient treated with ruxolitinib. Reactivation of the JAK-STAT pathway in inhibitor persistent cells was facilitated by stabilization of phosphorylated JAK2 by Type I inhibitors and associated with increased expression of JAK2. This inherent insensitivity of MPN cells to JAK inhibitors led us to evaluate the requirement of JAK2 in naïve and inhibitor persistent MPN cells. Genetic deletion of JAK2 in *in vivo* model of ET/MF revealed an indispensable role for JAK2 in MPN pathogenesis. Further, RNAi and genetic loss of function experiments revealed that inhibitor persistent cells remain dependent on JAK2 for their survival. Based on these data, we evaluated Hsp90 inhibitors, which target JAK2 degradation, and found that combination of JAK and Hsp90 inhibitors was more efficacious than JAK inhibitor monotherapy in murine models. Importantly, Hsp90

inhibition was able to overcome JAK inhibitor persistence in pre-clinical models and in primary samples. These findings indicate that JAK2 is a bona fide therapeutic target for MPN and combinatorial strategies or JAK inhibitors that can overcome persistence have the potential to improve therapeutic efficacy in patients with MPN.

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LIST OF ABBREVIATIONS

5-FU	Fluorouracil
ALL	Acute Lymphoblastic Leukemia
AMKL	Acute Megakaryocytic Leukemia
AML	Acute Myeloid Leukemia
ATP	Adenosine Triphosphate
B-ALL	B-cell Acute Lymphoblastic Leukemia
BM	Bone Marrow
BMT	Bone Marrow Transplant
BSA	Bovine Serum Albumin
ChIP	Chromatin Immunoprecipitation
CHX	Cycloheximide
CML	Chronic Myeloid Leukemia
CMML	Chronic Myelomonocytic Leukemia
CMP	Common Myeloid Progenitor
COMFORT	Controlled Myelofibrosis Study with Oral JAK Inhibitor Treatment
DNA	Deoxyribonucleic Acid
Epo	Erythropoietin
ET	Essential Thrombocythemia
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GFP	Green Fluorescent Protein
GMP	Granulocyte Macrophage Progenitor
H3K4me3	Histone 3 trimethyl Lysine 4
H3K9me3	Histone 3 trimethyl Lysine 9
H3Y41	Histone 3 Tyrosine 41
HDAC	Histone Deacetylase
Hh	Hedgehog
HSC	Hematopoietic Stem Cell
IFN	Interferon

IL	Interleukin
LSK	Lin- ckit+ sca1+
LT-HSC	Long Term Hematopoietic Stem Cell
MBP	Myelin Basic Protein
MEP	Megakaryocyte Erythroid Progenitor
MF	Myelofibrosis
MPN	Myeloproliferative Neoplasm
mRNA	Messenger RNA
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
Ph	Philadelphia Chromosome
pI:pC	polyI:polyC
PMF	Primary Myelofibrosis
PV	Polycythemia Vera
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNAi	RNA Interference
siRNA	Short Interfering RNA
ST-HSC	Short Term Hematopoietic Stem Cell
TNF	Tumor Necrosis Factor
WBC	White Blood Cell

CHAPTER ONE

INTRODUCTION

Myeloproliferative neoplasms (MPN) are clonal hematological disorders characterized by dysregulated proliferation of one or more mature myeloid lineages. They can be classified into two groups: Philadelphia chromosome positive (Ph+) MPN, which includes chronic myeloid leukemia (CML) and the Ph- diseases, which include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). The prevalence of non-CML MPN is estimated to be around 300,000 patients in the United States (Mehta et al., 2013). CML is characterized by the presence of the BCR-ABL fusion protein and neutrophilia. A hallmark feature of PV is an elevated hematocrit whereas ET is defined by an increased platelet count. PMF patients usually present with enlarged spleens, extramedullary hematopoiesis accompanied by bone marrow fibrosis and constitutional symptoms such as weight-loss, fevers and fatigue. A subset of patients with PV and ET can also progress to secondary myelofibrosis (MF). Due to elevated

blood counts, patients with MPN suffer complications including thrombosis, hemorrhage and infection. Over time, these patients develop progressive bone marrow failure and may also transform to acute myeloid leukemia (AML) with a particularly poor prognosis.(Mesa et al., 2005)

In 1951, the hematologist William Dameshek recognized that these distinct disorders actually share certain common features, which he stated as follows - 'It is possible that these various conditions—'myeloproliferative disorders'—are all somewhat variable manifestations of proliferative activity of the bone marrow cells, perhaps due to a hitherto undiscovered stimulus. This may affect the marrow cells diffusely or irregularly with the result that various syndromes, either clear-cut or transitional, result.' (Dameshek, 1951).

GENETIC ALTERATIONS IN PH- MPN

The genetic basis for this commonality observed by Dameshek and others became clear in 2005, with the identification of a recurrent somatic mutation in the cytosolic tyrosine kinase, JAK2. The Janus family of kinases (JAK) is involved in the transduction of cytokine-mediated signals in a number of cell types and regulates cytokine-dependent gene expression, in part by activating the signal transducers and activators of transcription (STATs). The JAK-STAT pathway can interact with the receptor tyrosine kinase/Ras/MAPK pathway and also result in activation of the PI3K signaling pathway leading to complex biological consequences (Rane and Reddy, 2000; Shuai et al., 1994).
The most common genetic mutation found in MPN is a guanine to thymine transversion that results in the substitution of a phenylalanine in place of a valine at position 617 in the

pseudokinase domain of JAK2 (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005b). The *JAK2V617F* mutation occurs in approximately 95% of patients with PV and 40% - 60% of patients with ET or MF (Levine et al., 2007). It has also been identified in a small proportion of patients with chronic myelomonocytic leukemia (CMML) and a subtype of myelodysplastic syndrome known as refractory anemia with ringed sideroblasts and thrombocytosis (Levine et al., 2005a; Schmitt-Graeff et al., 2008).

Gain of function mutations in the SH2 region in exon 12 of JAK2 were also identified in JAK2V617F negative cases of PV. Expression of these mutants in Ba/F3 cell lines and in retroviral bone marrow transplant models caused a phenotype similar to JAK2V617F (Scott et al., 2007). Taken together, mutations in the *JAK2* gene occur in almost 100% of patients with PV.

Mutational analysis of the JAK-STAT signaling pathway in *JAKV617F* negative cases of MPN led to the discovery of somatic mutations at codon 515 in the thrombopoietin receptor (*MPL*) in a small proportion of ET and MF patients (W515L/K) (Pikman et al., 2006). The W515 residue is located in an amphipathic region between the transmembrane and cytoplasmic domains of the receptor. This region is thought to be involved in maintaining the receptor in an inactive, closed conformation in the absence of ligand (Staerk et al., 2006). Similar to JAK2 alterations, mutation of this gene results constitutive activation of JAK2 and downstream STATs (Pikman et al., 2006).

Genetic aberrations in the adaptor protein, *LNK*, which is a known negative regulator of the JAK-STAT pathway, have also been reported in a small proportion of *JAK2/MPL* negative ET/MF patients (Oh et al., 2010). Mutations in the E3 ubiquitin ligase, *CBL,* have also been reported in MPN and have been associated with a poor prognosis (Aranaz et al., 2012; Grand et al., 2009; Schwaab et al., 2012).

The frequency of occurrence of the most common mutations found in the non-CML MPN is provided in Table 1. Aside from the JAK-STAT signaling pathway, other mutations in several epigenetic regulators have been observed recently in MPN patients (mostly at a frequency <10%) including, *IDH1*, *IDH2*, *TET2*, *EZH2*, *DNMT3A*, *ASXL1*, *SF3B1*, *IKZF1* and others (Shih et al., 2012). Most of these co-occur with the *JAK2/MPL* mutations and their role in disease pathogenesis remains under active investigation.

Table 1.1 Frequency of common mutations in non-CML MPN

MPN	Gene Mutation					
	*JAK2*V617F	*JAK2* Exon 12	*MPL*	*LNK*	*TET2*	*ASXL1*
Polycythemia vera (PV)	95%	3% - 5%	NR	Present	9.8% - 16%	2% - 5%
Essential thrombocytosis (ET)	55% - 60%	NR	3% - 5%	3% - 6%	4.4% - 5%	5% - 10%
Primary myelofibrosis (MF)	50% - 60%	NR	8% - 10%	3% - 6%	7.7% - 17%	13% 26%
Post-PV/ET MF	50% - 60%	NR	NR	NR	14%	22% - 38.5%

NR: Not Reported

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BIOLOGY OF JAK-STAT PATHWAY

Four mammalian JAKs have been identified, namely JAK1, JAK2, JAK3, and TYK2 (Wilks et al. 1991, Harpur et al. 1992, Takahashi et al. 1994, Krowleski et al. 1990). These proteins are characterized by the presence of two highly homologous domains at the carboxyl terminus: the catalytic kinase domain (JH1) along with a pseudokinase (JH2) domain. JAK1, JAK2 and TYK2 are ubiquitously expressed as compared to JAK3, which is primarily expressed in hematopoietic cells (Kawamura et al., 1994). Cytokine receptors that interact with JAK kinases include homodimeric and heterodimeric Type I receptors that bind hormones, interleukins or colony-stimulating factors, and heterodimeric Type II receptors that bind interferons and IL-10-family cytokines (Leonard and O'Shea, 1998). The different JAK family members can form heterodimeric as well as homodimeric complexes depending on the specific cytokine receptor and transduce downstream signaling.

The crucial function of the JAK kinases in development, and in hematopoiesis in particular, has been elucidated from knockout mouse models. JAK1 mediates signaling of several pro-inflammatory cytokines such as IL-1, IL-6 and tumor necrosis factor alpha (TNFα). JAK1 knockout mice die perinatally and have impaired lymphocyte development (Rodig et al., 1998). JAK3 associates only with the common gamma chain (γc) found on lymphocytes and JAK3 deficient mice have a severe defect of B, T and NK cells (Nosaka et al., 1995; Park et al., 1995; Thomis et al., 1995). TYK2 can associate with JAK1 and JAK2 and mediates signaling of cytokines including IL-12, IL-22 and

interferon alpha/beta. TYK2 knockout mice are viable but have impaired immunological responses to certain infections (Karaghiosoff et al., 2000)

JAK2 is the only member that signals as a homodimer and associates with single chain receptors such as erythropoietin, thrombopoetin, growth hormone and granulocyte colony stimulating factor (G-CSF). Mice lacking JAK2 die in embryogenesis due to failure of definitive hematopoiesis (Neubauer et al., 1998; Parganas et al., 1998). This result phenocopies loss of erythropoietin (Epo) or the Epo receptor (EpoR). Cells from JAK2-/- mice do not respond to thrombopoietin, IL-3, GM-CSF and IFNγ indicating the requirement for JAK2 for these cytokine receptors (Parganas et al., 1998).

The JH2 domain of JAK2 was recently shown to possess catalytic activity and autophosphorylate Ser523 and Tyr570, which are known negative regulatory sites in JAK2 (Ungureanu et al., 2011). There is also evidence that the JH2 domain can stimulate the catalytic activity of the JH1 kinase domain. Mutations in the JH2 domain of JAK3 lead to loss of kinase function and an immunodeficient phenotype (Russell et al., 1995). Further, although deletion of the pseusokinase domain of JAK2 results in increased basal activity of the kinase, the hypersensitivity to cytokine stimulation observed in *JAK2V617F* mutant cells is lost upon deletion of the entire JH2 domain. Also, the basal activity of the deletion mutants is significantly lower than that of the cytokine stimulated full length JAK2 kinase (Saharinen and Silvennoinen, 2002; Saharinen et al., 2000). These observations suggest an additional positive regulatory role of the pseudokinase domain of the JAK kinases. The V617F mutation abrogates the catalytic activity of the

pseudokinase domain, thereby relieving the autoinhibitory regulation on JAK2. Interestingly, MPN patients with the *JAK2V617F* mutation have decreased phosphorylation at Tyr570, one of the negative regulatory sites in JAK2. (Ungureanu et al., 2011).

The crustal structure of the wild type and JAK2V617F mutant pseudokinase structure showed that the V617F mutation rigidified a critical helix in the JH2 domain and stabilized the stimulatory interaction necessary for activation of the JH1 domain (Bandaranayake et al., 2012). Thus, both loss of the inhibitory function along with gain of the stimulatory function of the JH2 domain might contribute to constitutive pathway activation in JAK2 mutant cells. The recently resolved crystal structure of the JAK1 pseudokinase domain included the polypeptide chain connecting the SH2 domain with the pseudokinase domain (SH2-PK linker) (Toms et al., 2013). This linker encompasses the region where PV-associated exon 12 mutations are found in JAK2. Structural and mutational analysis revealed that this linker plays a critical role in mediating a conformational change that is required for kinase activation upon cytokine stimulation (Toms et al., 2013; Zhao et al., 2009b). Thus, the JH2 domain acts as a cytokine-inducible switch that can regulate the catalytic activity of the JH1 kinase domain.

JAK-STAT PATHWAY ACTIVATION IN MPN

The critical role of this pathway in disease pathogenesis has been borne out in several pre-clinical models. Expression of the JAK2V617F allele in vitro transforms hematopoietic cells to cytokine independent growth as well as makes them hypersensitive to cytokine stimulation (Levine et al., 2005b). Transformation is dependent on coexpression of a cognate receptor such as erythropoietin (EPO-R), thrombopoietin (MPL) or the granulocyte colony-stimulating factor (GCSF-R) receptor (Lu et al., 2008). *In vivo* expression of JAK2V617F, either in a bone marrow transplant or genetic knockin model results in fully penetrant myeloproliferative disease, marked by elevated hematocrit/platelets and extramedullary hematopoiesis leading to splenomegaly, comparable to human PV. (Akada et al., 2010; Bumm et al., 2006; Lacout et al., 2006; Li et al., 2010; Marty et al., 2010; Mullally et al., 2010; Wernig et al., 2006; Zaleskas et al., 2006). Bone marrow and spleen cells isolated from diseased mice are able to form erythroid colonies in methylcellulose in the absence of cytokines and are hypersensitive to erythropoietin, which is a clinical feature of PV (Wernig et al. 2006, Lacout et al. 2006). Mutational analysis of patient samples as well as functional studies in genetic models have revealed the cell of origin in this disease to be a long-term hematopoietic stem cell (Jamieson et al., 2006; Mullally et al., 2010).

Expression of MPLW515L/K in murine Ba/F3 and 32D cells led to cytokine independent growth and constitutive phosphorylation of JAK2 and downstream effectors, STAT5, STAT3, AKT and p44/42 MAPK in the absence of ligand stimulation (Pikman et al., 2006). Reconstitution of MPLW515L in vivo in a bone marrow transplant model results

in a myelofibrosis-like disease characterized by following features: (i) Short latency (ii) Severe leukocytosis and thrombocytosis (iii) Marked splenomegaly and increased reticulin fibrosis (iv) Expansion of megakaryocyte/erythrocyte progenitors (Koppikar et al., 2010; Pikman et al., 2006). Primary cells isolated from diseased mice are able to form megakaryocytic colonies in the absence of cytokines in methycellulose culture, which is a feature of clinical ET. (Pikman et al., 2006).

In addition to activating the STAT family of transcription factors, JAK2 can also regulate gene expression via epigenetic mechanisms (Fig 1.1). Both wild type and mutant JAK2 have been found to translocate into the nucleus in hematopoietic cell lines and primary cells (Dawson et al., 2009; Rinaldi et al., 2010). It can directly phosphorylate tyrosine 41 on histone H3 and disrupt the binding of the repressive factor, HP1α, to this site (Dawson et al., 2009). This mechanism has been shown to regulate expression of several oncogenic target genes, including JAK2 itself (Rui et al., 2010a). Another study showed that mutant JAK2 can bind and phosphorylate the protein arginine methyltransferase, PRMT5, with a much higher affinity than wildtype JAK2 (Liu et al., 2011). This phosphorylation disrupts its interaction with methylosome protein 50 (MEP50), which leads to significant reduction of global histone arginine methylation and gene expression changes that affect erythroid differentiation and clonogenic activity. These studies demonstrate additional non-canonical roles of JAK2 that might also contribute to oncogenesis.

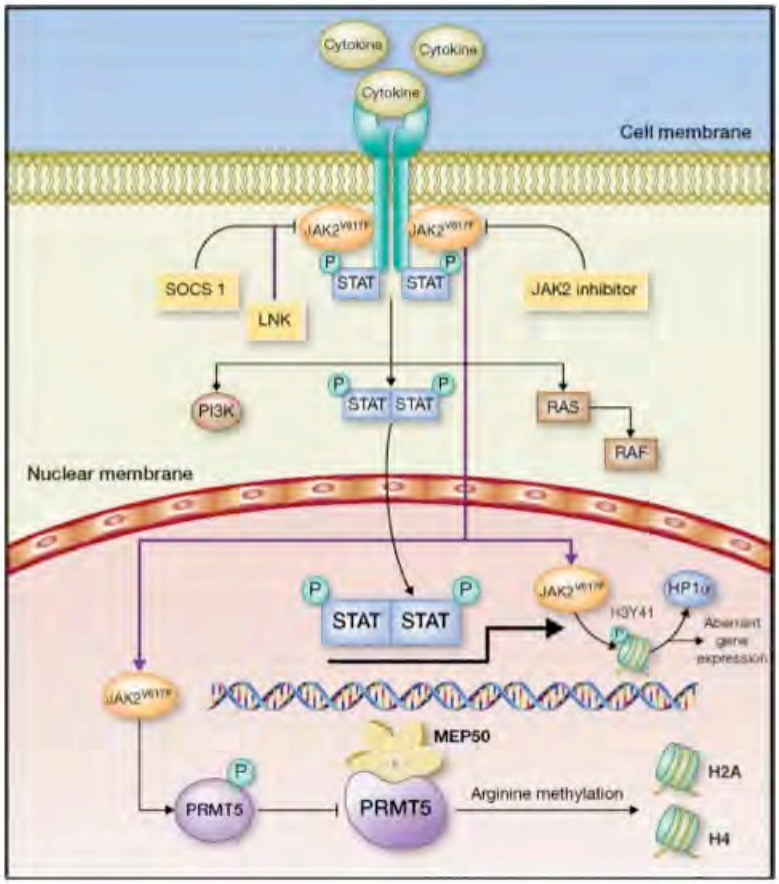

Figure 1.1: JAK-STAT signaling in MPN. In the presence of $JAK2^{V617F}$ mutations, the JAK/STAT pathway is constitutively activated, which leads to activation of the STAT family of transcription factors as well as activation of MAPK and PI3K pathways. The activity of the JAK2/STAT pathway is negatively regulated by SOCS1 and LNK. JAK2 can also localize to the nucleus and phosphorylate histone H3 (H3Y41), thus preventing its binding to the repressor HP1α. $JAK2^{V617F}$ also binds and phosphorylates the arginine methyltransferase PRMT5, which hampers its interaction with methylosome protein 50 (MEP50), thus decreasing global arginine methylation of histones H2A and H4.(Adapted from Clinical Cancer Research, Copyright 2013, 19(8), 1933-40, Quintás-Cardama A, Verstovsek S., Molecular pathways: Jak/STAT pathway: mutations, inhibitors, and resistance, with permission from AARC)

JAK-STAT PATHWAY ACTIVATION IN OTHER MALIGNANCIES

In addition to MPN-associated mutations, which have been extensively described and studied, the JAK-STAT pathway is aberrantly activated in a number of other malignancies. Activating translocations involving JAK2 have been described in both lymphoid and myeloid cancers(Lacronique et al., 1997; Peeters et al., 1997). A recurrent mutation at residue 683 (*JAK2 R683G/S*), is present in a significant proportion of patients with Down syndrome associated acute lymphoblastic leukemia (ALL) (Bercovich et al., 2008; Kearney et al., 2009; Mullighan et al., 2009). Similar to the *V617F* mutation, this residue lies in the pseudokinase domain of JAK2 and results in constitutive pathway activation when mutated. Along with *JAK2*, gain of function mutations in *JAK1* and *JAK3* have been reported in Ph- pediatric and adult ALL and are associated with a high-risk disease subtype with a poor overall prognosis(Flex et al., 2008; Mullighan et al., 2009). Mutations in *JAK2* and *JAK3* have also been identified in cell lines as well as patients with acute megakaryoblastic leukemia (AMKL), a rare subtype of myeloid leukemia (Mercher et al., 2006; Walters et al., 2006).

Activating mutations and overexpression of cytokine receptors like *CRLF2* and *IL7R*, which lead to hyperactivation of the JAK-STAT pathway have recently been reported in ALL(Harvey et al., 2010; Shochat et al., 2011; Yoda et al., 2010; Zenatti et al., 2011). Additionally, JAK2 is found to be amplified by gain of chromosome 9p24 in a significant proportion of Non-Hodgkin's lymphoma. (Joos et al., 2000). The JAK-STAT pathway is dysregulated in non-hematological cancers as well, including breast, lung and head and neck. Elevated cytokine secretion by tumor and stromal cells and increased receptor

expression seem to be the underlying mechanism for pathway activation in this context (Sansone and Bromberg, 2012). These reports point to an increasingly important role of the JAK-STAT pathway in the pathogenesis of a variety of cancers, thus making it an attractive therapeutic target.

CLINICAL ADVANCES IN JAK INHIBITORS

Prior to the discovery of these activating mutations in the JAK-STAT pathway, patients with MPN were treated with conventional therapies including phlebotomy, hyroxyurea, anagrelide or splenectomy, which help alleviate symptoms. However, the only curative option is allogeneic bone marrow transplant, which is associated with significant morbidity and mortality (Ballen et al., 2010).

Following the identification of activating mutations in the JAK-STAT pathway, there was considerable effort put into the development of small molecule inhibitors that can target the kinase activity of JAK2. The remarkable success of Abl kinase inhibitors such as imatinib in the treatment of CML provided a strong rationale for pursuing a similar therapeutic strategy in other MPN. Further, since *JAK2/MPL* negative patients with ET and MF also display an activated JAK-STAT gene expression signature, targeting this pathway has broad therapeutic implications in MPN. The clinical experience with some of the more promising drugs is described below

Ruxolitinib

The dual JAK1/JAK2 inhibitor ruxolitinib (INCB18424, Jakafi®) is FDA-approved for the treatment of myelofibrosis and is in late-stage clinical trials for intermediate and high-risk PV. It potently inhibits kinase activity of JAK1 and JAK2 in cell-free assays and reduces downstream STAT3/STAT5 signaling in MPN cell lines. It also inhibits cell proliferation and induces apoptosis in cells lines and hematopoietic progenitor cells isolated from MPN patients (Quintas-Cardama et al., 2010). In a MPLW515L-driven bone marrow transplant model of ET/MF, treatment with INCB16562 (a structurally related JAK2 inhibitor) significantly reduced blood counts, splenomegaly, serum cytokines and improved survival (Koppikar et al., 2010). The clinical efficacy of ruxolitinib was evaluated in two Phase II/III trials for PMF and secondary post-ET/PV MF: the Controlled MyeloFibrosis study with ORal JAK inhibiTor) trials, which compared ruxolitionib to either placebo (COMFORT-I) or best available therapy (COMFORT-II). Patients treated with ruxolitinib experienced significant alleviation of constitutional symptoms, reductions in splenomegaly and levels of circulating inflammatory cytokines (Harrison et al., 2012; Verstovsek et al., 2010; Verstovsek et al., 2012b). Ruxolitinib also improved overall survival with sustained responses with continued treatment and manageable side-effects (Verstovsek et al., 2012a). Further, preliminary data from a Phase II study of ruxolitinib in PV patients also suggests significant reductions in white blood counts and improvement in constitutional symptoms (Verstovsek et al., 2012d)

SAR302503

Another drug in late-stage clinical testing is SAR302503 (formerly TG101348), which is more specific towards JAK2 and JAK2V617F as compared to other JAK kinases. Further, the compound inhibited *ex vivo* hematopoietic colony growth in MPN patients (Lasho et al., 2008)and was efficacious in a mouse bone marrow transplant driven by JAK2V617F (Wernig et al., 2008). The clinical experience with SAR302503 in a Phase II study showed improvements in splenomegaly and constitutional symptoms, and durable reduction in mutant allele burdens in patients with intermediate or high-risk myelofibrosis, PV, or ET, and in patients with ruxolitinib-resistant or intolerant myelofibrosis (Pardanani et al., 2011b). SAR302503 is also being investigated in a Phase III trial in patients with intermediate or high-risk myelofibrosis.

CYT387

CYT387 is a Type I JAK1/2 inhibitor that was shown to suppress growth and downstream signaling in MPN cell lines as well as inhibit growth of erythroid colonies from PV patients (Pardanani et al., 2009). It was also efficacious in murine models of PV causing reductions in blood counts, spleen sizes and circulating cytokines (Tyner et al., 2010). In a phase I/II clinical trial for myelofibrosis, Pardanani and colleagues reported improvements in patient constitutional symptoms and reduction in splenomegaly. Additionally, about 70% of the patients enrolled in this trial became transfusion independent for prolonged periods suggesting this agent may have different effects on erythroid response compared to other agents in this class (Pardanani et al., 2013).

In addition to these compounds, there are a number of other inhibitors including CEP701, SB1518, LY2784544, NS018, AZD1480, and BMS911543 undergoing pre-clinical and clinical testing primarily in hematological malignancies but there is little data available regarding the efficacy of these inhibitors thus far (Table 2).

All the JAK inhibitors tested thus far in clinical trials have been ATP mimetic type I inhibitors, which are defined by their ability to bind the region occupied by the adenine ring of ATP in the active conformation of the kinase (Zhang et al., 2009). Treatment with type I JAK inhibitors leads inhibition of kinase activity accompanied by a paradoxical increase in activation loop phosphorylation of JAK2 (Andraos et al., 2012). On the other hand, type II inhibitors target the ATP-binding pocket as well a hydrophobic region that is only exposed when the DFG motif in the activation loop is in the 'out' or inactive conformation. BBT-594, a type II inhibitor of JAK2 can stabilize its inactive conformation and lead to decreased JAK2 phosphorylation and inhibition of downstream signaling (Andraos et al., 2012). However, this class of inhibitors has not been investigated in pre-clinical models so far.

Table 1.2: JAK inhibitors in clinical development for MPN

Agent	Company	Activity	Status
Ruxolitinib (INCB018424)	Novartis/Incyte	JAK1/JAK2	FDA approved for MF, Phase II clinical trials for PV
TG101348/SAR302503	Sanofi Aventis	JAK2, Flt3	Phase III for MF
CYT387	Cytopia	JAK1/JAK2/TYK2	Phase II for MF
Pacritinib (SB1518)	Sbio	JAK2, Flt3	Phase II for MF
AZD1480	Astra Zeneca	JAK1/JAK2	Phase I for MF
LY2784544	Eli Lily	JAK2 V617F	Phase I for MF
Lestaurtinib (CEP701)	Cephalon	JAK2, Flt3	Phase I for MF
BMS-911453	Bristol- Myers Squibb	JAK2	Phase I for MF
NS-018	Nippon-Shinyaku	JAK2, Src	Phase I for MF

MF: Myelofibrosis, PV: Polycytehmia vera

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LIMITATIONS OF JAK2 INHIBITORS

JAK inhibitors have been remarkably effective at ameliorating constitutional symptoms, reducing splenomegaly and improving survival in MPN patients. However, they have had limited efficacy in significantly reducing the mutant allele burden. They have also not successfully reduced cytopenias or reversed bone marrow fibrosis.

Similar results have also been observed in pre-clinical murine models of MPN. Mullally et al. (Mullally et al., 2010) generated a JAK2V617F knock-in mouse that had disease features of human PV. Treatment of the primary JAK2V617F knock-in mice with TG101348 for 6 weeks reduced spleen weights and improved histopathology in inhibitor treated mice compared to vehicle treated mice. The authors then purified Lin- ckit+ Sca1+ cells, which are enriched in hematopoietic stem cells; from the vehicle treated and JAK inhibitor treated primary mice and transplanted equal number of cells into lethally irradiated secondary recipients. All the secondary recipients however showed complete hematological reconstitution along with increased hematocrits, suggesting that inhibitor treatment was not effective in eradicating or even reducing the number of MPN-initiating cells. Further, longer treatment duration of 10 weeks was also not enough to eliminate the disease initiating cells as seen by increased hematocrits in tertiary recipients three weeks after transplantation suggesting that JAK inhibitor therapy was not curative in this model. We observed similar results in the MPLW515L GFP driven mouse bone marrow transplant model that mimics many features of human ET/PMF (Koppikar et al., 2010). Four weeks of treatment with INCB016562, another JAK2 inhibitor, reduced blood counts, improved survival and histopathology of treated mice; however, it did not reduce

GFP percentage (a measure of the mutant allele burden) in peripheral blood or bone marrow in treated mice. Further, disease rapidly relapsed following cessation of treatment, again demonstrating the absence of long-term cure (Koppikar et al., 2010). There are numerous hypotheses regarding the limited efficacy of JAK inhibitors in MPN, some of which are discussed below:

Acquisition of secondary mutations

One of the best-studied mechanisms of resistance to tyrosine kinase inhibitors is the acquisition of secondary mutations in the protein being targeted. Instances include Abl-kinase inhibitors in CML (Gorre et al., 2001), EGFR inhibitors in lung cancer (Kobayashi et al., 2005; Pao et al., 2005) and others. Most commonly, these mutations occur in the kinase domain and interfere with drug binding. Based on this assumption, several groups conducted *in vitro* studies to identify possible genetic mechanisms that might develop with long-term usage of JAK inhibitors. A saturation mutagenesis screen performed in JAK2V617 mutant cells identified five non-synonymous mutations in the JAK2 kinase domain that conferred resistance to ruxolitinib (Deshpande et al., 2012). Further, these mutations displayed cross-resistance to other JAK2 kinase inhibitors such as CYT387, TG101348, CEP701 and AZD1480. Another group isolated several other mutations in TEL-JAK2 mutant cells, in which JAK2 is constitutive activated via the fusion of its pseudokinase and kinase domain to the PNT oligomerization domain of TEL (Marit et al., 2012). These alterations primarily conferred resistance to JAK Inhibitor I, a commercially available pan-JAK inhibitor but did not affect response to other clinical inhibitors indicating that these might be compound specific mutations. Neither group

isolated the putative gatekeeper mutations at position M929, which is predicted to confer resistance to ATP-competitive inhibitors, suggesting that these screens did not achieve complete saturation. Upon testing the gatekeeper mutation, they found it conferred modest resistance to ruxolitinib and JAK inhibitor I.

Since JAK2 is mutated in other hematological malignancies including B-ALL, Weigert et al. utilized a similar approach in *JAK2R683G* mutant cell lines using a novel JAK2 inhibitor, NVP-BVB808 and identified the same alleles as previous studies (Weigert et al., 2012b). They also demonstrated that these alterations conferred varying degrees of resistance to other clinically relevant JAK inhibitors in JAK2V617F mutant cells.

All the mutations identified thus far are located in the kinase domain of JAK2. A number of the mutations occur at residues located in the ATP binding pocket of JAK2 that have been shown to interact with JAK inhibitor I based on the crystallographic analyses (Lucet et al., 2006) and presumably would interact with other Type I JAK inhibitors. They are also relatively few in number compared to those identified in BCR-ABL mutant cells in response to imatinib treatment (Azam et al., 2003) suggesting that a few critical residues might be involved in mediating resistance. Of note, no mutations in *JAK2* have been reported in MPN patients treated with JAK inhibitors to date.

Insufficient pathway inhibition

The JAK-STAT pathway is a crucial regulator of hematopoiesis and JAK2 is the major kinase required for erythropoietin receptor signaling and normal red blood development (Neubauer et al., 1998; Parganas et al., 1998). The JAK inhibitors in clinical development

are not specific for mutant JAK2 and can also efficiently inhibit wild type JAK2. Therefore, using doses that are capable of inhibiting mutant JAK2 activity is bound to also have adverse effects on normal hematopoiesis. This has been borne out in the clinic, where JAK2 inhibitors have been associated with dose limiting toxicities including anemia and thrombocytopenia (Verstovsek et al., 2010). This is unlike other highly efficacious kinase inhibitors, which are highly specific for the mutant protein such as vemurafenib in BRAFV600E mutant melanoma, or others like imatinib in CML that can be tolerated at high doses. This inability to sufficiently inhibit the pathway at clinically tolerable doses might also explain the lack of second site mutations in patients.

Activation of alternate pathways

Another strategy adopted by cancer cells to overcome targeted therapies is the activation of alternate, bypass pathways. In EGFR mutant non-small cell lung cancer, treatment with EGFR inhibitors can lead to amplification of c-MET, which activates downstream PI3K signaling in and EGFR-independent manner (Engelman et al., 2007). EGFR mutant cell lines can also persist in the context of chronic EGFR and downstream pathway inhibition by signaling via the IGF-1 receptor pathway (Sharma et al., 2010). Such a mechanism is a possibility in MPN as well and is under active investigation.

SCOPE OF THESIS

In order to gain a better understanding of the underlying mechanism of effects of JAK2 inhibitors in MPN, we attempted to model this phenomenon in the laboratory. We chronically exposed *JAK2/MPL* mutant cell lines to JAK inhibitors and demonstrated that

MPN cells can survive in the context of chronic JAK inhibitor exposure by reactivating the JAK-STAT pathway via the formation of heterodimers between JAK2 and other JAK kinases. This finding was recapitulated in murine models as well as in samples from MPN patient treated with ruxolitinib. Reactivation of the JAK-STAT pathway in inhibitor persistent cells is facilitated by stabilization of phosphorylated JAK2 by Type I inhibitors, which can be overcome by novel Type II inhibitors that engage JAK2 in its inactive conformation. These findings led us to evaluate the requirement of JAK2 in naïve and inhibitor persistent MPN cells. Genetic deletion of JAK2 in *in vivo* model of ET/MF revealed an indispensable role for JAK2 in MPN pathogenesis. Further, RNAi and genetic loss of function experiments revealed that inhibitor persistent cells remain dependent on JAK2 for their survival. Based on these data, we evaluated Hsp90 inhibitors, which target JAK2 degradation, and found that combination of JAK and Hsp90 inhibitors was more efficacious than JAK inhibitor monotherapy. Importantly, Hsp90 inhibition was able to overcome JAK inhibitor persistence in pre-clinical models and in primary samples. These findings indicate that JAK2 is a bona fide therapeutic target for MPN and combinatorial strategies or JAK inhibitors that can overcome persistence have the potential to improve therapeutic efficacy in patients with MPN.

CHAPTER TWO

MATERIALS AND METHODS

REAGENTS AND CELL LINES

The pan JAK inhibitor, JAK Inhibitor I, was purchased from Calbiochem (catalogue no. 420097). The JAK1 and JAK2 specific inhibitor INCB18424 was purchased from Chemietek. PU-H71 (8-(6-iodobenzo[d][1.3]dioxol-5-ylthio)-9-(3-(isopropyl amino)propyl)-9H-purine-6-amine) was synthesized as reported previously(Marubayashi et al., 2010). BBT-594 was a gift from T. Radimerski. Stock aliquots (1 mM) were prepared in DMSO and diluted in appropriate medium before use. Antibodies used for western blotting and immunoprecipitation included phosphorylated and total JAK2, STAT3, mitogen-activated protein kinase, AKT and phosphoSTAT5 (Cell Signaling Technologies). Total STAT5 antibody was purchased from Santa Cruz Biotechnology, and actin from EMD Chemicals. JAK1 and TYK2 antibodies were purchased from BD Transduction. Pan phosphotyrosine antibody was purchased from Millipore. The

generation and maintenance of Ba/F3 hMPLW515L and Ba/F3 EpoR-V617F cells have been described previously(Pikman et al., 2006). The *JAK2V617F*-positive human leukemic cell line SET-2 was grown in RPMI 1640 medium with 20% heat-inactivated serum, whereas UKE-1 (also *JAK2V617F*-positive) cells were grown in RPMI 1640 with 10% fetal calf serum, 10% horse serum and 1 μM hydrocortisone. Cycloheximide was purchased from Sigma. Patient mononuclear cells were grown in MEM Alpha + 20% fetal calf serum.

