Functional Interrogation of JAK2 and ASXL1 Mutations in Myeloproliferative Neoplasms

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Functional Interrogation of \textit{JAK2} and \textit{ASXL1} Mutations in Myeloproliferative Neoplasms
by
Taylor Brost Collins

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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Taylor Collins

Washington University in St. Louis

January 2021
Dedicated to my husband, Casey.
ABSTRACT OF THE DISSERTATION

Functional Interrogation of JAK2 and ASXL1 Mutations in Myeloproliferative Neoplasms

by

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Doctor of Philosophy in Biology and Biomedical Sciences
Molecular Cell Biology
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Professor Stephen T. Oh, Chair

JAK2 V617F is the most frequent mutation found in myeloproliferative neoplasms (MPNs), with 50-60% of myelofibrosis (MF) patients harboring this mutation. Mutations in ASXL1 often co-occur with JAK2 V617F and are associated with decreased survival and increased risk of transformation to secondary acute myeloid leukemia. How mutant ASXL1 contributes to the MPN disease phenotype and confers poor prognosis is not fully understood. Asxl1 mutation knock-in mouse models present with a relatively modest phenotype, following a long latency period and ASXL1 mutations have not been modeled in combination with JAK2 V617F. The field lacks human model systems that focus on how combined JAK2 V617F and ASXL1 mutations affect myelofibrosis pathogenesis.

The studies presented here describe two complementary pluripotent stem cell models utilized to study how JAK2 V617F and ASXL1 mutations influence MPN disease biology. Induced pluripotent stem cells were derived from a patient harboring both JAK2 and ASXL1 mutations. In parallel, the ASXL1 mutation present in the patient derived iPSC lines as well as the most
common \textit{ASXL1} mutation were both introduced in isolation and in combination with \textit{JAK2} V617F into H1 human embryonic stem cells. Following hematopoietic differentiation, colony forming assays and RNA sequencing were performed utilizing differentiated hematopoietic progenitors. The \textit{JAK2} mutant lines generated significantly more erythroid colonies than WT, \textit{ASXL1}, and double mutant lines. The \textit{ASXL1} mutation resulted in almost exclusively myeloid colony growth and the double mutant line presented with an intermediate erythroid colony growth phenotype. When expression of genes involved in erythropoiesis were assessed, the double mutant progenitor expression profiles were more similar to \textit{ASXL1} mutant profiles than gene expression patterns found in \textit{JAK2} mutant progenitors. This data suggested that the \textit{ASXL1} mutation blunted the erythroid proliferation phenotype associated with \textit{JAK2} V617F.

The observed phenotypes were validated utilizing myelofibrosis patient cells sorted from patients harboring \textit{JAK2} V617F and double mutant patients harboring both \textit{JAK2} V617F as well as an \textit{ASXL1} mutation. \textit{JAK2} mutant patient cells generated more erythroid colonies than \textit{JAK2/ASXL1} double mutant cells, while the double mutant cells differentiated into predominately myeloid lineage colonies. This study has demonstrated that \textit{JAK2} V617F induces an expansion of the erythroid lineage, while \textit{ASXL1} impairs erythroid colony growth and skews differentiation toward the myeloid lineage utilizing both human pluripotent cells as well as primary myelofibrosis patient samples. Future studies include utilizing the mutant pluripotent stem cell lines to assess potential mechanisms as to how \textit{JAK2} and \textit{ASXL1} mutations influence differentiation via signaling mass cytometry and manipulation of gene targets of interest revealed by the RNA sequencing data.
Chapter 1: Introduction and significance

Myeloproliferative Neoplasms

Myeloproliferative neoplasms (MPNs) are chronic myeloid malignancies clonally derived from hematopoietic stem or progenitor cells (HSPCs). MPNs are characterized by abnormal hematopoiesis where overproduction of one or more myeloid lineages is observed.\textsuperscript{2, 3} The classic BCR-ABL1-negative MPNs are categorized to include polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF). PV is primarily characterized by an increase in erythrocyte production and ET is characterized by overproduction of platelets. MF is diagnosed based on the presence of fibrotic tissue in the bone marrow as well as morphological changes in the megakaryocytes.\textsuperscript{4} In 1951, William Dameshek was the first to recognize that although each MPN presents with distinct clinical features, they are phenotypically related and should be classified together as “myeloproliferative disorders.” He noted that many MPN patients have several overlapping features including pancytosis and splenomegaly. He also noticed that patients with PV can develop bone marrow fibrosis as seen in myelofibrosis patients. He argued that because there were often difficulties distinguishing between the MPN subtypes, they should be considered “closely interrelated” diseases each displaying bone marrow proliferation.\textsuperscript{2}

MPNs are considered rare diseases with the prevalence for PV being estimated at 45-57 cases per 100,000 patients, ET approximated at 39-57 cases per 100,000, and MF approximated at 3.6-5.7 cases per 100,000 patients in the United States.\textsuperscript{5} The age adjusted incidence rates in the United States measured by cases per 100,000 patients per year are estimated at 1.57 cases for PV, 1.55
cases for ET, and 0.44 cases for PMF.\textsuperscript{6} Patients are typically diagnosed later in life at a mean age of 55-58 years old for PV patients, 53-57 years old for ET patients, and 61-67 for MF patients.\textsuperscript{5} PV and ET have the potential to transform into MF and all 3 MPNs have the potential to transform into secondary acute myeloid leukemia.\textsuperscript{7} One study found among MF patients, 35-40\% were primary myelofibrosis cases, 10\% were post PV patients, and 15\% were post ET patients with the remainder arising from another preexisting condition.\textsuperscript{5} According to another study approximately 4.9-6\% of PV patients transformed to MF 10 years after initial diagnosis and 6-14\% after 15 years. ET patients have a slightly lower risk of transforming to MF with 0.8-4.9\% transformed to MF 10 years after initial diagnosis and 4-11\% after 15 years.\textsuperscript{8} The transformation rate to leukemia is the highest in PMF patients, estimated at 10-20\% at 10 years and there is a lower risk for PV transformation to leukemia at 2.3\% at 10 years and 7.9\% at 20 years. It is even more uncommon for ET patients to transform to sAML at less than 1\% at 10 years.\textsuperscript{7}

MPN patients have a lower life expectancy as compared to healthy individuals, however PV and ET patients have relatively longer survival estimates than MF patients. Upon diagnosis PV patient median survival is estimated at 13.5 years and ET patient median survival is estimated at 19.8 years, however PMF patient survival is less at 6 years.\textsuperscript{9} All three of these classic MPNs share proliferation of hematopoietic stem cells independent of growth factors, hypercellular bone marrow, splenomegaly, and increased risk of thrombosis and hemorrhages.\textsuperscript{10} PV patients can experience headaches, tiredness, dizziness, sweating, and pruritus. A higher percentage of ET patients are asymptomatic with one third to one quarter of patients experiencing symptoms such as headaches, fainting, or chest pain. MF patients may present with more severe symptoms as approximately 75\% of patients experience symptoms such as weight loss, night sweats, fever,
joint pain, anemia, extramedullary hematopoiesis, in addition to the symptoms already mentioned.\textsuperscript{10}

Currently there are no drug therapy options that are known to cure MPNs. Drug therapy is also unlikely to reverse bone marrow fibrosis, prolong survival, or affect disease progression to sAML or MF.\textsuperscript{11,4} PV and ET treatment regiments are directed toward alleviating symptom burden and preventing clotting or bleeding. Phlebotomy is often utilized as a technique to lower the aberrantly high hematocrit in PV patients. Very low-risk PV and ET patients defined by lack of thrombosis history, less than 60 years old, and \textit{JAK2} unmutated (for ET patients) are either recommended for observation alone, or once daily aspirin if they have cardiovascular risk factors. ET patients who follow the same criteria, but harbor the \textit{JAK2} mutation are considered low-risk and are either recommended once or twice daily aspirin depending on whether or not they have cardiovascular risks. Patients are considered intermediate-risk if they have no history of thrombosis, unmutated \textit{JAK2} (for ET patients), but are over the age of 60 and may be treated with hydroxyurea and aspirin. High risk PV and ET patients are categorized as such because they have a history of thrombosis or are over the age of 60 and harbor the \textit{JAK2} mutation (for ET patients). These patients are often treated with hydroxyurea as well as aspirin or a systemic anticoagulant.\textsuperscript{11}

The Dynamic IPSS-plus or DIPSS-plus scoring system is a clinical prognostic scoring system that estimates survival from time of diagnosis and that plays a role in how MF patients are treated and/or decisions regarding allogenic stem cell transplantation. The DIPSS-plus score is based on age greater than 65 years, hemoglobin less than 100g/L, leukocyte count greater than 25x10\textsuperscript{9}/L, circulation blasts greater than or equal to 1%, constitutional symptoms, transfusion
dependency, platelet count less than 100 x 10^9/L and unfavorable cytogenetics. Myelofibrosis patients who are considered very low or low risk disease patients without symptoms are typically recommended for observation alone. Very low-risk patients fall into the DIPSS-plus low risk group and low risk patients are characterized by their low/intermediate-1 DIPSS-plus score. The key difference between very low and low-risk is that very low risk harbor CALR type 1 or type 1-like mutations and low-risk patients lack these “good prognosis” mutations. If the very low or low-risk patients are anemic, experiencing splenomegaly, or experiencing other symptoms they will often be treated with hydroxyurea or conventional drug options such as ruxolitinib.

Intermediate-risk or high-risk myelofibrosis patients are considered for allogenic stem cell transplantation, which is the only known treatment option that has the potential to cure or prolong survival for MF patients. Patients are considered intermediate-risk if their DIPSS-plus score is intermediate-2 risk and they lack an ASXL1 or other high-risk mutation. MF patients are considered to have high-risk disease if they are assigned a high-risk DIPSS-plus score and harbor a molecularly high-risk mutation such as an ASXL1 mutation. Although transplant is the only known curative option, the decision of whether or not to transplant a patient is made even more complex due to the fact that there is a substantial risk for transplant related mortality and morbidity. Some consider patients transplant candidates if they have intermediate-2 or high-risk disease according to the DIPSS-plus score and are under the age of 70 years old. Another group that may be considered candidates are patients with intermediate-1 risk disease according to their DIPSS-plus score and are under the age of 65, if they present with transfusion dependent anemia, or if their blast percentage is greater than 2% in the peripheral blood, or they present with adverse cytogenetics. If these higher risk patients are not transplant candidates, they may
be treated with pharmacological intervention either conventional drug therapy or investigational
drug options if the first choice conventional options are not beneficial for the patient.11

**JAK-STAT Signaling**

JAK-STAT signaling dysregulation is observed in all MPNs, due to somatic JAK-STAT
pathway activating mutations.14 The JAK-STAT pathway is a signaling cascade involved in
regulating cell proliferation and hematopoiesis.15 JAK-STAT signaling activation begins with the
binding of a ligand such as interferons, interleukins, or growth factors to a transmembrane
receptor such as a cytokine receptor. JAK binding sites are in close proximity to the membrane
and allow JAK to associate with the cytoplasmic domains of these receptors. JAK family
proteins consist of seven different homology (JH) domains, JH5, 6, and 7 make up the FERM
(four-point-one, ezrin, radixin, moesin) domain and JH3 and 4 make up the SH2 (Src homology
2) domain. FERM and SH2 domains located on the N-terminus are responsible for associating
JAK with the cytokine receptor cytoplasmic tail. JH1 encompasses the kinase domain and JH2
contains the pseudokinase domain. Binding of the ligand to the cytokine receptor changes the
structure of the receptor/JAK dimers and brings the JAKs in close enough proximity to each
other to allow one JAK protein to transphosphorylate the other JAK protein in the dimer at the
JH1 domain. The activated JAKs then phosphorylate the cytokine receptor cytoplasmic tail to
facilitate recruitment of the STAT family proteins. Individual receptors activate specific JAK
family proteins to affect downstream signaling. The activated JAKs then phosphorylate the
tyrosine residue on the STAT proteins which in turn causes the STATs to form homodimers or
heterodimers with other STAT proteins. Similar to the receptor/JAK relationships, specific
STATs are activated by particular JAKs. The activated dimerized STATs then translocate into
the nucleus via facilitated transport, where they act as transcription factors (Figure 1.1). STATs are capable of activating transcription of many genes, for example STAT3 has been shown to activate genes involved in cancer cell proliferation, migration, survival, and suppression of antitumor immune response. 16

The JAK2 V617F mutation is the most studied mutation in the JAK family proteins and it is also the most common mutation found in MPNs. 16 The JAK2 V617F mutation is an acquired somatic mutation, which results in constitutive JAK2 activation. 2 This mutation was discovered in 2005 by four separate groups. 17,18,19,20 The JAK2 V617F mutation is a somatic mutation in which a G to T substitution at position 1849 results in the substitution of valine for phenylalanine at codon 617. This mutation is found in 95% or more of patients diagnosed with PV, and 50-60% of patients diagnosed with ET or MF. Since the JAK2 V617F mutation is present in PV, ET, and MF, it is unclear as to how a single mutation contributes to three clinically distinct phenotypes. The majority of remaining MPN patients harbor either CALR or MPL mutations and they are found in 20-25% and 3-4%, respectively, of patients with ET and 20-25% and 6-7% of patients with PMF. 10-15% of MPN patients that do not harbor one of these three mutations are known as triple negative. 11 The JAK2 V617F mutation alters the JH2 or pseudokinase domain, which is known to negatively regulate the kinase domain and is capable of switching off the kinase activity of JAK2. There has been additional evidence published that the JH2 pseudokinase domain also has catalytic activity that regulates JH1 activity through auto-phosphorylation. 3 Dysregulation of JAK-STAT pathway signaling due to the JAK2 V617F mutation leads to an increase in cellular proliferation without the requirement for cytokine receptor binding by a
ligand, therefore the pathogenesis of these malignancies are independent of or hypersensitive to numerous cytokines.\textsuperscript{4,18}

Ruxolitinib, a JAK1 and JAK2 small molecule inhibitor, has been developed as a treatment for myelofibrosis patients. Myelofibrosis patients treated with ruxolitinib experience a reduction in spleen size and in symptom burden.\textsuperscript{21} However, ruxolitinib treatment does not eradicate the malignant clone, substantially reduce bone marrow fibrosis, or substantially improve overall survival of the patient.\textsuperscript{22,23} Ruxolitinib is a nonspecific JAK1 and JAK2 inhibitor, therefore it is plausible that the drug is not efficiently inhibiting the mutant form of the JAK2 protein or that constitutive JAK2 activity is not required for the maintenance of the MPN phenotype. This could also suggest other signaling pathways may be dysregulated that promote survival of the malignant cells.\textsuperscript{24} The observation that the \textit{JAK2 V617F} mutation is the most common mutation in MPNs, but JAK inhibitors fail to resolve the phenotype demonstrates the need to better understand the role of JAK2 as well as to determine the role of other mutations in disease pathogenesis and progression.

\textbf{MPN Mouse Models}

Since the discovery of the \textit{JAK2 V617F} mutation, numerous MPN mouse models have been developed as tools to study how this mutation as well as other JAK/STAT pathway activators contribute to disease pathogenesis.\textsuperscript{25} Several retroviral transduction models have been generated where \textit{ex vivo} bone marrow cells were transduced with virus expressing \textit{Jak2 V617F} and then the bone marrow cells were transplanted back into irradiated recipient mice. While this is an efficient method to study the function of the mutant protein it is an overexpression system and
expression can be 10 to 40-fold higher than physiologic wild-type protein expression levels. All of the groups that have generated a \( \text{Jak2 V617F} \) retroviral model have seen a phenotype that resembles PV with erythrocytosis, leukocytosis, and splenomegaly. After several months reticulin bone marrow fibrosis was observed with disease progression.\(^{26,27,28,29}\)

Transgenic MPN mouse models have been established via genomic integration of a construct containing murine or human \( \text{Jak2 V617F} \) cDNA into murine oocytes during embryogenesis. The construct integrates randomly into genomic DNA and expression can be controlled under a tissue-specific promoter. While this method allows for stable and transmissible expression, transgene products can be randomly inserted in the genome which may alter expression levels depending on placement. \( \text{JAK2 V617F} \) transgenic mouse models result in an MPN-like phenotype with some variability between groups. When expressing \( \text{JAK2 V617F} \) under either the \( H-2Kb \) or \( Vav \) promoters, the observed phenotype depended on the mutant protein expression level. Some of the lower expression mutant mice (although still greater expression level than \( \text{WT-Jak2} \)) presented with an ET-like phenotype and some presented with a PV-like phenotype after 9 months. In the higher expressing mice a more robust MPN disease phenotype was observed which included thrombocytosis, leukocytosis, hypercellular bone marrow, splenomegaly, and bone marrow fibrosis.\(^{30,31}\) Tiedt et al. generated a conditional model system that allowed for inducible expression of the \( \text{Jak2 V617F} \) transgene using the Cre-lox system under control of either the \( Mx \) or \( Vav \) promoter for hematopoietic compartment specific expression. Low level constitutive \( \text{Jak2 V617F} \) expression under the \( Vav \) promoter resulted in an ET-like phenotype with thrombocytosis while the leukocyte and hemoglobin levels were found to be close to the normal range. In contrast, they found mice developed a PV-like phenotype
when expression was under control of the Mx promoter characterized by elevated hemoglobin and hematocrit as well as leukocytosis and thrombocytosis.\textsuperscript{32,33}

Several knock-in mouse models have also been developed as a method to study endogenous mutant \textit{Jak2} expression specifically in the hematopoietic compartment. Akada et al. induced expression of \textit{Jak2} V617F via an inducible Mx-Cre system. Upon induction of mutant Jak2 expression the mice, a severe PV-like phenotype was observed with elevated erythrocyte, leukocyte, and platelet counts. Splenomegaly, extramedullary hematopoiesis, and progressive fibrosis were also characteristics of this model. They also found homozygous mutant mice presented with an even more robust phenotype with higher counts and accelerated bone marrow fibrosis.\textsuperscript{34} Another group recapitulated these findings with a similar knock in model.\textsuperscript{35} Mullally et al. also observed a PV-like phenotype utilizing a conditional knock-in mouse model where excision was achieved by Cre-recombinase mediated by the \textit{E2a} promoter. Similar to the previously described knock-in model erythrocytosis, leukocytosis, extramedullary hematopoiesis, and splenomegaly were features of this knock-in mouse. However, in contrast to the previous model, platelet counts were not elevated despite increased megakaryopoiesis found in the spleen and these mice did not develop bone marrow fibrosis when aged past 6 months. This model also presented with a more marked decrease in overall survival compared to other models.\textsuperscript{36}

