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Janus kinase (JAK) 2 regulation and the novel activation mutation, JAK2R$_{564}$Q

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Lana Marie Corbo

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Abstract of the Thesis

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Janus kinase (JAK) 2 is a critical secondary signaling protein and is ubiquitously expressed in hematopoietic cells. One of the major roles of JAK2 is to trigger signal transduction following binding of the hematopoietic growth factor thrombopoietin (TPO), to its receptor c-Mpl, which initiates megakaryopoiesis. Previous findings focused on other hematopoietic growth factor receptors suggest that JAK2 is critical for translocation of the receptor to the membrane. Using hematopoietic cell lines, in which we transiently overexpressed human JAK2, we found that increased JAK2 expression had no effect on cell surface membrane localization. We also studied the effects of c-Mpl cell surface localization following over-expression of mutant forms of JAK2 (JAK2V$_{617}$F and JAK2R$_{564}$Q) which have been shown to cause myeloproliferative diseases in humans. Interestingly, contrary to previous reports, we found no difference in cell surface localization, following overexpression of mutant JAK2. Finally, we determined the effects of the JAK2R$_{564}$Q mutation on cellular proliferation, survival, and signaling, comparing its effect to those of the more common JAK2V$_{617}$F mutation. Although the JAK2R$_{564}$Q mutation occurs within the same pseudokinase domain as JAK2V$_{617}$F, the functionality of the mutations differs. Our results suggest that the JAK2R$_{564}$Q mutation has the ability to prevent apoptosis while the JAKV$_{617}$F contributes to hyperproliferation.

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List of Abbreviations

BHK (Baby Hamster Kidney)
BrdU (Bromodeoxyuridine)
CRM (Cytokine Receptor Modules)
(fET) (familial Essential Thrombocythemia)
FBS (Fetal Bovine Serum)
FERM (band Four point one (F), Ezrin, Radixin (E), Moiesin (M))
HSC (Hematopoietic Stem Cell)
IL-3 (Interleukin-3)
JAK2 (Janus kinase 2)
JH (Jak homology)
LB (Lubria Bertani)
MPL (Myeloproliferative leukemia)
PV (Polycythemia Vera)
PMF (Primary Myelofibrosis)
Rpm (Rotations per minute)
SH (Src homology)
SOC (Super Optimal broth)
TPO (Thrombopoietin)
WT (Wild-type)

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Chapter 1

INTRODUCTION

Hematopoiesis is the process by which all blood cells are produced[1]. Hematopoietic stem cells (HSC) originating in the yolk sac, travel to the liver between the third and seventh gestation months and reside in the spleen until the later stages of gestation and early birth when they localize to the bone marrow[3]. HSCs yield progenitor cells that no longer have the “stem-like” capability of self-renewal. A myeloid progenitor cell differentiates into linear specific cell types including megakaryocytes that mature into platelets (Figure 1.1)[3]. During megakaryopoiesis, the megakaryocyte progenitor undergoes DNA replication in the absence of cell division. This process (endomitosis) results in megakaryocytes with a higher than normal complement of DNA, up to a maximum of 128N[1, 4]. A fully mature megakaryocyte then undergoes thrombopoiesis (platelet production), in which platelets are released from the bone marrow into the circulation. With the help of actin and myosin, branch-like projections extend from megakaryoctyes through blood vessels and fragment into 1000-3000 platelets that are released into the blood stream. On average, 10^{11} platelets are produced every 24 hours[4, 5]. The factor that is necessary for the growth and differentiation of megakaryocytes is called thrombopoietin (TPO)[6].

TPO, an acidic glycoprotein, contains 332 amino acids that make up two domains[7]. The amino terminal end binds to its receptor, c-Mpl and the carboxyl-terminal domain consists of N- and O-linked carbohydrates[6]. It binds to its receptor via residues within the first and forth alpha helices and in between the first and second helices. TPO signaling is regulated by c-Mpl localization to the membrane. Both an intracellular and extracellular form of the TPO receptor are expressed; an intracellular form at 80 kDa and a larger 85 kDa form that is expressed on the cell surface. The larger 85 kDa form is N-glycosylated and is actively involved in signaling[6]. When it is present on the cell surface, it must be able to bind TPO in order to function properly[8].

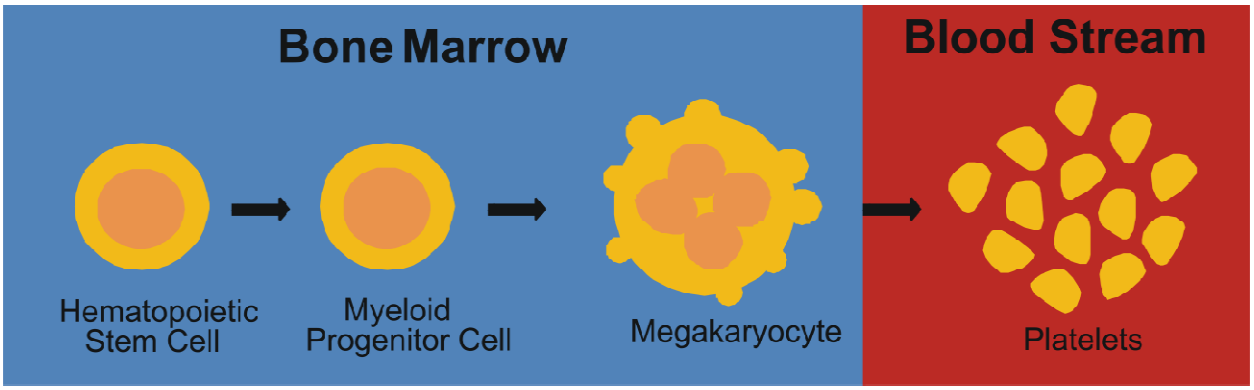

Figure 1.1. **Diagrammatic representation of hematopoiesis.** A hematopoietic stem cell turns into a committed megakaryocyte that ultimately matures into platelets [1].

Adapted from Geddis 2010

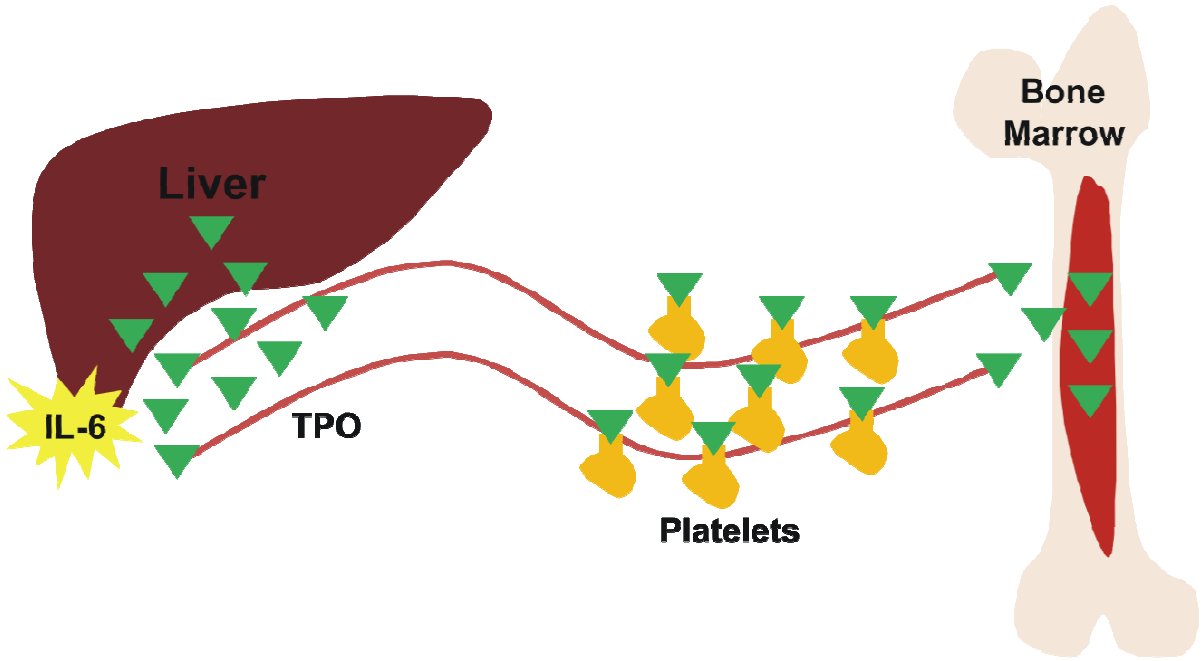

Figure 1.2. Thrombopoietin (TPO) production in the liver and bone marrow. TPO produced in the liver travels through the bloodstream and binds to platelets. Any unbound TPO will stimulate the production of more TPO in the bone marrow [1] [2] [4].