IN VITRO INHIBITOR ASSAYS

Viable cells were plated in triplicate at 10,000 cells per well in 96-well tissue culture treated plates in 200 μl medium with increasing concentrations of the JAK2 inhibitor or PU-H71. Inhibitor assays at 48 h were assessed with the cell viability luminescence assay CellTiter-Glo (Promega; catalogue no. G7571). Results were normalized to growth of cells in medium containing an equivalent volume of DMSO. The effective concentration at which 50% inhibition in proliferation occurred was determined with GraphPad Prism 5.0 software.

WESTERN BLOTTING AND IMMUNOPRECIPITATIONS

For Western blot analysis, cells were harvested after treatment inhibitor, washed in ice-cold PBS containing sodium orthovanadate, and collected in lysis buffer (150 mM NaCl, 20mM Tris (pH 7.4-7.5), 5mM EDTA, 1% Triton-X, 10% Glycerol) containing Protease Arrest (G-Biosciences), Phosphatase Inhibitor Cocktail II (EMD Chemicals). Protein was quantified using the Bio-Rad Bradford protein estimation and 30 – 50ug was loaded per well in 4%–12% Bis-Tris electrophoresis gels (Invitrogen). Protein was transferred on to

0.45micron nitrocellulose membranes and blocked in TBS with 0.5% Tween-20 with 5% non-fat milk.

For immunoprecipitation experiments, cells were harvested either at steady-state conditions or after 4 h of incubation with a JAK2 inhibitor. Protein was normalized with the Bradford dye, and 500–1,000 μg of total protein was incubated overnight with the appropriate antibody, followed by incubation with Protein G-agarose beads (EMD Chemicals) for a further 2 h. After incubation, cells were washed three times with cold PBS and boiled with Laemmli buffer for 10 min. Supernatant was loaded onto gels processed similar to western blotting.

KNOCKDOWN EXPERIMENTS

siRNA oligonucleotides targeting JAK1 and TYK2 were purchased from Invitrogen and used in accordance with the manufacturer's instructions. The two siRNA oligonucleotides used for JAK1 knockdown were 5′-GCACAGAAGACGGAGGAAAUGGUAU-3′ (JAK1VHS41387) and 5′-GCCUUAAGGAAUAUCUUCCAAAGAA-3′ (JAK1VHS41388). The siRNA sequence for TYK2 included a combination of two oligonucleotides (5′-UUCUCAUGGACUGUCUUCAGAAUGG-3′ (TYK2VHS41729) and 5′-GCAGCAAGUAUGAUGAGCAAGCUUU-3′ (TYK2VHS41246)). Scrambled siRNA was purchased from Dharmacon (D-001206-13-20). Cells were transfected with 20uM of scrambled siRNA, siJAK1, siTYK2, or both siJAK1 and siTYK2. Viability assays were set up 24 h after transfection and harvested after 48 h. Cells were harvested at 72 h after transfection to verify knockdown and assess downstream signalling. Persistent cells were

cultured in the presence of inhibitor during the entire experiment. shRNA oligonucleotides against JAK2 and TYK2 were gifts from L. Staudt and T. Look, respectively. shRNA target sequences used for knockdown of JAK2 were 5′-CTCTTCGAGTGGATCAAATAA-3′ (shRNA 1) and 5′-GCAGAATTAGCAAACCTTATA-3′ (shRNA 2). The target sequence for shRNA against TYK2 was 5′-CGTGAGCCTAACCATGATCTT-3′. Lentiviral particles were generated with the use of standard procedures. Cells were spinfected with virus and selected with puromycin. Cell viability was monitored with trypan blue (for JAK2 knockdown studies), and cells were harvested 10 days after selection in puromycin. Persistent cells were cultured in the presence of respective inhibitors during the entire experiment.

QUANTITATIVE RT–PCR ANALYSES

Total RNA was extracted with the RNeasy Mini Kit (Qiagen), and cDNA was synthesized with the Verso cDNA Kit (Thermo Scientific). Quantitative PCR was performed with FastStart Universal SYBR Green Master (Roche) with the following primer sequences: mouse JAK2, 5′-GATGGCGGTGTTAGACATGA-3′ (forward) and 5′-TGCTGAATGAATCTGCGAAA-3′ (reverse); mouse β-actin, 5′-GATCTGGCACCACACCTTCT-3′ (forward) and 5′-CCATCACAATGCCTGTGGTA-3′ (reverse); human JAK2, 5′-TCTTTCTTTGAAGCAGCAAG-3′ (forward) and 5′-CCATGCCAACTGTTTAGCAA-3′ (reverse); human HPRT1, 5′-AGATGGTCAAGGTCGCAAG-3′ (forward) and 5′-GTATTCATTATAGTCAAGGGCATATC-3′ (reverse).

IN VITRO KINASE ASSAYS

Protein was harvested from naive and persistent SET-2 cells and used for *in vitro* kinase assays. Endogenous JAK2 protein was precipitated with anti-JAK2 antibody (Santa Cruz; catalog no. sc-34480) and Protein G-Sepharose gel. For JAK2 activity assay, the immunoprecipitated JAK2 was incubated with myelin basic protein in a buffer containing 25 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 μM ATP and 2 mM dithiothreitol. The reaction was incubated at 25 °C with 1 and 10 nM INCB18424 for 1 h and stopped by addition of the SDS sample loading buffer. Samples were run under reducing conditions on SDS–PAGE gels and immunoblotted using a pan phosphotyrosine antibody (Millipore).

FLOW CYTOMETRY

Bone marrow and spleen cells were filtered; red blood cells were lysed and washed in phosphate-buffered saline (PBS). Cells were incubated with the following antibodies for 30 minutes on ice in PBS + 2% BSA. For staining of myeloid progenitors, the antibodies used were CD11b, Gr-1, Ter119, CD3, CD4, NK1.1, B220, CD19 conjugated to APCCy7, c-kit-PE, Sca-1-PECy7, e450-CD16/32, e660-CD34. For chimerism and mature leukocyte staining, the antibodies used were CD45.1-e450, CD45.2-APC, CD11b-PECy7, Gr-1-PE. Data was collected on LSRFortessa (BD Biosciences) and analysis was performed on FlowJo.

PATIENT SAMPLES

The Institutional Review Boards of Memorial Sloan Kettering Cancer Center and M. D. Anderson Cancer Center approved sample collection and all experiments. Informed

consent was obtained from all human subjects before study. Mononuclear cells were freshly extracted using Ficoll separation from peripheral blood and used for studies. Cells were treated with 150nm ruxolitinib for 6 hours or 500nM PU-H71 for 16 hours and used for western blot analysis.

MURINE MODELS AND ANALYSIS OF MICE

JAK2$^{f/f}$ mice were a kind gift from Kay-Uwe Wagner. They were back-crossed into C57BL/6 for 7 generations and then crossed to Mx1-Cre also in a C57BL/6 background. For JAK2 deletion studies, bone marrow cells from CD45.2 JAK2$^{f/f}$ Mx1-Cre positive and negative mice were enriched using CD117 microbeads from Miltenyi and transduced with viral supernatants containing MSCV-*hMPLW515L*-GFP. 1 million transduced cells along with 500,000 CD45.1 c-kit+ bone marrow cells were injected into the tail veins of lethally irradiated female CD45.1 mice. Nonlethal submandibular bleeds were performed 14-21 days after transplantation to assess engraftment and chimerism. For initiation experiments, mice received 4 intra-peritoneal doses of 100ul polyI:polyC (1mg/ml) every other day starting at 14 days post-tail vein injection. For maintenance experiments, mice received pIpC injections starting 19 days following tail vein injection. All mice were sacrificed 3 months after tail vein injection for histological analysis and flow cytometry. For ruxolitinib experiments, mice were randomized to receive vehicle (20% Captisol in citrate buffer), 60mg/kg ruxolitinib twice daily by oral gavage or polyI:polyC by IP injection at day 18 after tail vein injection. All mice were sacrificed 6 weeks later for further analysis.

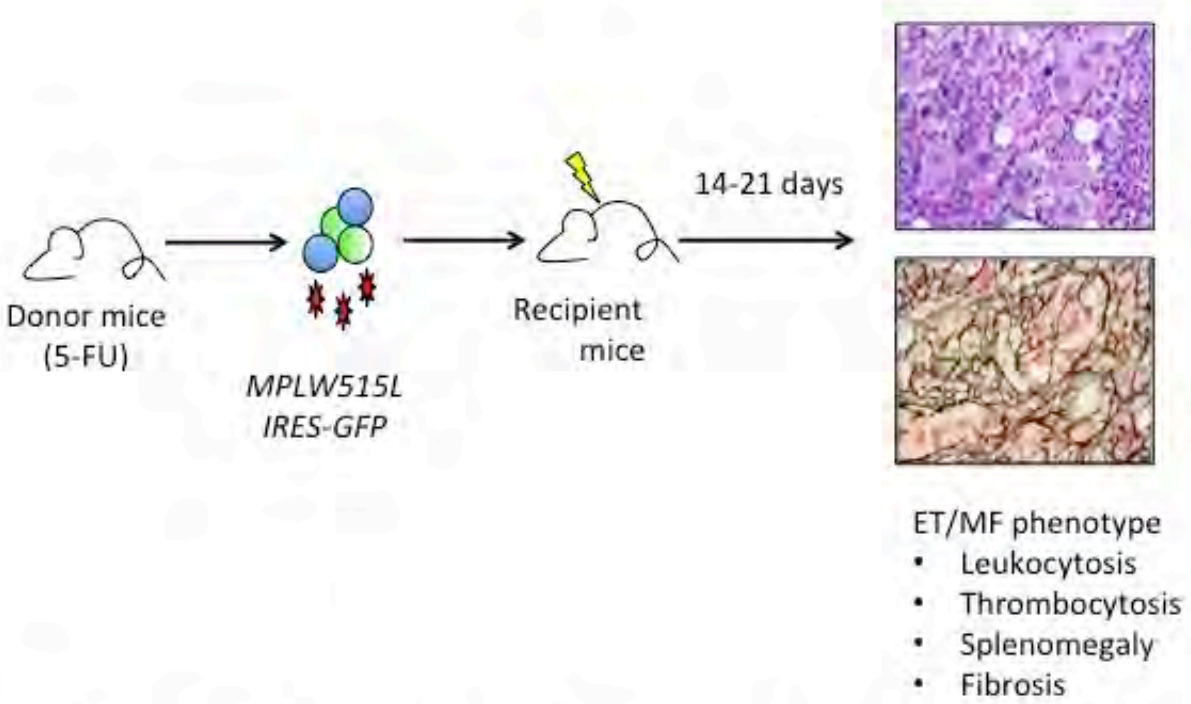


Figure 2.1 Schematic of retroviral bone marrow transplant model. Bone marrow cells are isolated from 5-FU treated donor mice and transduced with MPLW515L-IRES-GFP retrovirus. The cells are then tail-vein injected into lethally irradiated recipient mice. Within 2-3 weeks, all mice develop MPN characterized by elevated blood counts, splenomegaly and bone marrow fibrosis.

For ruxolitinib and PU-H71 combination studies, bone marrow cells were isolated from 5-FU treated Balb/C donor mice, transduced with *hMPLW515L-IRES-GFP* retrovirus and injected into lethally irradiated Balb/C female recipients. Disease establishment was assessed at Day 14 based on blood counts from submandibular bleeds. Mice were randomized to receive vehicle, 30mg/kg ruxolitinib twice daily by oral gavage, 90 mg/kg ruxolitinib twice daily by oral gavage and 30mg/kg ruxolitinib with 75mg/kg PU-H71 thrice weekly by intra-peritoneal injection. All mice were bled at day 14 following start of treatment. Two mice from each arm were sacrificed for further analysis. At the two-week time point, a subset of mice receiving 30mg/kg ruxolitinib alone also started receiving 75mg/kg PU-H71. Also, the ruxolitinib dose was increased to 90mg/kg in a subset of mice receiving combination treatment. At 4 weeks from the start of drug treatment, all mice were sacrificed for further analysis. For in vivo experiments, ruxolitinib was synthesized by the Bradner laboratory at the Dana-Farber Cancer Research Institute and PU-H71 was synthesized by the Chiosis laboratory at Memorial Sloan-Kettering Cancer Center.

Animal care was in strict compliance with institutional guidelines established by the Memorial Sloan-Kettering Cancer Center, the Guide for the Care and Use of Laboratory Animals and the Association for Assessment and Accreditation of Laboratory Animal Care International. For histopathology, tissues were fixed in 4% paraformaldehyde and then embedded in paraffin for analysis. Tissue samples were stained using hematoxylin and eosin as well as Gordon and Sweeds stain for reticulin fibers (ammoniacal silver procedure).

CHAPTER THREE

HETERODIMERIC TRANSACTIVATION OF JAK2 AS A MECHANISM OF PERSISTENCE

JAK2 inhibitors have been approved for the treatment of myelofibrosis (MF) and are in late-stage clinical testing for treating patients with intermediate and high-risk polycythemia vera. These drugs have been remarkably effective at alleviating constitutional symptoms, reducing spleen size, decreasing levels of circulating inflammatory cytokines and improving overall quality of life. However, they do not significantly affect the mutant allele burden or reverse cytopenias and bone marrow fibrosis in MF. Additionally, they are not curative and patients must continue on drug treatment for durable responses. Similar results have been observed in pre-clinical models of MPN. This lack of an initial response or development of genetic resistance argues for inherent insensitivity of MPN cells to JAK inhibitors, a phenomenon we termed

'persistence'. In order to understand this process, we began to model how JAK2/MPL mutant cells might survive in the context of chronic JAK2 inhibitor exposure, which is described in this chapter.

DEVELOPMENT OF JAK INHIBITOR PERSISTENT CELL LINES

We cultured SET-2/UKE-1 (*JAK2V617F*-positive leukemia) cells and Ba/F3 cells expressing JAK2V617F (EporVF) or MPLW515L (WL) cells in increasing concentrations of ruxolitinib (INCB18424) or a commercially available pan-JAK inhibitor, JAK Inhibitor I, for 4–6 weeks. In each case we found that JAK2/MPL-mutant cells could survive and proliferate at inhibitor concentrations that were 5-10 fold higher than the IC_{50} values of the parental naïve cell line (Fig 3.1). JAK2-inhibitor-persistent ($JAK2^{Per}$) cells had an attenuated apoptotic response to ruxolitinib as compared to the naïve cells (Fig 3.2). *JAK2* resequencing confirmed the absence of second-site mutations in all $JAK2^{Per}$ cell lines. $JAK2^{Per}$ cells were also insensitive to structurally divergent JAK inhibitors, including SAR302503 (also known as TG101348), a JAK2-selective inhibitor in late-stage clinical trials (Fig 3.3). These data indicate that $JAK2^{Per}$ cells are insensitive to different JAK inhibitors regardless of previous exposure to that inhibitor.

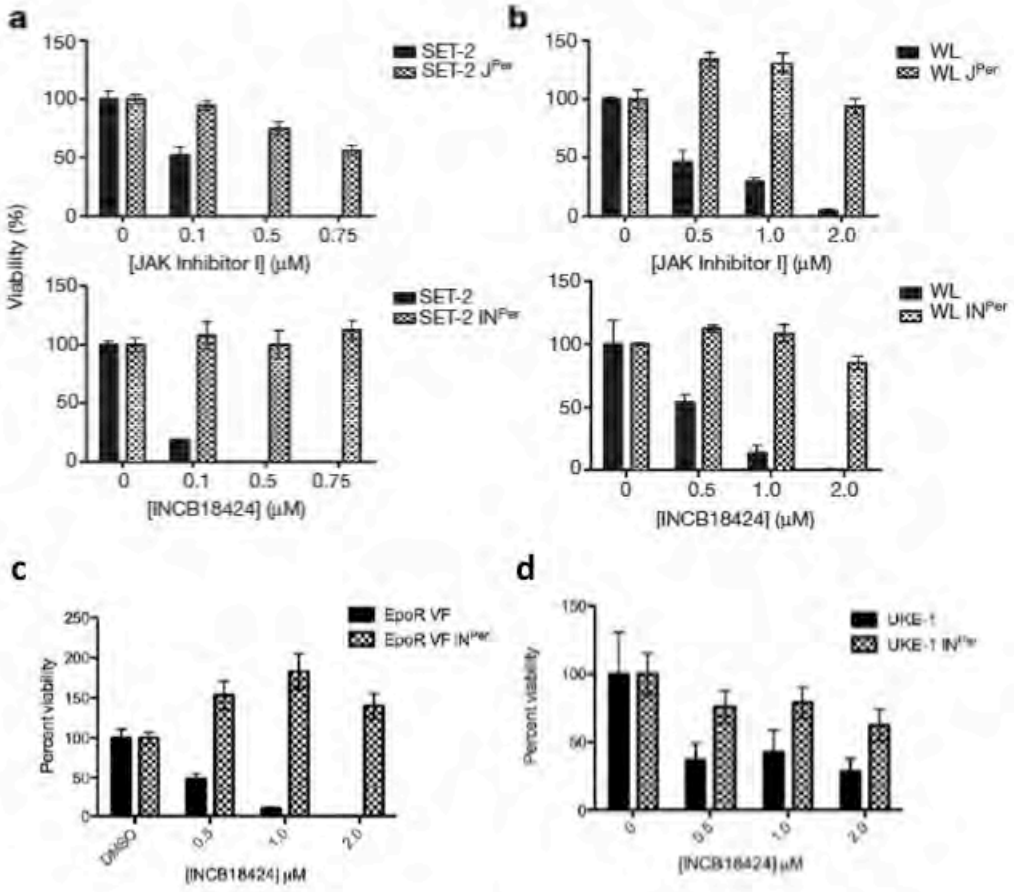


Figure 3.1: Generation of JAK inhibitor persistent cells. Proliferation of naive and persistent (a) SET-2 (b) WL (c) EpoRVF and (d) UKE-1 cells with JAK2 inhibitors. Data (mean±s.d.) are from wells plated in triplicate and are representative of three independent experiments.

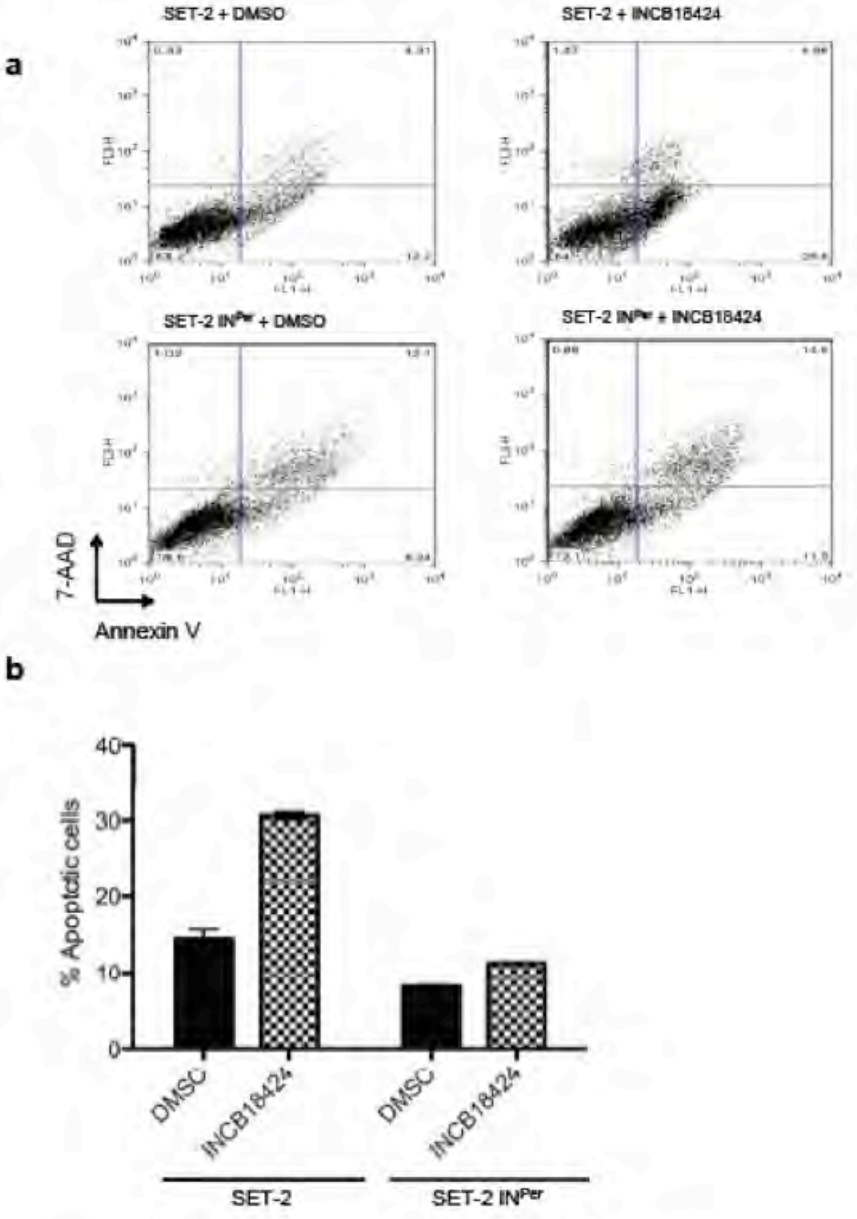


Figure 3.2: JAK Inhibitor persistent cells show lower apoptosis upon treatment with JAK2 inhibitor. (a) A representative plot of SET-2 naïve and persistent (IN^{Per}) cells treated with DMSO or 0.5μM INCB18424 for 24 hours. (b) Results from three biological replicates (mean ± S.E.M)

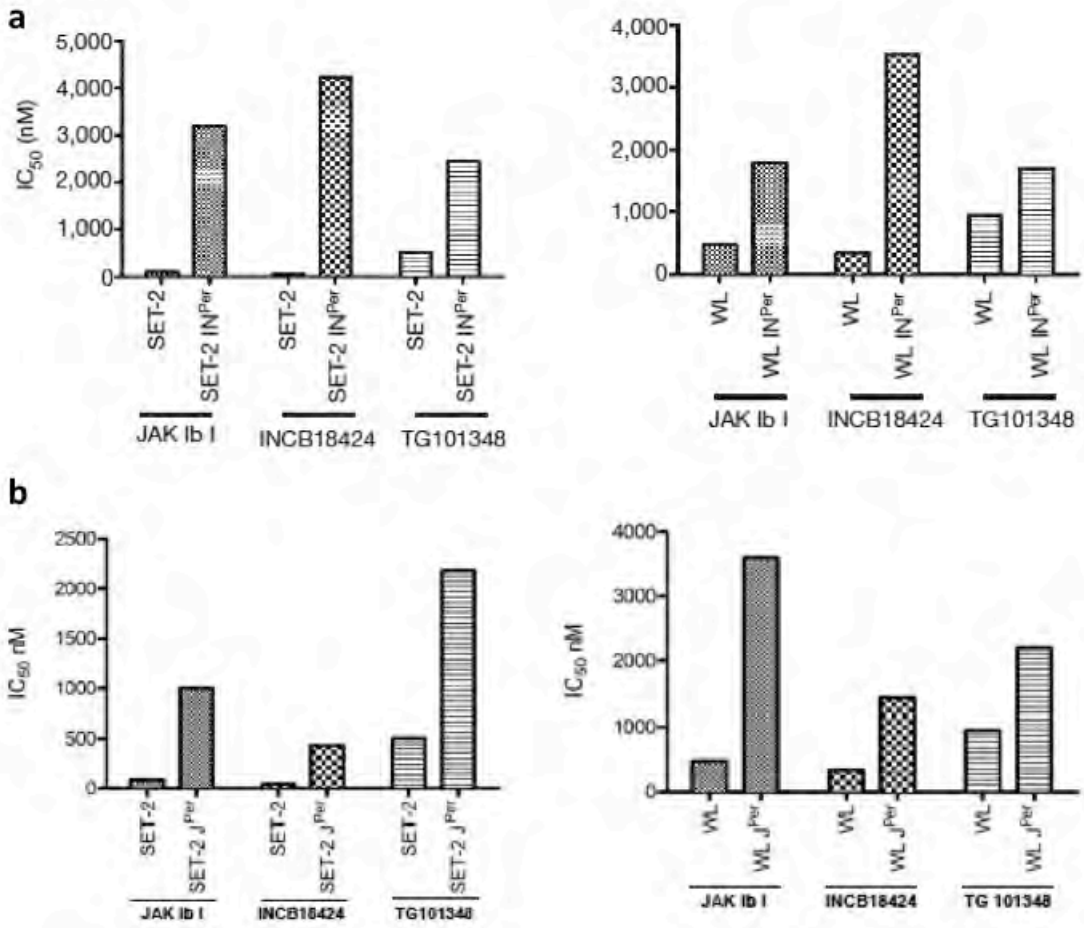


Figure 3.3: Persistent cell lines are insensitive to multiple JAK inhibitors. IC_{50} values of (a) SET-2 and WL IN^{Per} and (b) SET-2 and WL J^{Per} cells exposed to INCB18424, JAK Inhibitor I (Jak Ib I) and TG101348.

Our findings are consistent either with the selection of a subpopulation of pre-existing, persistent cells, as previously posited for epidermal growth factor receptor (EGFR) inhibitor-insensitive 'drug-tolerant persisters' (Sharma et al., 2010), or with the acquisition of persistence by naive, inhibitor-sensitive cells. To distinguish between these possibilities, we derived single-cell clones of inhibitor-naive JAK2/MPL mutant cell lines. Each clonally derived naive cell line was sensitive to JAK inhibitors and retained the capacity to become persistent over time to different JAK inhibitors (Fig 3.4). These data depict a general capacity for persistence in the absence of clonal selection.

REACTIVATION OF JAK-STAT SIGNALING IN PERSISTENT CELLS

Activation of a parallel pathway in order to circumvent inhibition of the main driver pathway is a common mechanism of resistance to targeted therapies in cancer (Engelman et al., 2007; Johannessen et al., 2010). Therefore, we examined downstream STAT signaling in the persistent cells. We observed robust reactivation of STAT3, STAT5 and MAPK in the persistent cells at drug concentrations sufficient to abrogate signaling in the naïve cells (Fig 3.5). We were able to validate these findings in primary MPN samples as well. *Ex vivo* treatment of mononuclear cells from patients chronically treated with ruxolitinib demonstrated sustained downstream signaling at inhibitor concentrations that inhibited signaling in naive MPN patient samples (Fig 3.6).

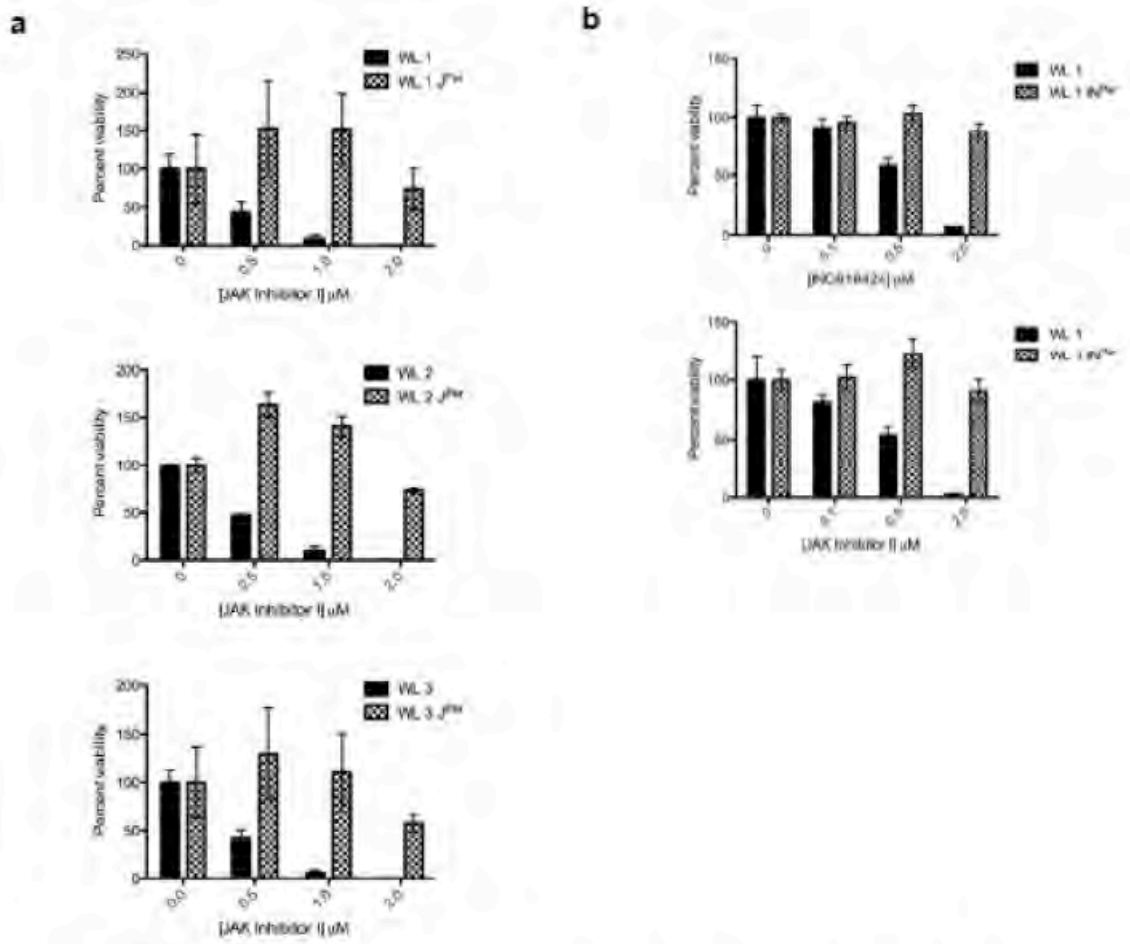


Figure 3.4: WL single cell clones can be made persistent to JAK inhibitors and these are cross resistant to other JAK Inhibitors. WL single cell clones were generated by culturing polyclonal WL cells in methylcellulose agar media. Inhibitor persistent derivatives of single cell clones were generated. (a) Viability assays of JAK Inhibitor I persistent (WL J^{Per}) WL 1, WL 2 and WL 3 single cell clones. (b) WL 1 IN^{Per} cells (INCB18424 persistent WL 1 cells) were not only resistant to INCB18424 but also cross resistant to JAK Inhibitor I. Data are from wells plated in triplicate (means ± S.E.M).

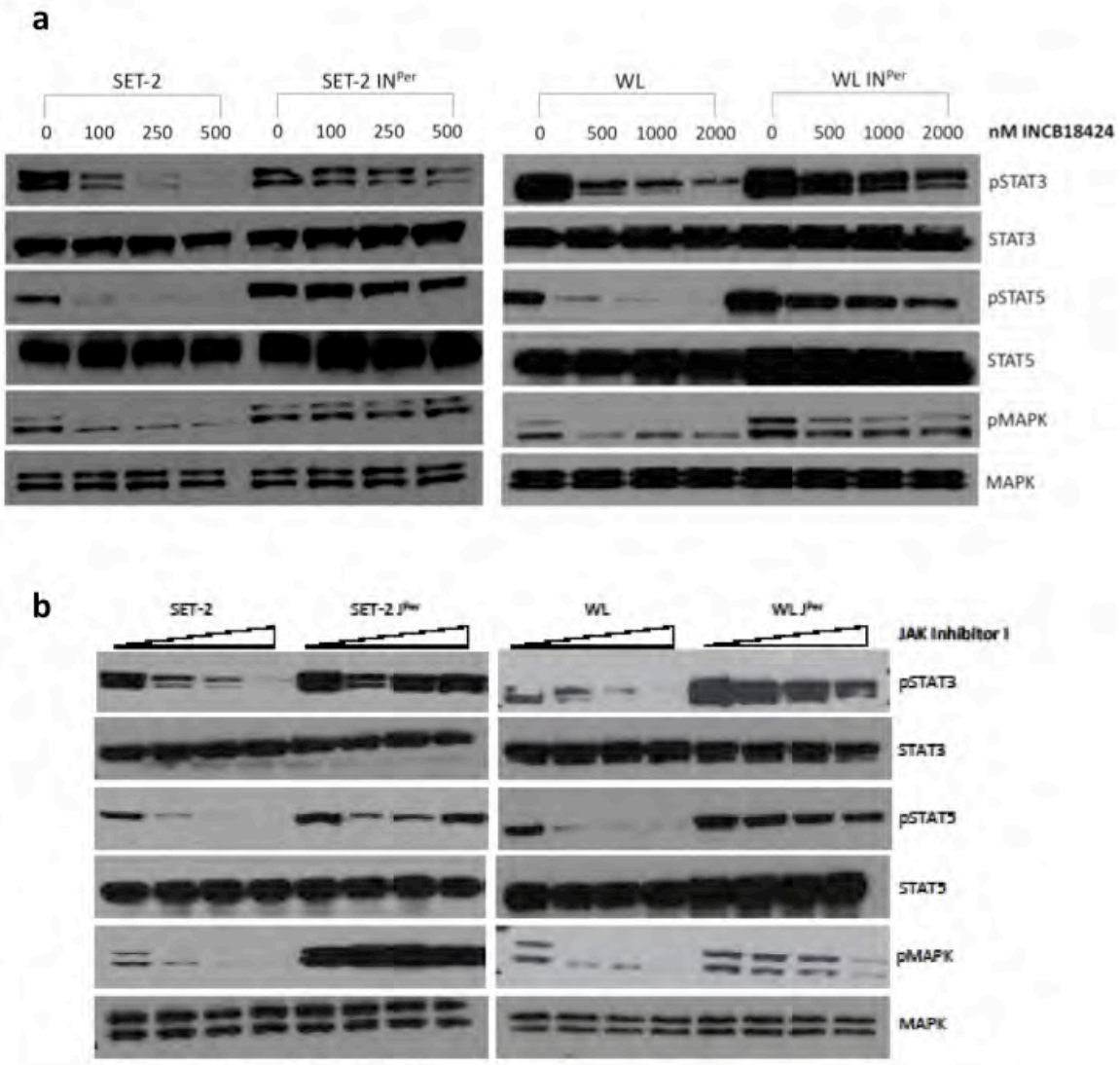


Figure 3.5: JAK inhibitor persistent cells show reactivation of downstream signaling. Naïve and persistent SET-2 and WL cells were washed and treated with increasing concentrations of (a) INCB18424 and (b) JAK inhibitor I for 4 hours.

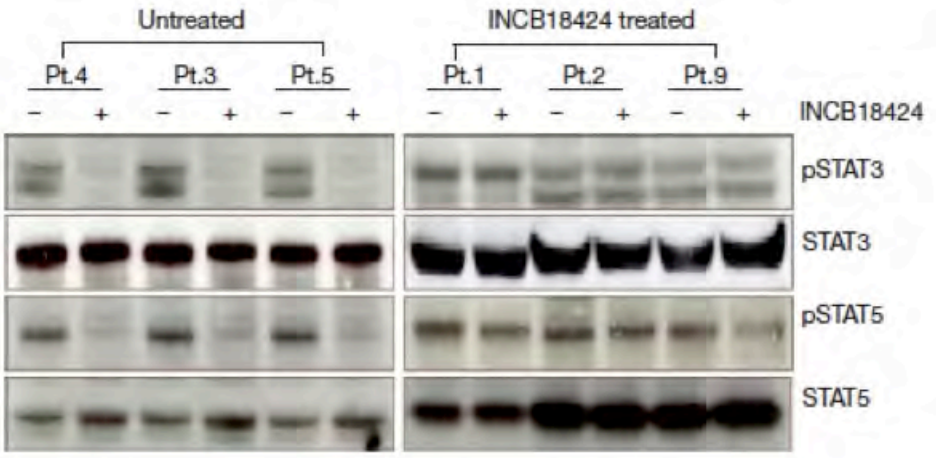


Figure 3.6: Cells from ruxolitinib (INCB18424) treated MPN patients are insensitive to JAK inhibitors *ex vivo*: Mononuclear cells from MPN patients were isolated and treated with DMSO or 150nM INCB184242 for 6 hours and western blotted.