Li et al. generated an Mx-Cre knock-in model system expressing human \textit{JAK2} V617F. Following induction of expression, the mice developed an ET-like phenotype with mild erythrocytosis while these mice lacked leukocyte count changes and splenomegaly. In addition to the majority
of mice that developed an ET-like phenotype, approximately 10% of the mice presented with a PV-like disease. It remains unclear as to why this model presented with a different phenotype than the PV-like phenotype observed in the knock-in models previously described, however there is speculation that it could be related to the acquisition of additional mutations, or effects of expressing human JAK2 V617F in a murine background. This mouse model most closely resembles what is seen in human MPN patients where heterozygous JAK2 V617F mutations associate more with an ET phenotype while homozygous JAK2 V617F mutations associate more frequently with a PV phenotype.\textsuperscript{37,38,33}

\textbf{ASXL1 Mutations}

\textit{Additional sex combs-like 1 (ASXL1)}, an epigenetic regulator, is one of the most frequently mutated genes in myeloid malignancies, including MPNs.\textsuperscript{39,40} ASXL1 mutations are found in 5-25\% of PV patients, 5-10\% of ET patients, and 13-23\% of PMF patients.\textsuperscript{41,42} These mutations also frequently occur in CHIP (clonal hematopoiesis of indeterminant potential), which is a premalignant state in which a somatic mutation has been acquired during normal human aging that causes clonal expansion, however the patient lacks clinical features required to diagnose a hematologic malignancy.\textsuperscript{43,44} ASXL1 mutations are associated with a poor prognosis involving decreased survival and increased risk of transformation.\textsuperscript{45,40}

\textit{ASXL1} is ubiquitously expressed, maps to chromosome 20q11, and encodes a protein that is comprised of 1541 amino acids (\textbf{Figure 1.2}).\textsuperscript{46} The vast majority of \textit{ASXL1} mutations are somatic frameshift or nonsense mutations located within exon 12, which is the last exon of the protein. These mutations result in a C-terminal truncation and loss of the plant homeodomain
(PHD) which is important for histone or DNA binding. Controversy remains as to whether ASXL1 mutations found in patients confer a loss of function, gain of function, and/or dominant negative phenotype. Evidence that ASXL1 mutations may be loss of function mutations has been published by Abdel-Wahab et al, where they have shown lack of ASXL1 protein in ASXL1 homozygous mutant leukemia cell lines via western blot analysis. They were unable to detect protein using an N-terminus or a C-terminus antibody, suggesting complete loss of protein. In contrast, Inoue et al. has published data suggesting these mutations may be gain of function or dominant negative. They show that overexpressed mutant truncated ASXL1 in mouse bone marrow cells followed by transplantation into recipient mice resulted in an MDS phenotype with similar features displayed by human MDS patients. The same group generated an N-terminal ASXL1 antibody and were able to detect ASXL1 protein of the expected size based on the truncation mutations found in two different leukemia cell lines that harbor homozygous ASXL1 mutations.

There are two distinct potential mechanisms of action that have been proposed as to how ASXL1 mutations may contribute to disease pathogenesis. The first mechanism suggests that ASXL1 mutations result in complete loss of ASXL1 protein and therefore cause the inability of ASXL1 to recruit the polycomb repressive complex 2 (PRC2) for PCR2-mediated H3K27 tri-methylation, resulting in increased transcription. ASXL1 is also known to bind to BAP1 (BRCA1-associated protein 1) the catalytic subunit of the polycomb repressive deubiquitinase complex (PR-DUB) that deubiquitinates H2AK119Ub. The second proposed mechanism suggests that ASXL1 mutations result in the translation of a functional truncated protein that enhances the DUB activity of the ASXL1-BAP1 complex causing increased transcription.
**ASXL1 Mouse Models**

Several *Asxl1* mouse models have been published, including knock-out and knock-in models. A constitutive *Asxl1* KO mouse model has been published by Wang et al. showing *Asxl1*+/- mice developed a mild MDS-like phenotype. They compared young (3-6 weeks old) and old (6-12 months old) *Asxl1*+/- mice and although they found no significant differences in blood counts, a subset of the old mice displayed mild leukopenia, anemia, and thrombocytopenia when compared to the younger animals. They also noted that the old *Asxl1*+/- mice exhibited more severe myeloid lineage dysplasia than the young *Asxl1*+/- mice. Upon performing competitive repopulation assays they found that Asxl1 loss impairs hematopoietic repopulating capacity by showing a decline in CD45.2 chimerism in animals that received *Asxl1*+/- BM cells.\(^{51}\)

Abdel-Wahab et al. published a conditional *Asxl1* KO model utilizing *Vav-cre* and *Mx1-cre* to delete *Asxl1* specifically in the hematopoietic compartment. *Asxl1* KO mice developed BM and splenic hypocellularity beginning at 6 weeks of age. The aged 6-12 month-old *Asxl1* KO mice presented with leukopenia and anemia. The aged *Asxl1* KO mice also exhibited an increase in CD71+/Ter119- erythroid precursors in both the BM and spleen, suggesting impaired erythroid differentiation. Myeloid cell dysplasia was also apparent in the aged *Asxl1* KO animals. Competitive serial transplants revealed *Asxl1* KO HSPCs had a significant disadvantage suggesting loss of Asxl1 causes a reduction in self-renewal capacity. *Asxl1* KO mice display features of an MDS-like disease.\(^{52}\)
A retroviral mutant ASXL1 expression mouse model was published by Inoue et al. in which they transduced mouse BM cells with a mutant ASXL1 (E635RfsX15) expressing retroviral vector and transplanted the cells into sublethally irradiated recipient mice. Post transplantation, the percentage of GFP-positive ASXL1 mutant (ASXL1-MT) cells gradually increased in the peripheral blood over a one year time period while the ASXL1 WT or empty vector chimerism decreased over time. The majority of GFP-positive ASXL1-MT cells were CD11b-positive while the GFP-negative, nontransduced cells were comprised of equal numbers of CD11b and B220-positive cells 6 months post-transplant, suggesting ASXL1-MT may confer a myeloid bias. The ASXL1-MT mice presented with an MDS-like disease after one year as shown by anemia, leukopenia, thrombocytopenia, hypercellular BM, and an enlarged spleen. The mice also displayed dysplasia in granulocytes as well as in erythroid cells. Expression of mutant truncated ASXL1 in this model system resulted in an MDS-like phenotype after a long latency.48

There are also several published KI mouse models all of which demonstrated development of very mild phenotypes after long latency periods, if a phenotype was observed at all. One of the published models inserted the p.E635RfsX15 Asxl1 mutation into the Rosa26 locus and then crossed these mice with Vav-cre transgenic mice to generate a conditional KI model. The Asxl1-MT KI mice presented with anemia and thrombocytosis at 70 weeks of age, however the mice did not develop an MDS-like phenotype.53 Two separate groups published a constitutive Asxl1 G643WfsX12 KI mouse model, which is a similar mutation to the most commonly found ASXL1 mutation in patients, p.G646WfsX12. One study determined that the Asxl1 KI was not sufficient for the development of a hematologic malignancy in mice within 18 months of age,54 while the
other study found that a subset of their Asxl1 KI animals developed an MDS/MPN like disease after 2 years based on the presence of leukocytosis, anemia, and thrombocytosis.55

Because JAK2 and ASXL1 mutations often co-occur in MF, Guo et al. developed a JAK2 V617F/Asxl1+/- mouse model by crossing JAK2V617F transgenic mice with Asxl1+- mice. They found JAK2 V617F/Asxl1+/- mice had significantly shorter overall survival rates than JAK2 V617F or Asxl1+/- mice. 5 of the 19 JAK2 V617F/Asxl1+/- mice developed MF after 2-6 months while only 1 of the 18 JAK2 V617F mice developed MF at 6 months of age. 3 of the 26 JAK2 V617F/Asxl1+/- mice progressed to secondary acute myeloid leukemia, but this was not seen in the littermate controls. JAK2 V617F/Asxl1+/- mice presented with higher peripheral blood leukocytes, neutrophils, and platelets compared to WT mice. The JAK2 V617F/Asxl1+/- group also displayed the lowest Hb levels compared to the other groups of mice at 8-10 months of age.

When CFU assays were preformed using spleen cells harvested from the four genotypes, the JAK2 V617F/Asxl1+/- cells generated the most BFU-E, CFU-E, and CFU-MK colonies. In this study the JAK2 V617F/Asxl1+/- mice presented with the most severe phenotype suggesting that the Asxl1 KO may accelerate the disease phenotype caused by the JAK2 V617F mutation.1

**Pluripotent Stem Cell Models**

While mouse models are utilized as powerful tools in studying MPNs, they do not fully recapitulate the spectrum of clinical features observed in human MPN patients. Jak2 V617F knock-in models exhibit profound erythrocytosis and splenomegaly, similar to phenotypic characteristics observed in human PV, however, rarely do these mice develop MF. Additionally, Asxl1 mutation knock-in mouse models present with a relatively modest phenotype, following a
very long latency period. We hypothesized that the use of a human pluripotent stem cell system has the potential to provide a more faithful model of the human disease to understand how these mutations contribute to MPN disease pathogenesis.

More broadly, studying human cancer can be made challenging by limited access to patient samples and the attainable patient samples are a finite resource. Tumor heterogeneity associated with patient samples also has the potential to confound interpretation of experimental results. Pluripotent stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) provide a self-renewing, unlimited source of cells that can be differentiated into any relevant cell type necessary to study disease biology or to investigate potential therapeutic options. ESCs were first harvested from human blastocysts by Thomson, et al. in 1998 and they were also able to develop an *in vitro* culture system to maintain and expand these cells. In 2007, the Yamanaka and Thomson laboratories both developed induced pluripotent stem cells by reprogramming somatic cells to an ES-like cell state in which the cells were able to self-renew as well as differentiate into any human cell type. Each group overexpressed their own transcription factor cocktail in order to reprogram terminally differentiated cells; Yamanaka used OCT4, SOX2, KLF4, and c-MYC, while Thomson used OCT4, SOX2, NANOG, and LIN28. Reprogramming patient somatic cells provides an unlimited supply of patient-derived research resource that harbors the pathogenic mutations in the context of the patient’s genetic background. This is a very powerful model system to study human disease mechanisms as well as to experiment with drug therapies in the context of the patient’s mutation status and genetic background. Embryonic stem cell models may not be patient derived, however with genetic engineering technologies such as CRISPR-Cas9, mutations can be introduced into pluripotent
stem cells. This system allows for the generation of an unlimited supply of human cell lines that harbor pathogenic mutations without the need for patient samples. These cell lines can be differentiated into any cell type of interest in order to study how these mutations may be differentially affecting cell populations.

Kotini et al. modeled the multistep process of leukemogenesis using pluripotent stem cells by generating iPSC lines from a spectrum of myeloid malignancy disease states including, familial predisposition, low-risk MDS, high-risk MDS, and MDS/AML. The hematopoietic phenotypes upon differentiation were characterized for each stage of disease severity. They observed that differentiated preleukemic cell-iPSC lines generated CD45+ hematopoietic cells that matured, based on CD34 expression loss, at a similar rate to that of normal cells. Differentiated low-risk MDS-iPSCs generated CD45+ hematopoietic progenitors at a slower rate, and they matured later as compared to the normal cells. High-risk MDS-iPSCs also produced CD45+ cells with a delay, however they were also produced at a much lower efficiency than CD45+ cells produced by low-risk MDS. While MDS/AML-iPSCs were able to give rise to CD45+ progenitors as efficiently as normal cells, they presented with a differentiation block as shown by their continued CD34 expression much longer than the other iPSC disease stages. They also observed reduced erythroid and myeloid colony growth via colony forming assays in all disease stages, and MDS/AML differentiated iPSCs gave rise to mostly immature myeloid colonies. When hematopoietic progenitor cells derived from differentiated iPSCs of the various disease states were transplanted into NSG mice, only MDS/AML progenitors were able to engraft and the transplanted mice showed characteristics of myeloid leukemia. They also were able to make stepwise genetic alterations to the iPSC lines to recapitulate the stepwise disease progression
from normal to preleukemia to high-risk MDS. Finally, they used their system for drug testing at each stage of disease progression and found differential sensitivities to particular drugs depending on disease severity and mutation burden. This publication demonstrated the utility and versatility of pluripotent model systems for studying disease biology. They were able to model disease stages from normal to AML through an MDS stage and transition between stages with the use of genetic engineering.

Chao et al. published another important example demonstrating the utility of iPSC modeling in studying disease pathogenesis.\(^6\) iPSC lines were generated by reprogramming AML blasts that harbored 11q23/MLL rearrangements and upon targeted sequencing they identified the original genetic aberrations were retained in the iPSC lines. The AML-iPSC lines differentiated into non-hematopoietic cell types did not exhibit any abnormal phenotypes, however when differentiated into hematopoietic progenitors, CD43+CD45+ cells gave rise to predominantly myeloid colonies as compared to control iPSC lines that gave rise to both myeloid and erythroid colonies. AML-iPSC colonies were hyperproliferative and had the capability of serial replating. They also transplanted CD43+CD45+ cells differentiated from AML-iPSCs into immunodeficient NSG mice and observed that the mice developed myeloid leukemia with peripheral blood, bone marrow, and spleen engraftment. The mice also presented with enlarged spleens and the human cells isolated from the mice displayed AML blast morphology. The harvested AML cells that engrafted in the mice harbored the same mutations that were present in the original patients from which the iPSC lines were derived. The iPSC-derived AML cells were able to be serially transplanted into secondary recipients and the mice eventually succumbed to leukemia.
They next performed unsupervised hierarchical clustering utilizing 1,000 most variable CpG sites from DNA methylation arrays. All leukemia cells clustered together including primary patient AML cells, AML-iPSC differentiated hematopoietic cells, and patient AML blasts that engrafted *in vivo*. Undifferentiated iPSCs were globally hypermethylated compared to differentiated AML-iPSCs as well as primary AML cells. Similar to the methylation data, RNA sequencing analysis showed hematopoietic cells differentiated from AML-iPSCs and primary AML patient samples had similar gene expression signatures. Based on this data the authors claim that the reprogramming reset global DNA methylation and gene expression patterns, however the patterns from the original patient samples reemerge upon hematopoietic differentiation. They were able to use their model system to test pharmacologic agents targeted to specific subclones present in the original AML patient sample. For example, EPZ-5676 is a small molecule inhibitor that has been shown to target MLL-rearranged leukemias in clinical trials. AML-iPSCs with an MLL-rearrangement were differentiated into hematopoietic cells and treated with the inhibitor and they found inhibition of colony formation and replating capability, however the inhibitor did not affect hematopoietic cells differentiated from normal iPSC controls or undifferentiated AML-iPSCs. Lastly, the authors generated iPSC lines from distinct AML subclones within the patient, i.e. KRAS wild-type and KRAS mutant which allowed them to assess the phenotype induced by a specific subclone as well as to test therapeutic options. These published examples of pluripotent stem cell models of myeloid disease have demonstrated that pluripotent stem cell model systems do in fact recapitulate features of human disease and are valuable tools to study disease mechanisms.
MPN Pluripotent Stem Cell Models

Several groups have published human MPN model systems in which they utilized patient derived induced pluripotent stem cells. Most of these studies have been proof of principle studies and demonstrated iPSC model systems do recapitulate expected phenotypes observed in patients and are therefore reliable systems for disease modeling.

Ye, et al. generated iPSC lines from peripheral blood CD34+ cells collected from one PV patient and one PMF patient, both of which harbored a heterozygous JAK2 V617F mutation. The CD34+ cells were reprogrammed via retroviral transduction using retroviral vectors that encoded the Yamanaka reprogramming factors. All clones that were successfully expanded from both patients were found to be JAK2 V617F heterozygous. They also reprogrammed CD34+ cells from cord blood and adult bone marrow that served as control lines. Following directed hematopoietic differentiation, CD34+CD34+ hematopoietic progenitors were plated to perform colony forming assays as well as to be grown in liquid culture. As expected, PV-iPSCs generated more erythroid colonies than the normal iPSC lines as well as an increased proliferation rate which recapitulates what is observed clinically in PV patients. Genes that were found to be upregulated in PV CD34+ via whole-genome microarray were also found to be upregulated in CD34+ cells generated from the PV-iPSCs utilizing quantitative PCR, e.g. NFI-B, HBG, and HBB. This data suggested that MPN iPSC model systems faithfully recapitulated phenotypic characteristics observed in MPN patients.

To further demonstrate the utility of this iPSC system the same group published a secondary manuscript in which they used their model to test three JAK inhibitors; INCB018424
(ruxolitinib), TG101348, and CYT387. They differentiated WT, JAK2 V617F heterozygous mutant, JAK2 V617F homozygous mutant, iPSC lines derived from a PV patient into CD34+CD45+ hematopoietic progenitors. The progenitors were then cultured in medium containing SCF, IL-3, and EPO known to promote erythroid differentiation and expansion. As expected, JAK2 V617F mutant iPSC derived progenitors exhibited a proliferative advantage as compared to the JAK2 WT iPSC derived progenitors and all three JAK inhibitors inhibited the observed cell proliferation. They also sorted differentiated erythroid cells and found that JAK inhibitors inhibit erythroid differentiation, supporting evidence that JAK2 plays an imperative role in erythropoiesis. This model also provides further evidence of the clinical observation that some JAK inhibitors cause anemia in MF patients. In addition to erythroid promoting medium, CD34+CD45+ HSPCs were cultured in medium containing SCF, TPO, and FL that promotes HSPC self-renewal. JAK inhibitor treatment had little effect on HSPC self-renewal as the CD34 percentages remained the same. The malignant clone is known to persist upon JAK inhibitor treatment and this model system suggested that while JAK inhibitors caused erythroid differentiation impairment, they did not affect the HSPC population which are known to be the disease initiating cells.

