UNIVERSITY OF CALIFORNIA,
IRVINE

# Calreticulin Mutations activate MPL Signaling and Calreticulin Expression can modulate MPL Signaling

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

Doctor of Philosophy

in Cancer Biology

by

Stefan Brooks

Dissertation Committee:
Professor Angela Fleischman, Chair
Associate Professor Matt Inlay
Professor Xing Dai

2020

# DEDICATION

To

my wife, my parents and friends

# TABLE OF CONTENTS

# LIST OF FIGURES

# ACKNOWLEDGEMENTS

This dissertation is dedicated to all who have helped me along this journey including the following: Christie and Prentis Brooks III, Ruth McConnaughhay, Carlton and Diana Brooks, Megan Brooks, Lauren Brooks, Luke, Hannah, Georgia, and Bennett Zappa, Nina Zappa, Justin and Megan Grubisha, Luke Cypher, Keith Rumenser, Munan Singhal, Tyler and Jodi Owczarski, Nick and Alli Jasch, Chad and Kaylee Mesbergen, John Policarpio, Michael and Layna Parra, Tricia Hildago, Genesis Tenerio, Corey and Ellen Collins, Russell, Kristen and Everett Uchizono Dylan, Kelly and Jaxson Heyden, Devon Langston, Megan Magee, Ted, Krista and Kerrianne Sames, and anyone else that helped me along the way.

I would like to thank my P.I, Dr. Angela Fleischman, for her tremendous support during my time in her lab. She really champions you as a graduate student. She supports you in any endeavor you wish to accomplish inside or outside the lab. She has really set up an environment in the lab that feels like family. She sets such an example with how hard she works seeing patients, running a lab, applying for grants, helping to write and edit papers, and traveling all over the world presenting the research coming out of the lab. I honestly would not have completed this project without her support and guidance. I am excited to be the first graduate student graduating from her lab and hope to have left the lab better off than when I started.

Secondly, I would like to thank Sarah Morse. She was the lab manager when I first started in the lab. Her and her husband Zach were so welcoming to me when I first joined the lab and in short time became good friends. I am extremely thankful for their friendship and Sarah for her help during her time in the lab.

I would like to thank Hew Yeng Lai for the comedic relief during our many years in the lab. Hew Yeng better known as “Betty” and I had quite the sibling like relationship. We would disagree on so many things yet anytime one of us had a need, we were always there to provide it.

I would like to thank Bri Hoover. She consistently pushed me to be a better graduate student in every way during my time in the lab. We always had great times scouting for great food locations during trips to ASH. I still think about Porter’s Beer Bar all the time.

I would like to thank Gaja for her help. I was feeling a bit stuck on this project when she joined our lab as a post doc. Her work and experience in isolating fetal livers and assays analyzing megakaryocytes helped renew my interest in this project and really pushed it over the finish line.

I would like to thank Laura, Bin Gu, Hellen, Jenny, Dr. Kim, Summer, Roshan, Himanshu and Quy.

I would like to thank those from my CMB cohort such as Amber, Jessica, Bob, Matt and Christine and Armond. Although we didn’t spend as much time together as I think we all would’ve liked, the encouragement I felt every time we did, kept me going.

I would also like to thank any of my co-authors on the papers I have been a part of.

I would like to thank the Fellows program at the UCI Beall Applied Innovation, especially Casie, Lisa, and Stephen. Thanks for giving me an opportunity to discover that working in intellectual property is what I wanted to do after I graduated.

I would also like to thank Rutan & Tucker for hiring me and training me as a patent agent. I am excited to continue on in this journey.

Lastly, I would like to thank Dr. David Fruman. He was the first person I met from UC-Irvine when I came out to visit and has been so supportive of me in my time here. He has always been willing to write a letter of recommendation or give guidance on my project. I honestly am not sure if I would've come to UC-Irvine or stayed in graduate school without his belief in me.

I would be remiss if I didn't mention that part of this work was funded by the T-32 Training grant at UC-Irvine and would thus like to thank the T32 committee, mentors, Nita Driscoll and Krystina for their help.

# Curriculum Vita

## Stefan Brooks

## Experience:

Registered Patent Agent

Patent Registration Number: 78,795

*Fellow, Research Translation Group, Applied Innovation* September 2018-Present

University of California- Irvine (UCI) Irvine, CA

- Analyzed records of invention from UCI faculty/staff for patent eligibility e.g. 35 U.S.C. 101
- Conducted prior art searches using patent databases and literature to provide reports on patentability e.g. 35 U.S.C. 102, 103, 112
- Produced detailed written reports providing licensing officers with a snapshot of patentability by comparing and analyzing invention to prior art, publications and other disclosures for 35 inventions e.g. medical devices and diagnostics, biological molecules for cancer therapeutics, immune modulation and antimicrobial treatment, and methods for diagnosing and treating medical conditions

## Education:

University of California, Irvine May 2020

Ph.D. in Biological Chemistry

Diversity Recruitment Fellowship, UC-Irvine

M.S. in Biomedical Science June 2019

- Developed animal models for myeloproliferative neoplasms (MPN) to study how mutations in hematopoietic stem cells cause a MPN phenotype *in vivo*
- Designed calreticulin knockout animal model to understand calreticulin's role in how blood cells develop

University of Pittsburgh May 2013

B.A. in History

## Research:

***Brooks, S.A***., et al. "Upregulation of Endogenous Thrombopoietin Receptor (MPL) with in Vivo Passage of Calreticulin (CALR) Mutant Ba/F3 Cells, Highlighting MPL as the Requisite Cytokine Receptor for CALR Mediated Transformation." *Leukemia Research*, vol. 82, 2019, pp. 11–14., doi:10.1016/j.leukres.2019.05.003.

***Brooks, S. A*.,** et al. "JAK2V617I Results in Cytokine Hypersensitivity without Causing an Overt Myeloproliferative Disorder in a Mouse Transduction–Transplantation Model." *Experimental Hematology*, vol. 44, no. 1, 2016, doi:10.1016/j.exphem.2015.09.006.

Lai, Hew Yeng, ***Brooks, S.A.,*** Craver, B.M., Morse, S.J., Nguyen, T.K., Haghighi, N., Garbati, M.R., Fleischman, A.G., "Defective Negative Regulation of Toll-like Receptor Signaling Leads to Excessive TNF-α in Myeloproliferative Neoplasm." *Blood Advances*, vol. 3, no. 2, 2019, pp. 122–131., doi:10.1182/bloodadvances.2018026450.

Craver, B.M., Ramanathan, G., Hoang, S., Chang, E., Mendez Luque, L.F., ***Brooks, S.A.,*** Lai, H.Y., Flesichman, A.G. 2019 "N-acetylcysteine inhibits thrombosis in a murine model of myeloproliferative neoplasms", *Blood Advances* Accepted for Publication

# Curriculum Vita

## Stefan Brooks

## Experience:

Registered Patent Agent

Patent Registration Number: 78,795

*Fellow, Research Translation Group, Applied Innovation* September 2018-Present

University of California- Irvine (UCI) Irvine, CA

- Analyzed records of invention from UCI faculty/staff for patent eligibility e.g. 35 U.S.C. 101
- Conducted prior art searches using patent databases and literature to provide reports on patentability e.g. 35 U.S.C. 102, 103, 112
- Produced detailed written reports providing licensing officers with a snapshot of patentability by comparing and analyzing invention to prior art, publications and other disclosures for 35 inventions e.g. medical devices and diagnostics, biological molecules for cancer therapeutics, immune modulation and antimicrobial treatment, and methods for diagnosing and treating medical conditions

## Education:

University of California, Irvine May 2020

Ph.D. in Biological Chemistry

Diversity Recruitment Fellowship, UC-Irvine

M.S. in Biomedical Science June 2019

- Developed animal models for myeloproliferative neoplasms (MPN) to study how mutations in hematopoietic stem cells cause a MPN phenotype *in vivo*
- Designed calreticulin knockout animal model to understand calreticulin's role in how blood cells develop

University of Pittsburgh May 2013

B.A. in History

## Research:

***Brooks, S.A***., et al. "Upregulation of Endogenous Thrombopoietin Receptor (MPL) with in Vivo Passage of Calreticulin (CALR) Mutant Ba/F3 Cells, Highlighting MPL as the Requisite Cytokine Receptor for CALR Mediated Transformation." *Leukemia Research*, vol. 82, 2019, pp. 11–14., doi:10.1016/j.leukres.2019.05.003.

