The Role of Thrombopoietin Signalling in *JAK2*^{V617F}-positive Myeloproliferative Neoplasms

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Abstract

Thrombopoietin (TPO) is the primary regulator of megakaryocyte development, regulating proliferation and differentiation in addition to the number of circulating platelets through binding to and stimulation of the cell surface receptor MPL. Activating mutations in MPL constitutively stimulate downstream signalling pathways, leading to aberrant haematopoiesis and contribute to development of myeloproliferative neoplasms (MPNs). Several studies have mapped the tyrosine residues within the cytoplasmic domain of MPL that mediate these cellular signals; however, secondary signalling pathways are incompletely understood. Additionally, the identification of the *JAK2*^{V617F} mutation has profoundly increased our understanding of MPNs and although a role has been implicated *in vitro*, the *in vivo* role of MPL in *JAK2*^{V617F}-positive MPNs has yet to be determined.

In this thesis, a novel signalling pathway for the negative regulation of TPO signalling was identified whereby MPL^{Y591} is phosphorylated resulting in association of SYK which negatively regulates TPO-mediated ERK1/2 signalling. Additionally, genetic manipulation of an *in vivo JAK2*^{V617F}-positive MPN mouse model led to the identification of MPL as an essential molecular component for development of *JAK2*^{V617F}-postive MPNs. In the absence or reduction of *MPL*, the disease fails to develop. However, removal of the cytokine, TPO, was unable to prevent the disease from developing.

These findings provide novel insights not only into regulation of TPO-signalling but also the role of TPO and MPL in *JAK2*^{V617F}-positive MPN disease pathogenesis. Identification of the role of MPL in MPN pathogenesis, as well as insights into additional regulatory pathways, contributes to our understanding of normal and pathological TPO signalling. These new insights also provide a basis for development of novel therapeutics for the treatment of MPNs and other diseases resulting from aberrant of TPO signalling.

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Author's Declaration

All of the work presented in this thesis was performed by myself with the exception of the mass spectrometry and SH2/PTB domain arrays in Chapter 3 which were performed by Dr. Sebastian J. Saur and Dr. Alexis Kaushansky, respectively and neutrophil *JAK2*^{V617F} expression levels presented in Chapter 5 which was performed by Dr. S. Leah Etheridge. All sources are acknowledged as references. This thesis has not been submitted for any other degrees. Some of the data has been published in peer-reviewed journals as listed below.

Sangkhae, V., Saur, S. J., Kaushansky, A., Kaushansky, K., and Hitchcock, I. S. (2014) Phosphorylated c-Mpl tyrosine 591 regulates thrombopoietin-induced signaling. *Exp Hematol* **42**, 477-486 e474.

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Sangkhae, V., Etheridge, S. L., Kaushansky, K., and Hitchcock, I. S. (2014) The thrombopoietin receptor, MPL, is critical for development of a JAK2V617F-induced myeloproliferative neoplasm. *Blood* **124**, 3956-3963.

CHAPTER 1 INTRODUCTION

1.1 Haematopoiesis: An Overview

Haematopoiesis is a dynamic process by which all of the mature blood cells of the body are generated through successive differentiation of a haematopoietic stem cell (HSC)(Figure 1.1). HSCs are a rare population of cells found in the bone marrow of adult mammals and are responsible for production of all mature blood cells throughout a person's lifetime. HSCs are defined by their dual capacity to undergo self-renewal and differentiate into mature hematopoietic cells including lymphoid and myeloid cells.

1.1.1 Embryonic Haematopoiesis: Emergence and expansion of HSCs

Mammalian haematopoiesis initiates at embryonic day 7.5 (E7.5) in the yolk sac blood islands and subsequently occurs in the aorta-gonad-mesonephros (AGM) region and the foetal liver until the bone marrow (BM) develops and takes over as the primary site of haematopoiesis throughout adulthood(5-7)(Figure 1.2). Developmental haematopoiesis is separated into two sequential waves: primitive haematopoiesis and definitive haematopoiesis(8). Primitive haematopoiesis occurs in the mammalian yolk sac giving rise to a unique population of primitive erythroid progenitors(9) to facilitate transport of oxygen to the developing embryo(10). Additionally, macrophages are produced(11) to aid in tissue remodelling(12). Definitive haematopoiesis is marked by the emergence of functional HSCs(12), which initially arise in the AGM region at murine E10-11(13,14); this region is analogous to the ventral endothelium of the dorsal aorta in the 5-week human embryo(15). Runx1, also known as acute myeloid leukaemia 1 protein (AML1) or core-binding factor subunit alpha-2 (CBFA2), is a transcription factor essential for AGM-derived HSC formation(16). All embryonic HSCs were shown to express *Runx1*(17). Mice lacking functional Runx1 exhibit normal primitive or yolk-sac derived haematopoiesis but failed to establish normal foetal liver haematopoiesis resulting in death at E12.5(16). Additionally, HSCs have also been detected in the mouse placenta paralleling the timing of appearance in the AGM region(18,19).

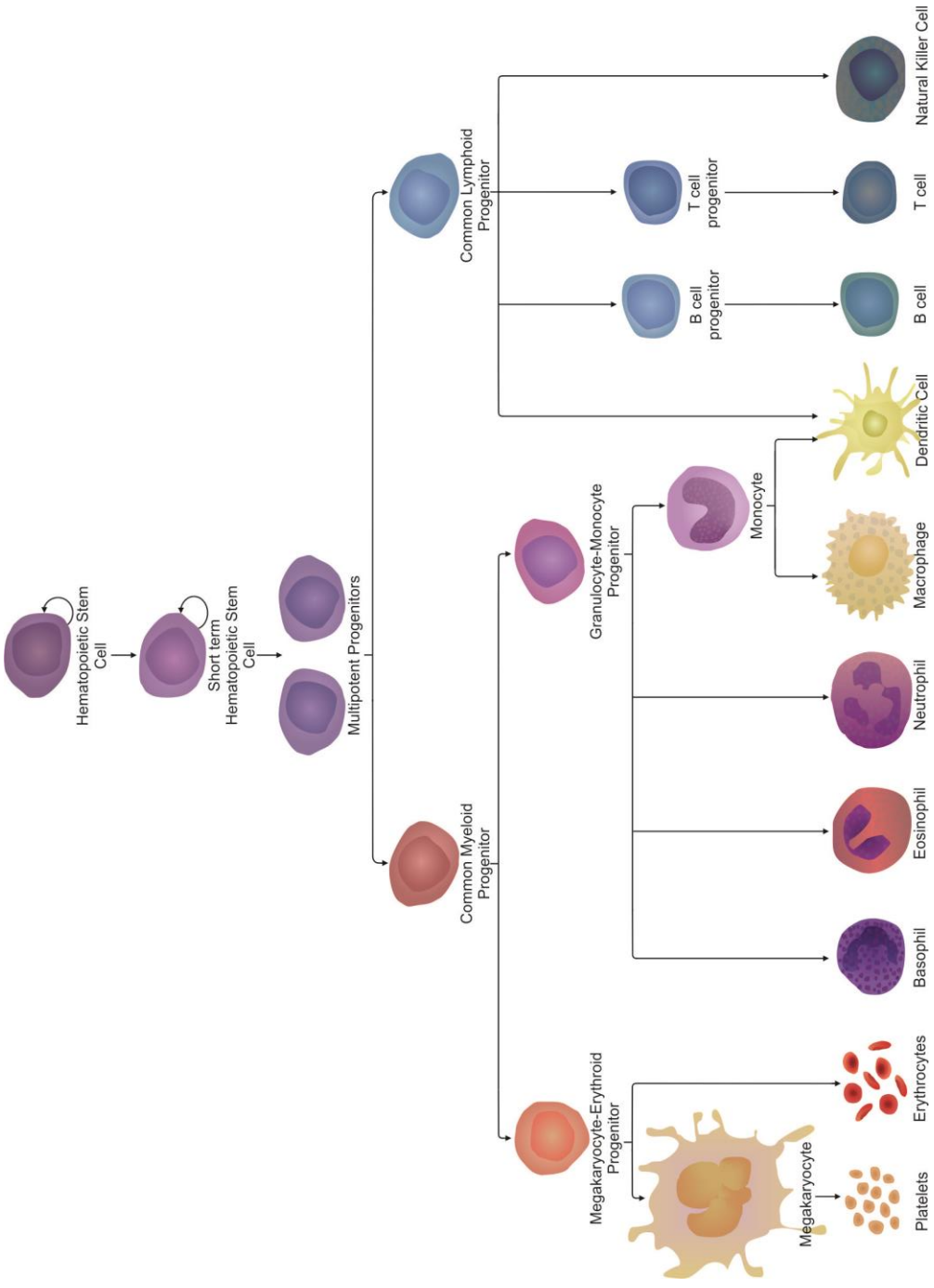


Figure 1.1 Overview of Haematopoiesis
Diagram representing differentiation of blood cells from the haematopoietic stem cell

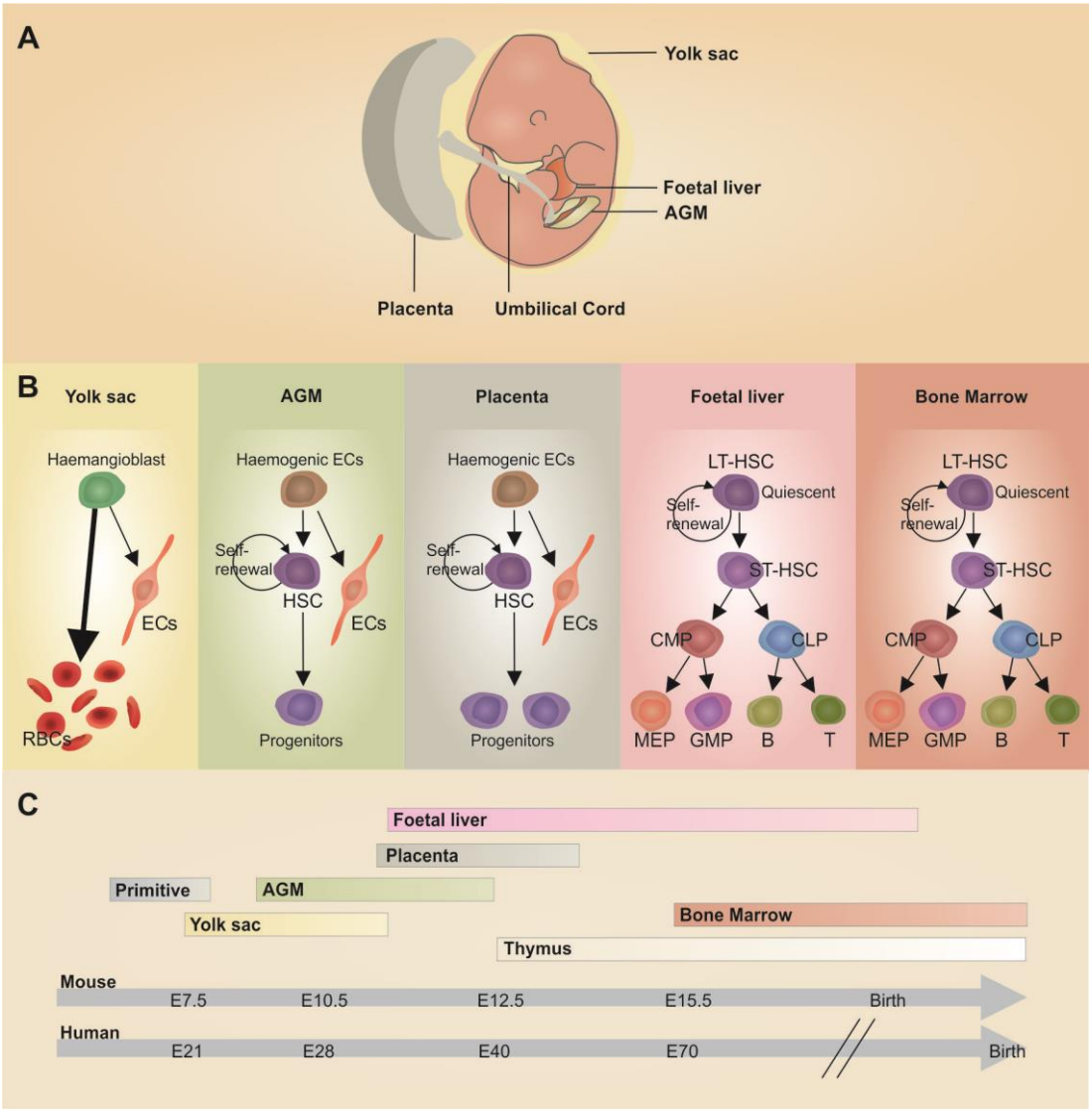


Figure 1.2 Embryonic Haematopoiesis

(A) Diagram of a mouse embryo at E11.5 with haematopoietic tissues labelled. (B) Diagram of cells generated within haematopoietic tissues within an embryo. (C) A timeline illustrating the primary regions of haematopoiesis during embryogenesis to birth in both mice and humans. Abbreviations: AGM – aorta-gonad-mesonephros; ECs – endothelial cells; RBCs – red blood cells; HSCs – haematopoietic stem cells; LT-HSC – long term haematopoietic stem cell; ST-HSC – short term haematopoietic stem cell; CMP – common myeloid progenitor; CLP – common lymphoid progenitor; MEP – megakaryocyte/erythroid progenitor; GMP – granulocyte/monocyte progenitor; B – B-cell; T – T cell.

Subsequent haematopoiesis occurs when HSCs migrate through the circulation where they colonize and expand within the foetal liver(12). HSCs ultimately colonize the thymus, spleen and bone marrow(5). It is believed that these sites provide niches to support HSC expansion rather than *de novo* generation(5). In adult mammals, the bone marrow is the primary site of haematopoiesis.

1.1.2 The Haemangioblast

In addition to initiation of primitive haematopoiesis, endothelial cells also differentiate from the mesoderm at E7.5. This concurrent development resulted in the hypothesis of a common precursor for blood and vascular cells called the haemangioblast (Figure 1.3). A number of cell surface markers are common between haematopoietic precursors and endothelial cells including the haematopoietic progenitor cell antigen CD34 (CD34), Foetal Liver Kinase 1 (Flk-1/CD135/fms-like tyrosine kinase 3 (FLT-3)) and the angiopoietin-1 receptor (Tie2)(20-22), further suggesting presence of a common progenitor. Both $Flk^{-/-}$ and $Tie2^{-/-}$ embryos are deficient in both haematopoietic and vascular development and die *in utero* between E8.5-9.5 or at E10.5, respectively(21,22). *In vitro* culture of individual blast colony forming cells derived from embryonic stem (ES) cells was able to generate both multi-lineage haematopoietic and endothelial cells, providing evidence for the existence of the elusive haemangioblast(23,24).

1.1.3 Haematopoietic Stem Cell

HSCs are a small subset of cells in the bone marrow responsible for production of all mature blood cells throughout a person's lifetime. HSCs are defined by their dual capacity to undergo self-renewal and differentiate into mature haematopoietic cells(25).

In 1961, Till and McCulloch observed in irradiated mice distinct nodules in the spleen of recipient mice which contained colonies of proliferating cells of multiple haematopoietic lineages(26), these colonies were later determined to be clones from single cells(27) capable of self-renewal(28). Due to this capacity for self-renewal in addition to their ability to expansively proliferate and differentiate, these cells were thought to be HSCs(29) and importantly, spleen colony formation provided an *in vivo* method to quantify these cells(26).

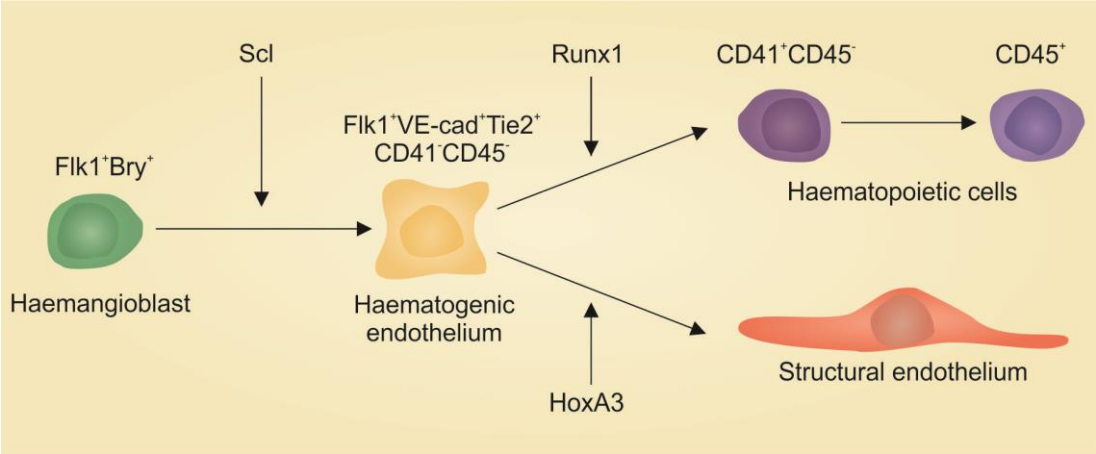


Figure 1.3 The Haemangioblast

A schematic depicting the differentiation of a haemangioblast into both HSCs and endothelial cells. Above the cells are cell associated cell surface markers. Transcription factors promoting lineage differentiation are indicated by arrows. Adapted from Medvinsky et al. *Development*, 2011(30).

The study of HSC biology is reliant on the ability to distinguish HSCs from other progenitor cells. To this end, there have been many studies aiming to identify markers capable of definitive identification of HSCs. The characterization of HSCs was vastly improved with the introduction of flow cytometry. As HSCs are capable of differentiation into multiple hematopoietic lineages, it was postulated that cells expressing lineage specific markers could be excluded as HSCs(31). In order to isolate a more pure population of HSCs from mouse bone marrow, monoclonal antibodies against B cells (B220), granulocytes (Gr-1), myelomonocytic cells (Mac-1) and T cells (CD4, CD8) were used to isolate lineage negative (Lin^-) cells(32). This isolated population was found to express low levels of thymocyte differentiation antigen 1 (Thy-1, CD90), consistent with previous reports characterizing rat(33) HSCs. This population is further divided based on stem cell antigen-1 (Sca-1) expression, to enrich for thymic repopulation ability(34), and it was reported that $Thy\text{-}1^{lo}Lin^-Sca\text{-}1^+$ cells (0.05% of whole bone marrow) comprised of a 50% pure long term HSC (LT-HSC) population(32). The $Thy\text{-}1^{lo}Lin^-Sca1^-$ population is enriched for short term HSCs (ST-HSC)(32). Later reports, however, found this fraction of cells to be heterogeneous with only 25% of the cells being capable to long term repopulation ability, the remainder providing only transient multilineage repopulation(35). In the search for a pure HSC population a number of additional cell surface markers were analysed for their potential to better distinguish the HSC. Morrison et al. found stem cell growth factor receptor (c-kit, CD117) expression to further enrich the $Thy\text{-}1^{lo}Lin^-Sca\text{-}1^+$ HSC population for LT-HSCs(35). Osawa et al. then utilized the human HSC marker, CD34, to further enrich the $c\text{-}kit^+Thy\text{-}1^{lo}Lin^-Sca\text{-}1^+$ HSC population. Unexpectedly, the $CD34^{lo/-}$ population was better at distinguishing LT-HSCs than the $CD34^+$ population(36). Christiansen et al. used a different tyrosine kinase, Flk-2 and found isolated that $c\text{-}kit^+Thy\text{-}1.1^{lo}lin^{-/lo}Sca\text{-}1^+Flk\text{-}2^-$ cells were functionally superior at long term multilineage reconstitution, reconstituting all irradiated recipients whereas $c\text{-}kit^+Thy\text{-}1.1^{lo}lin^{-/lo}Sca\text{-}1^+Flk\text{-}2^+$ cells provided only short term reconstitution(37). Thus, the $Thy\text{-}1^{lo/-}Lin^-Sca\text{-}1^+c\text{-}kit^+CD34^{lo/-}Flk\text{-}2^-$ mouse bone marrow population is highly enriched for LT-HSCs whereas the $Thy\text{-}1^{lo/-}Lin^-Sca\text{-}1^+c\text{-}kit^+CD34^+Flk\text{-}2^+$ is enriched for ST-HSCs. Although these markers are very good for distinguishing between LT-HSCs and ST-HSCs from the bone marrow, the number of antibodies necessary to isolate these

cells makes studying these cells *in vivo* within their microenvironment extremely difficult. To this end, the SLAM family of cell surface receptors was identified as markers of HSCs(38).

The SLAM family includes signalling lymphocytic activation molecule 1 (SLAMF1/CD150), natural killer cell receptor 2B4 (CD244) and signalling lymphocytic activation molecule 2 (SLAMF2/CD48) are differentially expressed on HSCs, multipotent progenitors (MPPs) and lineage restricted progenitors. Using only the SLAM family of markers, the $CD150^{+}CD244^{-}CD48^{-}$ population (0.0084%±0.0028% of whole bone marrow) contains 20% LT-HSCs while the $CD150^{-}CD244^{+}CD48^{-}$ population is indicative of MPPs and the $CD150^{-}CD244^{+}CD48^{+}$ population of lineage restricted progenitors(38). However, the HSC purity in this fraction of cells is further improved upon selection from the $Sca\text{-}1^{+}Lin\text{-}c\text{-}kit^{+}$ subset of HSCs, with 50% of the $Sca\text{-}1^{+}Lin\text{-}c\text{-}kit^{+}CD150^{+}CD244^{-}CD48^{-}$ population being LT-HSCs(38). Although this fraction is highly enriched for LT-HSCs, it still requires a lot of markers for identification and isolation. Kent et al. defined a modified set of markers, termed E-SLAM(39) which differs from SLAM HSCs ($CD150^{+}CD48^{-}$) in that it includes the murine endothelial protein C receptor (EPCR/CD201) which was found highly expressed in HSCs(40). Selection for the four $E\text{-}SLAM^{+}$ markers, $C150^{+}CD45^{+}CD201^{+}CD48^{-}$, yields 43% LT-HSCs(39).

1.2 Thrombopoietin Signalling

1.2.1 Discovery of MPL

The myeloproliferative leukemia virus (MPLV) was first described in 1986 by Wendling and colleages(41). They showed that mice infected with MPLV developed an acute and generalised myeloproliferative disorder, characterised by hepatosplenomegaly, granulocytosis, thrombocytosis and polycythemia(41). MPLV infection was sufficient to induce myeloid progenitor cell transformation resulting in the ability of these cells to grow and differentiate in the absence of exogenous growth factors *in vitro*(42,43). To conclusively demonstrate that MPLV was the molecular entity responsible for the disease, the full-length provirus was cloned and tested for transforming ability(44). Intravenous injection of viral supernatant into mice resulted in splenomegaly, leucocytosis and polycythaemia(44). Further analysis of haematopoietic progenitor cells from these mice revealed cells capable of proliferation and differentiation in the absence of exogenous colony-stimulating factors(44), demonstrating that MPLV was indeed the molecular entity necessary for the observed myeloproliferation. Sequence analysis of MPLV in 1990 revealed it was derived from Friend murine leukemia virus (F-MuLV) with 1.5kb of the F-MuLV envelope replaced by a novel 0.7kb non-viral sequence(44). The novel sequence, termed viral *mpl* (v-*mpl*), was found to be conserved among mammals, including humans and specific to cells within major hematopoietic compartments of mice(44). The predicted amino acid (aa) sequence of the MPLV *env-mpl* gene suggested that the v-mpl protein coded for a membrane spanning receptor with no predicted kinase activity and a conserved Trp-Ser-X-Trp-Ser (WSXWS) NH2-terminal ligand binding motif characteristic of the hematopoietic cytokine receptor superfamily(44,45). The human homolog, cellular MPL (c-MPL), was successfully cloned in 1992(46). In this body of work, MPL refers to c-MPL.

1.2.2 MPL structure

MPL belongs to the homodimeric type I cytokine receptor family. This family of receptors is characterised by the presence of four conserved cysteine residues forming a cytokine receptor homology module (CRM), the WSXWS motif, fibronectin type III domains and conserved Box1/Box2 motifs(47)(Figure 1.4).

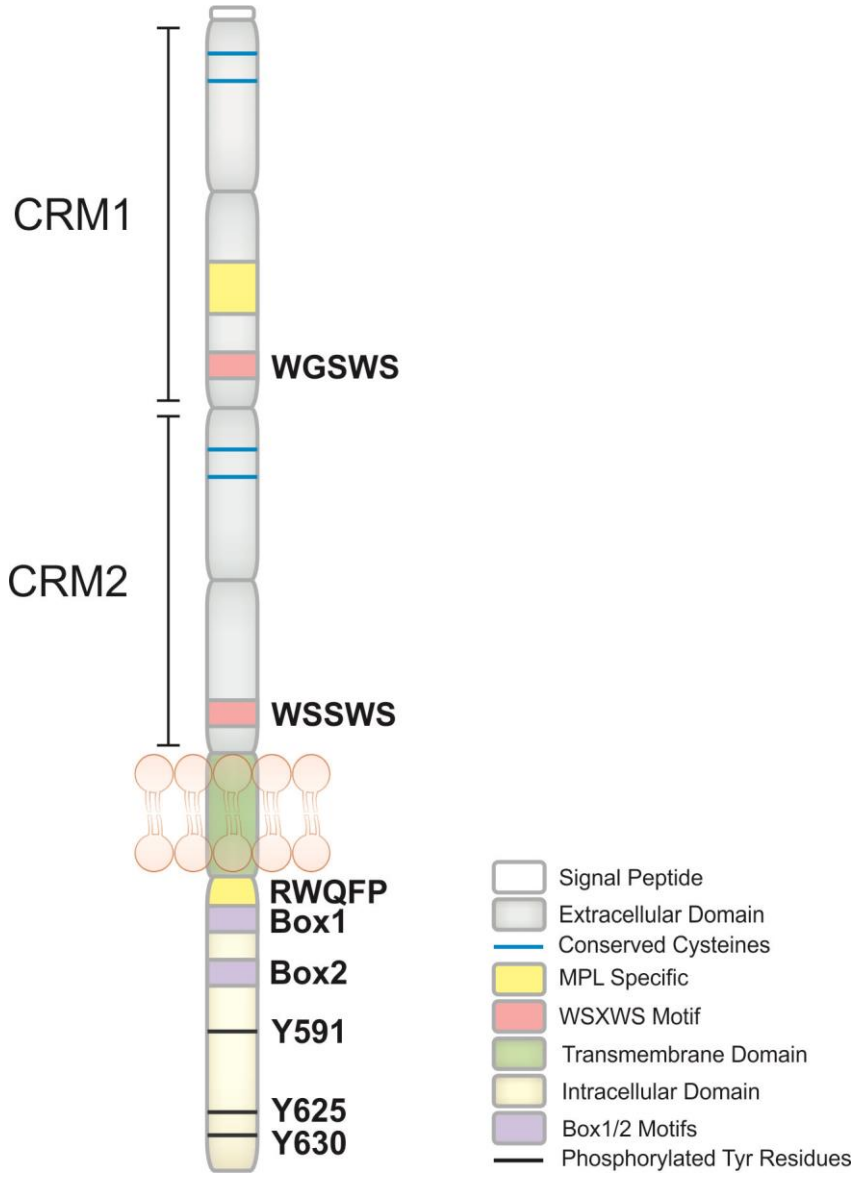


Figure 1.4 Schematic representation of human MPL monomer
Figure represents full length human MPLP isoform which is 635aa long. The location of important motifs is indicated. MPL contains an 18aa signal peptide, 2 CRMs, a single transmembrane spanning region, an amphipathic motif at the junction between the transmembrane and cytoplasmic regions, Box1/Box2 motifs for JAK2 binding and cytoplasmic residues that are phosphorylated in response to TPO.

The family can be further subdivided into groups based on sequence and structural homology of the cytokine and its receptor. MPL belongs to the group 1 receptors along with the erythropoietin receptor (EPOR), prolactin receptor (PRLR), growth hormone receptor (GHR) and orphan receptor CLF-3(48). Group 1 receptors and ligands are considered the prototypic type I cytokines. These receptors are comprised of a single chain, function as homodimers and are characterised by an extracellular domain consisting of only CRMs(48,49). Full length human MPL protein is comprised of 635aa with a large 485aa extracellular domain including a predicted 18aa signal peptide and two CRMs, a 22aa transmembrane domain and a short 122aa intracellular domain(46)(Figure 1.4). Human *MPL* was mapped to chromosome 1p34(50) and murine *MPL* to chromosome 4(51). MPL is highly conserved, the human and mouse proteins share 81% amino acid identity, conservation is highest for the cytoplasmic domain with 91% identity(52). Human *MPL* is encoded by 12 exons over 17kb of DNA with a single exon coding for the signal peptide, four exons for each of the two CRMs, a single exon for the transmembrane domain and two exons for the cytoplasmic domain(53). There are four *MPL* mRNA isoforms found in humans. The first isoform, *MPLP* contains 12 exons and encodes the full length functional receptor(46,53). Translation initiation is thought to occur at the methionoine codon at position 8(46). The second isoform, *MPLK*, encodes a truncated 572aa receptor resulting from premature transcript termination within intron 10(46,53). The resulting receptor contains an extracellular and transmembrane domain identical to MPLP but has a cytoplasmic domain of only 66aa which differs from MPLP after 9aa(46). The extracellular domains of both MPLP and MPLK contain four N-linked glycosylation sites(46). A third isoform, *MplS*, is the result of transcripts lacking exons 9 and 10, resulting in loss of CRM-2 and the transmembrane domain thus coding for a soluble form of MPL(53). A fourth isoform, termed *MPL-del*, lacks 24 amino acids within the extracellular domain resulting from alternative splicing between exons 8 and 9 and encodes a protein that is not expressed on the cell surface(54). Upon western blot detection of human MPL from Ba/F3-MPL cell lines, there are 2 glycoforms present, an 85kDa and 80kDa form representing the mature fully glycosylated and immature forms of the receptor, respectively(55,56).

1.2.3 MPL Biology

It was recognised early on that the cellular role of MPL was in haematopoiesis(41,44). The ability of the v-*mpl* to induce generalised myeloproliferation was the first line of evidence(41). Moreover, MPL expression is restricted to haematopoietic tissues including adult spleen, foetal liver and bone marrow(44). The ability of MPLV to induce growth factor independence within a broad range of hematopoietic lineages suggested its role in early HSCs(44). Antisense oligodeoxynucleotide targeting of *Mpl* prevented maturation of MK progenitors, revealing its role as a regulator of megakaryopoiesis(57). The proposed role of MPL in HSCs and megakaryopoeisis was supported by its expression on primitive hematopoietic progenitor cells and enriched expression on megakaryocytes and platelets(57). Although MPL was identified in 1990, it remained an orphan receptor until the cloning of its ligand thrombopoietin (TPO) in 1994, making it difficult to study its exact biological role. The biological roles of TPO and MPL are discussed together in detail in the subsequent sections.

1.2.4 Cloning of Thrombopoietin

Platelets were first described by Bizzozero in 1882 and in the 1900s, Wright reported that these cells, which arose from bone marrow megakaryocytes, were essential in coagulation (Reviewed in(58)). A number of studies following these seminal observations, led to the idea that platelet production was a regulated process (Reviewed in (58)), eventually leading Kelemen to the coin the term ‘thrombopoietin’(59). The cytokine was finally cloned almost simultaneously in 1994 by five independent groups and was shown to stimulate platelet production *in vivo* through expansion of megakaryocyte progenitors and promotion of megakaryocyte maturation as measured by increased cell size and polyploidy(60-64) and (Reviewed in (58)). Based off of the N-terminal sequence of purified porcine TPO, de Sauvage and colleagues were able to clone human TPO(62). Whereas, genetic removal of previously cloned cytokines capable of moderately increasing platelet counts did not result in thrombocytopenia (Reviewed in (58)), animals genetically deficient in the gene for TPO (*thpo*), were severely thrombocytopenic(65), validating TPO as the regulator of platelet production.

1.2.5 Thrombopoietin structure

Human TPO cDNA is 1,774 nucleotides in length with a poly(A)$^{+}$ tail with an open reading frame of 1,059 nucleotides(62). It is synthesized as a 353 amino acid long polypeptide which is cleaved to form a mature 332 amino acid long secreted protein(66). The human TPO gene was mapped to chromosome 3q27 and is 6.2kb in length comprised of six exons and five introns(67), although an additional exon was subsequently detected upstream of exon 1(68). The protein is encoded by exons 3-7(68). Only a single copy of the TPO gene was detected in the genome(67) but a number of splice variants have been reported. The first has a 4aa deletion of residues 112-115 at the junction of exon 6 and 7 but maintained the same reading frame whereas the second variant has an extra splice site within exon 7 and resulted in a frameshift. Studies in which cells were transfected with full-length TPO cDNA resulted in expression and secretion of biologically active TPO(68,69); however, both splice variants were expressed but not secreted(68) and was therefore unable to induce proliferation of MPL expressing Ba/F3 cells(69).

The protein consists of two distinct domains. The amino(N)-terminal domain of human TPO, which consists of 153-residues, is responsible for receptor binding(64). This region is also highly hydrophobic and is predicted to function as a secretory signal(69). The carboxyl(C)-terminal domain is less conserved; however, conserved glycosylation sites within this domain(69) undergo both N- and O-linked glycosylation(70,71), which promotes protein stability(70). Although independent studies found the C-terminal domain to be necessary for protein secretion, the role of glycosylation remains uncertain. One study found glycosylation at Asn213 and Asn234 to be necessary for proper secretion of the protein(72) whereas another study reported that N-linked glycosylation only played a minor role(71).

The receptor-binding domain or N-terminal region of TPO shares structural similarities and high sequence homology with EPO, maintaining 22% identity and 25% conserved substitutions; however, both bind to their cognate receptor with high fidelity(65,73,74). Sequence alignment of TPO against interleukin-4 (IL-4) resulted in prediction of 40 surface exposed residues that were subsequently tested for functional activity using alanine-scanning mutagenesis, monoclonal epitope mapping

and phage display binding assays(75). Mutation of two residues, Asp8 and Lys138, had the largest effect on receptor binding increasing the half-maximal effective concentration (EC_{50}) values approximately 20-fold. Additional residues affecting binding include Lys14, Lys52, Arg136 and Arg140 with an increased EC_{50} between 5- and 15-fold and Arg17, Ser24, Lys59, Arg98, Ser106, Leu129, Gln132, His133 and Leu144 which increased EC_{50} between 2- to 5-fold. The majority of these resides map to helix-1 and helix-4 and map to one side of the predicted protein(75). Recently, a clinical mutation of TPO, Arg17Cys, which maps to helix-1, was recently described in a family with congenital thrombocytopenia and aplastic anaemia(76) validating the importance of helix-1 for receptor association. The crystal structure of the receptor-binding domain of TPO revealed a four helix bundle with up-up-down-down topology, also known as an antiparallel four-helix bundle fold(73).

1.2.6 Thrombopoietin biology

1.2.6.1 Thrombopoietin regulation

It was discovered well before the cloning of human TPO that plasma, serum and urine from thrombocytopenic patients and animals contained the factor capable of promoting megakaryopoiesis (reviewed in(77)). In fact, this served as the basis for some of the strategies utilized to clone TPO(62-64). The cloning of TPO allowed for investigation into its regulation. Insight into transcriptional regulation came when a *Tpo*$^{-/-}$ mouse was generated by de Sauvage et al.(65). Unlike the *Mpl*$^{+/-}$ mouse(78), the heterozygous *Tpo* mouse displayed an intermediate platelet count between wild-type (*WT*) and *Tpo*$^{-/-}$, indicative of a gene dosage effect which was supported by reduced TPO mRNA in these mice(65). Therefore, TPO is constitutively expressed with regulation occurring predominantly at the post-transcriptional level. TPO is primarily synthesized in the liver and to a lesser extent the kidneys and subsequently released into the circulation (Figure 1.5)(79). Analysis of TPO levels relative to platelet mass revealed an inverse relationship between the two(80,81). The stimulatory effect of TPO rich serum from thrombocytopenic animals on megakaryopoiesis was eliminated after addition of platelets or soluble MPL(80), suggesting that MPL expressed on platelets was a likely candidate for sequestering

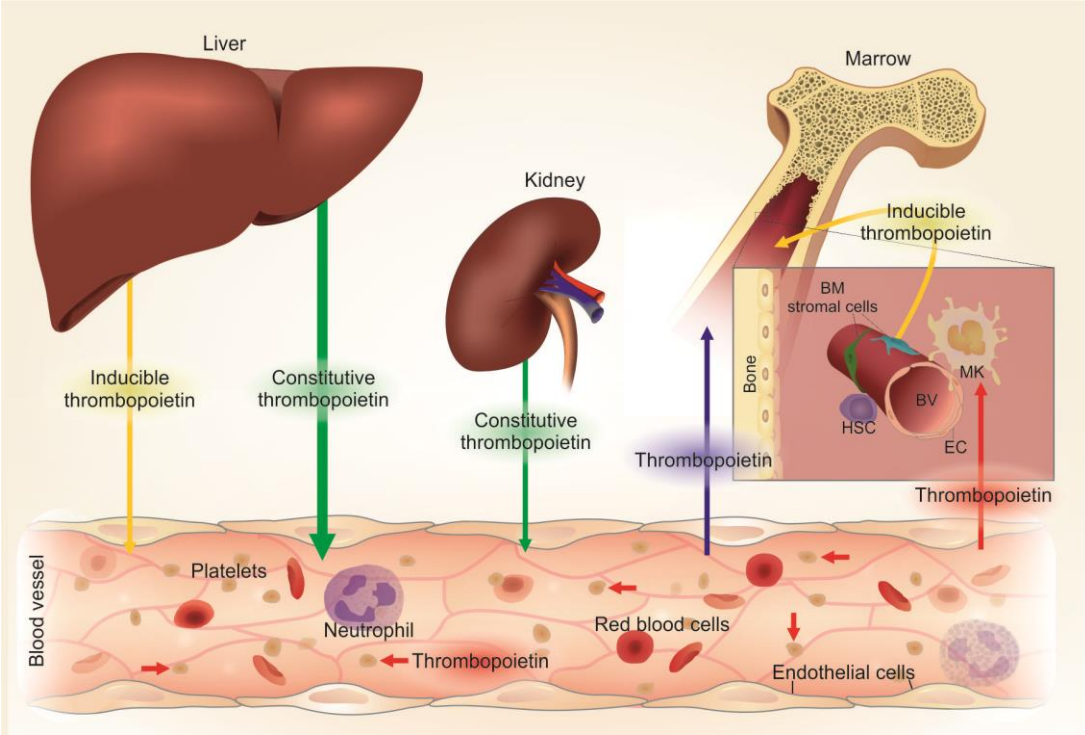


Figure 1.5 Regulation of thrombopoietin

Thrombopoietin (TPO) synthesis (green arrows) is both constitutive and inducible. Constitutive synthesis occurs primarily by the liver and to a lesser degree by the kidneys. Under physiological stress, such as thrombocytopenia or inflammation, TPO synthesis is induced in the liver and bone marrow stroma. Additionally, binding of desialylated platelets to hepatic Ashwell-Morell receptors is also thought to induce TPO synthesis in the liver. TPO acts primarily in the bone marrow (blue arrow), where receptor(MPL)-bearing cells bind and utilise the cytokine. Uptake of TPO by platelets and megakaryocytes regulates levels of circulating TPO (red arrows).Abbreviations: BM – bone marrow; HSC – haematopoietic stem cell; BV – blood vessel; EC – endothelial cell; MK – megakaryocyte.

circulating TPO. This mechanism for TPO clearance is known as the "sponge theory" and was further supported by the observation that *Mpl*$^{-/-}$ mice have elevated TPO levels despite normal levels of TPO mRNA(78). Similarly, in patients with congenital amegakaryocytic thrombocytopenia (CAMT) resulting from non-functional MPL, there are increased concentrations of circulating TPO(82). Additionally, transfusion of *WT* platelets into *Mpl*$^{-/-}$ mice was able to reduce plasma TPO levels through binding, internalization and degradation of the cytokine(83). Although some studies found no change in TPO mRNA levels in kidneys and livers of thrombocytopenic mice(65,83), McCarty and colleagues detected increased TPO mRNA in marrow and spleens of thrombocytopenic mice by semi-quantitative RT-PCR(84), suggesting that TPO can be regulated transcriptionally in response to platelet demand within these tissues. Additionally, expression can be regulated by the inflammatory mediator interleukin-6 (IL-6). Increases in IL-6 induce upregulation of hepatic TPO mRNA both *in vitro* and *in vivo*(85).

Conversely, numerous studies suggest megakaryocyte mass rather than platelet mass is responsible for TPO clearance. It is difficult to distinguish the role of platelets versus megakaryocytes in TPO clearance as megakaryocyte mass directly correlates with platelet mass. However, this theory is predominantly based on two disorders, immune thrombocytopenic pupura (ITP) and myelodyplastic syndrome (MDS), whereby megakaryopoiesis is maintained but platelets are prematurely cleared by the immune system or production of platelets is dysfunctional, respectively. Despite normal numbers of megakaryocytes in the marrow, patients with ITP had relatively normal TPO levels(86-88). Similarly, in patients with MDS, plasma TPO levels were similar to healthy control patients(88). Since TPO levels in these patients are not as high as those observed with aplastic anemia (AA) despite similar platelet levels, this supports the role of megakaryocytes in the clearance of plasma TPO.

A recent study suggests binding of desialyated platelets to the Ashwell-Morell receptor (AMR) as the key regulator of TPO production(89). Platelets become desialylated over time and are subsequently removed by the AMR. In mice lacking functional AMR (*Asgr2*$^{-/-}$), they reported increased platelet count, volume and half-life with a larger proportion of mature platelets consistent with lack of clearance by

the AMR(89). Additionally, hepatic TPO mRNA was decreased by approximately 45% in *Asgr2*$^{-/-}$ mice suggesting that the AMR is not only necessary for clearance of desialylated platelets but also plays a role in upregulating *TPO* expression(89). Specifically, platelet uptake by the AMR results in increased Janus kinase 2 (JAK2) and Signal Transducer and Activator of Transcription 3 (STAT3) activation in hepatocytes *in vivo* and results in increased TPO mRNA expression *in vitro*(89). However, induced immune thrombocytopenia was able to increase plasma TPO levels but not increase TPO mRNA levels in *WT* and *Asgr2*$^{-/-}$ mice(89), thereby suggesting that although the AMR may play a role in TPO production, there are additional mechanisms regulating levels of circulating TPO.

1.2.6.2 Thrombopoietin and megakaryopoiesis

Several lines of evidence suggested a role for MPL in megakaryopoiesis, firstly, reduction in MPL expression in CD34^{+} cells through use of antisense oligonucleotides to MPL resulted in decreased *in vitro* megakaryocytic colony formation (CFU-MK) without affecting other haematopoietic lineages(57). Additionally, expression of MPL is highly enriched in platelets and megakaryocytes(57,90). The cloning of TPO provided a crucial element in the study of megakaryopoiesis. When TPO was cloned it was shown that TPO was able to drive megakaryocyte expansion and maturation both *in vitro* and *in vivo*(61). Injection of recombinant TPO *in vivo* induces a significant increase in platelets(60,91) resulting from stimulation of megakaryopoiesis(61,91). Additionally, TPO works synergistically with interleukin-3 (IL-3) and stem cell factor (SCF) to support CFU-MK formation(61). Importantly, lack of TPO completely prevents CFU-MK maturation, demonstrating that TPO is the primary regulator of megakaryocyte development and expansion(92). However, its role in thrombopoiesis is less defined. Culture of megakaryocytes in TPO promotes demarcation of the membrane, indicative of platelet formation(92). However, CD34^{+} progenitor cells are able to produce platelets in the absence of TPO when cultured in IL-3, IL-6 and SCF(93). Additionally, mice deficient in TPO or MPL (*Tpo*$^{-/-}$ or *Mpl*$^{-/-}$) are still capable of producing a low level of platelets(65,78) which suggests additional pathways exist to compensate in the absence of TPO signalling.

1.2.6.3 Thrombopoietin and haematopoietic stem cells

The ability of MPLV to induce proliferation of multiple haematopoietic lineages(41) alluded to a possible role for MPL in HSCs. Furthermore, in addition to expression on megakaryocytes, MPL is expressed on $CD34^+$ progenitor cells(57). Culture of LT-HSCs in TPO, IL-3, IL-6 and SCF increased proliferation significantly compared to culture in IL-3, IL-6 and SCF(94). However, culture in TPO alone was unable to recapitulate this proliferative effect, although it was able to support survival of these cells(94). In ST-HSCs, culture in TPO was sufficient to induce CFU-MK(94). These data suggest that TPO is capable of promoting HSC survival and promoting megakaryopoiesis. As mentioned previously, additional insights into the role of TPO and MPL in haematopoiesis came from generation of $Mpl^{-/-}$ and $Tpo^{-/-}$ mice. In $Mpl^{-/-}$ mice, the number of haematopoietic progenitor cells is greatly reduced(95). In BM transplantation studies, the repopulation capacity of HSCs lacking MPL was reduced seven-fold(96). In another study, *WT* HSCs were transplanted into $Tpo^{-/-}$ or *WT* mice; HSC expansion was reduced 15-20-fold when transplanted into $Tpo^{-/-}$ mice compared to *WT*(97). The importance of MPL in HSC maintenance is highlighted in patients that harbour mutations in MPL resulting in CAMT; although patients initially present with thrombocytopenia, the disease progresses to complete bone marrow failure, presumably due to exhaustion of the stem cell pool, necessitating a bone marrow transplant(82,98). These data support a significant role for TPO and MPL in HSC biology.

1.3 Thrombopoietin Signalling

Cytokines binding to their cognate receptors induce phosphorylation of the receptors themselves in addition to downstream signalling targets(99). Receptor tyrosine phosphorylation is a critical step in growth factor-induced signal transduction(100) as evidenced by experiments demonstrating that tyrosine kinase inhibition reduces cytokine-induced growth(101) whereas inhibition of phosphatases sustains proliferation of cells in the absence of cytokine(102). Regulation of signal transduction is necessary for normal cellular function while unregulated activation can lead to disease. Consequently, it is important to develop a clear understanding of cytokine induced signalling cascades and the regulators in place for dissolution of these signals.

1.3.1 TPO-mediated signal transduction

Like other members of the type I cytokine receptor family, the cytoplasmic domain of MPL lacks intrinsic kinase activity but transduces a signal through recruitment of JAK2 to the conserved box1 and box2 domains of the receptor(103). Upon TPO binding, MPL monomers at the cell surface homodimerize and undergo a conformational change allowing the closer juxtaposition of bound JAK2 proteins, resulting in trans-auto-phosphorylation of the JAK2s(104-108). Phosphorylated JAK2 can then, in turn, phosphorylate tyrosine residues within the cytoplasmic domain of MPL in addition to signal transduction proteins including those of the JAK-STAT pathway, the mitogen-activated protein kinase (MAPK) pathway, photidylinositol-3-kinase (PI3K) and protein kinase C (PKC)(Figure 1.6)(3,109-112).

1.3.1.1 MPL cytoplasmic domain

There are 5 tyrosine residues located within the cytoplasmic domain of MPL (Figure 1.4) of which, 2 (Y625 and Y630 also termed Y112 and Y117, respectively when labeled based on intracellular residues) have been shown to undergo phosphorylation in response to TPO(3). Tyrosine 625 is essential for TPO mediated proliferation and is phosphorylated in response to TPO stimulation(3). Phosphorylation of Y625 is necessary for activation of downstream signal transduction proteins, including Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (SHIP1), SHC-transforming protein 1 (Shc), STAT3, GRB2-associated-binding protein 1 and 2 (Gab1/2) and Tyrosine-protein phosphatase non-receptor type 11 (SHP-2, PTPN11) resulting in downstream activation of the PI3K and MAPK pathways(3,110,113). Using receptor truncations, Drachman *et al.*(3) demonstrated that MPL, SHIP, Shc, STAT3, STAT5 and JAK2 were all phosphorylated in response to TPO stimulation. Phosphorylation state of these proteins was unaltered upon truncation just upstream of Y630; however, further truncation removing Y625 resulted in abolished MPL, SHIP, Shc and STAT3 phosphorylation suggesting a major role of Y625 in TPO dependent phosphorylation of these proteins. This study also showed that removal of the site via a tyrosine to phenylalanine point mutation (Y625F), severely impairs signalling and growth in response to TPO(3). Multiple studies also showed that Gab1/2 is phosphorylated in response to TPO followed by subsequent activation of

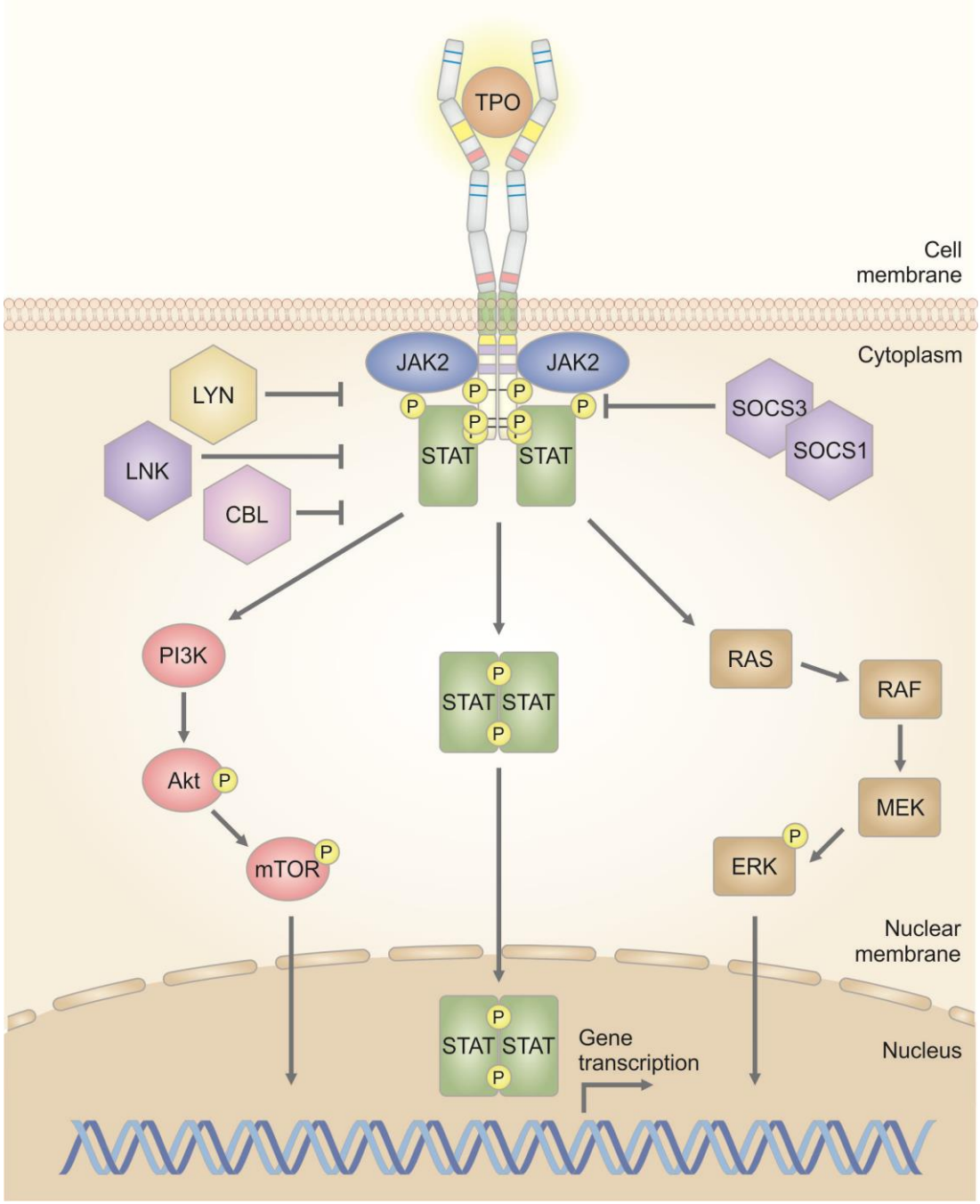


Figure 1.6 Thrombopoietin signalling

TPO binding to MPL results in activation of associated JAK2 molecules. Activated JAK2 phosphorylates distal MPL tyrosine residues; when these tyrosines are phosphorylated (P), they serve as docking sites for downstream signalling molecules and result in activation of the PI3K and MAPK pathways. Additionally, activated JAK2s phosphorylate STAT3 and STAT5 resulting in dimersation and translocation of these molecules into the nucleus where gene transcription is initiated. Subsequently, negative regulators such as SOCS proteins, LYN, LNK and CBL are activated which turn of TPO signalling.

the PI3K pathway, this activation was also dependent on Y625 of MPL(113) and regulated by SHP-2 as shown by overexpression of a dominant negative form of SHP-2(111). Additionally, Rojnukarin *et al*.(110) demonstrated that TPO-induced extracellular-signal-regulated kinase 1/2 (ERK1/2) phosphorylation was also dependent on the 10 carboxyl terminal residues of MPL, this was later shown in a different cell type to be Y625 dependent(114). Additionally, Y630 is also phosphorylated in response to TPO and like Y625, is involved in MPL phosphorylation and STAT3 activation(3). There are 3 additional tyrosine residues found in the cytoplasmic domain of MPL: Y521, Y542 and Y591 (also termed Y8, Y29 and Y78, respectively based on the intracellular numbering of residues). These residues were not found to be phosphorylated in response to TPO in early studies(3). Mutation of Y521 and Y542 to phenylalanine in a receptor missing the distal 3 tyrosine residues was unable to alter proliferation in response to TPO(3); however, it was possible these residues play a role in TPO signalling independent of sustaining proliferation. This was found to be the case with Y521, which is involved in targeting of MPL for lysosomal degradation(1). Conversely, cells expressing mutant MPL with a deletion encompassing Y591(115) or a Y591F mutation(3) exhibited an increased proliferative effect compared to cells expressing receptors with intact Y591, suggesting the location of a negative regulatory region within the cytoplasmic tail of MPL. It was also determined that the region encompassing Y591 is dispensable for proliferation yet necessary for differentiation(115). In Y591F mutant expressing cells, stimulation with TPO increased Protein kinase B (Akt, PKB) and ERK1/2 signalling relative to WT(1) further supporting Y591 as a negative regulator of TPO signalling. The role of Y591 extends even further as it also mediates MPL internalization(1). More recently, Pecquet *et al*.(116) reported Y591 phosphorylation in a constitutively active MPL W515L receptor, meriting re-analysis of the phosphorylation state of Y591 in a WT receptor utilizing more sensitive detection methods.