Saliba et al. studied heterozygous and homozygous JAK2 V617F states in MPNs utilizing an iPSC system. They generated iPSC lines from two PV patients, one harboring a homozygous JAK2 V617F mutation as well as a 20q deletion and an additional heterozygous ASXL1 mutation and from another patient harboring a heterozygous JAK2 V617F mutation without other present mutations identified. iPSC lines were generated by expressing retroviruses encoding the Yamanaka factors in CD34+ cells. Upon hematopoietic differentiation followed by colony
forming assays, a shift towards megakaryocytic colonies was observed with the JAK2 V617F heterozygous line iPSC line and the JAK2 V617F homozygous line skewed toward erythroid colony growth. These phenotypes recapitulate the phenomenon observed in the clinical setting in which JAK2 V617F homozygous mutations correlate with a PV phenotype and heterozygous JAK2 V617F mutations correlate with an ET phenotype in patients. They also tested different inhibitors to determine if genotype effects drug sensitivity in colony growth assays, however sensitivity differences were not observed between genotypes.63

Hosoi et al. generated iPSC lines from CD34+ cells collected from a primary myelofibrosis patient as well as a secondary myelofibrosis patient. The CD34+ cells were reprogrammed using a retroviral system containing the Yamanaka factors and the resulting iPSC clones were differentiated into hematopoietic cells. CD34+CD45+ hematopoietic progenitors were plated in methylcellulose to assess colony formation and significant differences were not observed when MF-iPSC colony output was compared to normal iPSC colony output. They demonstrated that the MF-iPSCs as well as the normal iPSCs could be successfully differentiated into CD41a+CD42b+ megakaryocytes. Morphologic differences were not observed when normal, pMF, and sMF derived megakaryocytes were compared. However, when the iPSC derived megakaryocytes were subjected to RT-PCR to assess gene expression patterns, IL-8, PDGFA, and TGFB1 were upregulated to some extent in the MF derived iPSCs with IL-8 having the greatest significance. This manuscript demonstrated that iPSC lines can be generated from MF patient CD34+ cells and they were able to be differentiated into functional megakaryocytes that upregulate genes involved in cytokine production of cytokines that are known implicated in myelofibrosis.64
These studies provided evidence that patient derived iPSC lines can be differentiated into lineages known to be dysregulated in MPNs and they do faithfully recapitulate features observed in MPN patients, e.g. PV derived iPSC lines presented with an erythroid proliferation phenotype as seen in PV patients. These studies also demonstrated that patient derived iPSC lines can be utilized for therapeutic testing. iPSC lines have been shown to be reliable human disease model systems, however much disease biology remains that can potentially be uncovered utilizing this patient derived system.

**In vitro Blood Differentiation**

During vertebrate development the hematopoietic system emerges in two waves, beginning with the primitive wave, which occurs in the yolk sac and gives rise to primitive erythrocytes and some myeloid lineage cells. A progenitor called the hemangioblast gives rise to hematopoietic and vascular cells during the primitive wave that transitions through hemogenic endothelium, which is known as a subset of specialized endothelial cells that can differentiate into hematopoietic cells. The second wave or definitive wave originates in the embryo proper and will continue as the permanent wave throughout adulthood. Definitive hematopoiesis emerges from specific sites of hemogenic endothelium scattered throughout the vasculature in the embryo. Definitive hematopoietic stem cells that are able to give rise to all mature blood cells including erythroid, myeloid, and lymphoid lineage cells. Cells are shown to be true hematopoietic stem cells if they are able to reconstitute the full suite of hematopoietic cells upon transplantation into irradiated adult recipient mice. While generating a transplantable hematopoietic stem cell that is capable of reconstituting the mouse hematopoietic system via *in
vitro differentiation has proven to be challenging, Sturgeon et al. has developed an in vitro differentiation system that recapitulates stages of embryonic hematopoiesis and allows for the separation of primitive and definitive programs. Upon signaling pathway analysis they found that primitive hematopoiesis is transiently dependent on the Activin-nodal pathway as opposed to definitive hematopoiesis. While this pathway is required for primitive specification, manipulation of this pathway did not affect definitive hematopoiesis, therefore they characterized the role of the Wnt-β-catenin pathway during hematopoietic development in search for a way to distinguish the definitive program from the primitive program.

Manipulation of the Wnt-β-catenin pathway during mesoderm specification, which occurs during day 2-3 of the in vitro differentiation process, allowed the differentiation process to be driven toward primitive or definitive hematopoietic progenitors. The CD235a+ primitive population increased upon inhibition of this pathway with the small molecule IWP2. In contrast, when the cultures were treated with a GSK-3 inhibitor, CHIR99021, which is a Wnt agonist, the CD235+ population was inhibited. A significant proportion of the IWP2-treated mesoderm gave rise to primitive hematopoietic progenitors that express CD43, while CHIR99021 was shown to inhibit primitive hematopoiesis shown by the absence of CD43+ cells in the CHIR99021-treated cultures. The hallmark of definitive hematopoiesis is the generation of lymphoid lineage cells as only myeloid and erythroid lineage cells emerge from the primitive program, therefore T cell potential was used as an indication of definitive hematopoiesis in this system. CD34+CD43-progenitors derived from KDR+CD235a- mesoderm treated with CHIR99021 on day 2 during mesoderm specification were capable of T- cell generation, in contrast to the CD43+ progenitors derived from the IWP2 treated KDR+CD235a+ mesoderm. For in vitro definitive
hematopoietic differentiation, the CHIR99021 treated cultures are sorted on day 8 to isolate CD34+CD43-CD73-CD184- hemogenic endothelium as well as to isolate CD34+CD43-CD73-CD184+ cells that provide NOTCH signaling that is required for the endothelial-to-hematopoietic transition. CD34+CD45+ progenitors emerge from the endothelial-to-hematopoietic transition that have erythroid, myeloid, and lymphoid potential. Modulation of the Wnt-β-catenin pathway during mesodermal patterning allows for the generation of definitive hematopoietic progenitors which is important for adult human disease modeling because unlike the published pluripotent stem cell models of MPNs, this model system recapitulates the definitive hematopoietic program that is present throughout adulthood and most relevant to the disease biology.
Figure 1.1. Schematic of the JAK-STAT signaling pathway.
Figure 1.2. Schematic of ASXL1 gene located on chromosome 20. *ASXL1* is composed of 12 exons and 1541 amino acids. The plant homeodomain (PHD) is located on the C-terminus and is important for DNA binding. The majority of *ASXL1* mutations found in myeloid malignancies are located in exon 12.
Chapter 2: Generation and differentiation of pluripotent stem cell lines harboring \textit{JAK2} and \textit{ASXL1} mutations

Introduction

Pluripotent stem cell lines were utilized as a model system to study how \textit{JAK2} and \textit{ASXL1} mutations influence MPN disease pathogenesis. Patient derived induced pluripotent stem cell lines as well as human embryonic stem cell lines harboring \textit{JAK2} and \textit{ASXL1} mutations were generated. Stem and progenitor cells were sorted from an MF patient who had acquired both a \textit{JAK2} mutation as well as an \textit{ASXL1} mutation and the sorted cells were reprogrammed in order to revert them back to a pluripotent state. In addition, \textit{JAK2} and \textit{ASXL1} mutations were introduced into the H1 embryonic stem cell line via CRISPR-mediated gene editing to generate stable pluripotent cell lines expressing these mutations. Both model systems have strengths as the iPSC lines provide an unlimited supply of physiologically relevant patient cells and the ESC model allows for a human system in which the observed phenotype is due to the introduced mutations alone and not other disease modifying factors that may be unknowingly present in the patient derived cells. iPSC lines and ES lines have the capacity to differentiate into any cell type in the human body and specifically for the focus of this project they can be directed towards specific hematopoietic lineages.

A hematopoietic differentiation protocol has been developed that directs differentiation towards either the primitive or definitive program.\textsuperscript{67} This is relevant because the definitive program gives rise to the hematopoietic stem cell that is responsible for differentiating into all cell types that
comprise the hematopoietic system in vivo. The patient derived iPSCs as well as the H1 embryonic stem cells were differentiated into primitive and definitive progenitors separately to characterize and compare hematopoietic phenotypes induced by JAK2 and ASXL1 mutations. While primitive experiments were performed and an erythroid expansion phenotype was observed in response to the JAK2 mutation, the primary focus of this project was on definitive hematopoiesis as this program gives rise to the full suite of hematopoietic cells in the adult human.

**Methods**

**Patient sample sorting**

Cryopreserved patient peripheral blood mononuclear cells (PBMCs) were thawed in the 37 degree C water bath. The thawed cells were transferred to a 50 mL conical tube and 9 mL of RPMI plus containing 10% FBS + 1% penicillin-streptomycin + 1-2 mM L-glutamine + 20 U/mL Na heparin (Sigma 2106) + 0.025 U/mL benzonase was added dropwise. The cells were spun at 250 X g for 5 min with 50% brake. The supernatant was removed and the pellet was resuspended in 10 mL of RPMI plus. The cells were then filtered using a 70 µM filter and 10 uL was collected to count the cells. 100,000 cells were transferred to 6 FACS tubes to stain as unstained and fluorescence minus one (FMO) controls; unstained, unstained + 7AAD, FMO for CD34 on FITC, FMO for CD38 on APC, FMO for PE, and FMO for 7AAD. The samples and controls were spun down and resuspended in live cell buffer (LCB) containing DPBS + 2% FBS + 1mM EDTA. The samples and controls were resuspended in 49 µL per 1.5 million cells. 20 µL of Fc receptor binding inhibitor per 1.5 million cells was added to the cell suspensions and the
samples were incubated on ice for 20 minutes. The antibodies were added according to the table below and incubated for 30 minutes on ice.

<table>
<thead>
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<th>Antibody</th>
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<th>Volume per 1.5 million cells</th>
<th>Vendor</th>
<th>Catalog number</th>
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</thead>
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<td>10 µL</td>
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</tr>
</tbody>
</table>

The samples were then washed twice with LCB. The samples were resuspended in a minimum volume of 500 µL and filtered through strainer caps before taken to the Synergy or MoFlo fluorescence-activated cell sorting instruments for sorting. CD34+ cells were collected. Representative gating strategy is shown in Figure 2.1.

**Human iPSC line generation**

The CytoTune-iPS 2.0 Sendai Reprogramming kit from Life Technologies (#A16517) was utilized to reprogram sorted patient CD34+ cells into induced pluripotent stem cell lines. The protocol provided by Life Technologies was followed. CD34+ cells were plated into 2 wells of a 24-well plate in complete StemPro-34 medium containing cytokines including 100 ng/mL SCF, 50 ng/mL IL-3, and 25 ng/mL GM-CSF on day -3. On day -2 0.5 mL of medium was replaced with 0.5 mL of fresh StemPro-34 containing cytokines. Similarly, on day -1 0.5 mL of medium was replaced with 1 mL of fresh StemPro-34 with cytokines. The transduction was performed on day 0 using the CytoTune 2.0 Sendai reprogramming vectors at the recommended MOI in StemPro-34 containing cytokines with the addition of 4 µg/mL of Polybrene. After an overnight
incubation on day 1, the medium was replaced with fresh StemPro-34 containing cytokines to remove the reprogramming vectors. On day 3 the cells were plated on mouse embryonic fibroblast (MEF) cultures that were prepared the day prior utilizing StemPro-34 without cytokines. Half of the medium was replaced with fresh StemPro-34 without cytokines every other day for 3 days. 7 days post transduction the cultures were transitioned to iPSC/hES medium by replacing half of the StemPro-34 without cytokines with iPSC/hES medium. iPSC/hES medium was utilized for pluripotent stem cells grown on MEFs and contained DMEM/F12 + Glutamax, 20% knock out serum, 0.1 mM non-essential amino acids, 1X beta-mercaptoethanol, 1% penicillin-streptomycin, and 10 ng/mL bFGF. All medium was replaced with fresh iPSC/hES medium daily 8 to 28 days post transduction and the cultures were monitored for the emergence of iPSC colonies. Individual iPSC colonies were then picked and transferred to individual wells of MEF cultures for expansion and cryopreservation.

**Human ESC line generation**

The following methods were provided by Yi-Hsien Chen, the assistant director of the GEiC. hJAK2 and hASXL1 mutant clones were generated by the Genome Engineering and iPSC Center (GEiC) at Washington University in St. Louis utilizing the human H1 embryonic stem cell line (WA01, WiCell) as the parental line. 1x10^6 single cells were washed in DPBS and resuspended in P3 primary buffer (Lonza) containing gRNA/Cas9 ribonuclease protein (RNP) complex (200 pmol gRNA + 80 pmol SpCas9). The CA-137 program was then utilized for electroporation with a 4D-Nucleofector (Lonza). Mutated cells were confirmed with junction PCRs following nucleofection and then single-cell sorted. Targeted deep sequencing analysis using primer sets specific to the target regions of interest were utilized to screen the single cell clones. 

**Synthetic gRNA (IDT):**

hJAK2(V617F): 5’ AATTATGGAGTATGTGTCTG 3’
hASXL1(p920T): 5’ CCAACCTGGGGCTCAACAGA 3’
hASXL1(dupG): 5’ CGGCCACCACTGCCATCGGA 3’

hJAK2(V617F) ssODN (IDT):
5’tttttgaagcagcagatgagcagctttctcacaageatttggtttaattaCggagtatgtTctctggagacgaagtaagtaaaactacagtttctaatgcctctcagagcatcttg 3’
hASXL1(p920T) ssODN (IDT):
5’cccatcccatgagatgagcagctttctcacaageatttggtttaattaCACgtagctttctcagagcatcttg 3’
hASXL1(dupG) ssODN (IDT):
5’tgctgctgcctctgccacctccctcatcggtggccccgccacctccacccctcagttggggatttg 3’

**Pluripotent stem cell maintenance**

Human pluripotent stem cells (hPSCs) were cultured in DMEM/F12 + Glutamax, 20% knock out serum replacement, 0.1mM non-essential amino acids, 1X beta-mercaptoethanol, 1% penicillin-streptomycin, and 10 ng/mL bFGF and were grown on gamma-irradiated MEFs for expansion prior to differentiation. Every 24 hours the media was replaced until 70-80% confluency was reached every 5-7 days. The cells were passaged using 0.25% trypsin for one minute. 24 hours prior to passaging for a differentiation assay, 6-well plates were coated with matrigel and placed in the incubator overnight. The hPSCs were then collected and distributed onto matrigel coated plates and placed in the incubator overnight for differentiation to begin the following day.

**Immunofluorescence Staining**

iPS cells were grown on mouse embryonic fibroblasts in 12 or 24 well plates until 50-70% confluent. The media was removed and the cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature. The cells were washed with 0.1% tween-20 in PBS for 5 minutes at room temperature twice. For intracellular antibodies (Oct-4) the cells were permeabilized with 1% triton X in PBS for 30 minutes at room temperature. All wells were then blocked using 4% goat serum diluted in PBS for 30 minutes at room temperature. The primary antibodies were added at 1:200 dilution in PBS and incubated overnight at 4 degrees C. The cells were then
washed with 0.1% tween-20 in PBS for 5 minutes at room temperature 3 times. For detection the cells were then stained with the secondary antibodies at a dilution of 1:300 in PBS for 1 hour at room temperature. Again, they were washed with 0.1% tween-20 in PBS for 5 minutes at room temperature 3 times. The antibodies utilized are in the table below:

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<thead>
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<th>Primary Antibody</th>
<th>Vendor</th>
<th>Catalog Number</th>
<th>Secondary Antibody</th>
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</table>

**Pluripotent stem cell differentiation**

The hematopoietic differentiation protocol utilized in this study was published by Andrea Ditadi and Christopher Sturgeon. On differentiation day 0, the hPSCs were harvested as cell clumps from matrigel plates and cultured in suspension in serum-free differentiation (SFD) media with 10 ng/mL BMP4 to form embryoid bodies (EBs) directed towards mesoderm specification. These cultures were placed in an incubator set to hypoxic conditions (5% CO2, 5% O2, 37°C) and they continued to be cultured in hypoxia for the duration of the differentiation protocol. 24 hours later on differentiation day 1, SFD containing 10 ng/mL BMP4 and 10 ng/mL bFGF was added to the cultures. On day 2, the media was replaced with SFD containing 3 µM CHIR99021, a Wnt agonist, to generate definitive hematopoietic progenitors or containing 3 µM IWP2, a Wnt antagonist to drive differentiation towards the primitive program. On day 3, the media was replaced with StemPro-34 (SP34) media to specify hemogenic endothelium. SP34 media containing several hematopoietic promoting cytokines was added to the cultures on day 6. On day 8 the EBs were collected and digested using trypsin-EDTA and collagenase II.
CD34+CD43-CD73-CD184- hemogenic endothelium as well as CD34+CD43-CD184+ cells, to provide notch signaling, were isolated via FACS. Following the sort, 5,000 CD34+CD43-CD73-CD184- cells and 5,000 CD34+CD43-CD184+ cells were plated in 50uL of cytokine rich SP34 media into 96-well LoCluster plates and placed in the hypoxic incubator overnight. On the same day as the sort, a 24-well plate was coated with matrigel and placed in the incubator overnight. On the following day, the 50uL cell suspensions were spotted onto each well of the 24-well plate and 4 to 6 hours later flooded with 1mL of the cytokine rich SP34 media. These cells were cultured in the hypoxic incubator for 7 to 9 days and then CD34+CD45+ hematopoietic cells were isolated by FACS and plated in methylcellulose for colony forming assays. Below is a table of the antibodies utilized for FACS.

<table>
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<th>Volume (100uL reaction)</th>
<th>Vendor</th>
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**Colony forming assays**

20,000 bulk culture primitive cells or 2,500 sorted CD34+CD45+ definitive progenitors were resuspended in 1 mL of methocult per plate. 1mL was plated per 35mm plate using a 16 gauge needle. Definitive progenitors were plated in methocult #2 and primitive cells were plated in the methocult indicated per experiment. (Table 2.1) Colonies were scored and counted after 10-14 days of incubation.
Results

CD34+ cells were FACS-sorted from peripheral blood mononuclear cells collected from a 64-year-old, female MF patient who was diagnosed with primary MF (Figure 2.1). The patient harbored the JAK2 V617F mutation with a variant allele frequency of 57% as well as an ASXL1 P920Tfs*4 mutation with a variant allele frequency of 28% and lacked other known driver mutations, verified by targeted sequencing utilizing a panel of known MPN driver mutations performed in the clinic. The sorted CD34+ cells were reprogrammed using the Sendai virus reprogramming method and 16 distinct iPSC clones were generated, including 3 JAK2 homozygous mutant/ASXL1 heterozygous mutant clones, 9 JAK2 heterozygous mutant/ASXL1 heterozygous mutant, and 4 JAK2 heterozygous mutant/ASXL1 wild type. These iPSC line genotype ratios were expected based on the variant allele frequencies of these mutations. The JAK2 mutation burden was greater than 50% which might suggest that all of the patient cells had a heterozygous JAK2 mutation and some had acquired a homozygous JAK2 mutation. We also expected fewer double mutant lines compared to JAK2 mutant only lines because the ASXL1 mutation burden was 28% compared to the 57% mutant JAK2 burden. Upon pilot differentiation assays utilizing several of the iPSC lines, clone 19 (JAK2 Het/ASXL1 WT) and clone 11 (JAK2 HET/ASXL1 Het) were chosen as the primary experimental lines to be assessed based on their ability to generate hematopoietic progenitors. Both clone 19 and clone 11 were karyotypically normal (Figure 2.2A,B) and expressed several pluripotency markers that were assessed via immunofluorescence assay (Figure 2.2C). The original banked patient-specific iPSC line inventory lacked a JAK2 WT control iPSC line for the differentiation assays because neither reprogramming generated a JAK2 WT iPSC clone. Again, the inability to generate a WT control
line was anticipated based on the JAK2 allele burden being greater than 50% which would suggest that there may not have been JAK2 WT cells within the patient sample. Clone 19 (JAK2 Het/ASXL1 WT) was submitted to the Genome Engineering and iPSC Center (GEiC) at Washington University and the core facility performed the karyotyping and reverted the JAK2 mutation via CRISPR-mediated gene editing in order to generate an isogenic JAK2 WT/ASXL1 WT iPSC control line. To eliminate the potential of any clone-to-clone variability, clone 11 (JAK2 Het/ASXL1 Het) was also submitted to the GEiC and all of the possible genotypes were derived from this single parental clone. Both mutations were reverted and JAK2 Het/ASXL1 WT, JAK2 WT/ASXL1 Het, and JAK2 WT/ASXL1 WT lines were each generated from clone 11 via CRISPR-mediated gene editing.