Next, we examined whether persistence was associated with constitutive JAK2 activation. We observed persistent phosphorylation of JAK2 in persistent cells at drug concentrations that inhibited JAK2 activation in naïve cells (Fig 3.7). Further, gene expression analysis showed that the expression of known JAK–STAT target genes was maintained in JAK^{Per} cells, whereas these genes were suppressed with acute treatment of inhibitor-naive parental cells (Fig 3.8). These data indicate that the persistent cells continue to signal via the JAK-STAT pathway and do not activate a bypass pathway as a mechanism of resistance.

HETERODIMERIC TRANSACTIVATION OF JAK2 BY OTHER JAK KINASES

Given that JAK inhibitors should inhibit JAK2 autophosphorylation, we reasoned that other kinases might associate with and phosphorylate JAK2 in persistent cells. Although the erythropoietin receptor (EpoR) and MPL predominantly signal through JAK2, previous studies have shown that many cytokine receptors signal through JAK kinase heterodimers (Ihle and Gilliland, 2007). We therefore assessed the activation status of JAK1, JAK3 and TYK2 in naive and persistent SET-2 and WL cells. We observed increased phosphorylation of JAK1 in $JAK2^{Per}$ cells in comparison with parental cells, whereas TYK2 was constitutively phosphorylated in both parental and $JAK2^{Per}$ cells (Fig 3.9). Accordingly, immunoprecipitation studies demonstrated that JAK1 and TYK2 associated with phosphoJAK2 in persistent cells, but not in the respective parental cells (Fig 3.10).

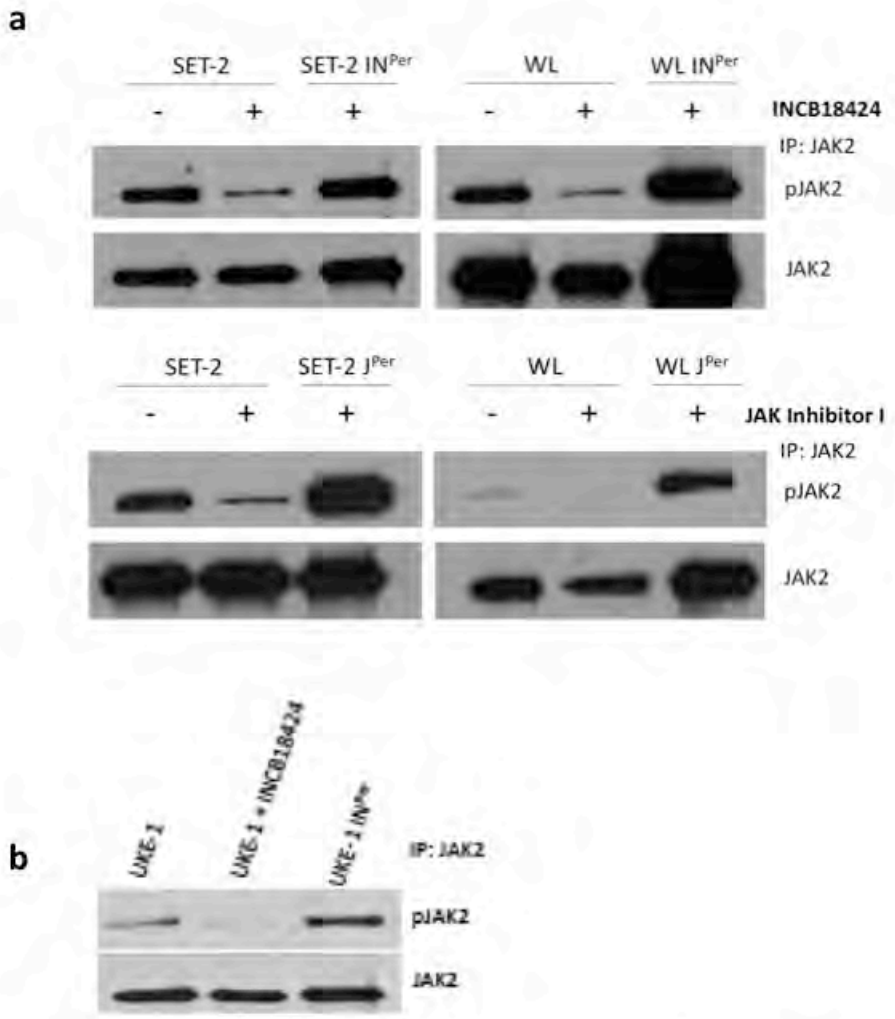


Figure 3.7: Reactivation of JAK2 in persistent cell lines: (a) SET-2 and WL cells and (b) UKE-1 cells were washed in PBS to remove inhibitor followed by exposure to JAK Inhibitor I or INCB18424 for 4 hours, followed by immunoprecipitations with JAK2 and Western blot analysis as noted. Cells were incubated with same concentration of inhibitor in which persistent cells were cultured chronically.

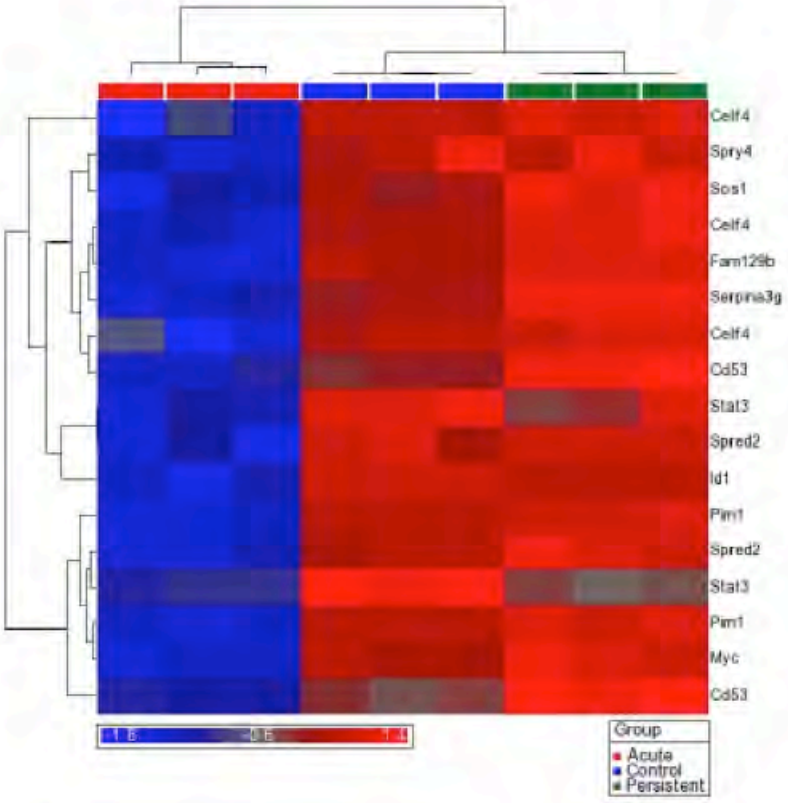


Figure 3.8: **Reactivation of JAK-STAT pathway in JAK inhibitor persistent cells**. WL naïve cells were acutely treated with either DMSO or 0.8 μM INCB18424 for 4 hours in triplicates. RNA was extracted from these cells along with WL IN^{Per} cells and gene expression was analyzed using Affymetrix microarray version MOE 430

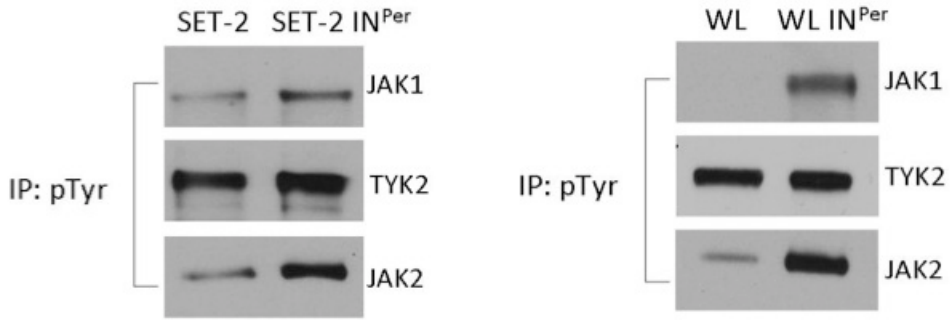


Figure 3.9: Activation of JAK1 and TYK2 in persistent cells: Increased phosphorylation of JAK1 in persistent cells and constitutive TYK2 phosphorylation in both naive and persistent cells.

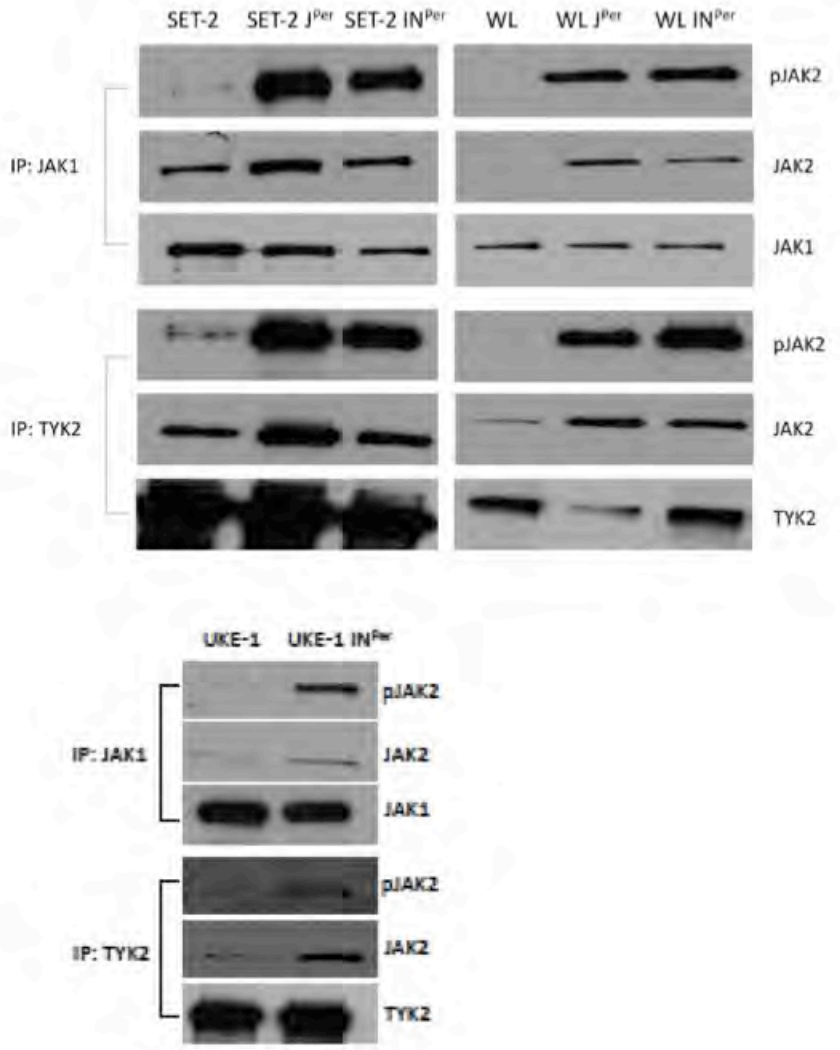


Figure 3.10: Heterodimers between JAK1 and TYK2 with activated JAK2 in persistent cells: Immunoprecipitation with JAK1 and TYK2 revealed increased association between phosphorylated JAK2 and JAK1/TYK2 in persistent cells as compared to naïve cells.

We then assessed whether this phenomenon was observed *in vivo* in a murine model of ET/MF. We treated mice engrafted with MPLW515L-mutant murine bone marrow (Koppikar et al., 2010) with vehicle or with ruxolitinib. Treatment with ruxolitinib was associated with decreased splenomegaly; however, the proportion of malignant cells was not decreased on treatment with JAK inhibitor (Fig 3.11a), in concordance with our previous results (Koppikar et al., 2010). Further, we noted increased JAK2 phosphorylation and increased association between JAK1 and JAK2 in hematopoietic cells from diseased mice treated with ruxolitinib (Fig 3.11b and c). We were able to extend this observation in primary samples as well where we saw a similar association between phosphoJAK2 and JAK1 or TYK2 in ruxolitinib-treated patient samples but not in inhibitor-naive patient samples (Fig 3.12).

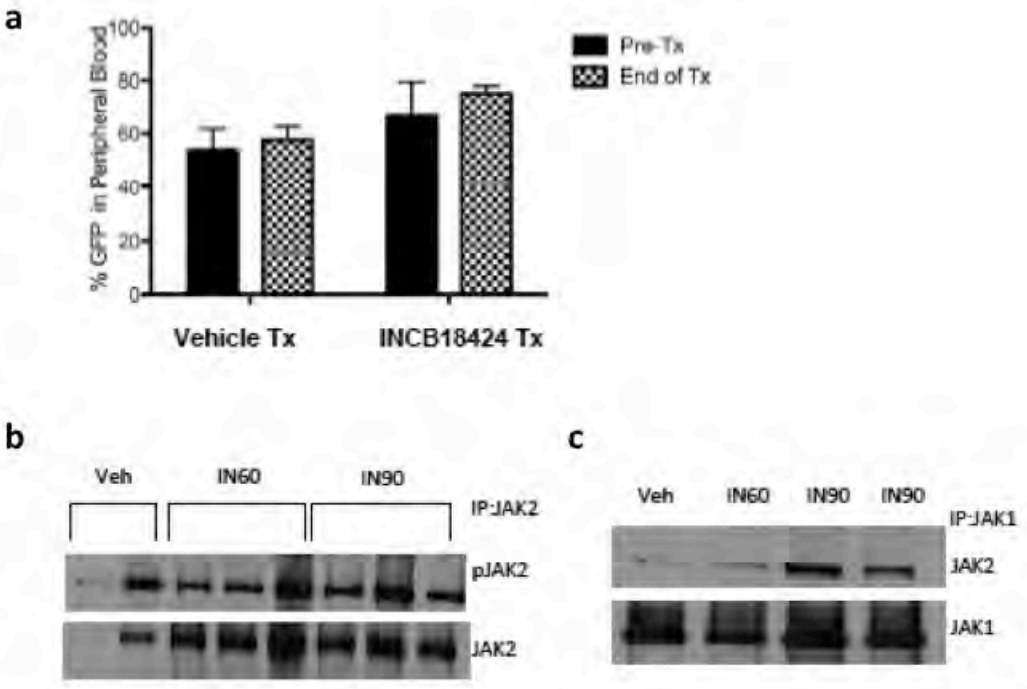


Figure 3.11: JAK inhibitor persistence in a murine model of ET/MF: (a) Mutant allele burden in terms of GFP+ cells is unchanged with ruxolitinib treatment. (b) JAK2 is reactivated in splenocytes of mice treated with ruxolitinib (c) Increased association between JAK1 and JAK2 in splenocytes of inhibitor treated mice.

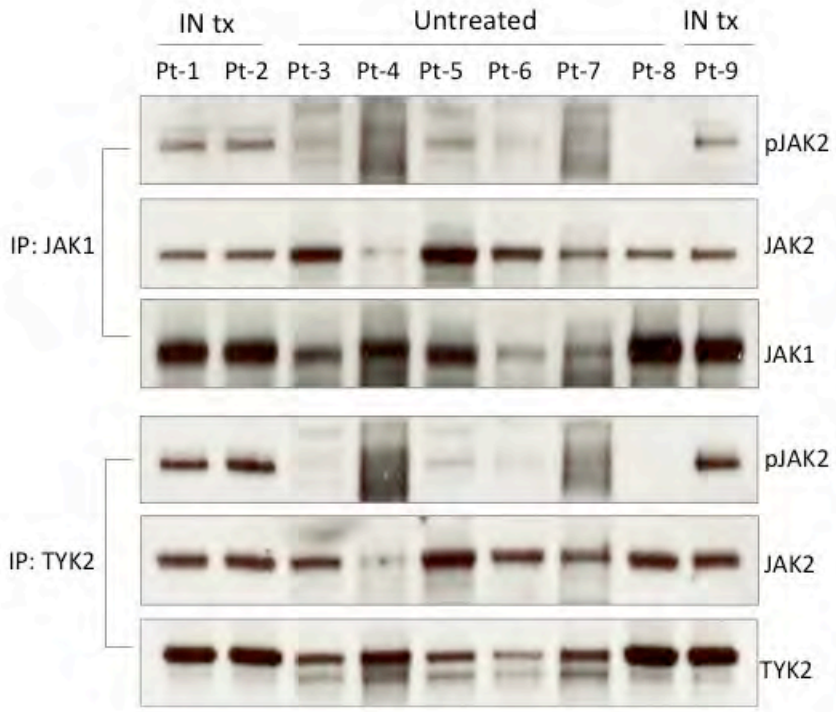


Figure 3.12: Heterodimers in primary MPN samples: JAK1/TYK2 association with phosphoJAK2 in granulocytes from three INCB18424-treated (IN Tx) patients, which is not observed in INCB18424-naive MPN samples.

We then asked how dependent the persistent cells were on other JAK kinases by using siRNA targeting JAK1 and TYK2. Knockdown of JAK1 and TYK2 increased the sensitivity of persistent cells to JAK inhibitors (Fig 3.13a), whereas the parental cells remained unaffected by JAK1 and TYK2 knockdown (Fig 3.13b). Additionally, loss of JAK1 and TYK2 led to decreased downstream signaling and decreased JAK2 phosphorylation in the persistent cells (Fig 3.13c).

We performed *in vitro* kinase assays to examine kinase activity of JAK2 in inhibitor persistent cells. JAK2 was immunoprecipitated from naïve and ruxolitinib persistent SET-2 cells and its catalytic activity was assessed based on the phosphorylation of a generic substrate, myelin basic protein (MBP). This assay revealed that the JAK2 heterodimeric complex was more active in persistent cells as compared to the parental naïve cells (Fig 3.14a). Further, JAK2 in persistent cells could phosphorylate MBP at concentrations of ruxolitinib sufficient to inhibit JAK2 kinase activity in naive SET-2 cells (Fig 3.14b). To determine whether JAK1-mediated phosphorylation of JAK2 was insensitive to ruxolitinib, we co-expressed a constitutively active mutant form of JAK1 (JAK1V658F) with kinase-dead JAK2 (JAK2K882E) in JAK2-deficient γ2A cells. We observed that JAK1 could transphosphorylate JAK2 in this context and this phosphorylation could not be completely inhibited by ruxolitinib treatment (Fig 3.14c). These data suggest that the heterodimer complex in persistent cells retains kinase activity that is relatively insensitive to JAK inhibitors

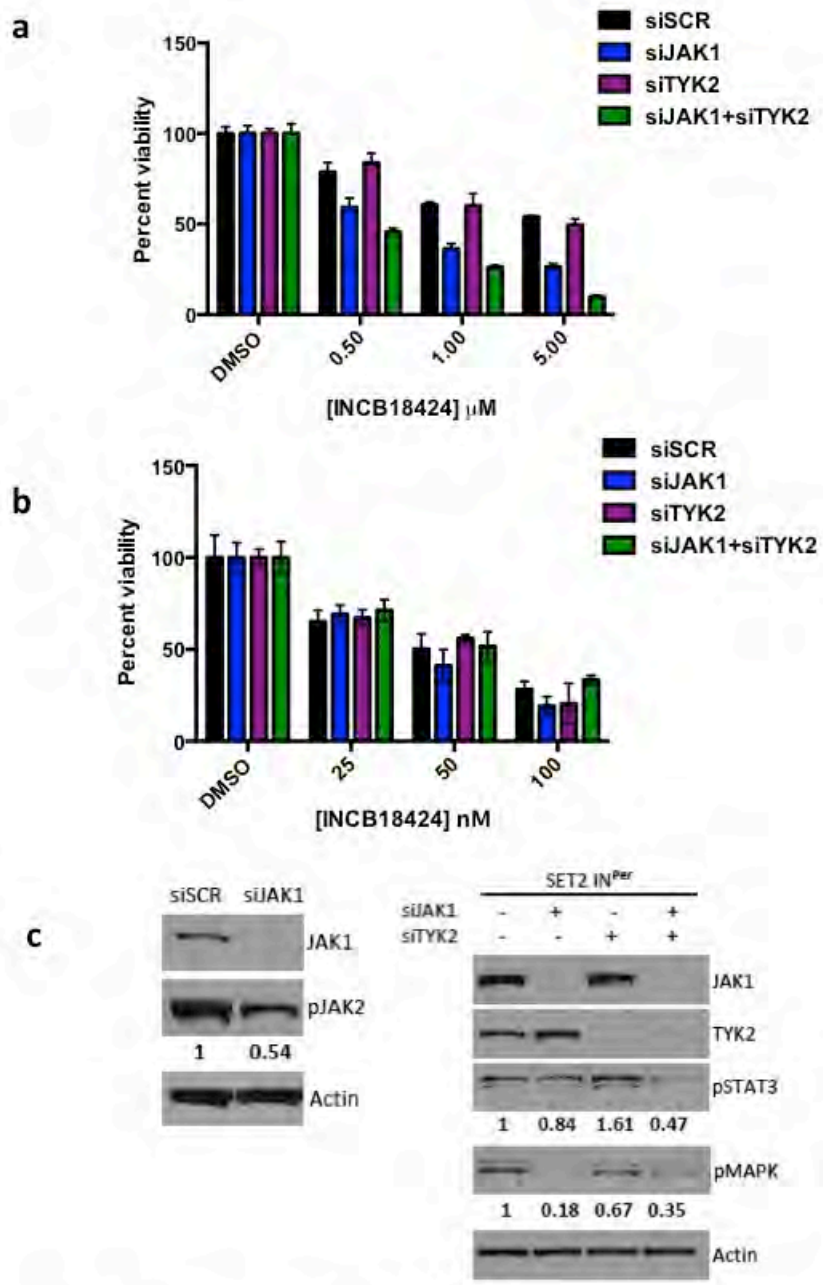


Figure 3.13: Knockdown of JAK1 and TYK2 can partially overcome persistence: Dual knockdown of JAK1 and TYK2 in SET IN^{Per} using siRNA resensitizes them to INCB18424 treatment (a) but has no effect in naïve SET-2 cells (b). (c) Knockdown also inhibits phosphoJAK2 and downstream STAT-MAPK signaling in persistent cells

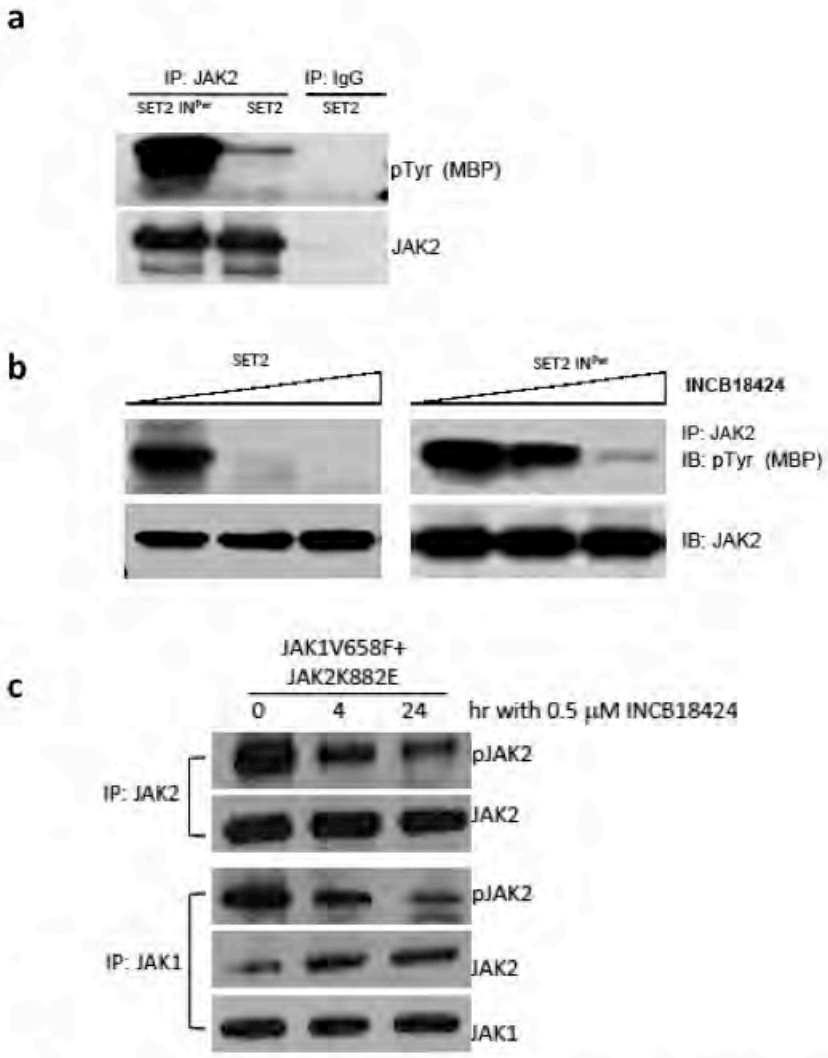


Figure 3.14: The JAK2 heterodimer complex in persistent cells is relatively insensitive to in vitro inhibitor treatment. (a) Immunoprecipitation of JAK2 in SET-2 and SET-2 IN^{Per} cells demonstrates increased activation of JAK2 in persistent cells in comparison to naïve cells. (b) In vitro kinase assays with increasing concentrations of INCB18424 (1 and 10 nM) for 1 hour shows that JAK2 from naïve SET-2 cells is inhibited at 1 nM INCB18424 while JAK2 isolated from IN^{Per} cells remains active. Kinase activity of JAK2 was evaluated using myelin basic protein (MBP), a generic substrate. (c) JAK1- dependent phosphorylation of JAK2 is insensitive to inhibitor treatment in JAK2-null γ2A cells overexpressing a kinase-dead JAK2 and activated JAK1 allele

REVERSIBILITY OF PERSISTENCE WITH DRUG WITHDRAWAL

Another important observation we made was that persistence was reversible. After washing out the drug, the persistent cells became sensitive to inhibitor in 2-4 weeks (Fig 3.15). Treatment with inhibitor led to reduction in phosphorylated JAK2, similar to naïve cells (Fig 3.16). Furthermore, the resensitized cells no longer showed JAK1 or TYK2 association with phosphoJAK2 (Fig 3.17). Resensitized ($JAK2^{Resens}$) cells were sensitive to all three JAK inhibitors, suggesting that patients with MPN may respond to retreatment with the same or different inhibitor following a brief drug withdrawal.

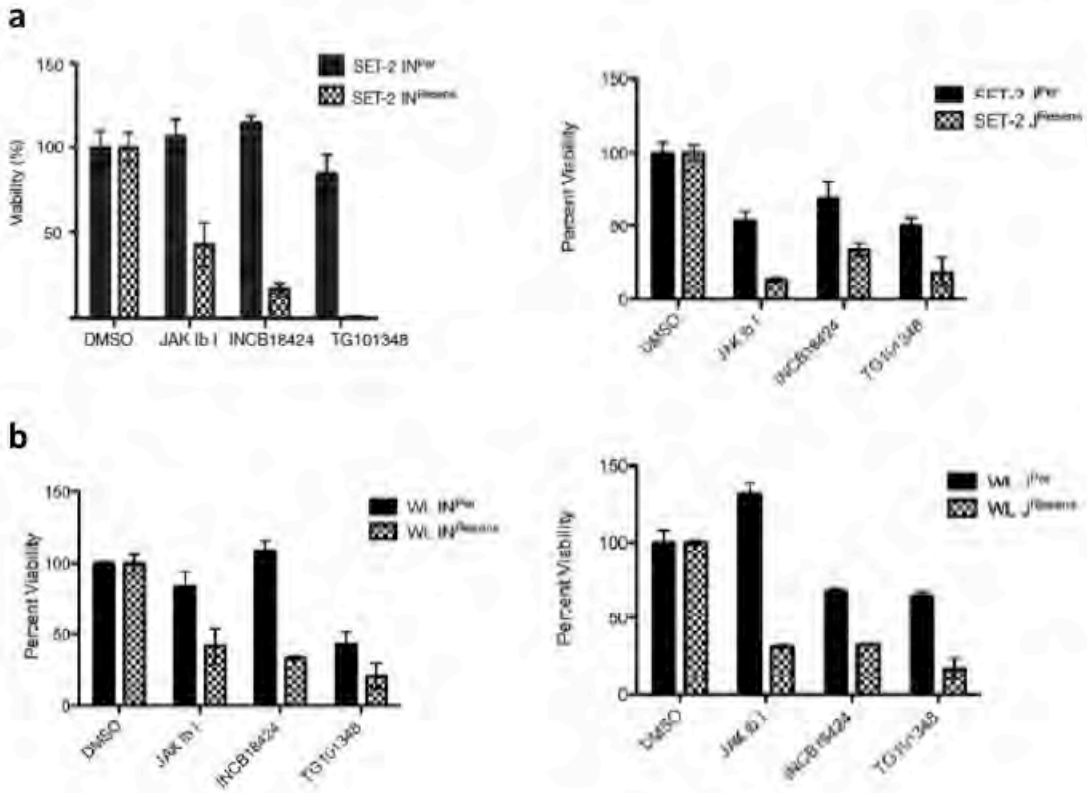


Figure 3.15: Persistence is reversible with drug withdrawal. (a) SET-2 and (b) WL persistent cells become resensitized to different JAK inhibitors in 2-4 weeks following drug withdrawal. Percent viability of SET-2 cells at 1μM JAK Inhibitor I, 0.25μM INCB18424 and 2μM TG101348 and of WL cells at 1μM JAK Inhibitor I and INCB18424 and 2μM TG101348 is shown.

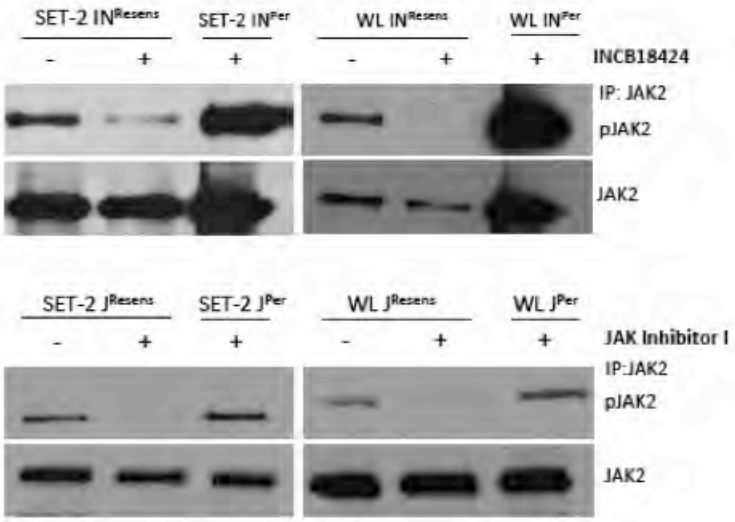


Figure 3.16: Resensitized cells have lower levels of activated JAK2. Resensitized cells were exposed to JAK Inhibitor I or INCB18424 for 4 hours, followed by immunoprecipitations with JAK2 and Western blot analysis as noted. Cells were incubated with same concentration of inhibitor in which persistent cells were cultured chronically.

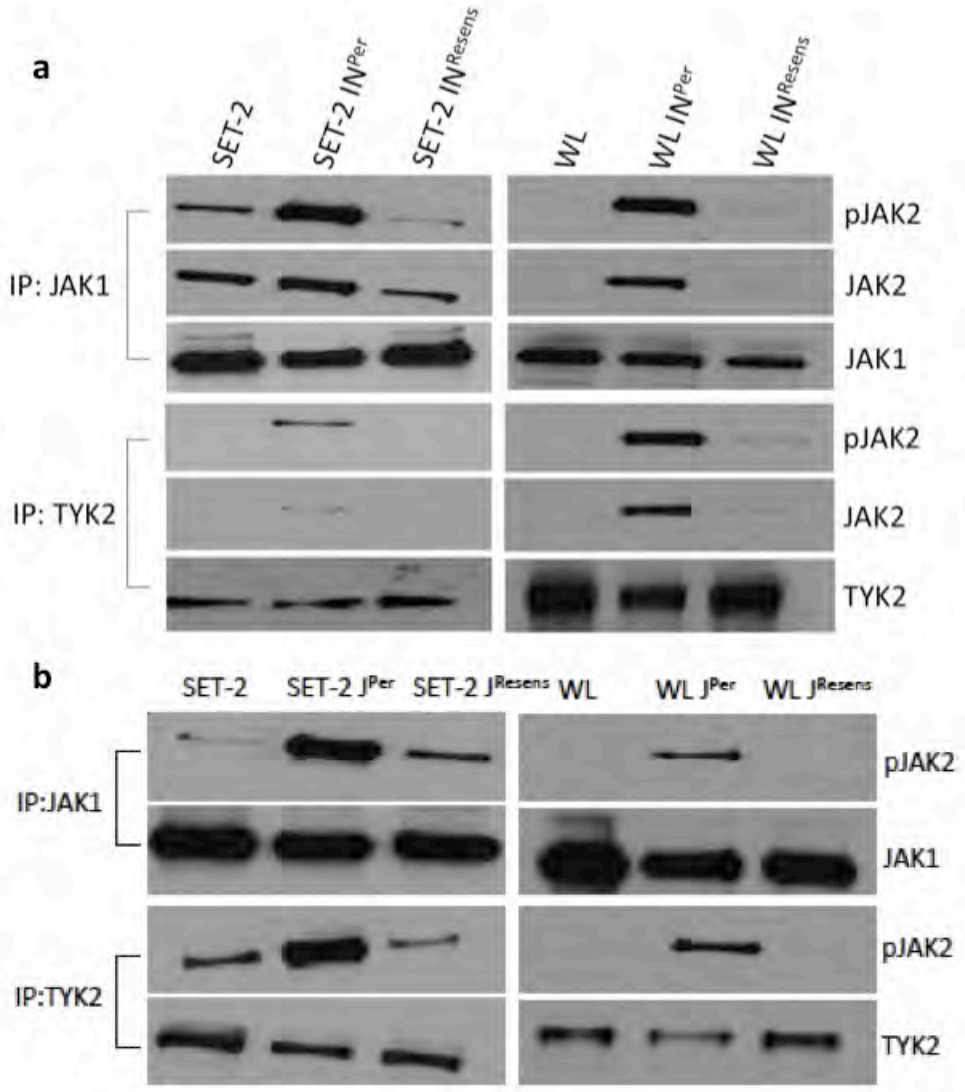


Figure 3.17: Resensitized cells lose heterodimers between activated JAK2 and JAK1/TYK2. Immunoprecipitation with JAK1 and TYK2 in inhibitor resensitized SET-2 and WL cells revealed loss of heterodimers with phosphorylated JAK2, which is similar to naïve cells. (a) INCB184242 persistent cells (b) JAK Inhibitor I persistent cells

We also observed that *JAK2* messenger RNA and JAK2 protein levels were higher in persistent cells than in parental cells, and returned to lower levels with resensitization (Fig 3.18). Treatment with ruxolitinib was associated with an increase in *JAK2* mRNA and JAK2 protein expression in MPLW515L-transduced mice. Similarly, comparison of expression of JAK2 in granulocytes from MPN patients prior to and during ruxolitinib treatment revealed an increase in *JAK2* mRNA levels following drug exposure. (Fig 3.19) The reversible nature of this phenomenon led us to speculate that there might an epigenetic basis to the mechanism. Chromatin immunoprecipitation (ChIP) analysis of the JAK2 locus showed a significant increase in H3K4me3, a modification associated with active promoters, and a decrease in H3K9me3, a mark more typically associated with inactive heterochromatin, in persistent cells in comparison with parental cells (Fig 3.20a), which is consistent with a change to a more active chromatin state at the *JAK2* locus. However, global H3K4me3 levels in naive and persistent cells remained unchanged, which is consistent with specific effects on H3K4me3 at the *JAK2* locus in persistent cells (Fig 3.20b).