C-Mpl is expressed in hematopoietic stem cells, megakaryocytes, and platelets. It is composed of 635 amino acids encoded at chromosome 1p34 by the *MPL* gene[1]. A unique amphipathic residue enables it to span the membrane. The intracellular domain is composed of a box 1 and box 2 motif which are involved in Janus Kinase (JAK) 2 binding. C-Mpl lacks enzymatic activity and recruits JAK2 for downstream signaling and activation[1]. JAK2 via its FERM (band Four point one (F), Ezrin, Radixin (E), Moiesin (M)) domain binds to c-Mpl at the box 1 motif[1, 9]. Disassembly of the two proteins can occur following phosphorylation of the FERM domain which will disrupt signaling[1, 9]. Its extracellular domain consists of two cytokine receptor modules (CRM) about 200 amino acids long that bind TPO[1, 9]. Ligand stimulation causes a conformation change which brings two JAK2 molecules juxtaposition allowing for cross-phosphorylation and activation to occur (Figure 1.3)[1].

JAK2 is a critical secondary signaling protein ubiquitously expressed in hematopoietic cells. Seven regions, the JAK Homology domains (JH), within the protein are conserved within the JAK family. Two domains, JH1 and JH2 correspond to the kinase domain and the pseudokinase domain, respectively. The kinase domain lies adjacent to the catalytically inactive pseudokinase domain[9]. The JH2 domain was identified due to the absence of important glycine and aspartic acid residues which account for its lack of catalytic activity[9]. The inactive pseudokinase domain consists of three inhibitory regions that interact with the kinase domain to inhibit basal JAK2 activity and maintain the activation loop (A-loop)[9]. Previous studies have shown that the pseudokinase domain may have a regulatory role in kinase inhibition[9]. Mutations within these regions result in increased basal JAK2 activity. JAK2 associates with c-Mpl via JH domains 3-7 at the N-terminal FERM domain[9]. JAK2 associates with the receptor via proline-rich regions within the FERM domain at the box 1 motif of c-Mpl[1]. This causes activation and phosphorylation of the receptor and allows translocation to the membrane, which is essential for receptor functioning. Mutations within these domains as well as mutations that result in the opposite phenotypes such as platelet deficits seen in thrombocytopenia and anemia which result from mutations in the erythroid transcription factor, Gata binding factor-1 (GATA1), as well as *MPL* mutations will lead to malfunction and myeloproliferative disorders[10].

Myeloproliferative disorders are the result of congenital or acquired mutations which perturb the regulatory function of myelopoiesis. Polycythemia vera (PV), a clonal disease that causes hyperplasia in platelets, red blood cells, and white blood cells can lead to complications of thrombosis, hypertension, hemorrhage, and leukemia[11]. It arises from an acquired single point mutation within the pseudokinase domain, JAK2V_{617}F [2] [12]. This point mutation causes a highly conserved valine change to a phenylalanine occurs in over 90% of patients with PV and more than 50% of other common myeloproliferative disorders including essential thrombocytosis (ET) and primary myelofibrosis (PMF). The mutation interferes with the inhibitory interaction between the pseudokinase and kinase domain which results in hypersensitivity to growth factors [2] [13] [14]. The mechanisms by which myeloproliferative disorders arise and thrive may provide insight for future therapies.

We have identified a novel mutation in JAK2, JAK2R_{564}Q, which causes familial essential thrombocythemia (fET). This mutation results from a single nucleotide base change from G to A at base 1691, leading to the arginine to glutamine change at amino acid 564. This mutation was first identified in a 6 year old male patient with elevated platelet levels of 961 k/mcL after being tested for other common alterations in JAK2 and c-Mpl. Both his mother and sister also had the mutation with platelet counts of 550-600/mcL. The father did not test positive for the mutation nor did he have high platelet counts.

Our studies present a potential mechanism by which JAK2R_{564}Q causes familial thrombocytosis that differs from the way the JAK2V_{617}F mutation leads to polycythemia vera . We hypothesize that the JAK2R_{564}Q mutation gives hematopoietic stem cells and megakaryocyte progenitors an anti-apoptotic ability which may contribute to elevated platelet levels. Intriguingly, although the two mutations occur in the in the same pseudokinase domain, they exert different effects on cell survival and proliferation.

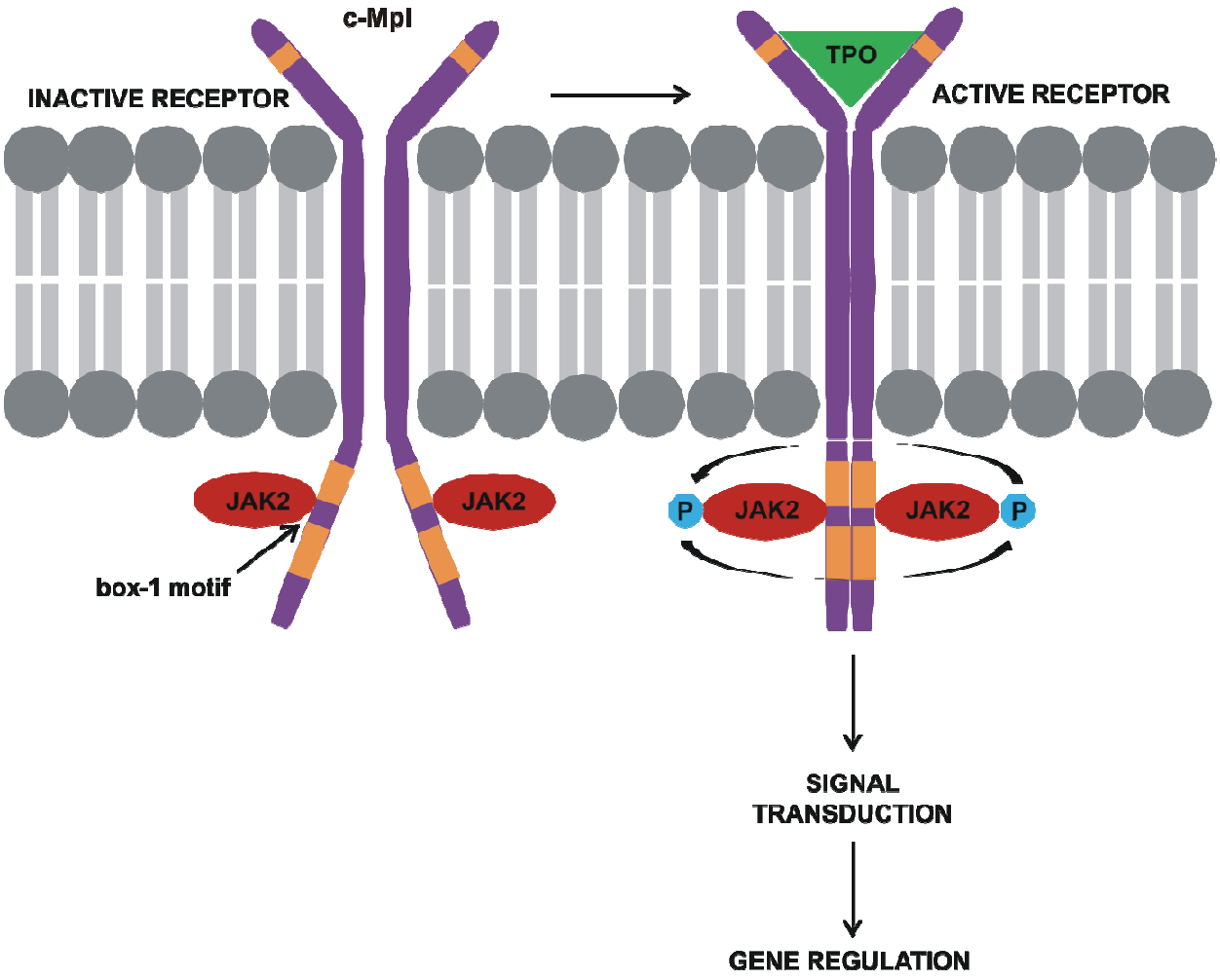

Figure 1.3. Schematic diagram of JAK2 activation. Ligand binding results in a c-Mpl conformational change which brings two JAK2 molecules in juxtaposition allowing cross-phosphorylation, signal transduction, and gene regulation [1].