JAK2 V617F mutant clone 19 was compared to JAK2 WT clone 1D11, i.e. corrected clone 19, utilizing primitive differentiation assays (Figure 2.3). Following differentiation, 20,000 primitive hematopoietic progenitors were plated in methylcellulose to assess colony growth. Myeloid colony output was variable from experiment to experiment with either the JAK2 mutant line producing more myeloid colonies than the JAK2 WT line or the two lines producing similar numbers of myeloid colonies (Figure 2.4). However, in each experiment the JAK2 mutant line generated more erythroid colonies as compared to the JAK2 WT corrected line (Figure 2.4 B,C,D). Optimization experiments were required to observe the primitive erythroid phenotype, because the first two primitive differentiation assays did not yield or yielded very few erythroid colonies from either the JAK2 WT or JAK2 mutant iPSC lines (Figure 2.4 A,B). In order to optimize primitive erythroid colony growth, several methocult formulations were tested (Table 2.1). Differentiated H1 embryonic stem cell primitive progenitors were plated into three different
methocult cocktails to dissociate from variability that the iPSC lines may impose (Figure 2.5). Myeloid and erythroid colonies were generated in all three methocult formulations, however methocult #1 generated nearly double the number of erythroid colonies when compared to the other two methocult formulations. The JAK2 WT and JAK2 mutant primitive progenitors differentiated from the iPSC lines were plated in all three methocult formulations and as seen previously, erythroid progenitors were generated by the JAK2 mutant cells in methocult #1 predominantly (Figure 2.4 C,D).

The patient-derived iPSC lines were also differentiated into definitive hematopoietic progenitors that were generated via endothelial to hematopoietic transition (Figure 2.3). The definitive hematopoietic progenitors were plated in methylcellulose to assess colony formation potential of the WT, JAK2 mutant, ASXL1 mutant, and double mutant iPSC lines. Consistently across experiments, lack of erythroid colony growth was observed in all of the iPSC lines irrespective of genotype. To verify that lack of erythroid colony growth was not due to a technical or human error, a differentiation was performed in tandem with Stephanie Luff, a post-doc in the Sturgeon lab who is an expert in the hematopoietic differentiation protocol, utilizing H1 embryonic stem cells. A subset of each of our differentiated progenitors were plated by the other person in order to confirm that variability within the plating process did not cause a loss in erythroid colony potential. Stephanie and I differentiated human H1 embryonic stem cell lines into definitive hematopoietic progenitors that generated myeloid as well as many erythroid colonies successfully (Figure 2.6). Since the H1 line differentiation experiment resulted in erythroid colony growth, the same technique was utilized to repeat the differentiation assays with the iPSC lines. Several subsequent definitive differentiation experiments were performed with the iPSC lines.
lines following the side-by-side experiment and the iPSC lines continued to only generate myeloid colonies which confirmed the lack of erythroid colony formation was specific to the patient-derived iPSC lines.

As a complementary approach and an attempt to characterize an erythroid phenotype, the mutations of interest were also knocked into H1 embryonic stem cells. Two different ASXL1 mutations were introduced into H1 embryonic stem cells including the same mutation that the patient-derived iPSC lines harbor (P920Tfs*4) as well as the most commonly found ASXL1 mutation in myeloid malignancies (G646Wfs*12). Each mutation was introduced via CRISPR-mediated gene editing by the GEiC either in isolation or in combination with the JAK2 V617F mutation generating single and double mutant ES cell lines.

Three primitive differentiation experiments were performed utilizing mutant H1 ES cell lines including the ASXL1 mutant and double mutant lines harboring ASXL1 P920Tfs*4. Following stepwise differentiation regulated by a Wnt antagonist, the primitive progenitors were plated in methylcellulose to assess how JAK2 and ASXL1 mutations influence colony output. While the total colony number phenotype was inconsistent between experiments, the JAK2 mutant differentiated primitive progenitors consistently generated the greatest number of primitive erythroid colonies as well as the highest proportion of erythroid colonies as compared to WT, ASXL1 mutant, or double mutant primitive progenitors. The ASXL1 mutant line generated less erythroid colonies than the JAK2 mutant line in all three experiments and less than the double mutant line in two of the experiments (Figure 2.7). The focus was then shifted to definitive differentiation experiments because the definitive hematopoietic program is more
physiologically relevant as the adult hematopoietic system arises as a result of the definitive program.

Discussion

While mouse models are powerful tools to study disease biology, *Asxl1* mutation knock in mouse models present with a relatively modest phenotype, following a very long latency period. Neither *JAK2* nor *ASXL1* mouse models fully recapitulate the full spectrum of human disease phenotypes, therefore a human model system has the potential to provide a useful tool to better understand how these mutations affect disease pathophysiology. Two complementary pluripotent stem cell approaches were generated to study how *ASXL1* mutations influence MPN pathogenicity. iPSC lines provide a physiologically relevant disease model system because they are MPN patient cell derived and they also provide a potentially unlimited supply of cells. Upon primitive hematopoietic specification, MF patient derived *JAK2* mutant iPSC lines generated an increase in erythroid colonies as compared to WT iPSC lines. This observed erythroid phenotype is supported by previously published studies that utilized MPN iPSC lines to demonstrate an erythroid phenotype associated with mutant *JAK2*.61,63 *JAK2* mutant patient derived iPSC lines have been shown to generate enhanced erythroid colony output *in vitro* in several studies which recapitulate the expansion of erythroid cells observed clinically in *JAK2* mutant PV patients. These data suggest modeling the *JAK2* mutation utilizing iPSC lines is a biologically relevant system to study disease causing mutations *in vitro*.

While the published *JAK2* mutant iPSC studies recapitulated clinical features found in PV patients, they did not specify primitive or definitive hematopoietic programs. The lack of Wnt
modulation during mesodermal patterning is an indication that the hematopoietic progenitors that were generated as a result of the differentiation process were a combination of primitive and definitive progenitors. It may be necessary to model the definitive program in order to better understand the disease biology because hematopoietic stem cells that give rise to all hematopoietic cells in the adult human emerge from the definitive program while the primitive wave is hematopoietic stem cell independent. In contrast to previously published MPN iPSC studies, this thesis project focused on the definitive program as an attempt to more accurately model adult hematopoiesis as MPNs are adult diseases caused by acquired somatic mutations. The differentiation protocol published by Christopher Sturgeon and Andrea Ditadi\textsuperscript{69} allows for hematopoietic differentiation to be directed toward either the primitive or definitive program. The MF patient derived iPSC lines were directed toward the definitive hematopoietic program as evidenced by lack of CD43, a primitive progenitor marker, expression on day 8 of differentiation.

Several differentiation experiments resulted in almost exclusively myeloid lineage cells with a striking lack of erythroid colony formation. It is essential to assess the erythroid phenotype associated with these mutations \textit{in vitro} because of the strong \textit{in vivo} phenotype correlated with the \textit{JAK2} mutation in patients. For the \textit{in vitro} model system to be an accurate portrayal of the disease biology, the erythroid expansion phenotype observed with the expression of mutant \textit{JAK2} in patients should be recapitulated. For these reasons, several different manipulations to the protocol were tested in attempt to grow erythroid colonies from the differentiated iPSC derived hematopoietic progenitors. The progenitors were plated in two individual methylcellulose formulations (H4435 and H4034) and they yielded similar results with
predominantly myeloid colony generation, as seen previously. Perhaps a temporal discrepancy was causing erythroid cells to emerge earlier or later than myeloid colonies in the methylcellulose cultures. To determine if an adjusted time point might capture the erythroid progenitors, multiple methylcellulose plating time points were tested following endothelial to hematopoietic transition culture. None of the tested time points resulted in erythroid colony formation following differentiation of the patient derived iPSC lines. In addition, the cells were cultured in another incubator post methylcellulose plating in order to confirm incubator specifications in relation to settings or gas composition were not negatively influencing colony output. Wilson Fok, a post-doc in Luis Batista’s lab, plated a subset of my differentiated hematopoietic progenitors in methylcellulose to rule out any technical differences when plating that may be causing the loss of erythroid potential. These described experimental adjustments that failed to result in erythroid colony formation led to a differentiation experiment performed in tandem with Stephanie Luff, who is an expert in regard to this specific hematopoietic differentiation protocol. Stephanie and I each completed a differentiation experiment simultaneously using H1 ES cells and then plated some of our own cells as well as the other person’s differentiated progenitors in methylcellulose. Both experiments resulted in a high proportion of erythroid colony growth which suggested the lack of erythroid colony formation was an issue specific to the iPSC lines and not due to a technical error. Exome sequencing was performed and there were no obvious acquired mutations found in the iPSC lines that might explain erythroid differentiation impairment. Because the iPSC lines did not allow for the erythroid phenotype to be assessed despite multiple optimization attempts, the mutations were introduced into the H1 embryonic stem cell line as an alternative human in vitro approach.
As mentioned in the results section, \textit{JAK2} mutant, \textit{ASXL1} mutant, and double mutant H1 ES cell lines were generated via CRISPR-mediated gene editing at the GEiC at Washington University in St. Louis. Two specific \textit{ASXL1} mutations were introduced, including the \textit{ASXL1} mutation found in the patient derived iPSC lines which allowed for comparison of two different pluripotent stem cell lines (iPSC and ESC) harboring the same mutation. The most common \textit{ASXL1} mutation found in myeloid malignancies was also introduced to validate that two distinct \textit{ASXL1} mutations behaved in a similar fashion in this pluripotent stem cell model system. Corroborating phenotypes induced by the \textit{ASXL1} mutations with at least two \textit{ASXL1} mutations was important to ensure the reported phenotypes were not specific to a single \textit{ASXL1} mutation. We also received two clones for each genotype to confirm any observed phenotypes are not specific to a particular clone that may be an outlier.

Three differentiation experiments were performed in which the lines were directed toward the primitive hematopoietic program. The \textit{JAK2} mutant ESC line generated more primitive erythroid colonies than the WT, \textit{ASXL1} mutant, and double mutant lines. The \textit{JAK2} mutation consistently presented with an erythroid expansion phenotype when expressed in both the iPSC lines as well as the ESC lines when directed toward primitive hematopoietic differentiation, which demonstrated the reliability of both of these systems as human \textit{in vitro} disease models. While the \textit{ASXL1} mutant lines consistently generated fewer primitive erythroid colonies than the \textit{JAK2} mutant lines, the primitive differentiation phenotype induced by the \textit{ASXL1} mutation was much less consistent when compared to the WT and double mutant lines. Fewer erythroid colonies were generated by the \textit{ASXL1} mutant line than the \textit{JAK2} and \textit{ASXL1} double mutant line in two out of the three replicates. \textit{ASXL1} mutant line differentiation also resulted in primitive
erythroid colonies comprising a smaller proportion of the total colonies when compared to the
double mutant line in two of the replicates. While the primitive mutant ESC line differentiation
experiment results were not entirely consistent amongst replicates, the data suggested the JAK2
mutation caused an increase in erythroid colony generation while the ASXL1 mutation blunted
total colony output as well as resulted in a loss of erythroid colony formation even in the
presence of mutant JAK2. JAK2 mutant hematopoietic progenitors generated more erythroid
colonies than the ASXL1/ JAK2 double mutant progenitors in each experiment which suggested
the ASXL1 mutation may partially rescue the erythroid phenotype induced by the JAK2 mutation.
To fully characterize the primitive hematopoietic phenotype associated with JAK2 and ASXL1
mutations, more primitive differentiation experiments are required, however the project shifted
toward definitive hematopoietic differentiation assays to more accurately model these disease-causing mutations that are acquired in the stem and progenitor compartment. Definitive
hematopoiesis occurs in the embryo proper and continues throughout adulthood to generate the
full suite of hematopoietic cells and therefore this hematopoietic stem cell dependent program
may be the more appropriate focus as MPNs are adult diseases caused by acquired somatic
mutations.
Table 2.1. **Methocult formulations tested in primitive differentiation assays.** Methocult #1 is a recipe developed by Christopher Sturgeon and Methocult #2 and #3 were purchased from Stem Cell Technologies.
Figure 2.1. Representative gating strategy utilized to sort lineage negative CD34+ cells from patient PBMCs.
Figure 2.2. Karyotyping and immunofluorescence staining of induced pluripotent stem cell clones. (A) Clone 19 (JAK2 het mutant/ASXL1 WT) and (B) clone 11 (JAK2 het mutant/ASXL1 het mutant) exhibited normal karyotypes confirmed by karyotyping analysis performed by the GEiC. (C) Immunofluorescence staining revealed 4 iPSC clones as well as the H1 embryonic stem cell control line express the indicated pluripotency factors.
Figure 2.3. Schematic of hematopoietic differentiation protocol. (A) On day 0 pluripotent stem cells were trypsinized and cultured in BMP4 to form embryoid bodies. Wnt pathway manipulation on day 2 directed differentiation to generate CD43+ primitive progenitors or CD34+CD43- definitive progenitors. (B) On day 8 of differentiation 5000 CD34+CD43-CD184-
CD73- and 5000 CD34+CD43-CD184+ cells were sorted and plated onto matrigel on day 9. As endothelial to hematopoietic transition occurred CD34+CD45+ definitive progenitors emerged and were sorted on day 15.
Figure 2.4. Colony forming assays utilized primitive hematopoietic progenitors differentiated from patient derived iPSC lines. (A)(B) Colony output from 20,000 $JAK2$ mutant and $JAK2$ WT primitive differentiated progenitors plated in methocult. (C)(D) Colony output from 20,000 $JAK2$ mutant and $JAK2$ WT primitive progenitors plated in 3 different
methocult formulations described in Table 2.1. Black represents myeloid colonies and grey represents erythroid colonies. Error bars represent SEM of technical replicates plated in triplicate.
Figure 2.5. 20,000 primitive progenitors differentiated from H1 embryonic stem cells were plated in 3 different methocult formulations. Black represents myeloid colony number and gray represents erythroid colony number. Error bars represent SEM of technical replicated plated in triplicate.
Figure 2.6. Colony forming assay of differentiated definitive progenitors derived from H1 ES cells performed in tandem with Stephanie Luff. The first set of initials indicates the person who was responsible for differentiating the cells and the second indicates who was responsible for plating them. Colony counts are shown as colony number per 2000 CD34+CD43-CD184-CD73- cells. Colors represent the colony type. Error bars show SEM of technical replicates plated in duplicate.
Figure 2.7. Colony forming assays utilizing primitive hematopoietic progenitors differentiated from mutant H1 ES cell lines. Colonies counted per 20,000 cells plated in methocult. DKI is double knock in which harbors both the JAK2 as well as the ASXL1 mutation. Black represents myeloid colonies and grey represents erythroid colonies counted. Error bars represent SEM of technical replicates plated in duplicate.


Chapter 3: *ASXL1* mutations impair erythroid differentiation in a pluripotent stem cell model system

**Introduction**

As previously discussed, pluripotent stem cells provide a human disease model system in which mutations can be introduced and/or corrected as a way to study the impact specific genetic alterations have on disease biology. An advantage to using pluripotent stem cell lines is individual mutations can be studied on the same human parental cell background as an isogenic, controlled system. Human embryonic stem cell lines harboring *JAK2* V617F, *ASXL1* P920Tfs*4*, *ASXL1* G646Wfs*12 as well as each *ASXL1* mutation in combination with *JAK2* V617F that have been generated by the GEiC at Washington University were differentiated toward the definitive hematopoietic program. CD34+CD45+ hematopoietic progenitors were sorted from each genotype and characterized via colony forming unit assays and RNA sequencing analysis. Three independent sets of clones were utilized, each including a *JAK2* mutant, *ASXL1* mutant, and double mutant clone. Validating observed phenotypes with three sets of clones including two independent *ASXL1* mutations provided confidence that the observed phenotypes were an accurate representation of the introduced mutations and not a reflection of one or more abnormal clones. While the data from three sets of clones was not identical, similar trends were observed confirming that the phenotypes were due to *JAK2* V617F and *ASXL1* mutations.

*JAK2* V617F has been extensively studied in various MPN model systems, however less is known about how *ASXL1* mutations influence MPN pathogenesis, even though *ASXL1* mutations
correlate with a worse prognosis for patients. A lack of studies exists interrogating the impact of harboring both ASXL1 and JAK2 mutations in combination. These mutations often co-occur in MPN patients providing reason to study these mutations in isolation as well as in combination. CRISPR technology in conjunction with the described hESC derived hematopoietic differentiation system allowed JAK2, ASXL1, and double mutant human pluripotent stem cell lines to be phenotypically characterized as compared to an isogenic wild type control.

**Methods**

**Pluripotent stem cell maintenance**

Refer to chapter 2 methods

**Pluripotent stem cell differentiation**

Refer to chapter 2 methods. Definitive differentiation assays were performed. On day 2, the media was replaced with SFD containing 3 µM CHIR99021, a Wnt agonist, to generate definitive hematopoietic progenitors

**Colony forming assays**

2,500 sorted CD34+CD45+ definitive progenitors were resuspended in 1 mL of methocult per plate. 1 mL was plated per 35mm plate using a 16-gauge needle. Methocult #2 was utilized for definitive differentiation assays. (Table 2.1) Colonies were scored and counted after 10-14 days of incubation at 37 degrees C.