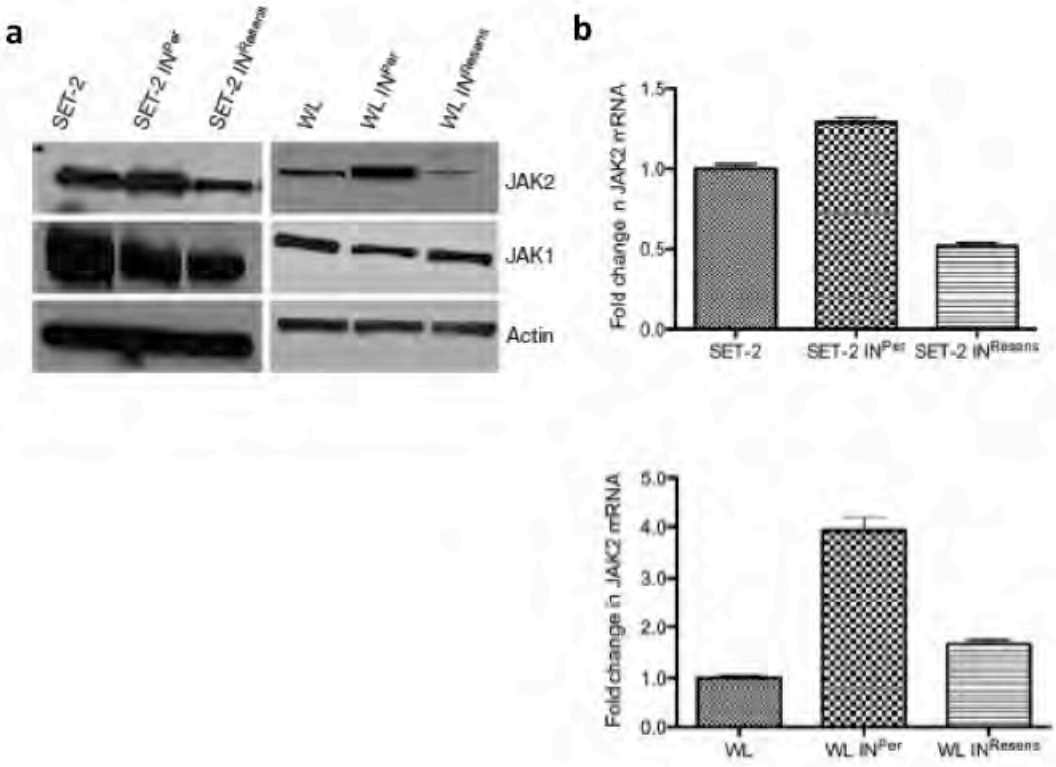


Figure 3.18: Changes in JAK2 expression levels. Persistent cells have increased JAK2 expression compared to naïve cells at the (a) protein and (b) mRNA level, which are reduced in resensitized cells.

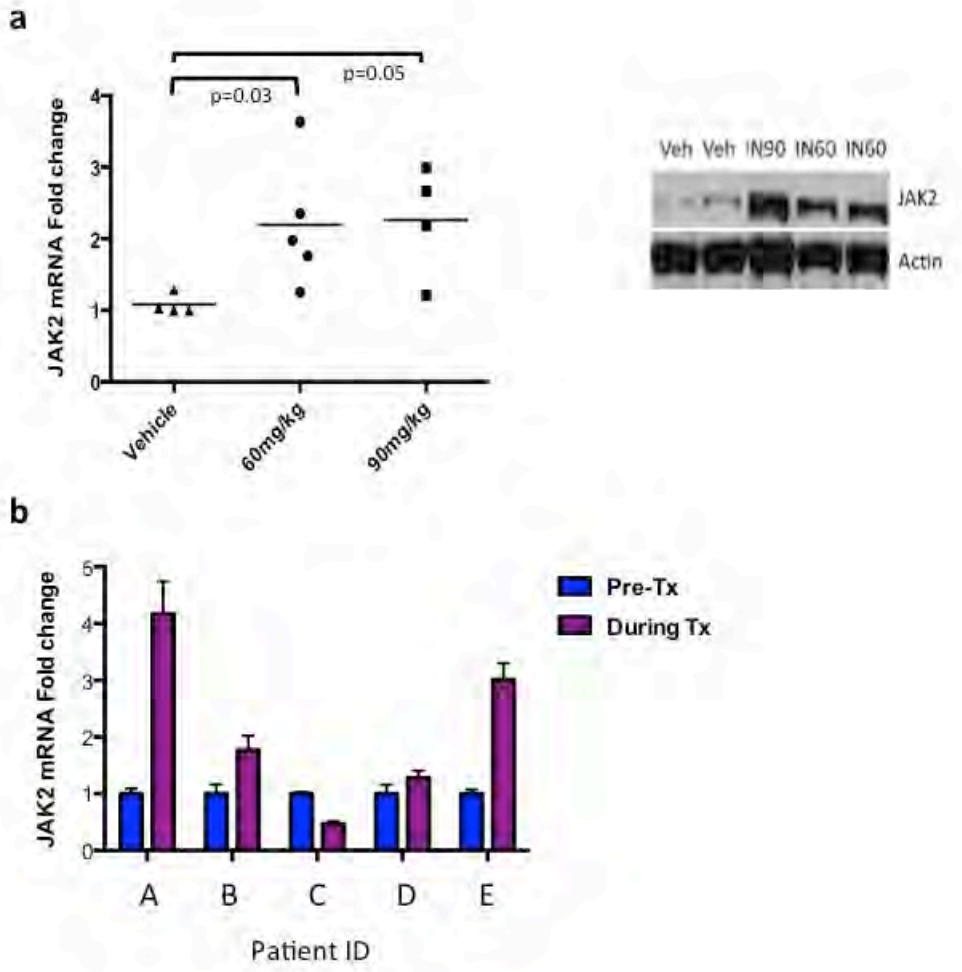


Figure 3.19: Increased JAK2 expression with inhibitor treatment *in vivo*. (a) JAK2 levels were assessed by q-PCR and immunoblotting in splenocytes from MPLW515L-transduced mice treated with INCB18424. (b) *JAK2* mRNA in granulocytes from MPN patients prior to and during ruxolitinib treatment.

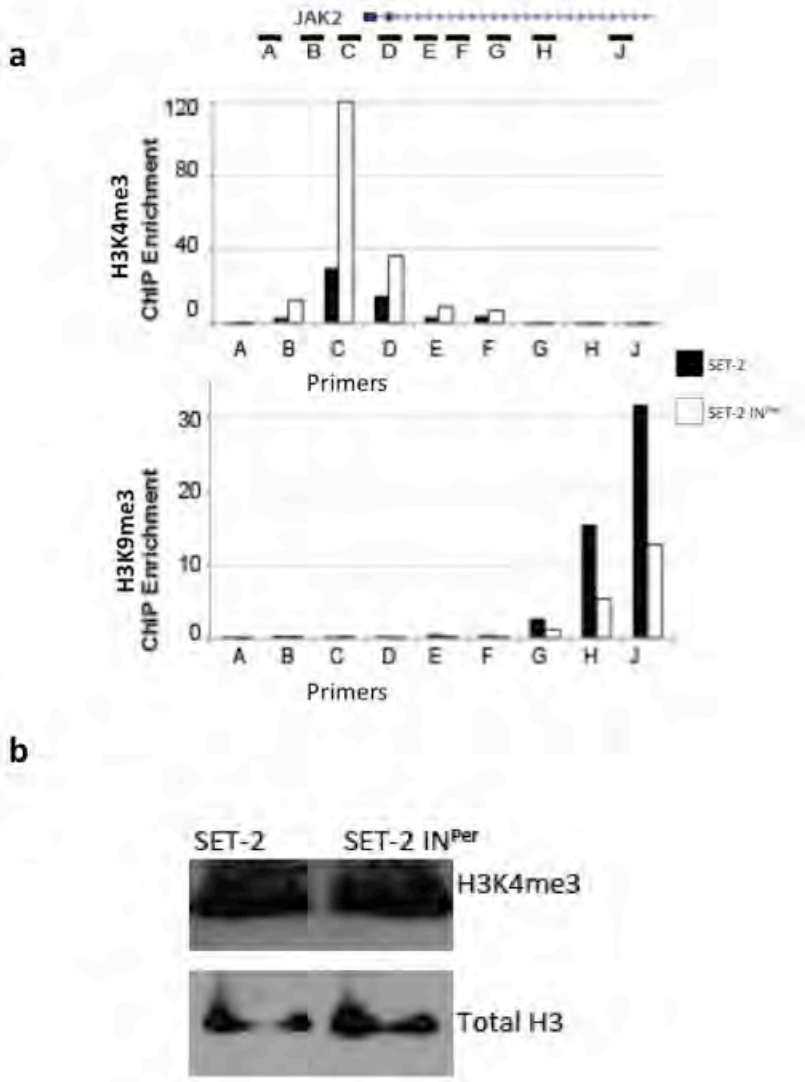


Figure 3.20: Epigenetic changes at *JAK2* locus.(a) ChIP-qPCR analysis of the JAK2 locus revealed increased H3K4me3 and decreased H3K9me3 in persistent cells compared to naïve cells. (b) Global H3K4me3 levels are unchanged in persistent cells.

Next, we asked whether overexpression of JAK2 was sufficient to induce persistence in MPN cells. We generated Ba/F3 stable cells overexpressing MPL-W515L with and without ectopic JAK2 expression and cultured them in increasing concentrations of ruxolitinib. Upregulation of JAK2 protein levels did not increase the IC_{50} of the parental cells. Although ectopic expression of JAK2 did result in a slight acceleration of the generation of JAK inhibitor persistence, the viability, growth characteristics, and IC_{50} of these cells were similar to cells expressing only MPLW515L with endogenous JAK2 by 4-5 weeks at which time both cell lines were fully persistent (Fig 3.21a) We also made single cell clones of Ba/F3 EpoR JAK2VF-HA-FLAG cells expressing different amounts of JAK2 from the transgene due to differences in integration/copy number. These cells had differing amounts of JAK2, but we did not find any correlation between levels of JAK2 and IC_{50} for ruxolitinib (Fig 3.21b). These data suggest that increased JAK2 expression contributes to persistence, but is not sufficient to cause rapid inhibitor persistence without chronic (2-4 week) JAK inhibitor exposure.

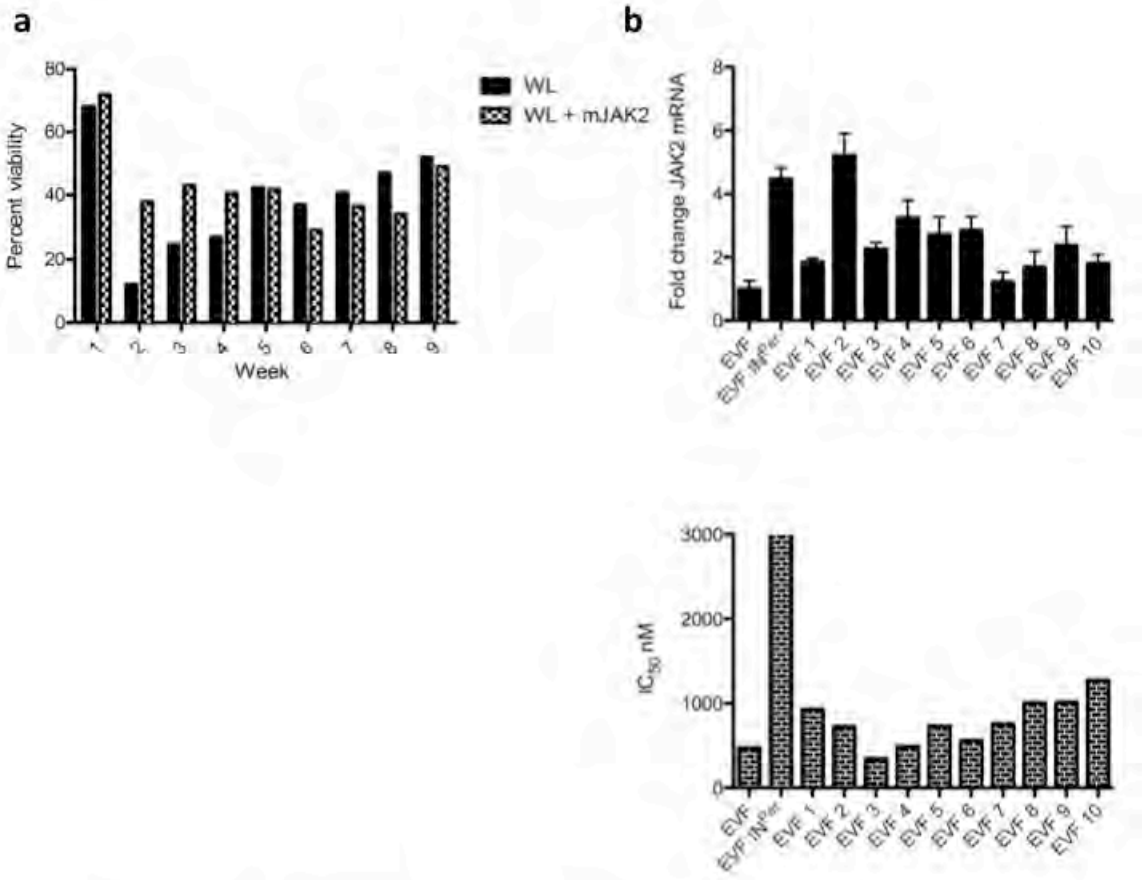


Figure 3.21: Overexpression of JAK2 is not sufficient to induce persistence. (a) Viability of Ba/F3 WL and WL + JAK2 upon addition of 1µM ruxolitinib. (b) JAK2 mRNA levels of single cell clones do not correlate with IC_{50} values of ruxolitinib

Given that JAK2 protein levels, and particularly phosphoJAK2 levels, increased with persistence, we examined whether JAK2 inhibitor persistence was also associated with post-transcriptional stabilization of total and activated JAK2. We have previously shown that JAK2 levels decline rapidly on treatment with cycloheximide in JAK2-mutant cells (Marubayashi et al., 2010). As expected, we noted a time-dependent decrease in phosphoJAK2 and total JAK2 levels in naive and resensitized WL/SET-2 cells; however, exposure to cycloheximide did not result in a significant decline in JAK2, or more notably in phosphoJAK2, in JAK inhibitor persistent cells (Fig 3.22). These data suggest that chronic treatment with inhibitor results in the stabilization of activated JAK2, which, combined with increased *JAK2* mRNA expression, facilitates the formation of heterodimers.

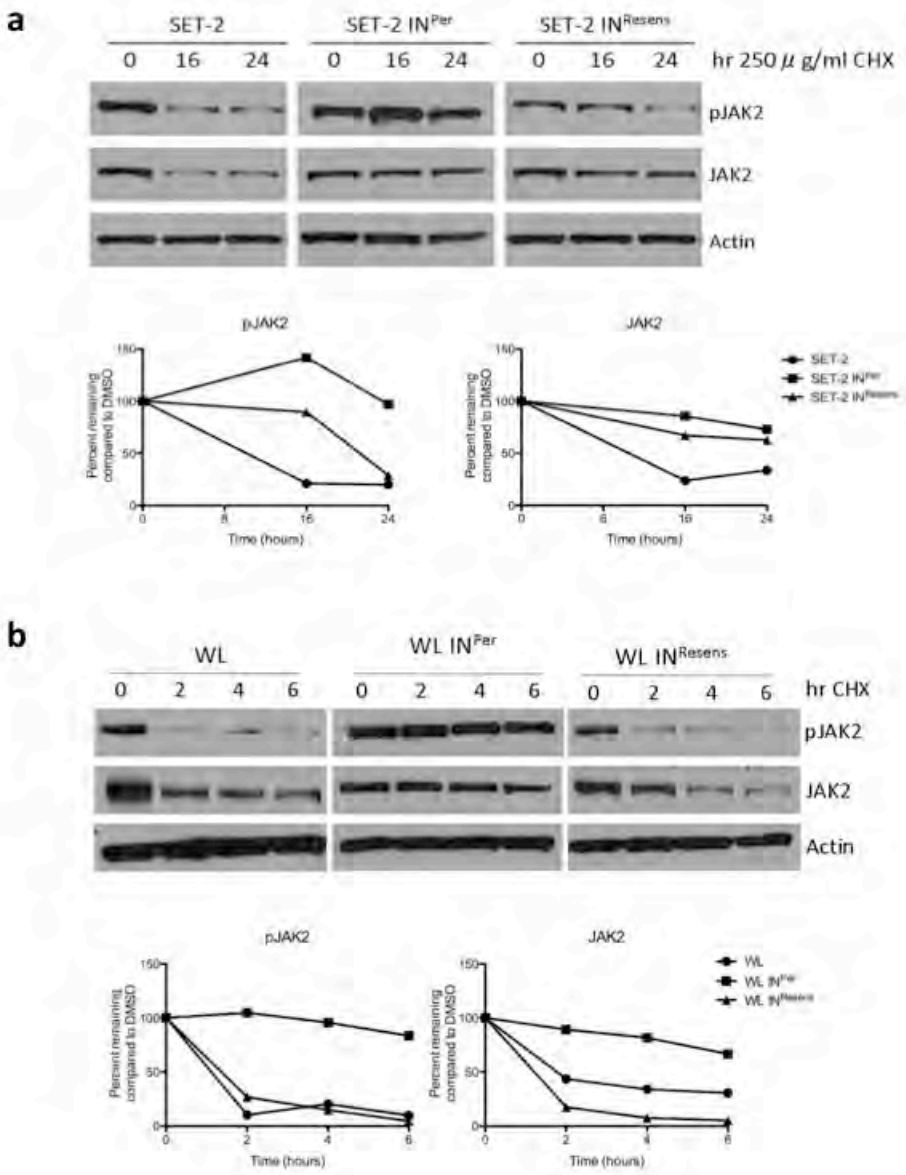
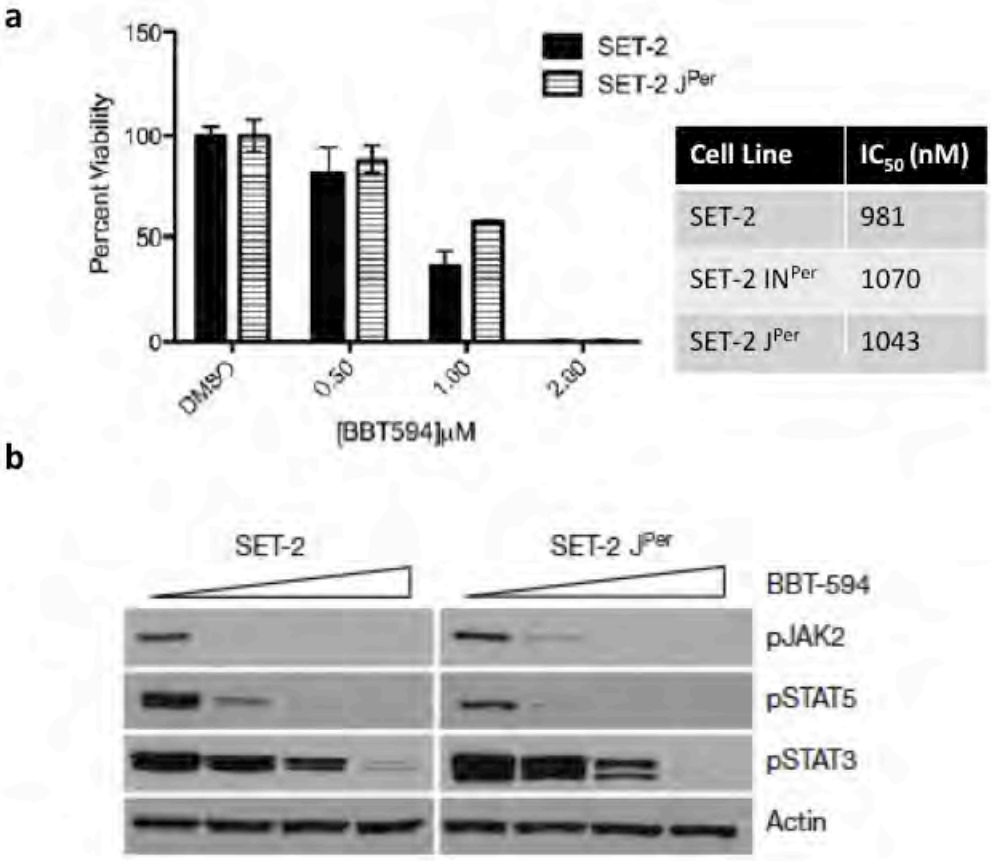


Figure 3.22: Post-transcriptional stabilization of JAK2 in persistent cells. PhosphoJAK2 and total JAK2 levels are degraded on treatment with cycloheximide (CHX; 500 μg/ml in naive and resensitized (a) SET-2 and (b) WL cells, but not in persistent cells.

THERAPEUTIC STRATEGIES TO OVERCOME PERSISTENCE

The JAK inhibitors currently in clinical development are Type I inhibitors, which bind the ATP-binding pocket of JAK2 in the 'active' conformation. A recent paper reported that this mode of binding leads to stabilization of activation loop phosphorylation, thereby resulting in increased levels of phosphorylated JAK2 (Andraos et al., 2012). Based on these findings, we asked whether this mechanism was contributing to development of persistence. We therefore tested the efficacy of BBT-594, a novel type II inhibitor that engages JAK2 in its inactive conformation and does not contribute to stabilization of activated JAK2. Treatment with BBT-594 inhibited the proliferation of persistent cells to a similar extent as the naïve cells (Fig 3.23a). Additionally, activation of JAK2 and downstream STAT signaling was efficiently inhibited in both naïve and persistent cells by this compound (Fig 3.23b). Thus, novel agents that bind JAK2 in a different conformation can be used to overcome JAK inhibitor persistence in MPN.

Taken together, our results suggest that kinase inhibitor persistence can occur through reversible changes in JAK2 expression and stabilization of activated JAK2 by Type I inhibitors, which facilitates transphosphorylation by other JAK kinases (Fig 3.24). The outstanding question remains whether these persistent cells remain dependent on JAK2 for their survival. The next chapter discusses the requirement of JAK2 in naïve and persistent MPN cells and how this can be leveraged therapeutically to overcome persistence.



Cell Line	IC_{50} (nM)
SET-2	981
SET-2 IN^{Per}	1070
SET-2 J^{Per}	1043

Figure 3.23: Persistent cells remain sensitive to type II inhibitors. (a) Naïve and JAK Inhibitor I persistent SET-2 cells have similar IC_{50} values for BBT-594, a type II JAK inhibitor. (b) Treatment of naive and persistent SET-2 cells with BBT-594 inhibits phosphorylation of JAK2 and downstream STAT signaling

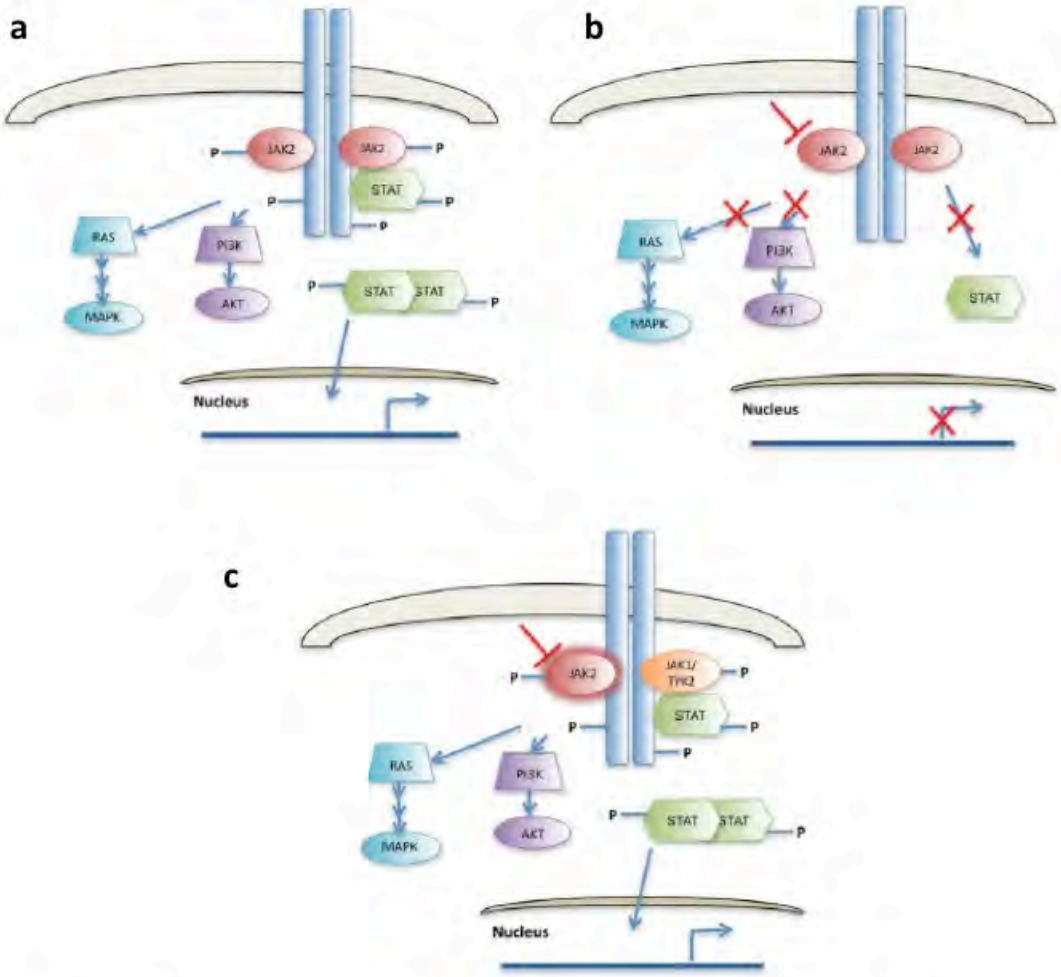


Figure 3.24: Model of JAK inhibitor persistence in MPN cells. Figure (a) depicts signaling in a naïve MPN cell downstream of JAK2. (b) Upon acute treatment with JAK2 inhibitor, signaling is shut down. (c) In a persistent cell, JAK2 levels are upregulated, post-transcriptionally stabilized can now be transphosphorylated by JAK1 and TYK2.

CHAPTER FOUR

REQUIREMENT OF JAK2 IN NAÏVE AND PERSISTENT MPN CELLS

Pre-clinical and clinical studies have shown that JAK inhibitors are not curative in MPN and do not effectively reduce the size of the MPN clone. This might be due to incomplete pathway inhibition at clinically tolerable doses, presence of other disease alleles or incomplete dependence on JAK2 by the MPN clone. Second site mutations in JAK2, which might explain the limited efficacy of these drugs, have not been reported in patients chronically treated with ruxolitinib. In vitro mutagenesis screens have not identified recurrent resistance alleles of JAK2 at a significant frequency; a majority of cells can persist in the presence of chronic exposure to a JAK inhibitor (Deshpande et al., 2012; Marit et al., 2012). The previous chapter elucidated the underlying mechanism for development of persistence, where JAK2 is activated via the formation of heterodimers with other JAK kinases including JAK1 and TYK2 (Koppikar et al., 2012). This phenomenon was observed in cells lines, mouse models as well as in primary samples.

This inherent insensitivity of MPN cells to JAK inhibitors led us to evaluate the requirement of JAK2 in MPN pathogenesis in an *in vivo* murine model of ET/MF. We also tested whether JAK inhibitor persistent cells remain dependent on JAK2 for their survival. Finally, we assessed the efficacy of therapeutic strategies that target degradation of total JAK2 protein rather than simply inhibition of its kinase activity.

JAK2 IS REQUIRED FOR INITIATION OF MPLW515L-INDUCED DISEASE

Retroviral expression of mutant MPLW515L in hematopoietic cells *in vivo* results in the development of a highly penetrant, lethal MPN, characterized by leukocytosis, thrombocytosis, extramedullary hematopoiesis and extensive bone marrow fibrosis (Pikman et al., 2006). We decided to evaluate the effect of loss of JAK2 on disease development in this model. Germline deletion of JAK2 results in embryonic lethality due to lack of definitive hematopoiesis (Neubauer et al., 1998; Parganas et al., 1998). We therefore utilized a conditional knockout approach in which JAK2 could be deleted in an inducible and hematopoietic-specific manner by Cre-recombinase expressed under the control of the Mx1 promoter (Khn et al., 1995). Bone marrow cells from $JAK2^{f/f}$ Mx1-Cre+ and Mx1-Cre- mice expressing the CD45.2 congenic marker were transduced with a GFP-tagged MPLW515L retrovirus and transplanted into irradiated CD45.1 recipients along with equal number of CD45.1 support bone marrow. Two weeks following transplantation, we determined engraftment by the presence of GFP positive cells in peripheral blood. Before the mice developed overt disease in terms of elevated blood counts, JAK2 was deleted by injection of polyI:polyC (pI:pC). Evaluation of peripheral blood chimerism revealed that JAK2 deleted cells had a significant survival disadvantage

against the CD45.1 wildtype bone marrow (Fig. 4.1). In these mice, white blood cell and platelet counts remained normal (Fig 4.2a and b) and mutant allele burden measured by percentage of GFP positive cells was significantly reduced compared to controls (Fig 4.2c). Spleen and liver sizes were also significantly reduced in mice with bone marrow lacking JAK2 (Fig 4.2d,e). Additionally, bone marrow fibrosis, a hallmark feature of this MF model, was absent in JAK2 deleted mice (Fig 4.2f). One mouse in the Mx+ cohort had incomplete deletion of JAK2 as can be seen by presence of the floxed allele in peripheral blood (Fig 4.3a). This mouse had elevated blood counts and an enlarged spleen (Fig 4.3b,c) indicating that any residual disease in this model was due to transduced cells with intact JAK2. These data suggest that JAK2 function is required for all aspects of disease development in MPL-mediated disease.

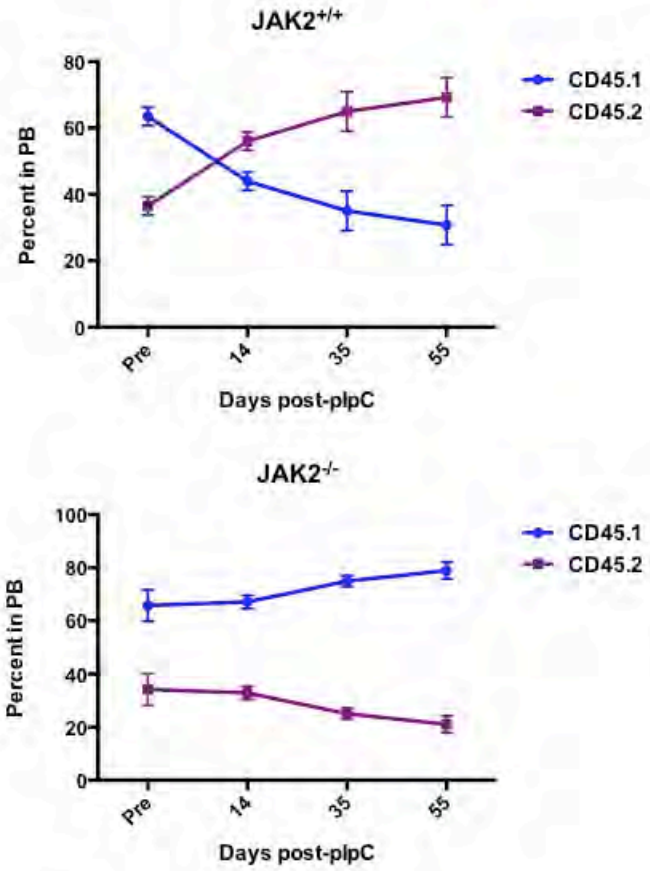


Figure 4.1: JAK2-/- cells have a survival disadvantage in competitive transplants. CD45.2 *MPLW515L*-transduced JAK2$^{f/f}$ Mx1-Cre+ and Cre- cells were transplanted into CD45.1 recipient mice along with equal number of CD45.1 support marrow. Following engraftment (Day 14 following transplant), JAK2 was excised by administration by polyI:polyC and chimerism in peripheral blood was assessed.

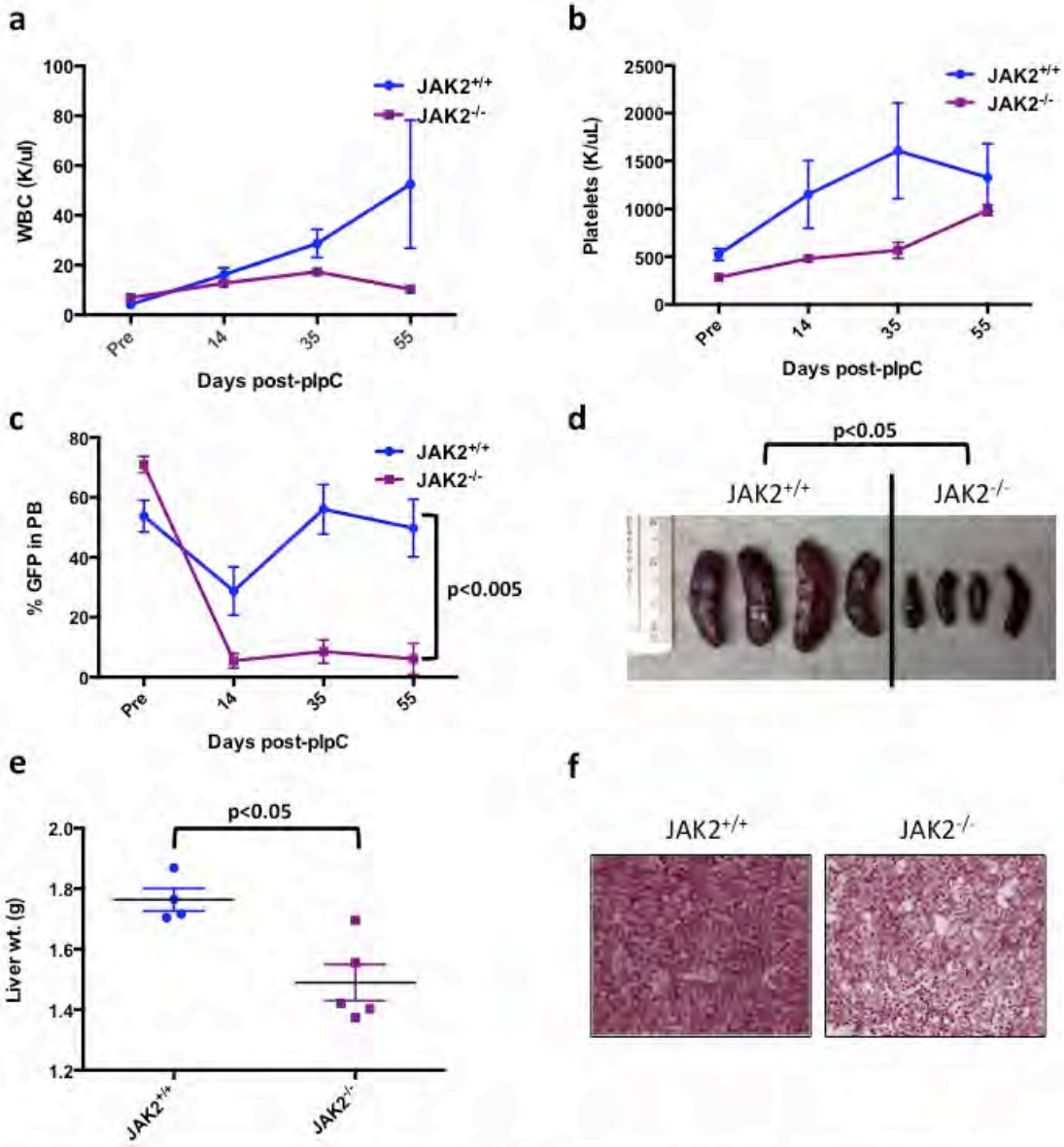


Figure 4.2: JAK2 is required for initiation of MPLW515L-mediated disease. Deletion of JAK2 following engraftment of MPLW515L transduced bone marrow leads normalization of (a) leukocytosis (b) platelets. It also leads to significant reduction in (c) mutant allele burden in terms of GFP positive cells in peripheral blood (PB) and (d) spleen size (e) liver size as compared to JAK2+/+ controls. (f) Bone marrow fibrosis assessed by reticulin staining is absent in JAK2 deleted bone marrow. Data is shown as mean±S.E.M

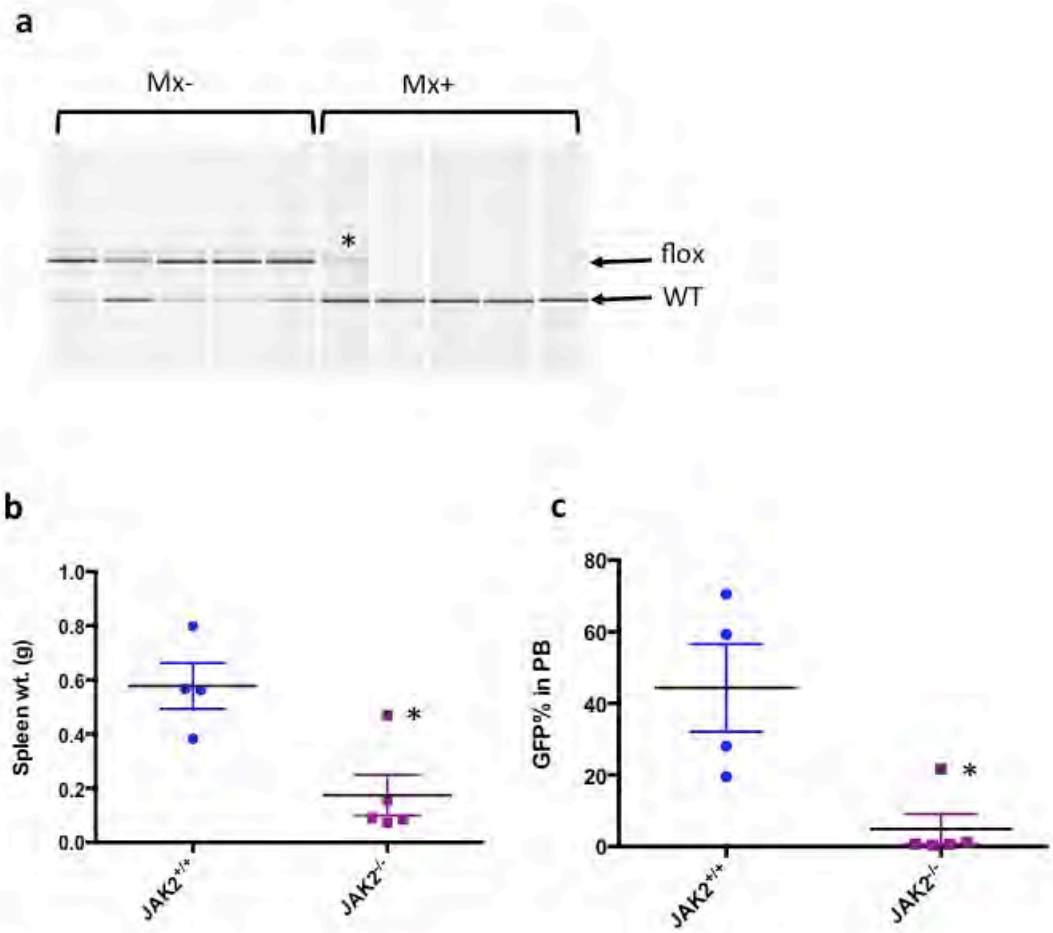


Figure 4.3: Residual disease is due to cells with intact JAK2. DNA from peripheral blood from Mx1-Cre+ mice revealed incomplete excision in one mouse (marked by *). This mouse had (a) an enlarged spleen and (b) higher proportion of GFP+ cells in peripheral blood as compared to other mice in this cohort.