***Brooks, S. A*.,** et al. "JAK2V617I Results in Cytokine Hypersensitivity without Causing an Overt Myeloproliferative Disorder in a Mouse Transduction–Transplantation Model." *Experimental Hematology*, vol. 44, no. 1, 2016, doi:10.1016/j.exphem.2015.09.006.

Lai, Hew Yeng, ***Brooks, S.A.,*** Craver, B.M., Morse, S.J., Nguyen, T.K., Haghighi, N., Garbati, M.R., Fleischman, A.G., "Defective Negative Regulation of Toll-like Receptor Signaling Leads to Excessive TNF-α in Myeloproliferative Neoplasm." *Blood Advances*, vol. 3, no. 2, 2019, pp. 122–131., doi:10.1182/bloodadvances.2018026450.

Craver, B.M., Ramanathan, G., Hoang, S., Chang, E., Mendez Luque, L.F., ***Brooks, S.A.,*** Lai, H.Y., Flesichman, A.G. 2019 "N-acetylcysteine inhibits thrombosis in a murine model of myeloproliferative neoplasms", *Blood Advances* Accepted for Publication

# Curriculum Vita

## Stefan Brooks

## Experience:

Registered Patent Agent

Patent Registration Number: 78,795

*Fellow, Research Translation Group, Applied Innovation* September 2018-Present

University of California- Irvine (UCI) Irvine, CA

- Analyzed records of invention from UCI faculty/staff for patent eligibility e.g. 35 U.S.C. 101
- Conducted prior art searches using patent databases and literature to provide reports on patentability e.g. 35 U.S.C. 102, 103, 112
- Produced detailed written reports providing licensing officers with a snapshot of patentability by comparing and analyzing invention to prior art, publications and other disclosures for 35 inventions e.g. medical devices and diagnostics, biological molecules for cancer therapeutics, immune modulation and antimicrobial treatment, and methods for diagnosing and treating medical conditions

## Education:

University of California, Irvine May 2020

Ph.D. in Biological Chemistry

Diversity Recruitment Fellowship, UC-Irvine

M.S. in Biomedical Science June 2019

- Developed animal models for myeloproliferative neoplasms (MPN) to study how mutations in hematopoietic stem cells cause a MPN phenotype *in vivo*
- Designed calreticulin knockout animal model to understand calreticulin's role in how blood cells develop

University of Pittsburgh May 2013

B.A. in History

## Research:

***Brooks, S.A***., et al. "Upregulation of Endogenous Thrombopoietin Receptor (MPL) with in Vivo Passage of Calreticulin (CALR) Mutant Ba/F3 Cells, Highlighting MPL as the Requisite Cytokine Receptor for CALR Mediated Transformation." *Leukemia Research*, vol. 82, 2019, pp. 11–14., doi:10.1016/j.leukres.2019.05.003.

***Brooks, S. A*.,** et al. "JAK2V617I Results in Cytokine Hypersensitivity without Causing an Overt Myeloproliferative Disorder in a Mouse Transduction–Transplantation Model." *Experimental Hematology*, vol. 44, no. 1, 2016, doi:10.1016/j.exphem.2015.09.006.

Lai, Hew Yeng, ***Brooks, S.A.,*** Craver, B.M., Morse, S.J., Nguyen, T.K., Haghighi, N., Garbati, M.R., Fleischman, A.G., "Defective Negative Regulation of Toll-like Receptor Signaling Leads to Excessive TNF-α in Myeloproliferative Neoplasm." *Blood Advances*, vol. 3, no. 2, 2019, pp. 122–131., doi:10.1182/bloodadvances.2018026450.

Craver, B.M., Ramanathan, G., Hoang, S., Chang, E., Mendez Luque, L.F., ***Brooks, S.A.,*** Lai, H.Y., Flesichman, A.G. 2019 "N-acetylcysteine inhibits thrombosis in a murine model of myeloproliferative neoplasms", *Blood Advances* Accepted for Publication

# ABSTRACT OF THE DISSERTATION

Calreticulin Mutations activate MPL Signaling and Calreticulin Expression can modulate MPL Signaling

by

Stefan Brooks

Doctor in Philosophy

University of California, Irvine, 2020

Professor Angela Fleischman, Chair

Myeloproliferative Neoplasms are a collection of blood cancers in which there is a dysregulation of hematopoiesis leading to an over proliferation of myeloid cells. These are often caused by activating mutations in non-receptor tyrosine kinases. Recently, mutations in the C terminus of calreticulin have been discovered in MPN patients.

Our lab has confirmed that in the presence of the TPO receptor MPL, these mutations constitutively activate the JAK/STAT pathway. Our lab has also discovered that overexpression of wild type calreticulin may suppress MPL signaling leading to impairment of self-renewal in vivo, a reduction in colony formation from sorted LKS cells that overexpress calreticulin and a physical interaction between MPL and calreticulin. Furthermore, our lab has demonstrated that loss of calreticulin leads to an increase in frequency of cell populations in the bone marrow that express MPL including LKS, LKS-SLAM and MEPs, loss of calreticulin leads to an increase of colony formation for LKS cells and discovered the absence of calreticulin in LKS cells leads to a competitive advantage, in vivo, further suggesting that loss of calreticulin leads to enhanced MPL signaling. This dissertation helps to demonstrate a role for calreticulin in hematopoiesis.

# Chapter 1: Introduction

## 1.1 Hematopoiesis

Hematopoiesis is the process by which all blood cells are created. Hematopoiesis involves hematopoietic stem cells (HSCs) that are multipotent. These cells can differentiate into any type of blood cell including HSC, progenitor or mature blood cell as needed (1). This process of differentiation from an HSC to a mature blood cell has been described as a hierarchical structure in which HSCs exist at the top of the structure.

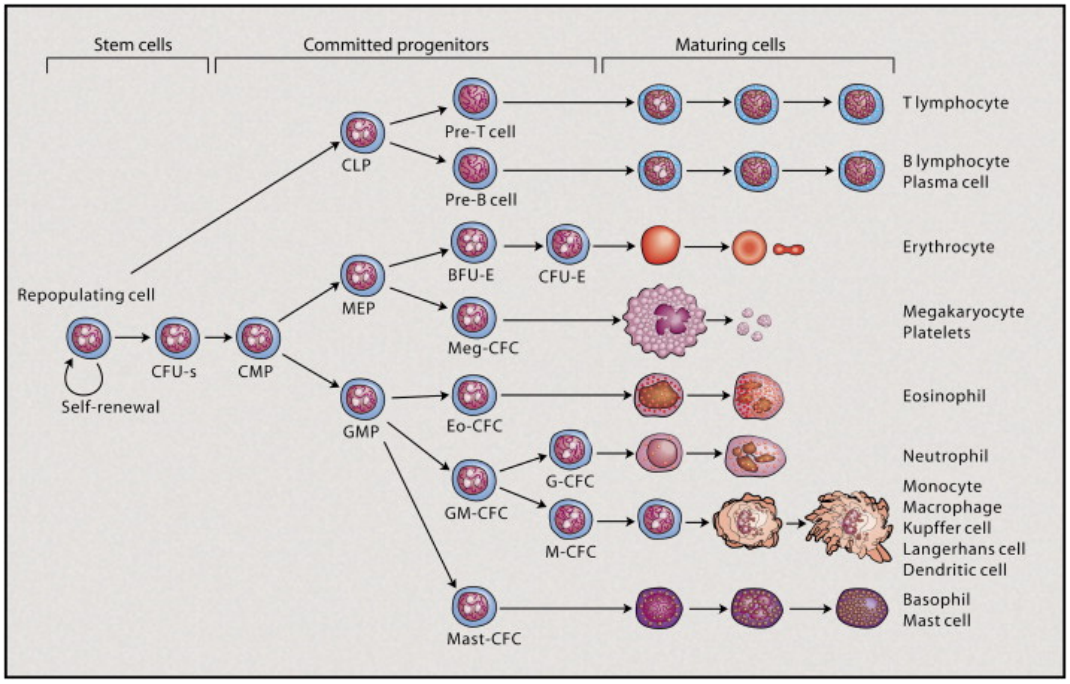



Figure 1.1: Hematopoietic Family Tree Showing how the Mature Cells in the Various Lineages Are Generated by Self-Generating **(Metcalf, Immunity 2007)**

HSCs are low in frequency in the body, approximately 0.01% of total nucleated cells, but all cells differentiate from them. As a cell moves down the hierarchy due to differentiation, the cells increase in frequency in the body and are more committed to a single cell fate. HSCs differentiate to more cell type specific progenitors such as a common lymphoid progenitor

(CLP), committing the cell to the lymphoid lineage, or a common myeloid progenitor (CMP), committing the cell to the myeloid lineage. From these progenitors, the cells can further differentiate to a more cell specific progenitor such as a granulocyte-macrophage progenitor (GMP) or a megakaryocyte-erythrocyte progenitor (MEP). From these further differentiated progenitors, the cells differentiate to the terminal or mature cell such as a B cell, T cell, platelet, red blood cell or macrophage.