1.3.2 Negative regulation of TPO signalling

Negative regulation of TPO-mediated signalling is controlled by a number of proteins, including protein phosphatases, suppressors of cytokine signalling (SOCS) proteins, src family kinases (SH2B adapter protein 3 (LNK), Tyrosine-protein kinase

Lyn (LYN)) and Focal Adhesion Kinase (FAK)(Figure 1.6)(117-119). SOCS3 is upregulated in response to TPO(117) and provides a negative feedback mechanism in the control of TPO signalling(120). Interferon-alpha (IFN-α) is able to inhibit megakaryopoiesis in primary cells and reduce proliferation of MPL-expressing cells cultured in TPO, an effect postulated to result from IFN-α induction of SOCS1 which in turn inhibits TPO signalling(117). In megakaryocytes isolated from *lnk* null mice, Akt, STAT3, STAT5 and ERK1/2 phosphorylation in response to TPO was increased compared to WT cells(118). Similarly, megakaryocytes isolated from *lyn* null mice exhibited increased Akt and MAPK activation but reduced SHIP1 phosphorylation following TPO stimulation(119). Furthermore, in FAK-null megakaryocytes, Lyn kinase activity was reduced and similar to *lyn* null megakaryocytes, ERK1/2 and Akt phosphorylation was increased suggesting FAK as an upstream regulator of Lyn in the regulation of TPO signalling(121). Additionally, in *lnk* and *lyn* null mice and in mice with *Fak* null megakaryocytes, there was a marked increase in the number of megakaryocytes, demonstrating that these proteins function to suppress TPO mediated signalling necessary for megakaryopoiesis. However, the region of MPL necessary for each of these proteins to inhibit TPO signalling has yet to be determined. Additional negative regulatory control occurs through receptor internalization via clathrin-dependent endocytosis dependent and E3 ubiquitin protein ligase casitas B-lineage lymphoma(Cbl)-mediated ubiquitination and degradation through the proteasome in addition to degradation by lysosomal pathways(1,122), which have been attributed to Y591 and Y521, respectively. We and others have reported an increased proliferative capacity in response to TPO stimulation in cells lacking Y591, supporting the role of Y591 as a negative regulator of TPO-mediated proliferation(1,3). Aberrant positive signalling results in cellular hyperproliferation leading to development of haematological malignancies(123-125). Therefore, a thorough understanding of the regulation of TPO-mediated signalling is important for protection against uncontrolled cellular proliferation.

1.4 Myeloid Malignancies

Myeloid malignancies (MM) are a group of clonal diseases of HSC origin. MMs include acute myeloid leukaemia (AML), myeloproliferative neoplasms (MPN) and myelodysplastic syndromes (MDS) (Figure 1.7). AML is characterised by excessive proliferation of myeloid blast cells within the BM resulting in disruption of normal haematopoiesis (Reviewed in (126)) and is the second most prevalent leukaemia in the United Kingdom (UK)(Haematological malignancy research network (HMRN), www.hmrn.org). MDS are characterised by ineffective haematopoiesis due to improper maturation of myeloid progenitor resulting in cytopaenias (Reviewed in (127)). Whereas AML and MDS are characterised by overproduction of immature cells, MPNs are characterised by overproduction of mature myeloid cells(128). Of the three, prevalence of MPNs is highest in the UK (HMRN) and both MDS and MPNs can both progress to AML. Despite increased molecular insight into these diseases, overlapping clinical features makes exact classification difficult resulting in overlapping classifications such as MDS/MPN (Figure 1.7) (Reviewed in (129)). Further studies are necessary to better understand and characterise these disorders.

1.5 Myeloproliferative Neoplasms

In 1951, William Dameshek coined the term ‘myeloproliferative disorders (MPDs)’(128). Dameshek recognized that hyperproliferation of bone marrow cells, specifically erythroblasts, granulocytes and megakaryocytes were often a concurrent event. Due to the similarities and overlapping clinical features of some myeloproliferative syndromes, he grouped together chronic myelogenous leukemia (CML), polycythemia vera (PV), primary myelofibrosis (PMF) and essential thrombocythemia (ET), speculating that they shared a common cellular origin that was being overstimulated by a ‘myelostimulatory factor’. The clonal nature of these disorders was confirmed in female PV patients using X-linked chromosomal inactivation patterns (XCIP)(130). The nomenclature of MPDs was changed to myeloproliferative neoplasms (MPNs) in the 2008 World Health Orgainzation (WHO) re-classification of haematological malignancies(129)(Figure 1.7). However, the classification of MPNs still largely resembles that which Dameshek described in 1951.

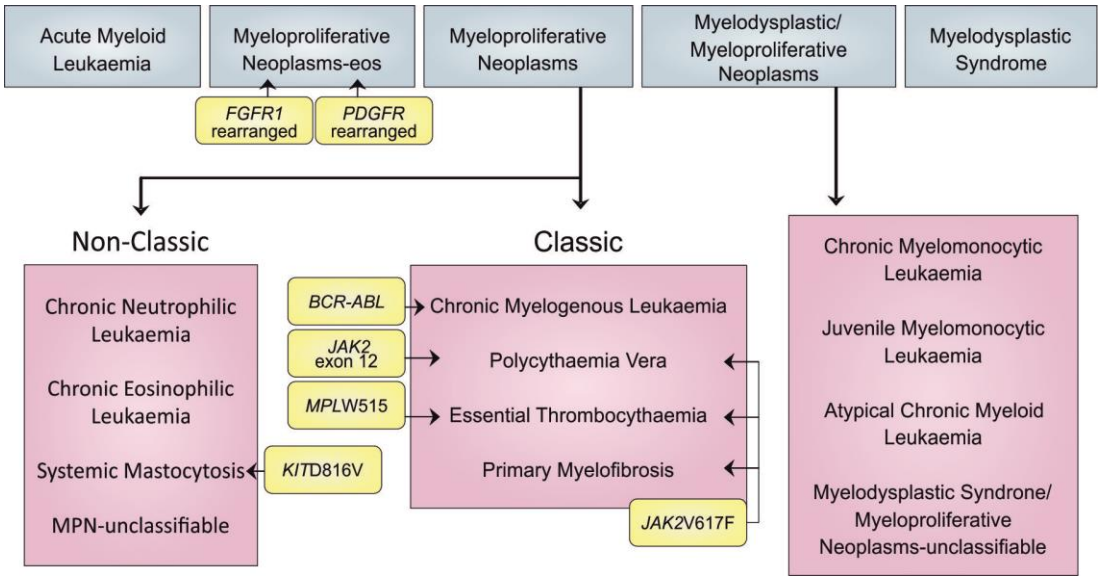


Figure 1.7 2008 WHO Classification of Myeloid Malignancies
Myeloid malignancies are grouped into five major categories shown in blue: Acute myeloid leukaemia (AML), myeloproliferative neoplasms-eos (MPN-eos), myeloproliferative neoplasms (MPN), myelodysplastic/myeloproliferative neoplasms (MDS/MPN) and myelodysplastic syndrome (MDS). Subcategories are listed in pink and common mutations associated with each disease are listed in yellow.

In 1960, Nowell and Hungerford described a chromosomal abnormality in several cases of CML(131), which was later termed the Philadelphia (Ph) chromosome. The observed cytogenetic abnormality was determined to have resulted from a translocation between the long arm of chromosomes 22 and 9 (t(9;22)(q34;q11))(132) resulting in a breakpoint cluster region and Abelson murine leukaemia viral oncogene homolog 1 (*BCR-ABL1*) fusion gene(133). The *BCR-ABL1* gene encodes an active tyrosine kinase(134) and its expression is sufficient for induction of CML *in vivo*(135). The Ph chromosome was the first cytogenetic factor identified in haematological malignancies. This discovery led to further classification of the classical MPNs into two distinct classes: Ph-positive MPNs which consists of CML and Ph-negative MPNs which is comprised of PV, ET and PMF.

1.5.1 Ph-positive MPNs

1.5.1.1 Chronic Myelogenous Leukaemia (CML)

CML accounts for approximately 1.5% of all haematological malignancies in the UK with a median diagnosis occurring at approximately 59 years of age and a 5-year survival rate of 87.1% (HMRN). The disease is typically characterised by increased cells of the granulocytic lineage including blasts in both the PB and BM (Reviewed in (136) and (137)). Symptoms associated with CML include fatigue, weight loss, night sweats and abdominal discomfort resulting from splenomegaly(138); however, between 20% to 40% of patients are diagnosed without presentation of any symptoms(138). CML can undergo acute transformation resulting in AML(139,140), the transformation rate has been reported at approximately 28%(141). Presence of the Ph chromosome is a hallmark of CML(129) and it is present in 99% of all CML patients(142).

1.5.2 Ph-negative MPNs

Ph-negative MPNs include PV, ET and PMF. Together, Ph-negative MPNs account for approximately 8.7% of total haematological malignancies in the UK. Around 3,200 new cases are diagnosed each year in the UK (HMRN). Similar to CML, these MPNs predominantly affect the elderly population with the median age at diagnosis being approximately 72 years (HMRN). Symptoms associated with Ph-negative

MPNs include fatigue, concentration problems, early satiety, inactivity, night sweats, itching, abdominal discomfort, bone pain, weight loss and fever(143).

1.5.2.1 Polycythaemia Vera (PV)

PV is characterised by an increase in red cell volume, haemoglobin, haematocrit and blood viscosity resulting from hyperproliferation of the erythroid lineage(128). Its annual incidence is 2.8 cases per 100,000 people per year(144). The 5-year survival rate is high at approximately 93% (HMRN). The most common clinical complications associated with PV are increased risk of arterial and venous thrombosis(145), with thrombosis at clinical presentation reported in 13-60% of patients(146). Although less common, PV patients are also at risk for haemorrhagic events. Risk of transformation to myelofibrosis or AML occurs at a rate of 6% and 7%, respectively at 15 years(147). Thrombosis is the major cause of mortality in PV patients(147,148).

1.5.2.2 Essential Thrombocythaemia (ET)

ET is characterised by a sustained increase in platelet count resulting from proliferation of cells within the megakaryocytic lineage. The annual incidence of ET has been reported at 1.5 cases per 100,000 people per year(144) with the 5-year survival rate at approximately 93% (HMRN). Similar to patients with PV, ET patients are also at risk of both thrombotic and haemorrhagic events and thrombosis at clinical presentation was reported in 11-51% of patients whereas bleeding accounts for 0.3% of events per year(146). Despite the low percentage, haemorrhagic events account for approximately 4% of deaths of ET patients(146). The incidence of haemorrhagic events is associated with extremely high platelet counts(146), which is typically attributed to platelet sequestration of von Willebrand factor (vWF); although, recent evidence suggests that endothelial cells may also play a role in these haemorrhagic complications(149). The risk of progression to myelofibrosis or AML is 4% and 2%, respectively at 15 years(147,150).

1.5.2.3 Primary Myelofibrosis (PMF)

PMF is characterised by multilineage expansion of the myeloid lineage which is accompanied by BM fibrosis(151). The annual incidence of PMF has been reported

at 0.4 cases per 100,000 people per year(144). The 5-year survival rate is 43% (HMRN). Symptoms include pancytopenia, bleeding and infection resulting from progressive bone marrow failure and pain, weight loss and sweating due to marked splenomegaly resulting from extramedullary haematopoiesis(137). PMF is difficult to diagnose due to broad symptoms and varying levels of multilineage expansion; additionally, depending on the stage of the disease, peripheral blood counts change(137). Although all MPNs are capable to transformation to AML, the risk is highest in PMF with approximately 25% of patients developing AML(137). PMF has the worst outcome with morality due to infections or haemorrhage resulting from bone marrow failure(137).

1.6 JAK2 in MPN pathogenesis

Identification of the Ph chromosome in CML prompted a search a similar cytogenetic cause for other haematological malignancies. In early studies of PV, ET and PMF patients, mutations in EPOR and MPL could not be identified, suggesting that the cause of cytokine hypersensitivity in these diseases was due to an effector downstream of these receptors(152). In 2005 several groups identified the *JAK2*^{V617F} mutation in most patients with PV and approximately half of patients with ET and PMF. Subsequently, additional mutations in exon 12 of *JAK2* were identified in patients with PV and idiopathic erythrocytosis(153). These discoveries identified mutations in JAK2 as the main genetic lesions of Ph-negative MPNs providing significant insight into the molecular pathogenesis of these diseases.

1.6.1 JAK-STAT signalling

Hematopoietic cytokine receptor signalling is largely dependent on the JAK-STAT signalling axis (Reviewed in (154)). Cytokine receptors lack intrinsic kinase activity and often rely on JAKs for signal transduction (Reviewed in (154)). There are four members of the JAK family: JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2). The JAK family of proteins is composed of four functional domains (Figure 1.8), the 4-point, ezrin, radixin, moesin (FERM) domain, a SRC homology 2 (SH2)-like domain and two JAK homology (JH) domains.

Both JH domains, JH1 and JH2, share high homology with tyrosine kinase domains; however, only JH1 has was initially believed to harbour catalytic kinase activity. JAK2 catalytic activity is stimulated upon phosphorylation of Tyr1007 within the activation loop(155). Regulation of kinase activity is an intrinsic property whereby the JH2 domain, also termed the pseudokinase domain, regulates the kinase activity. It was thought that this was occurring through interaction of the pseudokinase domain with the kinase domain(156,157). Additional JAK2 regulation involves phosphorylation at Tyr570 which inhibits JAK2-dependent signalling(158). Recent evidence suggests that the pseudokinase domain does, in fact, possess kinase activity and negatively regulates catalytic activity of JAK2 through phosphorylation of negative regulatory sites, Tyr570 and Ser523(159). The crystal structure of the JH2 domain revealed that JH2 domain phosphorylates Ser523 in *cis* whereas Tyr570 is phosphorylated in *trans*(160). Phosphorylation at these sites is JAK2 specific and possibly acts as an additional inhibitory regulator(160). Disruption of the pseudokinase domain results in increased basal JAK2 activity(159) and cytokine independent activation of STATs(156). The Src Homology 2(SH2)-like domain of JAKs was predicted through computational modelling(161,162) and functional assays with JAK3 show that this domain is capable of association with phosphorylated tyrosine residues(162). However, functional studies with JAK1 utilizing a common loss of function mutation within the SH2 domain did not affect cell surface localization at the plasma membrane or signalling(163). The N-terminal FERM domain is necessary for receptor association. Experiments utilizing shortened fragments of JAK FERM domains consisting of only the JH6 and JH7 regions showed that these fragments are sufficient for receptor association(164,165). Additionally, it was shown that the JAK2 FERM domain acts as a chaperone promoting EPOR cell surface expression, which is accomplished in the absence of an active kinase domain(166). Moreover, the JAK FERM domains are responsible for receptor specificity(167). Mutation of conserved hydrophobic resides within the FERM domain, Y107 of JAK1(168), L98, I102 and clinically relevant mutation of Y100 of JAK3(169) disrupt association with cytokine receptors utilizing gp130 and IL-2Rγ, respectively. These data demonstrate the biological function of the N-terminal domain of JAK which is independent of kinase activity.

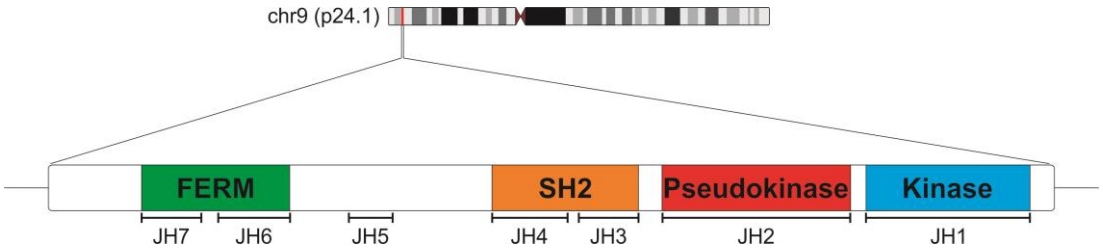


Figure 1.8 Functional domains of JAK2 protein

Diagram depicting the seven Janus homology (JH) domains of JAK2. JH1 is the C-terminal kinase domain and possess catalytic tyrosine kinase activity. JH2 is the pseudokinase domain which is responsible for regulating catalytic activity. JH3 and JH4 comprise the SRC homology 2(SH2)-like domain; its function is not fully understood. JH6 and JH7 comprise the 4-point, ezrin, radixin, moesin (FERM) domain which facilitates association with cytokine receptors.

Different JAKs preferentially activate different cytokine receptor complexes (Figure 1.9), the specificity arising from differences in JAK FERM domains(167). Homodimeric receptors such as EPOR, GHR, MPL, PRLR or granulocyte-macrophage colony-stimulating factor receptor (GMCSFR) preferentially activate JAK2. Whereas heterodimeric and heterotrimeric receptors can activate different JAKs depending on the receptor subunits (Reviewed in (170)).

Cytokine binding to their respective receptors results in receptor aggregation or in the case of pre-formed dimers, a conformational change that results in JAK tyrosine phosphorylation and subsequent activation. The activated kinase can then in turn phosphorylate tyrosine residues within the cytoplasmic tail of the receptor in addition to downstream STATs. Phosphorylated STATs are able to dimerize and translocate to the nuclease where they activate gene transcription (Figure 1.6) (Reviewed in (154,170,171)).

1.6.2 Negative regulation of JAK2 signalling

As with any signalling cascade, tight regulation is necessary to prevent aberrant signalling and deregulated proliferation which could result in development of disease. JAK2 signalling is controlled by a number of regulatory mechanisms including kinase inactivation, ubiquitination and inhibition of downstream signal transducers.

Inactivation of JAK2 kinase activity is accomplished through interactions of the kinase domain with the pseudokinase domain. The inhibition is thought to occur through phosphorylation of specific inhibitory residues, Ser523 and Try570, within the pseudokinase domain. Phosphorylation at these sites is accomplished through both *cis* (Ser523) and *trans* (Tyr570) phosphorylation. Importantly, JAK2 is the only JAK that utilizes homodimeric cytokine receptors, which assures presence of a second JAK2 molecule to facilitate the *trans* phosphorylation(160). Phosphorylation at these sites prevents activation of the kinase; however, once the kinase is activated there are a number of proteins involved in suppressing the signalling cascade including phosphotryosine phosphatases (PTP), SOCS and LNK.

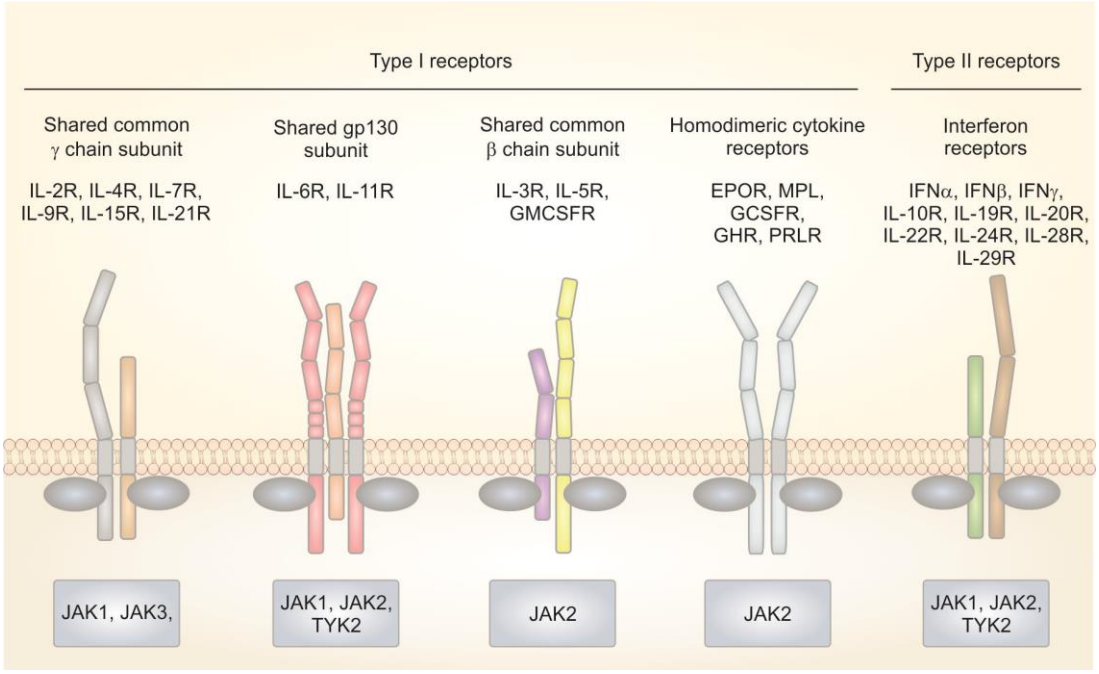


Figure 1.9 Cytokine receptor differential JAK activation
The JAK family of kinases differentially associate with cytokine receptors and are activated upon cytokine binding to receptors. This schematic shows the different families and subgroups of cytokine receptors and their associated JAKs. IL – interleukin; GMCSFR – granulocyte-macrophage colony stimulating factor receptor; EPOR – erythropoietin receptor; MPL – thrombopoietin receptor; GCSFR – granulocyte colony stimulating factor; GHR growth hormone receptor; PRLR – prolactin receptor; IFN – interferon.

A PTP involved in regulation is Src homology region 2 domain-containing phosphatase-1 (SHP-1). SHP-1 associates with either the receptor or to JAK2 itself and dephosphorylates the activated proteins(172) thereby disrupting signalling. SOCS proteins are part of a negative feedback loop that inhibits JAK-STAT signalling. SOCS proteins are transcriptionally regulated by STATs and function by binding to either activated receptors or JAK2 proteins to turn off the pathway(172). Specifically, SOCS3 binds directly to JAK2 and the receptor and occludes the substrate-binding groove of JAK2 effectively inhibiting the kinase(173). The adaptor protein, LNK, is a negative regulator of cytokine signalling regulating signal transduction downstream of IL-3, SCF, TPO, EPO, platelet-derived growth factor (PDGF) and tumour necrosis factor (TNF)(174). Specifically, LNK attenuates signalling downstream of TPO and EPO through interaction and attenuation of JAK2 activity resulting in decreased STAT3, STAT5, Akt and MAPK signalling(118,174,175).

Removal of JAK2 associated receptors from the cell surface and targeting of them for recycling or degradation is another mechanism of control. This is accomplished through ubiquitination of receptors. Specifically, for MPL, ubiquitination of the receptor occurs after cytokine-induced activation at two intracellular lysine residues, MPL^{K553} and MPL^{K573}(122). Ubiquitination results in receptor internalization and degradation through both the lysosomal and proteosomal pathways(122), effectively preventing additional signal transduction.

Immediately downstream of JAK2 in the canonical signalling cascade is the activation of STAT proteins. Inactivation of these signal transducers will also inhibit JAK2 signal transduction. A family of proteins known as protein inhibitor of activated STAT protein (PIAS) inhibit STAT signalling by binding to activated STAT dimers and inhibiting their association with DNA for induction of transcription(172) thereby terminating additional downstream activation. PIAS1(176) and PIASy(177) both inhibit STAT1 whereas PIAS3 inhibits STAT3 signalling(178).

1.6.3 The JAK2^{V617F} mutation

Given its role in signal transduction of multiple haematopoietic cytokine receptors, JAK2 was a promising candidate as a molecular driver for MPNs. Indeed, sequencing of *JAK2* from granulocytes of MPN patients in three independent studies identified a single G to T nucleotide transition in exon 14 of *JAK2*, resulting in a Val to Phe substitution at amino acid residue 617 located in the pseudokinase domain(179-181). The mutation was absent in control patients and in T-cells from patients with *JAK2*^{V617F}-positive peripheral-blood granulocytes revealed that the mutation was MPN specific and that it was an acquired mutation(179). The *JAK2*^{V617F} mutation was detected in 98% of patients with PV and approximately 50% of patients with ET and PMF(179-181). Although the *JAK2*^{V617F} mutation predominates in classic Ph-negative MPNs, it can also be detected in approximately 5% of patients with CML, MDS or denovo AML(152). In agreement with its characterisation as a clonal stem cell disorder, the *JAK2*^{V617F} mutation was detected in haematopoietic progenitors(179) in addition to the $CD34^{+}CD38^{-}CD90^{+}Lin^{+}$ subset of HSCs(182).

Presence of the *JAK2*^{V617F} mutation in only half of patients with ET and PMF attest to the heterogeneity of these diseases. Although identification of this mutation has profoundly increased the precise categorisation of these diseases based on molecular pathogenesis, questions still remain regarding how a single point mutation can result in three different clinical pathologies in addition to the molecular basis of disease in *JAK2*^{V617F}-negative MPN patients.

1.6.3.1 One mutation, three phenotypes

Although identification of the *JAK2*^{V617F} mutation significantly increased our understanding of the molecular pathogenesis of Ph-negative MPNs, it is still not completely understood. One central question that remains unanswered is how a single mutation yields three distinct clinical phenotypes. There are a number of hypotheses that have been explored to try and answer this outstanding question. They include differences in *JAK2*^{V617F} dosage, presence of additional somatic mutations, differences in the cell type where the mutation is acquired and differential STAT signalling, all of which are discussed in this section (Figure 1.10).

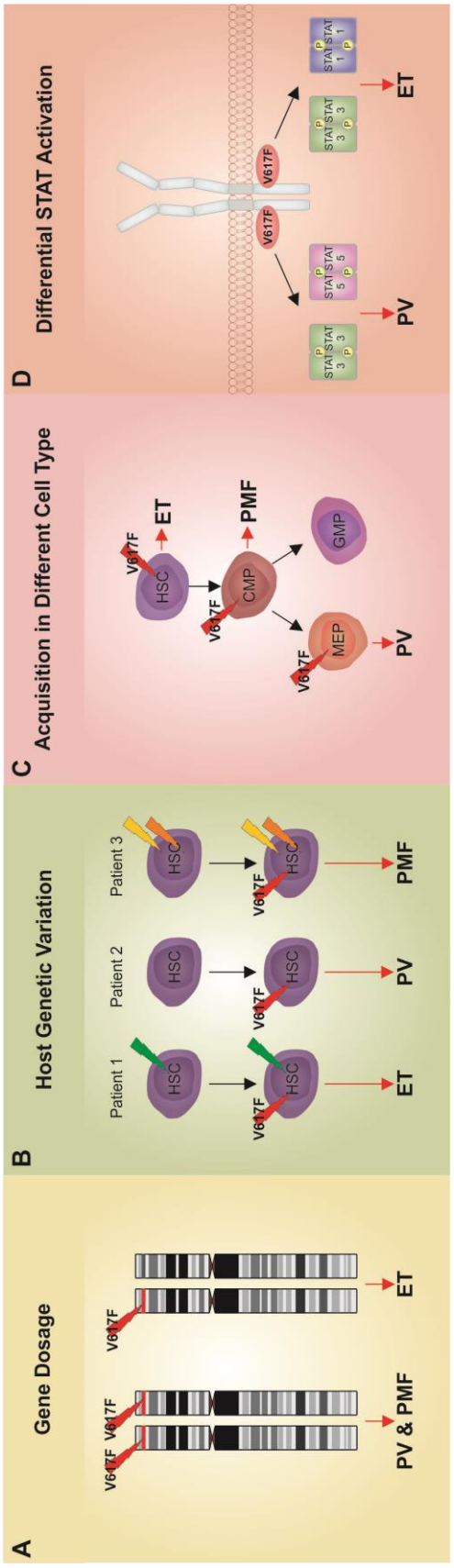


Figure 1.10 One mutation, three phenotypes

This diagram provides a graphical summary of the theories explaining how the *JAK2*^{V617F} mutation is able to cause three distinct phenotypes. (A) Gene dosage. Evidence suggests that the allelic burden of *JAK2*^{V617F} dictates the disease phenotype. (B) Host genetic variation. This theory suggests that genetic variations between patients that are present prior to acquisition of the *JAK2*^{V617F} mutation dictates the disease phenotype. (C) Acquisition of *JAK2*^{V617F} in different cell types. The *JAK2*^{V617F} mutation needs to be acquired in an HSC or early progenitor to cause disease, at different stages of differentiation receptors are differentially expressed. It is possible that depending on the cell type in which *JAK2*^{V617F} is acquired, the mutant kinase associates with preferentially expressed receptors thereby dictating the disease. (D) Differential STAT activation. For this theory, it is thought that activation of downstream STAT proteins by *JAK2*^{V617F} is what determines disease phenotype.

1.6.3.1.1 Gene dosage

In this model, it is thought that the phenotypic variation observed between PV, ET and PMF patients is a continuum that depends on level of mutant kinase present within the cell. *JAK2*^{V617F} was detected in patients with PV, ET and PMF; however, the mutation was found either in a homozygous or heterozygous state in these patients(179). Homozygosity for *JAK2*^{V617F} is a consequence of a mitotic recombination event(179-181), the rate of which was highest in granulocyte DNA from PV patients at 25% compared to 3% and 9% in ET and PMF, respectively(179). Studies of individual myeloid progenitor colonies from PV and ET patients found homozygous *JAK2*^{V617F} clones in almost all progenitors from PV patients but rarely in ET patients(183). Additionally, not only is homozygosity more characteristic of PV, even within PV, higher levels of *JAK2*^{V617F} burden correlates with significantly higher haemoglobin levels and progression to myelofibrosis(184,185). These clinical data support the idea of a phenotypic continuum dependent upon level of *JAK2*^{V617F} expression and is further supported by *JAK2*^{V617F} mouse models. In models generated through transplantation of retrovirally tranduced BM cells, *JAK2*^{V617F} expression was superphysiological resulting in development of PV-like phenotypes(186-189). In later models where human *JAK2*^{V617F} was expressed under the control of the mouse *Jak2* locus, the ratio of mutant *JAK2* to WT *Jak2* was similar and this yielded an ET-like phenotype(190). Tiedt and colleagues developed a mouse model where copy number and consequently expression of human *JAK2*^{V617F} could be modulated through induction of recombination by different *Cre* recombinases. They showed that lower levels of *JAK2*^{V617F} expression correlated with an ET-like phenotype whereas high expression resulted in PV(191).Taken together, these data strongly support that *JAK2*^{V617F} expression levels play a role in determining MPN phenotype.

1.6.3.1.2 Host genetic variations

Another theory in the explanation of varying phenotypes resulting from *JAK2*^{V617F} expression is the idea that genetic variations predispose patients to development of a particular MPN phenotype. In experiments utilizing *JAK2*^{V617F} mouse models, expression of *JAK2*^{V617F} in C57Bl/6 mice resulted in trilineage hyperplasia and splenomegaly characteristic of PV whereas Balb/C mice developed PV but also marked leukocytosis(186,189). These results suggest that the genetic variations

between C56Bl/6 and Balb/C mice caused the varying phenotypes. The influence of host genetic variations has been tested in patients. Single nucleotide polymorphisms (SNP) for four candidate genes associated with JAK-STAT signalling: EPOR, MPL, granulocyte colony-stimulating factor receptor (GCSFR) and JAK2 identified correlations between SNPs in *JAK2* and *EPOR* and development of PV(192) suggestive of at a least a partial role of genetic background on MPN phenotype although no definitive link has been determined.

1.6.3.1.3 Acquisition of JAK2^{V617F} in different cell types

Ph-negative MPNs are clonal disorders(193) and their phenotypes are characterised based on the specific haematopoietic lineages that are being overproduced. It is thought that MPN phenotype is dependent on the cell in which the *JAK2*^{V617F} mutation is acquired. Different cells have varying capacities for lineage differentiation so it is possible acquisition of *JAK2*^{V617F} in cells capable of differentiation down only the megakaryocytic lineage leads to development of ET whereas acquisition in a cell capable of megakaryocytic, erythroid and granulocytic differentiation leads to PV or PMF. One study found *JAK2*^{V617F}-positive cells in granulocytic, erythroblastic, megakaryocytic and lymphoid lineages from PV and PMF patients(194) suggestive of acquisition in an early haematopoietic progenitor. Additional considerations include differences even within the HSC compartment which have been reported between PV and PMF patients, where the frequency of *JAK2*^{V617F}-positive HSCs is higher in PMF compared to PV(195). However, due to the difficulty in determining the specific cell where the *JAK2*^{V617F} was acquired, explicitly proving this model would be difficult.

1.6.3.1.4 Differential STAT activation

Another theory to explain how *JAK2*^{V617F} results in three distinct clinical phenotypes is based on the fact that JAK2 can activate a number of different STATs and proposes that differential activation of specific STATs drives a specific MPN phenotype. A study analysing the expression pattern of phosphorylated STAT3 and STAT5 found increased STAT3 and STAT5 phosphorylation in PV, an increase in STAT3 but decrease in STAT5 phosphorylation was observed in ET and both STAT3 and STAT5 phosphorylation was decreased in PMF(196). Additionally, another study analysing differences between clonally-derived *JAK2*^{WT} and *JAK2*^{V617F}

$CD34^+$ cells found increased phosphorylated STAT1 in *JAK2*^{V617F} cells compared to *JAK2*^{WT} cells from the same ET patient; however, this was not observed in cells from PV patients(197). Furthermore, modulation of STAT1 activation in heterozygous *JAK2*^{V617F} ET cells was able to induce a PV-like phenotype(197), providing strong evidence that STAT1 activation has a role in determining MPN phenotype.

1.6.4 JAK2 exon 12

Although 98% of PV patients harbour the *JAK2*^{V617F} mutation, there remains a subset of PV patients that are *JAK2*^{V617F}-negative. In this subset of patients, *JAK2* exon 12 mutations were identified; additionally, these mutations were not detectable in ET patients(153). Four different mutations in *JAK2* exon 12 were identified in the original study: F537-K539delinsL, H538QK539L, K539L and N542-E543del, all located within a conserved region predicted to lie within the linker region between the SH2 and pseudokinase domain of *JAK2*(153). The mutational frequency was low in granulocyte DNA but prevalent in erythropoietin (EPO)-independent erythroid colonies(153). Similar to *JAK2*^{V617F}, co-expression of each of the *JAK2* exon 12 mutations within EPOR in Ba/F3 cells was sufficient to induce cytokine-independent proliferation and activation of downstream signalling pathways(153,181). Expression of the most common exon 12 mutation, *JAK2*^{K539L}, *in vivo* was able to recapitulate an MPN in mice(153). Identification of these mutations provides additional evidence that the molecular pathogenesis of PV is highly dependent on JAK2 as mutations are found in essentially all PV patients. This also provides an additional level for diagnostic stratification which may make it easier for implementation of specific targeted therapies.

1.7 Additional Mutations Associated with MPNs

Although JAK2 is the predominant mutation in Ph-negative MPNs, it is not present in all MPN patients, specifically those with ET and PMF. Additional mutations have been identified and described in MPN patients, some of which are discussed below.

1.7.1 MPL

Activating mutations in exon 10 of *MPL* are found in the neoplastic cells of 5-10% of patients with ET or PMF(124,125,198). Although not common, *MPL* mutations are not mutually exclusive from *JAK2*^{V617F}(199). The most common mutation are *MPL*^{W515L}(124) and *MPL*^{W515K}(125) but the *MPL*^{S505N}(200) mutation has also been described. These mutations are sufficient to induce disease and are discussed in greater detail in Section 1.9.1.

1.7.2 Calreticulin (CALR)

Recent identification of somatic mutations of exon 9 of *CALR* in patients with ET and PMF(201,202) has not only provided important insight into the molecular pathogenesis of these diseases but also serves as an additional prognostic tool(201). Mutations in *JAK2* and *MPL* account for approximately 50% and 10%, respectively of mutations found in ET and PMF; within the subset of patients negative for both of these genetic lesions, *CALR* mutations accounted for 67% of patients with ET and 88% of patients with PMF(201). Additionally, *CALR* mutations are mutually exclusive with *JAK2* and *MPL* mutations(201,202); however, it was also found mutated in other myeloid cancers(202). The mutation is present in HSCs and progenitor cells suggestive of an initiating mutation(202).There are 2 types of *CALR* mutations, both resulting in frameshift mutations causing loss of the endoplasmic reticulum-retention motif (Lys-Asp-Glu-Leu [KDEL] amino acid sequence). The type I *CALR* mutation is able to induce cytokine independent proliferation *in vitro* and utilises the JAK/STAT signalling axis(201). Both ET and PMF patients with *CALR* mutation presented with higher platelet counts but lowered white-cell counts compared to patients with mutant *JAK2*(201,202). The *CALR* mutation also provides an additional means for prognostic stratification, with the *CALR* mutation correlating with increased survival among PMF patients relative to *JAK2* or *MPL* mutations and increased survival among ET patients relative to *JAK2* mutations(201), although transformation to PMF was increased(202). Unfortunately, 8.8% of patients, predominantly with ET, are triple negative for *JAK2*, *MPL* and *CALR* mutations(201) suggesting the possibility of an additional yet unidentified cytogenetic lesion or other non-genetic factors.

1.7.3 TET2

In 2009, mutations in tet methylcytosine dioxygenase 2 (*TET2*) were identified in a wide range myeloid malignancies including MPNs with 11% of *JAK2*$^{V617F+}$ patients harboring a *TET2* mutation(203,204). Analysis of progenitor cells from 5 *JAK2*^{V167F} and *TET2* double mutant MPN patients found that all cells expressing *JAK2*^{V617F} also possessed a *TET2* mutation, suggesting that *TET2* mutations preceded the JAK2 mutation(203); however, this was later challenged when subsequent studies showed acquisition of *TET2* mutations following acquisition of *JAK2*^{V617F}(205). Additional studies are needed to further elucidate the correlation between TET2 mutations and MPNs.

1.8 Treatments for MPNs

MPNs are a heterogeneous group of disorders with varying symptom profiles and clinical needs, making the choice of therapy difficult(143). A number of molecular insights have improved the understanding of the molecular pathogenesis allowing for more precise diagnosis and development of targeted therapies. There are currently a few categories of available treatments for MPNs including cytoreductive therapies, allogeneic stem cell transplantation and targeted therapies, namely imatinib and ruxolitinib, which are discussed below.

1.8.1 Imatinib

Imatinib (STI571 (4-[(4-methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]phenyl]benzamide methanesulfonate) is a small molecule tyrosine-kinase inhibitor marketed by Novartis as Glivec and Gleevec in the UK and United States (USA), respectively used specifically in for the treatment of CML(206). It is the most successful drug for targeted MPN therapy and the gold standard for treatment of CML; however, its efficacy is restricted to CML. Imatinib is able to inhibit the BCR-ABL1 tyrosine kinase through competitive inhibition at its ATP-binding site, preventing activation of the kinase and downstream signal transduction(207). Treatment results in cytogenetic remission in approximately 90% of all CML patients(208) with low levels of toxicity reported(209). Additionally, molecular remission is also achieved with low or undetectable levels of *BCR-ABL1* transcripts detected in imatinib treated patients in cytogenic remission(210).

However, despite the efficacy of the drug, acquired mutations within the kinase domain result in resistance to imatinib treatment (Reviewed in (211).

1.8.2 Allogeneic stem cell transplant

Allogenic stem cell transplants are currently the only curative option for PMF. However, the procedure is high-risk for patients with the possibility of graft failure, graft versus host disease and toxicity resulting from treatment. Additional factors such as the state of disease progression, availability of a suitable donor and age of the patient also complicate the decision to implement treatment especially as 3-year overall survival rate is approximately 50% with reports of life expectancy being lower with than without treatment. Allogenic stem cell transplants are therefore reserved for patients with a life expectancy of less than 5 years (Reviewed in (143)).

1.8.3 Aspirin and cytoreductive therapy

Thrombotic complications are leading cause of morbidity and mortality in untreated MPN patients(212); therefore many therapies aim to reduce these events. A study by Marchioli et al. reported a significantly lower rate of major thrombotic complications and associated mortality when haematocrits of less than 45% were maintained compared to those with a haematocrit target of 45-50%(213).Currently, the most common therapy for PV patients is a regimen of low-dose aspirin which reduces the risk of vascular events without significantly increasing the incidence of major bleeding; however its role in ET is less understood(143,212). Aspirin regimens in PV patients were initially reported to increase the incidence of gastrointestinal bleeding(214); however, it was later recognized that this was a result of improper dosage(212).

The aim of cytoreductive therapy is to reduce cancer cell burden. Phlebotomy is a non-drug therapy aimed at reducing haematocrit in PV patients to levels that lower the risk of thrombotic events(148,213). Surprisingly, phlebotomy alone resulted in increased survival compared to combinatorial therapies although there were increased incidences of thrombotic complications resulting in mortality within the first 2-4 years(148). In PV and ET patients hydroxycarbamide (HC), formerly hyroxyurea, is the most common cytoreductive treatment followed by

anagrelide(215,216). HC functions by inhibiting the biosynthesis of deoxyribonucleotides from ribonucleotides(217) preventing production of new cells. The mechanism of action of anagrelide is not well described but it has been shown to inhibit megakaryocytic differentiation(218) and is a known phosphodiesterase inhibitor(219). However, there are conflicting results regarding the advantages of HC compared to anagrelide on decreasing thrombotic risk. One study reported significantly increased vascular risk including thrombosis, haemorrhage and transformation to MF on an anagrelide regime compared to HC(215) whereas another study reported no significant difference in risk between the two treatments(216). HC is also used for treatment of PMF, although it has only resulted in modest clinical benefits(220). Interferon-α2 (IFN-α2) therapy is another successful regime in the treatment of MPNs, inducing an approximate 80% response in patients(221). However, its toxicity has led to discontinuation in approximately 20% of patients(221). A modified version of the drug, pegylated interferon α-2a (PEG-IFN-α-2) is an emerging treatment for both PV and ET with 76% and 77% of PV and ET patients, respectively exhibiting a haematological response to treatment(222). Notably, PEG-IFN-α-2 has also been reported to induce molecular remission in 18% and 17% of PV and ET patients, respectively(222). However, similar to other treatments(211), acquired mutations reduce responsiveness to therapy(222). Use of anti-cancer drugs, pipobroman and busulfan are reserved for PV and ET patients intolerant to hydroxycarbamide or anagrelide due to increased leukemogenic potential(143,220,223).

1.8.4 Splenectomy

Patients with PMF can develop marked splenomegaly, therefore therapeutic intervention includes splenectomy. It is typically reserved for patients with marked splenomegaly whose splenomegaly is not responsive to drug therapies(224) as perioperative mortality rates range from 7%-15%(225) and morbidity rates are approximately 25%(224). Additionally, splenectomy does not improve survival and serves a more palliative role(225).

1.8.5 Ruxolitinib

Given the large role JAK2 mutations have in driving Ph-negative MPNs and the effectiveness of the kinase inhibitor imatinib in treatment of CML, it was thought that inhibition of JAK2 would effectively treat Ph-negative MPNs. However, the efficacy of therapeutic JAK2 inhibitors has been limited due to specificity issues and adverse side effects, since JAK2 function is critical for normal haematopoiesis. Ruxolitinib (INCB018424) is a small molecular JAK inhibitor that functions by binding to the ATP-binding pocket of kinase(226) thereby limiting the availability of nucleoside triphosphate molecule for transfer to a tyrosine residue which would result in tyrosine phosphorylation. Preclinical studies determined that it was a potent inhibitor of JAK1 and JAK2 although it was also able to inhibit JAK3 and TYK2(227). *In vitro*, it was able to inhibit *JAK2*^{V617F} –induced signalling and proliferation of *EPOR* and *JAK2*^{V617F} expressing Ba/F3 cells and was also able to inhibit proliferation of haematopoietic progenitor cells from MPN patients(227). Additionally, in a *JAK2*$^{V617F+}$ MPN allotransplantation mouse model, ruxolitinib significantly reduced splenomegaly, decreased *JAK2*$^{V617F+}$ cell burden and increased overall survival compared to control mice without affecting peripheral blood counts(227). Of note, the MPN model used in this study did not display any changes in peripheral blood counts upon introduction of *JAK2*^{V617F}. These data strongly supported ruxolitinib for treatment of MPNs. In a phase 1-2 clinical trial, patients with myelofibrosis treated with ruxolitinib showed clinical improvement characterised by a prompt and significant reduction in spleen size, weight gain and observable functional improvements(228). Non-haematologic toxicity was reported in less than 10% of patients(228). The main haematologic side effect was thrombocytopenia which was decreased upon lowering dosage and reversed upon discontinuation of treatment(228). Additionally, the increase in circulating inflammatory cytokines, including IL-6, observed in myelofibrosis patients(229) was also reduced(228), possibly contributing to the observed decrease in splenomegaly. Similar results were obtained in 2 separate, randomised, phase 3 clinical trials of ruxolitinib(220,230). Median survival was increased in patients receiving ruxolitinib compared to placebo (8.4% vs 15.6%, respectively)(230); however median survival was similar for ruxolitinib treated patients compared to those receiving best available therapy (8% vs 5%, respectively)(220). However, in both studies, patients in the

ruxolitinib group reported decreased symptoms and increased quality-of-life(220,230). Despite the marginal increase in overall survival compared to placebo, ruxolitinib provides significant palliative care making it a useful drug in the treatment of MPN symptoms. Results from these trials prompted the U.S. Food and Drug Administration to approve ruxolitinib for treatment of myelofibrosis at the end of 2014.

Although not yet approved by the United States Food and Drug Administration (FDA) for treatment of PV, Ruxolitinib is currently undergoing clinical trials for treatment of PV(231). In general, it was well tolerated and was able to reduce haematocrit and splenomegaly in patients with advanced PV who were refractory or intolerant to HC and similarly to trials in myelofibrosis patients, thrombocythaemia and anaemia were the most adverse side effects(231).

Additional JAK inhibitors are currently under development as therapeutic compounds. TG101348, like ruxolitinib, is a ATP-competitive inhibitor which exhibits high selectivity for JAK2 and JAK2^{V617F} although FLT3 and RET were also inhibited(232). In contrast to ruxolitinib, there were no reported changes in pro-inflammatory cytokines(232), possibly due to more selective JAK2, rather than JAK1 inhibition. Additionally, *JAK2*^{V617F} allele burden was significantly reduced in patients with an initial allele burden higher than 20%(232). Another kinase inhibitor, Lestaurtinib (CEP-701) also exhibits selective inhibition of JAK2 and JAK2^{V617F} but similar to TG101348 it is also able to inhibit FLT3 and RET in addition to TRKA(233). However, JAK2^{V617F} allele burden was not reduced following treatment(234). Additional inhibitors under development include: XL019, SB518, CYT387, AZD1480, INCB028050, INCB16562, tascocitinib, NVP-BSK805; these compounds will not be discussed in this work but are reviewed in (235). In general these compounds are successful at inhibiting JAK activity both *in vitro* and *in vivo*; however, selectivity remains the largest obstacle. Although off target effects are undesired for the treatment of MPNs, JAK2 inhibitors that target additional JAKs, such as those involved in immunological or inflammatory responses, are being investigated for treatment of rheumatological disorders, autoimmune diseases, prevention of allograft rejection and inflammatory conditions(Reviewed in (235)).

1.9 *MPL* and *TPO*: their current and prospective roles in MPNs

MPL and TPO are essential for proper regulation of platelet production, dysregulation of either result in disease. Insufficient production or function of MPL or TPO results in thrombocytopenias whereas overproduction or unsolicited activation results in thrombocythaemias. Mutations can result in disease; however, they also provide insight into normal regulation of these proteins. This section focuses on the role of MPL and TPO and their association with MPNs.

1.9.1 Activating MPL mutations

Activing mutations within MPL typically result in thrombocythaemia: the overproduction of platelets. A number of activating mutations in MPL have been described. The first report of unsolicited MPL activation was the translocation of the v-mpl envelope protein to a portion of the intracellular domain of MPL resulting in myeloproliferation, which eventually led to the cloning of the receptor. Since then, a number of additional mutations have been reported. A study analysing a family with familial ET found a heterozygous serine to asparagine mutation within the transmembrane domain at codon 505 of MPL (MPL^{S505N})(200). All members of the family harbouring the mutation had increased platelet counts whereas counts from the members without the mutation were unaffected(200). In another study, sequence analysis of cytokine receptors from granulocyte DNA of myelofibrosis patients revealed a tryptophan to leucine mutation at codon 515 (MPL^{W515L})(124). Four of forty-five (9%) patients harboured this mutation which was absent from buccal cell control DNA from the same patients, suggesting a somatic origin(124). Additionally, in a larger study, the MPL^{W515K} mutation was later identified, also from granulocyes of MPN patients(125). Both, the MPL^{S505N} and MPL^{W515L} mutations confer cytokine-independent signalling and proliferation when expressed in cell lines(124,200); although this has not been shown for MPL^{W515K}, it is expected to exhibit a similar functional consequence. MPL^{W515} is located at the transmembrane-cytoplasm interface and resides within an α-helical amphipathic domain (KWQFP in murine and RWQFP in humans) that prevents unsolicited dimerization and subsequent activation of MPL(236). The W515 mutations were only found in patients with myelofibrosis or ET(125).

1.9.2 MPL expression in MPNs

Shortly after the cloning of *MPL* in 1992, its expression was assessed in a number of human haematological malignancies. The study reported low levels of *MPL* transcript levels detected by northern blot in bone marrow or peripheral blood from patients with myeloproliferative neoplasms, acute lymphoblastic leukaemia (ALL) and non-Hodgkin's lymphoma (NHL)(237). Subsequent studies supported these findings. Horikawa et al. reported decreased *MPL* mRNA transcript and protein expression in platelets from ET patients (238). Duensing et al. reported decreased *MPL* mRNA expression in peripheral blood from PV and ET patients (239). Harrison et al. and Li et al. reported that *MPL* expression was significantly decreased in ET patients, as measured using immmuno-labeling of cell surface *MPL* on platelets (240,241). Conversely, Moliterno et al. reported decreased *MPL* expression on platelets from PV patients but normal expression on platelets from an ET patient(242). This discrepancy could be explained by heterogeneity within MPNs as later studies also found reduced *MPL* expression in MPNs; yet, many of these studies reported heterogeneity of *MPL* expression in cells between and even within patients. Le Blanc et al. reported mixed *MPL* expression on platelets from PV patients as determined by western blot (243). Numerous groups reported mixed *MPL* staining intensity and pattern in bone marrow sections from ET(244,245) and PV(246) patients(247). A study by Moliterno and Spivak found 2 isoforms of MPL in PV patients that were differentially recognized by antiserum against the MPL extracellular domain and an antibody against the C-terminal domain, possibly an additional explanation for the heterogeneous staining previously reported(55). They found the isoform specific to PV platelets to be incompletely glycosylated and therefore more susceptible to endoglycosidase H (EndoH) digestion resulting in decreased expression, a defect which was exaggerated as the disease progressed(55). The dependence of MPL expression on disease duration makes precise delineation of its role difficult. Consequently, despite these studies, the role of MPL expression in MPNs remains to be determined. Many of these studies examined MPL expression as a prognostic marker for MPNs. However, recent discoveries have provided a better understanding of the molecular pathogenesis of MPNs; therefore, analysis of MPL expression based on these distinct subsets could help answer outstanding questions regarding the role on MPL expression in MPNs.

1.9.3 TPO expression in MPNs

Unlike MPL, mutations of the TPO protein have not been reported to cause MPNs. However, cytogenetic abnormalities in the region of chromosome 3q26, where the *THPO* gene is located, are associated with abnormal haematopoiesis, including thrombocytosis(248-250). Additionally, mutations within the *THPO* genes have been reported which result in more efficient translation of the protein(251,252). Affected individuals present with high circulating TPO levels resulting in hereditary ET(251,252). Taken together, these data suggests that TPO plays a role in MPN development, specifically, when plasma TPO levels are dysregulated, MPNs may develop.

This association has led a number of groups to study the relationship between TPO levels and MPNs. Harrison et al. report no significant differences in TPO levels between patients with ET, reactive thrombocytosis (RT) and other MPNs compared to normal patients(240). Similarly, Li et al. also found no difference between plasma TPO levels in ET patients compared to normal patients(241). Normal TPO levels were attributed to reduced MPL expression on platelets which offset the increase in platelet count characteristic of ET(241). Conversely, Tomita et al. report an inverse relationship between serum TPO levels and platelet count in ET patients. Successful therapeutic reduction of platelet count resulted in increased TPO levels. However, this can be explained by reports of lowered MPL expression on ET platelets resulting in decreased ability to sequester and remove circulating TPO(253). Furthermore, Wang and Hashimi investigated the reported increase in plasma TPO levels in MPN patients and found no abnormalities associated with the regulation of TPO production by BM mononuclear and BM stromal cells(254). Therefore the changes observed in plasma TPO levels is likely due to differences in MPL expression and thus ability to sequester TPO(254). Whereas there are reports of heterogeneity of *MPL* expression within the MPN patients, the consensus with TPO is clearer cut with the defect being specific to MPL rather than TPO.

1.9.4 Interplay between JAK2 and MPL

JAKs are not only necessary for signal transduction of cytokine receptors but also necessary for their cell surface localization and stability(166,255,256). Analysis of

proteins involved in the IL-6 signalling pathway revealed similar half-lives for the receptor component, gp130, and JAKs(257). Additionally, fluorescence recovery after photobleaching (FRAP) studies showed that once JAKs associate with a receptor, they cannot be exchanged(258) and it is only when JAKs are associated with a receptor are they localised to the plasma membrane(259). These studies demonstrate that JAKs and their receptors are highly interdependent.