JAK2 PLAYS A CRITICAL ROLE IN SURVIVAL OF MPN MUTANT CLONE

We then wanted to determine the requirement of JAK2 in maintenance of the disease clone in the MPLW515L model. Disease establishment usually takes four to six weeks following transplantation of *MPLW515L*-transduced bone marrow. JAK2 was excised by administration of pI:pC at this time point. Similar to previous results, the JAK2 deleted cells had a significant survival disadvantage as measured by peripheral blood chimerism. Loss of JAK2 at this stage of disease resulted in significant reduction in leukocytosis and platelet counts (Fig 4.4a,b). Spleen sizes were also significantly smaller (Fig 4.4d). We also observed a significant reduction in mutant allele burden, in terms of GFP+ cells (Fig 4.4c). Of note, this reduction in mutant allele burden is not seen even with maximal kinase inhibition in this same model. Examination of the remaining GFP+ mutant cells in the bone marrow revealed incomplete excision of JAK2 since we were able to detect the floxed JAK2 allele in GFP+ sorted cells by PCR (Fig 4.4e). Thus, similar to the previous result, residual disease was due to mutant cells with intact JAK2. Further, deletion of JAK2 led to significant decrease in extramedullary hematopoiesis, restoration of splenic architecture and complete loss of bone marrow fibrosis (Fig 4.5). There was also a reduction in the megakaryocytic-erythroid progenitor (MEP) compartment and CD11b+ Gr1+ myeloid lineages (Fig 4.6), which are expanded in this model of MPN. These data demonstrate that conditional deletion of JAK2 after establishment of disease can prevent further progression. Thus, JAK2 is required for maintenance of the mutant MPN clone in this model.

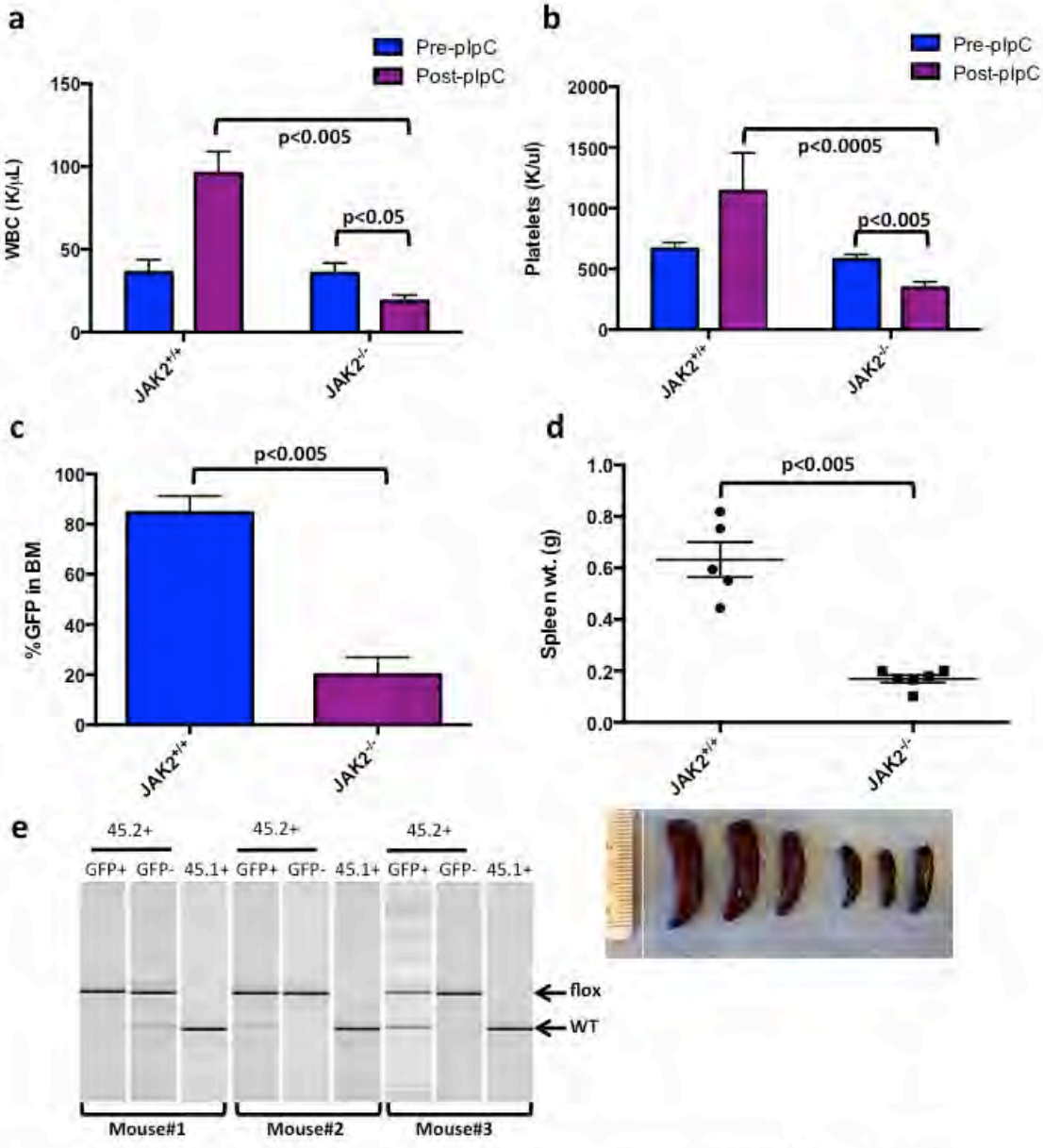


Figure 4.4: JAK2 plays a critical role in survival of MPN clone. Excision of JAK2 following establishment of MPLW515L-mediated disease led to a significant reduction in (a) leukocytosis (b) platelets (c) mutant allele burden as assessed by percentage of GFP positive cells in bone marrow (BM) and (d) spleen sizes. (e) DNA was isolated from sorted GFP+ and GFP- cells as well as CD45.1 cells from bone marrow from three Mx1-Cre+ mice. GFP+ cells retained JAK2 floxed allele, which was not detectable in unsorted cells (data not shown) indicating that residual GFP+ disease cells had incomplete excision of JAK2.

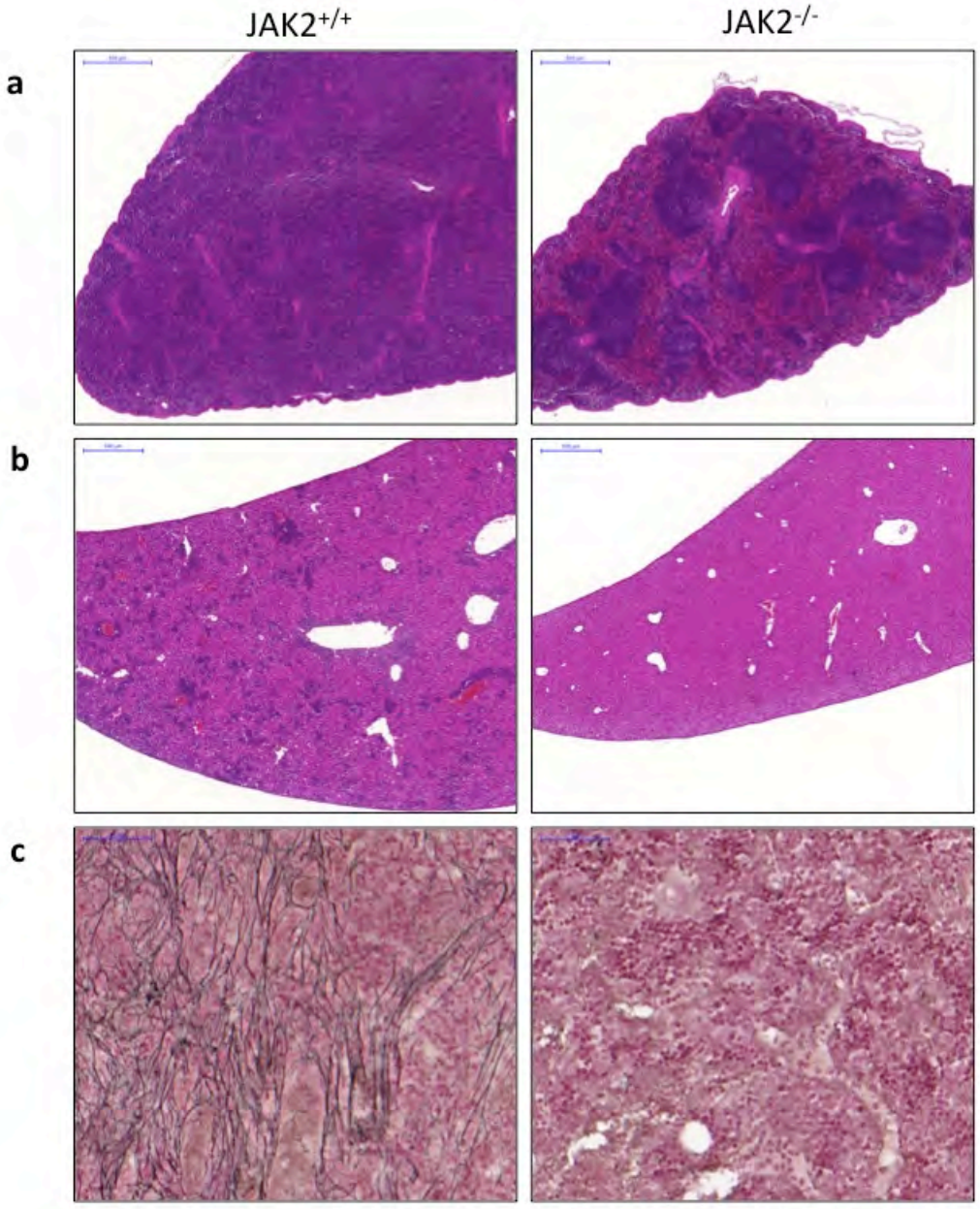


Figure 4.5: Deletion of JAK2 leads to significant histopathological improvement. Loss of JAK2 resulted in significant improvement in disease phenotype including (a) restoration of splenic architecture (b) decrease in myeloid infiltration in liver and (c) reduction in bone marrow fibrosis.

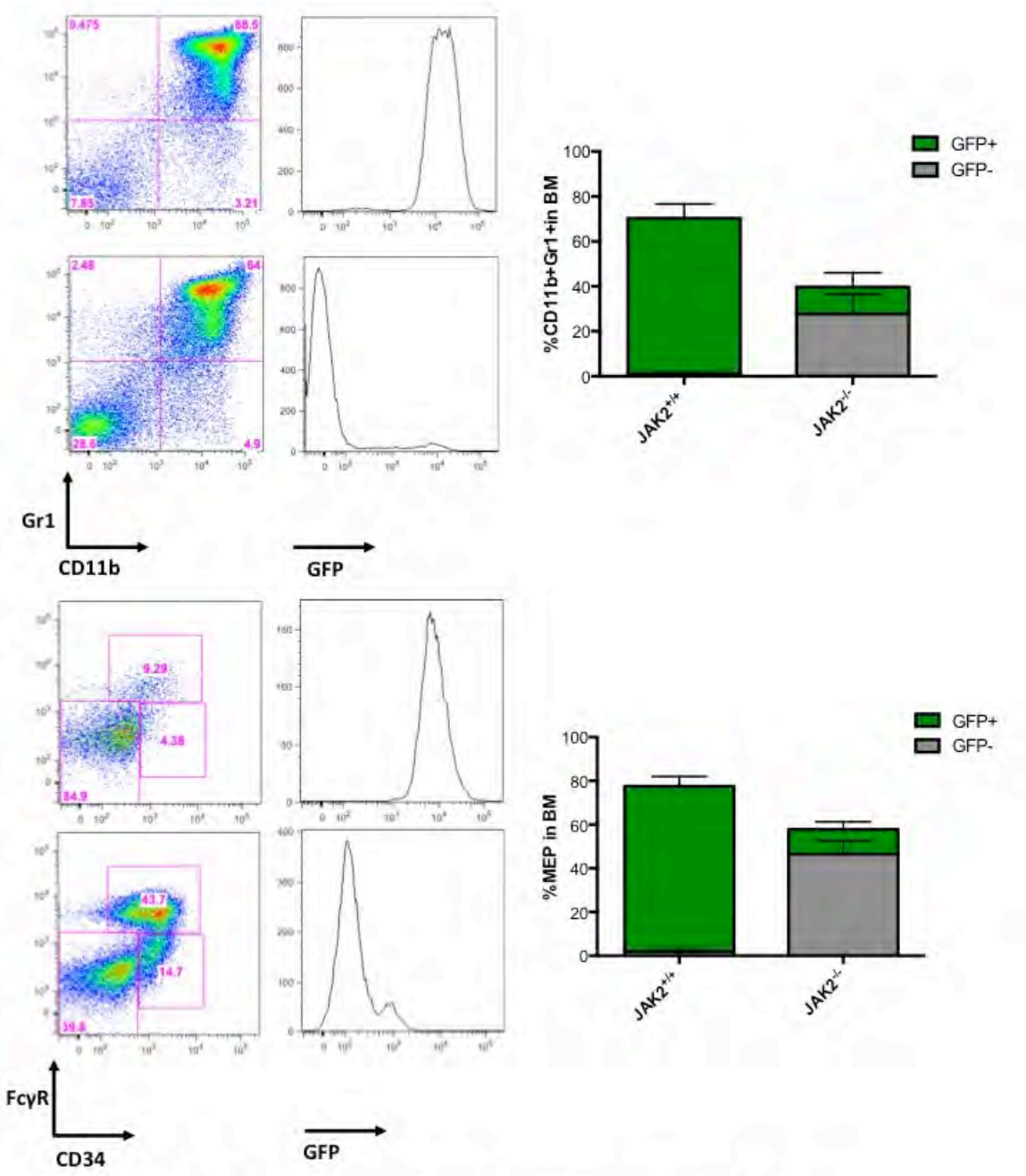


Figure 4.6: Loss of JAK2 leads to depletion of myeloid cells in myeloid lineages. Deletion of JAK2 resulted in a dramatic reduction in contribution of GFP+ cells to (a) CD11b+Gr1+ myeloid cells and (b) megakaryocyte-erythroid progenitors (MEP) in bone marrow. Data is represented as mean ± S.E.M, n=6)

DELETION OF JAK2 IS MORE EFFECTIVE THAN JAK INHIBITOR TREATMENT *IN VIVO*

We decided to directly compare the efficacy of genetic loss of JAK2 to JAK kinase inhibitor in our mouse model. Bone marrow from JAK2$^{f/f}$ Mx1-Cre+ mice was retrovirally transduced with MPLW515L-IRES-GFP and transplanted into lethally irradiated recipients. After disease establishment, mice were randomized to either receive vehicle, 60mg/kg ruxolitinib twice daily or pI:pC to delete JAK2. As reported previously (Koppikar et al., 2010), although drug treatment improved blood counts, there was no reduction in mutant allele burden in terms of GFP positive cells. In contrast, deletion of JAK2 led to significant decrease in the percentage of GFP+ cells in the bone marrow (Fig 4.7a). Deletion of JAK2 was also more effective at reducing blood counts and spleen size as compared to drug treatment (Fig 4.7b,c). Analysis of myeloid and progenitor populations revealed that loss of JAK2 leads to significant reduction in MEP and CD11b+Gr+ proportions with a significant decrease in the contribution of mutant (GFP+) cells (Fig 4.7d). These results indicate that deletion of JAK2 is superior to JAK2 kinase inhibitor treatment alone at reducing disease burden in this model.

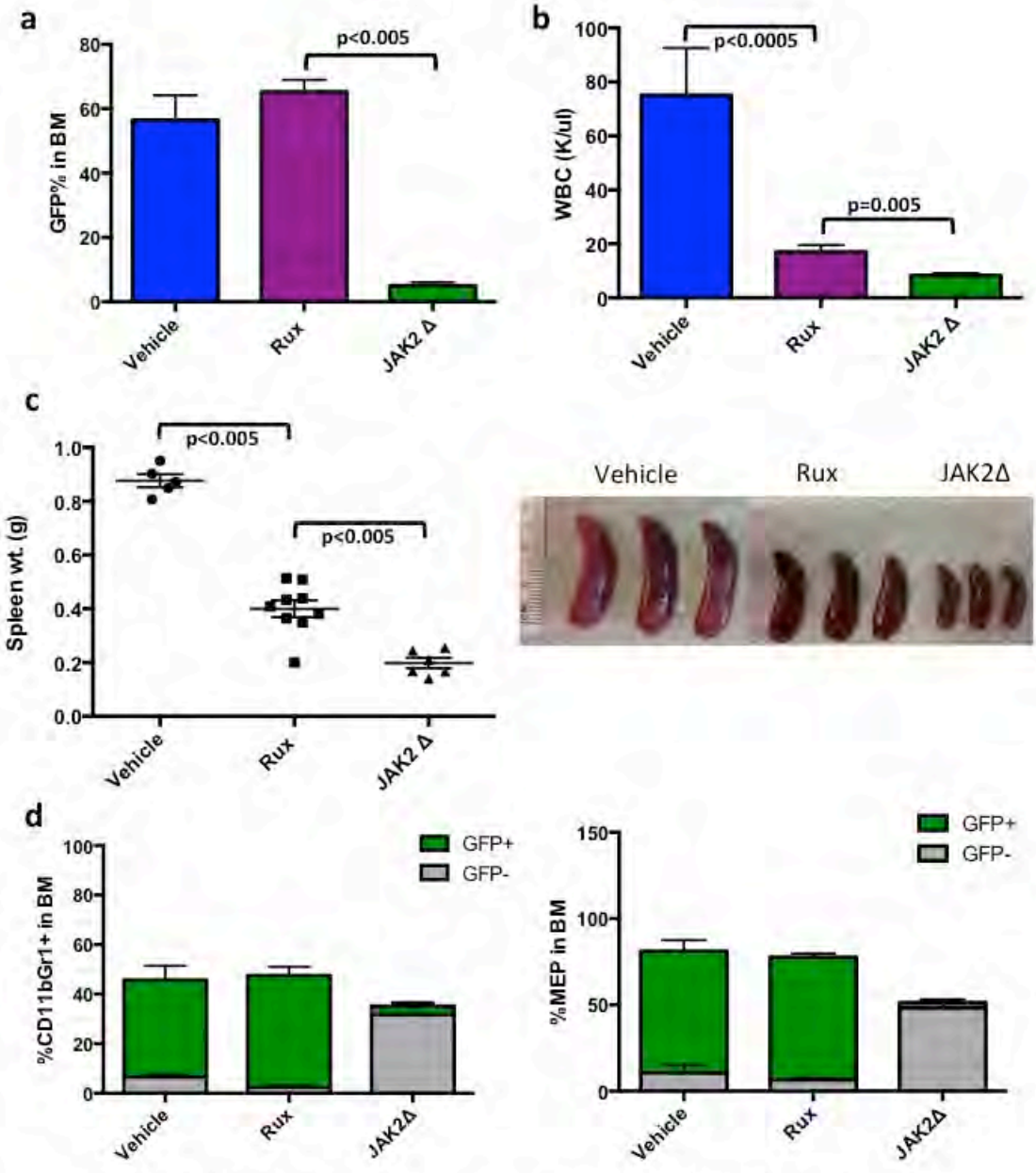
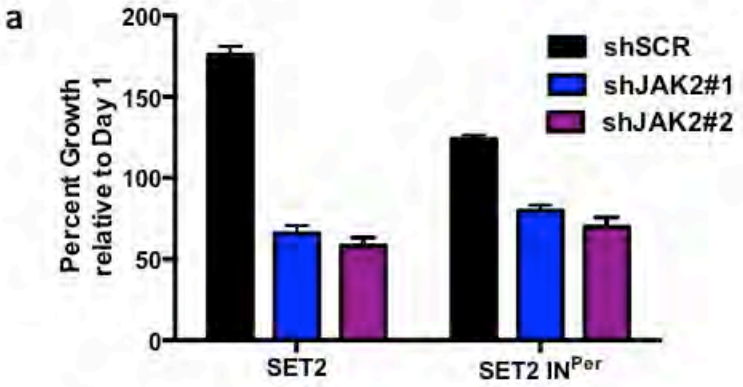


Figure 4.7: Deletion of JAK2 is more efficacious than kinase inhibition. *MPLW515L* transduced mice were treated with vehicle, 60mg/kg ruxolitinib or polyI:polyC to excise JAK2. (a) Deletion of JAK2 led to significant reduction in mutant allele burden in bone marrow (BM) as compared to inhibitor treatment. It also resulted in further decrease in (b) blood counts and (c) spleen size than ruxolitinib alone. (d) Loss of JAK2 leads to reduction in megakaryocyte-erythroid progenitor (MEP) and myeloid (CD11b+Gr+) proportions with a significant decrease in the contribution of mutant (GFP+) cells. (Mean ± S.E.M.)

PERSISTENT CELLS REMAIN DEPENDENT ON JAK2

Using a genetic loss of function model of JAK2, we demonstrated that JAK2 is indispensable for development and maintenance of MPLW515L-induced disease *in vivo*. We then investigated whether JAK inhibitor persistent cells that can survive in the presence of chronic drug exposure remain dependent on expression of JAK2. Knockdown of JAK2 using two different short hairpins in naïve and persistent cell lines led to growth suppression (Fig 4.8a) and inhibition of downstream STAT3/STAT5 signaling (Fig 4.8b).

We then asked whether we could leverage this dependency therapeutically to overcome inhibitor persistence. We have previously shown that JAK2 is an Hsp90 client protein and treatment with PU-H71, an Hsp90 inhibitor, leads to degradation of total and activated JAK2 and inhibition of downstream signaling in MPN cells (Marubayashi et al., 2010). We found that JAK inhibitor persistent cells remained sensitive to PU-H71 (Fig 4.9a) and drug treatment led to efficient degradation of JAK2 and abrogation of downstream signaling (Fig 4.9b). These data indicate that JAK2 can serve as a scaffold for transactivation and downstream signaling even in the context of inhibition of kinase activity.



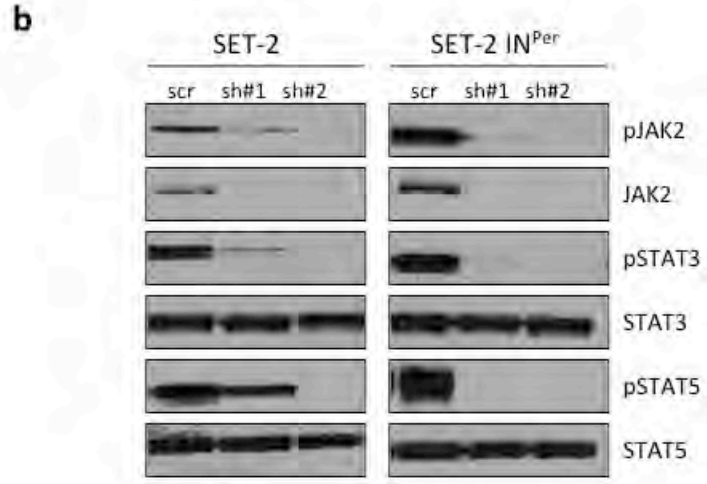


Figure 4.8: JAK inhibitor persistent cells remain dependent on JAK2. JAK2 was knocked down in naïve and ruxolitinib persistent SET-2 cells using two independent short hairpins. (a) Cell growth at day 5 following transduction is shown (mean±S.D.) (b) Knockdown of JAK2 leads to inhibition of downstream STAT signaling in naïve and persistent SET-2 cells.

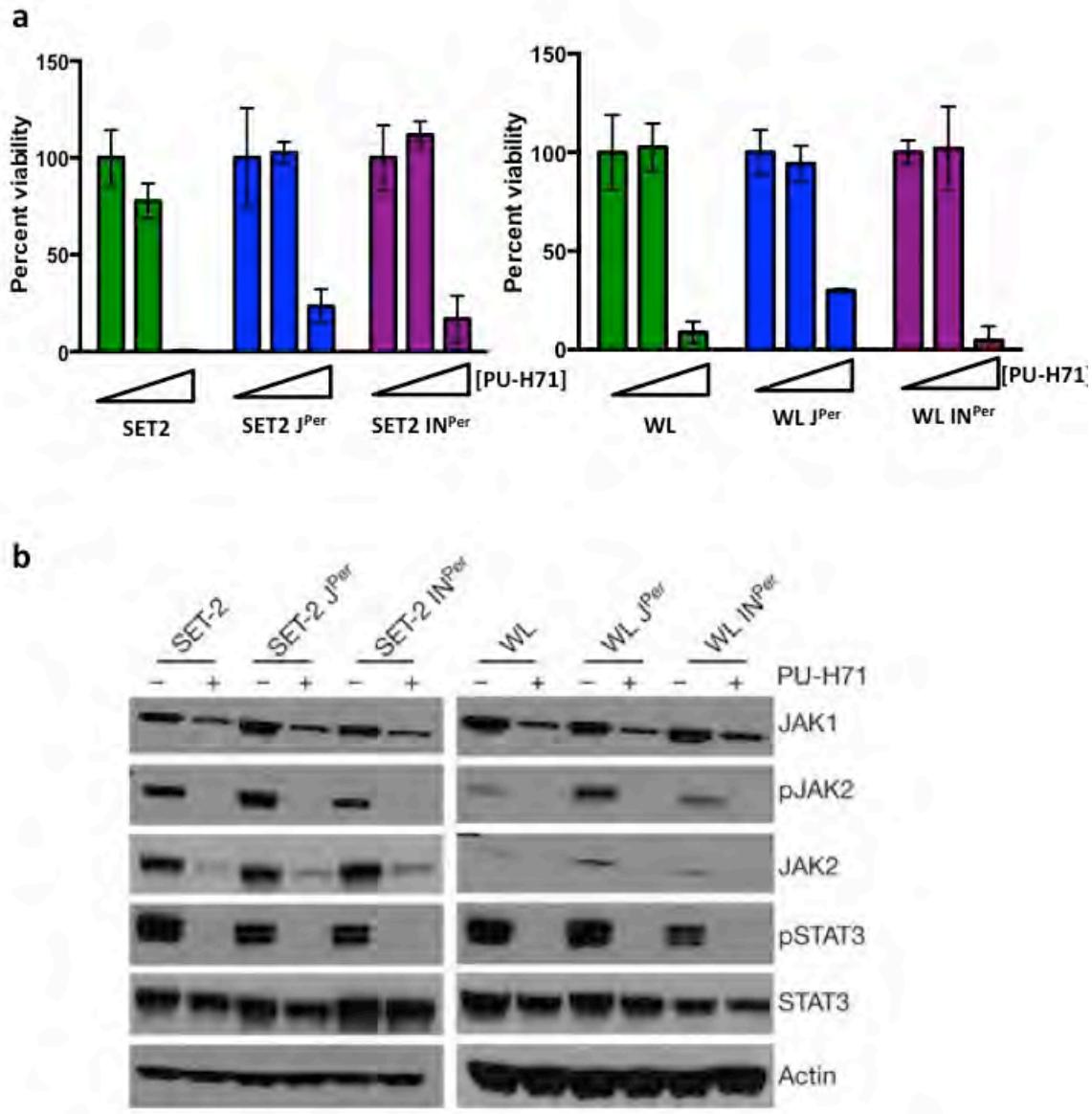


Figure 4.9: Persistent cells are sensitive to an Hsp90 inhibitor. (a) Viability of JAK inhibitor persistent and naïve SET-2 and WL cells treated with PU-H71. (b) PU-H71 degrades total and phosphoJAK2 and inhibits downstream signaling. Cells were treated with 2μM (SET-2) and 1μM (WL) PU-H71 for 16 hours.

COMBINATION OF JAK AND HSP90 INHIBITION IS MORE EFFICACIOUS THAN JAK INHIBITOR MONOTHERAPY: Based on cell line data and our previous work, we decided to test the efficacy of a combination of ruxolitinib and PU-H71 in the MPLW515L model of ET/MF. The dosing regimens tested included vehicle, two different doses of ruxolitinib monotherapy, combined JAK/HSP90 inhibition from the onset and ruxolitinib followed by the addition of PU-H71 after initial response (Fig 4.10a). At 2 weeks following start of treatment, the combination group displayed a significant reduction in white blood cell and platelet counts (Fig. 4.10b,c) compared to either low-dose or high-dose ruxolitinib alone. Combination treatment also led to further reduction in spleen size compared to ruxolitinib monotherapy (Fig 4.10d). We also observed a decrease in total and phosphorylated JAK2 levels and more potent inhibition of downstream signaling effectors including STAT3, STAT5 and MAPK by immunoblotting and immunohistochemistry in the combination treatment arm (Fig 4.11). Combination treatment also led to histopathological improvement in terms of reduction in bone marrow and spleen cellularity, decrease in megakaryocytes as well as a reduction in bone marrow fibrosis (Fig 4.12). These results were consistent over the entire 4-week drug trial, with significant improvements in blood counts and organomegaly seen in combination arms as compared to ruxolitnib alone. The ruxolitinib dose was increased from low dose (30mg/kg) to high dose (90mg/kg) in a subset of the combination treated mice, which proved to be the most efficacious strategy (Fig 4.13).

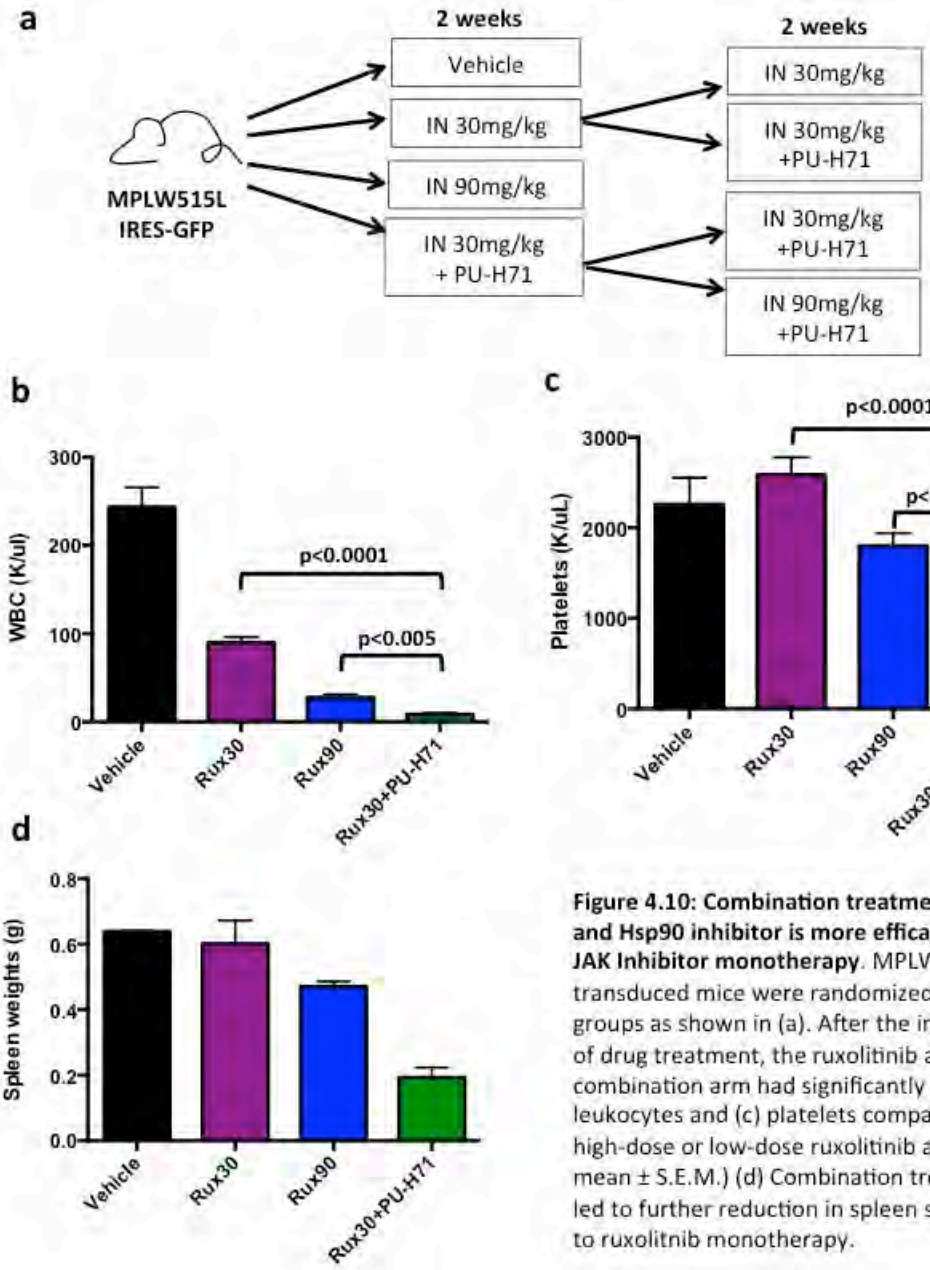


Figure 4.10: Combination treatment with JAK and Hsp90 inhibitor is more efficacious than JAK Inhibitor monotherapy. MPLW515L-transduced mice were randomized to five groups as shown in (a). After the initial 2 weeks of drug treatment, the ruxolitinib and PU-H71 combination arm had significantly lower (b) leukocytes and (c) platelets compared to either high-dose or low-dose ruxolitinib alone. (n=7, mean ± S.E.M.) (d) Combination treatment also led to further reduction in spleen size compared to ruxolitnib monotherapy.