This hierarchy or process of differentiation from an HSC to a mature blood cell has been worked out extensively in labs across the world. This is what is classically defined as the hematopoietic tree; however, some recently published work has demonstrated that some of these cells types may be able to skip differentiation stages such as HSCs being able to differentiate straight to a MEP without expressing markers native to a CMP (2).

The fantastic work done by many labs in this field have allowed researchers to be able to clearly identify and define HSC, progenitor and mature blood cells by a variety of proteins expressed on the cell surface (3). This allows researchers to use powerful techniques such as mass cytometry (CyTOF) and flow cytometry (FACS) to clearly define, analyze and even sort pure populations of blood cells. Furthermore, after isolating through FACS sorting we can perform limiting dilution transplants, competitive transplants, primary and secondary transplants into lethally irradiated recipient mice for functional analysis of HSCs.

HSCs can self-renew; in that they undergo mitosis to create identical daughter cells. The exact mechanism for whether this cell division is always symmetrical, meaning two identical daughters form with either having the capacity to differentiate or can be asymmetrical, meaning two daughter cells form but one is committed to differentiation from the start, has

been debated in the literature for quite some time (4). Regardless of how the exact mechanism works out, the fact that is not debated is that self-renewal occurs. This process of self-renewal preserves a pool of HSCs and allows the blood system to continue to exist throughout the life span of the organism. Physicians have exploited this occurrence in HSCs for the betterment of patients with bone marrow (BM) derived diseases such as leukemias, lymphomas, or sickle cell anemia through allogenic bone marrow transplants where healthy bone marrow from a donor is given to a bone marrow depleted patient. This allows the healthy bone marrow to home, engraft and expand in the bone marrow niche, creating healthy blood cells in the patient instead of diseased blood cells.

HSCs largely exist in a quiescent state (5). This allows HSCs to preserve their integrity as HSCs that frequently divide due to self-renewal can be subjected to various insults which may allow for mutations to occur in their genome. Furthermore, if HSCs divide too frequently, they can become exhausted, losing their self-renewal potential (6). It has been demonstrated that HSCs typically cycle ever 57 days (5). Subject to BM injury, stress of cytokines such as G-CSF, HSCs can be encouraged to divide. This delicate balance between differentiation, self-renewal and quiescence is highly regulated, involving a system of cytokines and chemokines that bind to cell surface receptors. When these ligands, cytokines or chemokines bind to cell surface receptors, it causes activation of the pathway including downstream effects of transcriptional changes inside the nucleus of the cell, translational changes and post translational modification changes. The specific transcriptional changes are beyond the scope of this dissertation but include DNA methylation, long non-coding RNAs, transcription factor activation and repression (7). Likewise, translational and post-translational changes occur during the differentiation

process and include upregulation or downregulation of key proteins as well as trafficking of key receptors to the cell surface (8).

Hematopoiesis occurs mostly in the bone marrow (9). During early murine embryonic development, there is a lack bone and bone marrow. Thus, hematopoiesis occurs in the yolk sac around 9.5 dpc (10). As the mouse embryo matures, hematopoiesis moves to the fetal liver around 14.5 dpc and finally moves to the bone marrow in the embryo around 18.5 dpc.

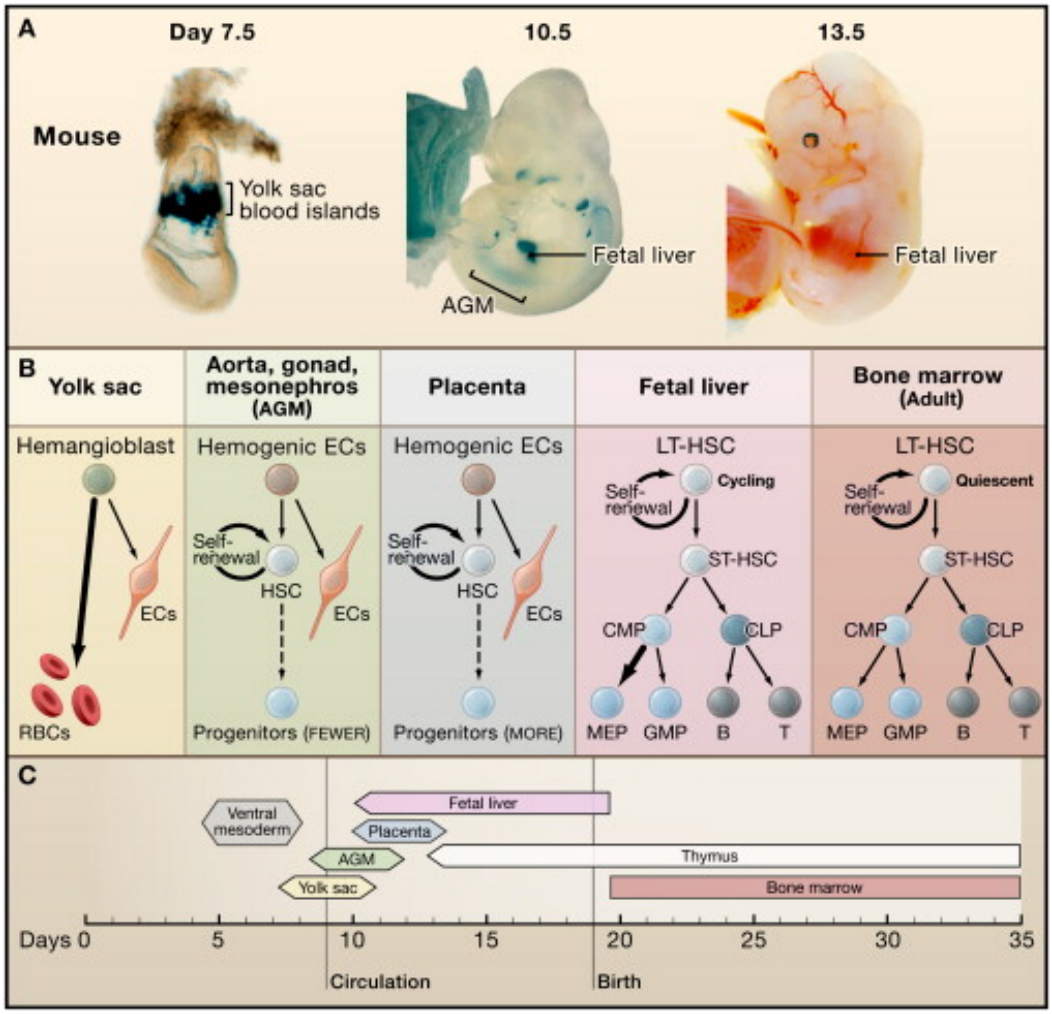



Figure 1.2: Developmental Regulation of Hematopoiesis in the Mouse (**Orkin et. al Cell 2008)**

This same process occurs in humans albeit at a different time scale as the human gestation period is much longer than the mouse gestation period. Within the bone marrow, HSCs physically reside in microenvironments called bone marrow niches. These niches are complex environments comprising of osteoblasts, stromal cells, chemokines, and endothelial cells (11). All of these cells help create a suitable environment for HSCs and can help either keep them in quiescence or provide the stimuli signals needed to proliferate and divide. Many researchers debate as to what are the necessary components to make up the bone marrow niche and research is being published every day adding additional elements to the bone marrow niche.