Adapted from Geddis 2010

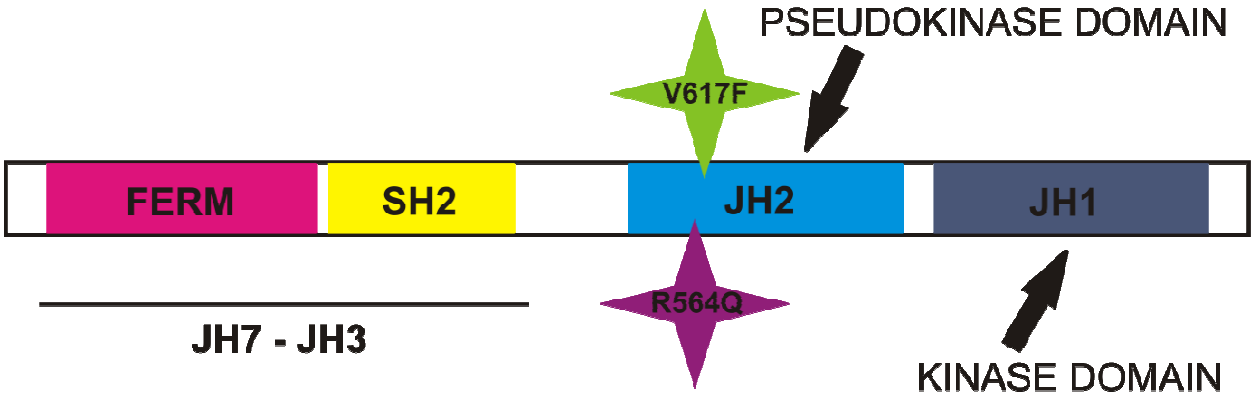

Figure 1.4. JAK2 Domains. The 4 JAK2 domains from N-terminus to C-terminus correspond to the FERM (band Four point one (F), Ezrin (E), Radixin (R), Moiesin (M)) domain and Src Homology (SH) domain also referred to as JAK homology (JH) domains 3-7, the pseudokinase(JH2) and kinase(JH1) domain. JAK2 mutations, $JAK2V_{617}F$ and $JAK2R_{564}Q$ occur in the JH2 domain which is important for the negative regulation of JAK2 activity[2].

Adapted from Baxter et al. 2005

Chapter 2

MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Cell culture plasticware and reagents

Tissue culture plasticware was purchased from Corning (Corning, NY) and reagents (RPMI-1640) were purchased from Mediatech (Manassas, VA). All cells were incubated at 37℃ in 5% CO_2/95% air atmosphere.

2.1.2 Cell line culture conditions

Ba/F3 cells (an immortalized murine bone marrow derived pro-B cell line) were previously stably transfected with c-Mpl. This cell line was maintained in RPMI-1640 (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin streptomycin glutamine (PSG), and interleukin-3 (IL3) produced by a baby hamster kidney (BHK) cell line(2μl/ml) in 75cm^2 flasks.

2.2 Site-directed Mutagenesis, Transformation, and DNA Purification

Wild-type (WT) human JAK2 was previously cloned into pEFV-5HisA (Invitrogen, Carlsbad, CA). This was used as a template to introduce the single base changes required to make the *JAK2*R_{564}Q and *JAK2*V_{617}F mutants. We also made a plasmid containing both $R_{564}Q$ and $V_{617}F$ mutations in order to see if these mutants signal through the same or distinct mechanisms. Site-directed mutagenesis was performed using a QuikChange kit (Agilent Technologies, Santa Clara, CA), according to manufacturer's instructions. Briefly, a master mix was made containing 10 x PCR buffer, 1.5 μg/μl pEFv5His *JAK2*WT plasmid, 1.25 μl of 125 ng forward primer, 1.25 μl of 125 ng reverse primer, 1 μl dNTPs, 40.5 μl water, and 1μl PFU Ultra (Agilent Technologies, Santa Clara, CA) polymerase. Concentration of DNA was measured using a SmartSpec 3000(BIO-RAD). Mutagenesis was performed with the appropriate primers for $R_{564}Q$, $V_{617}F$, and $R_{564}Q/V_{617}F$. Primers used to introduce point mutations were as follows:

R_{564}Q F: 5'-CAAAGATTTTTAAAGGCGTACAAAGAGAAGTAGGAGACTACGG-3'

R_{564}Q-R: 5’-CCGTAGTCTCCTACTTCTCTTTGTACGCCTTTAAAAATCTTTG-3’

V_{617}F-F: 5’-AGCATTTGGTTTTAAATTATGGAGTATGTTTCTTGTGGAGACGAGA-3’

V_{617}F-R: 5’-TCTCGTCTCCACAGAAACATACTCCATAATTTAAAACCAAATGCT-3’

The PCR reactions were performed using an MJ Research PTC-200 Peltier Thermal Cycler for 16 cycles at 95 ℃ for 30 seconds twice, 55 ℃ for 1 minute, 68 °C for 10 minutes (1 minute per kb) and ended with 60 °C for 5 minutes. 1μl DpnI (New England Biolabs, Ipswich, MA) was added after cycles were completed to degrade methylated template DNA. Samples were placed at 37 °C for 1 hour. 1μl of the master mix was transformed into 50 μl of XL1-Blue competent cells and was incubated on ice for 30 minutes. Cells were then heat shocked for 45 seconds in a 42 °C water bath and then transferred to ice for 2 minutes. 450 μl of super optimal broth (SOC) (Invitrogen, Carlsbad, CA) were added. Samples were shaken at 37 °C for 1 hour at 225 rpm. 100 μl were plated onto LB Agar plates containing 0.5 mM Ampicillin (Invitrogen Carlsbad, CA) and were incubated at 37 ℃ overnight. Mini preps cont aining 5 ml Lubria Bertani (LB), 0.5mM ampicillin, and 1 colony were then shaken at 37 ℃, 225 rpm overnight. DNA was isolated using a Qiagen Miniprep kit and sequenced at Stony Brook DNA Sequencing Core. 250 μl of correctly mutated colonies were then used to infect 250 ml of LB and 0.5 mM ampicillin and this was grown at 37°C, 225 rpm overnight. DNA was purified using a Qiagen Maxiprep kit.

2.3 Transfections

The cells were transfected using Neon Tranfection System (Invitrogen, Carlsbad, CA) by electroporation according to manufacturer’s instructions. Transfections were performed in 1 pulse at 1700v and 20ms.

2.3.1 JAK2 Expression

1, 2, 4, and 8 μg of pEF JAK2 cDNA containing a V5-His-A label (*pEF-JAK2-V5*) were transfected into 3 x 10^6 cells Ba/F3 cells expressing c-Mpl (Ba/F3-Mpl). The cells

were collected and protein was harvested 24 hours after transfection for flow cytometry and Western blotting, respectively.

2.3.2 Mutant *JAK2 Expression*

3 x 10^6 cells and 3μg of WT *JAK2*, JAK2R_{564}Q, *JAK2*V_{617}F, *JAK2*$R_{564}Q/V_{617}F$ cDNAs were used for transfection of a 25cm^2 flask of Ba/F3 cells expressing c-Mpl. The cells were collected and protein was harvested 24 hours after transfection for flow cytometry and Western blotting, respectively.