JAK2 is essential for haematopoiesis; deficient mice are embryonic lethal resulting from failure to develop definitive haematopoiesis(260). Additionally, inducible knockout of *Jak2* from the haematopoietic compartment of adult mice results in pancytopenia and death on average 36 days post treatment(261). Similarly, TPO and MPL are also important for haematopoiesis as clinical mutations of MPL results in pancytopenia(98). JAK2 is the predominant JAK activated in response to TPO stimulation(103,262,263); it binds to the Box1 and Box2 motifs of MPL via its FERM domain and in the absence of this association, TPO signalling is abrogated(104). Additionally, JAK2 enhances cell surface localisation and stability of MPL(255). Given the close relationship between JAKs and their receptors, in addition to the functional interdependencies of MPL and JAK2, MPL and JAK2 can be considered functionally coupled.

1.9.5 JAK2 and MPL in MPNs

Activation of the JAK-STAT signalling pathway has been shown to cause MPNs. The *JAK2*^{V617F} mutation predominates in the classical MPNs with approximately 95% of PV patients and 50% of patients with ET and PMF harbouring the mutation(179-181). Moreover, activating mutations in MPL, *MPL*$^{W515L/K}$ and MPL^{S505N}, result in development of *JAK2*^{V617F}-independent ET and PMF(124,125,200). These mutations independently result in uncontrolled activation of the same JAK-STAT pathway resulting in MPNs. Additionally, MPNs are a stem cell disorder thus acquisition of *JAK2*^{V617F} must occur in an early haematopoietic cell to result in disease. Importantly, MPL is expressed on HSCs and although a number of other receptors are also expressed, the IL-3 receptor (IL-3R) and MPL are the only two that are dependent on JAK2 for signal transaction(235). Additionally, just as JAK2 is necessary for TPO signalling, it has been shown *in vitro* that *JAK2*^{V167F}

requires expression of a homodimeric type I cytokine receptor to induce cytokine independent proliferation(264). IL-3R signals as a heterotetramer whereas MPL is a homodimer, thus it is likely that MPL is important for *JAK2*^{V617F} pathogenesis.

Given the role of TPO and MPL in HSCs, the requirement for *JAK2*^{V617F} acquisition in HSCs to cause MPNs, the reliance of *JAK2*^{V617F} on a homodimeric type I cytokine receptor and the ability of activating mutations of MPL to result in MPNs, it is likely that MPL is also involved in *JAK2*^{V617F}-positive MPN development. Although this has been investigated *in vitro*, there has been a relative failure to study the role of MPL on MPNs *in vivo*. Additionally, current treatments of MPNs are predominantly aimed at treating the symptoms rather than the disease itself. Specific inhibition of JAK2 by a small molecule inhibitor, ruxolitinib, is successful at treating the disease in some patients; however, due to the broad function of JAK2, treatment is often associated with undesired side effects. Therefore, additional research is required to identify additional targets for therapeutic intervention of MPNs.

1.10 Primary Aims

In order to develop therapeutics in the treatment of MPNs, a more thorough understanding of the components necessary for disease pathogenesis is required. Although identification of the *JAK2*^{V617F} mutation greatly improved our understanding of MPNs, JAK2 inhibitors have not been able to recapitulate the effects observed with the use of Gleevec. It is possible that development of a better JAK2 inhibitor may result in better patient outcomes; however, further identification of additional factors necessary for disease development provides an additional and potentially more relevant therapeutic target. The role of TPO and its receptor, MPL, in development of MPNs has yet to be determined. The following specific aims were designed to answer some of the outstanding questions surrounding this field of study.

- Characterise the additional regulatory elements within MPL necessary in the regulation of TPO signalling

- Determine the functional role of MPL tyrosine residues in JAK2^{V617F}-mediated signalling *in vitro*

- Generate an *in vivo* model to determine the role of TPO and MPL in JAK2^{V617F}-positive MPNs

- Determine the role of TPO and MPL in development of JAK2^{V617F}-positive MPNs *in vivo*

CHAPTER 2 GENERAL MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Cell culture plasticware and reagents

Tissue culture plasticware was purchased from BD Biosciences. Tissue culture media: Roswell Park Memorial Institute (RPMI) 1640, Dulbecco modified Eagle medium (DMEM) and Dulbecco's phosphate-buffered saline (DPBS) was purchased from Corning|cellgro, Penicillin-Streptomycin-Glutamine (PSG) (10,000 I.U./mL Pencillin, 10,000 µg/mL Streptomycin, 29.2 mg/mL L-glutamine in a 10mM citrate buffer) was purchased from Life Technologies and heat inactivated Fetal Bovine Serum (FBS) was purchased from Gemini Bio-products (West Sacramento, CA, USA). All cells were cultured at 37°C, 5% CO_2.

2.1.2 Isolation and culture of primary murine bone marrow cells

Mice were asphyxiated using CO_2 and cervical dislocation was performed as a secondary physical method of euthanasia. Femurs and tibias from mice were extracted and flushed with 7mL and 3mL DMEM +10% FBS, respectively using a 25G$^5/_8$ needle into a 50mL Falcon Tube. A single cell suspension is obtained by expelling cells through a 22G1½ needle into a 100µm nylon cell strainer (BD Biosciences, 352360). Cells were then pelleted at 300*g* and lysed for RNA or protein isolation or cultured in the appropriate medium as stated.

2.1.3 Cell Line Culture Conditions

Ba/F3 is an IL-3 dependent murine pro-B-cell line. These cells were cultured in RPMI 1640 containing 1% PSG, 10% FBS and 2µL/mL recombinant mouse(rm)IL-3 supernatant in 75cm^2 tissue culture flasks. The ecotropic retroviral packaging cell line, Platinum-E cells (Plat-E), were cultured in DMEM containing 1% PSG and 10% FBS in 10cm^2 tissue culture plates. F-36P is a human leukemia cell line derived from a patient with myelodysplastic syndrome. These cells were obtained from the

European Collection of Cell Culture and cultured as previously described(265), in 1% PSG, 5% FBS and 5ng/mL recombinant human(rh)IL-3 (PeproTech). The Phoenix-amphotropic retroviral packaging cell line (φNX) was cultured in DMEM containing 1% PSG and 10% FBS.

2.2 Total RNA Isolation

Total RNA was isolated from cells using an RNeasy Mini Kit (Qiagen). Cells were lysed in the suggested amount of RLT buffer supplemented with 10μL/mL 2-mercaptoethanol (β-ME) and homogenized using a QIAshredder homogenizer (Qiagen). 1 volume of 70% ethanol was added to the homogenized cells and this was then loaded onto a supplied RNeasy spin column, washed with Buffer RWI followed by 2 washes with Buffer RPE. RNA was eluted off the column using RNase-free water.

2.3 cDNA synthesis from total RNA

cDNA was synthesized from total RNA using a SuperScript™ II Reverse Transcriptase kit (Life Technologies). RNA, 1μL 10mM deoxynucleotide (dNTP) mix and 0.5μg oligo$(dT)_{12-18}$ and RNase-free H_2O were combined in a sterile 0.2mL polymerase chain reaction (PCR) tube and incubated at 65°C for 5 minutes, followed by a 4°C incubation for 1 minute. A 2X reaction master mix was prepared by combining 10X RT buffer, 25mM $MgCl_2$, 40U/μL RNaseOUT™ and was added to the RNA/primer mixture. The samples were then incubated for 42°C for 2 minutes, then 1μL of SuperScript™ II reverse transcriptase enzyme was added to each tube and cDNA synthesis reaction was performed by incubation at 42°C for 50 minutes. The reaction was terminated by increasing the incubation temperature to 70°C for 15 minutes, samples were then cooled to 4°C, 1μL of RNase H was added and incubated at 37°C for 20 minutes to hydrolyze phosphodiester bonds in RNA:DNA hybrid strands thereby removing the RNA templates from the reverse transcriptase reaction. Samples were diluted 1:5 in RNase-free H_2O and used for PCR or stored at -20°C. A minus RT control was run for each sample and was treated the same as the cDNA samples except RNase-free H_2O was added in place of SuperScriptII™ RT.

2.4 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

A reaction mixture comprising 1X PCR reaction buffer, 0.2mM of each dNTP, 2mM of forward and reverse primers, 1µL of diluted cDNA from Section 2.3 and 0.5U of PfuUltra HF DNA polymerase (Agilent Technologies) were brought up to a final volume of 50µL in a thin-wall PCR tube. The PCR reaction was performed using an MJ Research PTC-200 thermal cycler (Bio-Rad). Cycle parameters are listed in Table 2.1 and annealing temperatures were optimised for each gene target. 15µL of each reaction product was mixed with 3µL of 6X DNA loading dye (0.25% bromophenol blue(w/v), 0.25% xylene cyanol FF (w/v), 30% glycerol (v/v)) and analysed on a 1.5% agarose Tris/borate/EDTA (TBE: 89mM Tris base, 89mM boric acid, 2mM EDTA, pH 8.0) gel containing 0.2µg/mL ethidium bromide (EtBr). DNA products were visualized using an ultra-violet (UV) light box.

2.5 Western Blot Analysis

2.5.1 Protein extraction

Cultured or treated cells were harvested at 300*g* or 5 min at 4°C, washed three times in cold DPBS to remove any cytokines and media components and lysed in 500µL 1% NP-40 lysis buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1% NP-40) containing protease inhibitors (Protease inhibitor cocktail for mammalian cell and tissue extracts, containing 104nM AEBSF, 80µM aprotinin, 4mM bestatin, 1.4mM E-64, 2mM leupeptin, 1.5mM Pepstatin A; Sigma-Aldrich) and phosphatase inhibitors (1mM Na_3VO_4, 10mM NaF) at 4°C for 10 minutes. Cellular debris was removed by centrifugation of samples for 15 minutes at 20,500*g* at 4°C and the supernatants were collected and transferred into new microcentrifuge tubes.

2.5.2 Protein quantitation

Protein concentrations were determined using a DC™ protein assay (Bio-rad) according to manufacturer's instructions in a 96-well flat bottom microplate. This particular colormetric protein assay was chosen as it is compatible with protein samples containing detergent. It is a modified version of the Lowry assay and relies

Table 2.1 RT-PCR Cycling Parameters

Segment	**Cycles**	**Step**	**Temperature** (°C)	**Time (mm:ss)**
1	1	Polymerase activation	94	10:00
2	32 (unless otherwise stated)	Denature dsDNA	94	00:30
		Primer annealing	Varies, as listed	00:30
		Extension	72	01:00
3	1		4	hold

on the oxidation of aromatic residues, primarily tyrosine and tryptophan, by copper ions under alkaline conditions. Addition of Folin reagent results in its reduction by the modified aromatic residues resulting in development of a blue color which is then measured at an absorbance of 655nm on an iMark microplate absorbance reader (Bio-rad). Bovine serum albumin (BSA) protein standard solutions were prepared in 1% NP-40 lysis buffer, ranging from 0-4mg/mL. 5µL of each standard was added in triplicate and 5µL of each unknown sample was added in duplicate to wells of a 96-well plate. 1mL of working reagent A' was prepared (1mL of reagent A and 20µL of reagent S) and 25µL was added to each well followed by addition of 200µL of reagent B. The plate was mixed using the mixing function on the microplate reader and incubated at 25°C for 15 minutes. The plate was then read at an absorbance of 655nm. Any background absorbance was removed by subtracting the absorbance of the lysis solution and protein assay reagents. A standard curve was generated from the absorbance of the BSA standard solutions. From this, the concentration of each sample was calculated.

2.5.3 Sodium dodecyl sulfate (SDS)-Polyacrylamide gel electrophoresis (PAGE) and protein transfer

Protein samples were prepared using 10µg protein, 4x NuPAGE® LDS Sample Buffer (106mM Tris-HCl, 141 mM Tris base, 2% lithium dodecyl sulface (LDS), 10% glycerol, 0.51mM EDTA, 0.22mM SERVA blue G250, 0.175mM phenol red, pH 8.5) (Invitrogen) and 10x NuPAGE Sample Reducing Agent (50mM dithiothreitol (DTT)) (Invitrogen) brought to 20µL at 1x using 1% NP-40 lysis buffer. Samples were heated to 100°C for 5 minutes to denature the proteins. The samples were then loaded into a NuPAGE® Novex® 4-12% Bis-Tris pre-cast polyacrylamide gel and separated in 1x NuPAGE® MOPS SDS runnning buffer (50 mM MOPS, 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.7) (Invitrogen) supplemented with 500µL of NuPAGE Antioxidant in the inner chamber of the XCell SureLock® Mini-Cell running apparatus. NuPAGE® gels are at neutral pH thus some reducing agents including DTT do not always co-migrate with the protein allowing for the potential of protein re-oxidization so NuPAGE Antioxidant is added to ensure proteins remain in a reduced state during electrophoresis. Additionally, 3µL of Precision Plus Protein™ Kaleidoscope™ Standard (Bio-Rad) was loaded

onto the gel to monitor separation. Gels were run at 200V for 50 minutes. Gels were then transferred onto methanol activated polyvinylidene difluoride (PVDF) membrane (Bio-Rad) in transfer buffer (24mM Tris-base, 150mM Glycine, 20% Methanol (v/v)) using a Mini Trans Blot Cell transfer apparatus (Bio-Rad) at 110V for 1 hour.

2.5.4 Immunodetection of proteins

After proteins were immobilized onto PVDF, the membrane was incubated in blocking buffer (4% dry non-fat milk or BSA in Tris-buffered saline containing 0.1% Tween-20 (TBS-T)) at 60 rotations per minute on an orbital shaker for 1 hour at 25°C. The blocking solution was then discarded and the membrane washed for 1 minute in TBS-T before primary antibody diluted in blocking buffer was added. The membrane was incubated in primary antibody overnight at 4°C or 1 hour at 25°C. The blocking buffer, antibody concentration and time of incubation were optimized for each individual antibody used. Following primary antibody incubation, membranes were washed 3 times with TBS-T. For unconjugated antibodies, the membrane was then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody diluted in blocking buffer for 1 hour at 25°C followed by 4 washes in TBS-T. Membranes were then subjected to chemiluminescent detection with ECL-plus (GE Healthcare, Little Chalfont, UK) and imaged using radiography film or a FluorChem M imager (ProteinSimple, Santa Clara, CA, USA). Western blots were quantified by densitometry using ImageJ image processing and analysis software (http://rsbweb.nih.gov/ij/, National Institutes of Health, USA).

2.5.5 Membrane Stripping

Membranes were stripped of bound antibody to allow for detection of additional proteins and loading controls. Membranes were incubated in stripping buffer (SDS) with 6μL/mL 2-mercaptoethanol (β-ME) at 60°C for 10 minutes. Membranes were then rinsed 5X with dH_2O and incubated with agitation in TBS-T for 5 minutes before being used for additional immunodetection of proteins as described in Section 2.5.4.

2.6 Cloning

2.6.1 Site-directed mutagenesis

Site-directed mutagenesis was performed using a QuikChange II site-directed mutagenesis kit (Agilent Technologies, 200523-51) following the manufacturer's instructions. Vectors were mutagenized at various concentrations (25ng, 50ng 100ng) to ensure ideal reaction conditions. Each mutagenesis reaction contained 1X reaction buffer, dsDNA plasmid template, 125ng each of forward and reverse primers, 1µL of a proprietary dNTP mix, 2.5U of *PfuUltra* High-Fidelity DNA polymerase and ddH_2O to 50µL. Cycling parameters are listed in Table 2.2 and mutagenesis primer sequences and reaction annealing temperatures are listed in Table 2.3. Mutagenesis reactions were then Dpn *I* digested (10U/reaction) at 37ºC for 1 hour to digest the original dsDNA plasmid template.

2.6.2 Transformation

Mutagenized plasmids were transformed into Subcloning Efficiency™ DH5α™ competent *E.Coli* (Life Technologies). 2µL of the of the mutagenesis reaction was added to 50µL of the Subcloning Efficiency™ DH5α™ competent cells in a microfuge tube, the reaction was incubated on ice for 5 minutes, heat shocked for 45 seconds in a 42ºC water bath, placed back on ice for 2 minutes then 500µL of S.O.C. medium (#15544-034, Life Technologies) was added to each reaction and incubated at 37ºC for 1 hour at 225 rpm to allow the cells to recover. 100µL of the transformation reaction was then plated on pre-warmed Luria-Bertani (LB) plates containing 100µg/mL ampicillin and cultured overnight at 37ºC to allow for growth of colonies. A maximum of 5 individual colonies were selected from plates for validation.

2.6.3 Plasmid preparation

Individual colonies selected from LB ampicillin plates were individually cultured for 16 hours in 4mL of LB medium containing 100µg/mL ampicillin. Plasmids were then isolated from 3mL of each culture using a QIAprep Spin Miniprep Kit

Table 2.2 Site-directed Mutagenesis Cycling Parameters

Segment	Cycles	Step	Temperature (°C)	Time (mm:ss)
1	1	Polymerase activation	95	00:30
2	12*	Denature dsDNA	95	00:30
		Primer annealing	Varies, see Table 2.3	01:00
		Extension	68	10:00**

*recommended number of cycles for point mutations
**1 minute/kb of plasmid length. pMX-MPL = 9kb

Adapted from the Agilent Technologies QuikChange II Site-Directed Mutagenesis Kit user manual

Table 2.3 Site-directed Mutagenesis Primer Sequences and Annealing Temperatures

Vector Backbone	Protein	GenBank ID	Mutagenesis Primer Sequence	Tm (°C)	Anneal Temp (°C)	Mutation	Ref
pMX-puro	***MPL*^{WT}**	NM_005373.2					
	***MPL*^{Y591F}**		F-5'-CCAGATGGACTTCCGAAGATTGCAGCCTT-3' R-5'-AAGGCTGCAATCTTCGGAAGTCCATCTGG-3'	74.4	68.0	Y591 ↓ F591	(1,2)
	***MPL*^{Y625F}**		F-5'-CCAACCATTCCTTCCTACCACTAAG-3' R-5'-CTTAGTGGTAGGAAGGAATGGTTGG-3'	57.0	52.0	Y625 ↓ F625	(3)
	***MPL*^{Y630F}**		F-5'-TACCACTAAGCTTTTGGCAGCAGC-3' R-5'-GCTGCTGCCAAAAGCTTAGTGGTA-3'	59.9	55.0	Y630 ↓ F630	(3)
pQCXIN-neo	***JAK2*^{WT}**	BC039695.1					
	***JAK2*^{V671F}**		F-5'-AGCATTTGGTTTTAAATTATGGAGTATGTTTCTGTGGAGACGAGA-3' R-5'-TCTCGTCTCCACAGAAACATACTCCATAATTTAAAACCAAATGCT-3'	68.4	65.0	V617 ↓ F617	(4)

following the microcentrifuge spin protocol (Qiagen). Plasmids were then checked for the desired mutations as described in Section 2.6.4. For larger scale plasmid preparations, cultures in 400mL of LB medium containing 100µg/mL ampicillin were used for plasmid isolation with a QIAprep Maxi Kit (Qiagen) following the manufacturers' protocol.

2.6.4 Mutagenized plasmid validation

A diagnostic digest was performed on each plasmid to ensure isolation of the desired plasmid. Restriction enzymes used to clone in the gene of interest were used to digest the plasmid preparation product where each digestion reaction contained 1X NEB Buffer selected for maximal enzyme compatibility, 2µg BSA, 2U of each desired restriction enzyme, 1µg of the isolated plasmid DNA and ddH_2O was added to achieve a final reaction volume of 20µL. The reaction was then incubated at 37ºC for 1 hour then resolved on a 0.8% agarose (TBE) gel containing 0.2µg/mL EtBr (Figure 2.2). Correctly digested plasmids were sent to the Stony Brook Genomics Core for sequencing using primers to full length human *MPL*. Sequencing primers are listed in Table 2.4 and binding regions are shown in Figure 2.1. Plasmids with inserts containing the desired sequence underwent an additional plasmid preparation at a larger by Maxiprep (Section 2.6.3) and stored as a stock for experimental use.

2.7 Generation of stable cell lines

2.7.1 Generation of retroviral Particles

2.7.1.1 Ecotropic viral particles

Platinum-E (Plat-E) cells are an ecotropic retroviral packaging cell line used to generate vector containing retroviral particles used to make stable cell lines(266). These cells produce ecotropic retrovirus capable of infecting mouse or rat cells and were used to infect Ba/F3 cells. Plat-E cells are plated at 3×10^6 cells per 10cm tissue culture plate and cultured for 16 hours to allow for attachment and proliferation. Once cells reach approximately 50% confluency, they are transfected with a 2:1 mixture (µL:µg) of Lipofectamine 2000 (Life Technologies) and plasmid DNA. Generally, 2µg of plasmid DNA is used per transfection and each component

Table 2.4 MPL sequencing primers

Primer	Sequence
Reverse 1	5’-CACTTCCTCCTGGTCTGGAA-3’
Forward 1	5’-ATGCCCTCCTGGGCCCTCTTC-3
Forward 2	5’-CACGGTCATACAGCTGATTG-3
Forward 3	5’-CTGGATCCACCAGGCTGTGC-3’
Forward 4	5’-GCCACCGAGACCGCCTGGAT-3’
Forward 5	5’-ACCATGCCCCTGTCTGTGTGC-3’

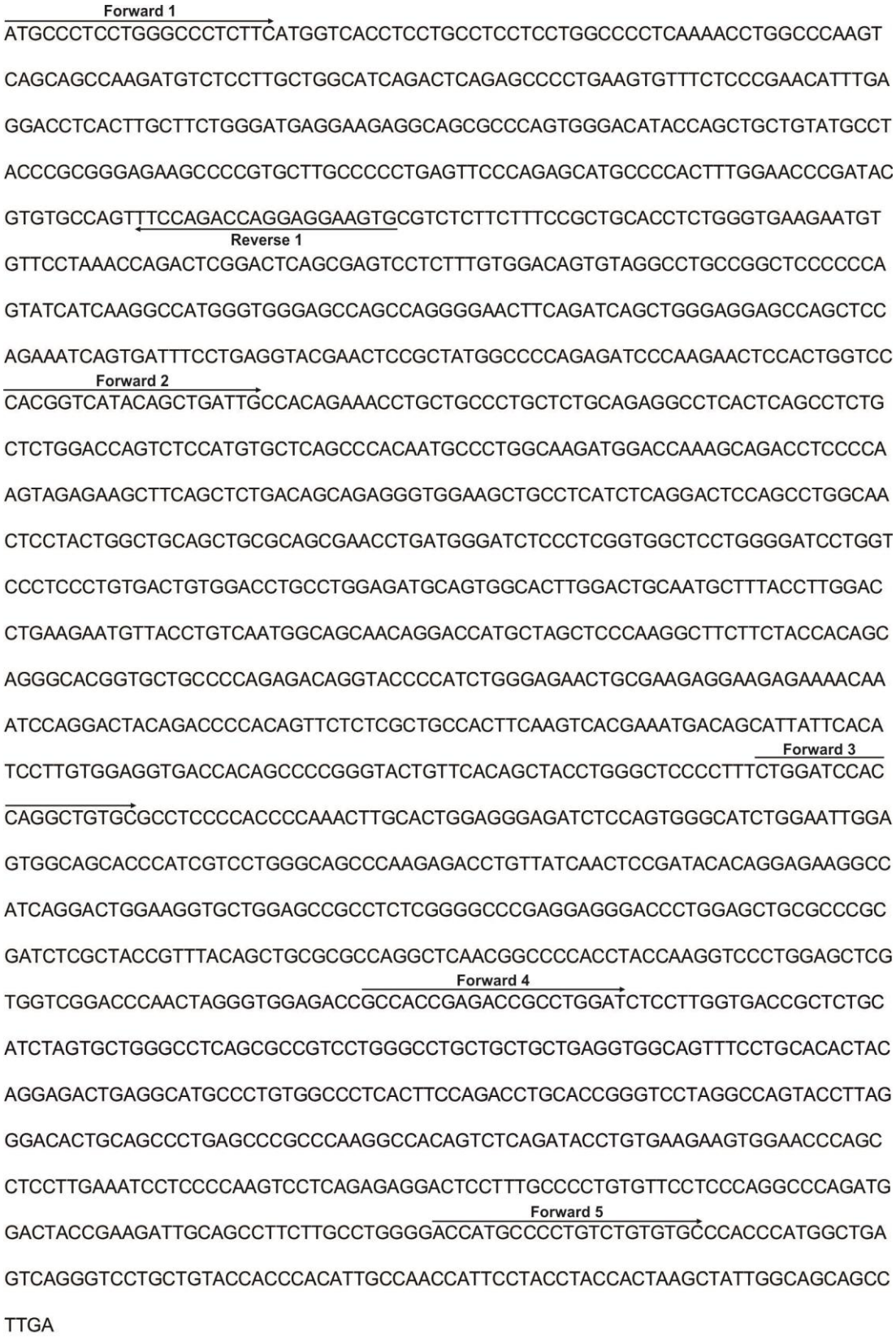


Figure 2.1 MPL sequencing primer binding sites
Figure showing binding regions for MPL sequencing primers listed in Table 2.1. Arrows dictate direction of sequencings reaction.

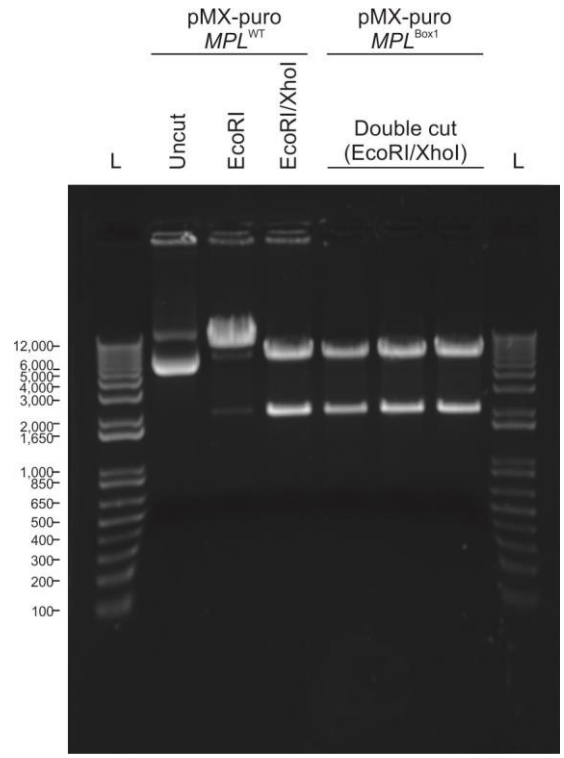


Figure 2.2 Restriction enzyme diagnostic digest

Mutagenized plasmids were initially tested for the presence of the gene of interested, MPL, by a restriction enzyme diagnostic digest. MPL was cloned into the multiple cloning site between EcoRI and XhoI sites; therefore, plasmids were treated with no restriction enzyme, EcoRI or a double digest with EcoRI and XhoI to test for the presence of the MPL insert. Digestion products were resolved on a 0.8% agarose TBE gel with ethidium bromide to visualise products. L – 1 kb plus ladder

is prepared separately in 250µL Opti-MEM media and mixed just prior to addition onto cells; Plat-E culture media is changed just prior to addition of the DNA-lipid complex. Culture media was replaced 32hrs after transfection and cultured for an additional 16hrs to allow for accumulation of retroviral particles. Media from these cells was then collected using a 10mL syringe and filtered with a 0.45µM syringe filter to remove any cells. Fresh media was added back onto the cells to allow for production of additional retroviral particles. These particles were harvested, same as previously, 8 hours later.

2.7.1.2 Amphotropic viral particles

φNX-amphotropic cells are an amphotropic retroviral packaging cell line developed by the Nolan laboratory at Standford University for efficient production of retroviral particles capable of infecting most mammalian cells (http://web.stanford.edu/group/nolan/_OldWebsite/retroviral_systems/phx.html). Retroviral particles were generated and harvested as described in Section 2.7.1.1. These viral particles were used to infect F-36P cells.

2.7.1.3 Retroviral transduction of cells

16 hours prior to harvest of the first batch of retroviral particles, Ba/F3 or F-36P cells were passaged 1:5 to ensure active proliferation of cells. Just after harvest of the first round of retroviral particles, 1 x 10^6 cells were counted and cultured in the harvested retroviral supernatnat with the addition of necessary culture cytokines as stated in Section 2.1.3 and 10µg/mL polybrene (Millipore). Following the second round of retroviral particle harvest, cells were pelleted and re-cultured in fresh retroviral supernatant, again with addition necessary cytokines and polybrene.

2.7.1.4 Antibiotic selection for infected cells

72 hours post transduction, cells were selected for plasmid uptake by culture with 5µg/mL puromycin for the PMX-puro plasmid or 400µg/mL geneticin (G418) for pQCXIN plasmid. Untransduced cells were also cultured in the respective antibiotic as a control. Cells were cultured under selection for 7 days following death of all the untransduced cells to ensure for complete selection.

2.8 Thrombopoietin ELISA

A Mouse thrombopoietin Quantikine® ELISA (R&D Systems) was used to quantify serum thrombopietin levels. Whole blood was collected via cardiac puncture using citrate (ACD Solution A of trisodium citrate, 22.0g / L; citric acid, 8.0 g / L; and dextrose 24.5 g / L, BD Biosciences) as an anticoagulant, samples were centrifuged at 10,000*g* for 10 minutes, platelet poor plasma (PPP) was collected and stored at -80°C. Samples were thawed on ice and diluted 5-fold in supplied Calibrator Diluent RD5-3 prior to being assayed. The assay was performed following the manufactures' protocol and standards, Control and samples were all assayed in duplicate.

2.9 Statistical Analysis

Data was analysed for statistical significance using GraphPad Prism 6 Software. Analysis of differences between means of multiple groups was performed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. Analysis of differences between means of multiple groups with greater than one independent variable was performed using two-way ANOVA followed by Dunnett's multiple comparisons test. Asterisks on graphs indicate significance compared to WT, ***$P \leq 0.001$, **$P \leq 0.01$ and *$P \leq 0.05$. A value of $P \leq 0.05$ was considered statistically significant.

CHAPTER 3 PHOSPHORYLATED MPL TYROSINE 591 REGULATES THROMBOPOIETIN-INDUCED SIGNALING

3.1 Experimental Rationale

In this chapter, we investigate the role of MPL^{Y591} in the negative regulation of TPO signalling. First, the phosphorylation state of Y591 will be determined using mass spectrometry. Then, utilizing an *in vitro* system where human MPL is ectopically expressed in Ba/F3 cells, we aim to determine the consequence of MPL^{Y591} removal on receptor phosphorylation and signal transduction in addition to identification of potential binding partners to Y591.

3.2 Materials and Methods

3.2.1 pMX-puro-MPL

pMX-puro is a retroviral vector generated to facilitate cDNA library generation. It is composed of a modified pBabe-puro vector, termed pBabeX, where the SV40 promotor and the puromycin resistance genes were removed and a multiple cloning site (MCS) was inserted(267). To maximize viral titers, the 5' long terminal repeat (LTR) of pBabeX was replaced with the 5' LTR and extended packaging signal of the MGF vector(268). For the purposes of our studies the SV40 promotor and puromycin resistance gene were reintroduced to the vector to allow for selection of transduced cells. Human *MPL* cDNA was cloned into a pMX-puro vector (Figure 3.1) using *EcoRI* and *XhoI* restriction sites in the multiple cloning site(Figure 3.2). pMX-puro-MPL^{Y591F} was generated by site-directed mutagenesis (Section 2.6.1 and Table 2.3) and the mutation was confirmed by sequencing (Figure 3.3).

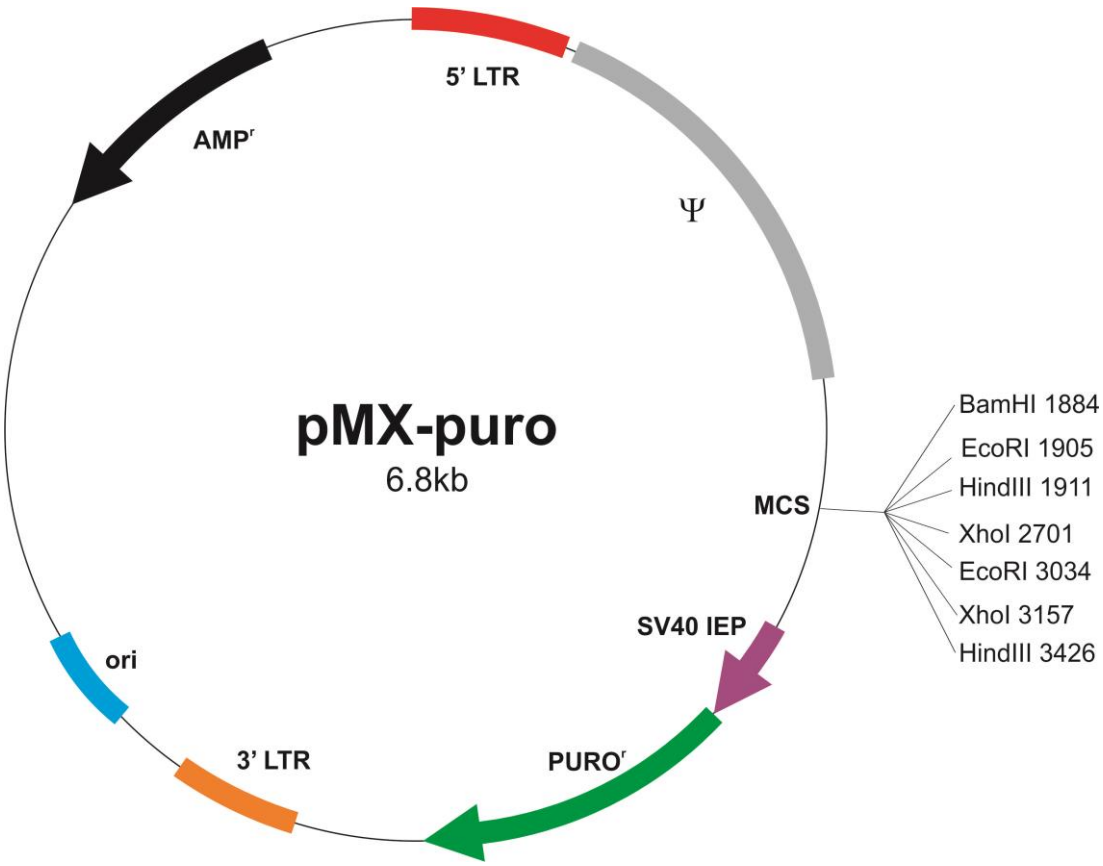


Figure 3.1 pMX-puro backbone vector
pMX-puro backbone contains a multiple cloning site (MCS) to allow for insertion of genes for expression. An ampicillin resistance gene allows for selection in *E.coli* and a puromycin resistance gene allows for selection in eukaryotic cells under the control of the SV40 immediate promoter (IEP).

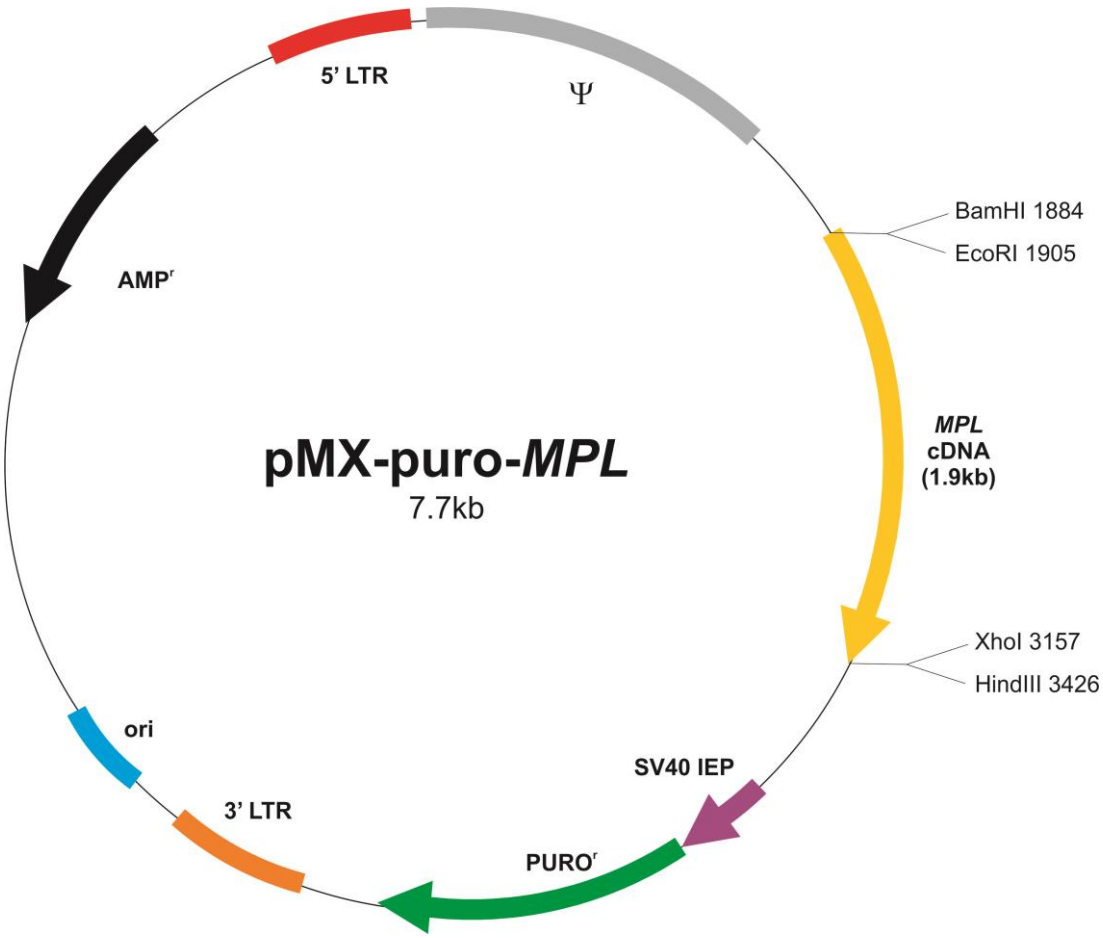


Figure 3.2 pMX-puro-MPL vector
MPL was cloned into the pMX-puro vector between EcoRI and XhoI restriction sites within the MCS.

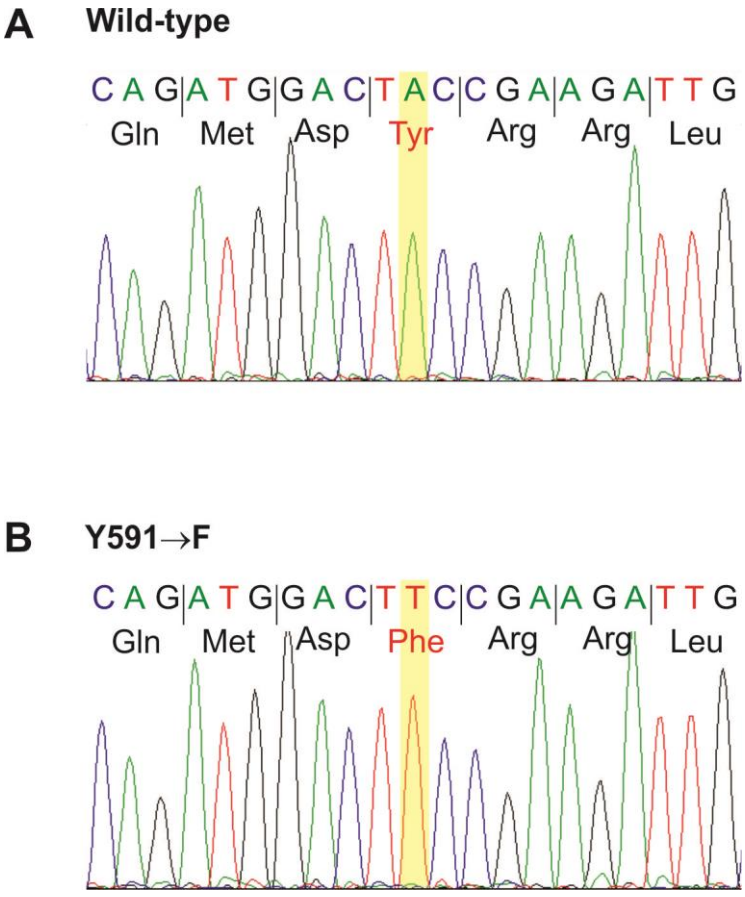


Figure 3.3 pMX-puro-MPL^{Y591F} Sequencing Results
Sequence analysis of WT and mutagenized MPL in the pMX-puro-MPL vector.

3.2.2 Immunoprecipitation

Protein lysate was obtained as described in 2.5.1. 500µg of protein was prepared to 1µg/µL and incubated with 20µL packed Protein A beads (Millipore) for 1 hour on an end-to-end rotator at 4°C to remove any proteins non-specifically binding to the beads. Samples were then incubated with 2µg of anti-MPL antibody (Millipore, #06-944) for 16 hours on an end-to-end rotator at 4°C. 20µL packed Protein A agarose beads (Millipore, 16-125) were then added for 4 hours on an end-to-end rotator at 4°C, MPL-antibody—bead complexes were washed 3 times with 1% NP-40 lysis buffer, protein was eluted using 2X NuPAGE® LDS sample buffer containing β-ME (Invitrogen) and boiled at 100°C for 5min. Eluted protein was then subjected to western blotting as described starting at Section 2.5.3.

3.2.3 Detection of active, GTP-bound Ras

Levels of activated Ras were detected using an Active Ras Pull-down and Detection Kit (Thermo Scientific, Rockford, IL, USA) following the manufacturers' protocol. Ba/F3-MPL cells were treated with or without 10ng/mL rhTPO for 5 minutes and lysed in the kit-provided lysis buffer supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich, P8340). Lysates were then subjected to pulldowns using the Ras binding domain of RAF proto-oncogene serine/threonine-protein kinase (RAF1) to isolate the active, guanosine-5'-triphosphate (GTP)-bound Ras, and analysed using western blot (Section 2.5) to detect levels of active Ras.

3.2.4 Peptide binding assays

Biotinylated peptides corresponding to MPL residues 582–601 including an aminohexanoic acid spacer (Biotin-Ahx-LCSSQAQMDY$_{591}$RRLQPSCLGT-NH2 and Biotin-Ahx-LCSSQAQMD(pY$_{591}$) RRLQPSCLGT-NH2) were synthesized by RS Synthesis (Louisville, KY). Lyophilized peptides were reconstituted to 10mM in DPBS. Peptide concentration was measured using a NanoDrop 2000 (Thermo Scientific) at 205nm rather than the typical method of protein quantification (Section 2.5.2.) due to lack of tryptophan residues and presence of only a single tyrosine residue within the peptides. 400µL of reconstituted peptide was conjugated to 400µL High capacity NeutrAvidin Agarose Resin (Thermo Scientific, 29200) for 4.5 hours at room temperature. F-36P-MPL^{WT} cells were treated with or without 1ng/mL

rhTPO for 5 minutes and lysed in NP-40 lysis buffer. Lysates were pre-cleared by incubation with 40µL High capacity NeutrAvidin agarose resin for 1 hour at 4°C. 1mg of pre-cleared lysate was incubated with 20µL peptide conjugated beads overnight at 4°C. The samples were centrifuged and washed 5 times with 1% NP-40 lysis buffer, resuspended in 2X NuPAGE® LDS sample buffer containing β-ME (Invitrogen) and subjected to western blotting as described starting at Section 2.5.3.

3.2.5 RNA interference

Syk siRNAs were purchased from Dharmacon RNAi Technologies as a SMARTpool, and *Ptpn6* siRNA and control siRNA were purchased from Invitrogen. All siRNAs were reconstituted according to the manufacturers' instructions. Cells were subjected to 1 pulse of 1700volts for 20ms using the Neon® Transfection System (Invitrogen). Ba/F3-MPL^{WT} and MPL^{Y591F} cells were washed in DPBS and resuspended in R buffer at 2.0x10^{7}/mL; 2.0x10^{6} cells were transfected with siRNA at a final concentration of 1µM. Cells were cultured for 24 hours post-transfection in RPMI 1640 +10% FBS supplemented with murine IL-3, they were then cytokine starved in RPMI 1640 +2% FBS for 16 hours, then stimulated with or without 10ng/mL rhTPO for 15min and lysed in NP-40 lysis buffer. Lysates were then subjected to western blotting (Section 2.5.3) or Ras pulldown (Section 3.2.3).

3.3 Results

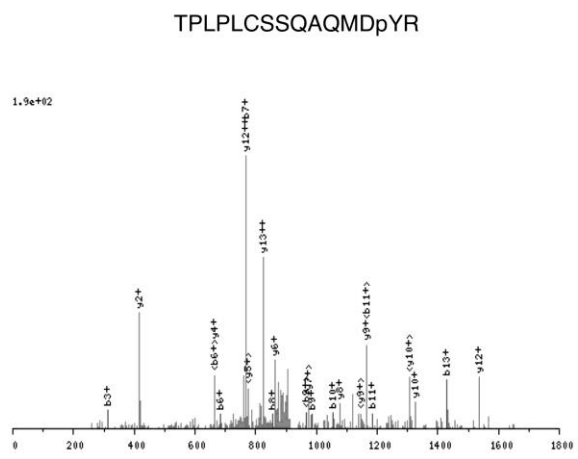
3.3.1 MPL tyrosine residue 591 (MPL^{Y591}) is phosphorylated in response to TPO

To develop a better understanding of MPL phosphorylation in response to TPO, tandem mass spectrometry was used to determine sites of TPO-mediated phosphorylation of MPL. MPL peptides were obtained by immunoprecipitating MPL from rhTPO stimulated Ba/F3-MPL cells followed by trypsin digest. Phosphorylated peptides were enriched using immobilized metal affinity chromatography (IMAC) and subjected to tandem mass spectrometry. Generated data was analysed using SEQUEST® and revealed the mass spectrum of the peptide TPLPLCSSQAQMDYR (Figure 3.4). The quantity computation with the help of the b- and y-ion series revealed that the mass of tyrosine (Y) 14 is 243Da, instead of the native 163Da. This is indicative of a phosphomodification (+80Da) of this tyrosine residue. Tyrosine 14

of the peptide is equivalent to MPL^{Y591} found in the intracellular domain of MPL, indicating that this residue is phosphorylated in response to TPO. This work was completed by Dr. Sebastian J Saur.

To begin assessing the role of MPL^{Y591}, we first studied receptor phosphorylation in the absence of MPL^{Y591} phosphorylation. Tyrosine residue 591was mutated using site-directed mutagenesis to phenylalanine to inhibit residue phosphorylation. This mutant form of MPL was ectopically expressed in Ba/F3 cells, which were then stimulated with 10ng/mL of rhTPO for 5 minutes, lysed and subjected to immunoprecipitation using an anti-MPL antibody. Immunoprecipitated protein was then analysed by western blot and probed for phospho-tyrosine, revealing the phosphorylation state of MPL.

In the absence of TPO stimulation, there is no detectable receptor phosphorylation; however, TPO stimulation induced a marked increase in WT receptor phosphorylation (Figure 3.5)(103). Consistent with previous reports, phosphorylation was comparable to WT when MPL^{Y630} was mutated and significantly diminished when MPL^{Y625}, a residue important for receptor activation, was mutated (Figure 3.5) (3). Mutation of MPL^{Y591} resulted in decreased total receptor phosphorylation to a level similar to MPL^{Y625F} (Figure 3.5). Additionally, to study changes in sensitivity to TPO and kinetics of receptor phosphorylation, Ba/F3-MPL^{WT} and MPL^{Y591F} cells were stimulated with varying concentrations of rhTPO and stimulated for different periods of time, respectively. In the absence of MPL^{Y591}, receptor phosphorylation was less sensitive to TPO stimulation (Figure 3.6). Additionally, the duration of MPL^{Y591F} receptor phosphorylation was decreased (Figure 3.7).



b+	#	AA	#	y+
102.0555	1	T	15	
199.1083	2	P	14	1745.739
312.1923	3	L	13	1648.686
409.2451	4	P	12	**1535.602**
522.3292	5	L	11	1438.549
682.3598	6	C	10	**1325.465**
769.3918	7	S	9	**1165.434**
856.4239	8	S	8	**1078.402**
984.4824	9	Q	7	991.370
1055.5196	10	A	6	**863.312**
1183.5781	11	Q	5	792.275
1314.6186	12	M	4	**664.216**
1429.6456	13	D	3	533.176
1672.6752	14	Y	2	**418.149**
	15	R	1	175.119

peptide sequence	pX site	SEQUEST Xcorr	Probability	z	observed m/z
1. TPLPLCSSQAQMDpYR	591	3.634	0.9979	+ 2	1847.78

Figure 3.4 Phospho-tyrosine peptide from the intracellular domain of MPL identified by LC-MS/MS following tandem IP[1]

Tandem mass spectra of the phosphopeptide TPLPLCSSQAQMDpYR were identified. The y and b series of the ions is indicated. The sequence resulting from these spectra is indicated above the panel. The mass of tyrosine (Y) 14 is 243 Da, instead of the native 163 Da. This indicates a phosphomodification (+80 Da) of this tyrosine residue. Tyrosine 14 of the peptide equals tyrosine 591 in the intracellular domain of MPL.

1Ectopically expressed human MPL protein in Ba/F3 cells recovered by tandem IP (anti-MPL→trypsin→IMAC). Peptide sequence is indicated along with the position of pY sites in MPL, the indicated peptide scores, charge state (z), and observed mass-to-charge ratios (m/z).

This work was completed by Dr. Sebastian J Saur.

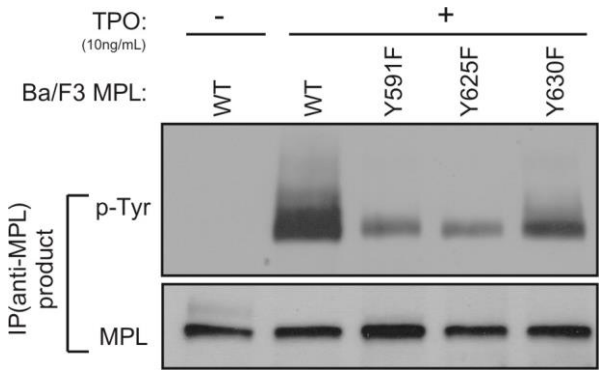


Figure 3.5 Mutant MPL receptor phosphorylation

Ba/F3 cells stably expressing human WT or mutant MPL were stimulated with rhTPO, lysed and subjected to immunoprecipitation with an anti- MPL antibody. Immunoprecipitates were analysed via western blot using a phospho-tyrosine probe to compare total receptor phosphorylation of the mutants to the wild-type receptor in response to TPO stimulation, blots were stripped and re-probed with an anti- MPL antibody to assess the level of immunoprecipitated protein. Data are representative of 3 independent experiments. Ba/F3 cells expressing different tyrosine to phenylalanine mutants of MPL were stimulated with 10ng/mL rhTPO for 5min. This is representative of 3 individual experiments.

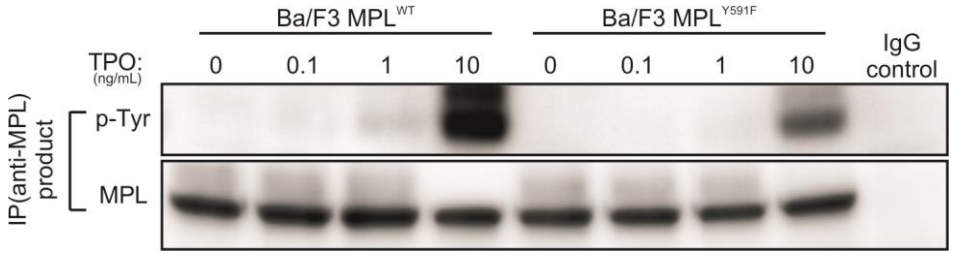


Figure 3.6 MPL^{Y591} receptor phosphorylation TPO dose response
Ba/F3 cells stably expressing human WT or mutant MPL were stimulated with rhTPO for 5min, lysed and subjected to immunoprecipitation with an anti- MPL antibody. Immunoprecipitates were analysed via western blot using a phospho-tyrosine probe to compare total receptor phosphorylation of the mutant to the wild-type receptor in response to TPO stimulation; blots were stripped and re-probed with an anti- MPL antibody to assess the level of immunoprecipitated protein.

Figure 3.7 MPL^{Y591} receptor phosphorylation TPO time course
Ba/F3 cells stably expressing human WT or mutant MPL were stimulated with 10ng/mL rhTPO, lysed and subjected to immunoprecipitation with an anti- MPL antibody. Immunoprecipitates were analysed via western blot using a phospho-tyrosine probe to compare total receptor phosphorylation of the mutant to the wild-type receptor in response to TPO stimulation; blots were stripped and re-probed with an anti-MPL antibody to assess the level of immunoprecipitated protein.

3.3.2 MPL ^{Y591F} exhibits hypersensitive, enhanced and prolonged ERK1/2 activation upon TPO stimulation

TPO binding to MPL results in receptor activation and subsequent initiation of numerous signalling cascades including the JAK/STAT, PI3K/Akt and MAPK pathways(106). To determine the role of MPL^{Y591} in TPO-mediated signalling, Ba/F3-MPL WT or MPL^{Y591F} cells were cytokine starved and stimulated with rhTPO. Consistent with previous reports, cells expressing MPL^{Y591F} exhibited a significant increase in the level and duration of ERK1/2 phosphorylation compared to MPL^{WT} (Figure 3.8 and Figure 3.9)(1). Cells expressing MPL^{Y591F} stimulated for 5min with increasing concentrations of rhTPO exhibited a significant hypersensitive response in ERK1/2 phosphorylation at low doses (0.1ng/mL) of rhTPO and enhanced maximal levels ERK1/2 phosphorylation (Figure 3.8). Akt phosphorylation is also increased in the MPL^{Y591F} mutant; however, activation of the JAK/STAT signalling was unaffected (Figure 3.8) (1). To study the mechanism behind the enhanced and prolonged ERK1/2 phosphorylation in MPL^{Y591F} expressing cells, the activation state of a stimulator upstream of the MAPK/ERK pathway, the small GTPase Ras was assessed. Ba/F3-MPL^{WT} or MPL^{Y591F} cells were cytokine starved for 18hr followed by 10ng/mL rhTPO stimulation. Cells were then analysed for active, GTP-bound Ras as described in Section 3.2.3. We observed a two-fold increase in the level of Ras-GTP in cells expressing the MPL^{Y591F} mutant receptor compared to WT (Figure 3.10).