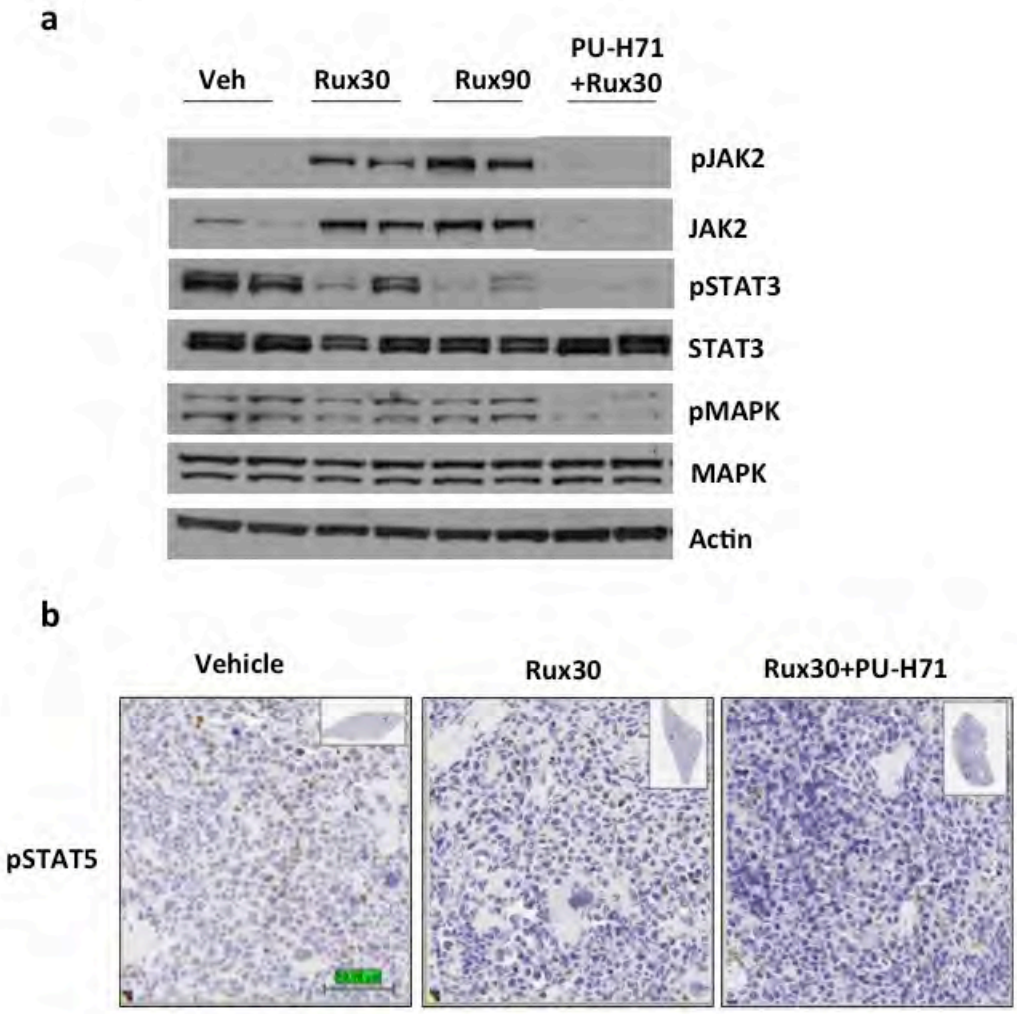


Figure 4.11: Combination treatment results in potent JAK-STAT pathway inhibition. Combination of ruxolitinib and PU-H71 is more effective at inhibiting downstream STAT3/STAT5 and MAPK signaling. Immunoblotting was performed on peripheral blood (a) and immunohistochemistry was performed on spleen sections (b).

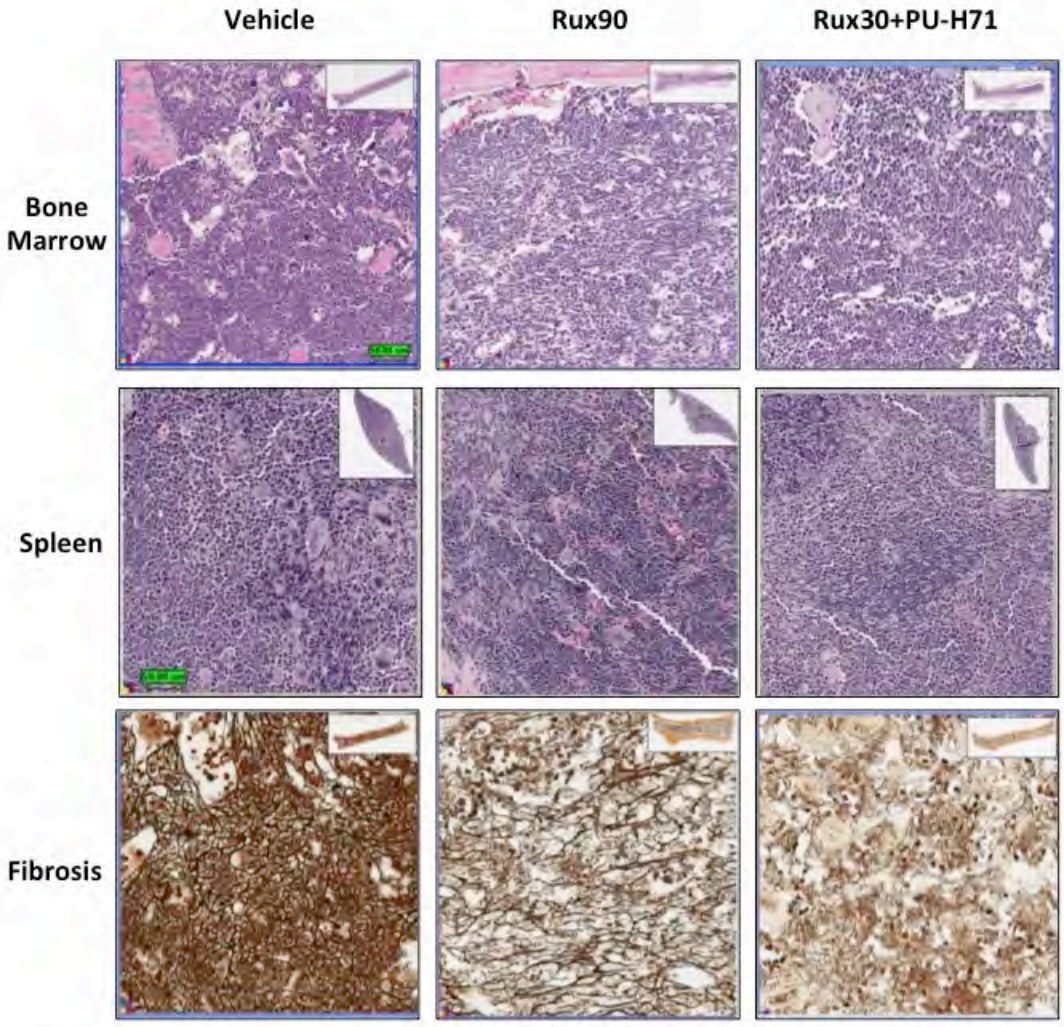


Figure 4.12: Combination treatment leads to further histopathological improvement compared to ruxolitinib monotherapy. Combination of ruxolitinib and PU-H71 resulted in further reduction in cellularity and number of megakaryocytes in bone marrow and spleen of treated mice. It also led to a further decrease in fibrosis as assessed by reticulin staining.

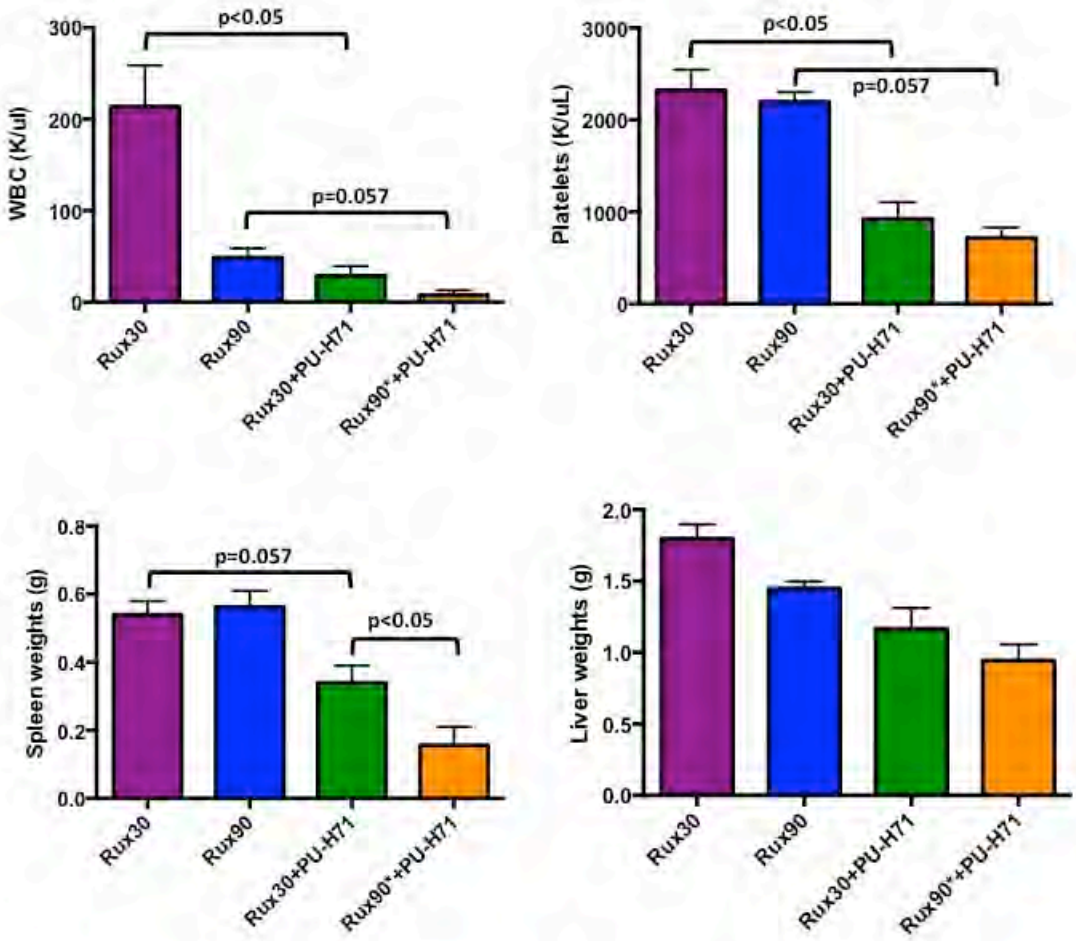


Figure 4.13: Long-term combination treatment with JAK and Hsp90 inhibitors is also efficacious. Treatment of MPLW515L mice with combination of ruxolitinib and PU-H71 for 4 weeks remained more effective than ruxolitinib monotherapy. After 4 weeks of drug treatment, the combination group that had ruxolitinib dose increased to 90mg/kg for 2 weeks (Rux90*+PU-H71) had lowest blood counts and spleen sizes compared to ruxolitnib alone.

GENETIC OR PHARMACOLOGICAL LOSS OF JAK2 CAN OVERCOME PERSISTENCE *IN VIVO:*

We then asked whether genetic deletion of JAK2 following initial ruxolitinib treatment could be effective at reducing disease burden. Although ruxolitinib treatment improved blood counts and reduced spleen size, we did not observe a reduction in mutant allele burden indicating persistence of MPN clone. Deletion of JAK2 following 2 weeks of ruxolitinib treatment led to significant reduction in mutant allele burden (Fig 4.14a), blood counts (Fig 4.14b) and spleen size (Fig 4.14c) as compared to mice that continued to receive drug. Further, deletion of JAK2 after long-term ruxolitinib treatment (6weeks) prevented disease relapse; blood counts and spleen weights remained low for up to 3 weeks following cessation of treatment (Figure 4.15a,b). The percentage of GFP positive cells, which remained high after 6 weeks of ruxolitnib treatment, was also decreased by deletion of JAK2 (Fig. 4.15c). These results indicate that deletion of JAK2 in vivo can successfully eliminate mutant MPN cells, which cannot be achieved solely by kinase inhibition of JAK2.

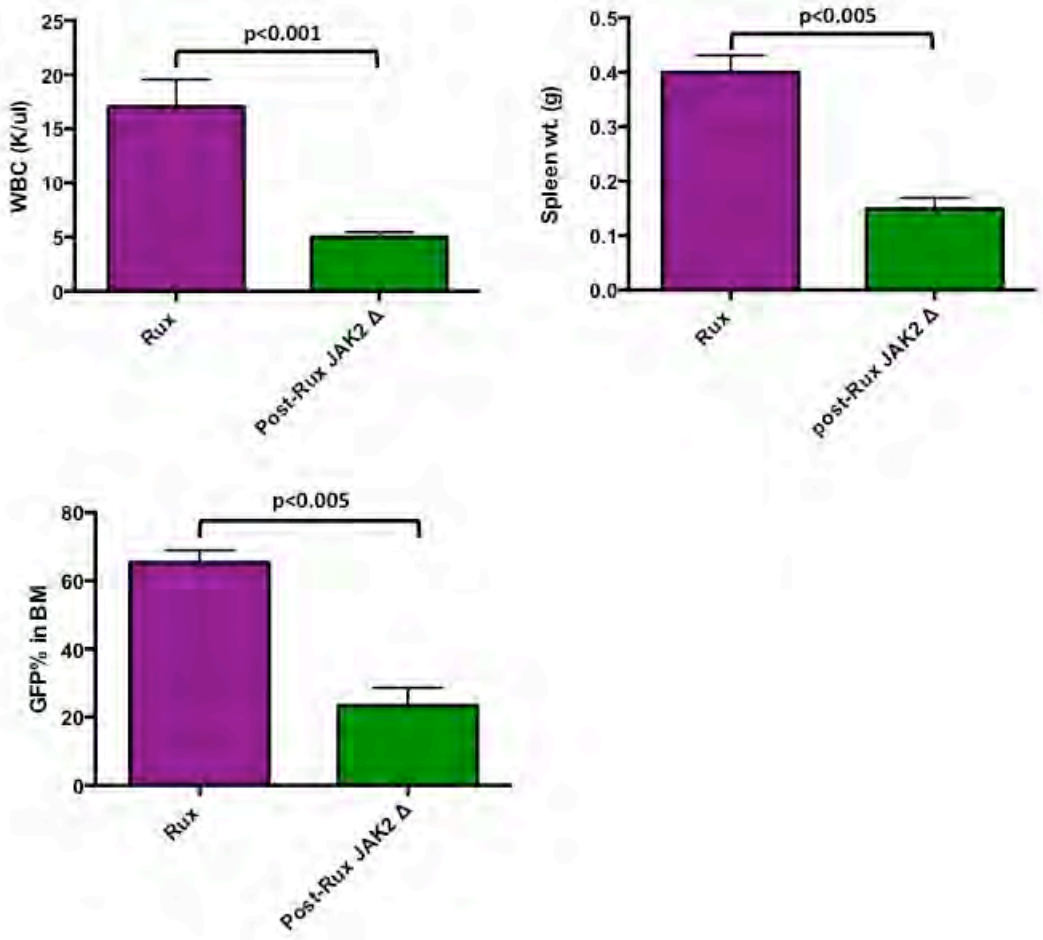


Figure 4.14: Deletion of JAK following ruxolitinib treatment can overcome persistence. Deletion of JAK2 following 2 weeks of ruxolitinib treatment resulted in reduction of (a) bloods counts, (b) spleen weights and (c) mutant allele burden compared to mice that continued to receive ruxolitinib.

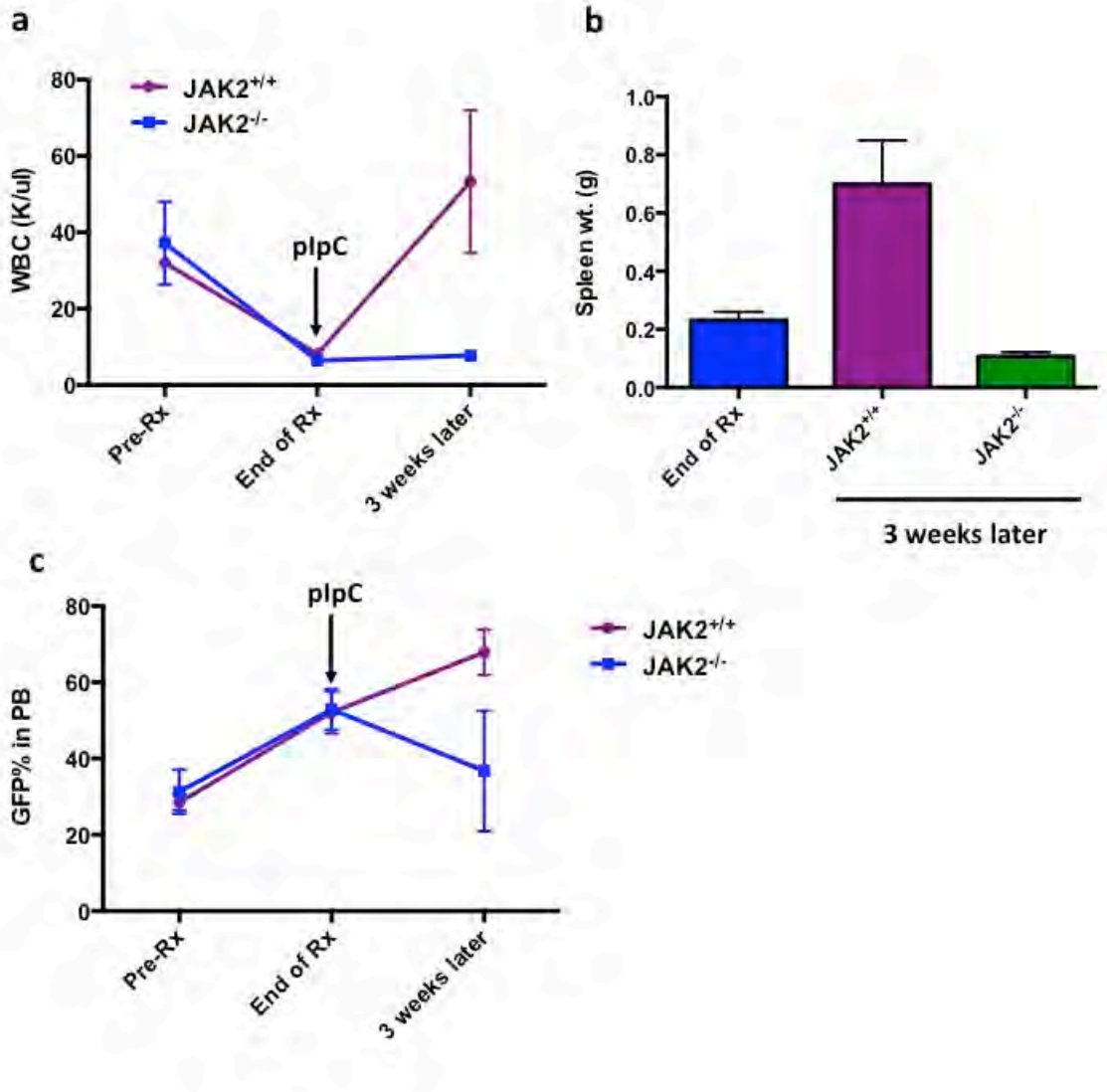


Figure 4.15: Deletion of JAK2 prevents disease relapse following cessation of treatment. Ruxolitinib treatment was stopped after 6 weeks and JAK2 was excised by administration of pIpC. Loss of JAK2 prevented rebound of (a) blood counts and (b) spleen sizes. Deletion of JAK2 also led to reduction in mutant allele burden (GFP+ cells) in peripheral blood (PB).

These results could also be recapitulated by targeting of JAK2 by pharmacological degradation. After two weeks of 30mg/kg ruxolitinib treatment, we added 75/mg PU-H71 treatment to a subset of mice. Two weeks of combination treatment resulted in significant reduction of leukocytosis and thrombocytosis (Fig 4.16a,b). Addition of PU-H71 to ruxolitnib monotherapy also led to a further decrease in splenomegaly (Fig 4.16c).

We have previously demonstrated that mononuclear cells from patients treated with ruxolitnib are insensitive to JAK2 kinase inhibition *ex vivo* and exhibit persistent downstream signaling even after treatment with a JAK inhibitor (Fig 3.6). We asked whether treatment with an Hsp90 inhibitor could overcome this persistence. Mononuclear cells were isolated from two different MF patients that were receiving ruxolitinib therapy. Treatment of these cells with PU-H71 led to degradation of total and activated JAK2 and abrogation of downstream STAT and MAPK signaling (Fig 4.16d). Thus, PU-H71 is effective in inhibiting JAK-STAT signaling in primary samples that are insensitive to JAK inhibition. Taken together, our results indicate that Hsp90 inhibition can overcome JAK inhibitor persistence in cell lines, pre-clinical models and primary MPN samples.

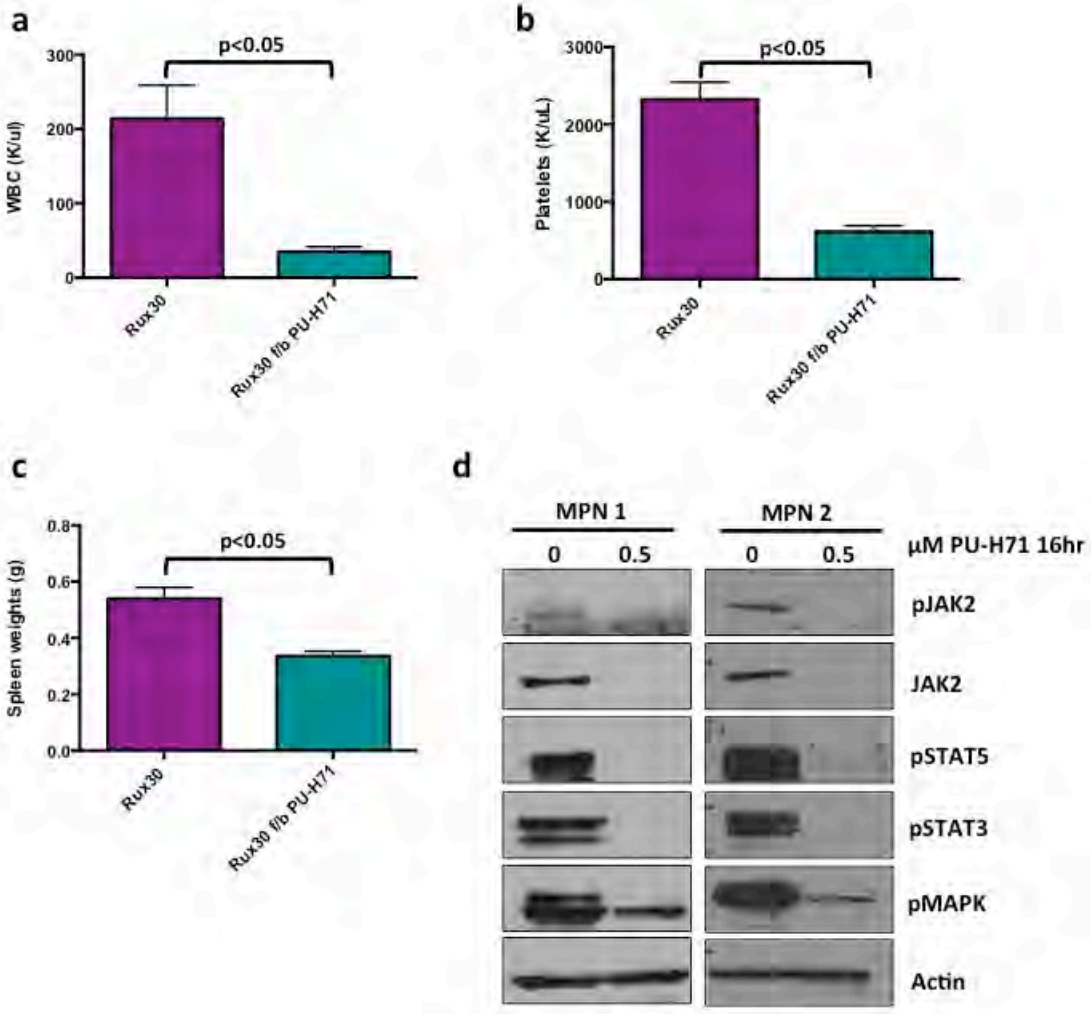


Figure 4.16: Hsp90 inhibition can overcome persistence *in vivo*. PU-H71 (@75mg/kg) was added to a subset of mice receiving 30mg/kg ruxolitinib after 2 weeks of treatment. Addition of PU-H71 resulted in significant reduction of (a) leukocytes (b) platelets and (c) spleen sizes as compared to mice receiving ruxolitnib monotherapy. Combination treatment (Rux30 followed by (f/b) PU-H71) lasted for 2 weeks. (d) Mononuclear cells from 2 MPN patients receiving ruxolitinib were isolated and treated with 0.5µM PU-H71 for 16 hours, which led to degradation of activated and total JAK2 and inhibition of downstream signaling.

JAK2 IS REQUIRED FOR NORMAL MYELOPOIESIS AND STEM CELL FUNCTION

Thus far, our data indicate that JAK2 has an indispensable role in the survival and maintenance of the MPN mutant clone. Complete loss of JAK2, as can be achieved by genetic deletion, can potentially be curative in this disease. However, loss of JAK2 in normal adult hematopoietic lineages has not been investigated in detail. This has important implications for JAK inhibitor therapy since in the clinic; JAK inhibition is associated with dose-limiting cytopenias. We therefore decided to use the conditional knockout model of JAK2 to evaluate the role of JAK2 in normal hematopoiesis. We performed competitive bone marrow transplant experiments in which CD45.2 $JAK2^{f/f}$ Mx1-Cre+ or Cre- cells along with equal number of CD45.1 wild type cells were injected into CD45.1 irradiated donor mice. JAK2 was deleted by administration of pI:pC following bone marrow engraftment and chimerism of mature myeloid, B and T lineages was monitored in peripheral blood for 16 weeks. Deletion of JAK2 led to significant reduction in overall CD45.2 chimerism (Fig 4.17a). Further examination revealed a dramatic reduction in CD11b+ Gr1+ myeloid lineages within 3 weeks of JAK2 deletion. B and T cell lineages showed a more gradual decline over the 16-week period (Fig 4.17 b,c,d,e). We also evaluated the effect of loss of JAK2 in hematopoietic stem and progenitor cells and found a significant reduction in CD45.2 contribution to the myeloid progenitor (Lin-ckit+sca1-), short-term (Lin-ckit+sca1+CD48-CD150-) and long-term (Lin-ckit+sca1+CD48-CD150+) stem cell compartment (Fig 4.17f). Taken together, these results suggest that JAK2 is required for survival of mature myeloid lineages and also plays an important role in maintenance of the stem cell and progenitor compartment.. Importantly, white blood cell counts, platelet counts and hematocrit remained normal in

these mice (data not shown) indicating that in this experimental system, support cells with intact JAK2 can sustain normal hematopoiesis. Since MPN patients are also chimeric for the JAK2V617F mutation (Jamieson et al., 2006), our results indicate that long-term treatment with mutant-specific JAK inhibitors or agents that degrade JAK2 in a tumor-specific manner will likely be well-tolerated and could eliminate primitive disease-initiating cells in MPN patients.

Taken together, these data demonstrate that despite the rapid development of persistence in MPN cells with chronic JAK inhibitor exposure, these cells still remain highly dependent on JAK2 for their growth and survival. Treatment with an Hsp90 inhibitor such as PU-H71, which degrades JAK2 specifically in mutant cells, can successful overcome persistence in cells lines, mouse models as well as in primary samples. Thus, JAK2 remains a bona fide target for therapy in MPN and strategies that combine JAK kinase inhibition with JAK2 degradation can be beneficial for patients.

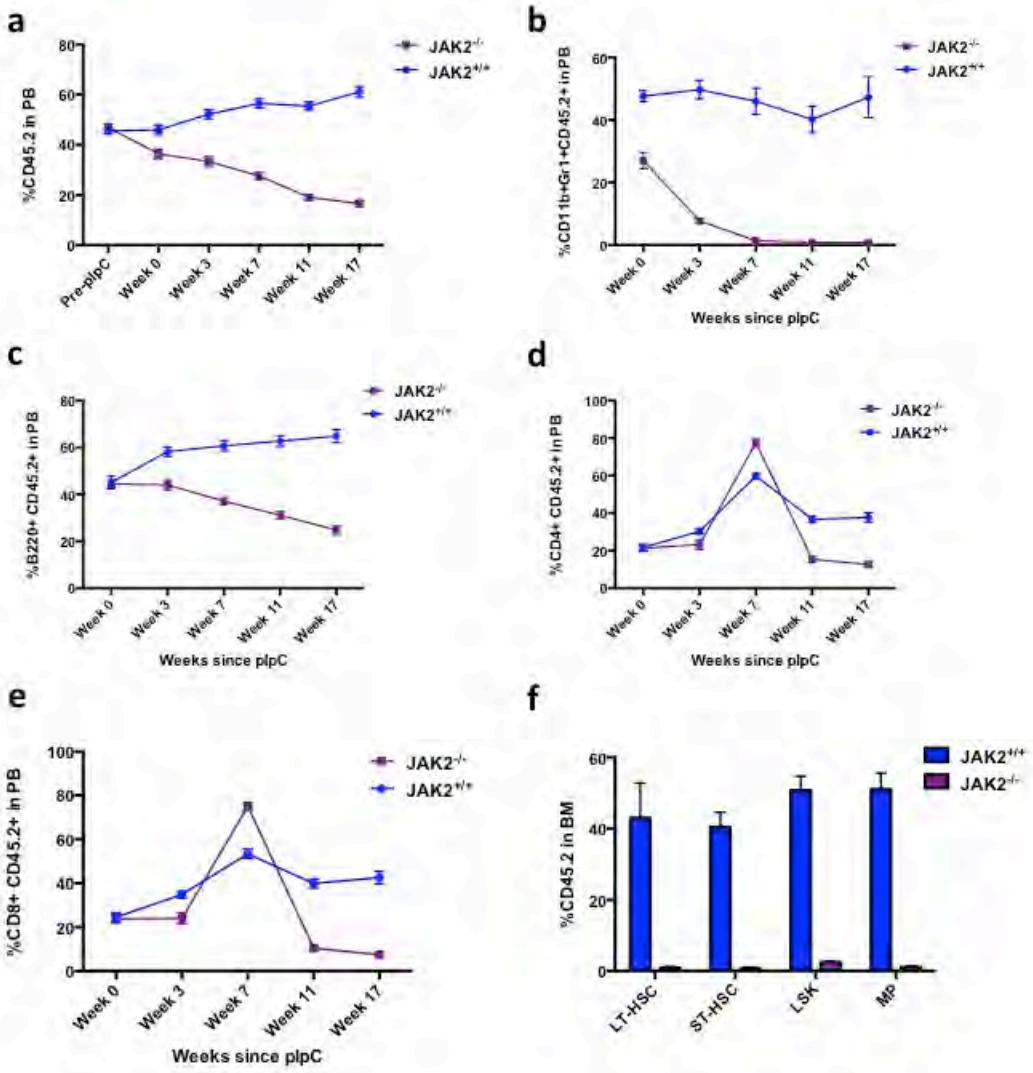


Figure 4.17: JAK2 is required in normal hematopoiesis. Competitive transplants were performed with JAK2f/f Mx1-Cre+ and Mx1-Cre- mice. JAK2 was excised by administration of polyI:polyC (plpC) at 2 weeks after injection. Loss of JAK2 led to reduction in (a) overall CD45.2 chimerism in peripheral blood as well as significant reduction in (b) myeloid cells (c) B cells and (d,e) T cells. (f) There was also a significant reduction in contribution of CD45.2 cells to hematopoietic stem and progenitor compartments. (n=7, mean ± S.E.M.)

CHAPTER FIVE

DISCUSSION

The majority of patients with the classical MPN; PV, ET and MF harbor somatic activating mutations in the tyrosine kinase JAK2, thereby making it an attractive therapeutic target in these diseases. However, the relative contribution of JAK2 signaling to disease phenotype, malignant proliferation, and disease progression has not been fully delineated. The dual JAK1/JAK2 inhibitor, ruxolitinib, was approved by the FDA in 2011 for the treatment of MF, and several other compounds are in late-stage clinical testing. Although these drugs alleviate many of constitutional symptoms in patients, they have been ineffective at reducing the MPN mutant clone. The previous two chapters discuss (i) mechanisms by which MPN cells can survive in spite of chronic inhibition of JAK2 kinase activity and (ii) establish that JAK2 plays a critical role in MPN pathogenesis and disease phenotype. In this chapter, I discuss some of the biological and clinical implications of these findings.

OVERCOMING JAK INHIBITOR PERSISTENCE

We show that MPN cells can persist in the presence of chronic JAK inhibition by reactivating downstream STAT-MAPK signaling via the formation of heterodimers between JAK2 and the JAK kinases JAK1 and TYK2. This phenomenon is observed in MPN cell lines, murine models and primary samples from patients treated with ruxolitinib. Additionally, this phenomenon is reversible suggesting that MPN patients could benefit from being re-exposed to drug after a brief withdrawal.

The formation of heterodimers is facilitated by the stabilization of phosphorylated JAK2 by Type I inhibitors such as ruxolitinib, which engage JAK2 in its active conformation. In MPN cell lines, persistence can be overcome by type II inhibitors of JAK2, which stabilize JAK2 in its inactive conformation and lead to a decrease in activation loop phosphorylation. In our studies, cells that were persistent to ruxolitinib and JAK Inhibitor I remained sensitive to treatment with BBT-594, a type II JAK inhibitor, and downstream signaling was inhibited at similar concentrations as the parental cells. Experiments evaluating the efficacy of newer, orally bioavailable type II JAK inhibitors in pre-clinical models of MPN are ongoing. It will also be important to investigate whether MPN cells can become persistent to this new class of inhibitors.

De novo mechanisms of resistance to JAK inhibitors should also be considered when assessing efficacy. We observed that mononuclear cells isolated from patients being treated with ruxolitinib are insensitive to *ex vivo* treatment with JAK inhibitors as compared to inhibitor-naïve controls. In a recent report, Kalota et al. reported similar findings in that granulocytes from myelofibrosis patients are relatively insensitive to *ex*

vivo JAK2 inhibition in terms of reduction in levels of phosphorylated STAT3 and STAT5 as compared to patients with PV and ET as well as normal controls (Kalota et al., 2013). Interestingly, this was observed in JAK2 inhibitor-naïve patients suggesting that certain subgroups of patients might have *de novo* mechanisms of resistance/persistence to JAK inhibitors. For example, pre-existing heterodimers between JAK2 and other JAK kinases in these patients might be contributing to this phenomenon, which should be further investigated.