2.4 Western blot analysis

Cells from 75cm^2 tissue culture flasks were centrifuged for 5 minutes at 1000rpm at room temperature. Supernatants were aspirated and pellets were resuspended in 10ml cold PBS and centrifuged again for 5 minutes at 1000 rpm. Supernatants were aspirated and pellets were resuspended in 1ml cold PBS and transferred into a 1.5 ml centrifuge tube and pelleted. Supernatants were aspirated and pellets were lysed in 100-250 μl Nonidet P40 (NP-40) lysis buffer (50 mM Tris-HCl, pH 7.4, 150mM NaCl, 1% NP-40, 1 mM Na_3VO_4) supplemented with protease inhibitors (Sigma-Aldrich). Protein concentrations were calculated using the BCA assay system (Bio-Rad, Hercules, CA) following the manufacturer's instructions. 20 μg of protein in 1% NP-40 lysis buffer and 4X Laemmeli loading dye (200 mM Tris, 400 mM DTT, 8% SDS, 0.4 % bromophenol blue, 40% glycerol, pH 6.3) were loaded onto a 4-15%, 10-well comb, 30 μl well Mini-PROTEAN TGX pre-cast gels (Bio-Rad), separated using SDS-PAGE, and run at 130 volts for 60-90 minutes in 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. Gels were transferred to polyvinylidene fluoride (PDVF) membrane at 110 volts for 1 hour in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol, 0.1% SDS). PVDF membrane was transferred into TBS-T (Tris-buffered saline containing 0.05% Tween-20 (TBS-T)) for 5 minutes and then to reduce non-specific binding, 10 ml of blocking buffer (4% dried milk or BSA, TBST) was added to PVDF membrane and incubated for 1 hour at room temperature. Immunodetections were performed with 1 hour incubations of specific primary antibodies (Table 1) and anti-mouse or anti-rabbit secondary antibodies with a horseradish peroxidase-conjugate (Table 2) in blocking buffer with 1 15-minute

wash and 2 5-minute washes with TBS-T in between incubations to remove excess or unbound antibody. A chemiluminescent detection reagent (ECL-plus; GE Health-care) was added for 5-minutes before the membrane was exposed to autoradiography film.

Primary Antibodies			
Name	Company	Dilution	Incubation Time (min)
JAK2	Cell Signaling	1:3000	60
V5	Invitrogen	1:5000	60
c-Mpl	Milipore	1:10000	60
Actin	Sigma-Aldrich	1:10000	60

Secondary Antibodies			
Name	Company	Dilution	Incubation Time (min)
α – rabbit HRP	Bio-Rad	1:20000 for c-Mpl	60
		1:3000 for JAK2	60
α – mouse HRP	Bio-Rad	1:3000 for V5	60

2.5 Flow Cytometry

2.5.1 c-Mpl Internalization

4 x 10^6 cells of each cell line were starved at 37 ℃ in RPMI-1640 with 2% FBS for 2 hours in a 24-well plate in quadruplicates with 1 x 10^6 cells per well. After 2 hours, cells were stimulated with 10ng/ml of recombinant TPO (kindly provided by Genentech, WA) for 0, 5, 15, and 30 minutes. 300 μl of each well were transferred to a micro centrifuge tube and pelleted. Cells were resuspended in 1ml of 0.1% BSA in PBS and incubated with a c-Mpl antibody recognizing the extracellular domain of c-Mpl (kindly provided by Amgen, Thousand Oaks, CA) for 15 minutes. Cells were then spun down, supernatants were aspirated, and pellets were resuspended in anti-mouse Q-DOT 585 antibody (Invitrogen, Carlsbad, CA) for 15 minutes. 1 ml of 0.1% BSA in PBS was added and cells were analyzed with an Accuri C6 flow cytometer (BD Accuri, Ann Arbor, MI) to measure c-Mpl surface expression.

2.5.2 Annexin V and Cell Counting/Survival

5 x 10^6 cells were starved at 37 ℃ in RPMI-1640 with 2% FBS for 24 hours in 25 cm^2 culture flasks. After 24 hours, 1 ml was removed and transferred to a micro centrifuge tube. 200 μl of cells were removed and mixed with 200μl PBS for cell counting using the flow cytometer. The remaining 800 μl were centrifuged, supernatant was aspirated and the pellet was resuspended in 300 μl of 1X annexin buffer (100 mM HEPES, 140 mM NaCl, 25 mM $CaCl_2$, pH 7.4). 100μl was removed and mixed with 5 μl FITC-conjugated annexin V antibody (BD Pharmingen, San Diego, CA). After incubating for 15 minutes, 400 μl of 1X annexin buffer were added. Samples were analyzed using an Accuri C6 flow cytometer (BD).

2.5.3 Bromodeoxyuridine (BrdU) Assay

Using a FITC/BrdU Flow Kit (BD Pharmingen, San Diego, CA) 5 x 10^6 cells were starved at 37 ℃ in RPMI-1640 with 2% FBS in 25 cm^2 culture flasks. A control was performed simultaneously under the same conditions except cell were maintained in RPMI-1640 with 10% FBS supplemented with IL-3. After 24 hours, 3 ml of cells were

removed and incubated with 30μl of 1mM BrdU for 1 hour at 37 ℃. Cells were spun down, supernatant was aspirated, and pellets were resuspended with 100 μl of BD cytofix/cytoperm buffer (paraformaldehyde, saponin) per cell line for 30 minutes on ice to fix cells. After incubation, cells were washed in 1ml of 1X BD perm/wash buffer (FBS, saponin) and pelleted. Supernatant was aspirated and pellets were resuspended with 100 μl BD cytoperm plus buffer (Dulbecco's Phosphate-Buffered Saline, 4% w/v paraformaldehyde). Cells were incubated for 10 minutes on ice. Cells were washed in 1ml of 1X BD perm/wash buffer and pelleted. Supernatant was aspirated and pellets were resuspended with 100μl of BD cytofix/cytoperm buffer and incubated on ice for 5 minutes to re-fix cells. Cells were washed in 1ml of 1X BD perm/wash buffer and pelleted. Supernatant was aspirated and pellets were resuspended in 100 μl DNase (300μg/ml) and incubated at 37 ℃ for 1 hour. Cells were washed in 1ml of 1X BD perm/wash buffer and centrifuged. Supernatant was aspirated and pellets were resuspended with 50 μl of anti-BrdU FITC at 1:50 (BD Bioscience) and incubated for 20 minutes at room temperature to stain BrdU-positive labeled cells. Cells were washed in 1ml of 1X BD perm/wash buffer and pelleted. Supernatant was aspirated and pellets were resuspended with 20 μl of 7-Amino-actinomycin D (7-AAD) to label DNA. 1ml of .1% BSA in PBS was added to each tube to stop reaction. Samples were analyzed using an Accuri C6 flow cytometer at 400 events/sec to measure BrdU expression.

Chapter 3

RESULTS

To observe JAK2 regulation and its effect on c-Mpl surface expression, 1, 2, 4, and 8 µg of pEF-JAK2-V5 were transfected into Ba/F3 cells expressing c-Mpl. Western blot analyses showed equal expression of c-Mpl in WTJAK2, JAK2R_{564}Q, JAK2V_{617}F, and JAK2R_{564}Q/V_{617}F (Figure 3.1). Similar results were observed after transfecting 3 µg of pEF WTJAK2 cDNA of mutant JAK2 into Ba/F3 cells expressing c-Mpl were comparable in each cell line (Figure 3.2a, 3.2b).

To analyze the differences in cell survival, cell proliferation, and cell cycling in the different mutants in comparison to WTJAK2, stably transfected Ba/F3 cells expressing c-Mpl with WTJAK2, JAK2R_{564}Q, JAK2V_{617}F, and JAK2R_{564}Q/V_{617}F were starved in 2% FBS for a total period of 72 hours. Cell viability was monitored every 24 hours over a 72 hour period by flow cytometry and revealed a large difference between WTJAK2 and mutants (Figure 3.3). About 60% of WTJAK2-expressing cells were dead after 48 hours in the absence of cytokine which was nearly double the amount of JAK2V_{617}F and JAK2R_{564}Q/V_{617}F-expressing cells. A decrease resulted in the number of viable cells in WTJAK2-expressing cells and an increase occurred in the number of cells expressing JAK2V_{617}F, confirming their factor independence. A modest decrease was observed in the JAK2R_{564}Q-expressing cells which only resulted in a 20% drop in cell number. After 48 hours, there was roughly a 2 fold difference in the number of viable cells in the JAK2R_{564}Q mutant in comparison to WTJAK2. Lastly, the double mutant, JAK2R_{564}Q/V_{617}F which was developed to examine any additive effects of the JAK2R_{564}Q mutation on JAK2V_{617}F, also demonstrated higher cell viability than the WTJAK2-expressing cells.