Hematopoiesis can occur outside the bone marrow and bone marrow niches. In mature adults, this is called extramedullary hematopoiesis. Most often this occurs during activated immune responses or when the bone marrow become uninhabitable for HSCs and progenitors due to bone marrow fibrosis. In extramedullary hematopoiesis, HSCs and progenitors will migrate to the sites of secondary hematopoiesis including the spleen and the liver (12). This can cause an enlargement of the spleen known as splenomegaly.

It is generally thought that as people age, they become more susceptible to various bone marrow ailments. This is thought to be due to a decrease in bone marrow cell number. Interestingly, it has been demonstrated that in humans and mice, HSC frequency in the bone marrow increases with age (13). The caveat of this is that although HSCs increase in the bone marrow, their competitive repopulation ability decreases, suggesting that although these cells have defined HSC cell surface markers, they are losing their HSC function. Furthermore, it has been published that in older age, in mice and humans, HSCs are actually skewed towards the

myeloid lineage and away from the lymphoid lineage during differentiation (13), which can lead to increases in myeloid cells and decreases in lymphoid cells.

**1.2 Cell Signaling Pathways in Hematopoiesis**

There are numerous cell signaling pathways that are important in hematopoiesis including Notch, Wnt, Hedgehog, JAK/STAT, TGF-B, MAPK, ERK and Smad (14). Most of these will be outside the scope of consideration for this dissertation but it is evidence that the cell signaling pathways important for hematopoiesis are complex and connected. Furthermore, many of these pathways can be secondarily activated via secondary messengers. For the purpose of this work, I will focus on the JAK/STAT pathway.

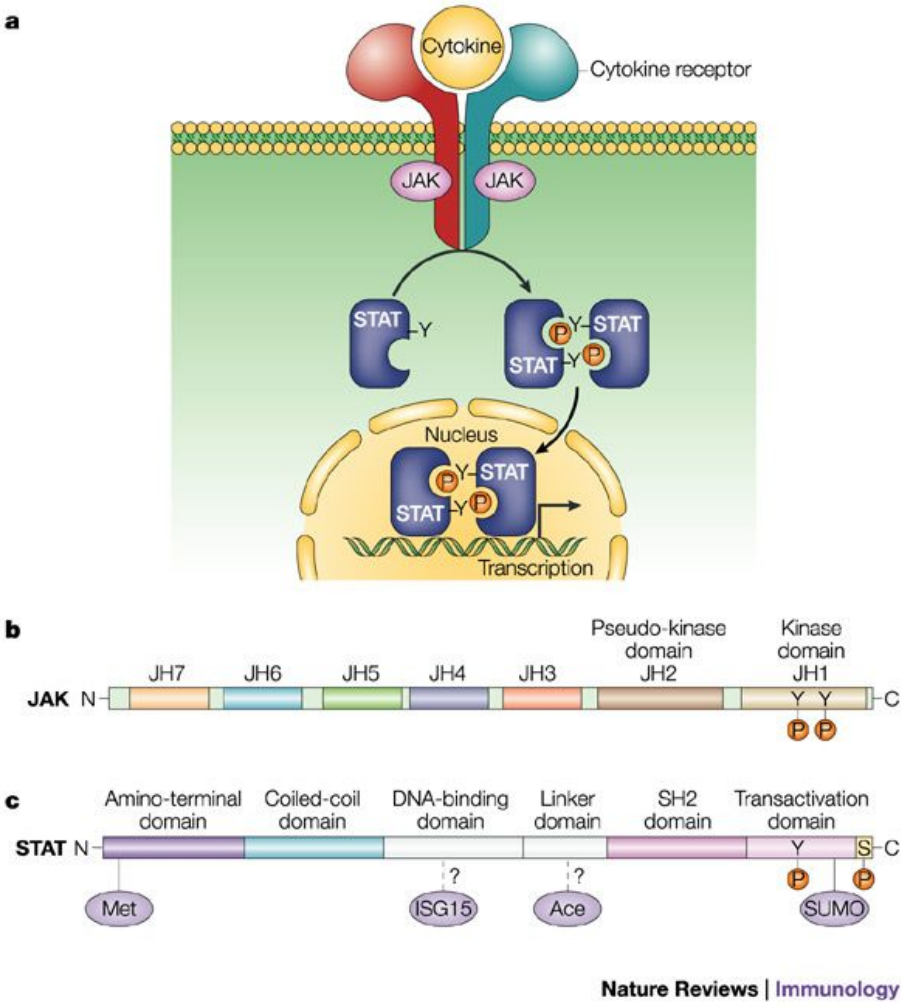



Figure 1.3: A schematic representation of the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway. **(Shuai et al Nature 2003)**

The JAK/STAT pathway activation occurs in four stages: cytokine receptor activation, tyrosine kinase activation, STAT dimerization and activation, and pathway regulation.

There are two classes of cytokine receptors that can activate the JAK/STAT pathway: class I and class II. These classes are divided by amino acid sequence similarity. Class I receptors contain cytokine receptors such as interleukin 1 (IL-1), interleukin 3 (IL-3), interleukin 6 (IL-6), erythropoietin (EPO), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), thrombopoietin (TPO) and growth hormone receptor (GHR).

Class II receptors contain mostly interferon receptors. In the cytokine receptor activation, ligands such as TPO or IL-3 bind to their respective receptors in a monomeric form (15). Once ligand and receptor are bound, the ligand receptor complex binds to another monomeric receptor to form a dimer. This dimerization activates the tyrosine kinases, stage two of JAK/STAT pathway activation.

There are 4 tyrosine kinases in the JAK/STAT pathway: such as JAK1, JAK2, JAK3 and Tyk2. The four kinases in the Janus kinase family are non-receptor tyrosine kinases meaning they must be activated by either class I or class II receptors. These kinases reside in the cytoplasm, close to the cytoplasmic regions of the cytokine receptors. Upon activation, they cross phosphorylate their respective kinases which recruits signal transducers and activators of transcription (STATs), a family of seven transcription factors that exist in the cytoplasm.

Stage three of JAK/STAT activation is the phosphorylation of STATs proteins. This causes their dimerization, activation and translocation to the nucleus to change transcription levels of key genes. Which genes are upregulated depends on which cytokine activated that pathway but canonical STAT5 genes include Tie-2, p57, CISH, Pim2, SOCS2 and CD 41 (16).

Within the JAK/STAT pathway there are a few regulatory steps to ensure this pathway is tightly regulated which is step four in JAK/STAT pathway activation. Within the JAK kinase, there exists a domain, JH2, that acts as a negative regulator of the JH1 kinase domain (17). The exact mechanism for how JH2 can regulate JH1 activity has not yet been elucidated but it is known that JH2 can prevent the JH1 domain from phosphorylating substrates. Another step of regulation in the JAK/STAT pathway is the presences of suppressor of cytokine signaling proteins known as SOCS. There are eight known SOCS proteins and their transcription is actually

upregulated by activation of the JAK/STAT pathway (18). SOCS3 specifically binds to JAK2 preventing further phosphorylation of target proteins. Other SOCS proteins can compete for phosphorylation sites on the STATs, shutting down the pathway. Furthermore, adaptor proteins such as Lnk, APS and tyrosine phosphatases such as CD 45 help negatively regulate the JAK/STAT pathway (19). All this demonstrates that the JAK/STAT pathway is extensively regulated.

**1.3 JAK/STAT's role in self-renewal.**

The JAK/STAT pathway is important for the self-renewal of HSCs. HSCs with an inactive or suppressed JAK/STAT pathway demonstrate reduced self-renewal potential (20). Knocking out JAK2 in mice is embryonic lethal due to impaired hematopoiesis (21). Conditional knock out of JAK2 at various stages of life, in a mouse model, is either embryonic lethal or can lead to severe impaired erythropoiesis, thrombopoiesis or even death (21). There are three other kinases that can be involved in the JAK/STAT pathway: JAK1, JAK3 and Tyk2. Conditional knockouts of JAK1 in a mouse model died shortly after birth and demonstrate reduced phosphorylation of STAT1, 3 and 6 (22). Knock out of JAK3 in a mouse model is not embryonic lethal but does lead to several T cell phenotypes due to their inability to respond to certain cytokines (23). Finally, knock out of Tyk2 in a mouse model is not embryonic lethal and $Tyk2^{-/-}$ mice show a reduced cytokine response unlike knockout of JAK1/2/3 (24).