ROLE OF JAK2 IN MPN PATHOGENESIS

The *JAK2V617F* mutation has been identified in the majority of patients with MPN. *In vivo* expression of this allele in murine bone marrow transplant systems and in genetic knockin models recapitulates many features of human MPN disease. This suggests that this pathway plays an important role in disease pathogenesis. However, kinase inhibition of JAK2 has not resulted in impressive molecular responses in preclinical and clinical setting, as has been observed with ABL kinase inhibitors in CML. Although MPN patients experience an improvement in constitutional symptoms, treatment with JAK inhibitors is not curative and does not decrease the size of the MPN clone. Additionally, the occurrence of secondary resistance mutations in response to chronic inhibition is often considered a hallmark of effective targeted therapy. This has been observed in numerous cases including ABL kinase inhibitors in CML (Gorre et al., 2001), EGFR inhibitors in lung cancer (Pao et al., 2005) and FLT3 inhibitors in acute myeloid leukemia (Smith et al., 2012). However, this has not been the case in the use of JAK2 inhibitors in the treatment of MPN. There have been no reports of second site mutations in JAK2 in

patients treated with ruxolitinib. *In vitro* genetic screens in JAK2 mutant cell lines have identified alleles that confer resistance to JAK2 inhibitors (Weigert et al., 2012a) (Deshpande et al., 2012; Marit et al., 2012). However, these do not occur at a significant frequency and a majority of cells can persist/survive in the presence of inhibitor without acquiring second site mutations. These observations led us to question if JAK2 represented a 'driver' lesion in MPN and whether targeting JAK2 remained a viable therapeutic strategy.

In our genetic studies, we demonstrate that JAK2 is essential in an *in vivo* model of ET/MF by deleting JAK2 in an inducible, tissue-specific manner at different stages of disease development. Loss of JAK2 leads to significant improvements in blood counts, organomegaly and bone marrow fibrosis. More importantly, deletion of JAK2 results in a dramatic reduction in mutant allele burden, which is not seen with maximal JAK2 kinase inhibition in this model (Koppikar et al., 2010). Further, JAK inhibitor persistent cell lines remain dependent on JAK2 expression for their growth and survival. Knockdown of JAK2 using short hairpins leads to decrease in cell proliferation and inhibition of downstream signaling in naïve and persistent MPN cells. These data suggest that MPN cell lines require JAK2, at least in part as a scaffold to maintain downstream signaling, even in the context of inhibition of its catalytic activity. Thus, targeting JAK2 remains a viable therapeutic option in the treatment of MPN and novel strategies that result in degradation/loss of total protein should be evaluated.

TARGETING JAK2 IN NAÏVE AND PERSISTENT MPN CELLS

There are several active agents that have been found to degrade JAK2 and inhibit downstream signaling in MPN. The following sections discuss some of these approaches.

Hsp90 inhibitors

Heat shock protein 90 (Hsp90) is a molecular chaperone protein that plays a critical role in maintaining protein homeostasis in response to various stimuli such as genomic instability, proteotoxic stress, changes in nutrient and oxygen levels. Cancer cells are particularly dependent on Hsp90 since this protein plays an important role in regulating the stability and function of mutated or amplified oncoproteins as well as protecting the cell from various stresses brought about by malignant transformation (Neckers and Workman, 2012). Hsp90 client proteins include several receptors, kinases and transcription factors involved in cancer such as HER2 (Miller et al., 1994), EML4-ALK, mutant EGFR (Normant et al., 2011; Shimamura et al., 2005), mutant BRAF (Grbovic et al., 2006) and activated AKT (Solit et al., 2003). Inhibition of Hsp90 by small molecules leads to degradation of its client proteins and in the case of oncogenic targets, growth inhibition or cytotoxicity in the cells that are dependent on them.

Our lab has previously demonstrated that JAK2 is a highly sensitive Hsp90 client protein and treatment of MPN cells with Hsp90 inhibitors leads to degradation of JAK2 and inhibition of downstream signaling at clinically achievable doses. PU-H71, a novel purine-scaffold Hsp90 inhibitor, was highly efficacious in pre-clinical models of MPN and resulted in lineage-specific reduction of myeloproliferation and increased survival.

Although both mutant and wild type JAK2 are Hsp90 client proteins, PU-H71 is preferentially retained in mutant cells, thus making it a tumor-selective inhibitor (Marubayashi et al., 2010). Another Hsp90 inhibitor, AUY922, has been shown to overcome genetic resistance to JAK inhibitors in cell lines and xenograft models of JAK2 mutant B-ALL (Weigert et al., 2012a). AUY922 was also synergistic with the JAK inhibitor, TG101348, in inducing apoptosis in primary MPN cells. It was also effective against JAK inhibitor resistant cell lines (Fiskus et al., 2011). We found that the JAK inhibitor persistent cell lines remained sensitive to PU-H71 with IC_{50} values similar to the parental naïve cells. These findings also spurred us to test the efficacy of combination treatment with PU-H71 and ruxolitinib in pre-clinical models of MPN. We found that addition of an Hsp90 inhibitor to ruxolitinib either at the beginning or following JAK inhibitor therapy led to significant improvements in leukocytosis, splenomegaly and bone marrow fibrosis as compared to JAK inhibitor monotherapy, without adverse side effects. We also observed more potent inhibition of downstream STAT-MAPK signaling in splenocytes from mice receiving PU-H71/ruxolitinib combination treatment. Importantly, we demonstrated that persistent signaling observed in cells from ruxolitinib treated MPN patients, could be inhibited by treatment with PU-H71. Of note, PU-H71 treatment led to degradation of JAK1 in MPN cells, suggesting that this drug could interfere with formation of heterodimers associated with persistence in these cells. Taken together, these findings suggest that PU-H71 can overcome JAK inhibitor persistence in MPN cells and provide a compelling rationale for combining these inhibitors in clinical trials for MPN.

HDAC inhibitors

Several studies have reported higher expression and enzymatic activity of histone deacetylases (HDACs) in primary MPN cells, which correlates with the degree of splenomegaly in patients (Skov et al., 2012; Wang et al., 2008). In addition to regulating gene expression via the deacetylation of histone substrates, HDACs can influence the function of myriad other proteins in cells including a number of transcription factors such as p53 (Luo et al., 2000; Murphy et al., 1999), STAT3 (Yuan et al., 2005) and hormone receptors (Gaughan et al., 2002; Wang et al., 2001). Importantly, Hsp90 is also a substrate of HDAC6 and aberrant Hsp90 acetylation has been show to affect maturation of client proteins (Kovacs et al., 2005) (Bali et al., 2005). HDAC inhibitor treatment results in degradation of JAK2 and inhibition of growth and downstream signaling in MPN cells (Guerini et al., 2007; Wang et al., 2009). This effect might be due to disruption of the binding of JAK2 and HSP90 and through additional mechanisms relating to the pleiotropic role of HDAC proteins on gene expression and protein trafficking in MPN cells. In separate phase I/II trials with the HDAC inhibitors panobinostat or givinostat, MF patients experienced improvement in systematic symptoms and reduction in splenomegaly (DeAngelo et al., 2013; Finazzi et al., 2013; Mascarenhas et al., 2011; Rambaldi et al., 2010). Cotreatment of MPN cells with the JAK2 inhibitor TG101209 and panobinostat led to synergistic induction of apoptosis in MPN cells (Wang et al., 2009). Similarly, combination of panobinostat with ruxolitinib showed greater activity than either agent alone in a JAK2V617 murine model (Evrot et al., 2013). There is an ongoing Phase I trial assessing the efficacy of combined ruxolitinib and panobinostat treatment.

Based on our studies, we hypothesized that epigenetic regulation of JAK2 expression might also contribute to development of persistence since we observed an increase in activated chromatin marks at the *JAK2* locus in persistent cells. JAK2 expression was also increased at the protein and mRNA level. This might be due to a positive feedback loop in which activated JAK2 can promote its own expression by phosphorylating histone H3Y41 at the *JAK2* locus (Rui et al., 2010b) Previous work attributed persistence in EGFR inhibitor-insensitive 'drug-tolerant persisters' to an altered chromatin state, which made these cells highly sensitive to HDAC inhibitors (Sharma et al., 2010). In the future, we plan on evaluating whether HDAC inhibitors can overcome persistence by altering transcriptional regulation of JAK2 and other target genes.

Inhibition of STATs

Expression of JAK2V617F and MPLW515L results in constitutive activation of the STAT family of transcription factors, mainly STAT3 and STAT5, leading to increased expression of STAT target genes involved in survival and proliferation including *Bcl-xL*, *Ccnd1* and *Myc*. Recently, several groups have reported that STAT5 is indispensable for development of JAK2V617F-mediated transformation and disease development (Funakoshi-Tago et al., 2010; Walz et al., 2012; Yan et al., 2012). Deletion of both isoforms of STAT5, STATa and STAT5b, resulted in normalization of blood counts, spleen size and myeloid progenitor expansion. Further, loss of STAT5 also led to abrogation of Epo-independent erythroid colony formation, a hallmark feature of PV (Yan et al., 2012). Conversely, *in vivo* expression of a constitutively active isoform of

STAT5 leads to a lethal myeloproliferative phenotype (Kato et al., 2005). Unpublished work from our laboratory shows that genetic deletion of STAT3 in the MPLW515L model reduces blood counts and splenomegaly and improves survival. Importantly, we demonstrate that JAK inhibitor persistent cells do not engage an alternate pathway but in fact reactivate downstream STAT signaling via formation of heterodimers between JAK2 and JAK1/TYK2. Taken together, these results suggest that STAT5 plays a crucial role in MPN pathogenesis and represents an attractive target for therapeutic intervention.

Cell-based screening assays have led to the identification of several molecule inhibitors of STAT5 (Nelson et al., 2011a). One of these compounds, pimozide, decreases STAT5 phosphorylation and expression of STAT5 target genes as well as induces cell cycle arrest and apoptosis in BCR-ABL and JAK2V617F mutant MPN cells (Bar-Natan et al., 2012; Nelson et al., 2011b). Importantly, it remains effective in the presence of the T315I/Bcr-Abl mutation, which confers resistance to most available Abl kinase inhibitors (Nelson et al., 2011b). Other strategies to target the STATs include decoy oligonucleotides that compete the protein away from its target sequences (Sen et al., 2012), (Wang et al., 2011), small molecules that prevent DNA binding (Turkson et al., 2005) and inhibitors of STAT dimerization, which is essential for DNA binding (Page et al., 2012; Schust et al., 2006). Experiments evaluating the efficacy of these approaches in pre-clinical MPN models, particularly in JAK inhibitor persistent cells, are warranted.

ROLE OF CYTOKINES IN RESPONSE TO JAK INHIBITOR THERAPY

Since our data suggest that MPN cells can rapidly become insensitive to currently available JAK inhibitors, understanding exactly how patients benefit from these drugs

remains an outstanding question in the field. MPN patients have high levels of pro-inflammatory serum cytokines, which predict response to therapy and correlate with shortened survival (Tefferi et al., 2011; Vaidya et al., 2012). An important observation made by several groups is that JAK kinase inhibitor treatment in MF patients and in murine models is associated with reduction in the elevated levels of cytokines (Koppikar et al., 2010; Tyner et al., 2010; Verstovsek et al., 2012c). This suggests that some of the clinical benefits of JAK inhibitors might be due to suppression of systemic inflammation and not from complete inhibition of constitutive signaling in MPN cells.

Increasing evidence suggests that both autocrine/paracrine as well as stromal secretion of cytokines plays an important role in the development of the MPN phenotype. Fleischman and colleagues reported that JAK2V617F mutant cells have increased expression of inflammatory cytokine tumor necrosis factor alpha (TNFα). Exposure to this cytokine *in vitro* leads to preferential expansion of *JAK2V617F* mutant cells compared to normal cells in mice and in primary samples. Further, reconstitution of JAK2V617 in a TNFα null background resulted in amelioration of disease (Fleischman et al., 2011). Cytokines like hepatocyte growth factor (HGF) and interleukin-11 (IL-11) were found to be overexpressed in PV patients and contributed to growth of erythroid colonies, although their effect was independent of JAK2V617F expression (Boissinot et al., 2011). Another study showed that JAK2 mutant myeloid cells have increased secretion of oncostatin M (OSM), which can stimulate production of angiogenic cytokines by fibroblasts and endothelial cells in the stroma (Hoermann et al., 2012). Ectopic overexpression of thrombopoietin in hematopoietic cells is sufficient to induce myeloproliferation and bone

marrow fibrosis in mice (Kakumitsu et al., 2005; Villeval et al., 1997; Yan et al., 1996). Recent studies have also shown that cytokines including interleukin-6 (IL-6), fibroblast growth factor (FGF) and chemokine C-X-C-motif ligand 10 (CXCL-10)/IFN-γ-inducible 10-kD protein (IP-10) can promote survival of JAK2V617F mutant cells. Using a coculture platform, this group demonstrated that these cytokines were secreted by bone marrow-derived stromal cells. Further, these cytokines were able to provide a significant survival advantage to MPN cells in response to JAK inhibitor treatment (Manshouri et al., 2011). Taken together, these studies suggest that cytokines secreted by both mutant and stromal cells play an important role in MPN pathogenesis and their pleiotropic effects must be considered when assessing the response to JAK inhibitors and other MPN therapies.

Since clinically available JAK inhibitors are not mutant specific, they might be inhibiting cytokine signaling in normal cells with wild type JAK2, which might also contribute to their efficacy. This hypothesis can be investigated by using inhibitor resistant JAK2 alleles, such as the *Y931C* mutation reported by Hornakova et al. (Hornakova et al., 2011) and others. Testing the efficacy of JAK inhibitors in murine bone marrow transplant models where either diseased or non-mutant cells coexpress an inhibitor resistance allele will allow us to distinguish between JAK2 inhibition in mutant versus normal cells. Further, this hypothesis might also explain the improved efficacy of JAK and Hsp90 inhibitor combination treatment where ruxolitinib might be inhibiting pro-inflammatory cytokines in the non-mutant stromal cells and PU-H71 might be providing mutant-specific inhibition of JAK2.

JAK1 is also an important mediator of signaling by several pro-inflammatory cytokines such as the interferons and IL-6 (Rodig et al., 1998). Although ruxolitinib can also efficiently inhibit JAK1 in cell-free assays, our data suggest that JAK1 forms a heterodimer with JAK2, which is insensitive to inhibition by ruxolitinib. However, further structural studies of the JAK1/JAK2 heterodimers will be required to fully understand this mechanism. Interestingly, SAR302503, a more specific JAK2 inhibitor, does not lead to a significant reduction in serum cytokines indicating that JAK1 might be the main mediator of cytokine signaling in MPN (Pardanani et al., 2011a). Conditional knockout murine models of JAK1 will also be useful in elucidating the contribution of JAK1 to disease phenotype and to the response to inhibitor therapy.

MAJOR HURDLES IN JAK INHIBITOR MONOTHERAPY

Our experiments with the genetic deletion of JAK2 in a murine model of ET/MF demonstrated the critical role of JAK2 in pathogenesis and phenotype of MPN. Thus, targeting JAK2 remains a viable option in the treatment of these diseases. However, the JAK-STAT pathway is a crucial regulator of hematopoiesis and JAK2 is the major kinase required for erythropoietin receptor signaling and normal red blood development (Neubauer et al., 1998; Parganas et al., 1998). Our studies demonstrate that JAK2 plays an important role in myelopoiesis and maintenance of the stem cell compartment in adult hematopoietic tissues. The JAK inhibitors in current clinical development are not specific for mutant JAK2 and can also efficiently inhibit wild type JAK2. Therefore, using doses that are capable of inhibiting mutant JAK2 activity is bound to also have adverse effects on normal hematopoiesis. This has been borne out in the clinic, where

JAK2 inhibitors have been associated with dose limiting toxicities including anemia and thrombocytopenia (Verstovsek et al., 2010). Thus, the limited efficacy of JAK inhibitors might also be due to insufficient inhibition of the pathway at clinically tolerable doses. However, the crystal structure of the wild type and *JAK2V617F* mutant pseudokinase domain was recently resolved. This should help inform rational drug design of molecules that can selectively inhibit mutant JAK2, thereby sparing normal hematopoiesis. The JAK2V617F mutation is particularly amenable to mutant-specific targeting since it occurs in a vast majority of patients with MPN.

There are some evidence from mouse models and from MPN patients that expression of JAK2V617 in hematopoietic stem cells does not lead to significant expansion of this compartment, but rather to increased proliferation of more differentiated myeloid and erythroid lineages (Anand et al., 2011; Li et al., 2010). However, several studies have shown that long-term hematopoietic stem cells are the disease-initiating cells in mice and in MPN patients (James et al., 2008; Mullally et al., 2012). Also, the J*AK2V617F* mutation has been shown to be a late genetic event in a subset of MPN patients (Kralovics et al., 2006) and mutations in other genes such as *TET2*, *ASXL1* and *EZH2* might be responsible for the early clonal expansion associated with these diseases (Vainchenker et al., 2011). Taken together, these findings suggest that JAK2 monotherapy might not be sufficient to eradicate the disease initiating cells in at least a subset of MPN patients.

TARGETING ALTERNATE PATHWAYS IN MPN

Pre-clinical and clinical data suggest that currently available JAK inhibitors have a limited therapeutic window and will likely not be curative for MPN. Therefore, there is a need to identify additional pathways that might be involved in the development and maintenance of the MPN mutant clone, which could then be targeted in combination with JAK2 for improved therapeutic benefit for MPN patients. In addition to enhanced JAK-STAT signaling, MPN cells display activation of other oncogenic pathways including MAPK and mTOR/PI3K signaling. In a phase I/II trial evaluating the efficacy of the mTOR inhibitor, everolimus, 60% of MF patients experienced improvement in constitutional symptoms and decrease in spleen enlargement albeit to a lesser degree than observed with JAK inhibitors. However, it did not lead to a decrease in mutant allele burden or significant changes in the cytokine profile of these patients (Guglielmelli et al., 2011). Cotreatment with an mTOR and JAK inhibitor had synergistic activity against JAK2 mutant cell lines and reduced Epo-independent colony formation of cells from PV patients (Bogani et al., 2013). In another study, treatment of cultured as well as primary MPN cells with the dual PI3K/mTOR inhibitor BEZ235 combined with the JAK inhibitor SAR302503 had synergistic effects on induction of apoptosis and inhibition of colony growth in cultured and primary MPN cells as compared to normal CD34+ cells (Fiskus et al., 2013). BEZ235 was also effective against a cell line that had been made resistant to TG101209 (Fiskus et al., 2013). Similar results were also reported with the combination of JAK2 inhibitors with a MEK inhibitor, AZD6244 (Fiskus, 2010; Suryani, 2012). Treatment with an allosteric Akt inhibitor, MK-2206 led to cell growth and induction of apoptosis in MPN cell lines, along with decreased colony formation in primary MF cells.

It was also efficacious in pre-clinical murine models, resulting in decreased organomegaly and megakaryocyte burden (Khan et al., 2013)

Recently, developmental pathways such as Hedgehog, Wnt and Notch have been shown to play a role in development of myeloid malignancies and remain an active area of research as possible therapeutic targets. Targeting β-catenin by either genetic deletion or pharmacological inhibition in combination with imatinib treatment abrogates disease-initiating cells in a Bcr-Abl model of CML (Heidel et al., 2012). The Hedgehog (Hh) pathway is also reported to play an important role in the maintenance of the stem cell compartment in CML. (Dierks et al., 2008; Zhao et al., 2009a). Combination treatment with a hedgehog inhibitor vismodegib and ABL kinase inhibitor, ponatinib was found to be efficacious in xenograft models of therapy-resistant BCR-ABL positive leukemia (Katagiri et al., 2013). Preliminary data from our lab indicates that this pathway is activated in the *MPLW515L* murine BMT model of ET/MF as well as in primary MPN samples. Importantly, in pre-clinical models, combination treatment with a Smoothened inhibitor, LDE-225 and ruxolitinib was more effective at reducing blood counts and bone marrow fibrosis as compared to ruxolitinib alone. In Phase I trials of the Hh inhibitor PF-04449913 in hematological malignancies including MPN, 4/5 MF patients attained stable disease while 1 experienced a clinical response including a reduction in spleen size (Jamieson, 2011). There are several early stage clinical trials testing the efficacy of combination therapies with JAK and Hh inhibitors in myelofibrosis, which might provide additional benefits for patients.

THE FUTURE OF MPN THERAPY

Although JAK kinase inhibitors have been approved for treatment of MPN, current agents are not effective at reducing the size of the MPN clone and therefore do not offer the potential for long term remissions or cure. MPN cells can rapidly develop persistent to chronic kinase inhibition but remain dependent on expression of JAK2 for growth and survival. Agents that lead to JAK2 degradation (Hsp90 inhibitors or histone deacetylase inhibitors), inhibition of downstream targets (STAT inhibitors) or that retain the ability to inhibit JAK2 in persistent cells (type II JAK inhibitors) have the potential to improve therapeutic efficacy in patients with MPN. However, since JAK2 plays a critical role in normal hematopoiesis, the development of mutant-specific JAK inhibitors that will spare normal cells might offer a viable therapeutic option to increase efficacy and therapeutic window. Targeting other proliferative and pro-survival pathways such as MAPK and Akt/PI3K/mTOR, which are activated in MPN cells, might also be beneficial. Since JAK inhibitors by themselves do not seem to eliminate disease-initiating MPN cells, drugs targeting developmental pathways that are involved in the maintenance of the stem cell compartment should also be evaluated. Finally, it would also be desirable to inhibit cytokine signaling by targeting JAK1 and other JAK kinases in normal and mutant cells to reduce systemic inflammation and constitutional symptoms associated with MPN, particularly MF. In conclusion, findings from our lab and others suggest that a combination treatment regimen that targets all these aspects of disease will likely be most beneficial and potentially curative for patients with MPN.

BIBLIOGRAPHY

Akada, H., Yan, D., Zou, H., Fiering, S., Hutchison, R.E., and Mohi, M.G. (2010). Conditional expression of heterozygous or homozygous Jak2V617F from its endogenous promoter induces a polycythemia vera-like disease. Blood *115*, 3589-3597.

Anand, S., Stedham, F., Gudgin, E., Campbell, P., Beer, P., Green, A.R., and Huntly, B.J. (2011). Increased basal intracellular signaling patterns do not correlate with JAK2 genotype in human myeloproliferative neoplasms. Blood *118*, 1610-1621.

Andraos, R., Qian, Z., Bonenfant, D., Rubert, J., Vangrevelinghe, E., Scheufler, C., Marque, F., Regnier, C.H., De Pover, A., Ryckelynck, H.*, et al.* (2012). Modulation of activation-loop phosphorylation by JAK inhibitors is binding mode dependent. Cancer discovery *2*, 512-523.

Aranaz, P., Hurtado, C., Erquiaga, I., Miguéliz, I., Ormazábal, C., Cristobal, I., García-Delgado, M., Novo, F.J., and Vizmanos, J.L. (2012). CBL mutations in myeloproliferative neoplasms are also found in the gene's proline-rich domain and in patients with the V617FJAK2. Haematologica *97*, 1234-1241.

Azam, M., Latek, R.R., and Daley, G.Q. (2003). Mechanisms of autoinhibition and STI-571/imatinib resistance revealed by mutagenesis of BCR-ABL. Cell *112*, 831-843.

Bali, P., Pranpat, M., Bradner, J., Balasis, M., Fiskus, W., Guo, F., Rocha, K., Kumaraswamy, S., Boyapalle, S., Atadja, P.*, et al.* (2005). Inhibition of histone deacetylase 6 acetylates and disrupts the chaperone function of heat shock protein 90: a novel basis for antileukemia activity of histone deacetylase inhibitors. The Journal of biological chemistry *280*, 26729-26734.

Ballen, K.K., Shrestha, S., Sobocinski, K.A., Zhang, M.J., Bashey, A., Bolwell, B.J., Cervantes, F., Devine, S.M., Gale, R.P., Gupta, V.*, et al.* (2010). Outcome of transplantation for myelofibrosis. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation *16*, 358-367.

Bandaranayake, R.M., Ungureanu, D., Shan, Y., Shaw, D.E., Silvennoinen, O., and Hubbard, S.R. (2012). Crystal structures of the JAK2 pseudokinase domain and the pathogenic mutant V617F. Nature structural & molecular biology *19*, 754-759.

Bar-Natan, M., Nelson, E.A., Walker, S.R., Kuang, Y., Distel, R.J., and Frank, D.A. (2012). Dual inhibition of Jak2 and STAT5 enhances killing of myeloproliferative neoplasia cells. Leukemia *26*, 1407-1410.

Baxter, E.J., Scott, L.M., Campbell, P.J., East, C., Fourouclas, N., Swanton, S., Vassiliou, G.S., Bench, A.J., Boyd, E.M., Curtin, N.*, et al.* (2005). Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. The Lancet *365*, 1054-1061.

Bercovich, D., Ganmore, I., Scott, L.M., Wainreb, G., Birger, Y., Elimelech, A., Shochat, C., Cazzaniga, G., Biondi, A., Basso, G.*, et al.* (2008). Mutations of JAK2 in acute lymphoblastic leukaemias associated with Down's syndrome. Lancet *372*, 1484-1492.

Bogani, C., Bartalucci, N., Martinelli, S., Tozzi, L., Guglielmelli, P., Bosi, A., and Vannucchi, A.M. (2013). mTOR inhibitors alone and in combination with JAK2 inhibitors effectively inhibit cells of myeloproliferative neoplasms. PloS one *8*, e54826.

Boissinot, M., Cleyrat, C., Vilaine, M., Jacques, Y., Corre, I., and Hermouet, S. (2011). Anti-inflammatory cytokines hepatocyte growth factor and interleukin-11 are over-expressed in Polycythemia vera and contribute to the growth of clonal erythroblasts independently of JAK2V617F. Oncogene *30*, 990-1001.

Bumm, T.G.P., Elsea, C., Corbin, A.S., Loriaux, M., Sherbenou, D., Wood, L., Deininger, J., Silver, R.T., Druker, B.J., and Deininger, M.W.N. (2006). Characterization of Murine JAK2V617F-Positive Myeloproliferative Disease. Cancer Res *66*, 11156-11165.

Dameshek, W. (1951). Some speculations on the myeloproliferative syndromes. Blood *6*, 372-375.

Dawson, M.A., Bannister, A.J., Gottgens, B., Foster, S.D., Bartke, T., Green, A.R., and Kouzarides, T. (2009). JAK2 phosphorylates histone H3Y41 and excludes HP1alpha from chromatin. Nature *461*, 819-822.

DeAngelo, D.J., Mesa, R.A., Fiskus, W., Tefferi, A., Paley, C., Wadleigh, M., Ritchie, E.K., Snyder, D.S., Begna, K., Ganguly, S.*, et al.* (2013). Phase II trial of panobinostat, an oral pan-deacetylase inhibitor in patients with primary myelofibrosis, post-essential thrombocythaemia, and post-polycythaemia vera myelofibrosis. British journal of haematology *162*, 326-335.

Deshpande, A., Reddy, M.M., Schade, G.O., Ray, A., Chowdary, T.K., Griffin, J.D., and Sattler, M. (2012). Kinase domain mutations confer resistance to novel inhibitors targeting JAK2V617F in myeloproliferative neoplasms. Leukemia *26*, 708-715.

Dierks, C., Beigi, R., Guo, G.-R., Zirlik, K., Stegert, M.R., Manley, P., Trussell, C., Schmitt-Graeff, A., Landwerlin, K., Veelken, H.*, et al.* (2008). Expansion of Bcr-Abl-Positive Leukemic Stem Cells Is Dependent on Hedgehog Pathway Activation. Cancer Cell *14*, 238-249.

Engelman, J.A., Zejnullahu, K., Mitsudomi, T., Song, Y., Hyland, C., Park, J.O., Lindeman, N., Gale, C.M., Zhao, X., Christensen, J.*, et al.* (2007). MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science *316*, 1039-1043.

Evrot, E., Ebel, N., Romanet, V., Roelli, C., Andraos, R., Qian, Z., Doelemeyer, A., Dammassa, E., Sterker, D., Cozens, R.*, et al.* (2013). JAK1/2 and pan-deacetylase inhibitor combination therapy yields improved efficacy in preclinical mouse models of JAK2V617F-driven disease. Clinical cancer research : an official journal of the American Association for Cancer Research.

Finazzi, G., Vannucchi, A.M., Martinelli, V., Ruggeri, M., Nobile, F., Specchia, G., Pogliani, E.M., Olimpieri, O.M., Fioritoni, G., Musolino, C.*, et al.* (2013). A phase II study of Givinostat in combination with hydroxycarbamide in patients with polycythaemia vera unresponsive to hydroxycarbamide monotherapy. British journal of haematology *161*, 688-694.

Fiskus, W., Manepalli, Rekha Rao, Balusu, Ramesh, Bhalla, Kapil N. (2010). Synergistic Activity of Combinations of JAK2 Kinase Inhibitor with PI3K/mTOR, MEK or PIM Kinase Inhibitor Against Human Myeloproliferative Neoplasm Cells Expressing JAK2V617F. Blood *ASH Annual Meeting Abstracts 2010 116: 798*

Fiskus, W., Verstovsek, S., Manshouri, T., Rao, R., Balusu, R., Venkannagari, S., Rao, N.N., Ha, K., Smith, J.E., Hembruff, S.L.*, et al.* (2011). Heat shock protein 90 inhibitor is synergistic with JAK2 inhibitor and overcomes resistance to JAK2-TKI in human myeloproliferative neoplasm cells. Clinical cancer research : an official journal of the American Association for Cancer Research *17*, 7347-7358.

Fiskus, W., Verstovsek, S., Manshouri, T., Smith, J.E., Peth, K., Abhyankar, S., McGuirk, J., and Bhalla, K.N. (2013). Dual PI3K/AKT/mTOR inhibitor BEZ235 synergistically enhances the activity of JAK2 inhibitor against cultured and primary human myeloproliferative neoplasm cells. Molecular Cancer Therapeutics.

Fleischman, A.G., Aichberger, K.J., Luty, S.B., Bumm, T.G., Petersen, C.L., Doratotaj, S., Vasudevan, K.B., LaTocha, D.H., Yang, F., Press, R.D., *et al.* (2011). TNFalpha facilitates clonal expansion of JAK2V617F positive cells in myeloproliferative neoplasms. Blood *118*, 6392-6398.

Flex, E., Petrangeli, V., Stella, L., Chiaretti, S., Hornakova, T., Knoops, L., Ariola, C., Fodale, V., Clappier, E., Paoloni, F., *et al.* (2008). Somatically acquired JAK1 mutations in adult acute lymphoblastic leukemia. The Journal of experimental medicine *205*, 751-758.

Funakoshi-Tago, M., Tago, K., Abe, M., Sonoda, Y., and Kasahara, T. (2010). STAT5 activation is critical for the transformation mediated by myeloproliferative disorder-associated JAK2 V617F mutant. The Journal of biological chemistry *285*, 5296-5307.

Gaughan, L., Logan, I.R., Cook, S., Neal, D.E., and Robson, C.N. (2002). Tip60 and histone deacetylase 1 regulate androgen receptor activity through changes to the acetylation status of the receptor. The Journal of biological chemistry *277*, 25904-25913.

Gorre, M.E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P.N., and Sawyers, C.L. (2001). Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science *293*, 876-880.

Grand, F.H., Hidalgo-Curtis, C.E., Ernst, T., Zoi, K., Zoi, C., McGuire, C., Kreil, S., Jones, A., Score, J., Metzgeroth, G., *et al.* (2009). Frequent CBL mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. Blood *113*, 6182-6192.

Grbovic, O.M., Basso, A.D., Sawai, A., Ye, Q., Friedlander, P., Solit, D., and Rosen, N. (2006). V600E B-Raf requires the Hsp90 chaperone for stability and is degraded in response to Hsp90 inhibitors. Proceedings of the National Academy of Sciences of the United States of America *103*, 57-62.

Guerini, V., Barbui, V., Spinelli, O., Salvi, A., Dellacasa, C., Carobbio, A., Introna, M., Barbui, T., Golay, J., and Rambaldi, A. (2007). The histone deacetylase inhibitor ITF2357 selectively targets cells bearing mutated JAK2V617F. Leukemia *22*, 740-747.

Guglielmelli, P., Barosi, G., Rambaldi, A., Marchioli, R., Masciulli, A., Tozzi, L., Biamonte, F., Bartalucci, N., Gattoni, E., Lupo, M.L., *et al.* (2011). Safety and efficacy of everolimus, a mTOR inhibitor, as single agent in a phase 1/2 study in patients with myelofibrosis. Blood *118*, 2069-2076.

Harrison, C., Kiladjian, J.J., Al-Ali, H.K., Gisslinger, H., Waltzman, R., Stalbovskaya, V., McQuitty, M., Hunter, D.S., Levy, R., Knoops, L.*, et al.* (2012). JAK inhibition with ruxolitinib versus best available therapy for myelofibrosis. The New England journal of medicine *366*, 787-798.

Harvey, R.C., Mullighan, C.G., Chen, I.M., Wharton, W., Mikhail, F.M., Carroll, A.J., Kang, H., Liu, W., Dobbin, K.K., Smith, M.A.*, et al.* (2010). Rearrangement of CRLF2 is associated with mutation of JAK kinases, alteration of IKZF1, Hispanic/Latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. Blood *115*, 5312-5321.

Heidel, F.H., Bullinger, L., Feng, Z., Wang, Z., Neff, T.A., Stein, L., Kalaitzidis, D., Lane, S.W., and Armstrong, S.A. (2012). Genetic and pharmacologic inhibition of beta-catenin targets imatinib-resistant leukemia stem cells in CML. Cell Stem Cell *10*, 412-424.

Hoermann, G., Cerny-Reiterer, S., Herrmann, H., Blatt, K., Bilban, M., Gisslinger, H., Gisslinger, B., Mullauer, L., Kralovics, R., Mannhalter, C.*, et al.* (2012). Identification of oncostatin M as a JAK2 V617F-dependent amplifier of cytokine production and bone marrow remodeling in myeloproliferative neoplasms. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *26*, 894-906.

Hornakova, T., Springuel, L., Devreux, J., Dusa, A., Constantinescu, S.N., Knoops, L., and Renauld, J.-C. (2011). Oncogenic JAK1 and JAK2-activating mutations resistant to ATP-competitive inhibitors. Haematologica *96*, 845-853.

Ihle, J.N., and Gilliland, D.G. (2007). Jak2: normal function and role in hematopoietic disorders. Current Opinion in Genetics & Development *17*, 8-14.

James, C., Mazurier, F., Dupont, S., Chaligne, R., Lamrissi-Garcia, I., Tulliez, M., Lippert, E., Mahon, F.-X., Pasquet, J.-M., Etienne, G.*, et al.* (2008). The hematopoietic stem cell compartment of JAK2V617F-positive myeloproliferative disorders is a reflection of disease heterogeneity. Blood *112*, 2429-2438.

James, C., Ugo, V., Le Couedic, J.-P., Staerk, J., Delhommeau, F., Lacout, C., Garcon, L., Raslova, H., Berger, R., Bennaceur-Griscelli, A.*, et al.* (2005). A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature *434*, 1144-1148.

Jamieson, C., Cortes, Jorge E., Oehler, Vivian, Baccarani, Michele, Kantarjian, Hagop M., Papayannidis, Cristina, Rice, Kristen N., Zhang, Xiaoxi, Shaik, Naveed, Courtney, Rachel, Levin, Wendy J., Martinelli, Giovanni (2011). Phase 1 Dose-Escalation Study of PF-04449913, An Oral Hedgehog (Hh) Inhibitor, in Patients with Select Hematologic Malignancies. Blood *ASH Annual Meeting Abstracts 2011 118: 424*

Jamieson, C.H.M., Gotlib, J., Durocher, J.A., Chao, M.P., Mariappan, M.R., Lay, M., Jones, C., Zehnder, J.L., Lilleberg, S.L., and Weissman, I.L. (2006). The JAK2 V617F mutation occurs in hematopoietic stem cells in polycythemia vera and predisposes toward erythroid differentiation. *103*, 6224-6229.

Johannessen, C.M., Boehm, J.S., Kim, S.Y., Thomas, S.R., Wardwell, L., Johnson, L.A., Emery, C.M., Stransky, N., Cogdill, A.P., Barretina, J*., et al.* (2010). COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. Nature *468*, 968-972.

Joos, S., Küpper, M., Ohl, S., von Bonin, F., Mechtersheimer, G., Bentz, M., Marynen, P., Möller, P., Pfreundschuh, M., Trümper, L*., et al.* (2000). Genomic Imbalances Including Amplification of the Tyrosine Kinase Gene JAK2 in CD30+ Hodgkin Cells. Cancer Research *60*, 549-552.