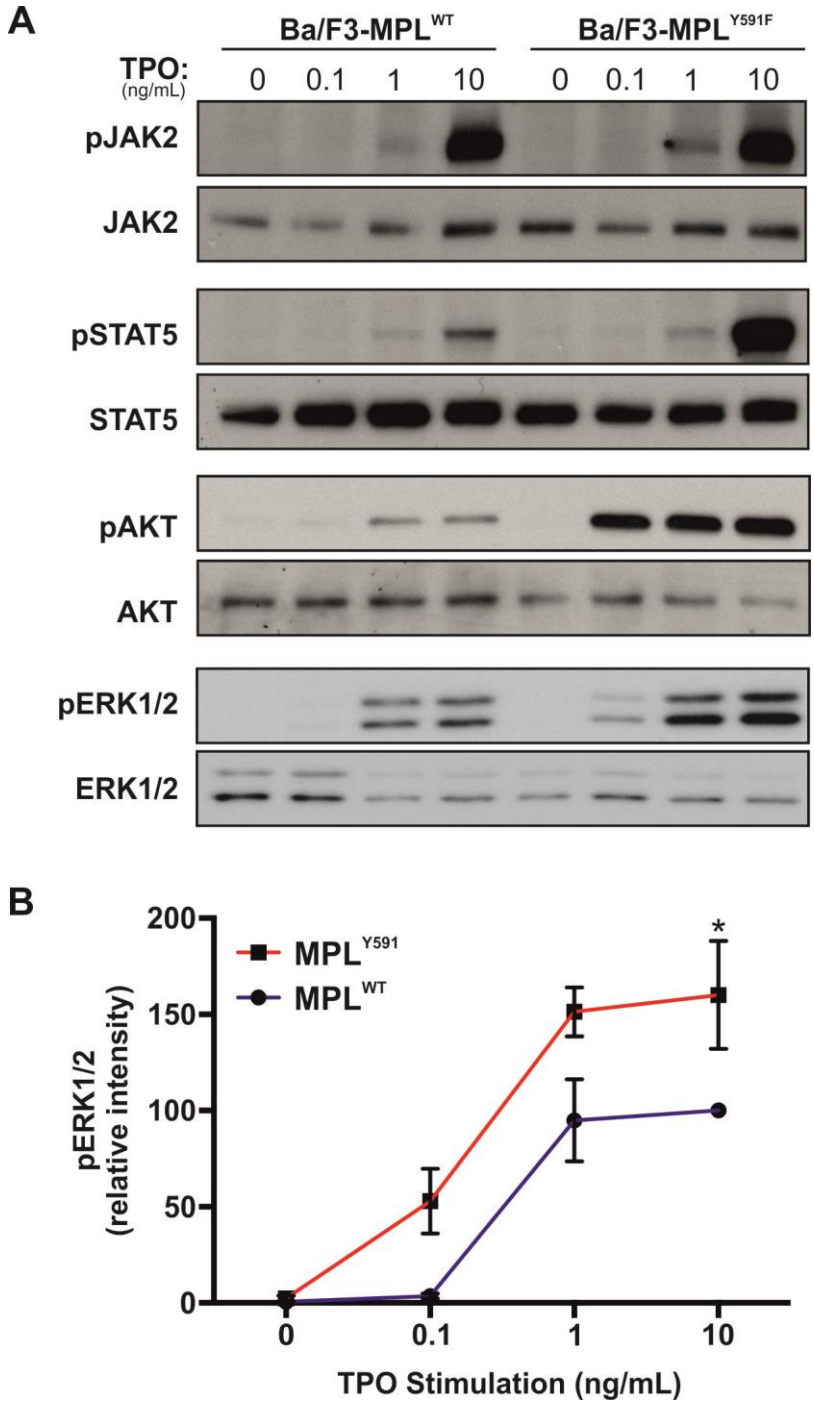


Figure 3.8 MPL^{Y591F} TPO Signalling Dose Response
(A)Western blot analysis of phosphorylation levels of JAK2, STAT5, AKT and ERK1/2 in Ba/F3-MPL^{WT}and MPL^{Y591F} cells stimulated with 0, 0.1, 1 and 10 ng/mL rhTPO for 5 min. (B) Densitometry of phospho-ERK1/2 blot. Error bars represent ± SEM. (n = 3) Densitometry was performed using ImageJ (National Institutes of Health, Bethesda, MD).

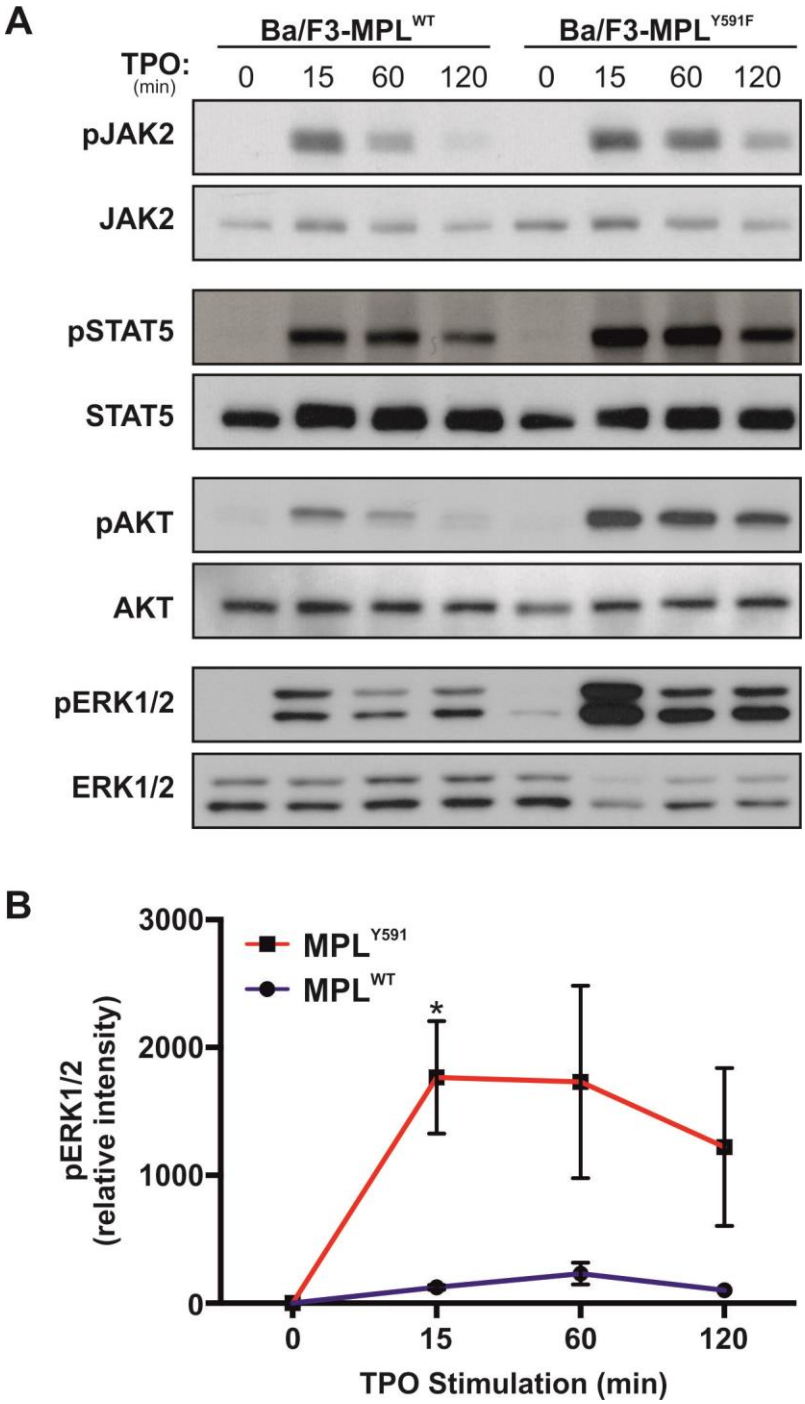


Figure 3.9 MPL^{Y591F} TPO Signalling Time Course
(A)Western blot analysis of phosphorylation levels of JAK2, STAT5, AKT and ERK1/2 in Ba/F3-MPL^{WT}and MPL^{Y591F} cells stimulated with 10 ng/mL rhTPO for 0, 15, 60, or 120 min. (B) Densitometry of phospho-ERK1/2 blot. Error bars represent ± SEM. (n = 3). Densitometry was performed using ImageJ (National Institutes of Health, Bethesda, MD).

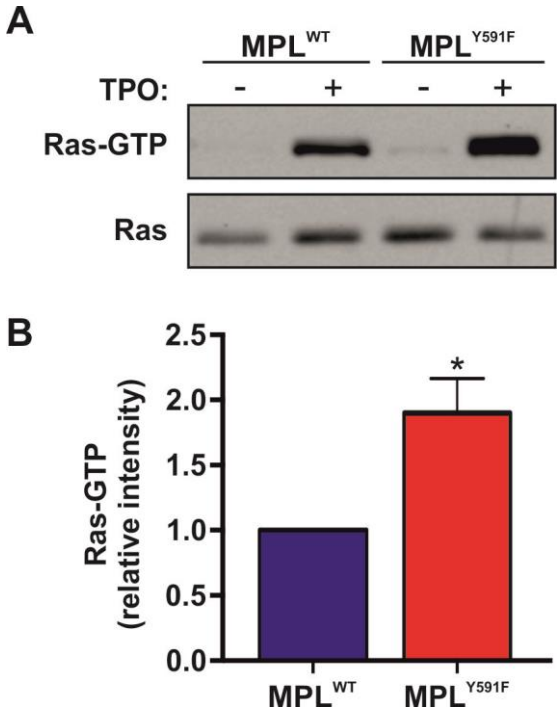


Figure 3.10 Activated Ras in Ba/F3 MPL^{Y591F} cells
(A) Detection of active, GTP-bound Ras in Ba/F3-MPL^{WT} and MPL^{Y591F} cells by immunoprecipitation and western blot. (B) Densitometry of GTP-bound Ras western blot. Error bars represent ±SEM. (n = 3). Densitometry was performed using ImageJ (National Institutes of Health, Bethesda, MD).

3.3.3 SHP-1 and SYK associate with phosphorylated MPL^{Y591} in vitro

An SH2/phosphotyrosine-binding(PTB) domain microarray performed in collaboration with Dr. Alexis Kaushansky (Harvard University) was used to identify potential binding partners for pY_{591}. A 5,(6)-TAMRA labeled pY_{591} sequence peptide was diluted to 8 varying concentrations (5μM, 3μM, 2μM, 1μM, 500nM, 200nM, 100nM and 10nM) and used to probe a protein array comprised of 136 SH2 or PTB domains (Table 3.1). Data was analysed using GraphPad Prism Software to determine specific binding. Peptides bound at <2μM, with $R^2 \geq 0.89$ were considered viable targets. The phospho-MPL^{Y591} peptide bound with high affinity in a manner consistent with saturation binding to domains from B lymphocyte kinase (BLK), Bruton agammaglobulinemia tyrosine kinase (BTK), Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog (FGR), interleukin-2-inducible T-cell kinase (ITK), SH2 domain-containing protein 1A (SH2D1A), SHP-1 (gene: *PTPN6*), spleen tyrosine kinase (SYK) and guanine nucleotide exchange factor VAV2 (VAV2) (Figure 3.11). mRNA expression of targets was confirmed in bone marrow, Ba/F3, CD41$^+$ cells and megakaryocytes by reverse transcriptase PCR as described in Section 2.4. Primers are listed in Table 3.2. FGR, ITK, SH2D1A, BTK, PTPN6 and SYK were all expressed in bone marrow cells (Figure 3.12A). To narrow down potential targets, expression of these genes was subsequently checked in Ba/F3 cells as our initial *in vitro* findings were performed in these cells, along with CD41$^+$ bone marrow cells and megakaryocytes. Only BTK, PTPN6 and SYK were expressed in these cell types (Figure 3.12B). Peptide pulldowns performed using synthesized MPL^{Y591} and phospho-MPL^{Y591} peptides were used to validate potential targets identified in the SH2/PTB domain microarray. As the peptides are based on human MPL, lysates used for pulldowns are from a human non-lymphoid leukaemia cell line, F-36P induced to ectopically express human MPL. A TPO stimulation time course was performed to determine the time at which MPL is maximally phosphorylated (Figure 3.13). Although maximal phosphorylation of MPL was achieved at 30 minutes post-TPO stimulation, lysates used for peptide pulldowns were stimulated for only 5 minutes to identify partners involved in receptor phosphorylation. MPL^{Y591} peptides were incubated with lysate in the absence of TPO stimulation and phospho-MPL^{Y591} peptide was incubated with lysate stimulated with 1ng/mL rhTPO for 5 min. Data shows that SHP-1 preferentially binds to

phosphorylated MPL^{Y591}, SYK binds to only phosphorylated MPL^{Y591} and BTK binds equally to phosphorylated and unphosphorylated MPL^{Y591} (Figure 3.14). The clathrin-mediated endocytosis adaptor protein, AP2, has been shown previously to interact with YXXØ motifs, where Y represents a tyrosine, X represents any amino acid and Ø represents an amino acid with a bulky hydrophobic side chain(269). There are 2 of these motifs found in MPL, Y591RRL being one of them, thus AP2 was used as a control for these pulldowns (Figure 3.14). Given these results, we continued to study both SHP-1 and SYK and their role in MPL^{Y591} regulation of TPO signalling.

Table 3.1 SH2/PTB Domain Binding Array Genes

ABL1 (c-abl oncogene 1, non-receptor tyrosine kinase)	**IRS4** (insulin receptor substrate 4)
ABL2 (c-abl oncogene 2, non-receptor tyrosine kinase)	**ITK** (interleukin-2-inducible T-cell kinase)
ANKS1 (ankyrin repeat and SAM domain containing 1)	**JAK2** (Janus kinase 2)
APBA1 (Amyloid beta A4 precursor protein-binding family A member 1)	**JAK3** (Janus kinase 3)
APBA3 (Amyloid beta A4 precursor protein-binding family A member 3)	**LCK** (lymphocyte-specific protein tyrosine kinase)
APBB1 (Amyloid beta A4 precursor protein-binding family B member 1)	**LCP2** (Lymphocyte cytosolic protein 2)
APBB2 (Amyloid beta A4 precursor protein-binding family B member 2)	**LNK** (CG17367 gene product from transcript CG17367-RC)
APBB3 (Amyloid beta A4 precursor protein-binding family B member 3)	**LYN** (Tyrosine-protein kinase Lyn)
APPL (adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1)	**MATK** (Megakaryocyte-associated tyrosine-protein kinase)
APS (CG6391 gene product from transcript CG6391-RC)	**NCK1** (non-catalytic region of tyrosine kinase adaptor protein 1)
BCAR3 (breast cancer anti-estrogen resistance 3)	**NCK2** (Cytoplasmic protein NCK2)
BLK (B lymphocyte kinase)	**NUMB** (numb homolog (Drosophila))
BLNK (B-cell linker)	**NUMBL** (numb homolog (Drosophila)-like)
BMX (BMX non-receptor tyrosine kinase)	**PIK3R1** (phosphoinositide-3-kinase, regulatory subunit 1)
BRDG1 (Signal transducing adaptor family member 1)	**PIK3R2** (phosphoinositide-3-kinase, regulatory subunit 2 (beta))
BTK (Bruton agammaglobulinemia tyrosine kinase)	**PIK3R3** (phosphoinositide-3-kinase, regulatory subunit 3 (gamma))
CBL (Cbl proto-oncogene, E3 ubiquitin protein ligase)	**PLCG1** (Phospholipase C, gamma 1)
CCM2 (cyclin-dependent kinase inhibitor 2A)	**PLCG2** (phospholipase C, gamma 2 (phosphatidylinositol-specific))
CHN2 (chimerin 2)	**PTK6** (protein tyrosine kinase 6)
CRK (Proto-oncogene c-Crk)	**PTPN11** (Tyrosine-protein phosphatase non-receptor type 11)
CRKL (v-crk avian sarcoma virus CT10 oncogene homolog-like)	**PTPN6** (protein tyrosine phosphatase, non-receptor type 6)
CTEN (tensin 4)	**RASA1** (RAS p21 protein activator (GTPase activating protein) 1)
DAB1 (Dab, reelin signal transducer, homolog 1 (Drosophila))	**RIN1** (Ras and Rab interactor 1)
DAB2 (Dab, mitogen-responsive phosphoprotein, homolog 2 (Drosophila))	**SH2B** (SH2B adaptor protein 1)
DAPP1 (Dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide)	**SH2D1A** (SH2 domain-containing protein 1A)
DOK1 (downstream of tyrosine kinase 1)	**SH2D2A** (SH2 domain containing 2A)
DOK2 (docking protein 2, 56kDa)	**SH2D3A** (SH2 domain containing 3A)
DOK4 (docking protein 4)	**SH2D3C** (SH2 domain containing 3C)
DOK5 (docking protein 5)	**SH3BP2** (SH3-domain binding protein 2)
DOK5L (docking protein 6)	**SHB** (Src homology 2 domain containing adaptor protein B)
E105251	**SHC1** (SHC (Src homology 2 domain containing) transforming protein 1)
E109111	**SHC3** (SHC (Src homology 2 domain containing) transforming protein 3)
E129946	**SLA2** (Src-like-adaptor 2)
E138606	**SRC** (Proto-oncogene tyrosine-protein kinase Src)
E169291	**STAT1** (Signal Transducers and Activators of Transcription 1)
E18941	**STAT2** (Signal Transducers and Activators of Transcription 2)
EAT2 (SH2 domain containing 1B)	**STAT3** (Signal Transducers and Activators of Transcription 3)
EB1 (cytochrome c oxidase subunit VIIa polypeptide 2 like)	**STAT4** (Signal Transducers and Activators of Transcription 4)
EPS8L2 (EPS8-like 2)	**STAT5** (Signal Transducers and Activators of Transcription 5)
FER (fer (fps/fes related) tyrosine kinase)	**STAT6** (Signal Transducers and Activators of Transcription 6)
FES (feline sarcoma oncogene)	**SYK** (Spleen tyrosine kinase)
FGR (Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog)	**TEC** (Tyrosine-protein kinase Tec)
FRS3 (fibroblast growth factor receptor substrate 3)	**TENC1** (tensin like C1 domain-containing phosphatase)
GRAP2 (GRB2-related adapter protein 2)	**TENS1** (Tensin 3)
GRB10 (growth factor receptor-bound protein 10)	**TNS** (Tensin 1)
GRB14 (growth factor receptor-bound protein 14)	**TRX** (trithorax)
GRB2 (Growth factor receptor-bound protein 2)	**TXK** (TXK tyrosine kinase)
GRB7 (growth factor receptor-bound protein 7)	**VAV1** (Proto-oncogene vav)
GULP1 (GULP, engulfment adaptor PTB domain containing 1)	**VAV2** (Guanine nucleotide exchange factor VAV2)
HCK (hemopoietic cell kinase)	**VAV3** (Guanine nucleotide exchange factor VAV3)
HSH2D (hematopoietic SH2 domain containing)	**YES1** (Proto-oncogene tyrosine-protein kinase Yes)
INPPL1 (inositol polyphosphate phosphatase-like 1)	**Zap-70** (Zeta-chain-associated protein kinase 70)
IRS1 (insulin receptor substrate 1)	

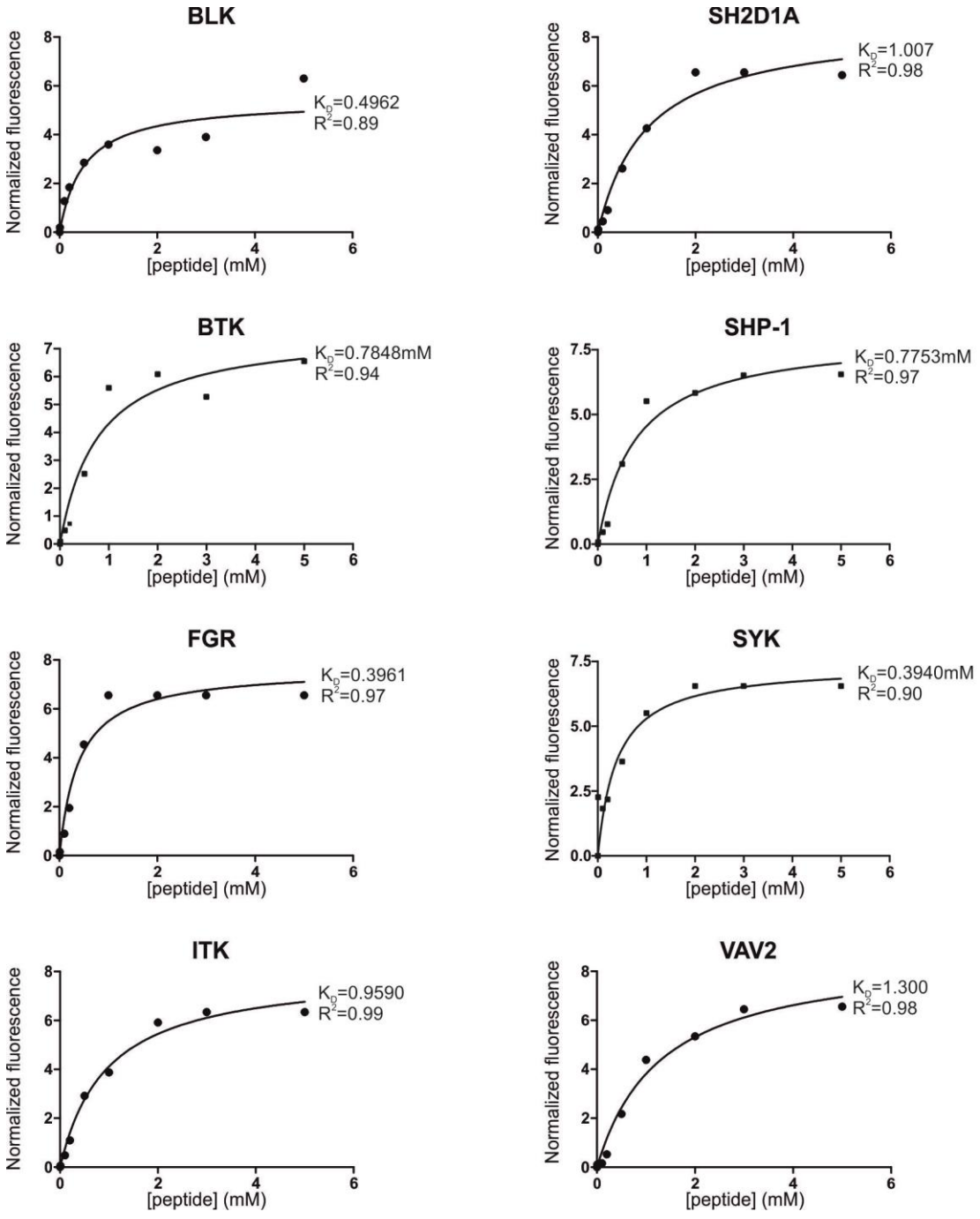


Figure 3.11 Saturation binding curves for MPL^{pY591} SH2 domain microarray targets

Saturation binding curves were generated from data gathered from an SH2 domain microarray probed with 8 varying concentrations of 5,6-TAMERA labeled MPL^{pY591} peptide. Graphs, equilibrium constants (K_D) and R^2 were generated using GraphPad Prism 4 software. Plots show normalized fluorescence as a function of peptide concentration

Table 3.2 Primers to validate expression of SH2 domain array targets

Gene	Sequence	Tm (°C)	Size (bp)	Accession
Blk	F-5'-AAGATCCGCACCCAGGACAA-3' R-5'-TTGTTCATCGGAGCCAGCAA-3'	65.12 65.08	353	NM_007549.2
Btk	F-5'-ATGGCGTCTGCACCAAACAA-3' R-5'-CCCCAAAAGCCCAGATGTCA-3'	64.87 65.43	372	NM_013482.2
Fgr	F-5'-TGCAGCACAAAGGTGGCAGT-3' R-5'-AGGATGTTGGCTGCCCTCAA-3'	65.24 65.29	317	NM_010208.4
Itk	F-5'-TAAAAGCATCAGCCGCGACA-3' R-5'-TGCCAATCTCCTGCACGAAC-3'	65.04 64.63	386	NM_001281965.1
Ptpn6	F-5'-TGCCCAGTTCATCGAAACGA-3' R-5'-TGCAGGGATCAAGGCTGATG-3'	64.96 64.61	301	NM_013545.3
Sh2d1a	F-5'-GGGCCCATCAAAGAGTTTGC-3' R-5'-ATGCCTTGATCCGGCTTCTG-3'	64.03 64.74	306	NM_011364.3
Syk	F-5'-TCCCCTTATGAGCCCCGTTT-3' R-5'-AGTGCCGTGAATGGGTGACA-3'	64.92 64.92	377	NM_011518.2
Vav2	F-5'-CGAGCCATCGATGTGTCCAT-3' R-5'-TGGCTTCCAGGGCTTCTTTG-3'	64.39 64.82	331	NM_009500.1

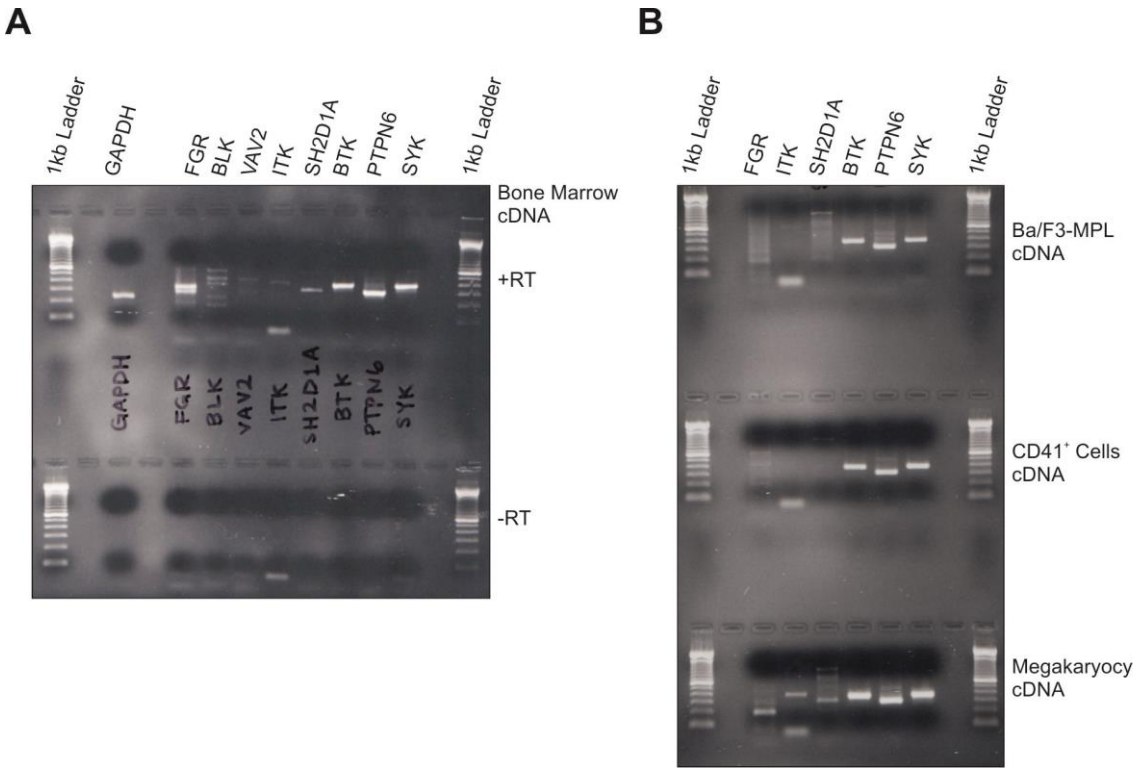


Figure 3.12 SH2 domain array target expression verification
(A) C57Bl/6 bone marrow was harvested, lysed, mRNA was isolated and cDNA was made using Invitrogen Superscript II. PCR was performed on cDNA, made with and without reverse transcriptase, using primers specific for the listed gene. (B) Ba/F3-MPL cells and $CD41^+$ cells and megakaryocytes isolated from cultured C57Bl/6 bone marrow cells were lysed, mRNA was isolated and cDNA was made. PCR was performed on cDNA using primers specific for genes that yielded PCR product in bone marrow cDNA.

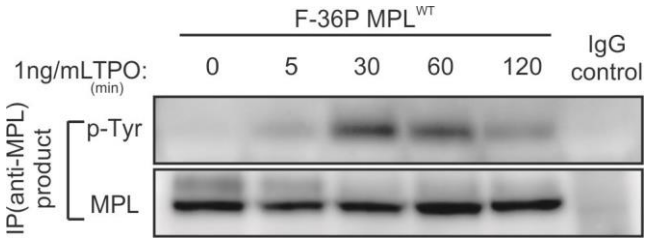


Figure 3.13 F36-P MPLWT receptor phosphorylation TPO time course
F36-P cells stably expressing human WT was stimulated with rhTPO, lysed and subjected to immunoprecipitation with an anti- MPL antibody. Immunoprecipitates were analysed via western blot using a phospho-tyrosine probe to compare total receptor phosphorylation of the mutant to the wild-type receptor in response to TPO stimulation; blots were stripped and re-probed with an anti--MPL antibody to assess the level of immunoprecipitated protein.

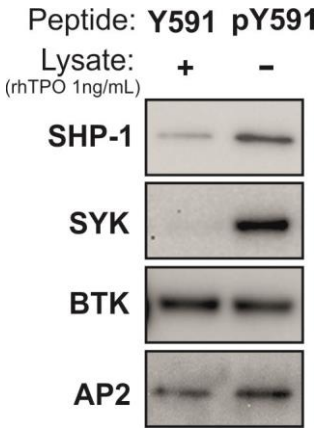


Figure 3.14 MPL^{Y591} peptide pulldowns
Biotinylated peptides corresponding to the amino acid sequence surrounding Y591 were synthesized (LCSSQAQMD(Y591/pY591)RRLQPSCLGT), conjugated to NeutrAvidin agarose resin and incubated with F-36P-MPL^{WT} whole cell lysate from cells treated with or without rhTPO. Proteins interacting with peptides were eluted and separated on SDS-PAGE and analysed via western blot. SHP-1, SYK and BTK antibodies were used to confirm potential partners and AP2 was used as a control.

3.3.4 SYK binds phosphorylated MPL^{Y591} to negatively regulate TPO-mediated ERK1/2 signalling

We sought to determine if SHP-1 and SYK were responsible for the increased ERK1/2 and Akt signalling observed in Ba/F3-MPL^{Y591F} cells. To do so, we used siRNAs to either *Ptpn6* (the gene encoding SHP-1) or *Syk* to reduce SHP-1 and SYK protein expression, respectively. In MPL^{WT} cells, reduction of SHP-1 protein expression resulted in no significant change in TPO induced ERK1/2 (Figure 3.15) or Akt phosphorylation (Figure 3.16), relative to treatment with control siRNA. Conversely, reducing SYK protein expression in MPL^{WT} cells resulted in significantly increased ERK1/2 phosphorylation with TPO stimulation compared to the control (Figure 3.17A-B). SYK siRNA treatment of MPL^{WT} cells was unable to induce ERK1/2 phosphorylation to the same extent as observed in MPL^{Y591F} cells; however, this is likely the result of incomplete knockdown of SYK (Figure 3.17A). We observed no difference in Akt phosphorylation as a result of SYK knockdown (Figure 3.18). To further understand the role of SYK in TPO-mediated ERK1/2 signalling, we assessed the phosphorylation state SYK including SYK^{Y317}, SYK^{Y346} and SYK$^{Y519/520}$ (SYK^{Y323}, SYK^{Y352} and SYK$^{Y525/526}$ in human SYK, respectively). Phosphorylation of SYK Y_{317}, a negative regulatory residue(270), was unaltered between MPL^{WT} and MPL^{Y591F} in response to TPO (Figure 3.19). However, baseline phosphorylation of SYK^{Y346} was higher in MPL^{WT} compared to MPL^{Y591F} (Figure 3.19). We were unable to detect phosphorylation of SYK$^{Y519/520}$. We found no difference in the phosphorylation state of SHP-1 (Figure 3.19).

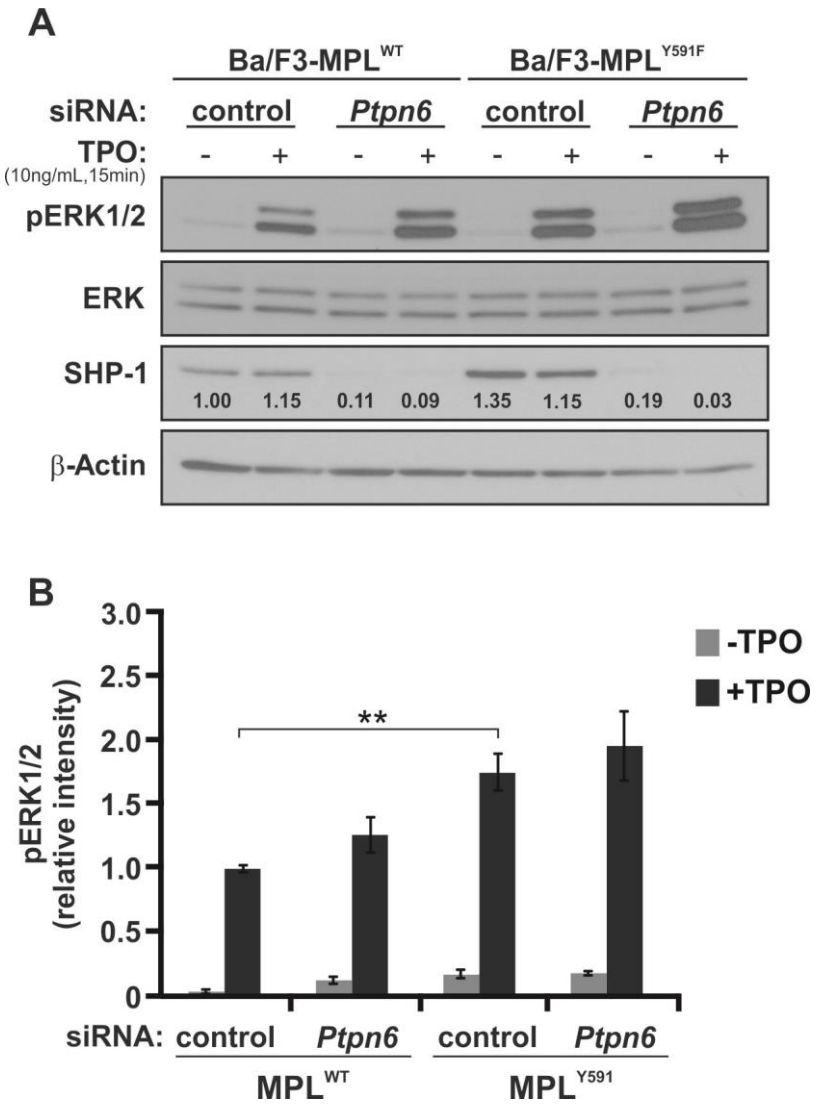


Figure 3.15 ERK1/2 phosphorylation following siRNA knockdown of *Ptpn6*
(A) Western blot analysis of phosphorylation of ERK1/2 in *Ptpn6* siRNA treated Ba/F3-MPL^{WT} and MPL^{Y591F} cells stimulated with 10ng/mL rhTPO for 15min. Knockdown efficiency was analysed by immunobloting for SHP-1, numbers represent densitometric quantification of protein levels. (B) Densitometric quantification of phospho-ERK1/2 blot using ImageJ software (**P < 0.01). Error bars represent ±SEM. (n=3).

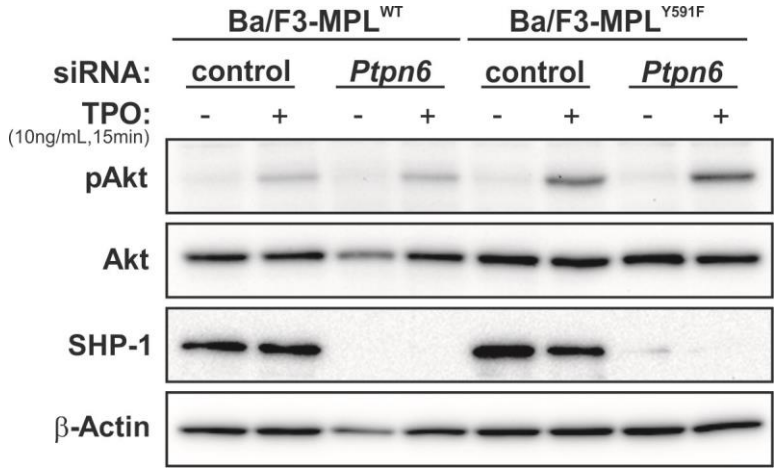


Figure 3.16 Akt phosphorylation following siRNA knockdown of *Ptpn6*
(A) Western blot analysis of phosphorylation of Akt in *Ptpn6* siRNA treated Ba/F3-MPL^{WT} and MPL^{Y591F} cells stimulated with 10ng/mL rhTPO for 15min.

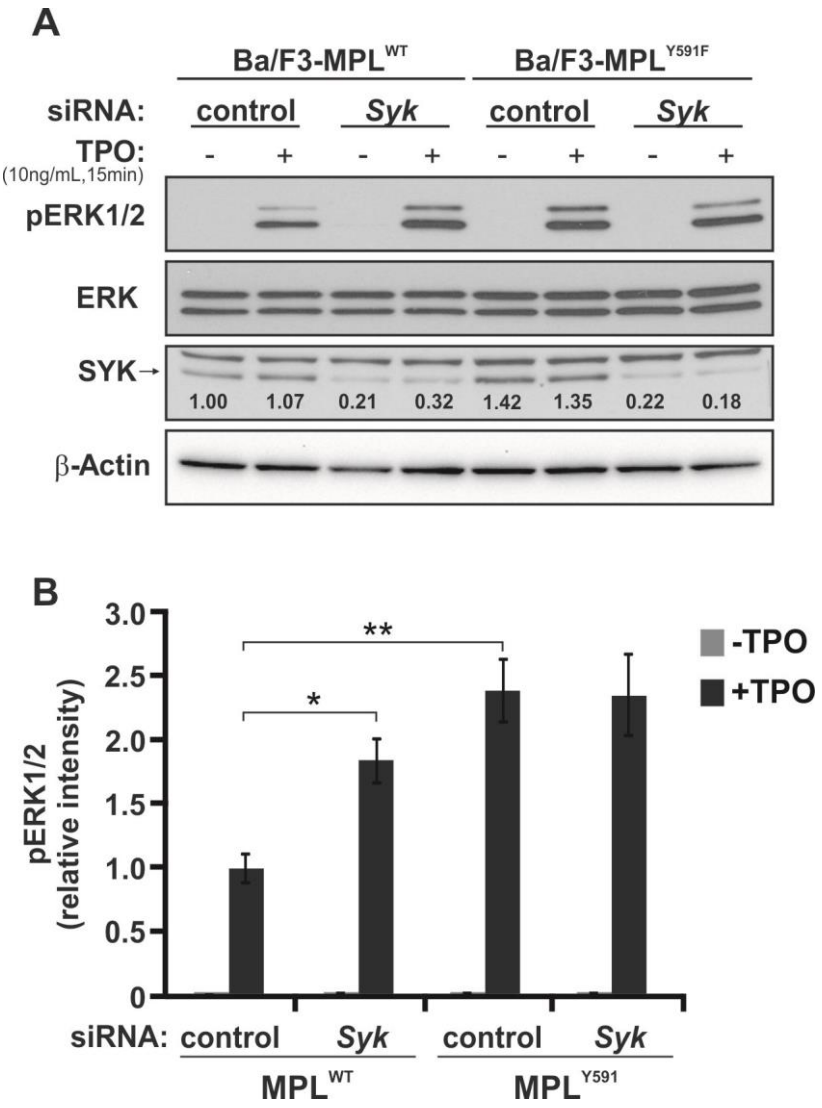


Figure 3.17 ERK1/2 phosphorylation following siRNA knockdown of *Syk*
(A) Western blot analysis of phosphorylation of ERK1/2 in *Syk* siRNA treated Ba/F3-MPL^{WT} and MPL^{Y591F} cells stimulated with 10ng/mL rhTPO for 15min. Knockdown efficiency was analysed by immunobloting for SYK, numbers represent densitometric quantification of protein levels. (B) Densitometric quantification of phospho-ERK1/2 blot using ImageJ software. Error bars represent ±SEM. (n=3).

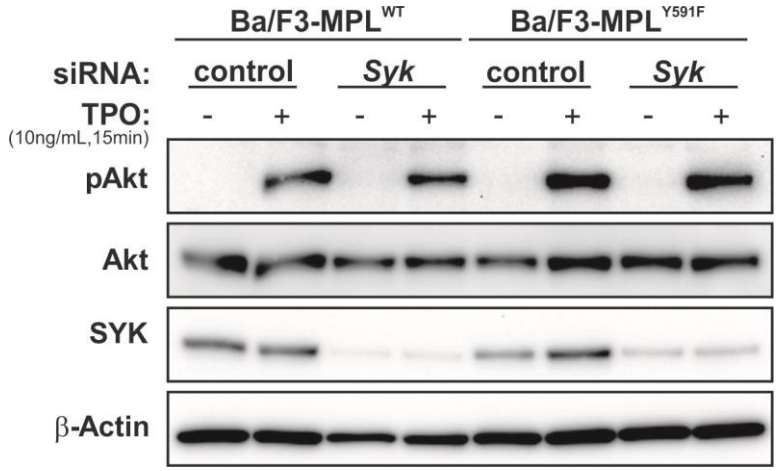


Figure 3.18 Akt phosphorylation following siRNA knockdown of *Syk*
(A) Western blot analysis of phosphorylation of Akt in *Syk* siRNA treated Ba/F3-MPL^{WT} and MPL^{Y591F} cells stimulated with 10ng/mL rhTPO for 15min.

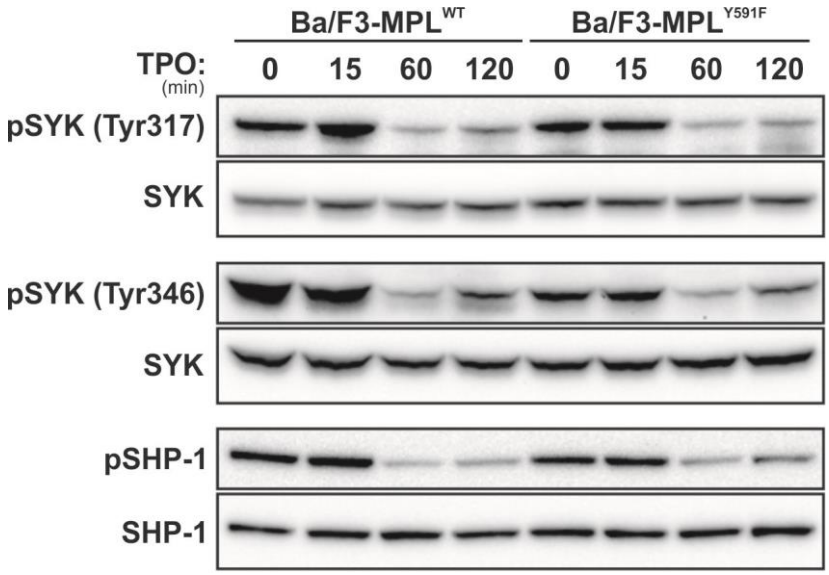


Figure 3.19 Phosphorylation of SYK and SHP-1
Western blot analysis of phosphorylation of SYK and SHP-1 from Ba/F3-MPL^{WT} and MPL^{Y591F} cells stimulated with 10ng/mL rhTPO for the indicated times.

3.4 Discussion

MPL is critical for the maintenance of haematopoietic stem cells, in addition to being the key regulator of megakaryopoiesis(62,103,271). Therefore, its regulation is essential to prevent uncontrolled activation of the receptor, which can result in the development of MPNs(124,200). In this chapter, we demonstrate for the first time that Y591 in wild-type MPL is phosphorylated in response to TPO and that removal of Y591 phosphorylation results in decreased total receptor phosphorylation. Y591 regulates the level of active Ras within the cell following TPO stimulation thus, its removal contributes to the higher levels of pERK1/2 observed in Y591F. Additionally, we identify SYK and SHP-1 as novel potential binding partners for pY591, and moreover, that knockdown of *Syk* mimics the Y591F phenotype, suggesting that SYK contributes to the negative regulatory effects of Y591 in wild-type cells. These findings suggest a significantly more important role than previously thought for Y591 in TPO signalling and receptor function.

In previous studies, using receptor truncations, Y591 was identified as a potential negative regulator of TPO-mediated proliferation(3). This has been further supported by our work showing that the Y591F point-mutation in MPL confers a proliferative advantage to cells in response to TPO(1). To determine whether the observed phenotype is the consequence of the removal of a phosphorylation site at Y591, within the intracellular domain of MPL, we determined the phosphorylation state of Y591 in wild-type MPL after TPO stimulation. Phosphorylation of MPL Y591 has been previously reported in the disease state associated with a W515A activating mutation of MPL(116) but here we report that MPL Y591 is also phosphorylated in response to TPO in the wild-type receptor. As such, we tested whether phosphorylation of Y591 provides a docking site for SH2 domain containing proteins to bind and act as direct downstream effectors or as a scaffold for additional effector proteins in TPO mediated signalling.

It has been known for many years that multiple residues of MPL are phosphorylated upon TPO binding. Previous studies have focused on Y625 and Y630(3); our demonstration in this study that MPL Y591 is also phosphorylated provides an additional potential point of regulation of receptor activity. Moreover, based on our

experiments using single tyrosine to phenylalanine mutations of MPL, the decrease in total receptor phosphorylation in the Y591F cells is greater than would be predicted based on loss of a single tyrosine site. This result also suggests that MPL Y625 and Y630 phosphorylation levels are, in part, dependent on the phosphorylation state of Y591 and that pY591 is required for maximal phosphorylation at Y625 and Y630. If so, these results suggest that Y591 might also serve as a docking site of a kinase responsible for phosphorylation of Y625 and Y630. To better explore this hypothesis the temporal phosphorylation profile of MPL would need to be explored.

Since TPO is the primary megakaryocyte growth factor(272), much has been discovered about the positive signals involved in its regulation. However, the mechanisms that negatively regulate its signalling are less well understood. The YRRL motif appears twice in the cytoplasmic domain of MPL and are important in negative regulation of TPO signalling. Y529RRL is required for lysosomal targeting and subsequent degradation and Y591RRL is important for MAPK pathway activation and suppression of proliferation(1). Our data suggest that Y591 can regulate MAPK pathway activation through modulation of active Ras levels within the cells. Active Ras is necessary for megakaryocyte differentiation(271), and therefore it is possible that a role of Y591 is to suppress megakaryocytic differentiation through downregulation of active Ras. The mechanism by which Y591 suppresses Ras activation is an important area for future studies as aberrant Ras signalling has been observed in a number of human cancers, including haematological malignancies(273).

Although attempts to co-immunoprecipitate MPL and SYK have been unsuccessful, which is a likely consequence of the transient nature of SH2 interactions, we found using an SH2-domain binding array followed by a peptide pulldown, that SYK may potentially binds to Y591 of MPL. SYK typically binds immunoreceptor tyrosine-based activation motifs (ITAMs), characterized by two YXXL/I sequences 6-9 amino acids apart(274). The region of MPL around Y591RRL does not contain additional YXXL/I motifs but MPL signals as a dimer; therefore, pY591RRL could serve as a hemi-ITAM for SYK binding. The role of SYK in immunoreceptor signalling has been well characterized and typically results in receptor activation and

positive signalling (reviewed in (274)). Surprisingly, however, our preliminary data shows that the elimination of this SYK binding site yields an increase in ERK1/2 signalling, suggesting that in wild-type cells, SYK is somehow downregulateting the phosphorylation of ERK1/2 in response to TPO. SYK phosphorylation decreases following TPO stimulation of both WT and Y591F MPL supporting its role as a negative regulator of TPO signalling. SYK Y346 has been linked to ERK activation in immune cells(270) and the decrease in SYK pY346 in MPL Y591F supports a link between the two proteins albeit a negative rather than the typical positive association. SYK function has been reported to interfere with epidermal growth factor (EGF)-mediated cellular responses through epidermal growth factor receptor (EGFR), thus in some cases, SYK functions as a negative regulator(275). However, the exact mechanisms by which MPL and SYK may interact and how this potential interaction affects TPO signalling requires further investigation. It is possible that there are a number of signalling intermediates between SYK and ERK including other negative regulatory proteins functioning as effectors facilitating negative regulation through Y591. However, the mechanisms through which SYK negatively regulates ERK signalling and its role in megakaryopoiesis have yet to be elucidated.

In addition to identifying SYK as a potential binding partner for pY591 and a regulator of ERK activation, peptide pulldowns identified two additional novel binding partners for Y591, SHP-1 and BTK. SHP-1 is a phosphatase that preferentially binds pY591, yet is not responsible for the increased ERK1/2 activation resulting from the Y591F mutation; however, SHP-1 could modulate other aspects of TPO signalling outside of ERK1/2 activation. SHP-1 negatively regulates signalling of type I cytokine receptors through binding of phosphorylated tyrosine residues on activated receptors and subsequent dephosphorylation of JAK2(276,277). We observed a slight increase in the level of phosphorylated JAK2 in the Y591F mutant; therefore, it is possible that in MPL, SHP-1 binding to pY591 mediates JAK2 activation. Additional experiments are required to further understand the role of Y591 in SHP-1 regulation.

In this report we show that MPL Y591 is phosphorylated in response to TPO, providing a novel docking site for SH2-domain containing proteins SHP-1 and SYK. Once bound to pY591, SYK negatively regulates ERK1/2 phosphorylation. Tight regulation of the cellular response to TPO is essential, as loss of this control seen in

clinical mutations of MPL (MPL W515L) results in development of haematological malignancies. Interestingly, a novel MPL Y591D mutation has been identified in a patient with JAK2V617F-positive polycythaemia vera(278). At the time of discovery, the function of the mutation was unknown; however, our data suggests that loss of Y591 in this patient may be facilitating the disease phenotype due to loss of a negative regulator. Thus, an understanding of the molecular details of TPO signalling is important for identification of areas for therapeutic intervention. A negative regulatory site such as Y591 and its associated effector proteins provide potential therapeutic targets for dysregulated cellular responses to thrombopoietin.

CHAPTER 4 DISSECTING THE ROLE OF MPL IN *IN VITRO* JAK2^{V617F}-POSITIVE SIGNALLING AND PROLIFERATION

4.1 Experimental Rationale

Taken together, the functional interdependence of MPL and JAK2 in maintenance of haematopoiesis and the ability of both proteins to induce MPNs, suggests that MPL may be capable of modulating the effects of *JAK2*^{V617F}. Therefore, in this chapter, we sought to determine the relationship between the phosphorylated tyrosine residues of MPL and *JAK2*^{V617F} pathogenesis *in vitro*.

4.2 Materials and Methods

4.2.1 Cloning of MPL and JAK2 containing plasmids

4.2.1.1 pMX-puro-MPL

pMX-puro-MPL was generated as described in Section 3.2.1 and mutagenesis was performed as described in Section 2.6 to generate MPL mutants. These new mutants include *MPL*^{Y625F} and *MPL*^{Y630F}. Mutagenesis primers annealing temperatures are listed in Table 2.3. For these studies, the previously generated *MPL*^{WT} and *MPL*^{Y591F} plasmids from Section 3.2.1 were also used.

4.2.1.2 pQCXIN-JAK2

pQCXIN is a retroviral expression vector Figure 4.1. It contains a CMV promotor, neomycin resistance gene as a selectable marker following an internal ribosome entry site (IRES), ampicillin resistance for bacterial selection. pQCXIN was obtained from Clontech and *JAK2*^{WT} cloned in using in-fusion cloning strategy between the BamHI and EcoRI restriction sites within the MCS (Figure 4.2)and subsequently mutagenized to *JAK2*^{V617F} using site-directed mutagenesis as described in Section 2.6 by Dr. S. Leah Etheridge to generate pQCXIN-*JAK2*^{WT} and pQCXIN-*JAK2*^{V617F} .

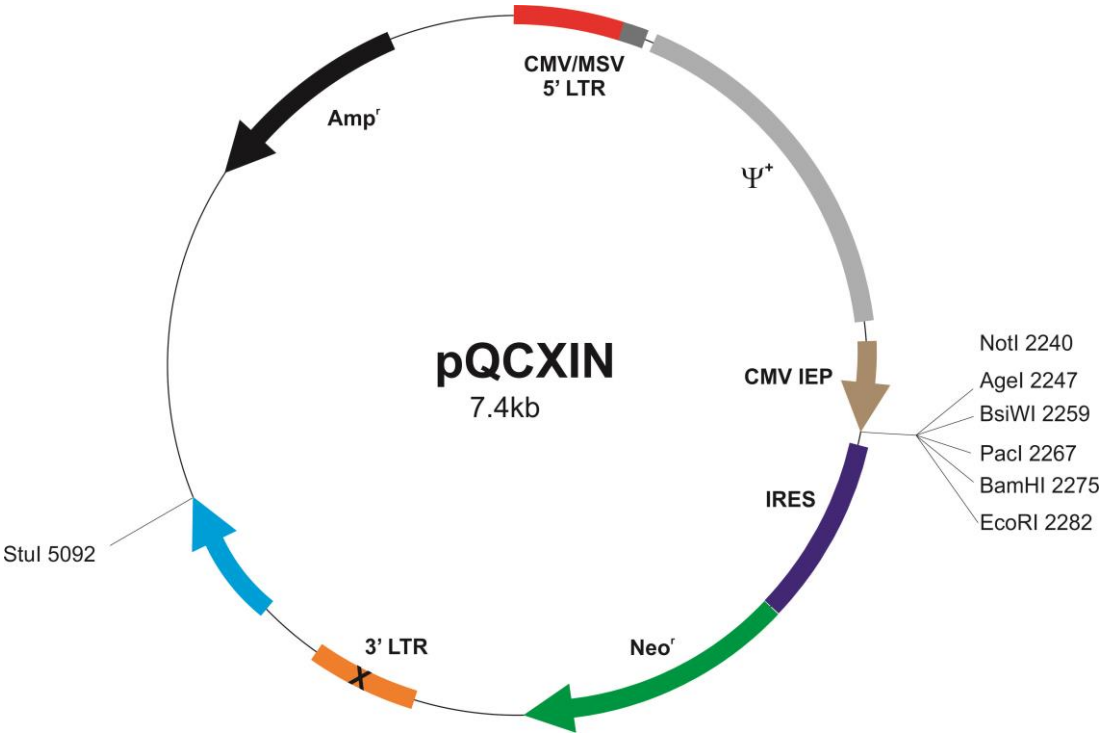


Figure 4.1 pQCXIN backbone vector
The pQCXIN vector contains an ampicillin resistance gene for selection in E.Coli and a neomycin resistance gene for selection in mammalian cells.