Rates of apoptosis were studied using an annexin V antibody by flow cytometry. After 48 hours, nearly 60% of the WTJAK2 expressing cells were apoptotic while the JAK2V_{617}F expressing cells were less sensitive to the absence of cytokine and showed significantly less cell death. Apoptosis was attenuated in JAK2R_{564}Q expressing cells. To further confirm cytokine dependence in the mutants, a BrdU assay was performed to

detect cells cycling in S-phase (Figure 3.5). Similar trends as seen in the annexin V assay were also observed. JAK2V_{617}F expressing cells continued to proliferate throughout the 48 hour period despite the absence of cytokine. However, in JAK2R_{546}Q expressing cells proliferation was unaltered. Within the first 48 hours, we found that JAK2R_{564}Q exhibited only a modest increase in the number of cycling cells compared to the control (Figure 3.6). Cells expressing the JAK2V_{617}F mutation however continued to actively proliferate for the first 48 hours. Proliferation decreased slightly compared to cells grown with cytokine. The number of apoptotic cells in WTJAK2 dramatically increased over the 72 hour starvation period in comparison to the mutants (Figure 3.4). The number of annexin V positive cells decreased 1.5 fold in the JAK2R_{564}Q mutants over the 72 hour period in comparison to WTJAK2. The apoptotic rate was about 1.2 times lower in JAK2R_{564}Q than in WT. Rates of apoptosis in JAK2V_{617}F decreased over 72 hours and were nearly 2 fold lower in comparison to WT. The double mutant also showed decreasing amounts of annexin V positive cells over time which were comparable to the JAK2V_{617}F mutant. Taken together, these data reveal a difference in both survival and proliferation behavior between the mutants.

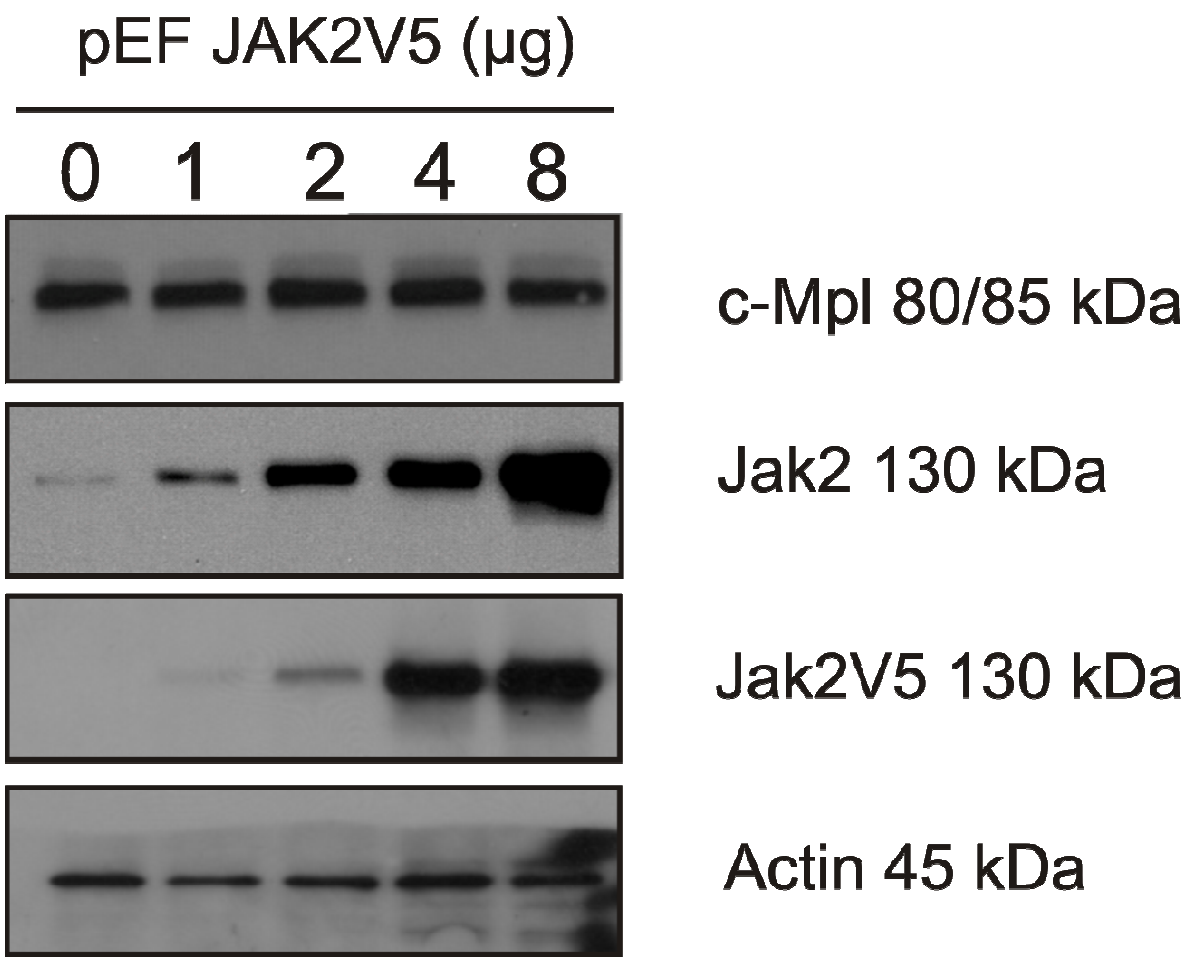

Figure 3.1. Western blot analysis of c-Mpl, JAK2, JAK2V5, and actin protein. (a) Equal levels of c-Mpl expression after transfection of 0, 1, 2, 4, and 8 µg of JAK2V5 into Ba/F3 cells expressing c-Mpl. (b) Endogenous levels of JAK2. (c) Expression levels of transfected 0, 1, 2, 4, and 8 µg of JAK2V5 (d) Actin.

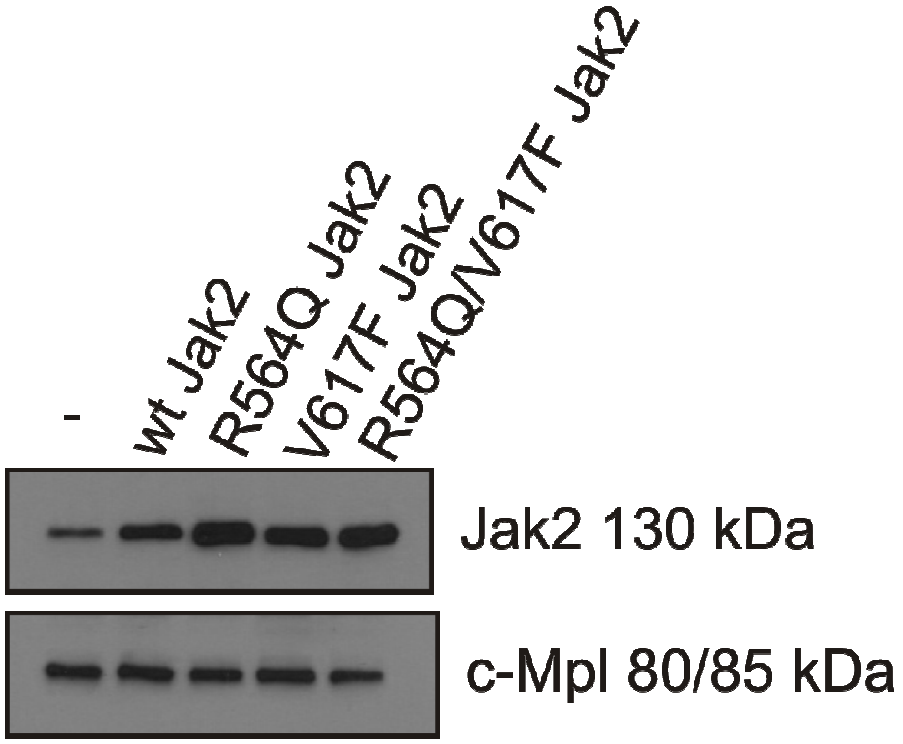

Figure 3.2a. Western blot analysis of Jak2 and c-Mpl protein after transient tranfections of 3ug pcDNA *WTJAK2, JAK2R$_{564}$Q, JAK2V$_{617}$F, JAK2R$_{564}$Q/V$_{617}$F*. Both (a) and (b) show equal expression levels of JAK2 and c-Mpl in *WT JAK2* and mutants.