The STAT transcription factors are downstream of the non-receptor tyrosine kinases involved in the JAK/STAT pathway. Deletion of Stat 3 is embryonic lethal between 6.5-7.5 dpc, demonstrating that STAT3 is necessary for early developmental hematopoiesis (25). STAT3 conditional knock out mice are viable and fertile but have elevated WBCs, reduced hematocrit

and hemoglobin and increased levels of circulating mature myeloid cells (26). This demonstrates STAT3's importance in hematopoiesis during the lifespan of the organism. STAT5 is not embryonic lethal but STAT5 deletion in HSCs leads to persistent cycling and reduced HSC number (27). In bone marrow and fetal liver cells lacking STAT5, there is a reduction in repopulation activity (28). This demonstrates STAT5's role in hematopoiesis and especially the self-renewal pathway.

Myeloproliferative leukemia protein (MPL), the TPO receptor, can activate JAK2 in this self-renewal pathway. As the TPO receptor, it is mostly responsible for thrombopoiesis but has a role in activating the self-renewal pathway due to its expression on HSCs. MPL knock out mice are viable but demonstrate significant reduction in platelet count (29). MPL knockout bone marrow cells also demonstrate to have a significant reduction in self renewal capacity and demonstrate an inability to secondarily transplant into lethally irradiated recipient mice (30). All this data taken together demonstrate critical roles for MPL, JAK1/2/3, STAT3 and STAT5 in the self-renewal pathway.

**1.4 Role of Mutations in JAK/STAT pathway in MPN Pathogenesis**

Like any signaling pathway used extensively in stem cells, the JAK/STAT pathway must be extensively regulated to ensure overactivation does not occur. However, somatic and germline mutations can occur, leading to constitutive activation or repression of this pathway. Patients that have mutations in this pathway can be diagnosed with a variety of myeloproliferative neoplasms (MPN) or myelodysplastic syndromes (MDS), an underproduction of bone marrow cells. Mutations in cell signaling pathways that cause MDS, an underproduction of bone marrow cells, are outside the scope of this dissertation. Myeloproliferative neoplasms can be

characterized by Philadelphia chromosome positive or negative. Philadelphia chromosome positive MPNs occur when a portion of chromosome 9 and chromosome 22 fuse together creating a fusion protein, BCR-ABL, a constitutive active tyrosine kinase that is found in chronic myeloid leukemia (CML) and a small subset of acute lymphoblastic leukemia (ALL) (31). Philadelphia chromosome negative MPNs usually occur when somatic point or frameshift mutations occur in signaling pathways leading to excessive production of myeloid cells (32). Patients usually present with one of six subtypes: polycythemia vera (PV), essential thrombocytosis (ET), primary myelofibrosis (PMF), chronic eosinophilic leukemia (CEL), chronic neutrophil leukemia (CNL), or mastocytosis. PV patients demonstrate excessive proliferation of red blood cells (RBCs) in their peripheral blood, demonstrated by high RBC and hematocrit counts in their CBCs (33). Patients may also experience splenomegaly. ET patients demonstrate excessive proliferation of platelets in their peripheral blood and their thrombotic risk can be significantly elevated compared to normal patients (34). In PMF, excessive scarring is produced in the bone marrow. This causes the bone marrow to become uninhabitable for HSCs to survive. This leads to a drastic drop in blood counts and hematopoiesis moving from the bone marrow to the spleen (35). CEL, a rare MPN, occurs when there is an excessive proliferation of eosinophil precursors. This causes eosinophil infiltration into the bone marrow and other tissues which can cause long term damage (36). CNL, also a rare MPN, occurs when there is an excessive proliferation of white blood cells, of which at least 80% are neutrophils (37). Finally, mastocytosis, another rare MPN, occurs when there is a proliferation of mast cells, an important cell type in the immune system, in the peripheral blood. These can infiltrate organs in great number, leading to organ failure (38).

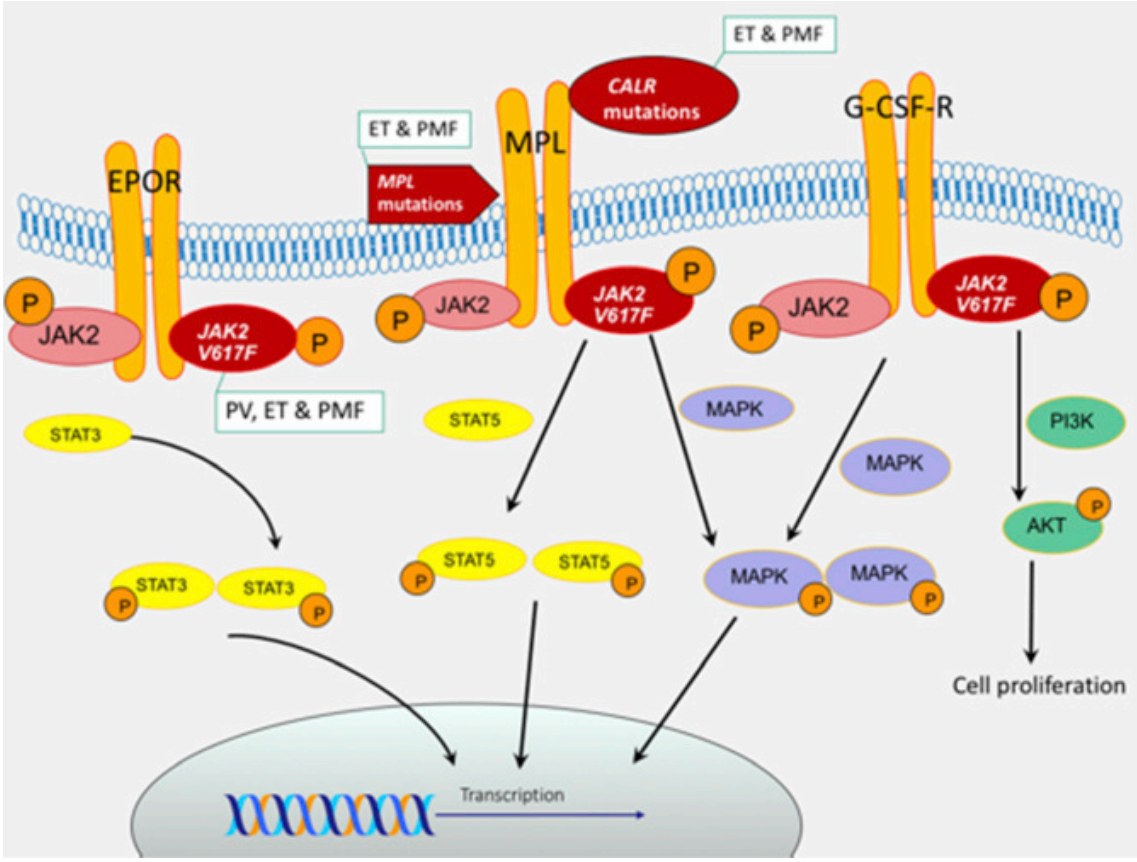



Figure 1.4: Mutations in the JAK/STAT pathway in MPN **(O'Sullivan et al Molecular and Cellular Endocrinology 2017)**

Due to excellent work by many different labs in the hematology/oncology field, key genetic mutations have been identified linked to most of the Philadelphia chromosome negative MPNs. In 2005, it was discovered that for patients diagnosed with PV, ET, and PMF, a majority of them have a point mutation in JAK2, JAK2V617F (39). This mutation occurs in the JH2 domain, changes a valine to a phenylalanine which leads to constitutive activation of the JAK/STAT pathway through phosphorylation of downstream STAT proteins leading to MPN. Somatic mutations in c-MPL have also been demonstrated to cause MPN, in patients testing negative for JAK2V617F. These mutations, MPLW515L/K, also lead to constitutive activation of the JAK/STAT pathway due to constitutive activation of the MPL receptor, upstream of JAK2. These

mutations are not as prevalent clinically as JAK2V617F and mostly present in patients diagnosed with ET or PMF (40). In 2013, somatic mutations in exon 9 of calreticulin were identified in ET and PMF patients that tested negative for mutations in JAK2 and MPL (41). In the last couple of years, the mechanism has been published for how these mutations in calreticulin lead to MPN. It is now understood that these mutations in calreticulin are able to act as a scaffolding protein for MPL. This interaction constitutively activates the JAK/STAT pathway, upstream of JAK2 (42). I will extensively detail the mechanism in later chapters. Mutations that have been linked to CEL and CNL have also been implicated in constitutively activating the JAK/STAT pathway. For CEL, mutations in platelet-derived growth factor receptor alpha (PDGFRA) have been shown to activate the JAK/STAT pathway (43). Mutations in granulocyte-colony stimulating factor 3 receptor (G-CSF3R) have been linked to CNL and these mutations also lead to activation of the JAK/STAT pathway (44).