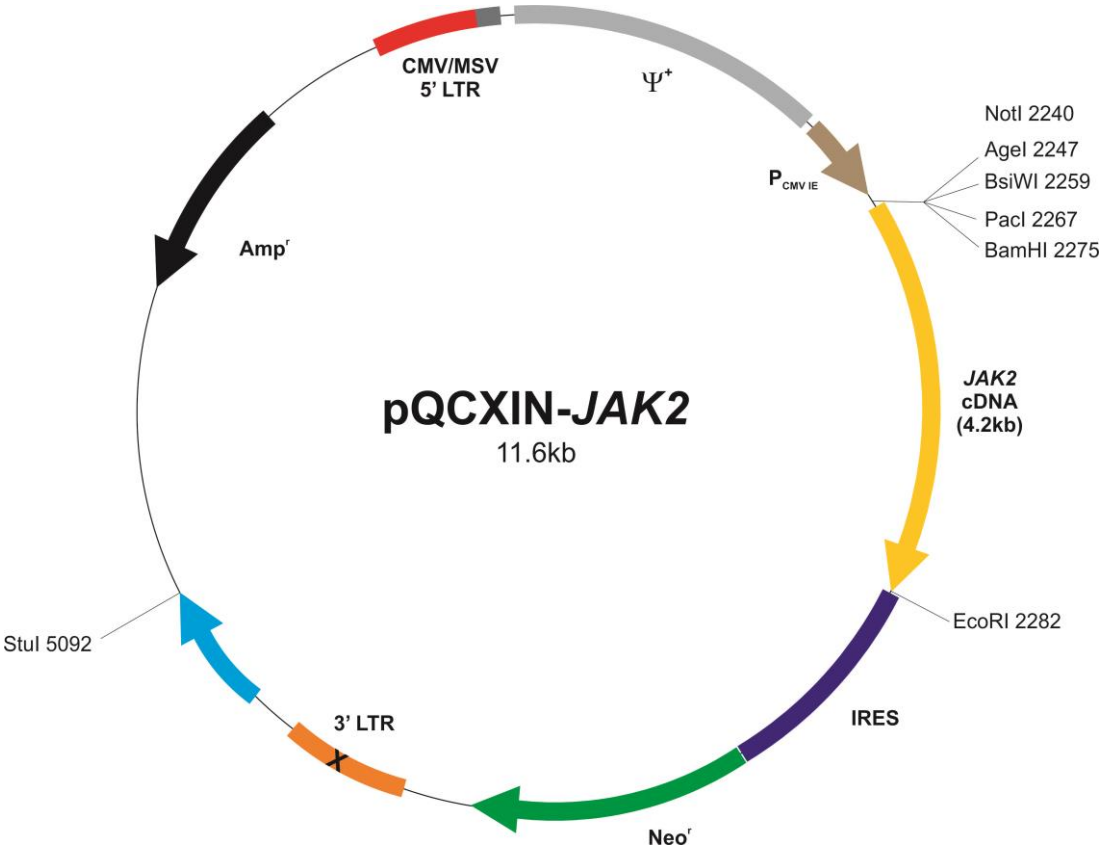


Figure 4.2 pQCXIN-*JAK2*^{WT} vector
Human JAK2 cDNA was cloned into the pQCXIN backbone vector between restriction sites for BamHI and EcoRI of the MCS.

4.2.2 XTT Cell Viability Assay

Tetrazolium salts are utilized in numerous biochemical assays and their widespread application is attributed to their ability to be reduced and form colored formazan dyes. Triphenyl tetrazolium chloride (TTC) was the first of these salts to be synthesized and since, scientists have been modifying this parent compound to produce a number of tetrazolium salts with specific properties for use in a wide range of biochemical applications (reviewed in (279)). The most common tetrazolium salt used for proliferation assays previously was 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT). However, acidified isopropanol used solubilize the MTT was dangerous and volatile leading increased health risk and increased likelihood of sample variation. Therefore, as a way to assess the necessity of MPL and *JAK2*^{V617F} for proliferation in cells, we performed 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide (XTT) proliferation assays. XTT is similar to MTT as their reduction is dependent on the rate of NAD(P)H production. However, XTT is not cell permeable and therefore, reduction occurs at the surface of the cell membrane(279). Cells were cultured in triplicate at $2.0x10^5$ cells/well of 96-well cell culture plates in the absence of 1ng/mL recombinant human TPO (rhTPO). At 24, 48 and 72 hours, XTT reagent was added to the cells and any proliferating cells would reduce the XTT compound resulting in a colorimetric change directly proportional to the number of metabolically active cells when read at 490nm. Although these assays do not directly measure the number of viable cells, studies have shown that data generated from these assays correlates with proliferation data gathered by cell counts and radioisotope assays(280).

4.3 Results

4.3.1 Generation of pMX-puro-*MPL* mutants

MPL mutants were generated by site directed mutagenesis as described in Section 4.2.1.1. Mutants were verified by sequencing using primers to full length human *MPL* (Figure 3.3, Figure 4.3 and Figure 4.4). *MPL* sequencing primers are listed in Table 2.4.

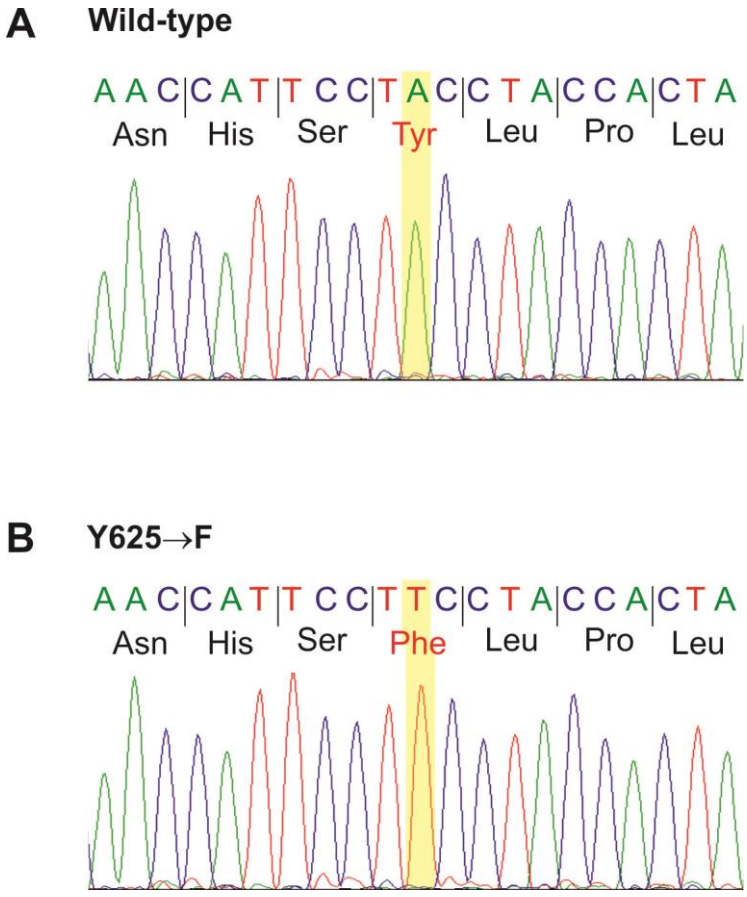


Figure 4.3 pMX-puro-MPL^{Y625F} Sequencing Results
Sequence analysis of WT and mutagenized MPL in the pMX-puro-MPL vector.

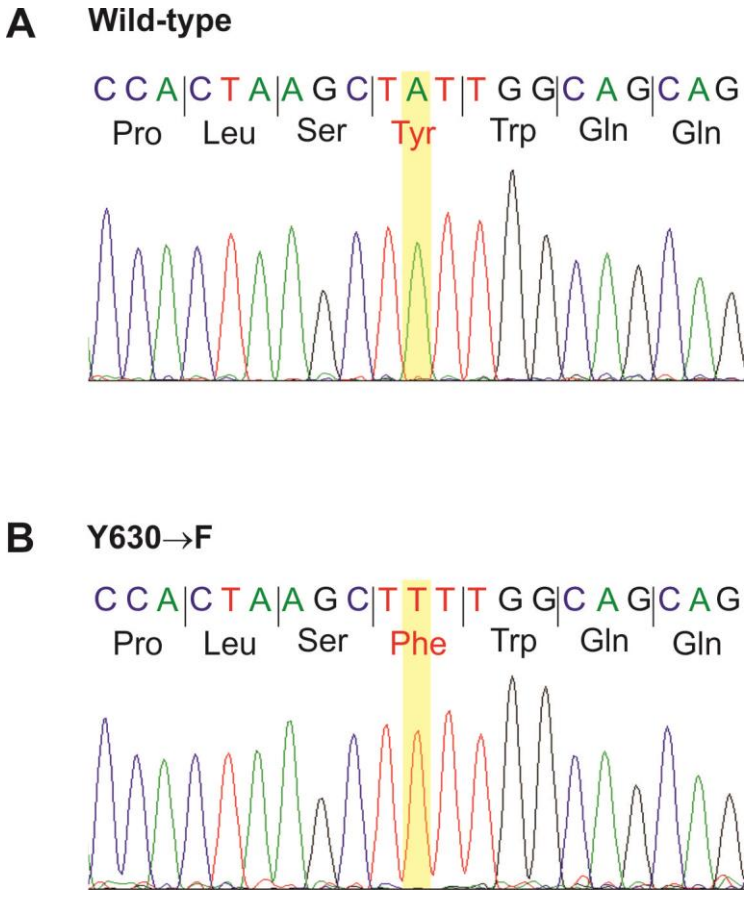


Figure 4.4 pMX-puro-MPL^{Y360F} Sequencing Results
Sequence analysis of WT and mutagenized MPL in the pMX-puro-MPL vector.

4.3.2 Total MPL and JAK2 expression in generated Ba/F3 cell lines

After cells were generated and selected for plasmid integration, cells were lysed and expression of MPL and JAK2 was determined by western blot (Figure 4.5). In lines not engineered to express MPL, there was no MPL expression detected (Figure 4.5). Total MPL expression varied between different MPL mutants with MPL^{WT} being maximally expressed. Total MPL^{Y591F} expression was approximately half that of MPL^{WT} (Figure 4.5). In both MPL^{WT} and MPL^{Y591F}, expression of $JAK2^{V617F}$ rather than $JAK2^{WT}$ did not affect total MPL expression (Figure 4.5). MPL^{Y625F} expression was absent when $JAK2^{WT}$ was expressed but rescued with $JAK2^{V617F}$ expression (Figure 4.5). The opposite was true for MPL^{Y630F} expression whereby expression was higher when co-expressed with $JAK2^{WT}$ rather than $JAK2^{V617F}$ (Figure 4.5). Overall, expression of JAK2 was unaltered between cells expressing $JAK2^{WT}$ or $JAK2^{V617F}$ (Figure 4.5). β-actin was used as a loading control (Figure 4.5).

4.3.3 Cell surface expression of MPL in generated Ba/F3 cell lines

MPL cell surface expression was assayed by flow cytometry. All cell lines express MPL at varying levels on their cell surface (Figure 4.6). However, MPL^{Y625F} exhibited decreased cell surface localization relative to $MPL^{WT}JAK2^{WT}$ (Figure 4.6). $MPL^{Y630F}JAK2^{WT}$ cells exhibited a bimodal distribution of MPL cell surface expression despite culture under puromycin selection (Figure 4.6).

4.3.4 Cell signalling in MPL/JAK2 Ba/F3 cell lines

Cell signalling was assessed in the MPL/JAK2 Ba/F3 cell lines. Cells were starved in RPMI 1640 supplemented with 2% FBS in the absence of IL-3 for 16 hours followed by stimulation with or without 10ng/mL rhTPO for 5min. Whole cell lysates were harvested and western blot analysis was performed as described in 2.5. Western blots are presented in Figure 4.7 and values determined by densitometry are presented in Figure 4.8. In the absence of MPL expression, JAK2 is not significantly phosphorylated. Co-expression of MPL^{WT}, MPL^{Y591F} and MPL^{Y630F} with $JAK2^{WT}$ results in TPO induced phosphorylation of JAK2. However, co-expression of MPL^{Y625F} and $JAK2^{WT}$ does not result increased JAK2 phosphorylation following TPO stimulation. When MPL^{WT} and $JAK2^{V617F}$ are co-expressed, JAK2 phosphorylation was present despite absence of cytokine. This is consistent with

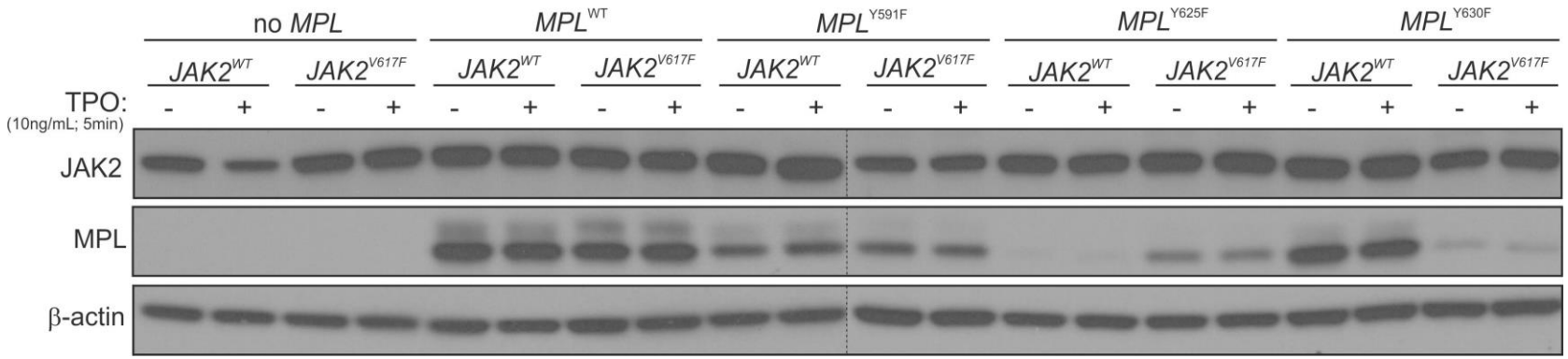


Figure 4.5 MPL and JAK2 protein expression in MPL Tyr to Phe mutants

Western blot analysis of MPL and JAK2 from stably transfected Ba/F3 cells stimulated with or without 10ng/mL rhTPO for 5 minutes.

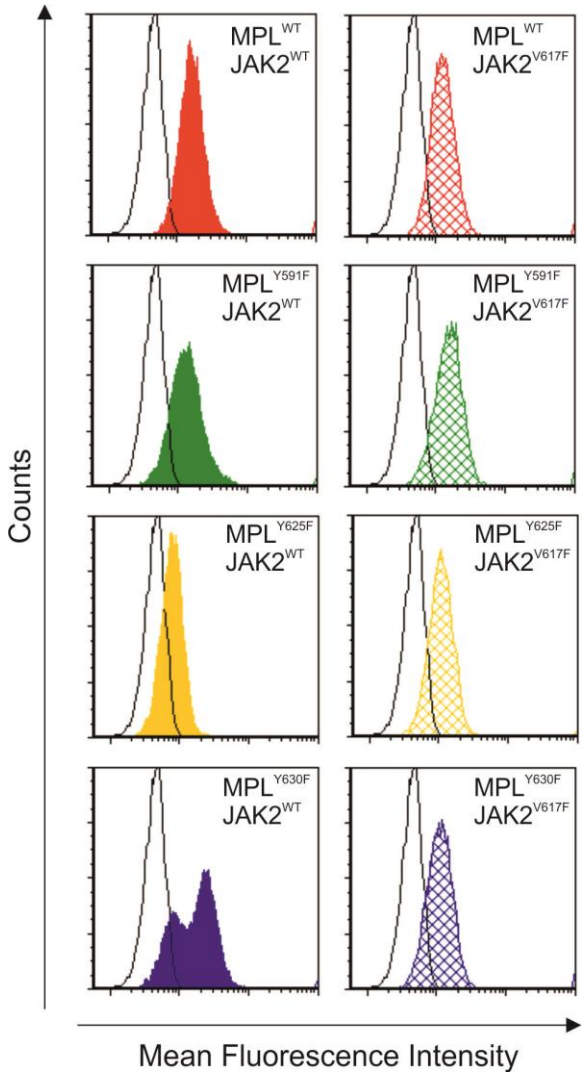


Figure 4.6 MPL cell surface expression

Flow cytometry analysis of MPL cell surface expression in BaF/3 MPL and JAK2 mutants. Pools of BaF/3 cells expressing various human MPL and JAK2 constructs were stained with an antibody against the extracellular domain of MPL (Amgen) followed by secondary staining with an anti-mouse Qdot 565 antibody. The open peak corresponds to cells stained with anti-mouse Qdot 565 secondary alone.

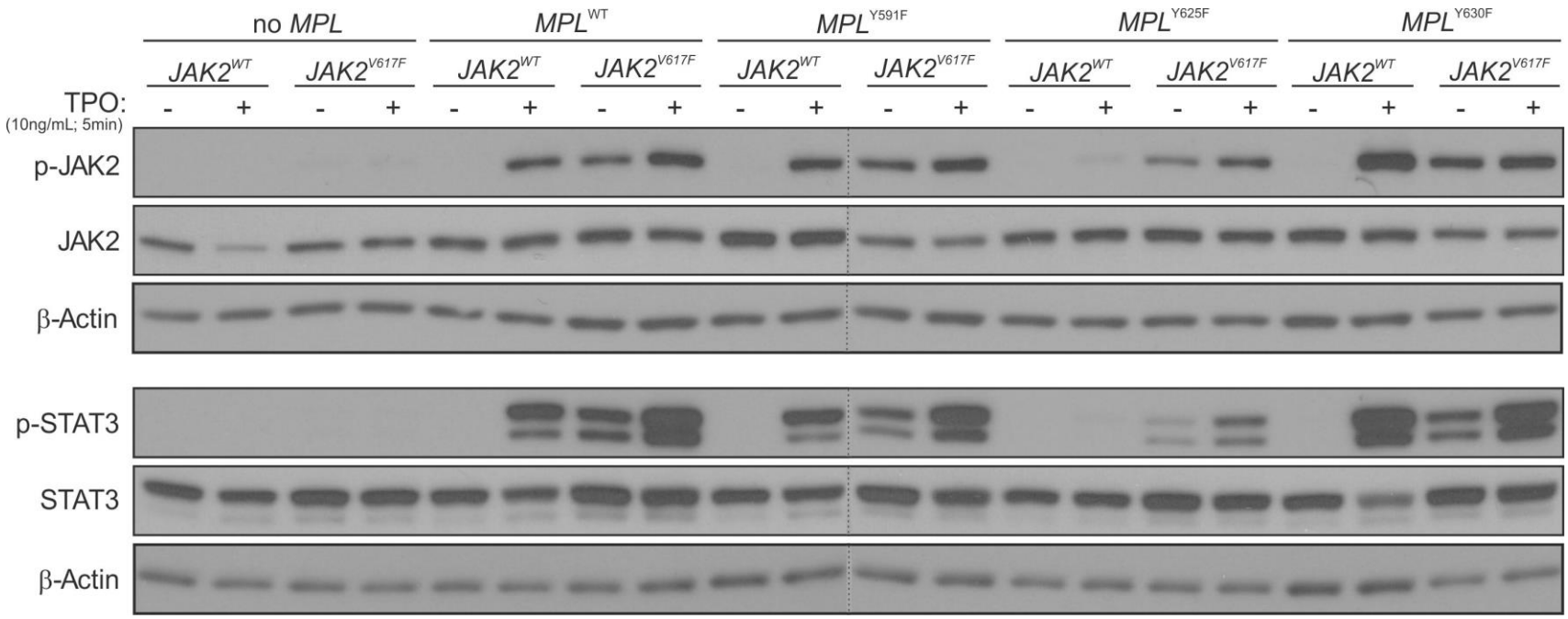


Figure 4.7 JAK/STAT Signaling in MPL and JAK mutant cell lines

Western blot analysis of phosphorylated JAK2 and STAT3 from MPL an JAK2 expressing Ba/F3 cells stimulated with 10ng/mL rhTPO for 5min. Total JAK2 or STAT3 and β-actin were used as loading controls.

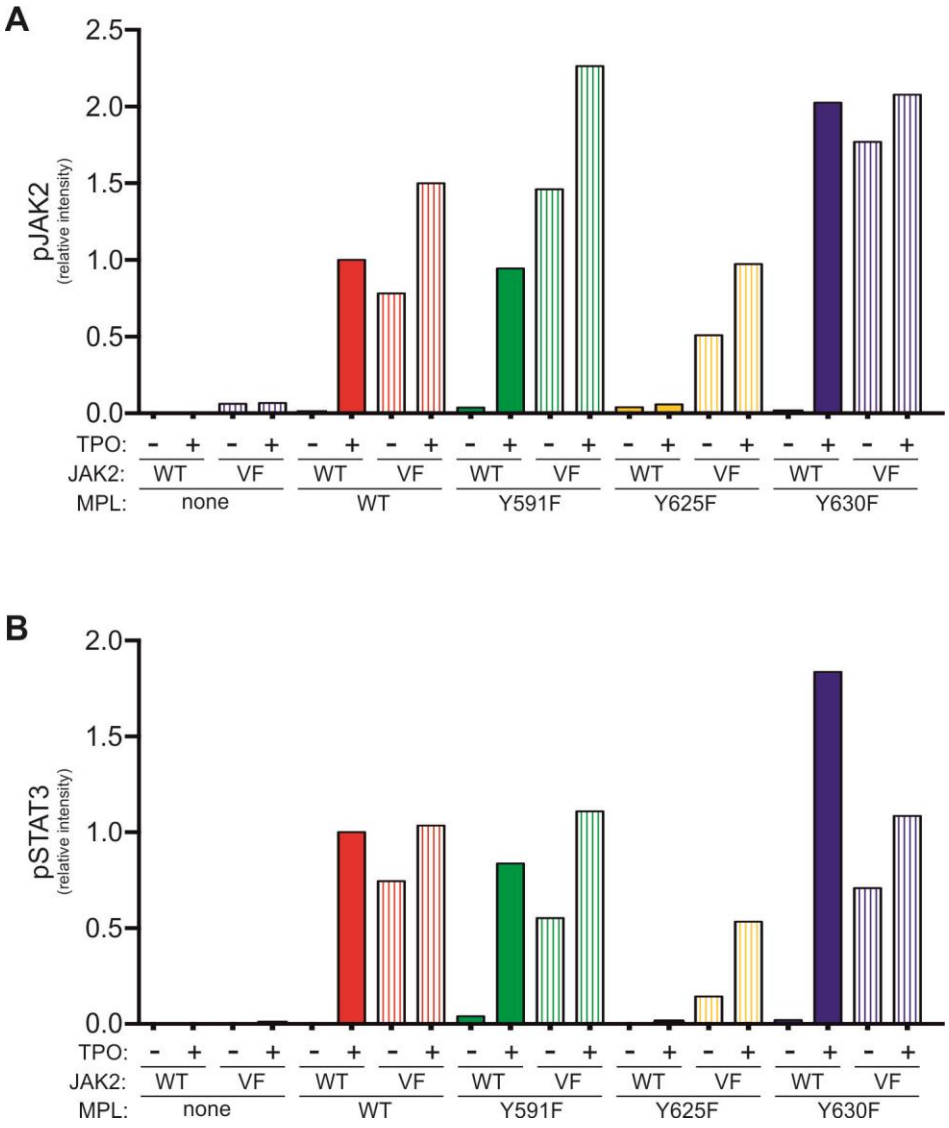


Figure 4.8 Densitometric quantification of pJAK2 and pSTAT3
Quantification of western blot from Figure 4.7. Values for phosphorylated protein are normalised to level of total (A)JAK2 or (B)STAT3 and are relative to $MPL^{WT}JAK2^{WT}$. Analysis was performed using ImageJ software.

previous reports demonstrating $JAK2^{V617F}$ is able to confer cytokine independent signalling in cell lines(181). Similarly, $JAK2^{V617F}$ was able to induce JAK2 phosphorylation when co-expressed with all MPL mutants (MPL^{Y591F}, MPL^{Y625F} and MPL^{Y630F}). This suggests that $JAK2^{V617F}$ activation occurs independently of and precedes MPL receptor phosphorylation. Cells expressing $JAK2^{V617F}$ and MPL are still able to respond to TPO as JAK2 phosphorylation increases following stimulation with TPO; however, this is potentially a result of the presence of endogenous murine $JAK2^{WT}$. As expected, phosphorylation of downstream target, STAT3(281), mirrors the phosphorylation pattern observed for JAK2 in both $JAK2^{WT}$ and $JAK2^{V617F}$ expressing cells.

4.3.5 Analysis of cell viability in MPL/JAK2 Ba/F3 cell lines

Cell viability was assessed in cells co-expressing WT or mutant MPL and WT or mutant JAK2 based on the percentage of cells capable of reducing XTT relative to those cultured in IL-3. XTT assays were performed on cells co-expressing MPL: WT, Y591F or Y625 and JAK2: WT or V617F. Y630F proliferative capacity was not assessed as cell surface expression was not uniform (Figure 4.6). Cells were cultured in the absence or presence of 1ng/mL rhTPO and XTT reduction capacity was measured at 24, 48 and 72hrs. Cells expressing $JAK2^{WT}$ or $JAK2^{V617F}$ in the absence of MPL were unable to maintain metabolic activity in the presence or absence of cytokine (Figure 4.9 and Figure 4.10). Similarly, cells expressing MPL^{WT} and JAK^{WT} were not viable in the absence of cytokine; however, co-expression of MPL^{WT} with $JAK2^{V617F}$ sustained metabolic activity in the absence of cytokine (Figure 4.9). As expected from previous studies, when cultured in the presence of cytokine both $MPL^{WT}JAK2^{WT}$ and $MPL^{WT}JAK2^{V617F}$ cells were metabolically active although mutant JAK2 was more effective at sustaining activity (Figure 4.10). These data support previous works showing that MPL expression is required for $JAK2^{V617F}$-mediated proliferation *in vitro* in the absence of cytokine(264). Analysis of MPL point mutants demonstrated that co-expression of MPL^{Y591F} with $JAK2^{WT}$ was able to sustain metabolic activity in the absence of TPO albeit to a lesser extent than $MPL^{WT}JAK2^{V617F}$ cells (Figure 4.9). Additionally, there was slightly increased metabolic activity in $MPL^{Y591F}JAK2^{V617F}$ cells

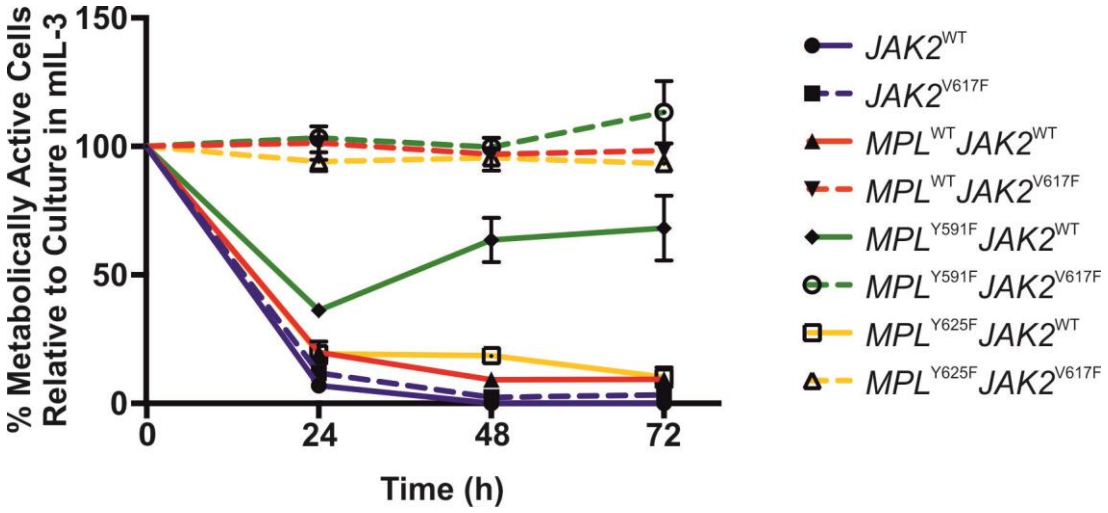


Figure 4.9 XTT assay with MPL point mutants in the absence of cytokine
Ba/F3-MPL-JAK2 cells were cultured in RPMI + 2%FBS in the absence of rhTPO. The ability of metabolically active cells to reduce XTT was measured at 24, 48 and 72 hours post-culture and is shown as a percentage relative to maximal activity when cultured in IL-3. Experiments were performed in triplicate and data are presented as mean ±s.e.m; n=3.

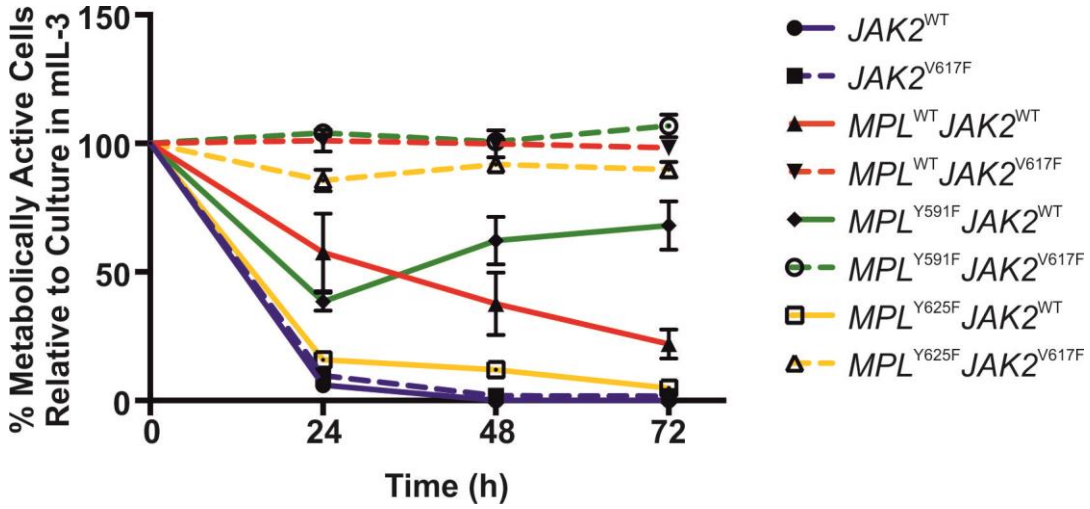


Figure 4.10 XTT assay with MPL point mutants with cytokine

Ba/F3-MPL-JAK2 cells were cultured in RPMI + 2%FBS and 1ng/mL rhTPO. The ability of metabolically active cells to reduce XTT was measured at 24, 48 and 72 hours post-culture and is shown as a percentage relative to maximal activity when cultured in IL-3. Experiments were performed in triplicate and data are presented as mean ±s.e.m; n=3.

compared to $MPL^{WT}JAK2^{V617F}$ cells observed at 72hours when cultured in TPO (Figure 4.10); however, the difference was not statistically significant. This was not surprising as MPL^{Y591} is a negative regulator of TPO-induced signalling and removal of this residue has been shown to increase proliferation as measured by MTT(1). Interestingly, metabolic activity for $MPL^{Y591F}JAK2^{WT}$ cells was almost identical in both the presence and absence of cytokine (Figure 4.9 and Figure 4.10). MPL^{Y625} was shown previously to be necessary for TPO mediated proliferation(3,282). Likewise, removal of MPL^{Y625} (MPL^{Y625F}) was unable to sustain activity when co-expressed with $JAK2^{WT}$ in either the presence or absence of cytokine; however, when co-expressed with $JAK2^{V617F}$, metabolic activity remained similar to maximally active cells cultured in IL-3(Figure 4.9 and Figure 4.10). These data suggest that removal of MPL^{Y591} and MPL^{Y625} within MPL is unable to overcome to proliferative effects induced by $JAK2^{V617F}$ expression in cells.

4.4 Discussion

Removal of MPL *in vitro* prevents cytokine independent signalling despite presence of $JAK2^{V617F}$. This is consistent with previous reports(264). Studies in cell lines expressing mutant MPL demonstrate the importance of receptor phosphorylation on downstream signalling(1,3,283). However, disruption of tyrosine phosphorylation sites within the cytoplasmic domain MPL had little effect on regulating $JAK2^{V617F}$-induced signalling. This is not entirely unexpected as platelets from mice expressing the MPL receptor lacking the distal 60 amino acids (Δ60), a region containing all three phosphorylated tyrosine residues, was still able to active the JAK/STAT pathway(284). In these mice, activation of the MAPK and Akt signalling pathways were disrupted; therefore, MPL phosphorylation plays a larger role in activation of these pathways and is unable to regulate $JAK2^{V617F}$-mediated signalling as demonstrated by our data. The box1/2 regions of MPL are important for association with JAK2 and disruption of these regions results in diminished TPO induced signalling and proliferation(104). These regions may be able to control $JAK2^{V617F}$-induced signalling through disruption of MPL/JAK2 association; however, this has yet to be examined.

Although numerous studies utilize the Ba/F3 cell line to study MPNs, it is important to note the limitations of using these cells. Ba/F3 cells are derived from murine pro-B cells. Being of lymphoid origin, intrinsic differences between these cells and those of the myeloid lineage may affect experimental outcomes and result in a number of experimental artefacts. For example, *JAK2*^{V617F} expression in these cell lines results in complete cytokine independence; however, *in vivo*, *JAK2*^{V617F} confers cytokine hypersensitivity(179,285). Nonetheless, studies utilizing cell lines provide preliminary evidence and rationale for investigation in a more relevant model. These data clearly demonstrate that MPL is required for JAK2^{V617F} signalling prompting further investigation into the role of MPL in JAK2^{V617F+} MPN development *in vivo*.

CHAPTER 5 GENERATION OF A HUMAN *JAK2*^{V617F}-POSITIVE MOUSE MODEL LACKING THROMBOPOIETIN OR MPL

5.1 Experimental Rationale

Results from the previous chapter showing that MPL cell surface expression is necessary for in vitro *JAK2*^{V617F}-induced transformation suggest that MPL may have an essential role in myeloproliferation. To further test the hypothesis that TPO and MPL are indeed necessary for MPN development, we generated *in vivo* models utilising crosses of transgenic mice and bone marrow chimeras. This chapter details the process of generating these mice.

5.1.1 Lineage restriction of *JAK2*^{V617F} expression in murine HSCs

5.1.1.1 *Flip-flop* mouse model

Flip-Flip (*FF1*) mice were generated by Tiedt et al using a bacterial artificial chromosome (BAC) transgene containing the first 12 exons of human *JAK2* in a forward orientation and exons 13-25 containing the V617F mutation in a reverse orientation flanked by antiparallel *lox71* and *lox66* sites(191). 9 consecutive copies of the transgene were integrated into the genome at chromosome 8 band A1, at a locus near the centromere where the authors report contains no known genes. In this orientation, the gene is not expressed. However, in the presence of *Cre*-recombinase, the antiparallel *lox71* and *lox66* sites recombined causing inversion of exons 13-25 into a forward orientation resulting in expression human *JAK2*^{V617F} under the endogenous human *JAK2* promoter. Additionally, upon inversion and ligation in the forward orientation, mutant *lox71* and *lox66* are ligated forming a new *lox71/66* site which is no longer recognized by *Cre*-recombinase and a normal *loxP* site(286). These *loxP* sites are important as they allow for excision of extra copies of transgene resulting in expression of only one copy of human *JAK2*^{V617F} (Figure 5.1 and Figure 5.2).

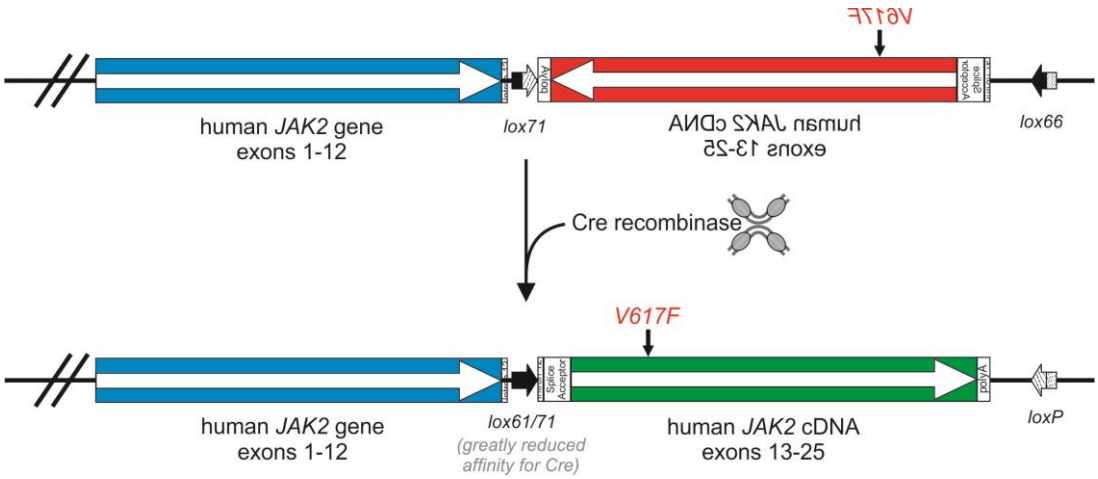


Figure 5.1 *Flip-flop* human *JAK2*^{V617F} transgene
Flip-flop (*FF1*) mice contain a human *JAK2*^{V617F} transgene. The first 12 exons are in the correct orientation while exons 13-25, which contains the V617F mutation, is in the reverse orientation flanked by anti-parallel mutant *loxP* sites. Presence of Cre recombinase results of 'flipping' of exons 13-25 to the correct orientation resulting in transcription of human JAK2V617F. Transcription is under the control of the native human *JAK2* promoter.

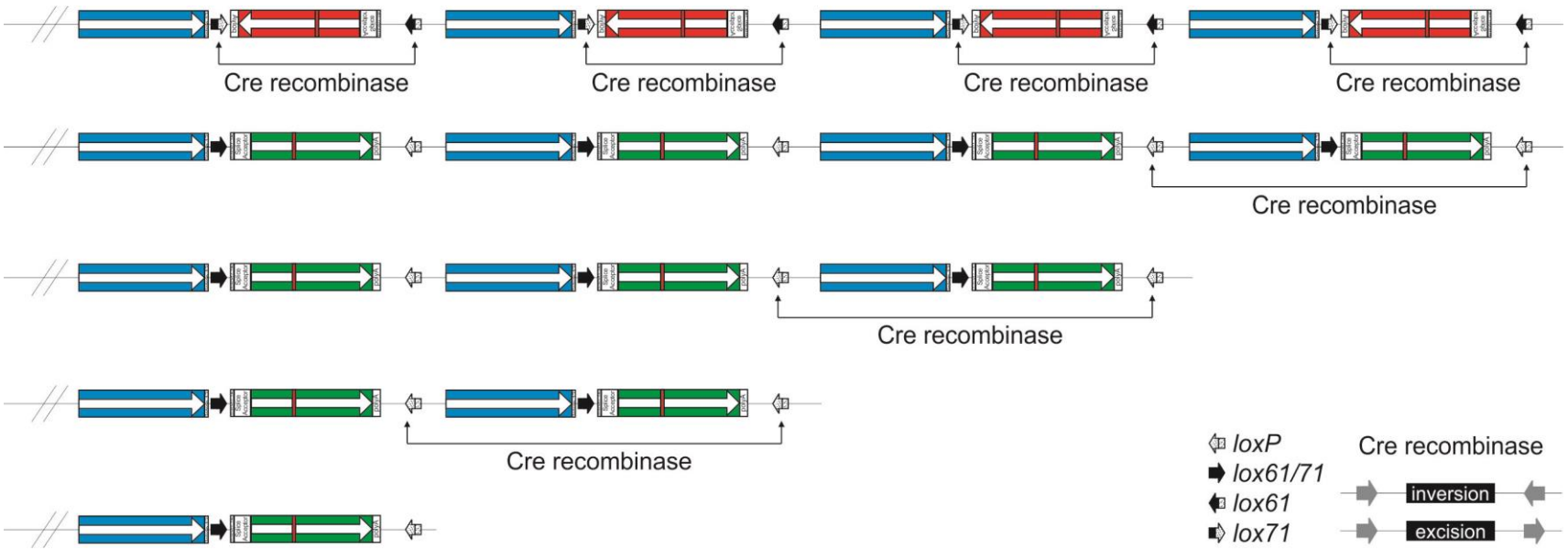


Figure 5.2 Cre-Lox recombination of the *Flip-flop* human *JAK2*^{V617F} transgene

The *Flip-flop* (*FF1*) transgene contains 9 copies of J*AK2* end on end; in this figure only 4 copies are shown. In the presence of *Cre*-recombinase, exons 13-15 are inverted resulting in multipl(191)e copies of human *JAK2*^{V617F} in the correct orientation. Inversion enables *Cre*-recombinase to subsequently excise out these copies of *JAK2*^{V617F} until only a single copy remains. The V617F mutation is represented as a red vertical bar. Adapted from Tiedt *et al*. Blood 2009(191).

5.1.1.2 *Tie2-Cre* mouse

Developed by Kisanuki et al., the Tie2-Cre transgene contains the *Tie2* promoter, *Cre* cDNA, *MT-1* polyA signal sequence and a *Tie2* intron 1 enhancer. The transgene was microinjected into C57BL/6 x $SJLF_1$ oocytes and offspring were tested for presence of the transgene by PCR. *Tie2-Cre* mice were crossed with *Cre* mediated *lacZ* expressing transgenic mice (*CAG-CAT-Z* or *R26R*) confirming that the *Tie2* promoter and enhancer regions were able to drive *Cre* transgene expression in a pan-endothelial fashion. Although they report no detectable *lacZ* expression in hematopoietic cells in E8.5 yolk sacs, they observe a subset of circulating lacZ-positive cells within the dorsal aorta(287). Tang et al. showed that the *Tie2-Cre* mouse was able to drive *Cre* transgene expression in 88% of primitive hematopoietic progenitor cells of *Tie2-Cre;Rosa26R-Enhanced yellow fluorescent protein* (*EYFP*) E9.5 embryos(288), suggesting the identity of the lacZ positive dorsal aorta cells identified by Kisanuki et al. to be primitive hematopoietic cells. In adult mice, 85% of bone marrow and 84% of splenic cells from *Tie2-Cre;Rosa26R-EYFP* mice were $EYFP^+$ indicating *Tie2* expression in adult hematopoietic cells from multiple lineages. Together these data support that *Tie2-Cre* induced recombination occurs within a primitive stem cell.

5.1.1.3 *Flip-flop/Tie2-Cre* mouse

The *FF1/Tie2-Cre* mice are generated from a cross between the *FF1* and *Tie2-Cre* mouse and was characterized by Etheridge et al. as a $JAK2^{V617F+}$ ET mouse model(149). This mouse expresses $JAK2^{V617F}$ in its hematopoietic and endothelial cells and develops pronounced thrombocytosis, neutrophilia and splenomegaly by 16 weeks of age. $JAK2^{V617F}$ was not grossly overexpressed in these mice and ranged from 10-50% of endogenous murine $Jak2^{WT}$. Expression of $JAK2^{V617F}$ was present in all colony forming cells (CFCs) tested from *FF1/Tie2-Cre* mice (Figure 5.3). Despite severe thrombocytosis, these mice exhibited attenuated thrombosis following injury due in part to dysregulated expression of vWF.

5.1.2 *Thpo*$^{-/-}$ mouse

The *Thpo*$^{-/-}$ mouse used in this study was generated by de Sauvage et. al.(65). To generate the *Thpo*$^{-/-}$ mouse, a targeting vector was engineered to remove 23 amino

acids of a fragment responsible for receptor binding and activation(62) and insert a neomycin-resistance (*neo*^{r}) gene to disrupt the mouse *Thpo* gene. The targeting vector was linearized and electroporated into a 129/Sv murine embryonic stem (ES) cell line(289) and selected for neomycin resistance. Selected cells were subsequently microinjected into C57Bl/6J blastocysts. Chimeric males were bred to C57Bl/6J females and offspring were checked for germline transmission of the mutant TPO allele by PCR and Southern blot analysis. Subsequently, mice positive for the mutant allele were interbred to generate homozygous *Thpo* gene-targeted (*Thpo*$^{-/-}$) mice. The authors reported normal Mendelian distribution of litters and adult *Thpo*$^{-/-}$ mice displayed no overt abnormalities. Northern blot analysis revealed no detectable *Thpo* transcripts in kidneys and livers of *Thpo*$^{-/-}$ mice and reported a reduction of *Thpo* transcripts in both tissues by half in heterozygotes (*Thpo*$^{+/-}$) compared to wild-type (*WT*). *Tpo*$^{-/-}$ mice showed an 88% decrease in the number of peripheral platelets accompanied by an increase in mean platelet volume (MPV). *Thpo*$^{+/-}$ mice however, exhibited a 33% reduction in peripheral platelets. There was no observed compensatory increase in IL-3, IL-6 or GMCSF resulting from removal of *Thpo*. Additionally, disruption of *Thpo* resulted in no significant difference in other hematopoietic cell lineages.

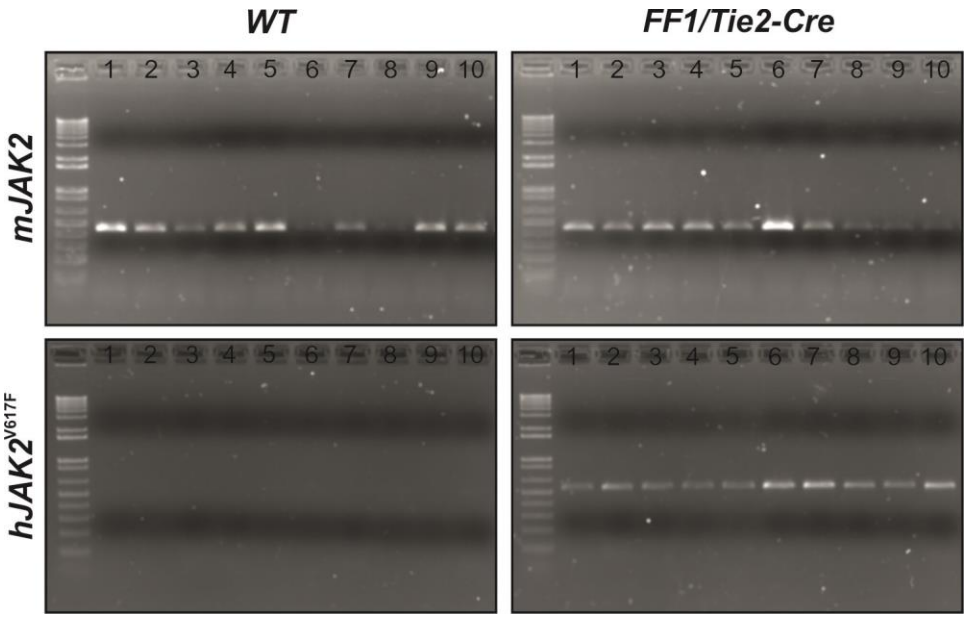


Figure 5.3 *JAK2*^{V617F} Expression in Individual Bone Marrow CFU-G Colonies
RT-PCR of individual bone marrow CFU-G colonies to show expression of mouse *JAK2* and human *JAK2*^{V617F}.

5.1.3 *Mpl*$^{-/-}$ mouse

The *Mpl*$^{-/-}$ mouse used in this study was generated by Gurney et al.(78). To generate the *Mpl*$^{-/-}$ mouse, a targeting vector was engineered to contain a neomycin-resistance (*neo*^{r}) gene under the mouse *phosphoglycerate kinase 1* (*pkg*) promoter inserted into the third exon of *Mpl* using a synthetic NotI restriction site. This vector was electroporated into murine ES cells and ES colonies positive for homologous recombination, as detected by a HindIII site from the *neo*^{r} gene, were subsequently microinjected into blastocysts. Once germline transmission was determined, two separate lines were interbred to generate homozygous *Mpl* gene-targeted (*Mpl*$^{-/-}$) mice. The authors reported no detectable *Mpl* transcripts through RT-PCR in spleens of *Mpl*$^{-/-}$ mice. *Mpl*$^{-/-}$ mice showed an 85% decrease in the number of peripheral platelets and reduced numbers of megakaryocytes in the spleen and bone marrow compared to wild-type, despite having increased levels of TPO in their serum. Additionally, heterozygous *Mpl* gene-targeted (*Mpl*$^{+/-}$) mice showed no significant changes in platelet and megakaryocyte numbers or TPO levels in serum. Disruption of *Mpl* resulted in no significant difference in other hematopoietic cell lineages.

5.1.4 B6.SJL-Ptprc^{a} Pep3^{b}/BoyJ mouse

The B6.SJL-*Ptprc*^{a} *Pep3*^{b}*/BoyJ* mouse strain in on a C57BL/6 background and carries a differential allele for protein tyrosine phosphatase, receptor type C (*Ptprc*), *Ptprc*^{a}, while *WT* C57BL/6 strains carry the *Ptprc*^{b} allele. *Ptprc*^{a} is also referred to as CD45.1 or Ly5.1 whereas *Ptprc*^{b} is referred to as CD45.2 or Ly5.2. The receptor is expressed on all leukocytes and because antibodies can readily distinguish between the two isotypes, this strain has become a widely used in transplantation studies (http://jaxmice.jax.org/strain/002014.html).

5.2 Materials and Methods

5.2.1 Mouse colony organization

Breeding pairs were set up and receive a non-redundant identifying breeding number. Males were considered sexually mature at 6 weeks of age and female mice at 8 weeks of age. At 10 days of age, mouse pups were sexed, tagged and catalogued using an ear punch method before being placed back into the cage with the mother.

Males were tagged and catalogued first followed by females. Each mouse identification number was based on the breeding number of the parents followed by a mouse number determined by the number of mice a breeding pair has already generated. The identification numbers were recorded and listed on cage cards. Pups receive ear punches using a 2mm thumb style EP-T 900 small animal ear punch (Braintree Scientific, Inc., Braintree, MA, USA) in the following order: single right and single left, two right, two left, two right and a single left, two left and a single right or two right and two left. The number of pups for each sex of a single litter has never surpassed the eight identifiable markings listed. The tissue is saved for subsequent genotyping as described in Section 5.2.2.1.

5.2.2 Genotyping of mice

5.2.2.1 Genomic DNA (gDNA) isolation for genotyping

Tissues from ear punches are placed into individually labelled microcentrifuge tubes, corresponding to the mouse identification number. Samples are then digested in 300µL Cell Lysis Buffer (Qiagen) with 0.2µg/mL Proteinase K in 10mM Tris-HCl, pH 8.0 at 55°C for 8 hours. Samples are then brought up to room temperature (25°C) and 100µL of Protein Precipitation solution is added and the sample vortexed. The sample is then centrifuged at 13,000*g* for 4 minutes to pellet the precipitate and supernatant then added to 300µL isopropanol to precipitate out the gDNA. The gDNA is then pelleted at 13,000*g* for 2 minutes and washed with 300µL of 70% EtOH and resuspended in 50µL of gDNA hydration solution. This gDNA is then used for the genotyping reaction.

5.2.2.2 Conventional polymerase chain reaction (PCR) of genomic DNA for genotyping

Genomic DNA is isolated as described in Section 5.2.2.1. Genotyping is performed using HotStarTaq DNA polymerase Kit (Qiagen). A final concentration of 1X PCR buffer, 1X Q-solution, 4.5mM $MgCl_2$, 200µM of each dNTP, 0.2µM of forward and reverse primer and 0.625U HotStarTaq DNA polymerase was brought up to 25µL with PCR grade H_2O in a thin walled 0.2mL PCR tube and run on a Veriti thermal cycler (Life technologies), primers and annealing temperatures are listed in Table 5.1. PCR products were mixed with DNA loading dye (6X: 0.25% bromophenol blue

Table 5.1 Primers for Conventional Genotyping

Gene	Sequence	Size (bp)	Tm* (°C)	Ref
FF1	F-5'-GAGCAAGCTTTCTCACAAGC-3' R-5'-AATTCTGCCCACTTTGGTGC-3'	~500	54.5 56.4	(191)
Tie2-Cre	F-5'-CTGCATTACCGGTCGATGCA-3' R-5'-ACGTTCACCGGCATCAACGT-3'	~300	57.8 59.9	(290)
Mpl WT	F-5'-GAGGAAGAGGCAGCACCCAGTG-3' R-5'-GGGAATACAGGGGGCATGCACG-3'	~350	62.2 62.8	
Mpl neo	F-5'-TAGCGGCTGATGTTGAACTG-3' R-5'-ACTATCCCGACCGCCTTACT-3'	~200	55.2 57.6	
Tpo WT	F-5'-GTCGACCCTTTGTCTATCCCT-3' R-5'-GGTGAATGTAACCTGGGATAA-3'	~300	56.1 51.9	(65)
Tpo neo	F-5'-AAGTATCCATCATGGCTGATG-3' R-5'-TAGCCAACGCTATGTCCTGATA-3'	~350	55.4 52.3	

*in 50mM NaCl

(w/v), 0.25% xylene cyanol FF (w/v), 30% glycerol (v/v)) to 1X and resolved using 1.5% agarose gels in TBE containing 0.2µg/mL EtBr at 120 volts for 50 minutes.