**Flow cytometry**

Methylcellulose cultures containing suspended colonies were scraped into 15 mL conical tubes and spun at 500 x g for 5 mins. The methylcellulose supernatant was aspirated and the cell pellets were washed three times with live cell buffer (LCB) containing DPBS + 2% FBS + 1mM
EDTA. The cell pellets were resuspended in 75 µL of LCB and stained with CD235 (5 µL PerCP/Cy5.5 BioLegend #306614) and CD71 (20µL PE BD #561938) antibodies for 30min at room temperature. The cells were washed twice and analyzed using a BD Fortessa flow cytometer. Data was analyzed using Cytobank.

**RNA sequencing**

RNA sequencing was performed by the Genome Technology Access Center (GTAC) at Washington University in St. Louis.

The following sequencing methods were provided by Toni Sinnwell at the GTAC.

Total RNA integrity was determined using Agilent Bioanalyzer. Library preparation was performed with 10ng of total RNA with a Bioanalyzer RIN score greater than 8.0. ds-cDNA was prepared using the SMARTer Ultra Low RNA kit for Illumina Sequencing (Takara-Clontech) per manufacturer’s protocol. cDNA was fragmented using a Covaris E220 sonicator using peak incident power 18, duty factor 20%, cycles per burst 50 for 120 seconds. cDNA was blunt ended, had an A base added to the 3’ ends, and then had Illumina sequencing adapters ligated to the ends. Ligated fragments were then amplified for 12 cycles using primers incorporating unique dual index tags. Fragments were sequenced on an Illumina NovaSeq-6000 using paired end reads extending 150 bases.

Bioinformatic methods were provided by Elliott Klotz at the GTAC.

Basecalls and demultiplexing were performed with Illumina’s bcl2fastq software and a custom python demultiplexing program with a maximum of one mismatch in the indexing read. RNA-seq reads were then aligned to the Ensembl release 76 primary assembly with STAR version 2.5.1a. Gene counts were derived from the number of uniquely aligned unambiguous reads by
Subread:featureCount version 1.4.6-p5. Isoform expression of known Ensembl transcripts were estimated with Salmon version 0.8.2. Sequencing performance was assessed for the total number of aligned reads, total number of uniquely aligned reads, and features detected. The ribosomal fraction, known junction saturation, and read distribution over known gene models were quantified with RSeQC version 2.6.2.

All gene counts were then imported into the R/Bioconductor package EdgeR and TMM normalization size factors were calculated to adjust for samples for differences in library size. Ribosomal genes and genes not expressed in the smallest group size minus one samples greater than one count-per-million were excluded from further analysis. The TMM size factors and the matrix of counts were then imported into the R/Bioconductor package Limma. Weighted likelihoods based on the observed mean-variance relationship of every gene and sample were then calculated for all samples with the voomWithQualityWeights. The performance of all genes was assessed with plots of the residual standard deviation of every gene to their average log-count with a robustly fitted trend line of the residuals. Differential expression analysis was then performed to analyze for differences between conditions and the results were filtered for only those genes with Benjamini-Hochberg false-discovery rate adjusted p-values less than or equal to 0.05.

For each contrast extracted with Limma, global perturbations in known Gene Ontology (GO) terms, MSigDb, and KEGG pathways were detected using the R/Bioconductor package GAGE to test for changes in expression of the reported log 2 fold-changes reported by Limma in each term versus the background log 2 fold-changes of all genes found outside the respective term.
The R/Bioconductor package heatmap3 was used to display heatmaps across groups of samples for each GO or MSigDb term with a Benjamini-Hochberg false-discovery rate adjusted p-value less than or equal to 0.05. Perturbed KEGG pathways where the observed log 2 fold-changes of genes within the term were significantly perturbed in a single-direction versus background or in any direction compared to other genes within a given term with p-values less than or equal to 0.05 were rendered as annotated KEGG graphs with the R/Bioconductor package Pathview.

To find the most critical genes, the raw counts were variance stabilized with the R/Bioconductor package DESeq2 and was then analyzed via weighted gene correlation network analysis with the R/Bioconductor package WGCNA. Briefly, all genes were correlated across each other by Pearson correlations and clustered by expression similarity into unsigned modules using a power threshold empirically determined from the data. An eigengene was then created for each de novo cluster and its expression profile was then correlated across all coefficients of the model matrix. Because these clusters of genes were created by expression profile rather than known functional similarity, the clustered modules were given the names of random colors where grey is the only module that has any pre-existing definition of containing genes that do not cluster well with others. These de-novo clustered genes were then tested for functional enrichment of known GO terms with hypergeometric tests available in the R/Bioconductor package clusterProfiler.

Significant terms with Benjamini-Hochberg adjusted p-values less than 0.05 were then collapsed by similarity into clusterProfiler category network plots to display the most significant terms for each module of hub genes in order to interpolate the function of each significant module. The information for all clustered genes for each module were then combined with their respective statistical significance results from Limma to determine whether or not those features were also found to be significantly differentially expressed.
Results

Human embryonic stem cells (hESC) expressing mutant JAK2, mutant ASXL1, or both mutant JAK2 and mutant ASXL1 were differentiated toward the definitive hematopoietic program. On day 8 of differentiation CD34+CD43-CD184-CD73- hemogenic endothelium was sorted (Figure 3.1 A) and further cultured to generate CD34+CD45+ hematopoietic progenitors which were isolated on day 15 via FACS and utilized for downstream assays including colony forming assays as well as RNA sequencing (Figure 3.1 B). There was variability in hemogenic endothelium as well as CD34+CD45+ progenitor percentages across genotypes, however there were no consistent differences in progenitor compartment frequencies in relation to mutation status.

CD34+CD45+ hematopoietic progenitors were plated in methocult to assess colony output. Two individual ASXL1 mutations as well as two independent sets of clones both of which included ASXL1 and double mutant lines harboring ASXL1 P920Tfs*4 were evaluated. When the first set of WT, JAK2 mutant, ASXL1 P920Tfs*4 and double mutant lines were characterized the JAK2 V617F induced an erythroid expansion phenotype as indicated by a significant increase in number of erythroid colonies or BFU-Es generated by the JAK2 mutant line as compared to all three other genotypes. The ASXL1 mutation blocked erythroid colony formation and the ASXL1 mutant line generated significantly fewer erythroid colonies than the JAK2 mutant and WT lines (Figure 3.2 A). Similar trends were observed when BFU-E colony proportion of total colonies was compared as ASXL1 mutant progenitors generated a significantly smaller percentage of erythroid colonies than the JAK2 mutant or WT hESC lines (Figure 3.2 B). Not only did the
JAK2 mutation result in an increase of BFU-E colony frequency, but morphologically the BFU-E colonies were larger than WT and double mutant BFU-E colonies (Figure 3.2 C). Colonies were collected and stained with CD71 and CD235 antibodies to validate the colony forming unit assay counting results with flow cytometry. In preliminary analysis there was a suggestion that the JAK2 mutant line derived colonies presented with a more prominent CD71+CD235+ population and this population was less pronounced in the ASXL1 mutant colonies (Figure 3.2 D). These flow cytometry experiments utilizing colonies will have to be repeated to generate more clear, convincing data.

The JAK2 mutant line generated the highest number of total colonies and significantly more than the ASXL1 mutant and double mutant hESC lines. ASXL1 mutant progenitors also generated significantly fewer overall colonies than the WT line (Figure 3.3 A,B). The overall number and percentage of myeloid colonies generated was also assessed. While there were no significant differences in overall myeloid colony number, when evaluated as percentage of total colony number the ASXL1 mutation skewed colony production toward the myeloid lineage with a significantly higher percentage of myeloid colonies than JAK2 mutant or WT cell lines (Figure 3.3 C,D).

The described phenotypes observed with the first set of clones were confirmed with a second set of independent clones including clones harboring JAK2 V617F, ASXL1 P920Tfs*4 (same as previous set), both mutations, as well as a WT clone. Similar trends as previously described were observed as the second JAK2 mutant clone validated the observed erythroid proliferation
phenotype and generated significantly more erythroid colonies either by overall BFU-E numbers or percentage, than the WT, ASXL1 mutant, or double mutant lines (Figure 3.4 A,B). When total colony numbers were assessed with the second set of clones, the JAK2 mutant line also generated the most colonies and the ASXL1 mutant line the fewest (Figure 3.4 C,D). Again, ASXL1 mutant progenitors skewed differentiation toward the myeloid lineage by generating a significantly higher proportion of myeloid colonies and significantly less erythroid colonies as compared to both WT and JAK2 mutant lines (Figure 3.4 A,B,E,F). There were no statistically significant phenotypic differences between the ASXL1 mutant and double mutant lines in either set of clones suggesting the impact of the ASXL1 mutation may be dominant over the proliferative effects of the JAK2 mutation (Figure 3.3 and 3.4).

hESC lines expressing the most commonly found ASXL1 mutation, G646Wfs*12, were also differentiated into hematopoietic progenitors and plated in methylcellulose to validate the phenotype associated with ASXL1 P920Tfs*4 described previously. As seen with the other two sets of clones, a trend was observed in which cells expressing JAK2 V617F produced an increase in erythroid colony growth and ASXL1 P920Tfs*4 severely limited erythroid colony formation. JAK2 V617F expression rescued the BFU-E phenotype caused by ASXL1 P920Tfs*4 to bring BFU-E colony numbers within range of WT numbers, however erythroid colony counts were still decreased as compared to JAK2 V617F expressing cells (Figure 3.5 A,B). Significant differences were not observed when total colony numbers were compared between genotypes (Figure 3.5 C,D). There was an outlier data point in which the JAK2 mutant line generated markedly more overall colonies than the JAK2 mutant line generated in other experiments (Figure 3.5 A,D,E). The ASXL1 mutation trended toward a higher fraction of myeloid colonies
as compared to the \textit{JAK2} mutation which corroborated the colony data generated by both clones harboring \textit{ASXL1} P920Tfs*4 (\textbf{Figure 3.5 F}). The blunted erythroid colony growth phenotype observed in association with mutant \textit{ASXL1} was confirmed utilizing three independent sets of hESC clones as well as two individual \textit{ASXL1} mutations.

RNA sequencing analysis was performed utilizing WT, \textit{JAK2} mutant, \textit{ASXL1} P920Tfs*4 mutant, and double knock-in H1 derived CD34+CD45+ hematopoietic progenitors to further characterize the erythroid phenotype found via colony forming assays. RNA sequencing analysis also has the potential to uncover a mechanistic avenue to pursue moving forward. \textit{ASXL1} mutant progenitors downregulated genes involved in erythrocyte membrane protein regulation when assessed utilizing a published curated list of genes.\textsuperscript{85} These genes were upregulated by \textit{JAK2} V617F, however the expression pattern of the double mutant cells was more similar to that of \textit{ASXL1} mutant cells, suggesting that the effects of the \textit{ASXL1} mutation may be dominant over the effects of \textit{JAK2} V617F (\textbf{Figure 3.6 A}). The RNA sequencing data was also analyzed utilizing a published gene set of \textit{GATA1} targets\textsuperscript{86}, which is a transcription factor required for erythropoiesis. Many \textit{GATA1} target genes, including glycophorin A and hemoglobin genes, were downregulated in \textit{ASXL1} mutant and double mutant progenitors relative to WT and \textit{JAK2} mutant progenitors (\textbf{Figure 3.6 B}). A third published curated list was utilized to evaluate transcription factors that are important for erythropoiesis\textsuperscript{87} and the gene expression profile corroborated the blunted erythroid phenotype found to be associated with \textit{ASXL1} mutations by demonstrating downregulation of many erythroid transcription factors in \textit{ASXL1} mutant cells. \textit{PRMT6} which has been shown to inhibit erythroid gene expression was downregulated in \textit{JAK2} mutant progenitors and upregulated in \textit{ASXL1} mutant and double knock-in progenitors. Several genes
that are suppressed by PRMT6 were upregulated in the presence of the \textit{ASXL1} mutation as well (Figure 3.6 C). RNA sequencing analysis provided further validation of the blunted erythroid differentiation phenotype caused by \textit{ASXL1} mutations and confirmed \textit{ASXL1} mutation effects were dominant over the erythroid proliferation phenotype associated with \textit{JAK2} V617F.

\textbf{Discussion}

A human model system has been developed that enables the interrogation of \textit{JAK2} and \textit{ASXL1} mutations and their impact on MPN disease pathogenesis by knocking these mutations into H1 embryonic stem cells. Upon definitive hematopoietic differentiation CD34+CD45+ progenitors were generated and colony forming assays as well as RNA sequencing were performed. Notably, differentiation experiments focused on definitive hematopoiesis as evidenced by lack of CD43 expression on day 8 of differentiation. Previously published pluripotent stem cell MPN model systems have not differentiated between primitive and definitive hematopoiesis,\textsuperscript{61,63,64} which may be an important distinction as the definitive hematopoietic program has been shown to generate the full spectrum of hematopoietic cells through the differentiation of hematopoietic stem cells.\textsuperscript{65} Modeling disease causing mutations in the setting of definitive hematopoiesis is a biologically relevant human model system as these mutations are acquired in the stem and progenitor cell compartment and lead to aberrant myeloid and erythroid lineage production. hESC derived \textit{JAK2} V617F CD34+CD45+ cells plated in methocult resulted in an increase in erythroid lineage colonies which recapitulates the erythrocytosis phenotype associated with \textit{JAK2} V617F conditional knock-in mice as well as \textit{JAK2} mutant PV patients.\textsuperscript{36} RNA sequencing data collected from differentiated \textit{JAK2} V617F CD34+CD45+ cells further validated the erythroid expansion phenotype as many genes involved in erythropoiesis were upregulated. The
observed erythroid proliferation phenotype was not a novel finding, however this phenotype provided confidence in the hESC hematopoietic differentiation system as an appropriate model to study MPN disease biology. The observed phenotypes were also validated utilizing three individual sets of clones for each genotype including two sets harboring the same \textit{ASXL1} mutation and the third expressing a different \textit{ASXL1} mutation. Replicating colony forming unit assay results with three sets of individual hESC clones confirmed that unexpected acquired mutations during the CRISPR, culturing, or differentiation processes were not responsible for the observed phenotypic differences. Both \textit{ASXL1} mutations blunted erythroid colony formation suggesting that although they are distinct \textit{ASXL1} mutations, both frameshift mutations induce similar phenotypes.

\textit{ASXL1} mutant hESC derived progenitors caused a severe depletion of erythroid colony formation as well as downregulation of many genes important for erythropoiesis. \textit{ASXL1} mutations have previously been shown to be associated with anemia in MPN patients\cite{45,88}, however there is very little known about the impact of harboring both \textit{JAK2 V617F} as well as an \textit{ASXL1} mutation on MPN pathogenesis. The field lacks reliable model systems in which to study both mutations in combination. While \textit{ASXL1} mutant hESC derived progenitors generated a significantly higher percentage of myeloid cells than WT lines, myeloid colony proportion in the double knock-in line was not significantly different from either WT or \textit{ASXL1} mutant lines. However, the double knock-in line produced a significantly higher myeloid percentage of total colonies than the \textit{JAK2} mutant line in one of the clone sets and trended in that direction in the other two clones sets. The double knock-in line generated significantly less BFU-E colonies than the \textit{JAK2} mutant line, however again there were not significant differences in BFU-E colonies.
when double knock-in colony output was compared to WT or ASXL1 mutant line colony numbers. CD34+CD45+ progenitors that express both mutations presented with a phenotype and gene expression profile that more closely resembled that of the ASXL1 expressing cells suggesting the effects of the ASXL1 mutations mask the erythroid expansion phenomenon associated with the JAK2 mutation.

Protein arginine methyltransferase 6 (PRMT6) has been shown to be a crucial chromatin modifier that regulates the megakaryocytic/erythroid branching point. PRMT6 is recruited to erythroid genes such as glycophorin A and inhibits erythroid gene expression in primary human CD34+ cells. PRMT6 overexpression was also found to reduce erythroid colony formation. Its expression was downregulated in JAK2 mutant hESC derived CD34+CD45+ cells and upregulated in ASXL1 mutant as well as double mutant CD34+CD45+ hESC derived progenitors. Through the use of published ChIP-Seq tracks, ASXL1 was found to bind the PRMT6 promoter in mouse bone marrow cells as well as human HEK293 cells (Figure 3.6 D). Mutant ASXL1 binding to the PRMT6 promoter and enhancing its transcription and therefore inhibiting erythroid gene transcription could provide a potential mechanistic explanation to be explored further.
Figure 3.1. Gating Strategy for differentiated hemogenic endothelium and CD34+CD45+ progenitor sorts. A) On day 8 of differentiation CD34+CD43-CD184-CD73- hemogenic endothelium were sorted from each genotype. B) CD34+CD45+ definitive progenitors were sorted on day 15 to utilize for CFU and RNA sequencing assays.
Figure 3.2 ASXL1 mutations blunt the erythroid expansion phenotype caused by JAK2 V617F. A) Total BFU-E counts per 10,000 input. B) BFU-E percentage of total colony number. Each point represents a biological replicate. Error bars represent SEM. Mann-Whitney test was used for statistical analysis. WT n=7, JAK2 n=6, ASXL1 n=5, DKI n=5 C) Representative BFU-
E colony morphology at 10X magnification. D) Flow cytometry of total colonies harvested from methylcellulose. The ASXL1 P920Tfs*4 mutation was utilized for this set of clones.
Figure 3.3. Colony forming unit assays utilized hESC derived CD34+CD45+ progenitors.