Since myeloproliferative neoplasms is a collection of diseases, there exists many more mutations in patients diagnosed with MPNs. There can be mutations in adaptor proteins responsible for shutting down JAK/STAT signaling such as Lnk that lead to MPNs. There can be mutations that occur downstream of JAK2 in MPN at the epigenetic level such as mutations in TET2, IDH1/2, or DMNT3. These mutations allow DNA methylation being altered, leading to increases of transcription.

### 1.5 Therapeutics for common mutations in MPN

With all these mutations leading to constitutive activation of JAK/STAT cell signaling pathway, there are a few therapeutics that are effectively used by clinicians for treated these diseases. Most of these therapeutics are kinase inhibitors. For Philadelphia chromosome

positive CML, imatinib (Gleevec) is the gold standard and one of the successful stories of targeted therapy. Imatinib is a tyrosine kinase inhibitor that specifically inhibits BCR-ABL fusion proteins while leaving all other tyrosine kinases untouched. Treatment with imatinib leads to complete cytogenic response (lack of Philadelphia chromosome detection in bone marrow) in a large percentage of patients with means of survival extended out to about 20 years (45).

For those mutations that activate the JAK/STAT pathway, specifically in MPN, ruxolitinib, a JAK1/2 inhibitor, is often prescribed. Since ruxolitinib is a non-selective JAK1/2 inhibitor and selective inhibitors for point mutations in kinases are much harder to engineer, the clinical results when using ruxolitinib are not as effective as imatinib for CML and the results can vary. The benefit of ruxolitnib is that is can be used in MPN patients if they have JAK2 mutations, MPL mutations or calreticulin mutations (46). Interferon, a naturally occurring signaling protein, has been shown to reduce JAK2V617F disease burden but has potent side effects. Pegylated interferon, a longer lasting and more widely tolerated version of interferon, is currently in clinical trials for FDA approval (47). However, the only demonstrated cure for Philadelphia negative MPNs is an allogenic bone marrow transplant. These can be risky and unnecessary for PV and ET patients but can be necessary for PMF patients suffering from anemia (48). There can be significant complications for bone marrow transplant recipients including finding a suitable match or suffering from graft versus host disease.

In this chapter, I have provided significant background on hematopoiesis, the different cell signaling pathways important in hematopoiesis and self-renewal and discussed mutations that cause MPN and therapies used to treat MPN patients. In chapter two, I will go into detail about calreticulin's role in the cell. In chapter three, I'll discuss work published from our lab

describing a mutation in JAK2 that leads to an intermediate MPN phenotype. In chapter four, I will discuss how our lab discovered how mutations in calreticulin lead to MPN and further work describing calreticulin's role in hematopoiesis. Chapter five will summarize all the work I've done in my time in the Fleischman lab, place that work in context of the current field and envision where this work could progress in the future.

# Chapter 2: The Role of Calreticulin in a cell

## 2.1 General Introduction of Calreticulin

This chapter is a further introduction of calreticulin, what is known and published in the field and what is not known, especially of calreticulin's role in the field of hematopoiesis. Calreticulin is a ubiquitously expressed ER residing chaperone protein and is conserved from plants to humans (49). It is responsible for sequestering $Ca^{2+}$ ions in the ER lumen has multiple other roles throughout the cell.

As its main function of a chaperone protein, calreticulin must prevent peptides from aggregating together during translation to ensure correct folding occurs. It has been demonstrated that it is able to associate with newly formed glycoproteins, including cell surface receptors, suppressing their aggregation and ensuring correct folding occurs with its association with ERp57 (50). Calreticulin itself can be glycosylated and improper glycosylation or lack of glycosylation of calreticulin when expressed on the cell surface can lead to drastic immune responses (51)

One of the ways to determine calreticulin's importance in the cell and in the organism as a whole is through global gene knockouts. Global calreticulin knock outs are embryonic lethal at d.p.c. 14.5 in an *in vivo* mouse model due to impaired heart development (52). This impaired heart development is due to an impairment of calcium induced NFAT3 transcription factor nuclear localization. This demonstrates not only the importance of calcium signaling in embryo development but also the importance of calreticulin in calcium signaling.

Loss of calreticulin has been demonstrated to lead to a disruption of lipid homeostasis. This is thought to be due not to calreticulin's direct role in lipid homeostasis but due the dysregulation of calcium signaling which is important for lipid homeostasis (53).

**2.2 Calreticulin's role in cancer**

Calreticulin has been demonstrated to be a critical cell survival factor in $BRAF^{V600E}$ melanoma cell lines as knock down of calreticulin with shRNA suppresses growth and colony formation. This has been demonstrated in other cancer cell lines such as breast (MDA-MB361), colon (HCT116), ovarian (SKOU1), and neuronal (H80) cancer cell lines and has been linked to an increase in activated p53 following suppression of calreticulin (54). Calreticulin expression inside the cell or presentation at the cell surface has been linked to other types of cancers. In chronic lymphoid leukemia (CLL), calreticulin is a novel B cell receptor antigen and time of treatment free survival decreases as soluble calreticulin expression in the plasma increases (55, 56). In breast tumor tissue, calreticulin expression was found to be upregulated, especially in malignant tissues (57). In oral squamous cell carcinoma, high calreticulin expression correlated to reduced survival of patients (58). Conversely in endometrial cancer, low expression of calreticulin was associated with a more aggressive cancer and poor prognosis in patients (59). For pancreatic cancer, calreticulin promoted the development of cancer through the ERK/MAPK pathway and further was responsible for the epithelial to mesenchymal transition of these cells (60). These data taken together demonstrate that calreticulin's role in cancers in the body is widely varied.

## 2.3 Calreticulin and Cell Stress

One of calreticulin's main functions in the cell is highlighted when cell stress occurs. As a chaperone protein, it must ensure proteins are folded correct during cell stress, more specifically heat shock or calcium depletion. In cells where calreticulin is knocked out, the effects of ER stress are made worse. When calreticulin is present, cells have proper ER stress response, demonstrating that calreticulin is necessary and sufficient to relieve ER stress (61). Furthermore, when cells are subjected to ER stress via thapsigargin, a non-competitive ER calcium ATPase or tunicamycin, a drug leading to the unfolded protein response and apoptosis, calreticulin expressed is increase significantly (62). The mechanism for calreticulin dealing with ER stress response has been demonstrated to be in an autophagy related manner (63).

## 2.4 The domains of calreticulin and how they correspond to its function

Calreticulin is a 46 kDa protein that can structurally and functionally be separated into three domains (64). There is an N terminal domain, a P terminal domain and a C terminal domain. As of now, the full crystal structure of calreticulin with all 3 domains expressed at the same time has not yet been solved, due to the disordered C terminus bit the structure of N terminal and P terminal together and separately have been solved via NMR and through crystallization (65).