5.2.2.3 Quantitative PCR (qPCR) of genomic DNA for genotyping

Genotyping of transgenic mice to determine allelic copy number was accomplished using a TaqMan® Copy Number Assay (Invitrogen) as per manufacturer's instructions. Reaction components include TaqMan® Genotyping Mastermix, TaqMan® Copy Number Assay, TaqMan® Copy Number Reference Assay and 20ng gDNA. The TaqMan Genotyping Mastermix contains AmpliTaq® Gold DNA Polymerase, UP (UltraPure) and dNTPs necessary for target gene amplification. The TaqMan® Copy Number Assay contains unlabeled forward and reverse primers for the gene of interest in addition to a 6-carboxyfluorescein (FAM™) dye-labeled minor groove binder (MGB) probe that binds a sequence of the target gene between the forward and reverse primers. The TaqMan® Copy Number Reference Assay used is similar to the TaqMan® Copy Number Assay but its forward and reverse primers and VIC® dye-labeled tetramethylrhodamine (TAMRA™) probe recognizes the mouse transferrin receptor gene (*trfc*) and serves as an internal control. The reaction is considered a duplex reaction as both sets of primers and probes are assayed in the same reaction mixture. Genomic DNA is isolated as described in 4.2.2.1, the concentration is then quantified using a NanoDrop spectrophotometer (Thermo Scientific) and diluted in H_2O to 5ng/µL. When all the components of the reaction probes are added to the PCR reaction, there is no detectable signal because the proximity of the quencher to the FAM™ or VIC® dye-labeled reporter prevents detection of the reporter signal. When the reaction proceeds, the gDNA template is denatured, the forward and reverse primers and the probes anneal to their target gene sequences. During the elongation step, the AmpliTaq® Gold DNA Polymerase amplifies both the target and reference sequences. However, because the probes bind to a sequence between the forward and reverse primers the AmpliTaq® Gold DNA Polymerase uses its 5' nuclease activity to cleave the probe in order to complete the elongation step. As a result, the dye is released and the signal is no longer quenched, allowing for detection with each round of amplification. Assays were performed in 96-well plates with samples assayed in duplicate. Each plate contained the unknown samples, a sample of a known copy number (calibrator sample) and a no template control sample. The *FF1* transgene was assessed with Copy Number Assay

Hs02201428_cn for *JAK2*. The *Tie2-Cre* transgene was assessed with Copy Number Assay Mr00635245_cn for *cre*. Mouse transferrin receptor (*Trfc*) was used as an internal reference gene (4458366, Invitrogen). The reaction was run on an Applied Biosystems 7300 Real-Time PCR System. Data was interpreted using 7300 System Software and analysed using CopyCaller™ Software v2.0 (Invitrogen). *Tie2-Cre* homozygosity was determined using Taqman Copy Number assays for *cre*.

5.2.3 Hematopoietic cell transplantation

5.2.3.1 Preparation of donor bone marrow

Primary murine bone marrow was isolated as described in Section 2.1.2, counted and resuspended in DPBS at 5.0 x 10^7 cells/mL. Prepared cells are kept on ice until needed.

5.2.3.2 Bone marrow transplantation

48 hours prior to transplantation, 6-8 week old recipient mice are placed on antibiotic water containing 400mg/L sulfamethoxazole and 80mg/L trimethoprim. Mice are lethally irradiated, receiving a single 1000cGy dose of gamma-irradiation (Stony Brook University Division of Laboratory Animal Resources (DLAR) cesium source). Following irradiation, mice are anesthetized using isoflurane and 5.0 x 10^6 cells, 0.1mL of the prepared cells described in Section 5.2.3.1, are injected retro-orbitally into each mouse using a 28G½ insulin syringe. Antibiotic water is replaced every other day for two weeks post-transplant and every 3-4 days thereafter until mice are at least 90% reconstituted.

5.2.3.3 Submandibular bleeds

Mice are anesthetized using isoflurane and venesection is performed along the submandibular vein using a mouse-bleeding lancet (Goldenrod, MEDIpoint, Inc., Mineola, NY). Approximately 100µL of blood is collected into an EDTA containing blood collection tube (BD Biosciences) to prevent clot formation. A sterile gauze pad is applied to the wound with little pressure and bleeding ceases in under a minute if not immediately.

5.2.3.4 Determination of bone marrow chimerism

50µL of peripheral blood collected via submandibular bleed as described in Section 5.2.3.3 is added to 450µL of DPBS and then RBCs are lysed by addition of 4.5mL of a 0.8% NH_4Cl, 0.1mM EDTA solution, vortexed and kept on ice for 5 minutes. The sample is then centrifuged at 500*g* and washed 3x with DPBS. Cells were then resuspended in 100µL DPBS + 1% BSA and dual stained with PE/Cy7 conjugated anti-mouse CD45.1 clone A20 (Biolegend, 110729) and Alexa Fluor 488® conjugated anti-mouse CD45.2 clone 104 (Biolegend, 109815) antibodies at 1:100 in the dark at 25°C for 30 minutes. The staining reaction was stopped by addition of 400µL of DPBS + 1% BSA and cells were then analysed using flow cytometry.

5.2.4 *Mpl* cell surface expression on platelets

Mouse platelets were obtained from blood collected from mice via cardiac puncture immediately following CO_2 asphyxiation. Blood was drawn into a syringe containing acid citrate dextrose (ACD) (BD Biosciences), at a 1:9 ratio and diluted with an equal volume of Wash Buffer (150 mM NaCl, 20 mM Pipes, pH 6.5) and centrifuged at 60*g* for 10 min to isolate platelet rich plasma (PRP). To subsequently isolate platelets PRP was centrifuged at 240*g* for 10 min and resuspended in 1% NP-40 lysis buffer with inhibitors and subjected to western blot analysis (Section 2.5).

5.2.5 *JAK2*^{V617F} Expression

Bone marrow neutrophils (BMNs) were isolated using a Neutrophil isolation kit according to the manufacturer's protocol (Miltenyi Biotec) and mRNA was obtained using an RNeasy mini kit following the manufacturer's protocol (Qiagen). cDNA was prepared as described in 2.3. Human/mouse *JAK2* cDNA ratios were calculated using Taqman Gene Expression assays Hs01078117_m1 and Mm01208495_m1 (Applied Biosystems) for human *JAK2* and mouse *Jak2*, respectively. Standard curves were generated from linearized pcDNA3.1 plasmids containing either human *JAK2* or mouse *Jak2*.

5.3 Results

5.3.1 Breeding of *FF1*$^{+/-}$*Tie2*$^{+/-}$ mice

To generate mice expressing *JAK2*^{V617F} in their HSCs, *FF1*$^{+/-}$ mice were mated with *Tie2-Cre*$^{+/+}$ mice. These matings generated desired *FF1*$^{+/-}$*Tie2*$^{+/-}$ mice and *Tie2*$^{+/-}$ littermate controls. Litters exhibit non-Mendelian patterns of inheritance with a prejudice against the *FF1*$^{+/-}$*Tie2*$^{+/-}$ genotype. Only 80 of 400 pups from 88 litters were *FF1*$^{+/-}$*Tie2*$^{+/-}$, 20% compared to the expected 50% (P<0.0001, two-tailed bionomial test) (Figure 5.4). Mean litter size was 4.8 with 54.75% females. This mouse has been previously characterized(149).

5.3.2 Breeding of *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{-/-}$ and *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{+/-}$ mice

Mice expressing *JAK2*^{V617F} in their HSCs in the absence of *Mpl* were generated as depicted in Figure 5.5. *FF1* and *Tie2-Cre* mice were crossed with *Mpl*$^{-/-}$ mice to generate *FF1*$^{+/-}$*Mpl*$^{+/-}$ and *Tie2*$^{+/-}$*Mpl*$^{+/-}$ mice. These mice were then bred to *Mpl*$^{-/-}$ mice to generate *FF1*$^{+/-}$*Mpl*$^{-/-}$ and *Tie2*$^{+/-}$*Mpl*$^{-/-}$ mice. To maximize the number of usable mice and to overcome the partial embryonic lethality of *FF1*$^{+/-}$*Tie2*$^{+/-}$ mice mentioned in 5.3.1, *Tie2*$^{+/-}$*Mpl*$^{-/-}$ mice were crossed to generate *Tie2*$^{+/+}$*Mpl*$^{-/-}$. Homozygosity for *Tie2-Cre* was determined using qPCR as described in 5.2.2.3. Finally, *Tie2*$^{+/+}$*Mpl*$^{-/-}$ mice were crossed with *FF1*$^{+/-}$*Mpl*$^{-/-}$ mice to generate *FF1*$^{+/-}$ *Tie2*$^{+/-}$*Mpl*$^{-/-}$ experimental mice along with *Tie2*$^{+/-}$*Mpl*$^{-/-}$ littermate controls as depicted in Figure 5.5. Litters exhibited non-Mendelian patterns of inheritance with only 14 pups born of 301 pups in 64 litters being *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{-/-}$, only 4.65% compared to the expected 50% (P<0.0001, two-tailed binomial test) (Figure 5.4). Mean litter size was 4.625 with 49.44% females. Additionally, to generate *FF1*$^{+/-}$ *Tie2*$^{+/-}$*Mpl*$^{+/-}$ mice, *FF1*$^{+/-}$*Mpl*$^{-/-}$ mice were crossed with *Tie2*$^{+/+}$ mice or *FF1*$^{+/-}$ were crossed with *Tie2*$^{+/+}$*Mpl*$^{-/-}$ mice to generate *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{+/-}$ experimental mice and *Tie2*$^{+/-}$*Mpl*$^{+/-}$ littermate controls as depicted in Figure 5.6. Litters exhibited non-Mendelian patterns of inheritance with only 8 pups born of 43 pups in 9 litters being *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{+/-}$, only 18.60% compared to the expected 50% (P=0.0013, two-tailed binomial test) (Figure 5.4). Mean litter size was 5 with 54.17% females.

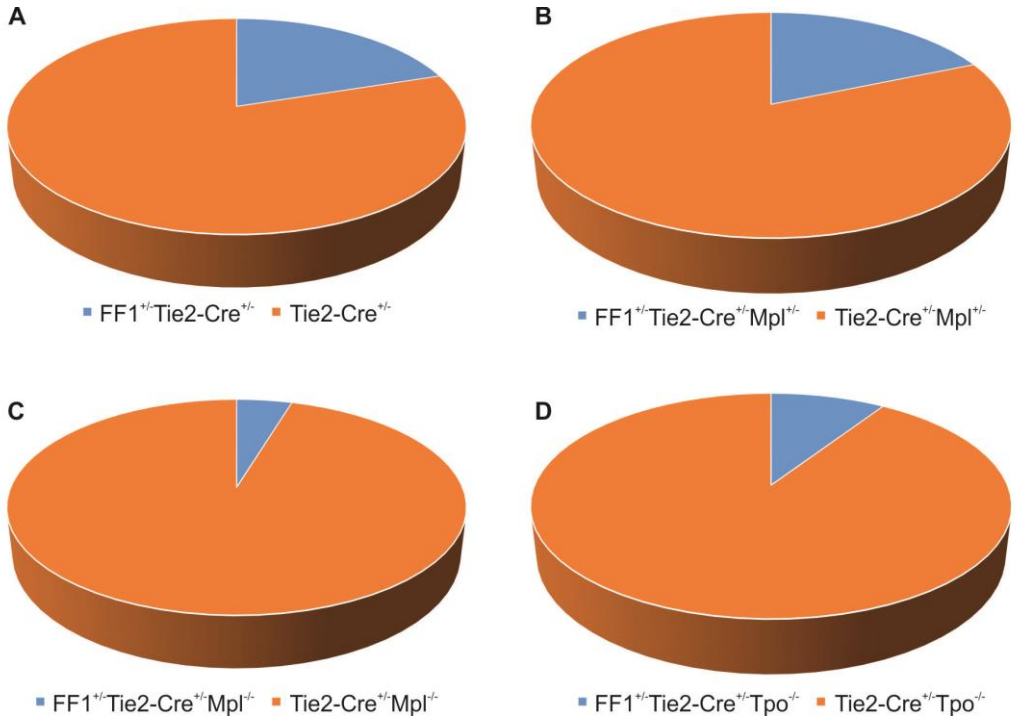


Figure 5.4 Proportion of experimental mice born
Proportion of (A) $FF1^{+/-}$Tie2-$Cre^{+/-}$ (B) $FF1^{+/-}$Tie2-$Cre^{+/-}Mpl^{+/-}$ (C) $FF1^{+/-}$Tie2-$Cre^{+/-}Mpl^{-/-}$ and (D) $FF1^{+/-}$Tie2-$Cre^{+/-}Tpo^{-/-}$ mice born are shown in blue. Based on the experimental design, experimental mice are expected to consist of 50% of the litter.

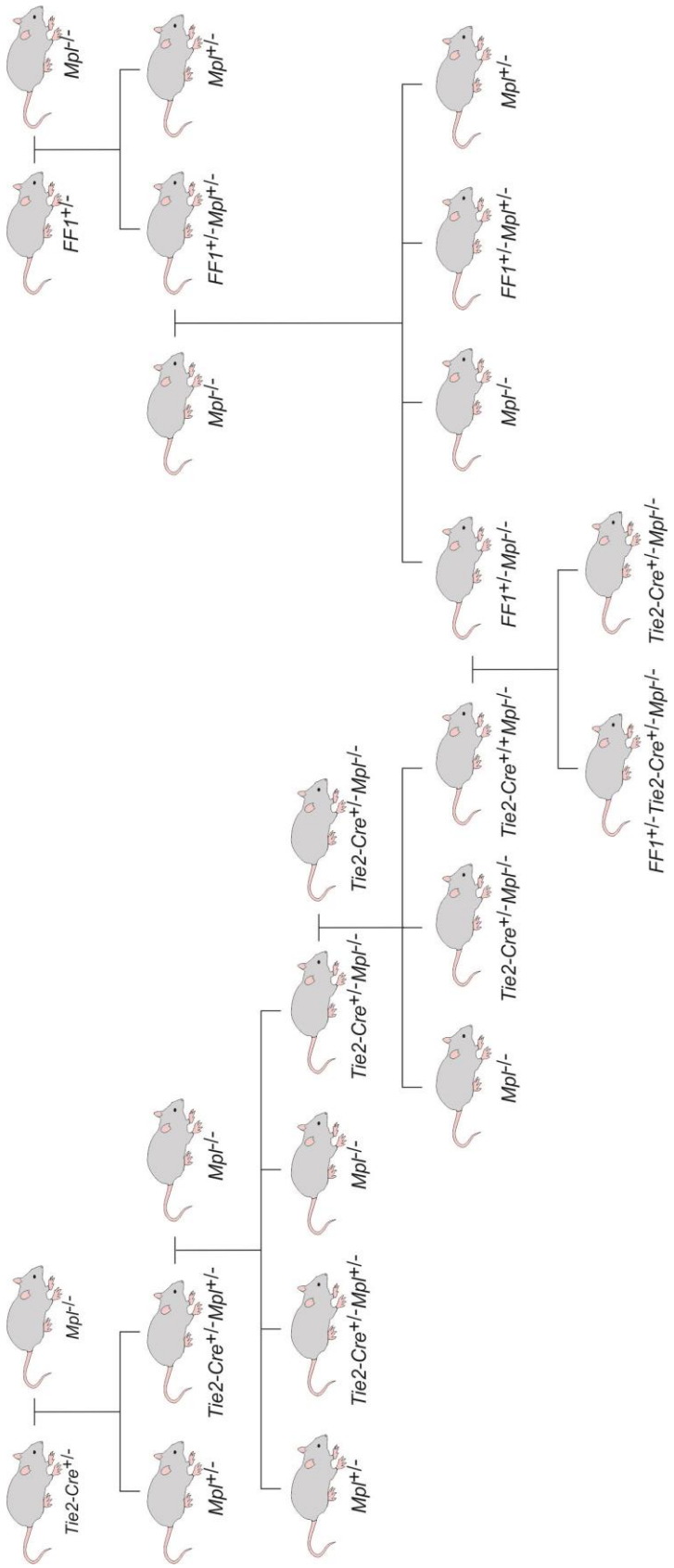


Figure 5.5 Schematic Depicting Breeding Strategy to Generate JAK2^{V617F+}Mpl$^{-/-}$ mice
Mice were bred to generate *FF1*$^{+/-}$*Tie2-Cre*$^{+/-}$*Mpl*$^{-/-}$ mice and *Tie2-Cre*$^{+/-}$*Mpl*$^{-/-}$ littermate controls

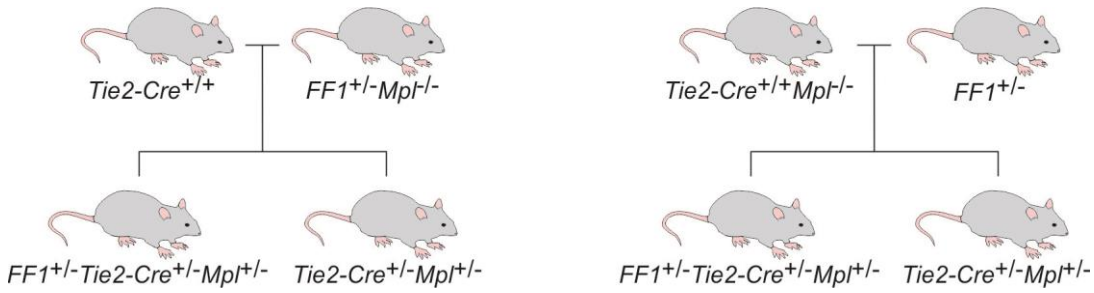


Figure 5.6 Schematic Depicting Breeding Strategy to Generate *JAK2*$^{V617F+}$*Mpl*$^{+/-}$ mice

Mice generated during breeding of *FF1*$^{+/-}$*Tie2-Cre*$^{+/-}$*Mpl*$^{-/-}$ mice were bred to generate *FF1*$^{+/-}$*Tie2-Cre*$^{+/-}$*Mpl*$^{+/-}$ mice and *Tie2-Cre*$^{+/-}$*Mpl*$^{+/-}$ littermate controls

5.3.3 *Mpl* expression in *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{-/-}$ and *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{+/-}$ mice

To confirm the phenotype of *Mpl*$^{-/-}$ and *Mpl*$^{+/-}$ mice, Mpl expression was analysed by western blot (Section 2.5) in platelets isolated from congenital *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{-/-}$, *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{+/-}$ and control mice. Platelet *Mpl* expression was halved in *Mpl* heterozygous (*Mpl*$^{+/-}$) mice compared to *WT* mice and *Mpl*$^{-/-}$ mice had undetectable levels of Mpl (Figure 5.7). Additionally, *JAK2*^{V617F} expression resulted in increased *Mpl* expression on both a *WT* and *Mpl*$^{+/-}$ background (Figure 5.7).

5.3.4 Breeding of *FF1*$^{+/-}$*Tie2*$^{+/-}$*Thpo*$^{-/-}$ mice

Mice expressing *JAK2*^{V617F} in their HSCs in the absence of TPO were generated as depicted in Figure 5.8. The strategy for generating *FF1*$^{+/-}$*Tie2*$^{+/-}$*Thpo*$^{-/-}$ mice is the same as discussed for *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{-/-}$ in 5.3.2 but in place of *Mpl*$^{-/-}$ mice, here *Thpo*$^{-/-}$ mice were used for breeding. Litters exhibit non-Mendelian patterns of inheritance with only 7 pups born of 76 pups in 22 litters being *FF1*$^{+/-}$*Tie2*$^{+/-}$*Tpo*$^{-/-}$, only 9.21% compared to the expected 50% ($P<0.0001$, two-tailed binomial test) (Figure 5.4). Mean litter size was 3.45 with 46.57% females.

5.3.5 Plasma TPO levels in *FF1*$^{+/-}$*Tie2*$^{+/-}$*Thpo*$^{-/-}$ mice

To confirm the phenotype of *Thpo*$^{-/-}$ mice, plasma TPO levels were measured by ELISA as described in Section 2.8. TPO levels in *FF1*$^{+/-}$*Tie2*$^{+/-}$*Thpo*$^{-/-}$ mice were below the limit of detection confirming the absence of circulating TPO in mice on a *Thpo*$^{-/-}$ background (Figure 5.9). TPO levels in *FF1*$^{+/-}$*Tie2*$^{+/-}$ mice were reduced compared to *WT* (Figure 5.9).

5.3.6 *JAK2*^{V617F} expression in neutrophils isolated from congenital *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{-/-}$, *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{+/-}$ and *FF1*$^{+/-}$*Tie2*$^{+/-}$*Thpo*$^{-/-}$ mice

Levels of *JAK2*^{V617F} expression in the *FF1*$^{+/-}$*Tie2*$^{+/-}$ MPN mouse model has been previously reported(149). To ensure that manipulation of either *Mpl* or *Thpo* expression does not alter the level of *JAK2*^{V617F} expression, *JAK2* expression in the generated models was tested as detailed in Section 5.2.5 by Dr. S. Leah Etheridge.

JAK2^{V617F} expression was comparable to endogenous *Jak2*. There was no significant difference in levels of h*JAK2* expression between *FF1*$^{+/-}$*Tie2*$^{+/-}$, *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{-/-}$, *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{+/-}$ and *FF1*$^{+/-}$*Tie2*$^{+/-}$*Thpo*$^{-/-}$ mice (Figure 5.10).

5.3.7 Generation of *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{-/-}$ mice through bone marrow transplantation

To generate *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ transplant mice, *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{-/-}$ donor marrow was transplanted into *Mpl*$^{-/-}$ recipients. Due to its critical role in hematopoiesis, *Mpl*$^{-/-}$ marrow has severely decreased repopulating activity(96). Therefore, to ensure engraftment of donor marrow and rather than recovery of recipient marrow, donor cells lacking *Mpl* were transplanted into *Mpl*$^{-/-}$ recipients rather than *WT*s. Control transplants were also performed and include *WT* into *WT*, *FF1*$^{+/-}$*Tie2*$^{+/-}$ into *WT*, and *Mpl*$^{-/-}$ into *Mpl*$^{-/-}$ as summarized in Figure 5.11. Chimerism was checked by flow cytometry and control mice were at least 90% reconstituted by 8 weeks post-transplant (Figure 5.12). Despite successful colonization of *Mpl*$^{-/-}$ or *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ donor marrow in irradiated *WT* recipients, there was a significantly increased death rate when *Mpl*$^{-/-}$ or *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ donor marrow was transplanted into *Mpl*$^{-/-}$ recipients compared to other transplant groups (Figure 5.13).

5.3.8 Generation of *FF1*$^{+/-}$*Tie2*$^{+/-}$*Thpo*$^{-/-}$ mice through bone marrow transplantation

To generate *JAK2*$^{V617F+}$*Thpo*$^{-/-}$ transplant model, *FF1*$^{+/-}$*Tie2*$^{+/-}$*Thpo*$^{+/+}$ bone marrow was transplanted into *Thpo*$^{-/-}$ recipients. As TPO is synthesized primarily in the liver, transplantation into a *Thpo*$^{-/-}$ background results in a functionally *Thpo*$^{-/-}$ mouse. Control transplants were also performed and include *WT* into *WT*, *FF1*$^{+/-}$*Tie2*$^{+/-}$ into *WT* and *WT* into *Thpo*$^{-/-}$ as summarized in Figure 5.11. Chimerism was checked by flow cytometry and control mice were at least 90% reconstituted by 8 weeks post-transplant (Figure 5.12).

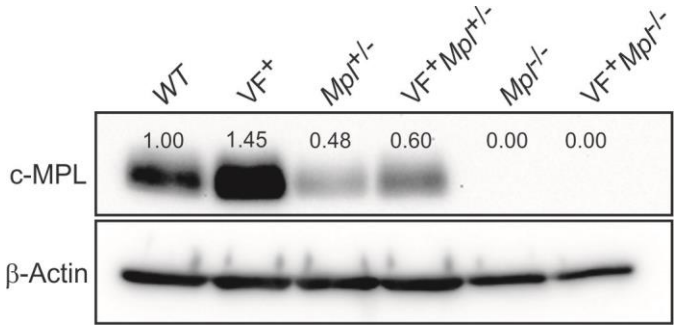


Figure 5.7 Murine platelet Mpl expression
Western blot analysis of MPL on platelets from *WT*, *JAK2*$V_{617}F^{+}$ (VF^{+}), *Mpl*$^{+/-}$, *JAK2*$V_{617}F^{+}$*Mpl*$^{+/-}$ (VF^{+}*Mpl*$^{+/-}$), *Mpl*$^{-/-}$ and *JAK2*$V_{617}F^{+}$*Mpl*$^{-/-}$ (VF^{+}*Mpl*$^{-/-}$) mice at 16 weeks of age. Numbers represent densitometric quantification of protein levels normalized to *WT*.

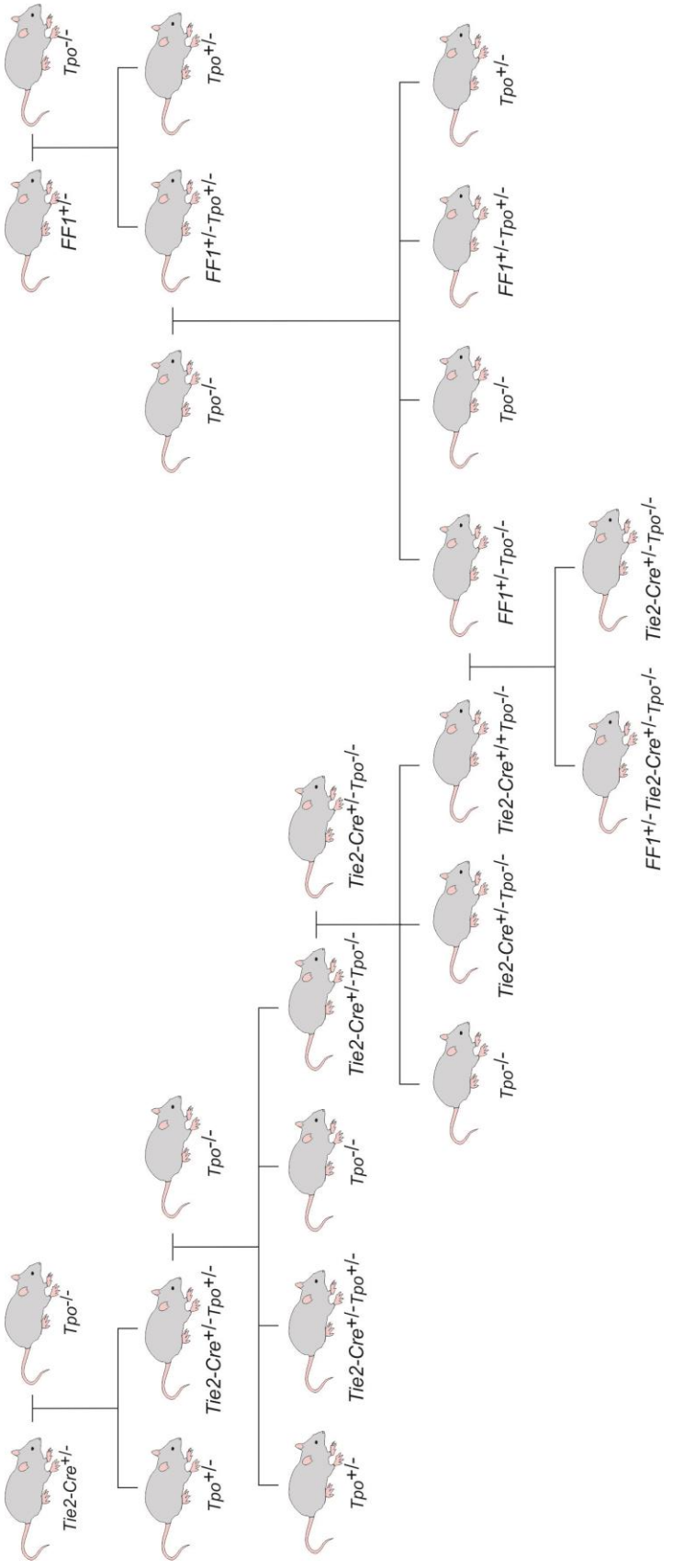


Figure 5.8 Schematic Depicting Breeding Strategy to Generate JAK$^{2V617F+}$Tpo$^{-/-}$ mice
Mice were bred to generate *FF1*$^{+/-}$*Tie2-Cre*$^{+/-}$*Tpo*$^{-/-}$ mice and *Tie2-Cre*$^{+/-}$*Tpo*$^{-/-}$ littermate controls

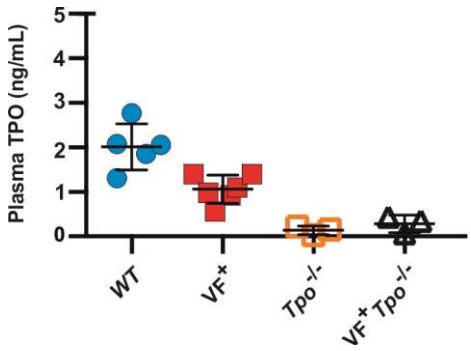


Figure 5.9 Plasma TPO levels

Plasma TPO levels in *Tpo* transgenic mice. Plasma was collected from 16-week old *WT*, *JAK2*$^{V617F+}$ (VF^{+}), *Tpo*$^{-/-}$ and *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice and TPO levels were quantified by ELISA. Each data point represents a single mouse and bars are presented as mean ±s.e.m; asterisks indicate significance compared to *WT*.

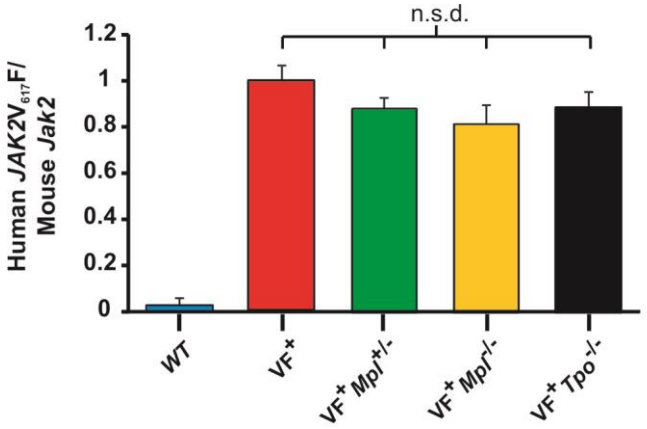
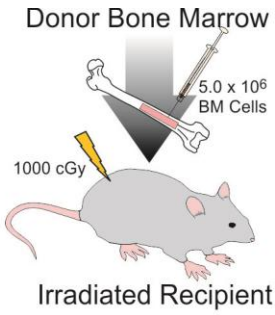


Figure 5.10 Human *JAK2*$^{V617F+}$ expression relative to endogenous mouse *JAK2*^{WT}
qPCR quantification of human *JAK2*V_{617}F expression relative to endogenous mouse *Jak2* expression in neutrophils from *WT*, *JAK2*$V_{617}F^{+}$ (VF^{+}), *JAK2*$V_{617}F^{+}$*Mpl*$^{+/-}$ (VF^{+}*Mpl*$^{+/-}$), *JAK2*$V_{617}F^{+}$*Mpl*$^{-/-}$ (VF^{+}*Mpl*$^{-/-}$) and *JAK2*$V_{617}F^{+}$*Tpo*$^{-/-}$ mice. Data is normalized to VF^{+} and presented as mean ±s.e.m. This work was performed by Dr. S Leah Etheridge.



	WT into *WT*	VF$^+$ into *WT*	*Mpl*$^{-/-}$ into *Mpl*$^{-/-}$	VF$^+$/*Mpl*$^{-/-}$ into *Mpl*$^{-/-}$	*WT* into *Tpo*$^{-/-}$	VF$^+$ into *Tpo*$^{-/-}$
Donor	*WT*	*JAK2*V$_{617}$F$^+$	*Mpl*$^{-/-}$	*JAK2*V$_{617}$F$^+$/*Mpl*$^{-/-}$	*WT*	*JAK2*V$_{617}$F$^+$
Recipient	*WT*	*WT*	*Mpl*$^{-/-}$	*Mpl*$^{-/-}$	*Tpo*$^{-/-}$	*Tpo*$^{-/-}$
Functionally	*WT*	*JAK2*V$_{617}$F$^+$	*Mpl*$^{-/-}$	*JAK2*V$_{617}$F$^+$/*Mpl*$^{-/-}$	*Tpo*$^{-/-}$	*JAK2*V$_{617}$F$^+$/*Tpo*$^{-/-}$

Figure 5.11 Generation of *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ and *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice by transplantation.

Bone marrow transplants were utilized to generate *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ and *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice to better simulate acquisition of the *JAK2*^{V617F} mutation.

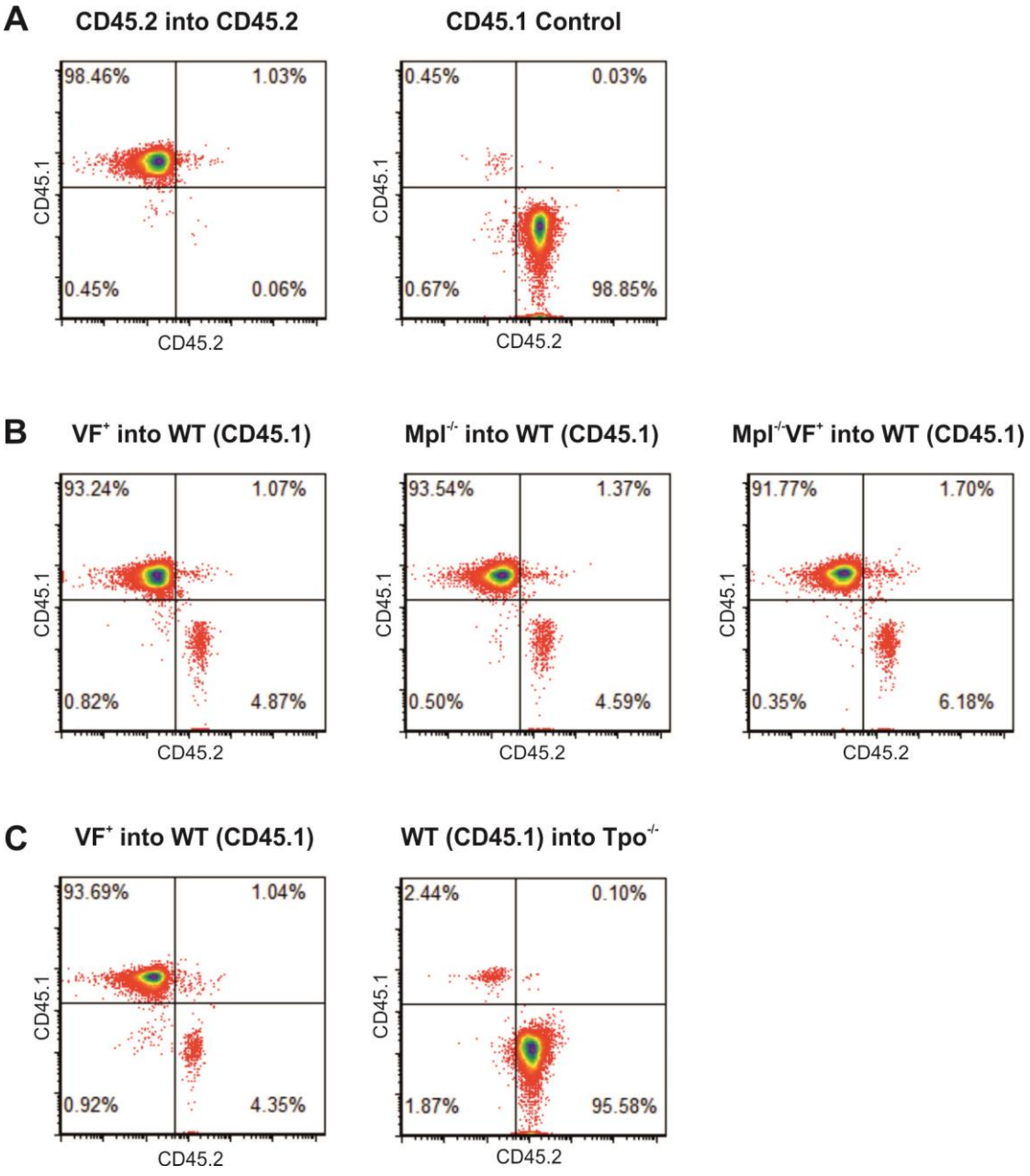


Figure 5.12 Representative chimerism plots for *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ and *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ transplant models

Peripheral blood was analysed by flow cytometry for CD45.1 vs CD45.2 expression in transplant models where possible. (A) Control samples. (B) Transplants for generation *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ and *JAK2*$^{V617F+}$*Tpo*$^{-/-}$

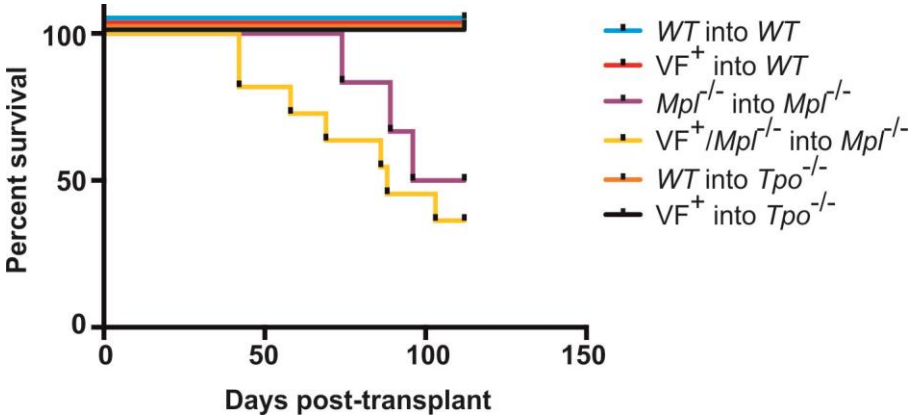


Figure 5.13 Survival proportions for *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ and *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ transplant mice.

Survival of transplant mice was monitored for 16 weeks post-transplant (112 days) and subjected to Kaplan-Meier survival analysis. Overlapping curves have been nudged for ease of viewing.

5.4 Discussion

5.4.1 *FF1*$^{+/-}$*Tie2*$^{+/-}$, *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{-/-}$, *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{+/-}$ and *FF1*$^{+/-}$*Tie2*$^{+/-}$*Tpo*$^{-/-}$ mice exhibit non-Mendelian patterns of inheritance

Although generation of *FF1*$^{+/-}$*Tie2*$^{+/-}$, *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{-/-}$, *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{+/-}$ and *FF1*$^{+/-}$*Tie2*$^{+/-}$*Tpo*$^{-/-}$ mice was successful, the number of viable experimental mice generated was vastly disproportionate from that expected based on the breeding scheme. There was no report of non-Mendelian inheritance patterns when the *FF1* mouse was originally generated or subsequently mated with *Vav-Cre* or *Mx-Cre* to generate *FF1*/*Vav-Cre* and *FF1*/*Mx-Cre* mice, respectively(191). Genetic analysis of *FF1*$^{+/-}$*Tie2*$^{+/-}$ mice reveals non-Mendelian inheritance. The average litter size reported for C57BL/6J mice by Jackson Laboratories is 4.9 (http://ko.cwru.edu/info/breeding_strategies_manual.pdf), which is similar to the average litter sizes observed from mating pairs generating *FF1*$^{+/-}$*Tie2*$^{+/-}$, *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{-/-}$ and *FF1*$^{+/-}$*Tie2*$^{+/-}$*Mpl*$^{+/-}$ mice. Therefore, the non-Mendelian pattern of inheritance exhibited for these matings may not be a result of embryos being prematurely resorbed *in utero*. In contrast, average litter size is reduced in matings generating *FF1*$^{+/-}$*Tie2*$^{+/-}$*Tpo*$^{-/-}$ mice. Thus, it is possible that a small amount of embryonic lethality is occurring. However, this average is similar to the average litter size for generation of *Tpo*$^{-/-}$ mice, which is 3.8 for our colony, suggesting that the decreased litter size is not due to embryonic lethality resulting from expression of *JAK2*^{V617F}. The decreased litter size for *Tpo*$^{-/-}$ compared to *Mpl*$^{-/-}$ mice is not unexpected as there are a number of clinical mutations that have been described in MPL(56,98) causing CAMT eventually resulting in complete bone marrow failure, yet there is only one report of a mutation in TPO able to recapitulate the disease(76). As TPO and MPL comprise of the same signalling axis, this suggests that although MPL mutations are detrimental, it is possible that mutations in TPO are lethal. Although it seems that introduction of the *JAK2*^{V617F} mutation is not causing abortion and resorption of embryos, it cannot be ruled out. The *Tie2* promotor is active in the haemangioblast(287), a precursor to HSCs and endothelial cells(Reviewed in (30)), and inducing *JAK2*^{V617F} expression so early on in our mouse model could be developmentally detrimental. Studies of angiogenesis in MPNs revealed increased microvessel density (MVD) in MPN patients(245). Additionally,

$CD105^{+}$-MVD, which are indicative of newly formed vessels, correlates with $JAK2^{V617F}$ allele burden(291). It is possible that because $JAK2^{V617F}$ is being expressed during early development in our congenital models, it results in dysregulated angiogenesis resulting in loss of embryos. Introduction of $JAK2^{V617F}$ during adulthood, as modelled via our transplant models, does not result in death resulting from introduction of the mutant *JAK2*. Although this suggests a defect resulting from early expression of $JAK2^{V617F}$; the actual effects on embryonic development have not been assessed.

It is notable that platelet counts for $FF1^{+/-}Tie2^{+/-}$ at 4 weeks are indistinguishable to counts from *WT* mice suggesting the possibility that $FF1^{+/-}Tie2^{+/-}$ mice having a more severe phenotype are naturally aborted prior to birth. Additional genetic experiments would need to be performed to determine the cause of the non-Mendelian inheritance pattern observed in $FF1^{+/-}Tie2^{+/-}$, $FF1^{+/-}Tie2^{+/-}Mpl^{-/-}$, $FF1^{+/-}Tie2^{+/-}Mpl^{+/-}$ and $FF1^{+/-}Tie2^{+/-}Tpo^{-/-}$ mice.

5.4.2 *Mpl* expression is increased when $JAK2^{V617F}$ is co-expressed

In $FF1^{+/-}Tie2^{+/-}$ mice, *Mpl* expression is increased when $JAK2^{V617F}$ is co-expressed. This is in contrast to a report that *Mpl* can be downregulated specifically by $JAK2^{V617F}$(292). Additionally, reduced *MPL* expression has been reported in platelets and megakaryocytes of MPN patients(238-241). However, *MPL* expression in these patients was assessed after development of the disease; therefore, it is difficult to distinguish if reduced MPL expression is a contributing factor or consequence of MPN development. Later studies reported heterogeneity of *MPL* expression within patients(243-247). Thus, the correlation between *MPL* expression in MPNs remains weak. It is possible that in our mice, the expression of human $JAK2^{V617F}$ in addition to expression of endogenous mouse *Jak2* results in the observed increase in MPL as JAK2 acts to stabilize the receptor(255). Another possibility is that *Mpl* expression decreases with disease progression. We have only examined expression at a single time point; however, monitoring MPL over time in our model or in patients will provide important information about the relationship between MPL and disease progression. Although this has not been performed in this study, it is currently under investigation.

5.4.3 *JAK2*^{V617F} expression levels in *FF1*$^{+/-}$*Tie2*$^{+/-}$ mice is physiological and is unaltered upon modulation of *Mpl* or *Tpo* expression

Since the identification of *JAK2*^{V617F} in 2005, a number of mouse models have been developed in an effort to better understand the underlying mechanisms associated with disease development and progression (Reviewed in (293)). The earliest models utilized BM transplantation of retrovirally transduced BM cells. Although these studies were able to show that *JAK2*^{V617F} expression is sufficient for MPN development in mice, expression levels were varied and sometimes super-physiological(186-189). However, in the *FF1* mouse generated by Tiedt et al, expression of *JAK2*^{V617F} remains under the control of the minimal *JAK2* promotor. Therefore, when the *FF1* mouse was crossed with either *VavCre* or *MxCre* mice, mutant *JAK2* expression was less than half of or equal to endogenous *Jak2*, respectively. This is in stark contrast to the three-fold higher expression of mutant *JAK2* reported in retrovirally transduced BM(191). *JAK2*^{V617F} expression in *FF1*$^{+/-}$*Tie2*$^{+/-}$ mice was comparable to endogenous *Jak2*, mirroring the ratio of *JAK2*^{V617F} to *JAK2*^{WT} reported in granulocytes of ET patients(191). This level of expression supports the ET-like phenotype observed in *FF1*$^{+/-}$*Tie2-Cre*$^{+/-}$ mice(149).

In approximately 30% of PV patients, a mitotic recombination event results in duplication event resulting in homozygosity for *JAK2*^{V617F}(179-181,285). Although these patients have progenitor cells that are homozygous for *JAK2*^{V617F}, overall they exhibit a combination of homozygous, heterozygous and WT progenitors(294). Based on the breeding strategy to generate *JAK2*^{V617F} (*FF1*$^{+/-}$*Tie2-Cre*$^{+/-}$) mice in this study, it is expected that the mice are heterozygous for the mutant allele. Homozygosity resulting from a gene duplication event was not analysed. The *JAK2*^{V617F} mutation however, was present in all granulocyte-macrophage progenitors tested. Although this is not common in MPN patients, it is conceivable that in a truly clonal disorder, expression of mutant *JAK2* is present in the majority of cells. Additionally, because there is a selective advantage for mutant progenitors(294), with time, the proportion of *JAK2*^{V617F}-positive cells predominates(183). In fact, there are clinical reports of *JAK2*^{V617F} expression in 100% of tested progenitors(183,295,296).

5.4.4 Transplantation models of *JAK2*^{V617F}*Mpl*$^{-/-}$ mice exhibit decreased survival

To mimic the conditions of an acquired mutation, BM transplants were performed to introduce *JAK2*^{V617F} in the bone marrow of adult *WT*, *Mpl*$^{-/-}$ or *Tpo*$^{-/-}$ mice. *WT* and *Tpo*$^{-/-}$ recipient mice were largely unaffected by irradiation and transplantation. In contrast, *Mpl*$^{-/-}$ mice exhibited decreased survival post-transplantation despite achieving at least 90% chimerism. It is likely that this is due to stem cell exhaustion. Although *Mpl*$^{-/-}$ mice are able to survive to adulthood without overt complications, humans lacking expression of functional MPL develop CAMT and suffer from stem cell exhaustion in childhood, necessitating a bone marrow transplant(98). When *Mpl*$^{-/-}$ bone marrow is transplanted into irradiated recipients, it must repopulate the BM compartment. As a high percentage of chimerism is achieved, these cells are capable of differentiation to fulfil this role. However, because MPL is necessary for maintenance of HSCs(94), it is possible that the capacity of the original pool of HSCs from the *Mpl*$^{-/-}$ mice to maintain normal haematopoiesis is diminished resulting in bone marrow failure and subsequent death.

Despite the unexpected non-Mendelian inheritance patterns observed for *JAK2*$^{V617F+}$ mice, in this chapter, we have generated viable *JAK2*$^{V617F+}$*Mpl*$^{+/-}$, *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ and *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice by genetic crossings. Additionally, we have generated *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ and *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ mice by transplantation to recapitulate acquisition of the mutation as with the human disease. These mouse models will allow us to study the roles of MPL and TPO in MPNs *in vivo*.

CHAPTER 6 THE ROLE OF THROMBOPOIETIN AND MPL IN MYELOPROLIFERATIVE NEOPLASM DEVELOPMENT IN VIVO

6.1 Experimental Rationale

Results from the previous chapter demonstrate that *MPL* expression is necessary for *JAK2*^{V617F}-mediated signalling and proliferation in cell lines, the *in vitro* correlate of myeloproliferation *in vivo*. However, its role in MPN development *in vivo* has yet to be demonstrated. Therefore, in this chapter, we sought to determine the role of MPL in MPN development utilizing a JAK2^{V617F}-positive MPN mouse model. Additionally, since TPO was able to induce a higher level of JAK/STAT signalling in the presence of JAK2^{V617F}, the role of TPO was also determined.

6.2 Materials and Methods

6.2.1 Complete blood counts

Whole blood was collected by submandibular bleeds, as described in 4.2.3.3, were used to perform complete blood counts. Counts were performed on a Hemavet®950 (Drew Scientific, Inc., Miami Lakes, FL, USA), which draws 20μL of whole blood for each count directly from the blood collection tube. To ensure accurate and consistent results, prior to each round of counts, a background read was taken followed by a count using a fixed mouse blood control from the manufacturer, which was then compared to known values. Complete counts include white blood cells (WBC), neutrophil (NE) count and percentage, lymphocyte (LY) count and percentage, monocyte (MO) count and percentage, eosinophil (EO) count and percentage, basophil (BA) count and percentage, red blood cells (RBC), haemoglobin (Hb), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelet count (PLT) and mean platelet volume (MPV).

6.2.2 Colony Forming Cell Assay

Myeloid colony forming assays were performed in methylcellulose-based medium containing 15% FBS, 2% BSA, 2mM L-glutamine, 5 x 10^{-5}M β-ME, 10µg/mL rh-insulin and 200µg/mL human transferrin in the presence or absence of 50ng/mL recombinant murine rmSCF, 10ng/mL rmIL-3, 10ng/mL rmIL-6 and 5IU/mL rhEpo (R&D Systems). Bone marrow cells were harvested as described in Section 2.1.2 and plated at 1 x 10^4 per dish in triplicate. Plates were incubated at 37ºC with 5% CO_2 for 7 days at which point colonies were scored by morphology. Single colonies were harvested from plates and lysed for mRNA analysis as previously described(149). Megakaryocyte colony forming assays were performed in collagen-containing medium containing 1% BSA, 10µg/mL rh insulin, 200µg/mL iron saturated human transferrin, 4% lipid solution, 1 x 10^{-4}M β-ME, 2mM L-glutamine (MegaCult™-C Collagen and Medium with Lipids; Stem Cell Technologies) in the presence or absence of 50ng/mL rhTPO and 10ng/mL rmIL-3. Bone marrow cells were plated at 1 x 10^5 per well of a double chamber slide (Stem Cell Technologies) in duplicate and incubated at 37ºC with 5% CO_2 for 7 days. Colonies were dehydrated, fixed in acetone and stained for 6 hours in acetylthiocholine iodide (Sigma-Aldrich) following the manufacturer's suggested protocol prior to scoring.

6.2.3 Flow Cytometry

E-SLAM HSC enumeration: Murine BM cells were harvested as described in Section 2.1.2. Red blood cells were lysed at 4ºC for 10min using a 1:10 DPBS:ammonium chloride solution (15.5mM NH4Cl, 1.2mM $NaHCO_3$, 0.1mM EDTA) followed by 3 washes with DPBS. 5 x 10^6 cells were stained using CD45 (Clone 104) (Biolegend, San Diego, CA, USA), EPCR(CD201) (Clone eBio1560), CD48 (Clone HM48-1) and CD150 (Clone mShad150) (eBioscience, San Diego, CA, USA) antibodies. $CD45^+EPCR(CD201)^+CD48^-CD150^+$ cells were considered E-$SLAM^+$. Spleen erythroblast determination: Murine spleens were obtained and homogenized. Single cell suspensions were obtained by syringe filter through a 100µm filter basket. A red cell lysis was performed and cells were stained with antibodies to murine TER119 and CD71 (Clone RI7217) (Biolegend). Flow cytometric analysis was performed on a BD Accuri C6 Flow Cytometer (BD Biosciences) using FCS express software (De Novo Software, Glendale, CA, USA).

6.2.4 Histology

For histological analysis of bone marrow, femurs were collected and epiphyses were removed, bones were then fixed in 4% paraformaldehyde for 24 hours and decalcified using Richard-Allan Scientific™ Decalcifying Solution (Thermo Scientific) following manufacturer's instructions. Paraffin sections (5-μm thickness) were stained with haematoxylin and eosin (H&E). For analysis of spleens, spleens were harvested and weighed. To control for differences resulting from intrinsic differences in size between males and females, spleens weights are represented as percent total body weight.

6.3 Results

6.3.1 *Mpl* and *Tpo* are necessary for development of *JAK2*^{V617F}-positive MPNs

Transgenic mice expressing human *JAK2*^{V617F}(149) were studied in an *Mpl* homozygous null (*JAK2*$^{V617F+}$*Mpl*$^{-/-}$), *Mpl* heterozygous (*JAK2*$^{V617F+}$*Mpl*$^{+/-}$) or *Tpo* homozygous null (*JAK2*$^{V617F+}$*Tpo*$^{-/-}$) background. Platelet MPL expression was halved in *Mpl* heterozygous (*Mpl*$^{+/-}$) mice compared to *Mpl* homozygous *WT* mice (*WT*) (Figure 5.7). Additionally, *JAK2*^{V617F} expression resulted in increased *Mpl* expression (Figure 5.7). Levels of *JAK2*^{V617F} cDNA ranged from 10% to 50% that of the endogenous murine *JAK2*(149), close to that expected in blood cells of patients with MPNs expressing a single copy of the mutant kinase.

JAK2^{V617F} expression in HSCs resulted in MPN development by 12 weeks of age with a significant increase in platelet (Figure 6.1A) and neutrophil count (Figure 6.1B) but no change in haematocrit or red blood cell count (Figure 6.1C and Figure 6.2) compared to *WT*.