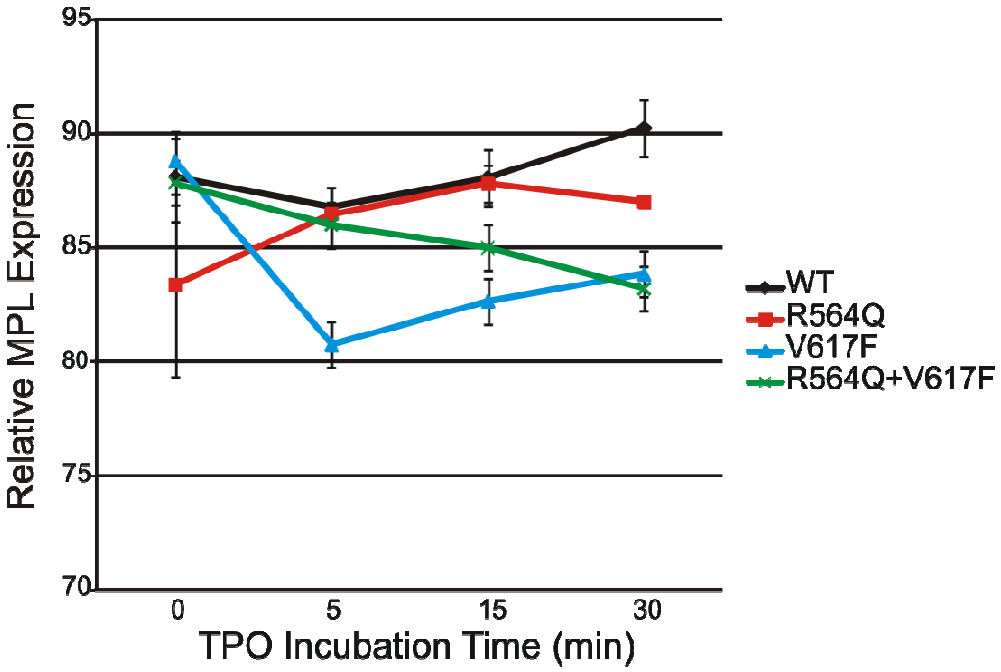

Figure 3.2b. Relative MPL surface expression after 0, 5, 15, 30 minutes of incubation with 10ng/ml TPO. MPL surface expression is similar in WT and mutant expressing cells.

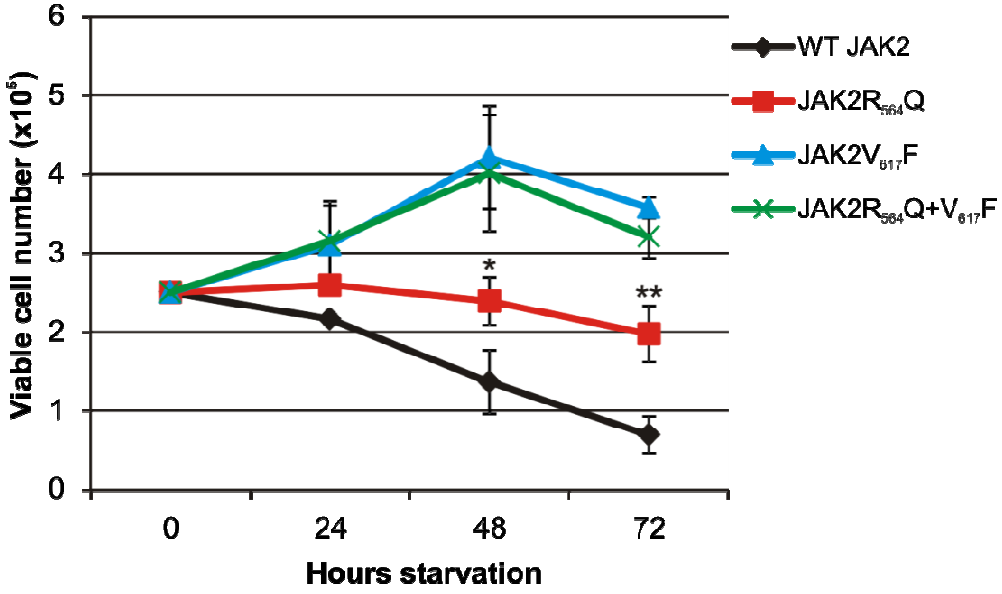

Figure 3.3. Cell survival in the absence of cytokine over a 72 hour period. WT JAK2 expressing cells continue to die in the absence of cytokine while both $JAK2V_{617}F$ and $JAK2R_{564}Q/V_{617}F$ expressing cells continue to proliferate. $JAK2R_{564}Q$ expressing cells show only a modest change in proliferation in comparison to the other mutants. (** $p<0.001$, * $p<0.05$).

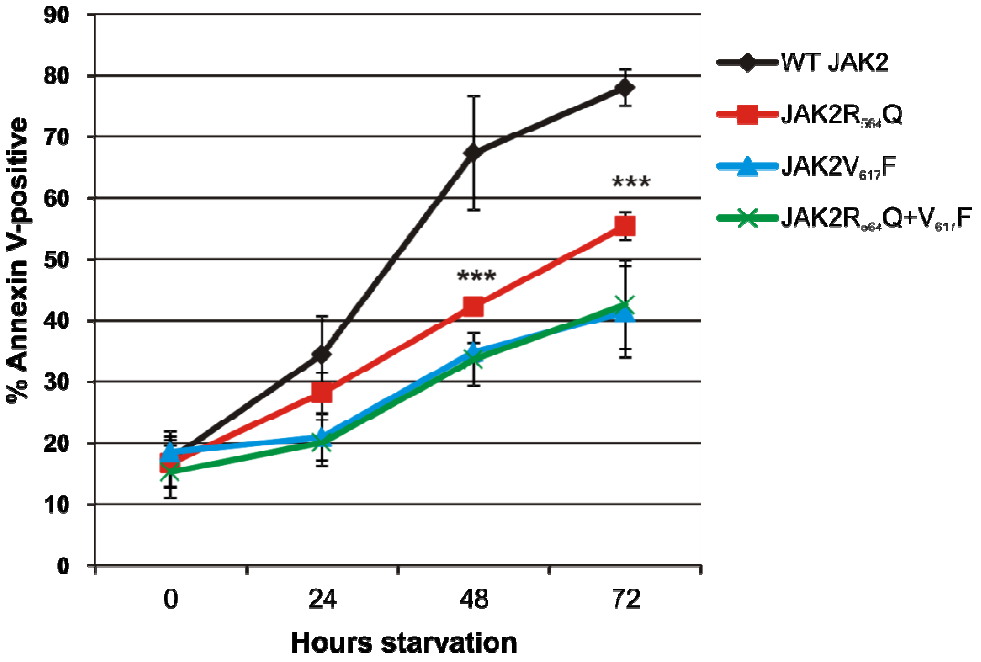

Figure 3.4. Apoptosis in the absence of cytokine over a 72 hour period. An increase of apoptotic cells is observed in WT JAK2 expressing cells whereas the rate of apoptosis in $JAK2V_{617}F$ and $JAK2R_{564}Q/V_{617}F$ expressing cells decrease throughout the 72 hour period. Apoptosis is modest in R564Q expressing cells. (*** $p<0.0001$, ** $p<0.001$, * $p<0.05$).