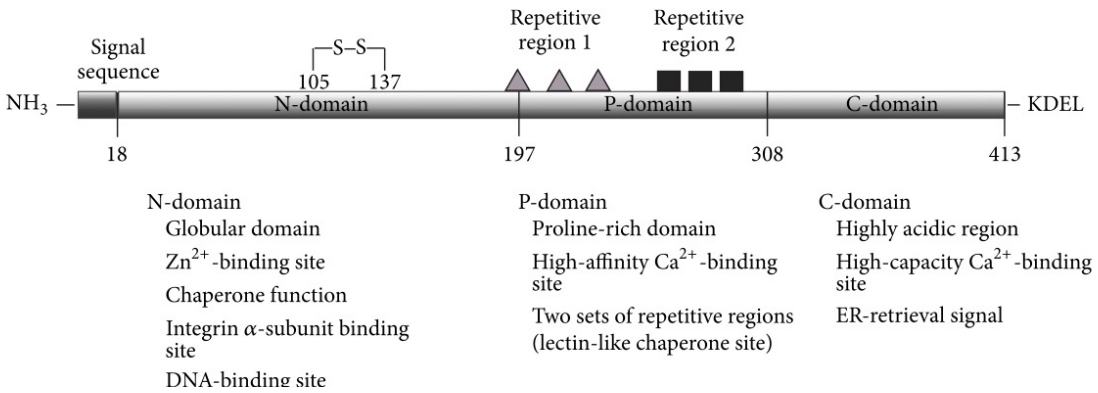



Figure 2.1. The protein structure and putative functions of calreticulin domains. **(Lu et al Biomedical Research International 2015)**

The N terminal region of calreticulin is 180 amino acids long. This region of the protein is highly conserved (66). The N terminal region shares identical sequence similarity to vasostatin, a peptide that is known to suppress angiogenesis (67). Furthermore, it can suppress growth of Burkitt lymphoma, colon carcinoma and endothelial cells (67). This N terminal region has also been identified as having the ability to catalyze the transfer of acetyl groups from polyphenolic acetates to certain proteins (68). In keeping with the variety of functions that calreticulin plays in the cell, the antiadhesive activity of thrombospondin, a secreted glycoprotein that mediates cell to cell contact, is mediated by the N terminal domain of calreticulin when it is at the cell surface (69). Lastly, the N terminal region of calreticulin has been found to associate with human CD69 at the cell surface (70).

The P domain of calreticulin is called the P domain due to its proline rich region. It is 100 amino acids long and has the function of being lectin like, although the sequence for the P domain is varied from usual lectin domains (71). The structure of the P domain has been solved via both crystal structure and NMR (72). This has demonstrated that the P domain has three antiparallel beta sheets and one hairpin loop (73). These beta sheets repeat in a sequence of

111222 which is quite similar to calnexin, a similarly structured protein with a similar function, that has a beta sheet repeat sequence of 11112222 (73).

With its role as a chaperone protein,it has been published that the P domain is where other co-chaperones can dock to help with protein folding such as ERp57 and ERp29 (74). It is thought that the P domain is like an adaptor and can bind live cells in a low affinity P domain dependent way.

The P domain is essential for the correct assembly and processing of MHC class 1 complexes on the cell surface (75). These complexes are essential for proper cytotoxic T cell response. The P domain of calreticulin is thought to be important for antigen presentation by MHC class I complexes at the cell surface as in a calreticulin deficient cell line expression of the P domain further suppresses MHC class 1 complex expression at the cell surface (75).

The C terminal domain of calreticulin is 110 amino acids long (76). It ends in an amino acid sequence of lysine, aspartic acid, glutamic acid and leucine. This sequence is known as the KDEL sequence and expressed at the c terminus of proteins for their retro translocation to the ER. Due to this sequence, it is thought that the C terminus is responsible for the intracellular localization of calreticulin (77).

The c terminus of calreticulin is very acidic, in terms of amino acids, which allows it to be very disordered and flexible (78). This prevents the structure of the C terminus from being crystalized and solved. This region binds $Ca^{2+}$ ions at high capacity but low affinity. It has been published that the binding of $Ca^{2+}$ ions stabilized the structure of calreticulin and stabilizes its thermostability during heat shock stress (79). The deletion of the C terminus actually enhances the binding of purified calreticulin to polypeptide substrates and increases its chaperone

activity, suggesting that in some confirmations, the C terminus can actually interfere with the chaperone function of the N and P domains (79). The c terminal acidic region of calreticulin mediates phosphatidylserine binding at the cell surface, leading to apoptotic cell phagocytosis (80).

There has been a lot of interest in the last couple of years in the C terminal domain of calreticulin since it was discovered that frameshift mutations occurring in exon 9 of calreticulin lead to MPN in patients negative for JAK2 and MPL mutations. A knock-in mouse model of these calreticulin mutations demonstrate as increase in HSCs number and frequency in the bone marrow but do not demonstrate a competitive advance in a competitive transplant assay (81). In iPSC cells derived from a patient with the five base pair insertion frameshift mutation, there was a marked increase in megakaryopoiesis compared to wildtype controls with elevated expression of the transcription factors GATA1 and GATA2 (82). This suggests an early commitment to the megakaryocyte lineage in progenitor cells with calreticulin mutations in the C terminus.

It has been demonstrated that these frameshift mutations lead to a novel C terminus that abolishes the KDEL sequence, leading to it being secreted from the ER and the cell through Golgi mediated exocytosis (83). This extracellular calreticulin can inhibit phagocytosis of dying cancer cells by dendritic cells (83). Furthermore, it is thought that this novel C terminus leads to impairment of calcium ion binding (84). In light of this, it is not surprising that cells harboring mutations in the c terminus of calreticulin do not undergo phagocytosis, even with calreticulin expressed at the cell surface, since these mutations lead to a novel C terminus and are thought to not be able to mediate binding with phosphatidylserine and lead to phagocytosis.

Pronier et al demonstrated quite sophisticatedly that mutations in calreticulin lead to an upregulation of MPL transcripts, leading to JAK/STAT activation and leukemic transformation (85). They also demonstrated that a peptide with the exact same sequence as the C terminus competitively inhibits calreticulin mutants from binding to interacting proteins, leading to a decrease in MPL transcripts. In human $CD34^{+}$ cells taken from an MPN patent with a calreticulin mutation, they demonstrated that treatment of these cells with the C terminal peptide reduces pSTAT5 and MPL transcripts. This suggests that calreticulin, in its wildtype form, could be used to suppress MPL signaling. They are currently evaluating this peptide as a novel therapeutic for MPN patients with calreticulin mutations.

# Chapter 3: JAK2V617I results in cytokine hypersensitivity without causing an overt myeloproliferative disorder in a mouse transduction- transplantation model

## 3.1 Introduction

A single gain-of-function somatic point mutation in the Janus kinase 2 (JAK2) gene is present in the majority of patients with Philadelphia-negative myeloproliferative neoplasm (MPN) (86-90). JAK2 is a cytoplasmic tyrosine kinase which is critical in intracellular signaling by cytokine receptors such as erythropoietin (EPO), thrombopoietin (TPO), interleukin-3 (IL-3), granulocyte colony stimulating factor (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF). The JAK2V617F mutation results in a constitutively active JAK2, with continual activation of downstream intracellular signaling cascades (91-93). Although there is a familial predisposition to acquire the MPN (94, 95) the JAK2V617F mutation is somatically acquired in a hematopoietic stem cell. However, a germline JAK2V617I mutation has recently been identified in a family with hereditary thrombocytosis (96, 97). The ability of JAK2 V617I to confer cytokine independence had previously been shown in Ba/F3 cells randomly mutated at position 617 of JAK2 (98). Like acquired MPN, family members with germline JAK2 have thrombocytosis and megakaryocytic hyperplasia in the marrow with increased risk of thrombosis. But unlike acquired MPN, individuals with the JAK2V617I germline mutation do not develop a fibrotic bone marrow, splenomegaly, or transform to acute leukemia. Why germline JAK2V617I recapitulates some aspects but not others of the MPN phenotype in humans is unclear. To delineate the differences between JAK2V617F and JAK2V617I we compared the phenotype of mice with hematopoietic cells expressing JAK2V617F or JAK2V617I.

### 3.2 JAK2V617I Expression Induces a mild MPN Phenotype

Lethally irradiated C57B/6 mice were transplanted with equal numbers of bone marrow cells expressing JAK2 V617F, JAK2 V617I, or empty MSCV-IRES-GFP (MIG) vector (101). As expected, JAK2V617F mice developed erythrocytosis and leukocytosis. However, JAK2V617I mice had peripheral blood counts similar to empty vector mice (Fig 1A–C). To rule out the possibility that the phenotypic differences we observed in JAK2V617F versus JAK2V617I mice was not simply due to lower expression of JAK2 in JAK2V617I mice, we confirmed equivalent expression of JAK2 in JAK2V617F and JAK2V617I mice by quantitative RT-PCR (data not shown) and Western Blot (Supplemental Figure 1). The lack of thrombocytosis in the JAK2V617I mouse model is not unexpected, as thrombocytosis is not commonly observed in JAK2V617F MPN mouse models. Therefore, we performed a more thorough assessment of the effect of JAK2V617I on hematopoietic progenitors and myeloid cells including megakaryocytes at time of sacrifice.