Despite *JAK2*^{V617F} expression, in the complete absence of *Mpl* (*JAK2*$^{V617F+}$*Mpl*$^{-/-}$) the mice remained thrombocytopenic, with platelet levels similar to that of *Mpl*$^{-/-}$ controls (Figure 6.1A). When *Mpl* expression was halved (*JAK2*$^{V617F+}$*Mpl*$^{+/-}$), platelet counts were significantly decreased compared to *JAK2*$^{V617F+}$ controls, to levels comparable with *WT* mice (Figure 6.1A). Neutrophil counts were also reduced

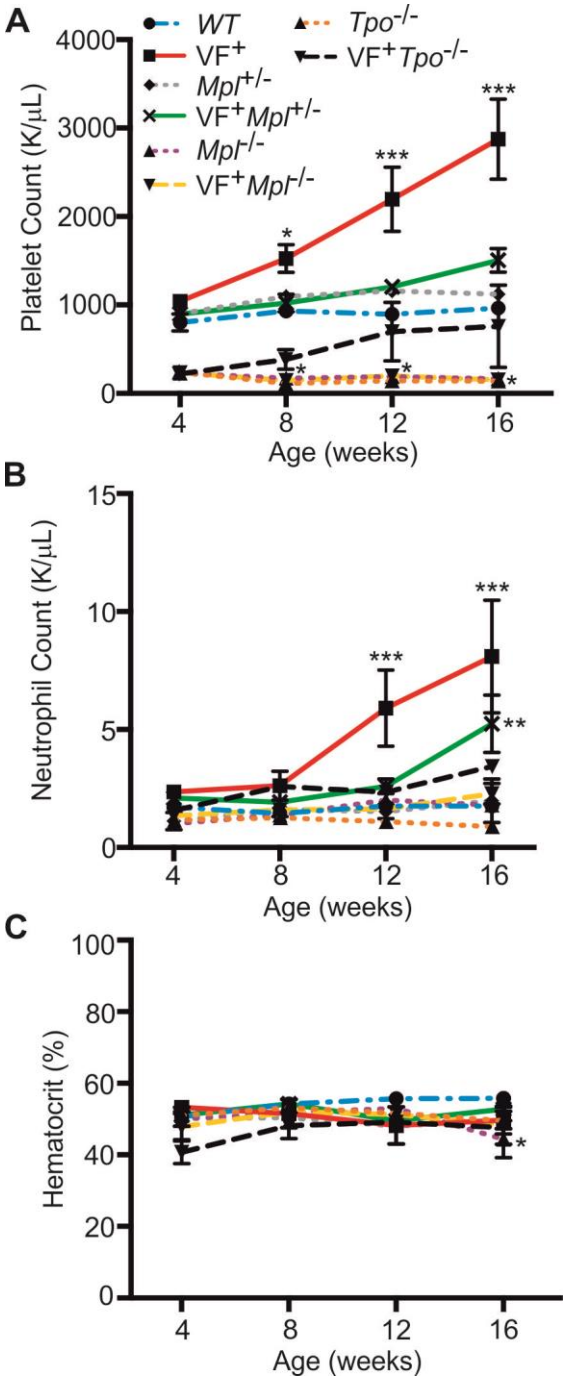


Figure 6.1 Platelets, neutrophils and haematocrit of congenital *JAK2*^{V617F}*Mpl* and *JAK2*^{V617F}*Tpo* mice

(**A**) Platelet (**B**) neutrophil counts and (**C**) haematocrit were monitored for 16 weeks after birth in *WT*, *JAK2*$V_{617}F^{+}$ (VF^{+}), *JAK2*$V_{617}F^{+}$*Mpl*$^{+/-}$ (VF^{+}*Mpl*$^{+/-}$), *JAK2*$V_{617}F^{+}$*Mpl*$^{-/-}$ (VF^{+}*Mpl*$^{-/-}$) and *JAK2*$V_{617}F^{+}$*Tpo*$^{-/-}$ (VF^{+}*Tpo*$^{-/-}$) mice. Data are presented as mean ±s.e.m; *n* = 3-9 mice per time point.

in *JAK2*$^{V617F+}$*Mpl*$^{+/-}$ compared to *JAK2*$^{V617F+}$ mice, but were significantly higher than *WT* controls at 16 weeks (Figure 6.1B). *Tpo* ablation (*JAK2*$^{V617F+}$*Tpo*$^{-/-}$) also significantly decreased platelet number compared to *JAK2*$^{V617F+}$ controls. However, expression of *JAK2*^{V617F} was able to increase platelet counts over time to levels comparable to *WT* levels (Figure 6.1A). Neutrophil counts, haematocrit and red blood cell counts in *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice were not significantly different from *WT* (Figure 6.1B-C and Figure 6.2).

The uptake and destruction of TPO by platelet MPL is a major regulator of circulating TPO blood levels(297). As expected, the thrombocythaemia observed in *JAK2*$^{V617F+}$ mice resulted in reduced TPO plasma levels, whereas *Mpl*$^{-/-}$ and *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ mice showed significantly increased plasma TPO levels (Figure 6.4). *Mpl*$^{+/-}$ and *JAK2*$^{V617F+}$*Mpl*$^{+/-}$ mice show a slight increase compared to *WT* and *JAK2*$^{V617F+}$ mice and *Tpo*$^{-/-}$ mice are completely TPO deficient (Figure 6.4). Increased bioavailability of TPO may provide a potential explanation as to why the *JAK2*$^{V617F+}$*Mpl*$^{+/-}$ mice display a moderate increase in platelet and neutrophil counts at 16 weeks (Figure 6.1A-B) and is also demonstrated by the increased baseline platelet count in *Mpl*$^{+/-}$ mice, compared to *Mpl*$^{-/-}$ mice.

We previously reported that this *JAK2*$^{V617F+}$ mouse model develops splenomegaly with abnormal splenic architecture, elevated number of megakaryocytes and significant expansion of primitive (TER-119^{+} CD71^{+}) and more mature (TER-119^{+} CD71^{-}) erythroblasts in the spleen(149). In the absence of, or with reduced expression of *Mpl*, splenomegaly was significantly reduced (P = 0.0166 and P = 0.0181, respectively compared to *JAK2*$^{V617F+}$ by one-way ANOVA; Figure 6.5E-F). Removal of *Mpl* (*JAK2*$^{V617F+}$*Mpl*$^{-/-}$) significantly decreased the number of splenic megakaryocytes (Figure 6.6A-B) and splenic erythrocyte expansion was reduced in *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ and *JAK2*$^{V617F+}$*Mpl*$^{+/-}$ mice (Figure 6.7). Conversely, removal of *Tpo* was not effective in reducing spleen size and weight as evidenced in our *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice (Figure 6.5). Splenic megakaryocytes were more prevalent in *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ compared to *JAK2*$^{V617F+}$ mice although average megakaryocyte size was reduced (Figure 6.6A-C). We also observed an expansion of splenic TER-119^{+} CD71^{+} erythroid progenitors in these mice (Figure 6.7).

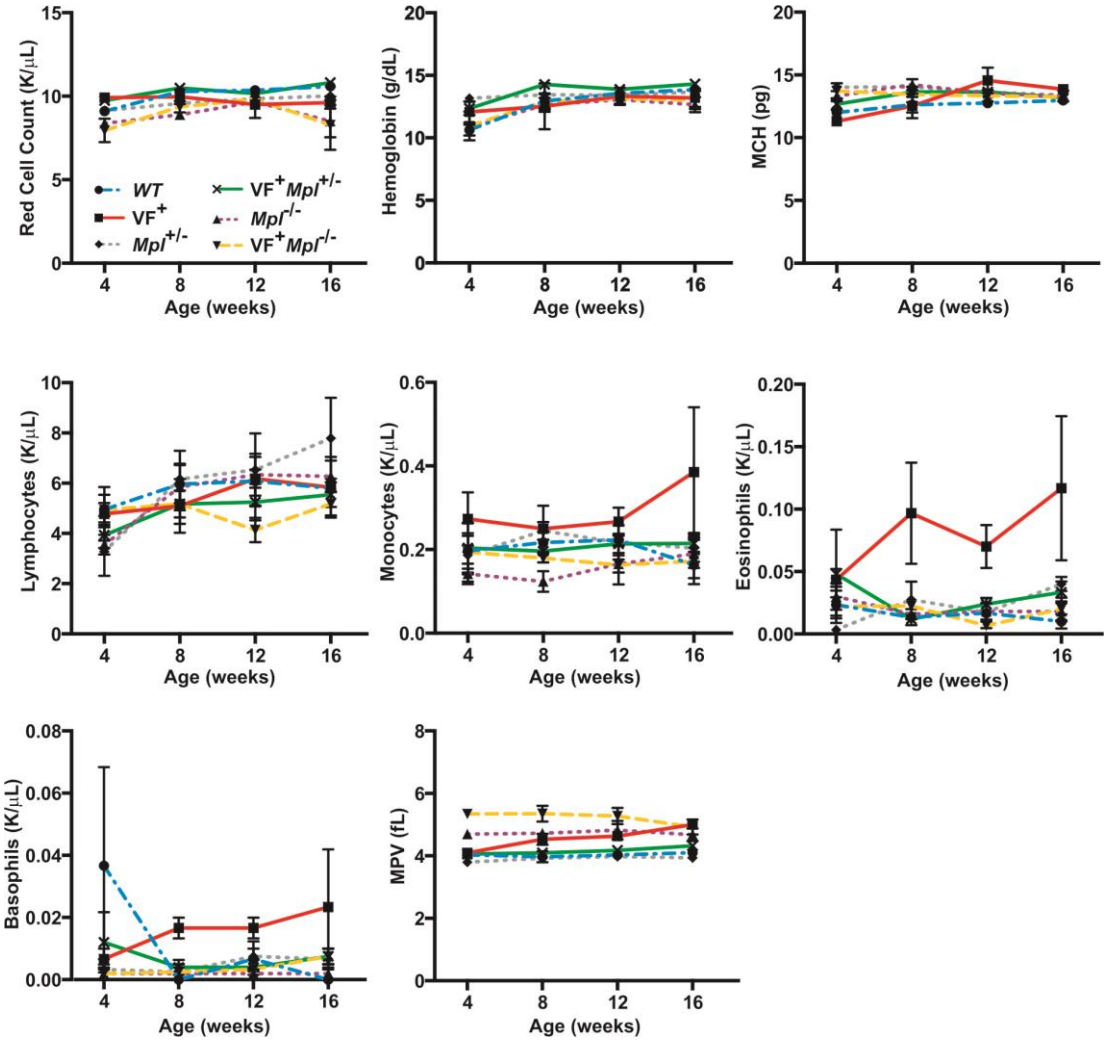


Figure 6.2 Blood counts for *JAK2*$^{V617F+}$*Mpl*$^{+/-}$ and *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ mouse models.

Blood counts were monitored in *WT*, *JAK2*$^{V617F+}$ (VF^{+}), *Mpl*$^{+/-}$, *JAK2*$^{V617F+}$*Mpl*$^{+/-}$ (VF^{+}*Mpl*$^{+/-}$), *Mpl*$^{-/-}$ and *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ (VF^{+}*Mpl*$^{-/-}$) mice over a 16-week period. Data are presented as mean ±s.e.m; n = 3-9 mice per time point.

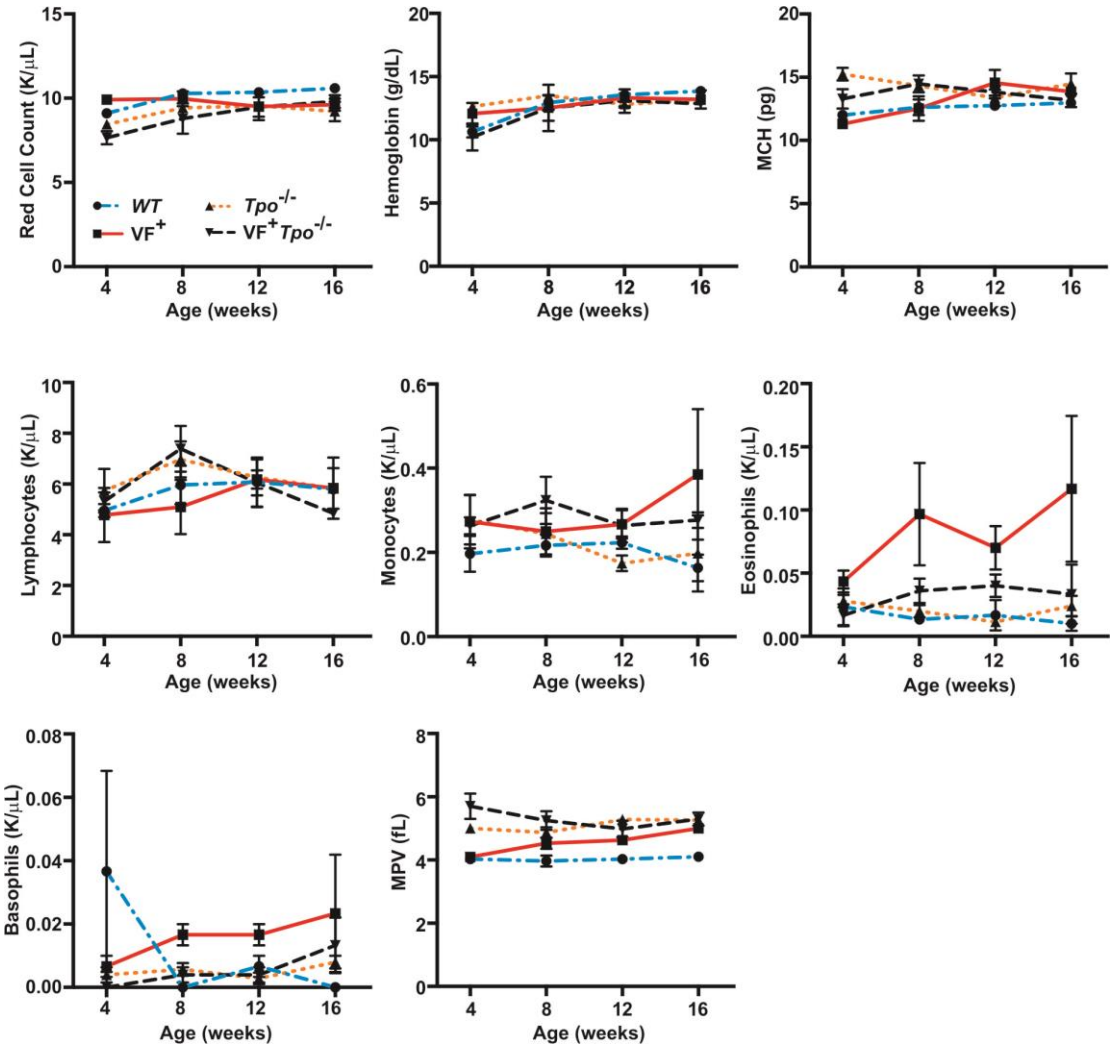


Figure 6.3 Blood counts for a *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ MPN mouse model.
Blood counts were monitored over a 16-week period in *WT*, *JAK2*$^{V617F+}$ (VF^{+}), *Tpo*$^{-/-}$ and *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice. *WT* and VF^{+} counts are the same in Supplemental Figure 2 for comparison purposes. Data are presented as mean ±s.e.m; n = 3-7 mice per time point.

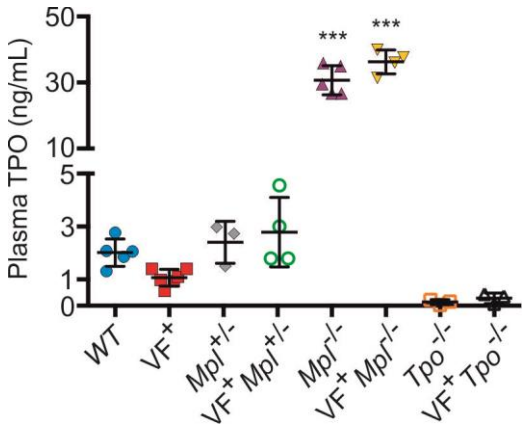


Figure 6.4 Plasma TPO levels in congenital *JAK2*^{V617F}*Mpl* and *JAK2*^{V617F}*Tpo* mice

Plasma TPO levels in *Mpl* and *Tpo* transgenic mice. Plasma was collected from 16-week old *WT*, *JAK2*$^{V617F+}$ (VF^{+}), *Mpl*$^{+/-}$, *JAK2*$^{V617F+}$*Mpl*$^{+/-}$ (VF^{+}*Mpl*$^{+/-}$), *Mpl*$^{-/-}$, *JAK2*V$_{617}$F^{+}*Mpl*$^{-/-}$ (VF^{+}*Mpl*$^{-/-}$), *Tpo*$^{-/-}$ and *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice and TPO levels were quantified by ELISA. Each data point represents a single mouse and bars are presented as mean ±s.e.m; asterisks indicate significance compared to *WT*.

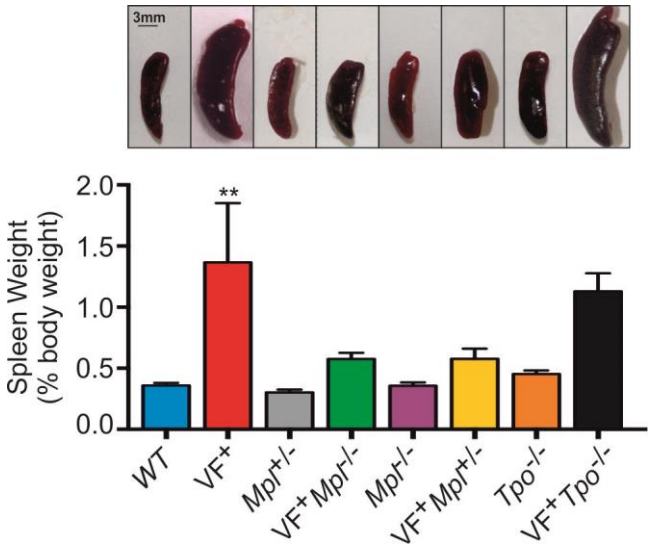


Figure 6.5 Spleens of congenital *JAK2*^{V617F}*Mpl* and *JAK2*^{V617F}*Tpo* mice
Representative spleens and weights of spleens harvested from 16 week old mice. Data are presented as mean ±s.e.m; *n* = 3-9 mice per time point. Scale bar, 3mm.

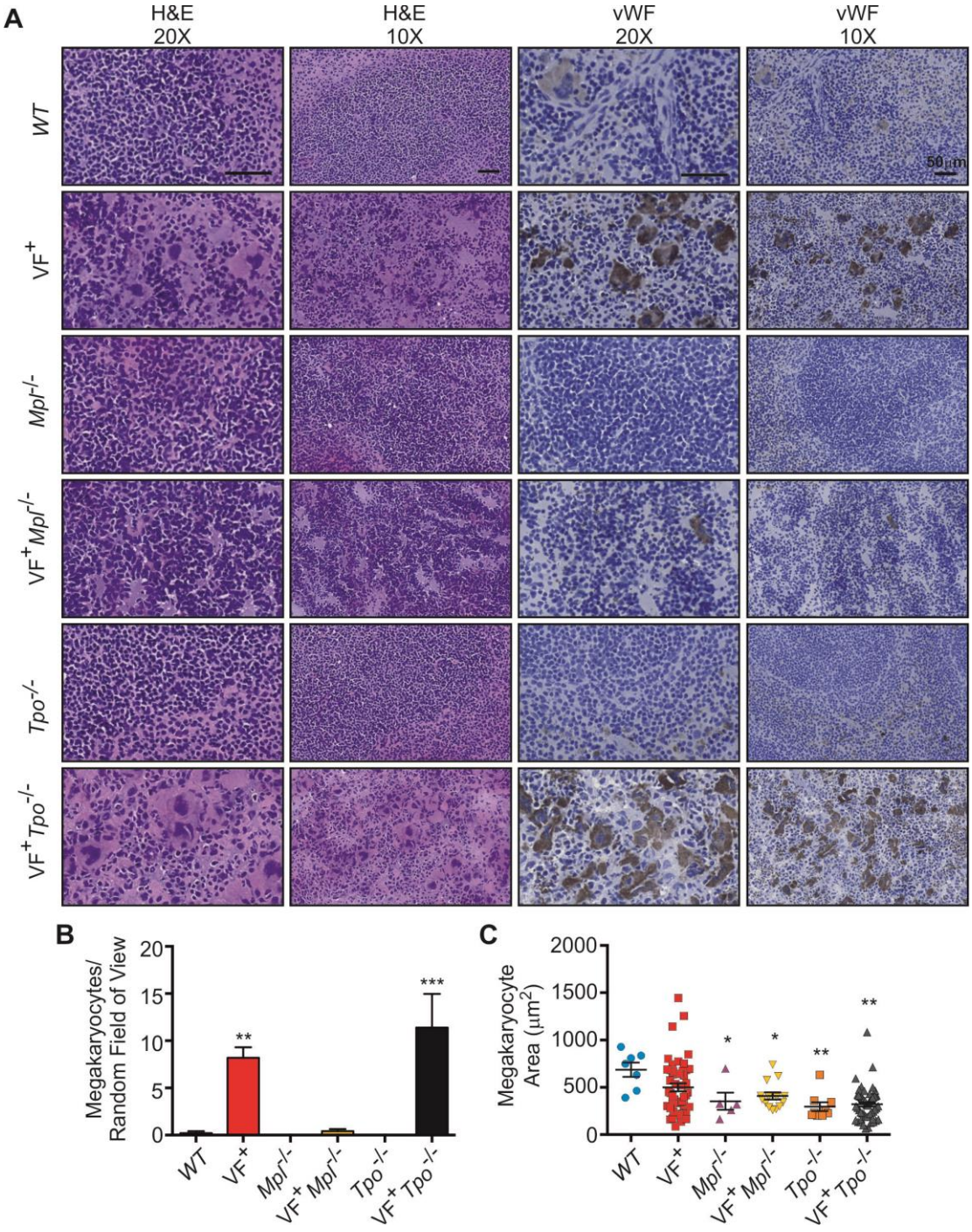


Figure 6.6 Spleen megakaryocyte analysis

(A) Representative H&E and vWF stained spleen sections from *JAK2*$^{V617F+}$ mouse models with megakaryocyte (B) count and (C) size. Scale bar, 50µm. Bars are presented as mean ±s.e.m ; asterisks indicate significance compared to *WT*, ***$P \leq 0.001$, **$P \leq 0.01$ and *$P \leq 0.05$ by one-way ANOVA.

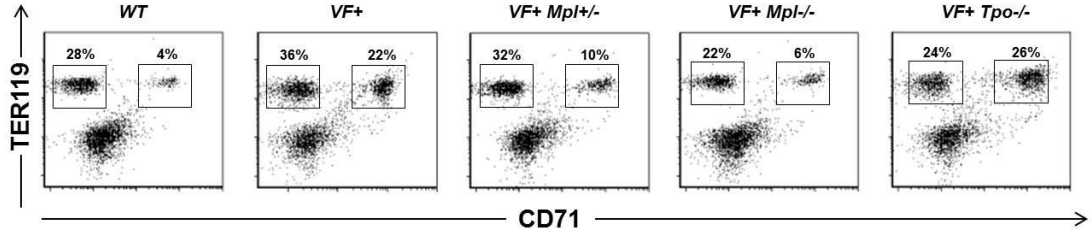


Figure 6.7 Analysis of mouse spleen erythroid progenitors
TER119 and CD71 antibody labeling and flow cytometric analysis revealed significant differences in the proportion of $TER119^{+}CD71^{+}$ cells in spleens of transgenic MPN mouse models at 16 weeks. An increase in expression of $TER119^{+}CD71^{+}$ cells indicates an increase in more primitive erythroblasts compared to $TER119^{+}CD71^{-}$ cells.

Bone marrow progenitor cells were analysed by colony forming unit (CFU) assays in the presence or absence of cytokine. In *WT* mice, the expression of *JAK2*^{V617F} significantly increased the number of cytokine-dependent (Figure 6.8A) and cytokine-independent (Figure 6.8B) marrow progenitor cells. However, removal of *Mpl* (*JAK2*$^{V617F+}$*Mpl*$^{-/-}$) resulted in CFU numbers being dramatically reduced both in the presence or absence of cytokine to levels not significantly different to those found in *WT* marrow (Figure 6.8A-B). Consistent with the observed thrombocytopenia, *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ mice exhibited an almost complete ablation of megakaryocyte progenitors, CFU-MK (Figure 6.8C). A similar phenotype was observed in factor-dependent colony formation in marrow from *JAK2*$^{V617F+}$*Mpl*$^{+/-}$ mice (Figure 6.8A), although in the absence of cytokine, CFU numbers were similar to *JAK2*$^{V617F+}$ controls (Figure 6.8B), potentially accounting for the neutrophilia observed in these mice at 16 weeks (Figure 6.1B). CFU-MK in *JAK2*$^{V617F+}$*Mpl*$^{+/-}$ mice and *Mpl*$^{+/-}$ controls were not significantly different to *WT* (Figure 6.8C). In the absence of cytokine, CFU numbers for *Tpo*$^{-/-}$ and *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice were significantly reduced compared to *JAK2*$^{V617F+}$ controls ($P < 0.0001$ and $P = 0.0014$, respectively compared to *JAK2*$^{V617F+}$ by one-way ANOVA; Figure 6.8B). Megakaryocyte progenitors of *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice were significantly increased compared to *WT*; however, there were still significantly less CFU-MK compared to *JAK2*$^{V617F+}$ mice ($P = 0.0013$ compared to *JAK2*$^{V617F+}$ by one-way ANOVA; Figure 6.8C). Of note, the observed reduction/elimination of *JAK2*^{V617F}-induced pathology was achieved despite expression of comparable levels of mutant *JAK2* in all of the *JAK2*$^{V617F+}$ mouse lines (Figure 5.10).

Histological analysis of bone marrow sections showed a dramatic increase in the number of megakaryocytes in *JAK2*$^{V617F+}$ mice compared to *WT*, an effect that was completely ablated in *JAK2*$^{V617F+}$*Mpl*$^{+/-}$ and *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ mice (Figure 6.9A-B). Similar to our previous results, *JAK2*$^{V617F+}$*Mpl*$^{+/-}$ and *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ appeared analogous to *WT* and *Mpl*$^{-/-}$, respectively. *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice showed an increase in the number of megakaryocytes compared to *WT*, which is consistent with the increase in CFU-MK. However, mature marrow megakaryocyte number was still significantly less than in *JAK2*$^{V617F+}$ marrow ($P < 0.001$ compared to *JAK2*$^{V617F+}$ by one-way ANOVA; Figure 6.9A-B). In the absence or reduction of *Mpl* and absence of *Tpo*, megakaryocyte size was significantly reduced (Figure 6.9C), consistent with

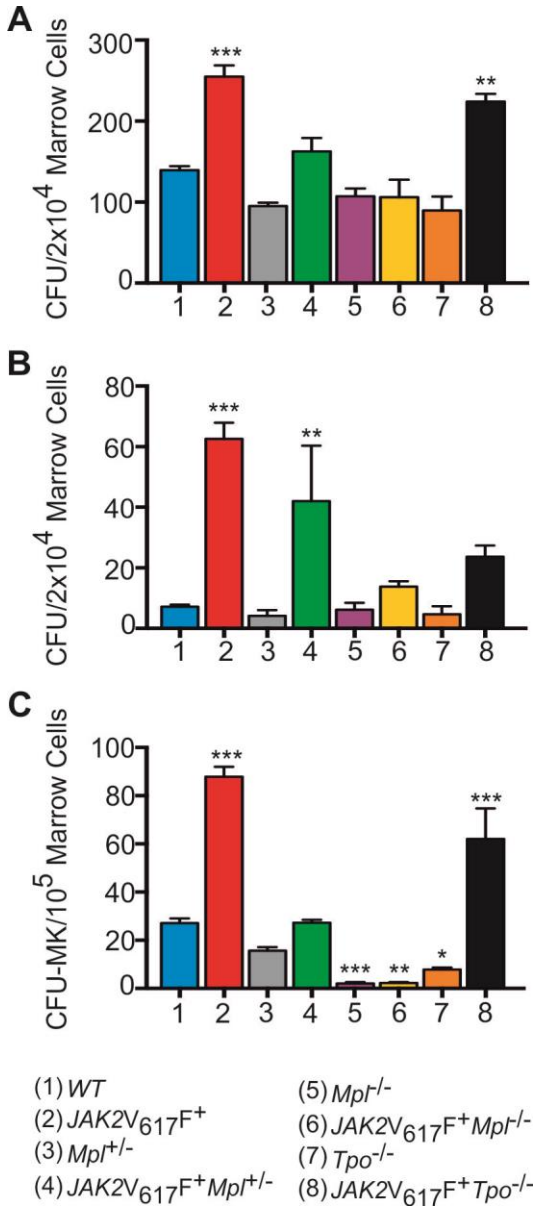


Figure 6.8 Progenitor cell analysis in congenital *JAK2*^{V617F}*Mpl* and *JAK2*^{V617F}*Tpo* mice

Data was collected from 16 week old *WT* , *JAK2*$^{V617F+}$, *Mpl*$^{+/-}$, *JAK2*$^{V617F+}$*Mpl*$^{+/-}$, *Mpl*$^{-/-}$, *JAK2*$^{V617F+}$*Mpl*$^{-/-}$, *Tpo*$^{-/-}$ and *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice. (**A**) Number of progenitor cells present per 2 x 10^4 marrow cells as determined by CFU assays in the presence and (**B**) absence of cytokine. Data are presented as mean ±s.e.m; *n* = 3-5. (**C**) CFU-MK per 1 x 10^5 marrow cells as determined by Megacult assays in the presence of cytokine. Data are presented as mean ±s.e.m. *n* = 3-5.

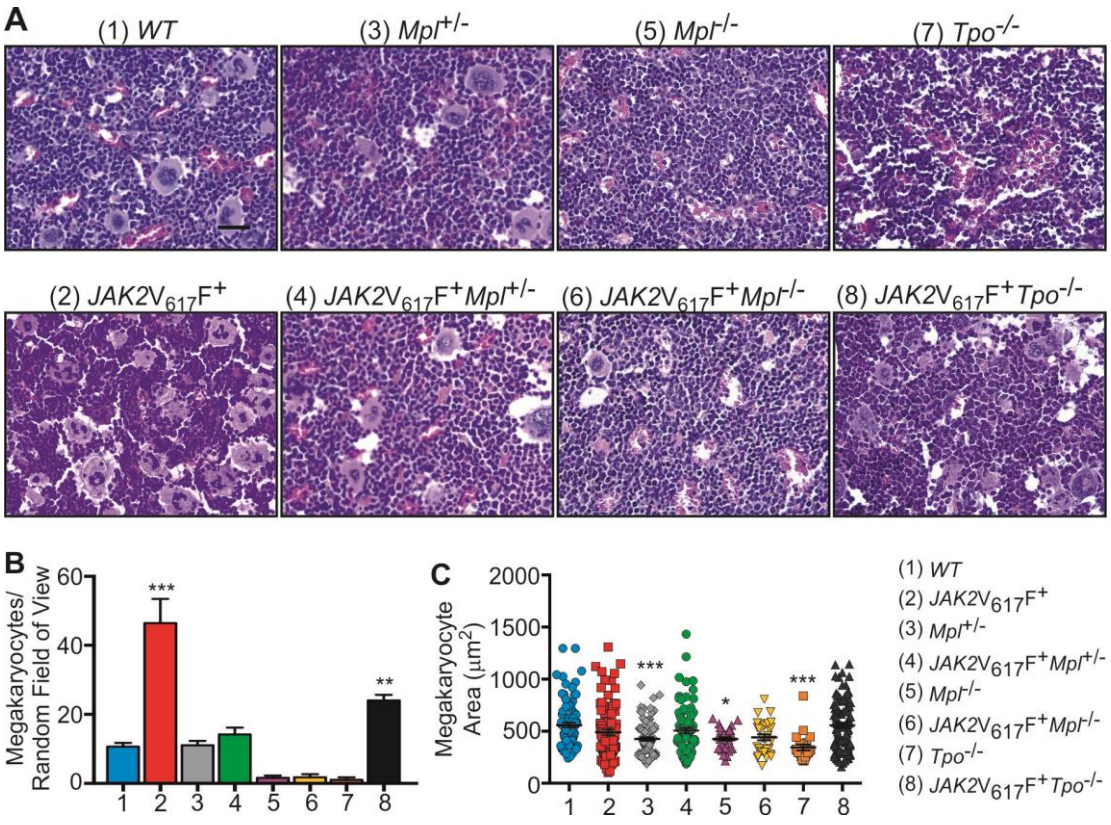


Figure 6.9 Bone marrow histology in congenital *JAK2*^{V617F}*Mpl* and *JAK2*^{V617F}*Tpo* mice

Data was collected from 16 week old *WT* , *JAK2*$^{V617F+}$, *Mpl*$^{+/-}$, *JAK2*$^{V617F+}$*Mpl*$^{+/-}$, *Mpl*$^{-/-}$, *JAK2*$^{V617F+}$*Mpl*$^{-/-}$, *Tpo*$^{-/-}$ and *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice. (A) Representative H&E stained sections of bone marrow with (**B**) megakaryocyte count and (**C**) size. Scale bar, 50μm.

previous reports(298). This effect was overcome by presence of *JAK2*^{V617F} (Figure 6.9C). Despite increases in megakaryocyte number in bone marrow of these mice, there was no detectable fibrosis of the marrow (Figure 6.10).

Taken together, these data clearly demonstrate that MPL is essential in MPN progression. Specifically, the *JAK2*^{V617F}-mediated MPN in this murine model fails to develop in the absence of *Mpl*; however, the disease was only nominally attenuated in the absence of *Tpo*. Importantly, we also demonstrate that reducing *Mpl* expression significantly attenuates MPN progression in *JAK2*$^{V617F+}$ mice, indicating that *Mpl* is a limiting factor for *JAK2*^{V617F}-driven MPNs.

6.3.2 Attenuation of *JAK2*^{V617F}-positive MPNs through removal of *Tpo* or *Mpl* is not a result of dysfunctional primitive haematopoiesis.

Mpl is expressed in primitive HSCs during embryogenesis and is also critical in the establishment of normal adult haematopoiesis(299). To ensure that the effects we observed in the *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ mice were not a result of dysfunctional primitive haematopoiesis, we performed a series of BM transplantation experiments using *JAK2*$^{V617F+}$ and *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ adult donor marrow cells. This allowed us to specifically determine the consequences of introducing the *JAK2*^{V617F} mutation during adulthood, therefore better reflecting the development of the human disease through acquisition of a somatic mutation in *JAK2*.

Due to its critical role in haematopoiesis, *Mpl*$^{-/-}$ marrow has severely decreased repopulating activity(96). Therefore, to ensure that the observed effects were due to engrafted donor marrow and not due to recovery of recipient marrow, donor cells lacking *Mpl* were transplanted into *Mpl*$^{-/-}$ recipients rather than *WT* recipients. To generate a *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ transplant model, *JAK2*$^{V617F+}$*Tpo*$^{+/+}$ marrow cells were transplanted into *Tpo*$^{-/-}$ recipients. As TPO is synthesized primarily in the liver, transplantation into a *Tpo*$^{-/-}$ background results in a functionally *Tpo*$^{-/-}$ mouse, although it is theoretically possible that engraftment of stromal cells from the *JAK2*$^{V617F+}$*Tpo*$^{+/+}$ marrow could supply a very low level of TPO in our mouse model(300). In control mice, chimerism was confirmed at 4 weeks and periodic monitoring verified it was maintained at an average of 95%±SD. Similar to the congenital *JAK2*$^{V617F+}$ mutants, *WT* recipient mice transplanted with *JAK2*$^{V617F+}$

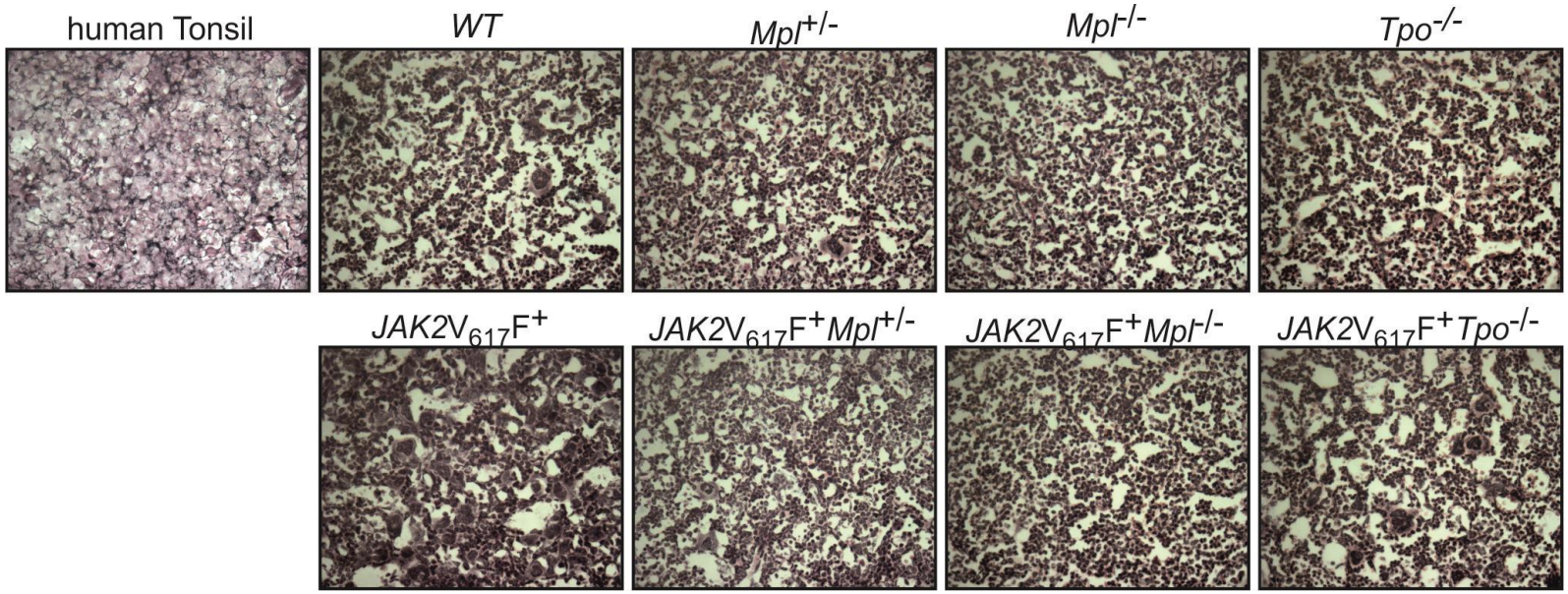


Figure 6.10 Bone marrow fibrosis analysis

Representative Gömöri reticulin stained bone marrow sections and a human tonsil stained control sample. Reticulin fibers – black; collagen – red; nuclei – grey/black and other tissues – yellow.

marrow developed a MPN by 12 weeks post-transplant, shown by increased platelet (Figure 6.11A) and neutrophil counts (Figure 6.11B). However, transplantation of *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ donor marrow (*VF*$^{+}$*Mpl*$^{-/-}$ *into Mpl*$^{-/-}$) failed to drive the development of a MPN (Figure 6.11A-B). Such transplant mice remained severely thrombocytopenic while neutrophil, haematocrit and red blood cells counts were not significantly different to *WT* (Figure 6.11A-C and Figure 6.12). In a *Tpo*$^{-/-}$ background, introduction of *JAK2*^{V617F} by transplantation (*VF*$^{+}$ into *Tpo*$^{-/-}$) was unable to generate a severe MPN, but did drive platelet production to levels comparable with *WT* (Figure 6.11A-C and Figure 6.13). The increased platelet count was accompanied by an increase in neutrophil count at 16 weeks post-transplant (Figure 6.11B).

Splenomegaly present in the *JAK2*$^{V617F+}$ into *WT* transplant mice was not observed in the *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ into *Mpl*$^{-/-}$ mice or *JAK2*$^{V617F+}$ into *Tpo*$^{-/-}$ transplants (Figure 6.14). Additionally, consistent with the blood counts, colony assays in the presence of cytokines demonstrated a significant increase in *JAK2*$^{V617F+}$ into *WT* bone marrow progenitors compared to *WT* into *WT*, *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ into *Mpl*$^{-/-}$ and *JAK2*$^{V617F+}$ into *Tpo*$^{-/-}$ mice (Figure 6.15A). The effect on the development of progenitor cells was even more apparent when cells were cultured in the absence of cytokine, with an approximate 10-fold decrease in the number of colonies developing from marrow derived from *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ into *Mpl*$^{-/-}$ transplant mice compared to marrow from *JAK2*$^{V617F+}$ into *WT* controls (Figure 6.15B). In the absence of cytokine, *JAK2*$^{V617F+}$ into *Tpo*$^{-/-}$ mice show a significant decrease in the number of colonies that developed compared to *JAK2*$^{V617F+}$ into *WT* animals ($P = 0.0068$; Figure 6.15B).

In *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ into *Mpl*$^{-/-}$mice, CFU-MK were depleted compared to *JAK2*$^{V617F+}$ into *WT* controls (Figure 6.15C), again demonstrating the importance of *Mpl* in *JAK2*$^{V617F+}$ MPNs. In *WT* into *Tpo*$^{-/-}$ mice, CFU-MK were similarly depleted; however, in *JAK2*$^{V617F+}$ into *Tpo*$^{-/-}$ mice, counts were comparable with *WT* into *WT* controls (Figure 6.15C). Consistent with the high platelet count, histological examination of *JAK2*^{V617F} into *WT* showed greatly elevated numbers of megakaryocytes present in the bone marrow (Figure 6.16A-B). However, this effect was absent in *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ into *Mpl*$^{-/-}$ and *JAK2*$^{V617F+}$ into *Tpo*$^{-/-}$ transplants (Figure 6.16 A-B). Megakaryocytes from *JAK2*$^{V617F+}$ mice were larger than *WT*,

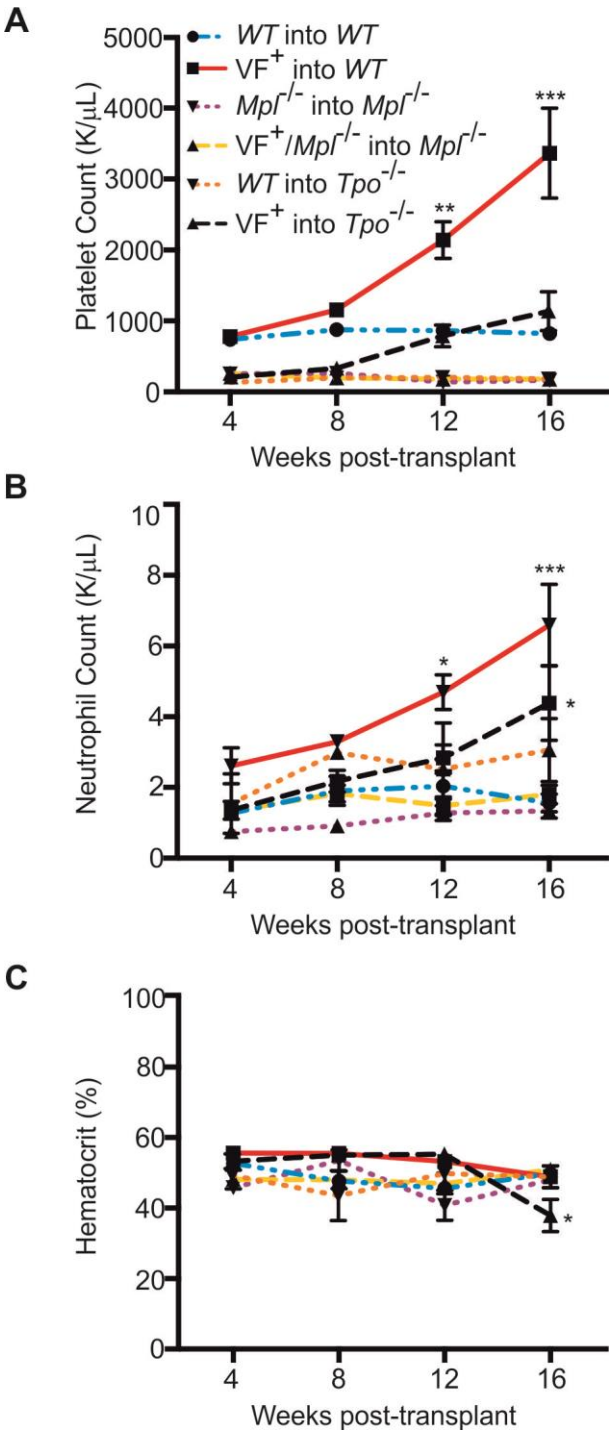


Figure 6.11 Platelets, neutrophils and haematocrit of transplant models of *JAK2*^{V617F}*Mpl*$^{-/-}$ and *JAK2*^{V617F}*Tpo*$^{-/-}$ mice

(A) Platelet (B) neutrophil counts and (C) haematocrit from *WT* into *WT*, *JAK2*$^{V617F+}$ into *WT* (VF^{+} into *WT*), *Mpl*$^{-/-}$ into *Mpl*$^{-/-}$, *JAK2*$^{V617F+}$ *Mpl*$^{-/-}$ into *Mpl*$^{-/-}$ (VF^{+} into *Mpl*$^{-/-}$), *WT* into *Tpo*$^{-/-}$ and *JAK2*$^{V617F+}$ into *Tpo*$^{-/-}$ transplant mice were monitored for 16 weeks post-transplant. Data are presented as mean ±s.e.m; *n* = 2-10 mice per time point.

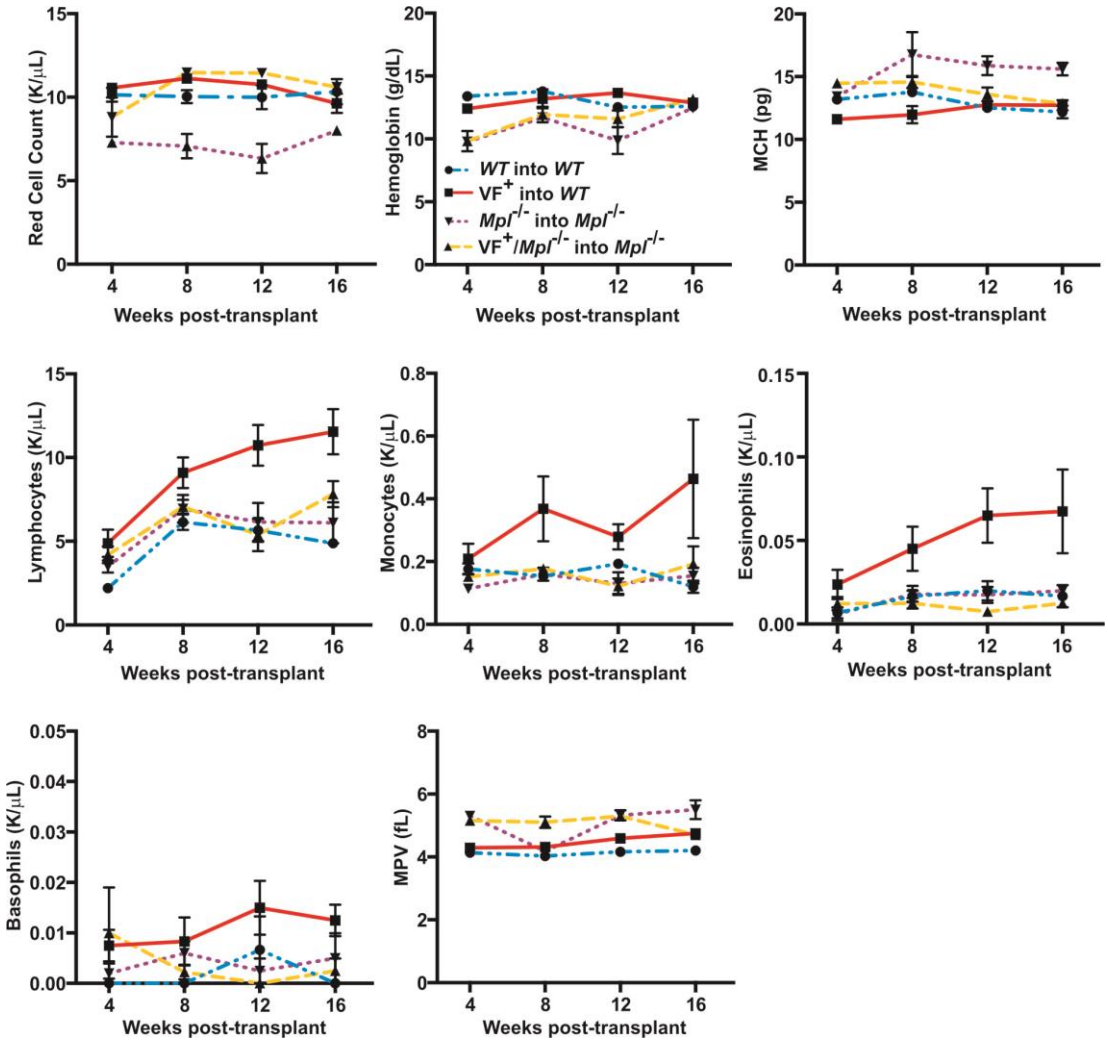


Figure 6.12 Blood counts for *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ transplant models
Blood counts were monitored in *WT* into *WT*, *JAK2*$^{V617F+}$ into *WT* (VF^{+} into *WT*), *Mpl*$^{-/-}$ into *Mpl*$^{-/-}$ and *JAK2*$^{V617F+}$ *Mpl*$^{-/-}$ into *Mpl*$^{-/-}$ (VF^{+} into *Mpl*$^{-/-}$) mice for 16-weeks post-transplant. Data are presented as mean ±s.e.m; n=2-10 mice per time point.

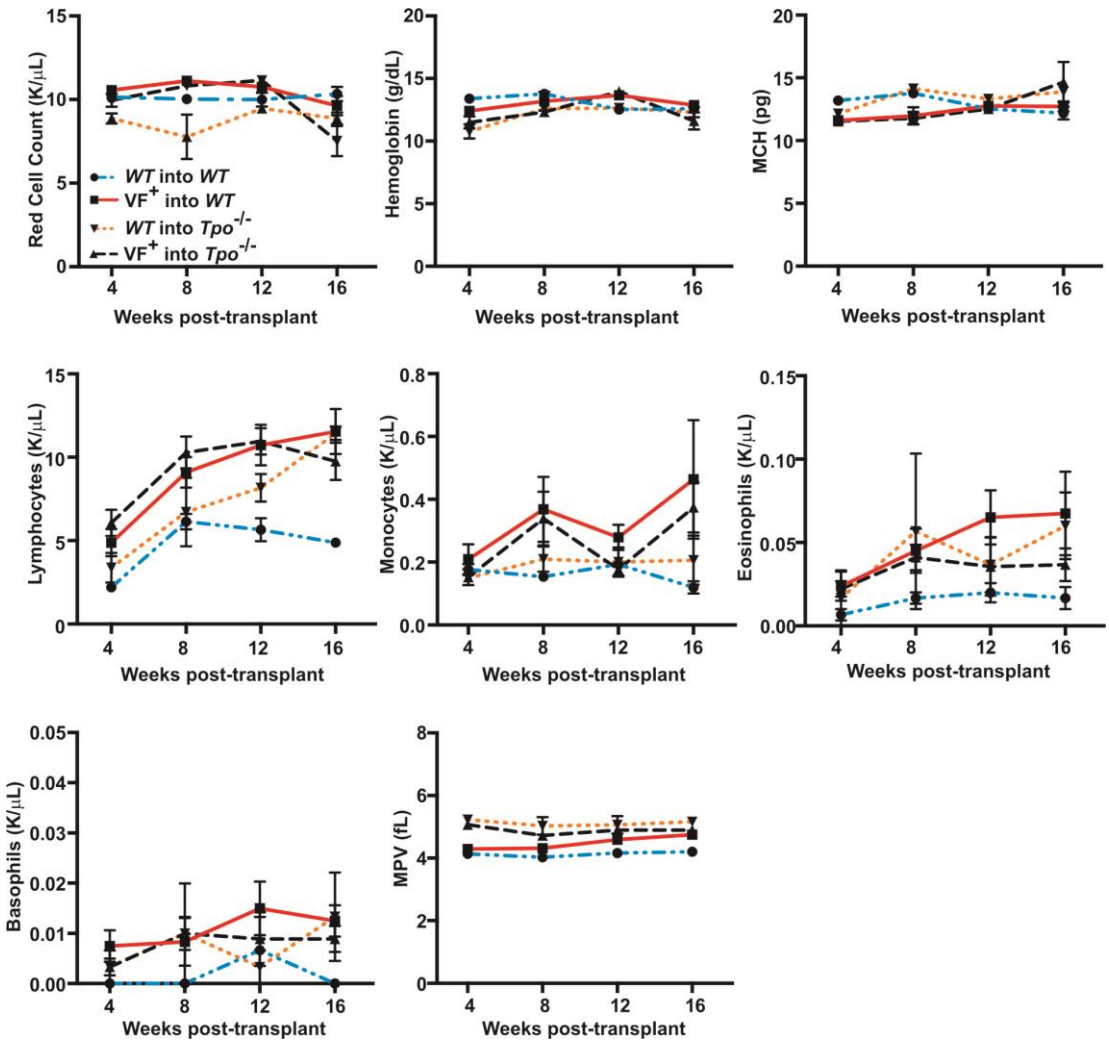


Figure 6.13 Blood counts for *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ transplant models
Blood counts were monitored in *WT* into *WT*, *JAK2*$^{V617F+}$ into *WT* (VF^{+} into *WT*), *WT* into *Tpo*$^{-/-}$ and *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice for 16-weeks post-transplant. Data are presented as mean ±s.e.m; *n*=3-9 mice per time point. *WT* into *WT* and *JAK2*$^{V617F+}$ into *WT* (VF^{+} into *WT*) counts are the same in Supplemental Figure 9 for comparison purposes.