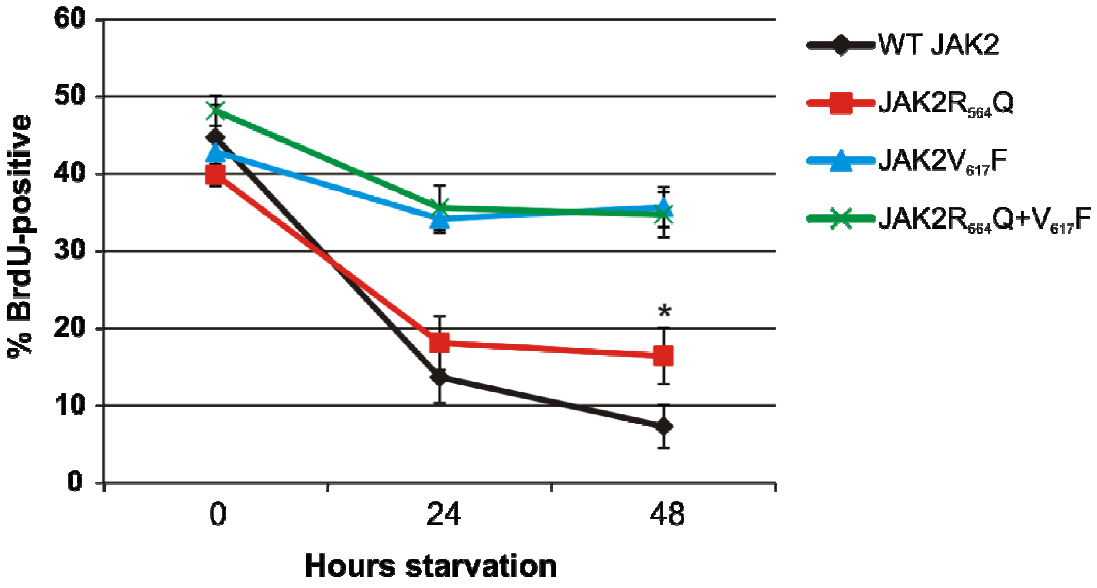

Figure 3.5. Proliferation in the absence of cytokine over a 48-hour period. A modest change in the number of cycling cells in JAK2R$_{564}$Q expressing cells was observed. Both JAK2V$_{617}$F and JAK2R$_{564}$Q/V$_{617}$F expressing cells continued to actively proliferate over the 48 hour period. (* $p<0.05$).

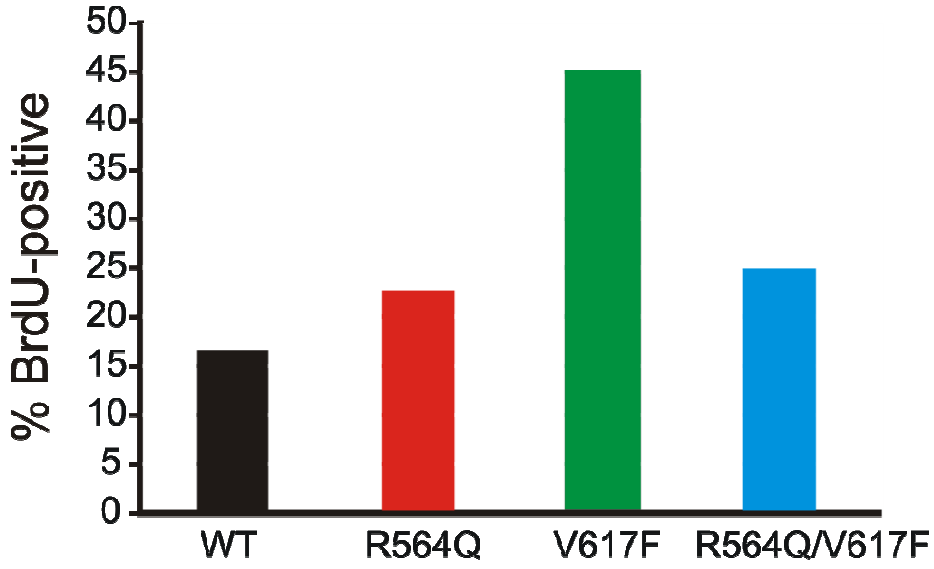

Figure 3.6. Proliferation in the presence of cytokine after 24 hours. JAK2V$_{617}$F expressing cells are hypersensitive to cytokine with proliferation rates 3 fold higher than WTJAK2 and nearly 2 fold higher than JAK2R$_{564}$Q and the double mutant.

CHAPTER 4

DISCUSSION

Since the identification of the JAK2V_{617}F mutation and its connection to PV, ET, and PMF, much has been discovered about the causes of hematopoietic dysfunction and the quest to find and understand new mutations has broadened. Other alterations in MPD determinants are also under critical examination for their clinical potential as drug targets including mutations and deletions in JAK2, c-Mpl, the tumor suppressor gene TET2, Additional Sex Combs-Like 1 (ASXL1), and Casitas B-lineage lymphoma proto-oncogene (CBL) [15]. There are inhibitors in clinical trials that target JAK2 deregulation caused by JAK2V_{617}F[16]. Although this alternative treatment is perceived to be a breakthrough, especially since current treatment options are limited, some issues exist with binding specificity. Inhibiting a critical secondary signaling molecule can result in severe side effects. Due to the important role of JAK2, blocking it has resulted in anemia and cytopenia in clinical trials[16]. Also, the mutation is outside the ATP binding pocket and is difficult to target. There are many research challenges involving TPO, c-Mpl, and JAK2 and the mechanisms by which mutations of JAK2 and other factors that contribute to disease needs further examining to improve diagnosis and treatment.

Due to its association with the TPO receptor and mutations within JAK2 being important determinants in myeloproliferative disorders, our initial studies began with the overexpression of WTJAK2 and JAK2 mutants to observe any changes in the level of c-Mpl expression. No apparent differences were observed in c-Mpl levels when comparing JAK2 mutations to wild type. These mutations are found in the pseudokinase domain, not in the FERM domain where c-Mpl binding occurs. These initial findings suggest that mutations to the pseudokinase domain do not affect c-Mpl expression in the cells. Western blot analysis is a method for visualizing total expression of c-Mpl in the cells but does not give accurate information about the localization of c-Mpl. In order to characterize any differences in localized c-Mpl expression caused by mutations to JAK2, flow cytometry was performed using anti-Mpl to probe for cell surface expression which would correlate to the 85kDa bands on the western blots. Anti-Mpl allows for specific measuring of surface c-Mpl only, which is the active form of the receptor.

Interestingly, we observed no rise in c-Mpl expression with the overexpression of WTJAK2 and JAK2 mutants. This further suggests that the levels of JAK2 may not be the cause of myeloproliferative disorders by directly increasing the membrane expressed form but rather that the mutations may be more significant in contributing to functionality.

To determine differences and similarities in functionality among the mutations, WTJAK2, JAK2R_{564}Q, JAK2V_{617}F, and a double mutant, JAK2R_{564}Q/V_{617}F were stably transfected into Ba/F3 cells expressing c-Mpl. Our cell survival and proliferation data showed that JAK2V_{617}F is cytokine independent which enables JAK2V_{617}F and the double mutant expressing cells to hyperproliferate in the absence of cytokine. This ability further contributes to the hyperfunction of JAK2 and the high platelet counts seen myeloproliferative disorders. Contrastingly, JAK2R_{564}Q expressing cells revealed an insignificant change in cell number while the double mutant showed even higher rates of cell proliferation than JAK2V_{617}F. This observation indicates that JAK2R_{564}Q contributes to myeloprolferative dysfunction through mechanisms that differ from JAK2V_{617}F. These results infer that JAK2R_{564}Q exhibits decreased apoptosis in the absence of cytokine, but not proliferation while JAK2V_{617}F and the double mutant shows both viability and proliferation in the absence of cytokine. Overall, this data supports that the JAK2R_{564}Q mutation inhibits apoptosis in the absence of cytokines while JAK2V_{617}F stimulates proliferation.