Colonies were counted per 10,000 cell input. A) Total colony counts fractionated by colony type. B) Total colony counts. C) Total myeloid colonies. D) Percent myeloid colonies of total colony number. The ASXL1 P920Tfs*4 mutation was introduced for this set of clones. Each point represents a biological replicate. Error bars represent SEM. Mann-Whitney test was used for statistical analysis. WT n=7, JAK2 n=6, ASXL1 n=5, DKI n=5
Figure 3.4. Colony forming unit assays utilized CD34+CD45+ progenitors derived from an additional set of clones. Colonies were counted per 10,000 cell input. A) Total colony counts fractionated by colony type. B) Total colony counts. C) Total myeloid colonies. D) Percent myeloid colonies of total colony number. E) Total BFU-E counts. F) BFU-E percentage of total colony number. The ASXL1 P920Tfs*4 mutation was introduced for this set of clones. Each point represents a biological replicate. Error bars represent SEM. Mann-Whitney test was used for statistical analysis. n=4 for each genotype.
Figure 3.5. Colony forming unit assays utilized CD34+CD45+ progenitors derived from an additional set of clones harboring an alternative ASXL1 mutation. Colonies were counted per 10,000 cell input. A) Total colony counts fractionated by colony type. B) Total colony counts. C) Total myeloid colonies. D) Percent myeloid colonies of total colony number. E) Total BFU-E counts. F) BFU-E percentage of total colony number. The ASXL1 G646Wfs*12 mutation was utilized for this set of clones. Each point represents a biological replicate. Error bars represent SEM. Mann-Whitney test was used for statistical analysis. WT n=3, JAK2 n=4, ASXL1 n=3, DKI n=3.
A. JAK2
   ASXL1
   DKI

   ACTB
   ADD1
   ADD2
   ADD3
   ANK1
   DMTN
   EPB41
   EPB42
   ERMAP
   SLC4A1
   SPTA1
   SPTB
   TMOD1
   TPM3

B. JAK2
   ASXL1
   DKI

   ABCB10
   ALAD
   ALAS2
   ANK1
   BACH1
   DMTN
   FTL
   GSTT2
   GYP
   HBA2
   HBB
   HBE1
   HBZ
   HMBS
   KLF1
   MAFG
   NFE2
   PPOX
   SLC4A1
   STOM
   TFRC
   UROD
   UROS
   ZFPM1

C. JAK2
   ASXL1
   DKI

   GATA1
   GATA2
   RUNX1
   TAL1
   KLF1
   PRMT6
   CEBPa
   ALAS2
   EPB42
   AHSP
   HOXA9
   ID2
   PBX1
   KIT
   SPTA1
   GYP

D. Asxl1 ChIP-Seq

   Mouse mm10

   c-Kit+ Bone Marrow

   BM-derived Lin-Kit+

   ASXL1 ChIP-Seq

   Human hg19

   HEK293

   PRMT6
Figure 3.6. Gene expression profiles of JAK2 and ASXL1 mutant hESC derived CD34+CD45+ progenitors. RNA sequencing analysis depicted as heatmaps showing log 2-fold expression relative to WT expression levels in differentiated CD34+CD45+ progenitors. Red represents upregulation and blue represents downregulation. WT n=2, JAK2 n=3, ASXL1 n=2, DKI n=3
A) Expression of erythrocyte membrane genes in curated list published by Steiner, et al.85
B) Expression of GATA1 target genes from gene list published by Welch, et al.86
C) Expression of transcription factors that are important for erythropoiesis published by Herkt, et al.87
D) Published ChIP-Seq tracks reveal ASXL1 binds to the PRMT6 promoter in mouse bone marrow cells as well as in human HEK293 cells.89,90,91 RNA sequencing analysis was done by Tim Kong.
Chapter 4: ASXL1 mutations impair erythroid differentiation in primary patient hematopoietic progenitors

Introduction

As previously described, human H1 stem cell lines engineered to express mutant JAK2 and mutant ASXL1 provided a model system utilized to study these mutations in vitro in the context of the hematopoietic system. They have been differentiated into hematopoietic progenitors to reveal JAK2 mutations resulted in a hyperproliferative erythroid phenotype and ASXL1 mutations caused a myeloid skewing as well as a lack of erythroid colony cell growth. While this is a powerful model system that has provided an unlimited supply of mutant cells that have the capability of being differentiated into any cell type in the human body, the human embryonic H1 cell lines were not derived from malignant patient cells. Our lab has access to hundreds of banked MPN patient samples that have been cryopreserved. For this study myelofibrosis samples were chosen as the focus and specifically myelofibrosis patients who harbored a JAK2 mutation without an ASXL1 mutation and patients who harbored both mutations. Normal bone marrow was utilized as a source of wild-type control cells because normal bone marrow has a much higher frequency of stem and progenitor cells than normal peripheral blood. To validate the previously described phenotypes observed with differentiated JAK2 and ASXL1 mutant progenitors in a more physiologically relevant cell type, colony forming unit assays were performed utilizing stem and progenitor cells sorted from patient PBMCs as well as normal bone marrow BMMCs. In both systems colony output of sorted CD34 positive cells was assessed. The blunted erythroid growth response to ASXL1 mutations observed with mutant ASXL1 and double
mutant JAK2/ASXL1 H1 cell lines was confirmed utilizing JAK2/ASXL1 double mutant myelofibrosis primary samples.

**Methods**

**Patient samples**

Patient peripheral blood and healthy donor bone marrow control samples were obtained with written consent according to a protocol approved by the Washington University Human Studies Committee. Peripheral blood or bone marrow mononuclear cells (PBMC or BMMC) were collected by Ficoll gradient extraction and cryopreserved in 80% FBS + 20% DMSO.

**Patient sample sorting**

Refer to chapter 2 methods.

**Colony forming unit assays**

CD34+ cells from primary patient PBMC or BMMC samples were single cell sorted into 96-well plates. The cells were individually sorted directly into 100µL of methocult #2 (Table 2.1). Colonies were scored, counted, and picked after 10-14 days of incubation at 37 degrees C.

**Colony genotyping**

Individual colonies were digested with lysis buffer (10mM Tris pH 8.0 + 100mM NaCl + 10mM EDTA pH 8.0 + 10% SDS in dH2O) containing proteinase K and glycogen for 2 hours at 50-55 degrees C. DNA was then extracted via isopropanol precipitation with vigorous shaking. Extracted DNA was then washed with 70% ethanol and diluted in TE buffer (10mM Tris pH 8.0 + 1mM EDTA in ddH2O). After incubating at 55 degrees C for a few hours the DNA was quantified and qPCR reactions were utilized to genotype the colonies for JAK2 and ASXL1 mutations utilizing the following primers and probes:
JAK2 F MCF37 primer - ATGAGCAAGCTTTCTCACAAGCATTTGGTTTTA
JAK2 R LevLong primer - GCTCTGAGAAGGCATTAGAAAGCCTGTAAGTT
JAK2 WT probe - VIC-TCTCCACAGACACATAC-MGBNFQ
JAK2 V617F probe - 6FAM-TCCACAGAAACATAC-NGBNFQ
ASXL1 F primer – TCCCCATCGAATGATGAGGTAGTGAAACAG
ASXL1 R primer - CCCAGGCAATGCAGGAGGG
ASXL1 WT probe - VIC- AGAACACATACCATCTG
ASXL1 Mutant probe- FAM- ACACATAACCATCTG

**Results**

Primary blood samples were collected from 5 normal bone marrow donors and 15 primary or secondary myelofibrosis patients. Of the myelofibrosis patients chosen, 8 patients harbored the JAK2 V617F mutation as well as an ASXL1 mutation and 7 patients harbored the JAK2 V617F mutation, but not an ASXL1 mutation. In most of the patients additional potentially disease causing or modifying mutations were found based on clinical sequencing results, however they were grouped based on their ASXL1 mutation status (Table 4.1). Lineage negative CD34 positive hematopoietic progenitors were sorted from normal bone marrow donors and myelofibrosis patient peripheral blood mononuclear cells that were isolated using a ficoll gradient protocol followed by cryopreservation. Equal numbers of lineage negative CD34 positive cells were plated in methocult and colony output was assessed. Across individual patients, similar total colony numbers were generated with the exception of a few outliers. Individual patient colony numbers were shown as a way to display the variability between patients, but also to visualize the increase in BFU-E colonies in the JAK2 only mutant patient samples (Figure 4.1 A). All but
one of the *JAK2* mutant patients generated a higher percentage of BFU-E colonies than all of the *JAK2/ASXL1* double mutant patients as well as normal bone marrow donors (*Figure 4.1 B*).

The primary samples were then pooled into 3 groups based on genotype for statistical analysis; normal bone marrow donors, *JAK2* mutant patients without an *ASXL1* mutation, and patients with both *JAK2* and *ASXL1* mutations (*Figure 4.2 A,B*). Total colony numbers were not statistically different between the three groups (*Figure 4.2 C*).

The patient cells expressing mutant *JAK2* and wild-type *ASXL1* generated the most erythroid colonies in terms of raw numbers as well as percentage of total colonies (*Figure 4.2 A,B*). *JAK2* mutant/*ASXL1* WT cells produced significantly more BFU-E colonies than *JAK2/ASXL1* double mutant cells (*Figure 4.3 A*). When calculated as erythroid colonies as percent of total colony output, BFU-E percentage was significantly higher in the *JAK2* mutant cohort compared to both normal bone marrow controls as well as *JAK2* mutant/*ASXL1* dual mutant colonies. Patient cells harboring both *JAK2* and *ASXL1* mutations generated a significantly lower proportion of BFU-E colonies than both normal bone marrow cells and *JAK2* mutant cells (*Figure 4.3 B*). Significant differences were not found when myeloid colony numbers were compared across genotypes (*Figure 4.3 C*). *JAK2* mutant patient cells produced a significantly lower percentage of myeloid colonies than both normal bone marrow and double mutant patient cells (*Figure 4.3 D*). Overall *ASXL1* mutations caused a blunted erythroid colony growth response even in the presence of *JAK2* V617F.
Sorted lineage negative CD34 positive cells from JAK2 mutant patient 220335, JAK2/ASXL1 dual mutant patient 153231, and a normal bone marrow donor were single cell sorted into 96 well methocult plates. Patient 153231 was chosen because JAK2 and ASXL1 mutations were the only mutations found as a result of clinical sequencing, therefore other mutations were not present that may potentially confound the results. This patient is also the same patient from which the induced pluripotent stem cell lines described in chapter 2 were derived. The colonies were enumerated and individually harvested. Genomic DNA was extracted and genotyped for JAK2 V617F and ASXL1 P920Tfs*4 mutations. As expected, all of the normal bone marrow donor colonies were JAK2 and ASXL1 WT (Figure 4.4 A). All of the colonies collected from patient 220335 who harbors JAK2 V617F, but not an ASXL1 mutation, were ASXL1 WT and JAK2 mutant (Figure 4.4 B). This result was also anticipated because of the high JAK2 V617F variant allele frequency (88%) found in these patient cells. The majority of colonies derived from JAK2/ASXL1 double mutant patient 153231 harbored both mutations (57% JAK2 mutant VAF and 28% ASXL1 mutant VAF) and a large majority of the double mutant colonies were myeloid colonies (Figure 4.4 C). A higher percentage of mutant JAK2 colonies were erythroid colonies compared to WT and double mutant colonies (Figure 4.4 D). The ASXL1 mutation caused a myeloid lineage skewing as JAK2/ASXL1 double mutant colonies were largely myeloid colonies in contrast to JAK2 mutant colonies that lack the ASXL1 mutation.

**Discussion**

Almost all of the patients selected for colony forming unit assays harbored mutations in addition to JAK2 and ASXL1 mutations because multiple potential driver mutations are often found when myelofibrosis patients are sequenced. The additional mutations in this study may be confounding
the results, however patients in each genotype group presented with the same trend and significance was reached when both BFU-E colony and myeloid colony percentages were compared between the three groups. The significant differences observed provided confidence that the phenotypes were a result of \textit{JAK2} and \textit{ASXL1} mutations as the status of these mutations were the only commonality separating the groups. The patient colony forming unit assay results also support the mutant H1-derived progenitor results described in chapter 2. Both the pluripotent stem cell system as well as the primary patient samples revealed an increase in erythroid colonies associated with mutant \textit{JAK2} as well as a myeloid lineage colony skewing in response to mutant \textit{ASXL1}. The primary sample experiments further confirmed the validity of the \textit{JAK2} and \textit{ASXL1} mutation expressing H1 embryonic stem cell lines as a model system to study MPNs because similar phenotypes are observed in the cells collected from myelofibrosis patients.

The studied patient cohort lacks patients with \textit{ASXL1} mutations that do not also harbor a \textit{JAK2} mutation. \textit{ASXL1} only mutant cells would serve as an additional control, however around 90\% of myelofibrosis patients harbor \textit{JAK2}, \textit{MPL}, or \textit{CALR} mutations which are all known to be JAK-STAT pathway activating mutations.\textsuperscript{11} Clonal hematopoiesis samples or myelodysplastic syndrome patient samples would have to be obtained in order to include \textit{ASXL1} only mutant controls, however, the result would be comparison between cells from different disease states. The erythroid colony depletion phenotype is apparent without an \textit{ASXL1} mutant only control even in the presence of \textit{JAK2} V617F.
Patient cells harboring both \textit{JAK2} V617F as well as an \textit{ASXL1} mutation generated significantly less BFU-E colonies than both normal bone marrow and \textit{JAK2} mutant only patient cells, which suggested that the \textit{ASXL1} mutation impairs erythroid differentiation. On average the \textit{JAK2} mutant only patients had a higher \textit{JAK2} allele burden (77\%) than the \textit{JAK2/ASXL} double mutant patients (52\%) and the erythroid growth in some cases was reflective of the \textit{JAK2} V617F variant allele frequency (VAF), however the VAFs do not completely explain the erythroid phenotype. For example, 729314 is a \textit{JAK2} mutant patient with a 96\% VAF and 398253 is a \textit{JAK2/ASXL1} double mutant patient also with a 96\% \textit{JAK2} V617F VAF, and 729314 generated much more overall erythroid colonies as well as a higher erythroid percentage relative to total colonies. In this case even though these two patients have the same \textit{JAK2} allele burden, the \textit{ASXL1} mutant patient generated fewer erythroid colonies, which suggested the \textit{ASXL1} mutation may impair erythroid differentiation. This phenotype supports and may be potentially part of an explanation for \textit{JAK2} mutations in combination with \textit{ASXL1} mutations being prognostically worse for patients than \textit{JAK2} mutations alone. The erythroid colony growth suppression observed in \textit{ASXL1} mutant primary patient samples also fits with an association with \textit{ASXL1} mutations and anemia that has been reported in the literature.\textsuperscript{45,88}
Table 4.1. List of myelofibrosis patient samples harboring JAK2 V617F and ASXL1 mutations. Specific JAK2 and ASXL1 mutations found are listed for each patient as well as any other additional mutations.
Figure 4.1. Colony forming unit assays utilized sorted CD34+ cells from myelofibrosis patients. Each column represents an individual patient. Colonies were single cell sorted and counted per 60 cell input. A) Raw colony numbers fractionated by colony type as indicated by
color. B) Colony type as percentage of total colony number. Error bars represent SEM. Normal bone marrow n=5, JAK2 patients n=7, JAK2/ASXL1 n=8
Figure 4.2. Colony forming unit assays utilized sorted CD34+ cells from myelofibrosis patients grouped by JAK2 and ASXL1 mutation status. A) Colony counts of combined normal bone marrow, JAK2 mutant patients, and JAK2/ASXL1 double mutant patients fractionated by colony type. B) Colony number depicted as percentage of total colony number. C) Total colony numbers with each individual patient represented. Error bars represent SEM.
Mann-Whitney test was used for statistical analysis. Normal bone marrow n=5, JAK2 patients n=7, JAK2/ASXL1 n=8
Figure 4.3. *JAK2/ASXL1* double mutant patient CD34+ cells generated less erythroid and more myeloid colonies than *JAK2* mutant patient CD34+ cells. Erythroid colony growth represented by A) BFU-E colony number and B) BFU-E colonies as a percentage of total colony number. C) Total myeloid colonies counted. D) Myeloid colonies as a percentage of total colony number.
number. Error bars represent SEM. Mann-Whitney test was used for statistical analysis. Normal bone marrow n=5, JAK2 patients n=7, JAK2/ASXL1 n=8
Figure 4.4. Colony genotyping revealed an ASXL1 mutation in combination with JAK2 V617F resulted in myeloid lineage skewing. Genotyping was performed via qPCR. Colors represent colony type. A) Normal bone marrow donor colonies were JAK2 V617F and ASXL1 WT. B) Colonies collected from JAK2 mutant MF patient 220335 were all JAK2 mutant.
Genotypes of colonies collected from MF JAK2/ASXL1 double mutant patient 153231 shown as by C) total colony number and D) colony type as a percentage of total colony number.
Chapter 5: Conclusions and future directions

Conclusions

JAK2 V617F is the most commonly found mutation in myeloproliferative neoplasms with 50-60% of myelofibrosis patients expressing mutant JAK2. While the majority of myelofibrosis patients harbor JAK2 V617F or another JAK-STAT activating mutation (i.e. MPL or CALR) they also often harbor mutations in epigenetic modifier genes such as ASXL1. ASXL1 mutations are associated with poor patient outcomes as well as increased risk of transformation to secondary acute myeloid leukemia. The mechanisms as to how ASXL1 mutations contribute to myelofibrosis pathogenesis and transformation remain unclear. JAK2 V617F and ASXL1 mutations often co-occur and these mutations have not been extensively studied in combination. As previously described, an Asxl1 heterozygous knock-out mouse crossed with a transgenic Jak2 V617F mouse has been studied. While this is the only reported JAK2 and ASXL1 model system, they focused on a complete ASXL1 knock-out as opposed to modeling mutations found in patients. The field lacks models that focus on ASXL1 mutations in combination with JAK2 V617F.

Two pluripotent stem cell model systems have been developed as in vitro approaches to study the individual and combined influence JAK2 V617F and ASXL1 mutations have on MPN disease pathogenesis. Initially, induced pluripotent stem cell lines were derived from myelofibrosis patient cells harboring JAK2 and ASXL1 mutations. While the rationale to use these lines was compelling because they were generated from physiologically relevant patient cells, complications arose with the lack of erythroid colony growth upon hematopoietic differentiation.
It was important to have the capability of characterizing an erythroid phenotype as the erythroid lineage is often dysregulated in MPN patients. While an alternative approach was pursued, the iPSC lines could still be utilized as a useful tool in future experiments, however they will require additional optimization experiments. Alternative iPSC lines will also be differentiated to determine if a different iPSC clone has the capacity to generate erythroid colonies. The iPSC lines will provide an unlimited model system to study JAK2 and ASXL1 mutations in the context of patient cells, unlike primary patient samples which are a finite resource. Patient-derived iPSC lines have been utilized by other groups as powerful tools to study various hematological disease states.\textsuperscript{59,60} They have been used to determine how mutations and disease severity influences hematopoietic progenitor differentiation. They have also been used to test pharmacologic agents as possible therapeutic options for myeloid malignancies. In addition to \textit{in vitro} studies, iPSC lines have been differentiated into hematopoietic progenitors and transplanted into NSG mice to study patient derived cells \textit{in vivo}, with the caveat that transplant experiments were only successful utilizing AML derived iPSC lines. Less severe disease states such as MPN or MDS patient derived cells have not successfully engrafted into mice. iPSC lines will provide an unlimited source of patient derived cells to study how ASXL1 and JAK2 mutation in combination drive pathogenesis in myelofibrosis patient cells.