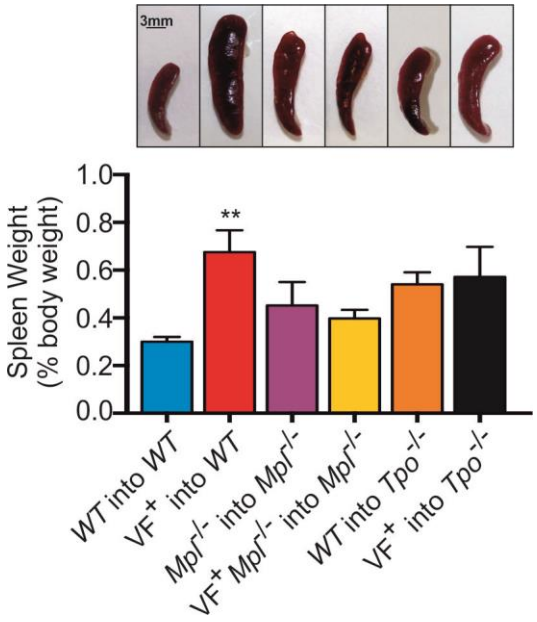


Figure 6.14 Spleens from *JAK2*^{V617F}*Mpl*$^{-/-}$ and *JAK2*^{V617F}*Tpo*$^{-/-}$ transplant models
Representative spleens and weights of spleens harvested 16 weeks post-transplant. Data are presented as mean ±s.e.m; *n* = 2-10 mice per time point. Scale bar, 3mm.

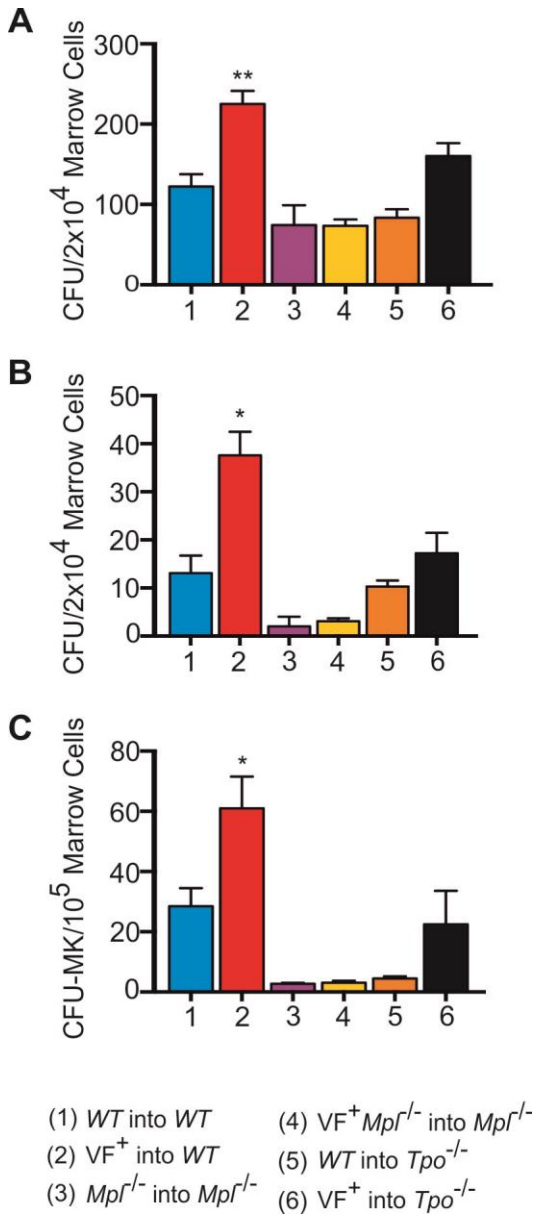


Figure 6.15 Progenitor cell analysis in *JAK2*^{V617F}*Mpl*$^{-/-}$ and *JAK2*^{V617F}*Tpo*$^{-/-}$ transplant models

Data was collected from *WT* into *WT*, *JAK2*$^{V617F+}$ into *WT* (VF^{+} into *WT*), *Mpl*$^{-/-}$ into *Mpl*$^{-/-}$, *JAK2*$^{V617F+}$ *Mpl*$^{-/-}$ into *Mpl*$^{-/-}$ (VF^{+} into *Mpl*$^{-/-}$), *WT* into *Tpo*$^{-/-}$ and *JAK2*$^{V617F+}$ into *Tpo*$^{-/-}$ mice 16 weeks post-transplant. (**A**) Number of progenitor cells present per 2 x 10^4 marrow cells as determined by CFU assays in the presence and (**B**) absence of cytokine. Data are presented as mean ±s.e.m; *n* = 2-12. (**C**) CFU-MK per 1 x 10^5 marrow cells as determined by Megacult assays in the presence of cytokine. Data are presented as mean ±s.e.m. *n* = 2-12.

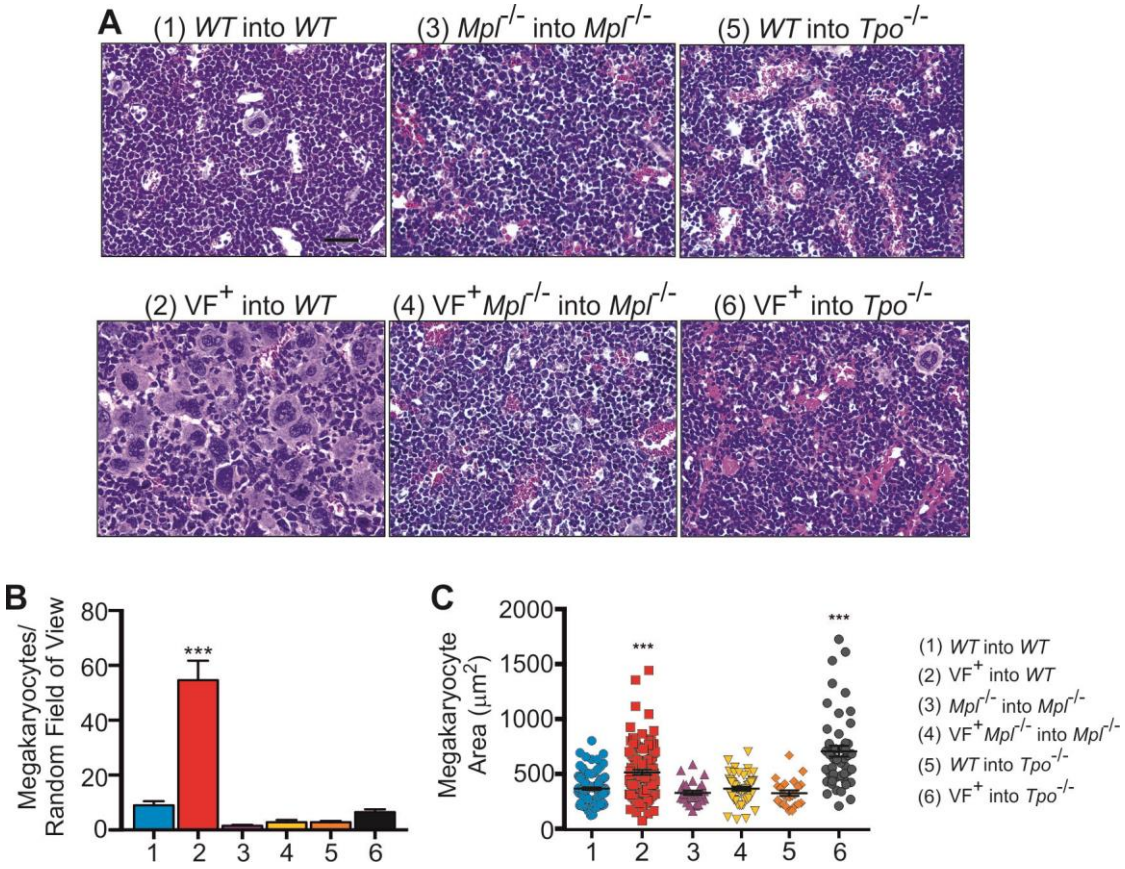


Figure 6.16 Bone marrow histology of *JAK2*^{V617F}*Mpl*$^{-/-}$ and *JAK2*^{V617F}*Tpo*$^{-/-}$ transplant models

Data was collected from *WT* into *WT*, *JAK2*$^{V617F+}$ into *WT* (VF^{+} into *WT*), *Mpl*$^{-/-}$ into *Mpl*$^{-/-}$, *JAK2*$^{V617F+}$ *Mpl*$^{-/-}$ into Mpl$^{-/-}$ (VF^{+} into Mpl$^{-/-}$), *WT* into *Tpo*$^{-/-}$ and *JAK2*$^{V617F+}$ into *Tpo*$^{-/-}$ mice 16 weeks post-transplant. (A) Representative H&E stained sections of bone marrow with (B) megakaryocyte count, where data are presented as mean ±s.e.m. n = 2-12 and (C) size where each data point represents a single megakaryocyte. Scale bar, 50μm.

consistent with the higher platelet count in these mice (Figure 6.16C). However, despite reduced numbers of megakaryocytes in *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice, their megakaryocytes were also larger compared to *WT* which may explain the gradual increase in platelet count (Figure 6.16C). Similar to results from our congenital mouse models, plasma TPO levels were high in mice with an *Mpl*$^{-/-}$ background and absent in *Tpo*$^{-/-}$ mice (Figure 6.17). Together, these data demonstrate that *Tpo* plays a less significant role in *JAK2*$^{V617F+}$ MPN development, while *Mpl* expression in HSCs is absolutely required for *JAK2*^{V617F}-mediated transformation. Additionally, introduction of *JAK2*^{V617F} during adulthood is sufficient for development of an MPN, but in this setting too, the disease is partially dependent on *Tpo* expression and entirely dependent on expression of *Mpl.*

6.3.3 *JAK2*^{V617F} promotion of HSC expansion is dependent on *Mpl* expression.

Given that the *JAK2*^{V617F} mutation is acquired in HSCs and the importance of both *Tpo* and *Mpl* in HSC maintenance, we determined whether the observed differences in pathology in our mouse models were due to a change in the number of primitive HSCs. Therefore, E-SLAM^{+} (CD45^{+}EPCR(CD201)$^{+}$CD48^{-}CD150^{+}) cells, a highly purified population of long-term repopulating (LTR) HSCs(39), were enumerated in marrow (Figure 6.18 and Figure 6.19). In our congenital models of MPNs we found that *JAK2*^{V617F} expression significantly increased the number of E-SLAM^{+} HSCs compared to *WT* (Figure 6.19A). *Mpl* ablation resulted in greatly decreased E-SLAM^{+} population, both in the presence and absence of *JAK2*V_{617}F (Figure 6.19A). However, *JAK2*$^{V617F+}$ *Mpl*$^{+/-}$ mice showed reduced numbers of E-SLAM^{+} cells compared to *JAK2*$^{V617F+}$ controls to a level similar to *WT*, demonstrating that both *JAK2*^{V617F} and *Mpl* expression are important for the pathologic increase of HSCs observed in MPNs (Figure 6.19A). Conversely, E-SLAM^{+} numbers in *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice are comparable to *JAK2*$^{V617F+}$ controls, thus *Tpo* is not necessary for *JAK2*^{V617F} to promote HSC expansion (Figure 6.19A). *Mpl* expression was also found to be critical in expanding the marrow E-SLAM^{+} populations in our transplant models of MPNs whereas removal of *Tpo* had little or no effect (Figure 6.19B). Our data indicate that *JAK2*^{V617F} significantly increases the pool of E-SLAM^{+} HSCs, likely promoting MPN development. This data shows that the proliferative effect of *JAK2*^{V617F} on HSCs is partially dependent on *Tpo* expression

and entirely dependent on *Mpl* expression. The decreased number of HSCs associated with *Mpl* reduction or ablation may provide a potential mechanism through which the absence of *Mpl* prevents MPN development.

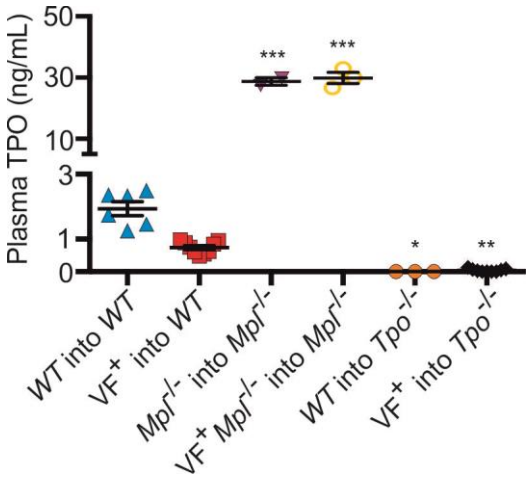


Figure 6.17 Plasma TPO levels in *JAK2*^{V617F}*Mpl*$^{-/-}$ and *JAK2*^{V617F}*Tpo*$^{-/-}$ transplant models

Plasma was collected 16 weeks post-transplant from *WT* into *WT*, *JAK2*$^{V617F+}$ into *WT* (VF^{+} into *WT*), *Mpl*$^{-/-}$ into *Mpl*$^{-/-}$, *JAK2*$^{V617F+}$ *Mpl*$^{-/-}$ into *Mpl*$^{-/-}$ (VF^{+} into *Mpl*$^{-/-}$), *WT* into *Tpo*$^{-/-}$ and *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice and TPO levels were quantified by ELISA. Each data point represents a single mouse and bars are presented as mean ±s.e.m; asterisks indicate significance compared to *WT* into *WT*.

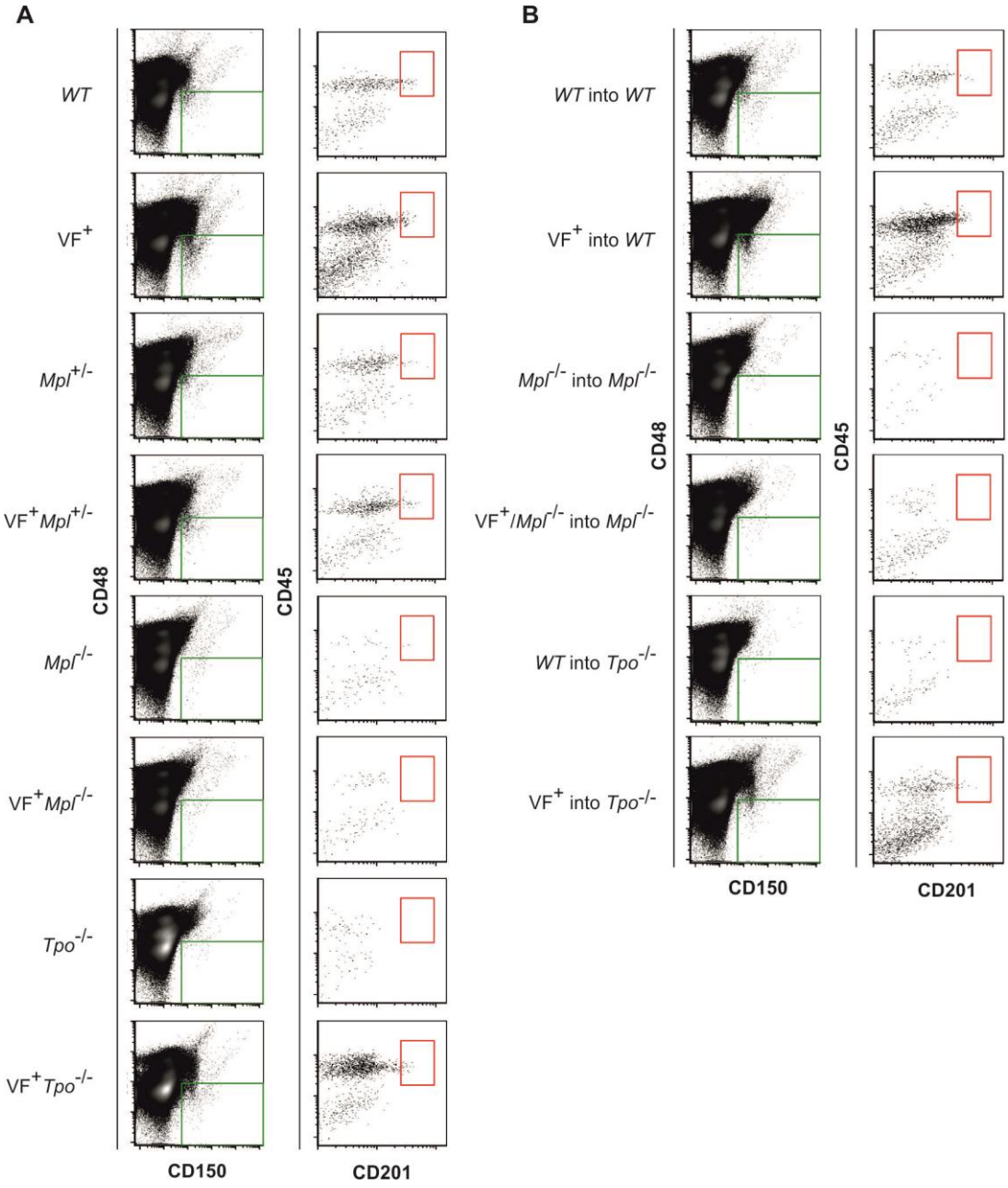


Figure 6.18 E-SLAM$^+$ (CD45$^+$EPCR(CD201)$^+$CD48$^-$CD150$^+$) cell identification.
Representative flow cytometry plots for the identification of E-SLAM$^+$ cells. 1 x 10^6 bone marrow cells from (A) 16 week old mice and (B) mice 16 weeks post-transplant were stained using CD45-AF488 (Clone 104), EPCR(CD201)-PE, CD48-PE/Cy7 (Clone HM48-1) and CD150-APC (Clone mShad150) antibodies and analysed. Live cells were first gated for CD48$^-$CD150$^+$ (green) then subsequently gated for CD45$^+$CD201$^+$ (red).

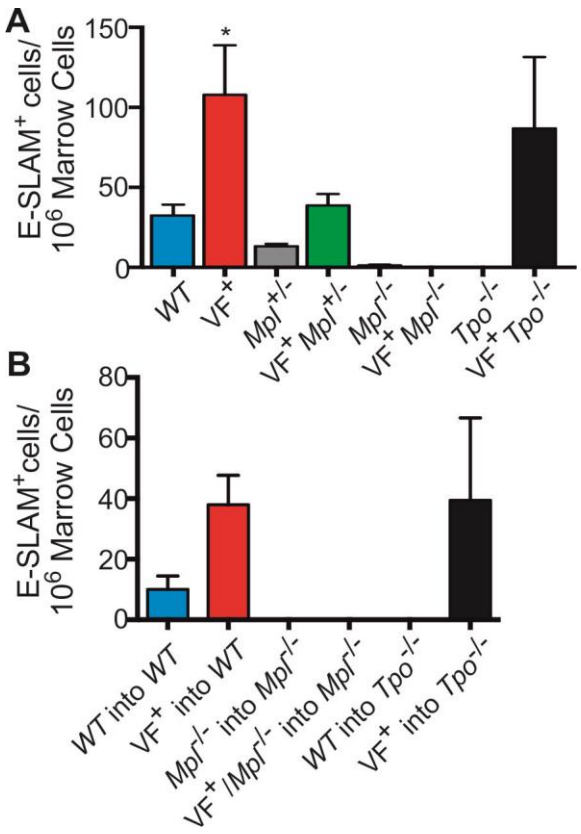


Figure 6.19 E-SLAM enumeration

(**A**) Bone marrow cells harvested from 16-week old congenital or (**B**) transplant mice 16-weeks post-transplant were analysed for number of E-SLAM$^+$ HSCs by flow cytometry. Data are presented as mean ±s.e.m; *n* = 2-9 mice per group.

6.4 Discussion

TPO and MPL are well known for their role in HSC maintenance and the regulation of platelet production(94,97,301). Like many type I cytokine receptors, MPL lacks intrinsic kinase activity and relies on JAK2 for signal transduction. Activating mutations in MPL cause ET and recent experimental data shows that JAK2 is required for this process(302), further highlighting the interdependency of MPL and JAK2. As MPNs are a stem cell disorder and given the role of MPL and TPO in HSC homeostasis and the dependency of MPL on JAK2, here we investigated the role of TPO and MPL in *JAK2*$^{V617F+}$ MPNs.

In addition to MPL, JAK2 is also responsible for signal transduction of the EPOR, G-CSFR, interleukin-3 receptor (IL-3R) and stem cell factor receptor (c-KIT)(303-305). Thus, it was possible that removal of *Mpl* could result in differential receptor interaction of JAK2. However, modulation of *Mpl* or *Tpo* expression resulted in changes to the severity of the MPN compared to our *JAK2*$^{V617F+}$ control model, but did not affect the type of MPN developed. For example, the drop in platelet and neutrophil counts resulting from modulation of *Mpl* or *Tpo* expression was not accompanied by an increase in haemoglobin, red cell count or haematocrit which would be indicative of PV. This finding would suggest that MPL is critical in the oncogenic transformation of HSCs in *JAK2*^{V617F}-positive MPNs. As the effect of MPL modulation is observed at the HSC level, we postulate that reduction or removal of MPL in a PV mouse model will also ameliorate the disease. However, as our mouse model recreates a disease similar to ET, future investigations are required to see if MPL is necessary for the development of other *JAK2*$^{V617F+}$ MPNs such as PV and PMF.

Although complete *Mpl* ablation in our model prevented MPN development, it resulted in thrombocytopenia. This is recapitulated in humans that lack functional MPL, resulting in defective responses to TPO causing CAMT(98). However, in CAMT patients, unlike our mouse models, thrombocytopenia progresses to pancytopenia and these patients require a bone marrow transplant, making complete removal of MPL an implausible means for therapeutic intervention. Nevertheless, our studies highlight the critical importance of *Mpl* in *JAK2*$^{V617F+}$ MPN development

in vivo. Indeed the necessity of MPL is highlighted in our *JAK2*$^{V617F+}$*Mpl*$^{+/-}$model, as reducing *Mpl* expression resulted in severely attenuated MPN progression, with platelet levels comparable to *WT*. Additionally, whereas HSCs were dramatically reduced in *JAK2*$^{V617F+}$*Mpl*$^{-/-}$ mice, expression of half the amount of *Mpl* in conjunction with *JAK2*^{V617F} decreases the neoplastic HSC pool.

Recent reports have provided new insights into the roles of TPO/MPL/JAK2 in megakaryopoiesis. Ng and colleagues have shown that knocking out *Mpl* specifically in megakaryocytes and platelets caused myeloproliferation as a result of increased TPO availability(306). In addition, Meyer et al demonstrated that ablation of *Jak2* specifically in megakaryocytes resulted in stem and progenitor cell expansion and thrombocytosis, presumably due to reduced internalization and degradation of the TPO/MPL complex in the absence of JAK2(307). Similarly, using UT7-MPL cell lines, Besancenot et al. showed that low levels of MPL expression increased TPO-stimulated proliferation, while low doses of JAK2 chemical inhibitors caused increased platelet production in mice(308). It would appear that some of these data contradict our findings that reduced *Mpl* expression prevents *JAK2*^{V617F}-mediated myeloproliferation. However, there are a number of differences between the studies which may explain the varying results. In our study, we reduced or ablated *Mpl* expression throughout haematopoiesis, not just in megakaryocytes or platelets as in the studies above. Therefore, in our system, the increases in circulating TPO caused by reduced platelet MPL expression, fails to stimulate myeloproliferation due to the reduction or absence of MPL in haematopoietic stem and progenitor cells. It would also appear that there are significant differences between the roles of TPO/MPL in megakaryopoiesis and their roles in haematopoietic progenitor expansion. Whereas the data presented by Meyer et al. and Besancenot et al. specifically investigate the function of MPL in megakaryopoiesis *in vivo* or megakaryocytic cell lines *in vitro,* we have focused more on the roles of MPL/*JAK2*^{V617F} in neoplastic progenitor cell expansion. Taken together, these data suggest that our understanding of TPO/MPL/JAK2 in haematopoiesis is still evolving and the biphasic roles of MPL should be taken into account when targeting MPL therapeutically.

Several groups have shown that expression of MPL or EPOR in conjunction with *JAK2*^{V617F} in cell lines was sufficient to stimulate proliferation in the absence of

cytokines(264,309,310), suggesting that the presence of *JAK2*^{V617F} and a receptor scaffold is sufficient for disease development. Although reducing *Mpl* had a far more significant effect on disease progression, it was surprising, and contradictory to previous *in vitro* findings, that *Tpo* ablation attenuated several aspects of MPN development. Despite demonstrating that *JAK2*^{V617F} and *Mpl* are sufficient for MPN development, our data also suggests that in the presence of TPO, the disease progression, specifically thrombocythaemia and neutrophilia, is greatly accelerated. However, although at 16 weeks *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice have platelet counts similar to *WT*, it is possible that these mice will eventually develop thrombocythaemia as platelet counts are conceivably still rising. Additionally, *JAK2*^{V617F} mediated splenomegaly and expansion of the HSC pool was not affected by the removal of TPO. We postulate that the unresolved splenomegaly in our *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice is a consequence of extramedullary erythrocytosis and megakaryocyte hyperplasia. The number of neoplastic HSCs in *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ mice is similar to *JAK2*$^{V617F+}$ suggesting that in the absence of TPO, *JAK2*^{V617F} is still able to exert a proliferative effect on HSCs. This demonstrates that MPL serves as a scaffold for *JAK2*^{V617F} and is sufficient for initiation of *JAK2*/*STAT* signalling in vivo.

Interestingly, we found that MPL protein expression was increased in platelets from *JAK2*$^{V617F+}$ and *JAK2*$^{V617F+}$*Mpl*$^{+/-}$ mice. These results are contradictory to previous findings with an alternative *JAK2*^{V617F} knock-in mouse model(116). It is not clear why the mouse models express different levels of MPL, but it should be noted that they exhibit significantly different phenotypes. While our *FF1/Tie2-Cre* mice present with thrombocytosis, neutrophilia and splenomegaly, the mice used by Pecquet et al, (which were originally generated by Marty and colleagues(311)), also develop high red blood cell counts, leukocytosis and myelofibrosis. Whether different disease phenotypes alter MPL expression is not currently known, but given our data demonstrating the significance of MPL levels in disease progression, this is an important subject for future investigations.

JAK2^{V617F} expression increases the number of E-SLAM^{+} HSCs, an effect which is severely attenuated by *Mpl* ablation. Increases in the HSC pool in *JAK2*$^{V617F+}$*Tpo*$^{-/-}$ and *JAK2*$^{V617F+}$*Mpl*$^{+/-}$ mice compared to *WT* could explain the delayed increase in neutrophils at 16 weeks. Whether or not *JAK2*^{V617F} gives HSCs a selective advantage

remains controversial(312). Our data supports previous reports that the *JAK2*^{V617F} mutation expands the HSC pool(313) possibly through increased cell cycling coupled with low apoptosis(314). However, other reports suggest that *JAK2*^{V617F} causes an HSC defect, reducing self-renewal thereby resulting in lowered HSC numbers(315). However, these discrepancies may be due to significant differences between mouse models, which develop various disease phenotypes over different periods of time(293).

In this report, we not only demonstrate that MPL is necessary for the development of a *JAK2*^{V617F}-mediated MPN in transgenic mouse models, but that the level of receptor expression directly affects disease severity and progression. We show that the *JAK2*^{V617F} oncogene requires the MPL transmembrane receptor to initiate the haematological malignancy in HSCs and that TPO contributes to disease progression but is not necessary for MPN development. The use of JAK2 inhibitors, such as ruxolitinib, has now been approved by the FDA to treat splenomegaly associated with myelofibrosis. However, clinical response to the drug has been mixed, warranting the exploration of other drug targets. Complete ablation of TPO only exerted a minor effect on MPN development; therefore, targeting of TPO in MPNs would likely require complete inhibition, making TPO a difficult target for clinical drug development. Instead, the data presented here highlight MPL as an excellent therapeutic target for the treatment of MPNs. Small molecule MPL agonists (eltrombopag) and TPO peptidomimetics (romiplostim) are now in clinical use for the treatment of idiopathic thrombocytopenic pupura (ITP), clearly demonstrating the feasibility of targeting drugs to modulate MPL activity. Therefore it is possible that modifying MPL levels through use of antibodies, small molecule inhibitors or peptide mimetics, or preventing its association with TPO in the presence of *JAK2*^{V617F}, may alter the clinical and pathological manifestations of MPNs.

CHAPTER 7 GENERAL DISCUSSION

Haematopoiesis is responsible for production of all the mature blood cells in the body which are essential for normal function. It is a tightly regulated process governed by a large network of cytokines and their receptors. Dysregulation at any point can result in a number of haematological disorders. Most of these disorders are the result of deregulation at the HSC level or within early progenitors. TPO and its receptor MPL are key regulators of megakaryopoiesis and maintenance of HSCs. Mutations resulting in non-functional MPL or TPO typically result in diseases characterised by pancytopaenias including CAMT. Survival rates associated with these diseases are low and often necessitate a bone marrow transplant. Conversely, activating mutations of MPL and mutations resulting in overproduction of TPO result in diseases characterised by overproduction of myeloid cells, most commonly of platelets resulting in thromocythaemia. Although the prognosis for these diseases is better than for those associated with inactivating mutations, myeloproliferation significantly increases thrombotic risk which can result in death. These diseases highlight the importance of MPL in maintenance of normal haemostasis. Therefore, a comprehensive understanding of the biology and regulation of TPO and MPL are biologically important. Although a number of studies have been undertaken to develop a better understanding of TPO biology, many of these studies occurred soon after the cloning of TPO in 1994. Over two-decades later, our understanding of TPO and MPL biology has increased yet remains incomplete.

The primary aim of this work was to determine additional negative regulatory mechanisms governing TPO signalling in addition to determining the role of MPL and TPO in MPN pathogenesis.

7.1 Negative regulation of TPO signalling

Activation of TPO signalling is important for HSC maintenance and haematopoiesis. However, unsolicited activation of the signalling cascade results in disease thus a thorough understanding of the mechanisms in place to prevent deregulation is of scientific importance. Although MPL^{Y591} was previously postulated as a negative

regulator of TPO signalling, the mechanism by which this occurred had not been investigated. In this body of work, novel binding partners of MPL were identified including BTK, SHP1 and SYK. SYK was shown to preferentially bind to phosphorylated Y591 of MPL to negatively regulate ERK1/2 signalling(Figure 7.1). Additionally, SHP1 also preferentially binds to MPL^{pY591} yet it did not exert an effect on ERK1/2 signalling. However, it has previously been shown to negatively regulate MPL signalling thus it is possible that SHP1 binds to MPL^{pY591} to regulate other pathways associated with TPO signalling. A number of negative regulatory mechanisms of TPO and MPL are known, such as involvement of negative regulators including SOCS proteins, LYN and LNK and pathways such as ubiquitination by CBL which results in receptor internalization and degradation. However, the mechanism of action, specifically the components of the receptor necessary in some of these pathways is less understood. In this work, not only was a negative regulatory pathway identified, the specific residue on the receptor, its phosphorylation state and an associated protein were all identified. This could prove important for development of therapeutics as many proteins involved in signal transduction are common between multiple pathways. Targeting of downstream effectors could result in undesired off target effects whereas targeting of specific components of a pathway such as a receptor would be more specific. Although this work has identified an additional element in the understanding of TPO signalling, much investigation is still required for a complete understanding of the biological roles of TPO and MPL.

7.2 *JAK2*^{V617F} and its effects on TPO signalling

A number of studies have shown that expression of *JAK2*^{V617F} is sufficient for cytokine independent signalling *in vitro* but only when a homodimeric type I cytokine receptor is also expressed. However, these studies were only performed with *WT* cytokine receptors including EPOR and MPL. In this work we sought to determine the role of phosphorylated tyrosine resides of the receptor, MPL, as they could possibly modulate *JAK2*^{V617F}-induced signalling. We found that removal of these resides, namely Y591, Y625 and Y630 had little effect on *JAK2*^{V617F}-induced signalling and proliferation. Therefore, phosphorylated tyrosine residues of MPL are unable to overcome cellular activation resulting from *JAK2*^{V617F} expression.

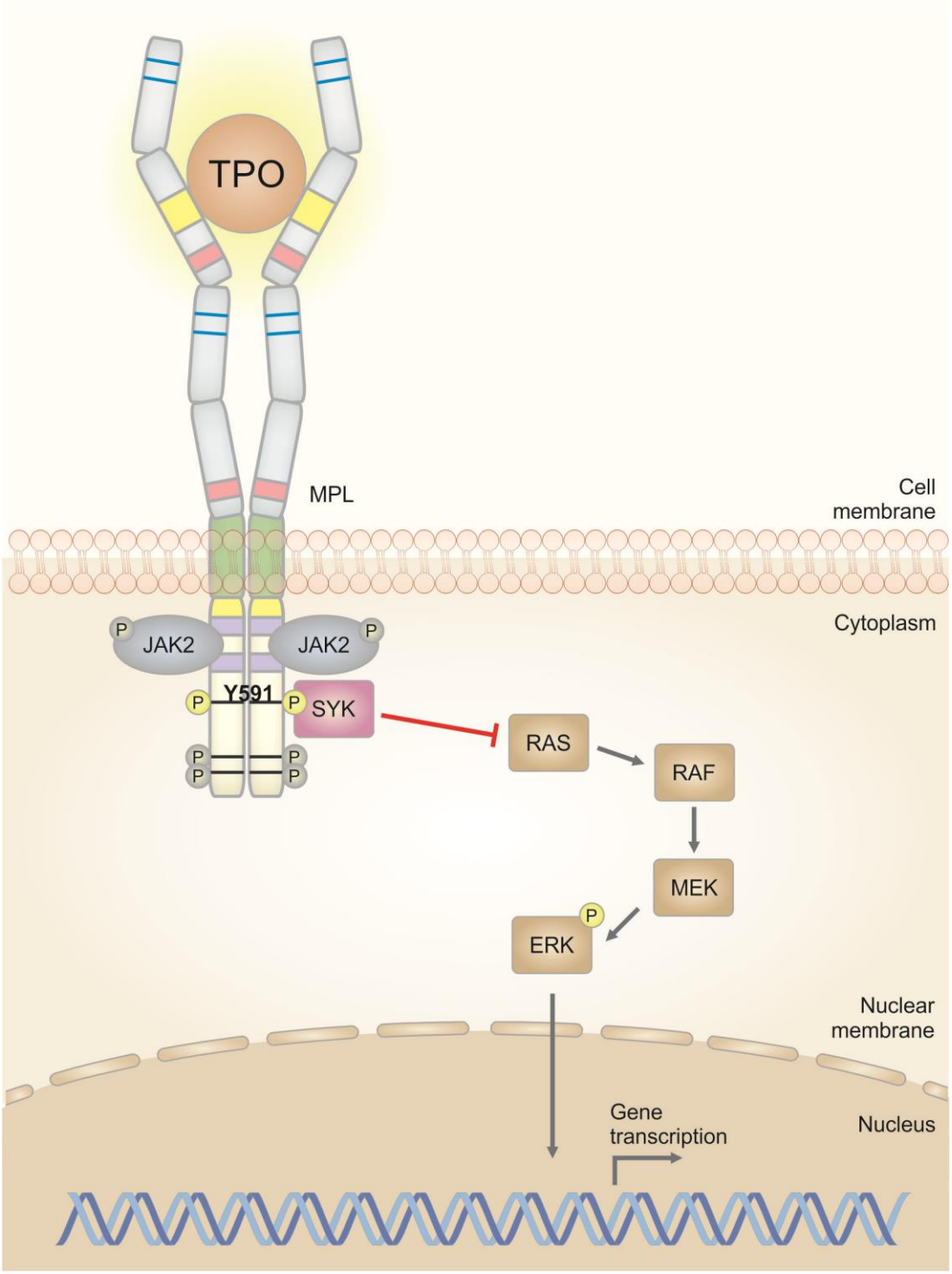


Figure 7.1 Summary of identified negative regulatory pathway
Thrombopoietin binding to MPL results in activation of the TPO signalling cascade. This work identifies a novel signalling pathway whereby TPO binding to MPL results in phosphorylation of Tyr591 resulting in recruitment and association of SYK to pTry591 which negatively regulates TPO-induced ERK1/2 signalling.

However, as others have shown, removal of the receptor completely prevents *JAK2*^{V617F} from inducing cytokine independent signalling and proliferation. Therefore, the specific tyrosine residues may not play a large role in inhibiting mutant kinase signalling but the receptor is still necessary, warranting further investigation. This work therefore also examines the role of MPL and TPO in an *in vivo* system.

7.3 Implications of MPL in *JAK2*^{V617F}-positive MPNs

Since its identification in 2005, much of the research focus has been on studying the role of *JAK2*^{V617F} in MPN development. *JAK2*^{V617F} is considered an oncogene; however, this work provides evidence that although *JAK2*^{V617F} is important for MPN development *in vivo*, in the absence of MPL the disease fails to develop. This work suggests that *JAK2*^{V617F} is in fact only one part of a 2 part oncogene, as disease development necessitates the expression of MPL(Figure 7.2). TPO plays a role in MPN development as disease progression is slowed in its absence. However, the data demonstrates that the disease can still develop in the absence of TPO; therefore, the role of TPO in MPN development is less significant than MPL. Due to its necessity for disease development, this work identifies MPL as a novel target for development of novel therapeutics for the treatment of MPNs. Specifically, because its function is at the HSC level, targeting of MPL could treat the disease rather than the symptoms. Importantly, our data suggests that targeting only a portion of MPL is necessary to prevent MPN development.

7.4 Therapeutic implications

Current treatments of MPNs include imatinib, allogenic stem cell transplantation, aspirin regimen, cytoreductive therapies, splenectomy and the JAK inhibitor ruxolitinib. Imatinib is a highly successful drug in the treatment of CML; however, treatment of Ph-negative MPNs (ET, PV and PMF) is less straight forward. Identification of mutations leading to different Ph-negative MPN pathologies such as mutations in JAK2, MPL and CALR has vastly improved our understanding of these disorders and development of more specific drug targets, namely JAK inhibitors such as ruxolitinib. During its development, there was much hope that ruxolitnib would be as successful in treating Ph-negative MPNs as imatinib was for CML.

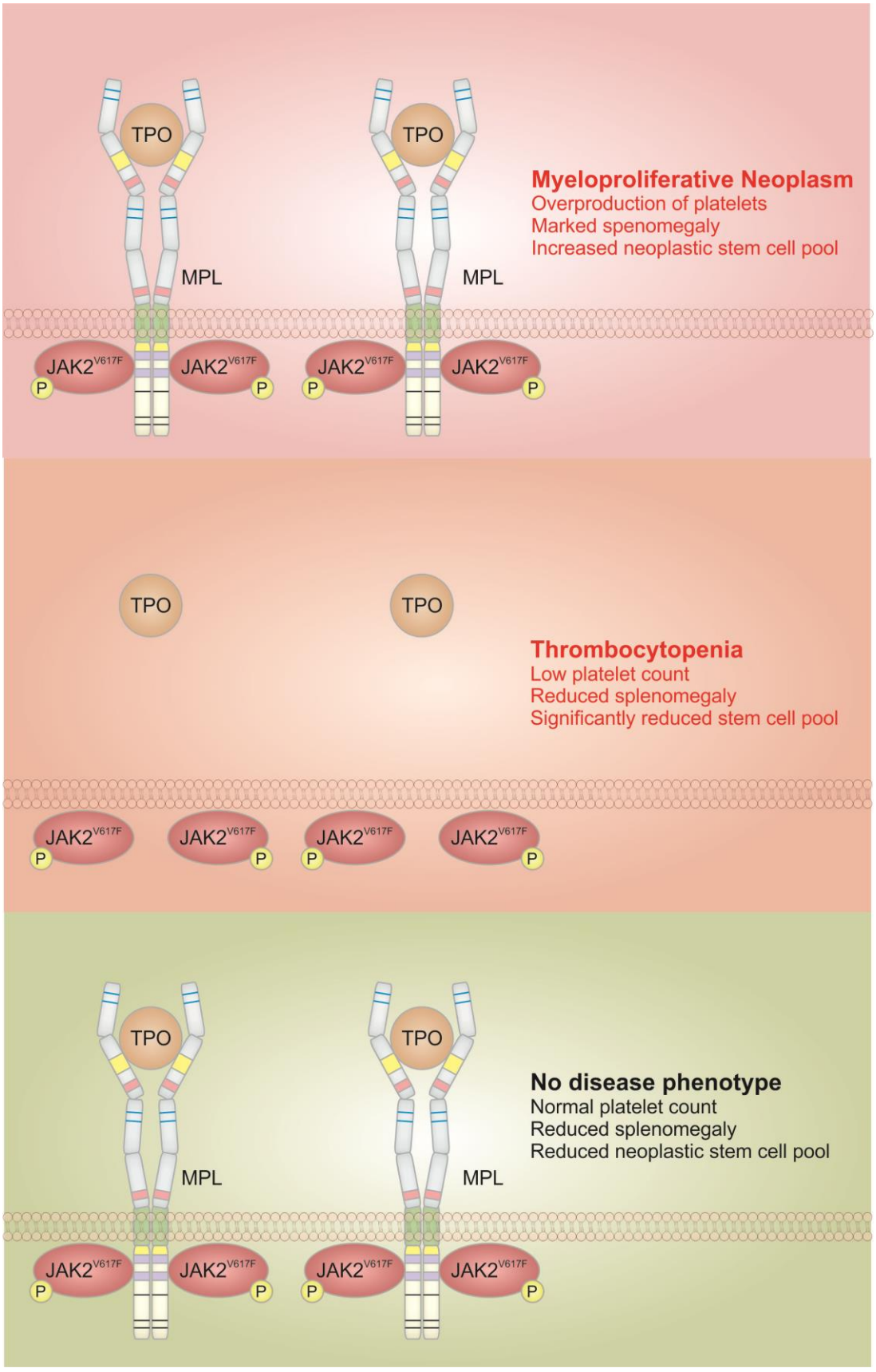


Figure 7.2 Summary of the role of MPL in MPN development
MPL is necessary for development of a myeloproliferative neoplasm (top panel), in the absence of MPL the disease fails to develop but thrombocytopenia also develops (middle panel); however the reduction of MPL completely prevents disease development without thrombocytopenia.

Patients report improvements following ruxolitinib therapy however, it is believed that much of the improvements are related to the ability of ruxolitinib to reduce inflammation. JAK2 is involved in numerous signalling pathways so targeting JAK2 results in off target effects; additionally, it has been shown that ruxolitinib also inhibits JAK1, which could be causing the reduction in inflammation. *JAK2*^{V617F} positive clones display selective advantage in patients, thus successful treatment with JAK2 inhibitors likely requires inhibition of all mutant kinases necessitating higher doses which are not tolerable. Our data demonstrates that reducing MPL levels is sufficient to prevent development of the disease. This would prove important therapeutically as doses of inhibitor sufficient to target only a portion of MPL could be successful in treating the disease. Additionally, as reduction of MPL exerts an effect at the HSC level by reducing the neoplastic stem cell pool, it is possible that targeting of MPL results in treatment of the disease itself rather than the symptoms. However, there are currently no available antagonists to MPL which would allow this to be tested experimentally. Therefore, the next step in understanding the role of MPL in MPN development and determining its feasibility as a novel therapeutic target for MPNs is development of an MPL antagonist.

7.5 Future research

Although this work identifies MPL at a potential and novel therapeutic target for the treatment of MPNs, there is still much work to be done to determine if targeting of MPL will be of clinical value.

We found MPL expression to be increased when *JAK2*^{V617F} was expressed which is in disagreement with some reports of MPL expression in patients(238-241). However, just as the homozygous *JAK2*^{V617F} clone increases with time, it is possible that MPL expression decreases as the disease progresses or as additional mutations are acquired. Previous studies analysing *MPL* expression in MPN patients reported an overall decrease in expression associated with disease. However, these studies were performed prior to identification of *JAK2*^{V617F} and they measure expression after disease development thus it is not clear whether *MPL* expression levels are a contributing factor or a result of the disease. Additionally, with the identification of additional mutation in MPNs such as *JAK2* exon 12, *MPL* and *CALR*, MPNs can

now be grouped based on mutational profiles thereby allowing for analysis to occur in clusters. With the help of HMDS, we are looking to analyse *MPL* expression in MPN patients and compare to mutations and prognostic outcome. Additionally, it would be of interest to monitor *MPL* expression over time to understand how the disease modulates expression.

Additionally, in these studies we show that expression of *JAK2*^{V617F} and half the amount of *MPL in vivo* was able to prevent disease development in an ET-like mouse model. It was shown that the reduction in MPL was able to reduce the neoplastic stem cell pool. However, the mechanism remains unknown and needs to be investigated. It is possible that pathological signalling is diminished due to a reduction in *MPL* expression. Additionally, analysis of differences between gene expression in *JAK2*$^{V617F+}$ compared to *JAK2*$^{V167F+}$*Mpl*$^{+/-}$ mice could also provide mechanistic insights; however, this remains to be investigated. Furthermore, the *in vivo* data presented in this work is based on a *JAK2*$^{V617F+}$ ET-like mouse model(149). If the effect of *MPL* reduction is being exerted at the HSC level, this could mean that MPL antagonists could be used to treat all subsets of *JAK2*$^{V617F+}$ MPNs. To validate this possibility it is necessary to perform similar studies in mouse models of PV and PMF. If indeed, modulation of *MPL* expression is able to prevent disease development, this would provide additional support for the development and use of MPL antagonists in treatment of MPNs.

This work identifies MPL as a novel therapeutic target for the treatment of MPNs; however, no antagonists are currently available thus future research includes development of an MPL antagonist. Promisingly, there are currently MPL agonists that are currently approved for treatment of IPT which have been successful with little to no off target effects (Reviewed in (316)); therefore showing that MPL is a viable therapeutic target. Unfortunately, there is currently no structural data for MPL making drug development difficult. However, a potential strategy for drug design is to modify the current MPL agonists and subsequently testing them for antagonistic ability.

7.6 Concluding remarks

TPO and MPL were discovered over 20 years ago and soon after were shown to be essential in megakaryopoiesis and HSC biology. Study of TPO/MPL biology is not only important for the understanding of normal haematopoiesis but also important in understanding of haematological disorders. The relationship between MPL and MPNs has been postulated previously and this was later confirmed when mutations in MPL resulted in development of MPNs. However, in this work it was shown that MPL is also important in $JAK2^{V617F+}$ MPNs and that it may be a useful therapeutic target. Future work will likely involve additional studies to determine the mechanism by which MPL prevents the disease from developing. Additionally, development of MPL antagonists is necessary for further examination of the potential of MPL as a therapeutic target. However, to do so structural studies of MPL would provide a better means for drug development and would provide an invaluable resource for further study of TPO/MPL biology. The results of this thesis identify a novel negative regulatory pathway in biological TPO signalling in addition to identifying MPL as an essential component for Ph-negative MPN pathogenesis; thereby paving the way for future studies of TPO and MPL in both a pathological and biological setting.

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LIST OF ABBREVIATIONS

A

aa	amino acid
AA	aplastic anemia
ABL1	Abelson murine leukaemia viral oncogene homolog 1
ACD	acid citrate dextrose
AGM	aorta-gonad-mesonephros
Akt	Protein kinase B, PKB
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
AMR	Ashwell-Morell receptor
ANOVA	one-way analysis of variance

B

BA	basophil
BAC	bacterial artificial chromosome
BCR	breakpoint cluster region
BLK	B lymphocyte kinase
BM	bone marrow
BMN	bone marrow neutrophils
BSA	bovine serum albumin
BTK	Bruton agammaglobulinemia tyrosine kinase

C

c-kit	stem cell growth factor receptor, CD117
C-terminal	carboxyl-terminal
CALR	calreticulin
CAMT	congenital amegakaryocytic thrombocytopenia
CBFA2	core-binding factor subunit alpha-2
Cbl	casitas B-lineage lymphoma
CD	cluster of differentiation

CD244	natural killer cell receptor 2B4
CFU	colony forming unit
CFC	colony forming cell
CFU-MK	megakaryocyte colony forming unit
CML	chronic myelogenous leukaemia
CRM	cytokine receptor homology module

D

DLAR	Division of Laboratory Animal Resources
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
DPBS	Dulbecco's phosphate-buffered saline
DTT	dithiothreitol

E

EC_{50}	half-maximal effective concentration
EDTA	ehylenediaminetetraacetic acid
EndoH	endoglycosidase H
EO	eosinophil
EPO	erythropoietin
EPOR	erythropoietin receptor
ERK	extracellular-signal-regulated kinase
ES	embryonic stem
ET	essential thrombocythaemia
EtBr	ethidium bromide
EtOH	ethanol
EYFP	enhanced yellow fluorescent protein

F

F-MuLV	Friend murine leukemia virus
FAK	focal adhesion kinase
FAM™	6-carboxyfluorescein
FBS	fetal bovine serum

FDA	United States Food and Drug Administration
FERM	4-point, ezrin, radixin, moesin
FF1	Flip-Flop
FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
Flk-1	foetal liver kinase-1, CD135, fms-like tyrosine kinase 3 (FLT-3)
FRAP	fluorescence recovery after photobleaching

G

G418	geneticin
Gab1	GRB2-associated-binding protein 1
Gab 2	GRB2-associated-binding protein 2
GCSFR	granulocyte colony-stimulating factor receptor
gDNA	genomic DNA
GHR	growth hormone receptor
GMCSF	granulocyte-macrophage colony-stimulating factor
GMCSFR	granulocyte-macrophage colony-stimulating factor receptor
GTP	guanosine-5'-triphosphate

H

H&E	haematoxylin and eosin
Hb	haemoglobin
HC	hydroxycarbamide
HCT	haematocrit
HMRN	haematological malignancy research network
HRP	horseradish peroxidase
HSC	hematopoietic stem cell
HU	hydroxyurea

I

IEP	immediate promoter
IFN-α	interferon-α
IL-3	interleukin 3
IL-6	interleukin 6
IL-3R	interleukin 3 receptor

IMAC	immobilized metal affinity chromatography
ITK	interleukin-2-inducible T-cell kinase
IRES	internal ribosome entry site
ITP	immune thrombocytopenic pupura

J

JAK2	janus kinase 2
JH	JAK homology

K

K_D	dissociation constant
KDEL	Lys-Asp-Glu-Leu

L

LB	Luria-Bertani
LDS	lithium dodecyl sulface
LIN	lineage
LNK	SH2B adapter protein 3
LT-HSC	long term haematopoietic stem cell
LY	lymphocyte
Lyn	Tyrosine-protein kinase Lyn

M

MAPK	mitogen-activated protein kinase
MCHC	mean corpuscular haemoglobin concentration
MCS	multiple cloning site
MCV	mean corpuscular haemoglobin
MDS	myelodysplastic syndrome
MGB	minor groove binder
Mk	megakaryocyte
MM	myeloid malignancies
MO	monocyte
MPL	myeloproliferative leukemia virus oncogene, c-MPL
MPLV	myeloproliferative leukemia virus

MPN	myeloproliferative neoplasm
MPP	multipotent progenitor
MPV	mean platelet volume
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
MVD	microvessel density

N

N-terminal	amino-terminal
NE	neutrophil
neo^r	neomycin resistance
NHL	non-Hodgkin's lymphoma

P

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEG-IFN-α-2	pegylated interferon α-2a
Ph	Philadelphia chromosome
PIAS	protein inhibitor of activated STAT protein
PI3K	photidylinositol-3-kinase
PKC	protein kinase C
pkg	phosphoglycerate kinase 1
Plat-E	platinum-E
PLT	platelet
PMF	primary myelofibrosis
PPP	platelet poor plasma
PRLR	prolactin receptor
PRP	platelet rich plasma
PSG	penicillin-streptomycin-glutamine
PTB	phosphotyrosine-binding
PTP	phosphotryosine phosphatases
PTPRC	protein tyrosine phosphatase, receptor type C
PV	polycythaemia vera

PVDF	polyvinylidene difluoride

Q

qPCR	quantitative polymerase chain reaction

R

RAF1	RAF proto-oncogene serine/threonine-protein kinase
RBC	red blood cell, erythrocyte
RDW	red blood cell distribution width
rh	recombinant human
rm	recombinant murine
RNA	ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute 1640 medium
RT	reactive thrombosis
RT-PCR	reverse transcriptase-polymerase chain reaction

S

Sca-1	stem cell antigen-1
SCF	stem cell factor
SDS	sodium dodecyl sulfate
SH2	Src Homology 2
SH2D1A	SH2 domain-containing protein 1A
Shc	SHC-transforming protein 1
SHIP1	phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1
SHP-1	Src homology region 2 domain-containing phosphatase-1, PTPN6
SHP-2	Tyrosine-protein phosphatase non-receptor type 11, PTPN11
SLAM	signalling lymphocytic activation molecule
SLAMF/1	signalling lymphocytic activation molecule 1, CD150
SLAMF/2	signalling lymphocytic activation molecule 1, CD48
SNP	single nucleotide polymorphism
SOCS	suppressors of cytokine signalling
ST-HSC	short term haematopoietic stem cell
STAT	Signal Transducer and Activator of Transcription
SYK	spleen tyrosine kinase

T

TAMRA™	tetramethylrhodamine
TBE	Tris/borate/EDTA
TET2	tet methylcytosine dioxygenase 2
Thy-1	thymocyte differentiation antigen-1, CD90
Tie2	tunica intima endothelial kinase 2, angiopoietin-1 receptor
TNF	tumour necrosis factor
TPO	thrombopoietin
trfc	transferrin receptor
TTC	triphenyl tetrazolium chloride
TYK2	tyrosine kinase 2

U

UK	United Kingdom
US	United States of America
UP	UltraPure
UV	ultra violet

V

v-mpl	viral myeloproliferative leukaemia virus
VAV2	guanine nucleotide exchange factor VAV2
VWF	von Willebrand factor

W

WBC	white blood cell
WSXWX	Trp-Ser-X-Trp-Ser
WT	wild-type

X

XCIP	X-linked chromosomal inactivation patterns
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide

β-ME	2-mercaptoethanol
φNX	Phoenix-amphotropic retroviral packaging cell line

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