Proper functioning of JAK2 is critical for downstream signaling and platelet production. JAK2 is regulated through interactions between the kinase and pseudokinase domain and phosphorylation of important tyrosine (Y) residues including Y1007/1008 which is responsible for JAK2 activation, Y570 which is associated with negative regulation and Y813 which positively regulates the kinase via SH2-Bβ[12]. We found that two mutations within the pseudokinase domain have different biological functions by which they contribute to cell survival and proliferation and cause myeloproliferative disorders. Our evidence reveals an anti-apoptotic function of JAK2R_{564}Q in comparison to JAK2V_{617}F, which appears to have pro-proliferative ability. First, we showed that regardless of the amount of transfected pEF JAK2 cDNA along

with mutant JAK2 cDNA, levels of total and cell surface c-Mpl are nearly equal which may suggest a highly regulated turn-over rate and regulation of JAK2 within the cell. Secondly, our cell counts suggest that the JAK2V_{617}F-expressing cells are cytokine independent. In the absence of cytokine, JAK2V_{617}F along with the double mutant expressing cells were still able to actively proliferate. Thirdly, our apoptosis data reveals an anti-apoptotic function of the JAK2R_{564}Q expressing cells. These data also further validates our claim that both the JAK2V_{617}F and double mutant expressing cells can stimulate proliferation in the absence of cytokine. Concurrent with the previous observations, incubations with BrdU further revealed that the JAK2V_{617}F and JAK2R_{564}Q/V_{617}F mutations are responsible for the hyperfunction of JAK2 activity and contribute to increases in proliferation in the absence of cytokine.

These results offer promising insights to the functionality JAK2R_{564}Q. Although JAK2R_{564}Q is localized in the same pseudokinase domain and hypothesized to be in relatively close proximity to V617 in structural models, its effect on cell survival is significantly different to JAK2V_{617}F[17]. Residues R564 and V617 are found in the pseudokinase domain, JH1, which binds the kinase domain, JH2, negatively regulating activation[18]. It has been shown that V617 is important for binding to the activation loop of JH1[17]. The JAK2V_{617}F mutation causes hyperproliferation in three hematopoietic cell lineages that leads to myeloproliferative disorders. It was shown that in the V_{617}F mutation, F617 exhibits a π-stacking interaction with F595 in the JH1 domain, which blocks the interaction with the activation loop of JH2[17]. Without this interaction, JH2 is not regulated and thus will continue to stay active and contribute to JAK2 hyperfunction in myleoproliferative disorders. JAK2R_{564}Q has also been shown to inhibit apoptosis and cause essential thrombocytosis. However these effects are not as additive to the JAK2V_{617}F mutation, with the JAK2V_{617}F being the dominant mutation. In order to derive a mechanism based on this information, structure and function relationships need careful analysis. In the JH1 domain, R564 forms a salt bridge interaction with two residues; E543 and D544[17]. Therefore any mutation to R564 to anything other than lysine would disrupt this salt bridge interaction. Through simulation, a similar mutation, R_{564}L, was shown to disrupt this salt bridge interaction and also have significant effects on the structure of the JH1/JH2 interactions[17]. Without this salt bridge interaction, a

loop region (residues 539-544) of the JH1 domain which appears to regulate the relative position of V617 is disrupted. These data suggest that altering this loop region disrupts the interactions of V617, which would then contribute to constitutive activation and hyperfunction of JAK2 activity. In the double mutant, the $V_{617}F$ mutation already disrupts the interactions between V617 and the activation loop, which dominates $R_{564}Q$ effects and accounts for the double mutant mirroring the functionality of the $V_{617}F$ mutation alone. Although the double mutant showed some ability to inhibit apoptosis due to $R_{564}Q$, the $V_{617}F$ phenotype dominated. X-ray crystal structure data on WTJAK2, JAK2$R_{564}Q$, JAK2$V_{617}F$, and JAK2$R_{564}Q/V_{617}F$ is needed to confirm these theories, elucidate interactions occurring within the protein, and provide more insight on structure and function.

JAK2$R_{564}Q$ has unique functionality from JAK2$V_{617}F$ and inhibits apoptosis. However, further studies need to be done on this novel and unique JAK2$R_{564}Q$ mutation. Originally found in a family with elevated platelet levels with no sign of a JAK2$V_{617}F$ mutation, it may also have a sex-linked phenotype. Higher platelet counts were found in the male in comparison to his sister and mother. This may be indicative of a sex-linked trait contributing to a higher platelet count phenotype. *In vivo* studies can provide more insights as well as validate previous in vitro findings to the biological properties and pathogenesis of the mutation. More patient samples containing the $R_{564}Q$ mutation are needed to further examine any predominant trends in phenotype, symptoms, and regional or environmental effects. Mouse models could be used to mimic myeloproliferative disorders and to observe whether similar anti-apoptotic functionality occurs due to the $R_{564}Q$ mutation. Studies also need to be done to determine the mechanisms by which a single mutation can cause multiple disorders. The $R_{564}Q$ mutation confirms the importance of JAK2 in hematopoietic homeostasis and its critical role in myeloproliferative disorders. The identification of JAK2 mutations aids in the prognosis of patients with $R_{564}Q$ and the quest for therapeutic targets and potential cures.

REFERENCES

1. Geddis, A.E., *Megakaryopoiesis.* Seminars in hematology. **47**(3): p. 212-219.
2. Baxter, E.J., et al., *Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders.* The Lancet, 2005. **365**(9464): p. 1054-1061.
3. Kindt, T.J., *Kuby immunology*. 6th ed. ed, ed. R.A. Goldsby2007, New York, N.Y: W.H. Freeman.
4. Geddis, A.E., *The regulation of proplatelet production.* Haematologica, 2009. **94**(6): p. 756-759.
5. Kaushansky, K., *Historical review: megakaryopoiesis and thrombopoiesis.* Blood, 2008. **111**(3): p. 981-986.
6. Kaushansky, K., *Molecular mechanisms of thrombopoietin signaling.* Journal of Thrombosis and Haemostasis, 2009. **7**: p. 235-238.
7. Kaushansky, K., *Thrombopoietin: a tool for understanding thrombopoiesis.* Journal of Thrombosis and Haemostasis, 2003. **1**(7): p. 1587-1592.
8. Kaushansky, K., et al., *Thrombopoietin, the Mp1 ligand, is essential for full megakaryocyte development.* Proceedings of the National Academy of Sciences, 1995. **92**(8): p. 3234-3238.
9. Saharinen, P., K. Takaluoma, and O. Silvennoinen, *Regulation of the Jak2 Tyrosine Kinase by Its Pseudokinase Domain.* Molecular and Cellular Biology, 2000. **20**(10): p. 3387-3395.
10. Nichols, K.E., et al., *Familial dyserythropoietic anaemia and thrombocytopenia due to an inherited mutation in GATA1.* Nat Genet, 2000. **24**(3): p. 266-270.
11. Spivak, J.L., *Polycythemia vera: myths, mechanisms, and management.* Blood, 2002. **100**(13): p. 4272-4290.
12. Kurzer, J.H., et al., *Tyrosine 813 Is a Site of JAK2 Autophosphorylation Critical for Activation of JAK2 by SH2-BÎ².* Molecular and Cellular Biology, 2004. **24**(10): p. 4557-4570.
13. Baxter, E.J., et al., *Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders.* Lancet, 2005. **365**(9464): p. 1054-1061.
14. Dai, C., et al., *Polycythemia vera. II. Hypersensitivity of bone marrow erythroid, granulocyte-macrophage, and megakaryocyte progenitor cells to interleukin-3 and granulocyte-macrophage colony-stimulating factor.* Blood, 1992. **80**(4): p. 891-899.
15. Tefferi, A., *Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1*, in *Leukemia (08876924)*, Nature Publishing Group. p. 1128-1138.
16. Lok, S., et al., *Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo.* Nature, 1994. **369**(6481): p. 565-568.
17. Lee, T.-S., et al., *Structural effects of clinically observed mutations in JAK2 exons 13-15: comparison with V617F and exon 12 mutations.* BMC Structural Biology, 2009. 9(1): p. 58.
18. Lee, T.-S., et al., *Mechanisms of constitutive activation of Janus kinase 2-V617F revealed at the atomic level through molecular dynamics simulations.* Cancer, 2009. 115(8): p. 1692-1700.