\textit{JAK2} V617F as well as two individual ASXL1 mutations were introduced into H1 embryonic stem cells as an additional pluripotent stem cell model system. Each ASXL1 mutation was introduced in isolation as well as in combination with \textit{JAK2} V617F. JAK2 mutant hematopoietic progenitors differentiated from mutant H1 pluripotent stem cell lines generated more overall colonies than WT, ASXL1 mutant progenitors, and double mutant progenitors. JAK2 mutant
progenitors also produced more erythroid colonies than each of the other lines. In contrast, ASXL1 mutant progenitors generated the fewest colonies and colony output was almost exclusively myeloid. These same trends were observed in three independent sets of clones, including two sets harboring the ASXL1 mutation found in the patient from which the induced pluripotent stem cell lines were derived and the third set harboring the most commonly found ASXL1 mutation in myeloid malignancies. The phenotypes were validated utilizing three sets of clones which provided confidence that erythroid proliferation associated with JAK2 V617F as well as the lack of erythroid colonies associated with ASXL1 mutant cells were consequences of the introduced mutations and not specific to an abnormal clone.

Ideally these mutations would be studied in the context of patient cells, however patient samples are limited and additional potential driver mutations of unknown significance are potentially present. The H1 pluripotent stem cell model system provided an unlimited supply of cells expressing only mutant JAK2 and/or mutant ASXL1 without other confounding mutations. While patient samples are limited, colony forming assays were performed utilizing select myelofibrosis patient samples to further verify the observed phenotypes. JAK2 mutant patient cells that lacked an ASXL1 mutation generated more overall as well as a higher percentage of erythroid colonies than normal bone marrow and double mutant JAK2/ASXL1 patient cells. Patient cells harboring both JAK2 V617F as well as an ASXL1 mutation produced very few erythroid colonies. ASXL1 mutations in both H1 pluripotent stem cells as well as in myelofibrosis patient samples resulted in impairment of erythroid differentiation.
The \textit{JAK2/ASXL1} double mutant pluripotent stem cell line presented with an intermediate erythroid phenotype, i.e. less BFU-E colonies than the \textit{JAK2} mutant line and more BFU-E colonies than the \textit{ASXL1} mutant line. The erythroid proliferation phenotype associated with the \textit{JAK2} mutant line was also observed in \textit{JAK2} mutant myelofibrosis patient samples as samples harboring \textit{JAK2 V617F} generated more erythroid colonies than patient samples with both \textit{JAK2} and \textit{ASXL1} mutations. When \textit{JAK2/ASXL1} double mutant samples were compared to normal controls, a discordance between the H1 pluripotent stem cell system and the patient samples was noted. Double mutant myelofibrosis patient samples generated less erythroid colonies than normal bone marrow controls, while \textit{JAK2/ASXL1} H1 pluripotent stem cell derived progenitors and WT progenitors generated similar numbers of erythroid colonies. However the lack of erythroid colonies generated by \textit{JAK2/ASXL1} patient cells compared to normal bone marrow controls recapitulated the reported association between \textit{ASXL1} mutations and anemia found in myelofibrosis patients.\textsuperscript{45} Perhaps increasing biological replicates of colony forming assays utilizing the double knock-in and WT pluripotent stem cell lines will provide a more accurate representation of the phenotype as variability exists amongst current double knock-in data points. Additional assays will also be performed such as flow/mass cytometry and serial replating assays in order to more comprehensively characterize the phenotype induced by \textit{JAK2} and \textit{ASXL1} mutations in combination. If the discordance in control comparisons remains after additional replicates and flow cytometry assays, one possible explanation is that the normal control in the pluripotent stem cell model system is an embryonic stem cell line and the normal control in the patient sample experiments is normal bone marrow lineage negative CD34 positive cells. The difference in normal control cells between the two model systems may explain the phenotype discordance. Another possibility is there may be differentiation differences between patient cells.
harboring JAK2 and ASXL1 mutations and JAK2/ASXL1 double mutant H1 derived progenitors. Perhaps there are additional factors (epigenetic, genetic, etc.) present in the myelofibrosis patient cells that influence colony output that are not factors in the H1 embryonic stem cell lines.

Mechanisms as to how ASXL1 mutations alone as well as in combination with JAK2 V617F influence erythroid differentiation remain unknown. Primary patient sample colony assay data described above in chapter 4 suggested ASXL1 mutations may have dominant effects over JAK2 V617F. Myelofibrosis patient cells harboring both mutations generated significantly less erythroid colonies than normal bone marrow as well as patient cells harboring JAK2 V617F without an ASXL1 mutation. While JAK2 V617F caused a substantial erythroid expansion, when combined with an ASXL1 mutation this erythroid phenotype was severely blunted to the extent that colony numbers were less than those generated by normal bone marrow control cells. Gene expression profiles of JAK2/ASXL1 double knock-in progenitors were similar to patterns found in ASXL1 mutant progenitors, specifically when gene signatures involved in erythropoiesis were assessed as described in chapter 3. This data supported the idea that altered hematopoiesis due to mutant ASXL1 may be dominant over the erythroid skewing phenotype observed in JAK2 mutant cells since the JAK2/ASXL1 erythroid gene expression patterns often resembled patterns seen in ASXL1 mutant cells and opposed patterns seen in JAK2 mutant cells. RNA sequencing analysis also identified possible relevant genes that were differentially expressed in ASXL1 mutant cells as compared to JAK2 mutant cells that could be targeted pharmacologically or genetically such as, PRMT6, TAL1, or CEBPA. These potential targets could be tested by treating JAK2 mutant, ASXL1 mutant, and JAK2/ASXL1 double mutant progenitors grown in colony forming assays with target specific inhibitors to assess how they affect erythroid differentiation.
Future Directions

While each of the three sets of H1 clones presented with similar trends, the erythroid proliferation associated with JAK2 V617F and impaired erythroid phenotype associated with mutant ASXL1 were statistically significant in the ASXL1 P920Tfs*4 clone sets, but not in the clone set expressing ASXL1 G646Wfs*12. Additional colony forming assay replicates need to be performed with the H1 pluripotent stem cell set expressing ASXL1 G646Wfs*12 in order to potentially achieve statistical significance. Gaining significance within the clone set expressing ASXL1 G646Wfs*12, which is the most commonly found ASXL1 mutation in myeloid malignancies, would strengthen the claim that the phenotypic differences are convincing and consistent across multiple ASXL1 mutations. We would expect both ASXL1 mutations to result in similar alterations to the protein because they are both frameshift mutations that result in a stop codon located in exon 12, however subtle differences may exist between the two mutations that influence how they impact protein function. If there are differences between the two mutations, the erythroid phenotype trends generated by each mutation may be distinct after biological replicates are added to the data set.

While colony forming assays as well as gene expression profiling demonstrated an impaired erythroid differentiation phenotype associated with ASXL1 mutations, flow cytometry or mass cytometry will be utilized to further confirm this phenotype without reliance on morphologic characterization. Repeat flow cytometry experiments assessing erythroid marker expression (CD71 and CD235) will be performed utilizing colonies grown from pluripotent stem cell derived mutant lines in methylcellulose cultures. Mass cytometry analysis will also be conducted
allowing for the assessment of surface markers as well as intracellular signaling markers. Surface antibodies will be used to assess the stem and progenitor compartment as well as erythroid and myeloid differentiation patterns of the \textit{JAK2/ASXL1} mutant lines. With simultaneous surface marker expression analysis, intracellular pathways including JAK-STAT signaling, NFκB signaling, MAPK signaling, proliferation, and apoptosis will also be interrogated to characterize how these mutations may be contributing to the observed hematopoietic phenotypes. These experiments will expose novel differentiation patterns or activated signaling pathways that may exist in \textit{JAK2/ASXL1} double mutant cells, but not in single mutant cells.

In addition to repeating colony assays utilizing the mutant pluripotent stem cell lines to increase sample size, serial replating assays will also be performed. Serial replating capacity will be assessed as a surrogate to interrogate the potential influence \textit{JAK2, ASXL1}, and double knock-in mutations have on self-renewal. One of the hallmark features of leukemic cells is unlimited self-renewal\textsuperscript{92} and therefore secondary acute myeloid leukemia cells will potentially have increased self-renewal capacity as compared to myelofibrosis cells. \textit{ASXL1} mutations have been shown to correlate with increased risk of leukemic transformation in myelofibrosis patients.\textsuperscript{45} Serial replating assays will be utilized as a distant proxy to provide insight into how these mutations may influence disease transformation. Perhaps double knock-in cells will have increased serial replating capacity as compared to either mutation alone suggesting \textit{JAK2} and \textit{ASXL1} mutations in combination may cause an increase in self-renewal. Increased self-renewal may partially explain the increased risk of transformation associated with \textit{ASXL1} mutations in myelofibrosis patients.
As previously described in chapter 1, it remains unclear whether ASXL1 mutations result in complete loss of function or if a truncated protein is translated that results in a gain of function or dominant negative effect. After unsuccessful attempts to address this question via western blot analysis in human cells, an alternative approach is necessary. ASXL1 knock-out lines will be generated by introducing an early frameshift mutation, in a similar fashion as to how the pluripotent stem cell lines with ASXL1 mutations in exon 12 were generated as described in chapter 2. To determine if these mutations are loss of function, rescue experiments will be performed by introducing mutant ASXL1 into the AAVS1 safe harbor locus of the ASXL1 knock-out pluripotent stem cell lines. Hematopoietic phenotypes upon differentiation of ASXL1 knock-out lines compared to ASXL1 knock-out lines expressing mutant ASXL1 will be utilized to address whether these mutations result in a loss of function. The phenotype associated with differentiation of the ASXL1 knock-out cells will not be affected or rescued by the expression of mutant ASXL1 if these mutations result in complete loss of protein function. As described previously in chapter 1, truncated ASXL1 has been shown to complex with BAP1 to enhance loss of H2AK119Ub, however ASXL1 knock-down in SET-2 cells did not have an effect on H2AK119Ub levels.\textsuperscript{50,47} ChIP-Seq will be utilized to assess H2AK119Ub levels in ASXL1 mutant differentiated progenitors to determine if ASXL1 mutations result in loss of H2AK119Ub suggesting ASXL1 mutations confer a gain of function. Additionally, ASXL1 knock-down was shown to upregulate HOXA gene expression, however depletion of H2AK119Ub did not activate HOX gene expression. HOX gene expression will be assessed via RNA-sequencing which may also suggest whether ASXL1 mutations result gain or loss of function. Pluripotent stem cell model systems subjected to genetic engineering are powerful tools that can be utilized to study how ASXL1 mutations affect protein function.
References


98. Milhollen MA, Traore T, Adams-Duffy J, et al. MLN4924, a NEDD8-activating enzyme inhibitor, is active in diffuse large B-cell lymphoma models: rationale for treatment of NF-


Appendix:

The manuscript below is an additional project that I contributed to and was submitted to Blood in May of 2020 as a Brief Report. It was rejected by Blood, but we have been given the opportunity to address the reviewers’ comments and resubmit to Blood Advances. Previous publications from our lab have shown the NFκB pathway to be hyperactive in myelofibrosis patients, therefore this study interrogated the efficacy of an NFκB pathway inhibitor, pevonedistat, in MPN cells both *in vitro* and *in vivo*. As previously described ruxolitinib has not been shown to eradicate the malignant clone which suggests targeting other dysregulated signaling pathways may provide therapeutic benefit for patients. My contributions to this manuscript included several experiments including RT-qPCR of NFκB-pathway related genes in HEL cells as well as in sorted MF patient lineage negative CD34 positive cells. I also performed flow cytometry utilizing pevonedistat treated HEL cells to demonstrate that pevonedistat targeted the NFκB pathway as well as mass cytometry analysis assessing the effect of pevonedistat treatment on MF lineage negative CD34 positive cells. I also wrote the initial draft of the manuscript. While we tested pevonedistat *in vitro* as well as in MPN mouse models, the primary concern raised by the reviewers was that pevonedistat was not tested in combination with ruxolitinib. We are in the process of testing the combination therapy in both *Jak2 V617F* knock-in mice and *MPLW515L* retroviral transplant mouse models. They also requested that experiments be completed to assess the effect of pevonedistat alone and in combination with ruxolitinib on activation of apoptosis or cell cycle arrest to provide a potential mechanistic explanation. Annexin-V staining has since been completed and we found pevonedistat and pevonedistat + ruxolitinib treatment induced apoptosis more robustly than ruxolitinib alone. After the addition of data assessing pevonedistat
and ruxolitinib combination treatment \textit{in vivo} the manuscript will be resubmitted to Blood Advances.

\textbf{Title}

Pevonedistat targets malignant cells in myeloproliferative neoplasms \textit{in vitro} and \textit{in vivo} via NFκB pathway inhibition

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\textbf{Abstract}

Targeted inhibitors of JAK2 (e.g. ruxolitinib) often provide symptomatic relief for myeloproliferative neoplasm (MPN) patients, but the malignant clone persists and remains susceptible to disease transformation. These observations suggest that targeting alternative dysregulated signaling pathways may provide therapeutic benefit. Previous studies identified NFκB pathway hyperactivation in myelofibrosis (MF) and secondary acute myeloid leukemia (sAML) that was insensitive to JAK2 inhibition. This study provides evidence that NFκB pathway inhibition via pevonedistat targets malignant cells in MPN patient samples as well as in MPN mouse models. Pevonedistat inhibitory activity on the NFκB pathway was confirmed utilizing
these models. Colony forming assays revealed preferential inhibition of MF colony growth compared to normal colony formation. In mass cytometry studies, pevonedistat blunted canonical TNFα responses in MF and sAML patient CD34+ cells. Pevonedistat also inhibited hyperproduction of inflammatory cytokines more effectively than ruxolitinib. Upon pevonedistat treatment, MPN mouse models exhibited reduced disease burden and improved survival. These studies demonstrating efficacy of pevonedistat in MPN cells in vitro as well as in vivo provide a rationale for therapeutic inhibition of NFκB signaling for MF treatment. Based on these findings, a Phase I clinical trial combining pevonedistat with ruxolitinib has been initiated.

**Introduction**

The identification of JAK2 V617F and other driver mutations (e.g. CALR, MPL) that activate JAK-STAT signaling in myeloproliferative neoplasms (MPNs)\(^1\) has led to the development of targeted inhibitors of JAK2 (e.g. ruxolitinib), which are now widely used for the treatment of patients with MPNs, in particular myelofibrosis (MF). These agents provide substantial symptomatic benefit, but they do not eradicate the underlying malignant clone, nor do they prevent transformation to secondary acute myeloid leukemia (sAML).\(^2,3\) These observations suggest that other signaling pathways may be dysregulated in a manner that promote survival of the malignant clone despite JAK2 inhibition.\(^4\)

Previous studies from our group utilized mass cytometry to identify a high prevalence of NFκB pathway hyperactivation in MF and sAML. CD34+ hematopoietic stem/progenitor cells (HSPCs) from MF and sAML patients were found to exhibit elevated levels of p65/RELA phosphorylation (p-p65/RELA), both basally as well as in response to TNFα stimulation.\(^5\) Treatment with
ruxolitinib did not ablate the activated NFκB signaling, nor did it fully ameliorate the hyperproduction of circulating inflammatory cytokines observed in MF. These observations suggest that targeting the NFκB pathways may provide therapeutic benefit for MF patients beyond JAK2 inhibition. In this study, we thus sought to investigate the therapeutic potential of NFκB pathway inhibition utilizing pevonedistat, a NEDD8-activating enzyme inhibitor which inhibits NFκB signaling by preventing the degradation of IκBα.

**Methods**

**Patient samples**

Patient and healthy donor control peripheral blood (PB) or bone marrow (BM) samples were obtained with written consent according to a protocol approved by the Washington University Human Studies Committee. Mononuclear cells (PBMC or BMMC) were obtained by Ficoll gradient extraction and cryopreserved according to standard procedures. Patient information listed in Supplemental Methods; Supplemental Table 1.

**RT-qPCR for NFκB target genes**

Quantitative RT-PCR was performed on RNA extracted from HEL cells or from MF patient or normal CD34+ cells using primers specific for NFκB target genes (Supplemental Methods; Supplemental Table 2).

**Colony forming unit (CFU) assays**

Lineage-negative CD34+ cells were sorted from MF patient PBMCs and normal BMMCs and plated in Methocult H4230 (StemCell) with pevonedistat, ruxolitinib, and/or TNFα at concentrations described in Figure 1B. Colonies were enumerated after two weeks in culture.
**Mass cytometry**

Cell staining was performed using methods and antibody panels as previously described.\(^5,6,10\) Mass-channel data was recorded on a CyTOF2 mass cytometer (Fluidigm). Debarcoded, normalized data was analyzed in in Cytobank (cytobank.org). Additional details provided in Supplemental Methods.

**Mouse studies**

All procedures were conducted in accordance with the Institutional Animal Care and Use Committee of Washington University. Pevonedistat treatment (60 mg/kg) was initiated following transplantation of Ba/F3-\(MPL\)W515L cells (\(10^6\) per mouse) into BALB/c mice (The Jackson Laboratories). GFP(+) cells in peripheral blood and bone marrow were analyzed by flow cytometry. 100,000 bead-enriched Kit+ cells from CD45.2 \(Jak2\) V617F donor mice were transplanted into CD45.1 lethally irradiated recipient mice. Pevonedistat (60mg/kg) was initiated after successful engraftment. Hematologic parameters were measured by Hemavet and flow cytometry. Additional details provided in Supplemental Methods.

**Data sharing statement**

Mass cytometry data will be made publicly available on Cytobank.org. All other data will be shared via email to the corresponding author.

**Results**

Initial experiments verified the inhibitory activity of pevonedistat on NF\(\kappa\)B signaling in MPN cells. NF\(\kappa\)B target gene expression induced by TNF\(\alpha\) was inhibited by pevonedistat in MF patient CD34+ cells (Figure 1A). In colony assays with CD34+ HSPCs, pevonedistat preferentially inhibited MF versus normal colony growth in a dose-dependent manner (Figure 1B). The addition
of TNFα led to decreased colony formation from both normal and MF HSPCs, while a greater reduction in colonies from MF CD34+ cells was observed upon co-treatment with TNFα and 0.2 µM pevonedistat. Similarly, treatment with 0.2 µM ruxolitinib led to a partial reduction in colonies, which was augmented by co-treatment with pevonedistat. Analogous effects of pevonedistat on NFκB signaling and cell proliferation were observed with JAK2 V617F-mutant HEL cells (Figure S1).