Kakumitsu, H., Kamezaki, K., Shimoda, K., Karube, K., Haro, T., Numata, A., Shide, K., Matsuda, T., Oshima, K., and Harada, M. (2005). Transgenic mice overexpressing murine thrombopoietin develop myelofibrosis and osteosclerosis. Leukemia research *29*, 761-769.

Kalota, A., Jeschke, G.R., Carroll, M., and Hexner, E.O. (2013). Intrinsic resistance to JAK2 inhibition in myelofibrosis. Clinical cancer research : an official journal of the American Association for Cancer Research *19*, 1729-1739.

Karaghiosoff, M., Neubauer, H., Lassnig, C., Kovarik, P., Schindler, H., Pircher, H., McCoy, B., Bogdan, C., Decker, T., Brem, G*., et al.* (2000). Partial impairment of cytokine responses in Tyk2-deficient mice. Immunity *13*, 549-560.

Katagiri, S., Tauchi, T., Okabe, S., Minami, Y., Kimura, S., Maekawa, T., Naoe, T., and Ohyashiki, K. (2013). Combination of ponatinib with Hedgehog antagonist vismodegib for therapy-resistant BCR-ABL1-positive leukemia. Clinical cancer research : an official journal of the American Association for Cancer Research *19*, 1422-1432.

Kato, Y., Iwama, A., Tadokoro, Y., Shimoda, K., Minoguchi, M., Akira, S., Tanaka, M., Miyajima, A., Kitamura, T., and Nakauchi, H. (2005). Selective activation of STAT5

unveils its role in stem cell self-renewal in normal and leukemic hematopoiesis. The Journal of experimental medicine *202*, 169-179.

Kawamura, M., McVicar, D.W., Johnston, J.A., Blake, T.B., Chen, Y.Q., Lal, B.K., Lloyd, A.R., Kelvin, D.J., Staples, J.E., and Ortaldo, J.R. (1994). Molecular cloning of L-JAK, a Janus family protein-tyrosine kinase expressed in natural killer cells and activated leukocytes. Proceedings of the National Academy of Sciences of the United States of America *91*, 6374-6378.

Kearney, L., Gonzalez De Castro, D., Yeung, J., Procter, J., Horsley, S.W., Eguchi-Ishimae, M., Bateman, C.M., Anderson, K., Chaplin, T., Young, B.D.*, et al.* (2009). Specific JAK2 mutation (JAK2R683) and multiple gene deletions in Down syndrome acute lymphoblastic leukemia. Blood *113*, 646-648.

Khan, I., Huang, Z., Wen, Q., Stankiewicz, M.J., Gilles, L., Goldenson, B., Schultz, R., Diebold, L., Gurbuxani, S., Finke, C.M.*, et al.* (2013). AKT is a therapeutic target in myeloproliferative neoplasms. Leukemia *27*, 1882-1890.

Khn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995). Inducible gene targeting in mice. Science *269*, 1427-1429.

Kobayashi, S., Boggon, T.J., Dayaram, T., Janne, P.A., Kocher, O., Meyerson, M., Johnson, B.E., Eck, M.J., Tenen, D.G., and Halmos, B. (2005). EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. The New England journal of medicine *352*, 786-792.

Koppikar, P., Abdel-Wahab, O., Hedvat, C., Marubayashi, S., Patel, J., Goel, A., Kucine, N., Gardner, J.R., Combs, A.P., Vaddi, K.*, et al.* (2010). Efficacy of the JAK2 inhibitor INCB16562 in a murine model of MPLW515L-induced thrombocytosis and myelofibrosis. Blood *115*, 2919-2927.

Koppikar, P., Bhagwat, N., Kilpivaara, O., Manshouri, T., Adli, M., Hricik, T., Liu, F., Saunders, L.M., Mullally, A., Abdel-Wahab, O.*, et al.* (2012). Heterodimeric JAK-STAT activation as a mechanism of persistence to JAK2 inhibitor therapy. Nature *489*, 155-159.

Kovacs, J.J., Murphy, P.J., Gaillard, S., Zhao, X., Wu, J.T., Nicchitta, C.V., Yoshida, M., Toft, D.O., Pratt, W.B., and Yao, T.P. (2005). HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. Molecular cell *18*, 601-607.

Kralovics, R., Passamonti, F., Buser, A.S., Teo, S.-S., Tiedt, R., Passweg, J.R., Tichelli, A., Cazzola, M., and Skoda, R.C. (2005). A Gain-of-Function Mutation of JAK2 in Myeloproliferative Disorders. The New England journal of medicine *352*, 1779-1790.

Kralovics, R., Teo, S.-S., Li, S., Theocharides, A., Buser, A.S., Tichelli, A., and Skoda, R.C. (2006). Acquisition of the V617F mutation of JAK2 is a late genetic event in a subset of patients with myeloproliferative disorders. Blood *108*, 1377-1380.

Lacout, C., Pisani, D.F., Tulliez, M., Gachelin, F.M., Vainchenker, W., and Villeval, J.-L. (2006). JAK2V617F expression in murine hematopoietic cells leads to MPD mimicking human PV with secondary myelofibrosis. Blood *108*, 1652-1660.

Lacronique, V., Boureux, A., Della Valle, V., Poirel, H., Quang, C.T., Mauchauffé, M., Berthou, C., Lessard, M., Berger, R., Ghysdael, J.*, et al.* (1997). A TEL-JAK2 Fusion Protein with Constitutive Kinase Activity in Human Leukemia. Science *278*, 1309-1312.

Lasho, T.L., Tefferi, A., Hood, J.D., Verstovsek, S., Gilliland, D.G., and Pardanani, A. (2008). TG101348, a JAK2-selective antagonist, inhibits primary hematopoietic cells derived from myeloproliferative disorder patients with JAK2V617F, MPLW515K or JAK2 exon 12 mutations as well as mutation negative patients. Leukemia, published online.

Leonard, W.J., and O'Shea, J.J. (1998). JAKS AND STATS: Biological Implications*. Annual Review of Immunology *16*, 293-322.

Levine, R.L., Loriaux, M., Huntly, B.J., Loh, M.L., Beran, M., Stoffregen, E., Berger, R., Clark, J.J., Willis, S.G., Nguyen, K.T.*, et al.* (2005a). The JAK2V617F activating mutation occurs in chronic myelomonocytic leukemia and acute myeloid leukemia, but not in acute lymphoblastic leukemia or chronic lymphocytic leukemia. Blood *106*, 3377-3379.

Levine, R.L., Pardanani, A., Tefferi, A., and Gilliland, D.G. (2007). Role of JAK2 in the pathogenesis and therapy of myeloproliferative disorders. Nature reviews Cancer *7*, 673-683.

Levine, R.L., Wadleigh, M., Cools, J., Ebert, B.L., Wernig, G., Huntly, B.J.P., Boggon, T.J., Wlodarska, I., Clark, J.J., Moore, S.*, et al.* (2005b). Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell *7*, 387-397.

Li, J., Spensberger, D., Ahn, J.S., Anand, S., Beer, P.A., Ghevaert, C., Chen, E., Forrai, A., Scott, L.M., Ferreira, R.*, et al.* (2010). JAK2 V617F impairs hematopoietic stem cell function in a conditional knock-in mouse model of JAK2 V617F–positive essential thrombocythemia. Blood *116*, 1528-1538.

Liu, F., Zhao, X., Perna, F., Wang, L., Koppikar, P., Abdel-Wahab, O., Harr, M.W., Levine, R.L., Xu, H., Tefferi, A.*, et al.* (2011). JAK2V617F-mediated phosphorylation of PRMT5 downregulates its methyltransferase activity and promotes myeloproliferation. Cancer Cell *19*, 283-294.

Lu, X., Huang, L.J.-S., and Lodish, H.F. (2008). Dimerization by a Cytokine Receptor Is Necessary for Constitutive Activation of JAK2V617F. Journal of Biological Chemistry *283*, 5258-5266.

Lucet, I.S., Fantino, E., Styles, M., Bamert, R., Patel, O., Broughton, S.E., Walter, M., Burns, C.J., Treutlein, H., Wilks, A.F.*, et al.* (2006). The structural basis of Janus kinase 2 inhibition by a potent and specific pan-Janus kinase inhibitor. Blood *107*, 176-183.

Luo, J., Su, F., Chen, D., Shiloh, A., and Gu, W. (2000). Deacetylation of p53 modulates its effect on cell growth and apoptosis. Nature *408*, 377-381.

Manshouri, T., Estrov, Z., Quintas-Cardama, A., Burger, J., Zhang, Y., Livun, A., Knez, L., Harris, D., Creighton, C.J., Kantarjian, H.M.*, et al.* (2011). Bone marrow stroma-secreted cytokines protect JAK2(V617F)-mutated cells from the effects of a JAK2 inhibitor. Cancer Res *71*, 3831-3840.

Marit, M.R., Chohan, M., Matthew, N., Huang, K., Kuntz, D.A., Rose, D.R., and Barber, D.L. (2012). Random mutagenesis reveals residues of JAK2 critical in evading inhibition by a tyrosine kinase inhibitor. PloS one *7*, e43437.

Marty, C., Lacout, C., Martin, A., Hasan, S., Jacquot, S., Birling, M.-C., Vainchenker, W., and Villeval, J.-L. (2010). Myeloproliferative neoplasm induced by constitutive expression of JAK2V617F in knock-in mice. Blood *116*, 783-787.

Marubayashi, S., Koppikar, P., Taldone, T., Abdel-Wahab, O., West, N., Bhagwat, N., Caldas-Lopes, E., Ross, K.N., xF, nen, M.*, et al.* (2010). HSP90 is a therapeutic target in JAK2-dependent myeloproliferative neoplasms in mice and humans. The Journal of Clinical Investigation *120*, 3578-3593.

Mascarenhas, J., Mercado, A., Rodriguez, A., Lu, M., Kalvin, C., Li, X., Petersen, B., Najfeld, V., Goldberg, J.D., and Hoffman, R. (2011). Prolonged Low Dose Therapy with a Pan-Deacetylase Inhibtor, Panobinostat (LBH589), in Patients with Myelofibrosis. ASH Annual Meeting Abstracts *118*, 794-.

Mehta, J., Wang, H., Iqbal, S.U., and Mesa, R. (2013). Epidemiology of myeloproliferative neoplasms in the United States. Leukemia & lymphoma.

Mercher, T., Wernig, G., Moore, S.A., Levine, R.L., Gu, T.L., Frohling, S., Cullen, D., Polakiewicz, R.D., Bernard, O.A., Boggon, T.J.*, et al.* (2006). JAK2T875N is a novel activating mutation that results in myeloproliferative disease with features of megakaryoblastic leukemia in a murine bone marrow transplantation model. Blood *108*, 2770-2779.

Mesa, R.A., Li, C.Y., Ketterling, R.P., Schroeder, G.S., Knudson, R.A., and Tefferi, A. (2005). Leukemic transformation in myelofibrosis with myeloid metaplasia: a single-institution experience with 91 cases. Blood *105*, 973-977.

Miller, P., Schnur, R.C., Barbacci, E., Moyer, M.P., and Moyer, J.D. (1994). Binding of benzoquinoid ansamycins to p100 correlates with their ability to deplete the erbB2 gene product p185. Biochemical and biophysical research communications *201*, 1313-1319.

Mullally, A., Lane, S.W., Ball, B., Megerdichian, C., Okabe, R., Al-Shahrour, F., Paktinat, M., Haydu, J.E., Housman, E., Lord, A.M.*, et al.* (2010). Physiological Jak2V617F Expression Causes a Lethal Myeloproliferative Neoplasm with Differential Effects on Hematopoietic Stem and Progenitor Cells. Cancer Cell *17*, 584-596.

Mullally, A., Poveromo, L., Schneider, R.K., Al-Shahrour, F., Lane, S.W., and Ebert, B.L. (2012). Distinct roles for long-term hematopoietic stem cells and erythroid precursor cells in a murine model of Jak2V617F-mediated polycythemia vera. Blood *120*, 166-172.

Mullighan, C.G., Zhang, J., Harvey, R.C., Collins-Underwood, J.R., Schulman, B.A., Phillips, L.A., Tasian, S.K., Loh, M.L., Su, X., Liu, W.*, et al.* (2009). JAK mutations in high-risk childhood acute lymphoblastic leukemia. Proceedings of the National Academy of Sciences *106*, 9414-9418.

Murphy, M., Ahn, J., Walker, K.K., Hoffman, W.H., Evans, R.M., Levine, A.J., and George, D.L. (1999). Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3a. Genes & development *13*, 2490-2501.

Neckers, L., and Workman, P. (2012). Hsp90 Molecular Chaperone Inhibitors: Are We There Yet? Clinical Cancer Research *18*, 64-76.

Nelson, E.A., Sharma, S.V., Settleman, J., and Frank, D.A. (2011a). A chemical biology approach to developing STAT inhibitors: molecular strategies for accelerating clinical translation. Oncotarget *2*, 518-524.

Nelson, E.A., Walker, S.R., Weisberg, E., Bar-Natan, M., Barrett, R., Gashin, L.B., Terrell, S., Klitgaard, J.L., Santo, L., Addorio, M.R.*, et al.* (2011b). The STAT5 inhibitor pimozide decreases survival of chronic myelogenous leukemia cells resistant to kinase inhibitors. Blood *117*, 3421-3429.

Neubauer, H., Cumano, A., M¸ller, M., Wu, H., Huffstadt, U., and Pfeffer, K. (1998). Jak2 Deficiency Defines an EssentialDevelopmental Checkpoint in DefinitiveHematopoiesis. Cell *93*, 397-409.

Normant, E., Paez, G., West, K.A., Lim, A.R., Slocum, K.L., Tunkey, C., McDougall, J., Wylie, A.A., Robison, K., Caliri, K.*, et al.* (2011). The Hsp90 inhibitor IPI-504 rapidly lowers EML4-ALK levels and induces tumor regression in ALK-driven NSCLC models. Oncogene *30*, 2581-2586.

Nosaka, T., van Deursen, J.M., Tripp, R.A., Thierfelder, W.E., Witthuhn, B.A., McMickle, A.P., Doherty, P.C., Grosveld, G.C., and Ihle, J.N. (1995). Defective lymphoid development in mice lacking Jak3. Science *270*, 800-802.

Oh, S.T., Simonds, E.F., Jones, C., Hale, M.B., Goltsev, Y., Gibbs, K.D., Merker, J.D., Zehnder, J.L., Nolan, G.P., and Gotlib, J. (2010). Novel mutations in the inhibitory adaptor protein LNK drive JAK-STAT signaling in patients with myeloproliferative neoplasms. Blood *116*, 988-992.

Page, B.D., Khoury, H., Laister, R.C., Fletcher, S., Vellozo, M., Manzoli, A., Yue, P., Turkson, J., Minden, M.D., and Gunning, P.T. (2012). Small molecule STAT5-SH2 domain inhibitors exhibit potent antileukemia activity. Journal of medicinal chemistry *55*, 1047-1055.

Pao, W., Miller, V.A., Politi, K.A., Riely, G.J., Somwar, R., Zakowski, M.F., Kris, M.G., and Varmus, H. (2005). Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS medicine *2*, e73.

Pardanani, A., Gotlib, J.R., Jamieson, C., Cortes, J.E., Talpaz, M., Stone, R.M., Silverman, M.H., Gilliland, D.G., Shorr, J., and Tefferi, A. (2011a). Safety and efficacy of TG101348, a selective JAK2 inhibitor, in myelofibrosis. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *29*, 789-796.

Pardanani, A., Gotlib, J.R., Jamieson, C., Cortes, J.E., Talpaz, M., Stone, R.M., Silverman, M.H., Gilliland, D.G., Shorr, J., and Tefferi, A. (2011b). Safety and Efficacy of TG101348, a Selective JAK2 Inhibitor, in Myelofibrosis. Journal of Clinical Oncology *29*, 789-796.

Pardanani, A., Laborde, R.R., Lasho, T.L., Finke, C., Begna, K., Al-Kali, A., Hogan, W.J., Litzow, M.R., Leontovich, A., Kowalski, M.*, et al.* (2013). Safety and efficacy of CYT387, a JAK1 and JAK2 inhibitor, in myelofibrosis. Leukemia *27*, 1322-1327.

Pardanani, A., Lasho, T., Smith, G., Burns, C.J., Fantino, E., and Tefferi, A. (2009). CYT387, a selective JAK1/JAK2 inhibitor: in vitro assessment of kinase selectivity and preclinical studies using cell lines and primary cells from polycythemia vera patients. Leukemia *23*, 1441-1445.

Parganas, E., Wang, D., Stravopodis, D., Topham, D.J., Marine, J.-C., Teglund, S., Vanin, E.F., Bodner, S., Colamonici, O.R., van Deursen, J.M.*, et al.* (1998). Jak2 Is Essential for Signaling through a Variety of Cytokine Receptors. Cell *93*, 385-395.

Park, S.Y., Saijo, K., Takahashi, T., Osawa, M., Arase, H., Hirayama, N., Miyake, K., Nakauchi, H., Shirasawa, T., and Saito, T. (1995). Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. Immunity *3*, 771-782.

Peeters, P., Raynaud, S.D., Cools, J., Wlodarska, I., Grosgeorge, J., Philip, P., Monpoux, F., Van Rompaey, L., Baens, M., Van den Berghe, H.*, et al.* (1997). Fusion of TEL, the ETS-Variant Gene 6 (ETV6), to the Receptor-Associated Kinase JAK2 as a Result of t(9; 12) in a Lymphoid and t(9; 15; 12) in a Myeloid Leukemia. Blood *90*, 2535-2540.

Pikman, Y., Lee, B.H., Mercher, T., McDowell, E., Ebert, B.L., Gozo, M., Cuker, A., Wernig, G., Moore, S., Galinsky, I.*, et al.* (2006). <italic>MPLW515L</italic> Is a Novel Somatic Activating Mutation in Myelofibrosis with Myeloid Metaplasia. PLoS medicine *3*, e270.

Quintas-Cardama, A., Vaddi, K., Liu, P., Manshouri, T., Li, J., Scherle, P.A., Caulder, E., Wen, X., Li, Y., Waeltz, P.*, et al.* (2010). Preclinical characterization of the selective JAK1/2 inhibitor INCB018424: therapeutic implications for the treatment of myeloproliferative neoplasms. Blood *115*, 3109-3117.

Rambaldi, A., Dellacasa, C.M., Finazzi, G., Carobbio, A., Ferrari, M.L., Guglielmelli, P., Gattoni, E., Salmoiraghi, S., Finazzi, M.C., Di Tollo, S.*, et al.* (2010). A pilot study of the Histone-Deacetylase inhibitor Givinostat in patients with JAK2V617F positive chronic myeloproliferative neoplasms. British journal of haematology *150*, 446-455.

Rane, S.G., and Reddy, E.P. (2000). Janus kinases: components of multiple signaling pathways. Oncogene *19*, 5662-5679.

Rinaldi, C.R., Rinaldi, P., Alagia, A., Gemei, M., Esposito, N., Formiggini, F., Martinelli, V., Senyuk, V., Nucifora, G., and Pane, F. (2010). Preferential nuclear accumulation of JAK2V617F in CD34+ but not in granulocytic, megakaryocytic, or erythroid cells of patients with Philadelphia-negative myeloproliferative neoplasia. Blood *116*, 6023-6026.

Rodig, S.J., Meraz, M.A., White, J.M., Lampe, P.A., Riley, J.K., Arthur, C.D., King, K.L., Sheehan, K.C., Yin, L., Pennica, D.*, et al.* (1998). Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. Cell *93*, 373-383.

Rui, L., Emre, N.C., Kruhlak, M.J., Chung, H.J., Steidl, C., Slack, G., Wright, G.W., Lenz, G., Ngo, V.N., Shaffer, A.L.*, et al.* (2010a). Cooperative epigenetic modulation by cancer amplicon genes. Cancer Cell *18*, 590-605.

Rui, L., Emre, N.C.T., Kruhlak, M.J., Chung, H.-J., Steidl, C., Slack, G., Wright, G.W., Lenz, G., Ngo, V.N., Shaffer, A.L.*, et al.* (2010b). Cooperative Epigenetic Modulation by Cancer Amplicon Genes. Cancer cell *18*, 590-605.

Russell, S.M., Tayebi, N., Nakajima, H., Riedy, M.C., Roberts, J.L., Aman, M.J., Migone, T.S., Noguchi, M., Markert, M.L., Buckley, R.H.*, et al.* (1995). Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. Science *270*, 797-800.

Saharinen, P., and Silvennoinen, O. (2002). The pseudokinase domain is required for suppression of basal activity of Jak2 and Jak3 tyrosine kinases and for cytokine-inducible activation of signal transduction. The Journal of biological chemistry *277*, 47954-47963.

Saharinen, P., Takaluoma, K., and Silvennoinen, O. (2000). Regulation of the Jak2 tyrosine kinase by its pseudokinase domain. Molecular and cellular biology *20*, 3387-3395.

Sansone, P., and Bromberg, J. (2012). Targeting the Interleukin-6/Jak/Stat Pathway in Human Malignancies. Journal of Clinical Oncology *30*, 1005-1014.

Schmitt-Graeff, A.H., Teo, S.S., Olschewski, M., Schaub, F., Haxelmans, S., Kirn, A., Reinecke, P., Germing, U., and Skoda, R.C. (2008). JAK2V617F mutation status identifies subtypes of refractory anemia with ringed sideroblasts associated with marked thrombocytosis. Haematologica *93*, 34-40.

Schust, J., Sperl, B., Hollis, A., Mayer, T.U., and Berg, T. (2006). Stattic: a small-molecule inhibitor of STAT3 activation and dimerization. Chemistry & biology *13*, 1235-1242.

Schwaab, J., Ernst, T., Erben, P., Rinke, J., Schnittger, S., Strobel, P., Metzgeroth, G., Mossner, M., Haferlach, T., Cross, N.C.*, et al.* (2012). Activating CBL mutations are associated with a distinct MDS/MPN phenotype. Annals of hematology *91*, 1713-1720.

Scott, L.M., Tong, W., Levine, R.L., Scott, M.A., Beer, P.A., Stratton, M.R., Futreal, P.A., Erber, W.N., McMullin, M.F., Harrison, C.N.*, et al.* (2007). JAK2 Exon 12 Mutations in Polycythemia Vera and Idiopathic Erythrocytosis. The New England journal of medicine *356*, 459-468.

Sen, M., Thomas, S.M., Kim, S., Yeh, J.I., Ferris, R.L., Johnson, J.T., Duvvuri, U., Lee, J., Sahu, N., Joyce, S.*, et al.* (2012). First-in-human trial of a STAT3 decoy oligonucleotide in head and neck tumors: implications for cancer therapy. Cancer discovery *2*, 694-705.

Sharma, S.V., Lee, D.Y., Li, B., Quinlan, M.P., Takahashi, F., Maheswaran, S., McDermott, U., Azizian, N., Zou, L., Fischbach, M.A.*, et al.* (2010). A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. Cell *141*, 69-80.

Shih, A.H., Abdel-Wahab, O., Patel, J.P., and Levine, R.L. (2012). The role of mutations in epigenetic regulators in myeloid malignancies. Nature reviews Cancer *12*, 599-612.

Shimamura, T., Lowell, A.M., Engelman, J.A., and Shapiro, G.I. (2005). Epidermal Growth Factor Receptors Harboring Kinase Domain Mutations Associate with the Heat Shock Protein 90 Chaperone and Are Destabilized following Exposure to Geldanamycins. Cancer Research *65*, 6401-6408.

Shochat, C., Tal, N., Bandapalli, O.R., Palmi, C., Ganmore, I., te Kronnie, G., Cario, G., Cazzaniga, G., Kulozik, A.E., Stanulla, M.*, et al.* (2011). Gain-of-function mutations in

interleukin-7 receptor-alpha (IL7R) in childhood acute lymphoblastic leukemias. The Journal of experimental medicine *208*, 901-908.

Shuai, K., Horvath, C.M., Huang, L.H., Qureshi, S.A., Cowburn, D., and Darnell, J.E. (1994). Interferon activation of the transcription factor Stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions. Cell *76*, 821-828.

Skov, V., Larsen, T.S., Thomassen, M., Riley, C.H., Jensen, M.K., Bjerrum, O.W., Kruse, T.A., and Hasselbalch, H.C. (2012). Increased gene expression of histone deacetylases in patients with Philadelphia-negative chronic myeloproliferative neoplasms. Leukemia & lymphoma *53*, 123-129.

Smith, C.C., Wang, Q., Chin, C.S., Salerno, S., Damon, L.E., Levis, M.J., Perl, A.E., Travers, K.J., Wang, S., Hunt, J.P*., et al.* (2012). Validation of ITD mutations in FLT3 as a therapeutic target in human acute myeloid leukaemia. Nature *485*, 260-263.

Solit, D.B., Basso, A.D., Olshen, A.B., Scher, H.I., and Rosen, N. (2003). Inhibition of Heat Shock Protein 90 Function Down-Regulates Akt Kinase and Sensitizes Tumors to Taxol. Cancer Research *63*, 2139-2144.

Staerk, J., Lacout, C., Sato, T., Smith, S.O., Vainchenker, W., and Constantinescu, S.N. (2006). An amphipathic motif at the transmembrane-cytoplasmic junction prevents autonomous activation of the thrombopoietin receptor. Blood *107*, 1864-1871.

Suryani, S., Sia, Keith CS, Bracken, Lauryn, Carol, Hernan, Evans, Kathryn, Kurmasheva, Raushan, Houghton, Peter J., Smith, Malcolm A., Lock, Richard B. (2012). Dual Inhibition of JAK/STAT and MAPK Pathways Results in Synergistic Cell Killing of JAK-Mutated Pediatric Acute Lymphoblastic Leukemia. Blood *ASH Annual Meeting Abstracts 2012 120: 3562*

Tefferi, A., Vaidya, R., Caramazza, D., Finke, C., Lasho, T., and Pardanani, A. (2011). Circulating interleukin (IL)-8, IL-2R, IL-12, and IL-15 levels are independently prognostic in primary myelofibrosis: a comprehensive cytokine profiling study. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *29*, 1356-1363.

Thomis, D.C., Gurniak, C.B., Tivol, E., Sharpe, A.H., and Berg, L.J. (1995). Defects in B lymphocyte maturation and T lymphocyte activation in mice lacking Jak3. Science *270*, 794-797.

Toms, A.V., Deshpande, A., McNally, R., Jeong, Y., Rogers, J.M., Kim, C.U., Gruner, S.M., Ficarro, S.B., Marto, J.A., Sattler, M.*, et al.* (2013). Structure of a pseudokinase-domain switch that controls oncogenic activation of Jak kinases. Nature structural & molecular biology.

Turkson, J., Zhang, S., Mora, L.B., Burns, A., Sebti, S., and Jove, R. (2005). A novel platinum compound inhibits constitutive Stat3 signaling and induces cell cycle arrest and apoptosis of malignant cells. The Journal of biological chemistry *280*, 32979-32988.

Tyner, J.W., Bumm, T.G., Deininger, J., Wood, L., Aichberger, K.J., Loriaux, M.M., Druker, B.J., Burns, C.J., Fantino, E., and Deininger, M.W. (2010). CYT387, a novel JAK2 inhibitor, induces hematologic responses and normalizes inflammatory cytokines in murine myeloproliferative neoplasms. Blood *115*, 5232-5240.

Ungureanu, D., Wu, J., Pekkala, T., Niranjan, Y., Young, C., Jensen, O.N., Xu, C.F., Neubert, T.A., Skoda, R.C., Hubbard, S.R.*, et al.* (2011). The pseudokinase domain of JAK2 is a dual-specificity protein kinase that negatively regulates cytokine signaling. Nature structural & molecular biology *18*, 971-976.

Vaidya, R., Gangat, N., Jimma, T., Finke, C.M., Lasho, T.L., Pardanani, A., and Tefferi, A. (2012). Plasma cytokines in polycythemia vera: phenotypic correlates, prognostic relevance, and comparison with myelofibrosis. American journal of hematology *87*, 1003-1005.

Vainchenker, W., Delhommeau, F., Constantinescu, S.N., and Bernard, O.A. (2011). New mutations and pathogenesis of myeloproliferative neoplasms. Blood *118*, 1723-1735.

Verstovsek, S., Kantarjian, H., Mesa, R.A., Pardanani, A.D., Cortes-Franco, J., Thomas, D.A., Estrov, Z., Fridman, J.S., Bradley, E.C., Erickson-Viitanen, S.*, et al.* (2010). Safety and Efficacy of INCB018424, a JAK1 and JAK2 Inhibitor, in Myelofibrosis. New England Journal of Medicine *363*, 1117-1127.

Verstovsek, S., Kantarjian, H.M., Estrov, Z., Cortes, J.E., Thomas, D.A., Kadia, T., Pierce, S., Jabbour, E., Borthakur, G., Rumi, E.*, et al.* (2012a). Long-term outcomes of 107 patients with myelofibrosis receiving JAK1/JAK2 inhibitor ruxolitinib: survival advantage in comparison to matched historical controls. Blood *120*, 1202-1209.

Verstovsek, S., Mesa, R.A., Gotlib, J., Levy, R.S., Gupta, V., DiPersio, J.F., Catalano, J.V., Deininger, M., Miller, C., Silver, R.T.*, et al.* (2012b). A Double-Blind, Placebo-

Controlled Trial of Ruxolitinib for Myelofibrosis. New England Journal of Medicine *366*, 799-807.

Verstovsek, S., Mesa, R.A., Gotlib, J., Levy, R.S., Gupta, V., DiPersio, J.F., Catalano, J.V., Deininger, M., Miller, C., Silver, R.T.*, et al.* (2012c). A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. New England Journal of Medicine *366*, 799-807.

Verstovsek, S., Passamonti, F., Rambaldi, A., Barosi, G., Rosen, P.J., He, S., Contel, N., Mookerjee, B., Rumi, E., Gattoni, E.*, et al.* (2012d). Long-Term Efficacy and Safety Results From a Phase II Study of Ruxolitinib in Patients with Polycythemia Vera. ASH Annual Meeting Abstracts *120*, 804-.

Villeval, J.L., Cohen-Solal, K., Tulliez, M., Giraudier, S., Guichard, J., Burstein, S.A., Cramer, E.M., Vainchenker, W., and Wendling, F. (1997). High thrombopoietin production by hematopoietic cells induces a fatal myeloproliferative syndrome in mice. Blood *90*, 4369-4383.

Walters, D.K., Mercher, T., Gu, T.L., O'Hare, T., Tyner, J.W., Loriaux, M., Goss, V.L., Lee, K.A., Eide, C.A., Wong, M.J.*, et al.* (2006). Activating alleles of JAK3 in acute megakaryoblastic leukemia. Cancer Cell *10*, 65-75.

Walz, C., Ahmed, W., Lazarides, K., Betancur, M., Patel, N., Hennighausen, L., Zaleskas, V.M., and Van Etten, R.A. (2012). Essential role for Stat5a/b in myeloproliferative neoplasms induced by BCR-ABL1 and JAK2(V617F) in mice. Blood *119*, 3550-3560.

Wang, C., Fu, M., Angeletti, R.H., Siconolfi-Baez, L., Reutens, A.T., Albanese, C., Lisanti, M.P., Katzenellenbogen, B.S., Kato, S., Hopp, T.*, et al.* (2001). Direct acetylation of the estrogen receptor alpha hinge region by p300 regulates transactivation and hormone sensitivity. The Journal of biological chemistry *276*, 18375-18383.

Wang, J.C., Chen, C., Dumlao, T., Naik, S., Chang, T., Xiao, Y.Y., Sominsky, I., and Burton, J. (2008). Enhanced histone deacetylase enzyme activity in primary myelofibrosis. Leukemia & lymphoma *49*, 2321-2327.

Wang, X., Zeng, J., Shi, M., Zhao, S., Bai, W., Cao, W., Tu, Z., Huang, Z., and Feng, W. (2011). Targeted blockage of signal transducer and activator of transcription 5 signaling pathway with decoy oligodeoxynucleotides suppresses leukemic K562 cell growth. DNA and cell biology *30*, 71-78.

Wang, Y., Fiskus, W., Chong, D.G., Buckley, K.M., Natarajan, K., Rao, R., Joshi, A., Balusu, R., Koul, S., Chen, J.*, et al.* (2009). Cotreatment with panobinostat and JAK2 inhibitor TG101209 attenuates JAK2V617F levels and signaling and exerts synergistic cytotoxic effects against human myeloproliferative neoplastic cells. Blood *114*, 5024-5033.

Weigert, O., Lane, A.A., Bird, L., Kopp, N., Chapuy, B., van Bodegom, D., Toms, A.V., Marubayashi, S., Christie, A.L., McKeown, M.*, et al.* (2012a). Genetic resistance to JAK2 enzymatic inhibitors is overcome by HSP90 inhibition. The Journal of experimental medicine *209*, 259-273.

Weigert, O., Lane, A.A., Bird, L., Kopp, N., Chapuy, B., van Bodegom, D., Toms, A.V., Marubayashi, S., Christie, A.L., McKeown, M.*, et al.* (2012b). Genetic resistance to JAK2 enzymatic inhibitors is overcome by HSP90 inhibition. The Journal of Experimental Medicine *209*, 259-273.

Wernig, G., Kharas, M.G., Okabe, R., Moore, S.A., Leeman, D.S., Cullen, D.E., Gozo, M., McDowell, E.P., Levine, R.L., Doukas, J.*, et al.* (2008). Efficacy of TG101348, a selective JAK2 inhibitor, in treatment of a murine model of JAK2V617F-induced polycythemia vera. Cancer Cell *13*, 311-320.

Wernig, G., Mercher, T., Okabe, R., Levine, R.L., Lee, B.H., and Gilliland, D.G. (2006). Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. Blood *107*, 4274-4281.

Yan, D., Hutchison, R.E., and Mohi, G. (2012). Critical requirement for Stat5 in a mouse model of polycythemia vera. Blood *119*, 3539-3549.

Yan, X.Q., Lacey, D., Hill, D., Chen, Y., Fletcher, F., Hawley, R.G., and McNiece, I.K. (1996). A model of myelofibrosis and osteosclerosis in mice induced by overexpressing thrombopoietin (mpl ligand): Reversal of disease by bone marrow transplantation. Blood *88*, 402-409.

Yoda, A., Yoda, Y., Chiaretti, S., Bar-Natan, M., Mani, K., Rodig, S.J., West, N., Xiao, Y., Brown, J.R., Mitsiades, C.*, et al.* (2010). Functional screening identifies CRLF2 in precursor B-cell acute lymphoblastic leukemia. Proceedings of the National Academy of Sciences of the United States of America *107*, 252-257.

Yuan, Z.L., Guan, Y.J., Chatterjee, D., and Chin, Y.E. (2005). Stat3 dimerization regulated by reversible acetylation of a single lysine residue. Science *307*, 269-273.

Zaleskas, V.M., Krause, D.S., Lazarides, K., Patel, N., Hu, Y., Li, S., and Van Etten, R.A. (2006). Molecular Pathogenesis and Therapy of Polycythemia Induced in Mice by JAK2 V617F. PloS one *1*, e18.

Zenatti, P.P., Ribeiro, D., Li, W., Zuurbier, L., Silva, M.C., Paganin, M., Tritapoe, J., Hixon, J.A., Silveira, A.B., Cardoso, B.A*., et al.* (2011). Oncogenic IL7R gain-of-function mutations in childhood T-cell acute lymphoblastic leukemia. Nature genetics *43*, 932-939.

Zhang, J., Yang, P.L., and Gray, N.S. (2009). Targeting cancer with small molecule kinase inhibitors. Nature reviews Cancer *9*, 28-39.

Zhao, C., Chen, A., Jamieson, C.H., Fereshteh, M., Abrahamsson, A., Blum, J., Kwon, H.Y., Kim, J., Chute, J.P., Rizzieri, D*., et al.* (2009a). Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. Nature *458*, 776-779.

Zhao, L., Dong, H., Zhang, C.C., Kinch, L., Osawa, M., Iacovino, M., Grishin, N.V., Kyba, M., and Huang, L.J. (2009b). A JAK2 interdomain linker relays Epo receptor engagement signals to kinase activation. The Journal of biological chemistry *284*, 26988-26998.