Signaling pathway analysis via mass cytometry revealed both p-p65/RELA elevation and IκBα degradation responses to TNFα in MF and sAML patient CD34+ cells, which were blunted with pevonedistat and TNFα co-treatment (Figure 1C-G, S2, S3A-C). Other signaling pathways analyzed via mass cytometry were minimally affected by pevonedistat: for example, CREB phosphorylation was induced by TNFα stimulation, but this response was not inhibited by pevonedistat, indicating specificity of pevonedistat for the NFκB pathway (Figure S3D). Pevonedistat also potently inhibited the aberrant hyperproduction of multiple inflammatory cytokines, including TNFα, from MF patient cells (particularly monocytes), more effectively than ruxolitinib (Figures S4,S5).

Potential effects of pevonedistat on MPNs in vivo were assessed in mouse models. BALB/c mice transplanted with Ba/F3-MPLW515L-GFP cells were treated with either vehicle or pevonedistat. Mice receiving vehicle exhibited a rapid increase in peripheral blood GFP(+) cells (Figure 2A). By day 15, vehicle-treated mice were moribund, with intestinal necrosis frequently observed postmortem (Figure S6), while all pevonedistat-treated mice remained viable (Figure 2B). Bone marrow GFP(+) cells were markedly higher in vehicle (23.99%) vs pevonedistat (0.23%) groups
(Figure 2C). Animals receiving vehicle exhibited hepatosplenomegaly, with spleen and liver mean weights of 788 mg and 1871 mg, respectively, compared to 127 mg and 1021 mg for pevonedistat-treated animals (Figure 2D-E).

Following stable engraftment, mice transplanted with Kit+ cells from Jak2 V617F knock-in mice were treated with pevonedistat versus vehicle for two weeks (Figure 2F). As expected, significant increases in WBC, hematocrit and platelets were observed over time in vehicle-treated mice (Figures 2G-I). In contrast, progressive development of these MPN disease features was absent in pevonedistat-treated mice. In addition, CD45.2(+) donor cells were significantly higher in animals receiving vehicle versus pevonedistat (Figure 2J). Expression analysis confirmed that pevonedistat treatment significantly decreased the expression of multiple NFκB target genes (Figure 2K).

**Discussion**

Our group previously demonstrated that MF and sAML patients frequently harbor hyperactive NFκB signaling that is not rectified by ruxolitinib treatment. In this study, pevonedistat was found to suppress growth of JAK2-mutant HEL cells, and to inhibit colony formation from MF patient HSPCs differentially versus normal bone marrow HSPCs. In addition, pevonedistat treatment significantly decreased disease burden and improved survival in MPN mouse models. Gene expression and mass cytometry analysis demonstrated specificity of pevonedistat for NFκB inhibition among intracellular signals activated by TNFα.
These findings indicate that pevonedistat can counteract several pathophysiologic parameters observed in MF and sAML patient cells \textit{ex vivo} and in relevant MPN mouse models, in a manner that is non-redundant with observed effects of ruxolitinib. These observations suggest that co-targeting of signaling mediated by JAK2 and NFκB could provide enhanced therapeutic benefit. Based on this hypothesis, our group has initiated a Phase I clinical trial, combining pevonedistat with ruxolitinib for MF treatment (NCT03386214). Phase I/II studies of pevonedistat in MDS and AML have shown tolerability, and a Phase III study of pevonedistat in combination with azacitidine in MDS/AML is currently ongoing (NCT03268954).\textsuperscript{12,13,14} Thus, targeting of NFκB via pevonedistat may have therapeutic applications in multiple myeloid malignancies.

While ruxolitinib remains the best available therapy for many MF patients, there remains an unmet need for a treatment that counteracts disease progression and reduces malignant clonal burden, in addition to improving patient symptoms. The studies presented here provide a rationale for targeting NFκB signaling via pevonedistat as a therapeutic modality for MF treatment.

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Authorship contributions

Disclosure of conflicts of interest
S.O. has served as a consultant for Incyte, Gilead Sciences, Novartis, Celgene/Bristol Myers Squibb, Blueprint Medicines, Kartos Therapeutics, Disc Medicine, and CTI BioPharma. All other authors disclose no competing interests.

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**Figure legends**

**Figure 1.** Pevonedistat suppresses TNFα-mediated NFκB signaling in myelofibrosis patient samples.

A) RT-qPCR of NFκB-pathway related genes in sorted CD34+ cells from normal bone marrow (BM) donor (n = 4) and myelofibrosis patient (n = 6) samples treated with 1 µM pevonedistat, 20 ng/mL TNFα, or in combination. CD34+ cells were treated for 4 hours. Relative NFκB-pathway related mRNA expression was determined by 2^dCt and normalized to ACTB. * p < 0.05; ** p < 0.01; by Mann–Whitney U test. NS = non-significant.

B) CFU assay of sorted CD34+ cells from normal bone marrow donor and myelofibrosis patient samples treated with pevonedistat, TNFα, ruxolitinib, or combinations at the indicated concentrations. 1000 CD34+ cells were plated in Methocult containing SCF and IL-3 in triplicate per condition. Colonies were counted after fourteen days in culture. The number of patients samples (n) per each condition is as follows: 7 NBM, 6 MF no inhibitor; 7 NBM, 6 MF 0.04 µM Pev; 7 NBM, 6 MF 0.2 µM Pev; 7 NBM, 7 MF 1 µM Pev; 3 NBM, 3 MF for all other conditions. * p < 0.05; ** p < 0.01; by Mann–Whitney U test. NS = non-significant.

C, D, E) viSNE plots of normal bone marrow donor (NBM 44) and two sAML patient samples (sAML1, sAML7) after signaling mass cytometry. C) CD34+ HSPC population identified
through extracellular surface staining with anti-CD34 antibody. Intracellular staining of phospho-p65/RELA (S529) (C) and IκBα (D) in patient samples treated with 1 μM pevonedistat for 1 hour, 20 ng/mL TNFα for 15 minutes, or in combination.

F) Heatmaps of signaling markers from CD34+ cells from 3 normal bone marrow donors, 5 primary MF patients, and 2 sAML patients analyzed by mass cytometry. Cells from patient samples were treated with 1 μM pevonedistat for 1 hour, 20 ng/mL TNFα for 15 minutes, or in combination. Signals from each patient sample per treatment condition were normalized to its basal signal.

G) Dual counts of intracellular phospho-p65/RELA and IκBα from CD34+ cells from the 10 patient samples in (F). * p < 0.05; by Mann–Whitney U test. NS = non-significant.

Figure 2. Pevonedistat improves outcomes in MPN mouse models.

A) Percentage of GFP+ cells (transplanted Ba/F3-MPLW515L cells) in the peripheral blood of BALB/c mice. Each week mice were treated 5 days in a row, followed by 2 untreated days with vehicle (n = 10) or pevonedistat (n = 10). Blood collected on date of sacrifice for vehicle-treated mice or day 19 for pevonedistat-treated mice. **** p < 0.0001; by Student’s t-test.

B) Kaplan-Meier survival plot of BALB/c mice transplanted with Ba/F3-MPLW515L cells treated with vehicle (n = 10) or pevonedistat (n=10).

C, D, E) % of GFP+ cells in the bone marrow (C), and spleen weights (D) and liver weights (E) of BALB/c mice transplanted with Ba/F3-MPLW515L cells and treated with vehicle (n = 10) or pevonedistat (n=10). Dashed grey lines representative of healthy, normal organ weights. **** p < 0.0001; by Student’s t-test
F) Schematic of the Jak2 V617F mouse experiment. Kit+ cells from Jak2 V617F Vav-Cre mice were transplanted into lethally irradiated recipient mice. Five weeks after transplantation, treatment with vehicle or pevonedistat was initiated.

G, H, I, J) Hemavet analysis of white blood cells (WBC; panel G), hematocrit (HCT; panel H), platelets (PLT; panel I), and transplanted CD45.2+ cells (panel J) of transplanted Vav-Cre mice treated with vehicle (n = 5) or pevonedistat (n = 6) at the indicated period. For relative comparisons, Hemavet values for each vehicle or pevonedistat treated mouse after 2 weeks of treatment were normalized to their respective value at pre-treatment value. * p < 0.05; ** p < 0.01; by paired Student’s t-test. NS = non-significant. Dashed lines representative of normal ranges.

K) RT-qPCR of NFκB target genes in mouse peripheral blood from Jak2 V617F transplanted mice after two weeks of treatment with vehicle (n = 5) or pevonedistat (n = 6). Relative NFκB target genes mRNA expression was determined by 2^dCt and normalized to ActB. * p < 0.05; ** p < 0.01; by Student’s t test.
Figure 1

(A) NFKBIA, NFKB1, NFKB2 expression levels in CD34+ cells from different conditions.

(B) Relative colony formation in normal BM and MF patient under treatment with Pev and TNFα.

(C) CD34+ cell surface markers in different conditions.

(D) p65 expression levels in basal, Pev, TNFα, Pev + TNFα conditions.

(E) IκBα expression levels in basal, Pev, TNFα, Pev + TNFα conditions.

(F) Heatmap showing expression levels of various signaling proteins in CD34+ cells under different conditions.

(G) Graphs showing dual counts for p-p65 and IκBα in basal, Pev, TNFα, Pev + TNFα conditions.
Supplemental Material

Supplemental Methods

RT-qPCR for NFκB target genes

Lineage-negative CD34+ cells were FACS-sorted from cryopreserved normal bone marrow mononuclear cell (BMMC) samples and myelofibrosis peripheral blood mononuclear cell (PBMC) samples. Following a 30 minute rest period, these cells (or HEL cells) were incubated with 1 µM pevonedistat and/or 20 ng/mL TNFα for 4 hours. The samples were then fixed for 10 minutes at room temperature with 1.6% formaldehyde. RNA extraction was performed using the Qiagen RNeasy kit, followed by reverse transcription using the Thermofisher SuperScript IV First-Strand Synthesis kit. The cDNA was then utilized as the template for the qPCR using primers specific to the transcript of interest (Supplemental Table 2).

Mass Cytometry

Cell staining for mass cytometry intracellular signaling experiments (Figure 1C-G, S2, S3), a protocol derived from Bendall et al., was used with a panel of antibodies previously utilized by Fisher et al. Briefly, cryopreserved cells were thawed and counted, stained with 2.5 µM cisplatin (Enzo Life Sciences, Farmingdale, NY, USA) for 1 min to identify non-viable cells for removal from subsequent data analysis, incubated for a 30 minute recovery period at 37 C, and then treated with pevonedistat for 1 hour, followed by stimulation TNFα for 15 min. Stimulated cells (after 15 minutes) were immediately fixed with 1.6% formaldehyde for 10 minutes at room temperature, washed with phosphate buffered saline (PBS), and stained with antibodies according to the protocol described by Bendall et al. Mass-channel data was recorded on a CyTOF2 mass cytometer (Fluidigm). Data were decoded using single cell debarcoder software.
(https://github.com/nolanlab/single-cell-debarcoder), and normalized with software from Fluidigm. Further analysis of data was performed in Cytobank (cytobank.org).

The intracellular cytokine protocol has been previously described. In contrast to the intracellular signaling mass cytometry protocol, the intracellular cytokine protocol includes surface marker staining of live cells, followed by fixation, a saponin-based cell permeabilization and intracellular cytokine staining. Briefly, cryopreserved cells were thawed and counted, incubated for a 30 minute recovery period at 37 C, and then incubated with intracellular signaling inhibitors (ruxolitinib, pevonedistat, or both in combination, or no inhibitor) for 4 hours at 37 C. After 2 hours of the four-hour incubation, cells were treated with secretion inhibitor cocktail (eBioscience Protein Transport Inhibitor Cocktail (500X), Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10 μM Brefeldin A and 2 μM monensin, for remaining 2 hours of incubation. Following incubation, live cells were resuspended in CyFACS buffer, counted, and stained on ice. The panel of antibodies used was previously described. Surface marker antibody staining was done on live cells on ice. Following surface marker staining, cells were stained with 2.5 μM cisplatin (Enzo Life Sciences) for 1 min, washed with CyFACS buffer, and fixed with 2% paraformaldehyde for 10 min at room temperature. Fixed cells were permeabilized with 1x eBioscience Perm Buffer (Invitrogen), and stained with the panel of intracellular cytokine antibodies diluted in eBioscience Perm Buffer. Cells were washed in CyFACS buffer. Cells were barcoded for batch run on CyTOF2 mass cytometer (Fluidigm), using metal barcode solutions diluted in 1x Maxpar Barcode Perm Buffer (Fluidigm), according to manufacturer’s instructions. Barcoded cells were fixed in 2% paraformaldehyde and labeled with Ir-intercalator (Fluidigm), to allow DNA-positive cell labeling.
Mass-channel data was recorded on a CyTOF2 mass cytometer (Fluidigm). Recorded mass cytometry raw data was further analyzed as described for intracellular signaling mass cytometry.

**Mouse studies**

Ba/F3-\textit{MPL}W515L-GFP cells (10^6 per mouse) were injected via tail vein into 7-week-years old female BALB/c mice (The Jackson Laboratories). Treatment with vehicle (20% 2-Hydroxypropyl-beta-cyclodextrin) or pevonedistat (60 mg/kg) began immediately after cell injection, twice daily by subcutaneous injection Monday through Friday. Peripheral blood was collected every Friday and GFP(+) cells were analyzed by flow cytometry. Survival was monitored daily, and moribund mice were humanely sacrificed. At the time of death liver and spleen were collected and weighed, and bone marrow was collected to analyze GFP(+) cells by flow cytometry. 100,000 bead-enriched Kit+ cells were collected from CD45.2 \textit{Jak2} V617F donor mice and transplanted via tail vein into CD45.1 lethally irradiated recipient mice. Four weeks after transplant PB was collected and hematologic parameters were measured by Hemavet and flow cytometry. Treatment, twice daily by subcutaneous injection Monday through Friday, with vehicle (20% 2-Hydroxypropyl-beta-cyclodextrin) or pevonedistat (60 mg/kg) began immediately after stable engraftment.

**HEL Cell Alamar Blue Viability Assay**

Human erythroid leukemia cells (HEL) were plated in triplicate in a 96-well assay plate at 0.05 M cells/mL in the presence of ruxolitinib and/or pevonedistat. Viability was assessed after 72 hours with Alamar Blue. Fluorescence was measured on a Biotek Microplate Reader.

**Phospho-Flow Cytometry**

HEL cells were incubated with the corresponding concentration of pevonedistat for 1 hour and a subset of the samples was stimulated with TNF\alpha for the last 15 minutes. The samples were fixed
with 1.6% formaldehyde for 10 minutes at room temperature, then permeabilized and stained with the intracellular antibodies. The samples were analyzed using a BD Fortessa flow cytometer.

**Statistical analysis**

Statistical significance was calculated with Prism software version 8 (GraphPad Software) utilizing two-tailed Student’s t test or Mann–Whitney U test as indicated. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

**Supplemental Tables**

**Supplemental Table 1. Patient information**

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<th>Patient Alias</th>
<th>Diagnosis</th>
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Supplemental Table 2. NFκB target gene RT-qPCR primer sequences

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Supplemental Figure Legends

Supplemental Figure 1. Pevonedistat suppresses TNFα-mediated NFκB signaling in HEL cells.

A) Cell viability assay of HEL cells treated with pevonedistat, ruxolitinib, or in combination. Cells were treated for 72 hours at the indicated drug concentration. Cell viability measured with Alamar Blue fluorescence detection.

B) Flow cytometry of IκBα in HEL cells treated with 20 ng/mL TNFα, pevonedistat, or in combination. HEL cells were treated with pevonedistat for 2 hours and TNFα for the last 30 minutes.

C) RT-qPCR of NFκB-pathway related genes in HEL cells treated with TNFα, pevonedistat, or in combination for the indicated durations. Relative NFκB-pathway related mRNA expression was determined by 2^-dCt and normalized to ACTB. Samples were run in duplicate for each experimental condition and timepoint.
Supplemental Figure 2. Heatmap of mass cytometry analysis of CD34+ cells from primary patient samples at basal conditions. Three mass cytometry runs were conducted and signals from CD34+ cells from primary MF/sAML patient samples were normalized to CD34+ cells from normal bone marrow (NBM 32 for run 1, NBM 43 for run 2, and NBM 44 for run 3).

Supplemental Figure 3. viSNE plots of drug-treated primary patient samples after intracellular signaling mass cytometry.

A) Identification of cell populations by cell-surface markers in two NBM donor samples and three MF/sAML patients.

B, C) Phospho-p65/RELA (S529) (B) and IκBα (C) signals in NBM 43, and MF 9, sAML patient samples after treatment with 1μM pevonedistat for 1 hour, 20 ng/mL TNFα for 15 minutes, or in combination.

D) pCREB signals in NBM 43, NBM 44, MF 9, sAML1 and sAML7 patient samples after treatment with 1 μM pevonedistat for 1 hour, 20 ng/mL TNFα for 15 minutes, or in combination.

Supplemental Figure 4. viSNE plots of drug-treated primary patient samples after intracellular cytokine mass cytometry.

A) Identification of cell populations by cell-surface markers in normal bone marrow donor (NBM 31), normal peripheral blood (LSR5 PB) and two MF (MF 2, MF 9) samples.

B) Intracellular cytokine signals after treatment with 5 μM ruxolitinib for 4 hours, 1 μM pevonedistat for 4 hours, or in combination.

Supplemental Figure 5. Intracellular cytokine mass cytometry analysis of CD14+ cells.
A) Mass cytometry showing reduced TNFα (upper panel) and IL-6 (bottom panel) in monocytes from primary patient samples treated with 5 µM ruxolitinib, 1 µM pevonedistat, or their combination. Cells were treated for 4 hours.

B) Intracellular cytokines of CD14+ cells from normal bone marrow (NBM 31), normal peripheral blood (LSR5 PB) and two MF (MF 2, MF 9) samples analyzed by mass cytometry. Cells from patient samples were treated with 5 µM ruxolitinib for 4 hours, 1 µM pevonedistat for 4 hours, or in combination. Signals from each patient sample per treatment condition were normalized to its basal signal.

**Supplemental Figure 6. Pevonedistat treatment of mice transplanted with Ba/F3 MPLW515L cells.**

A) Flow cytometry validation of GFP+ Ba/F cells expressing MPLW515L relative to parental Ba/F3 cells.

B) Body weights of mice treated with vehicle or pevonedistat at indicated dates.

C) Representative images showing sizes of excised spleens and livers from vehicle or pevonedistat-treated mice.

D) Representative images showing necrosis of internal organs from vehicle, but not pevonedistat-treated mice.
Supplemental Figure 1

A

B

C
Supplemental Figure 2
Supplemental Figure 4
Supplemental Figure 5
Supplemental Figure 6