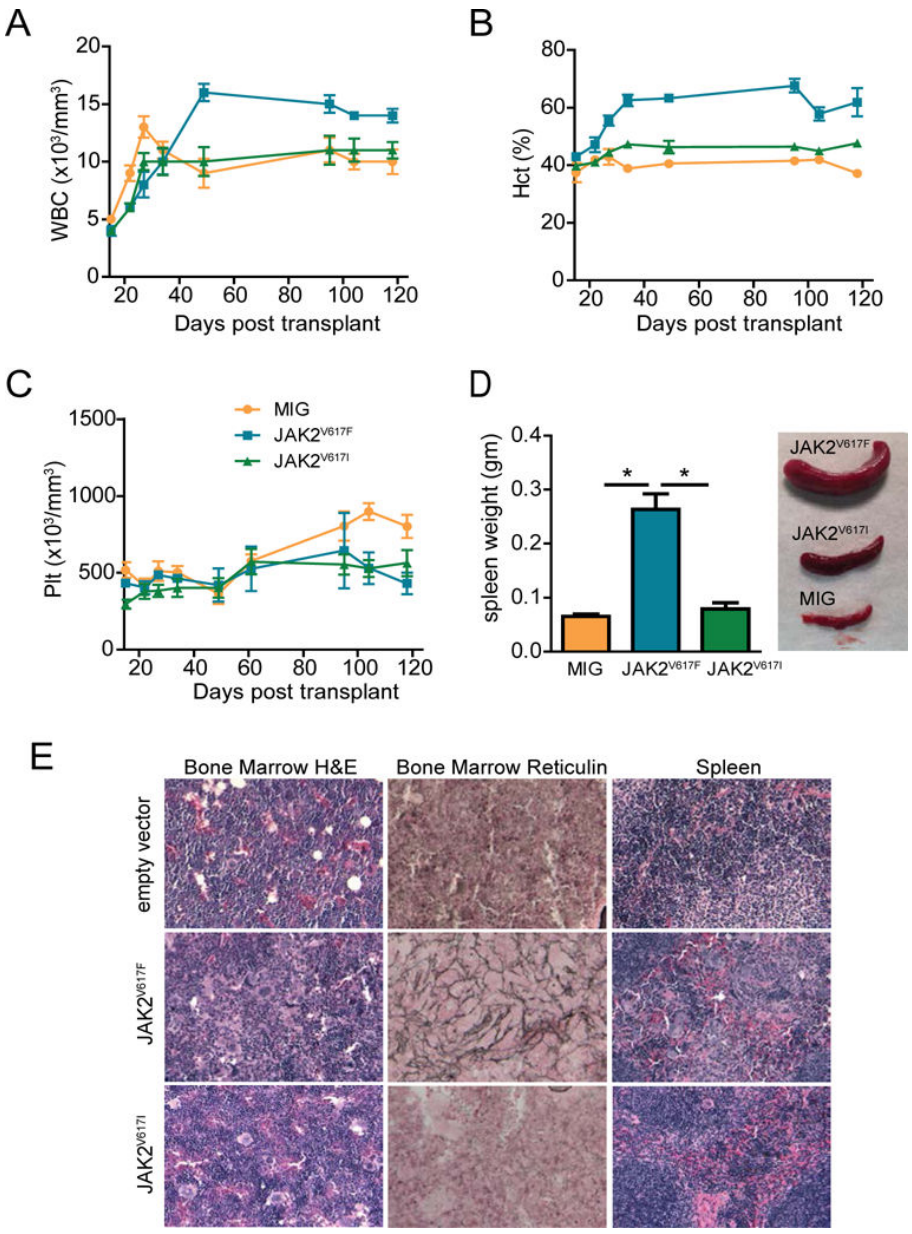



**Figure 3.1. JAK2V617I causes a mild MPN phenotype**

A–C. Peripheral blood was drawn weekly from MIG empty vector, JAK2 V617I, and JAK2V617F mice. WBC (A), HCT (B) and platelets (C) were measured using a VetABC hematology analyzer (scil). D. Spleen weight and a representative photo of MIG empty vector, JAK2 V617I, and JAK2 V617 mice. E. Bone marrow and spleen from each mouse was evaluated by a blinded pathologist. Representative H&E sections of bone marrow, spleen and reticulin stain of bone marrow are shown (20X magnification shown, Leica DC300 camera running IM50 Image Manager software).

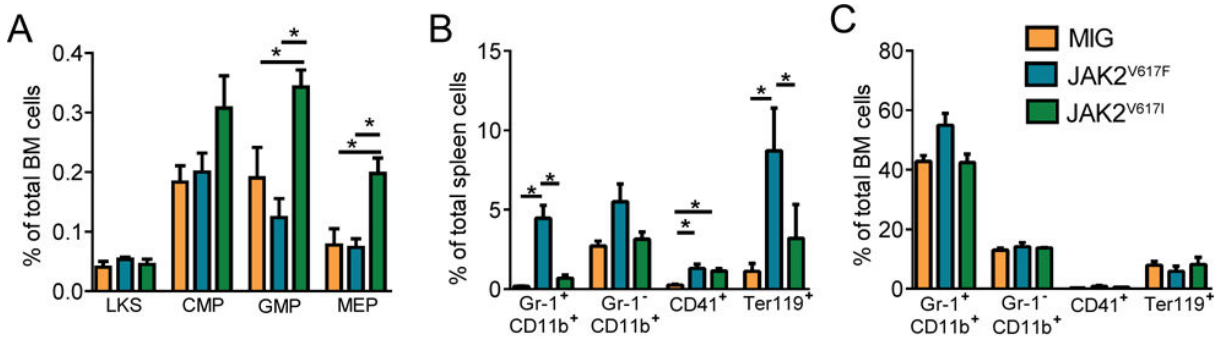
Mice were euthanized at 120 days post-transplant to fully assess the MPN phenotype. Spleen weight of JAK2V617I mice was not statistically different than empty vector mice (Figure 1D), however we found a positive correlation between spleen weight and percentage

of GFP-positive cells in the spleen (Supplemental Figure 2). As expected, spleen weight was increased in JAK2V617F mice (Figure 1D). On histologic inspection JAK2V617I and JAK2V617F mice had hypercellular marrows with increased numbers of megakaryocytes.

Mild fibrosis was seen in JAK2V617I mice, severe reticulin fibrosis was seen in JAK2V617F mice. The splenic architecture was preserved in JAK2V617I mice, whereas in JAK2V617F mice the splenic architecture was disrupted by invasion of myeloid cells including megakaryocytes (Figure 1E). These data demonstrate that ectopic expression of JAK2V617I in hematopoietic cells induces histologic evidence of MPN but with a milder phenotype as V617F compared to JAK2 as JAK2V617I expands myeloid progenitors and megakaryocytes and mobilizes myeloid progenitors to the spleen.

**3.3 JAK2V617I expands myeloid progenitors and megakaryocytes and mobilizes myeloid progenitors to the spleen**

To identify whether JAK2V617I affects the frequency of hematopoietic stem and progenitor V617F cells or expands mature myeloid lineage cells we compared these populations in JAK2V617I, JAK2V617I, and MIG empty vector mice (Figure 2A–C).



The bone marrow of JAK2 mice had expanded GMP and MEP populations as compared to MIG empty vector and JAK2V617F mice (Figure 2A). Mature granulocyte (Gr-1+CD11b+) and erythroid (Ter119+) populations were expanded in JAK2V617F but not JAK2V617I mice. To determine whether JAK2V617I had mobilized myeloid progenitors to the spleen we compared myeloid colony formation ability from harvested spleen cells of MIG, JAK2V617I and JAK2V617F. We found increased myeloid progenitor activity in the spleens from JAK2V617I and JAK2V617F mice as compared to MIG empty vector (Figure 2D). Both JAK2V617I and JAK2V617F mice had an increased fraction of megakaryocytes (CD41+) in the spleen as compared to empty vector mice (Figure 2B).

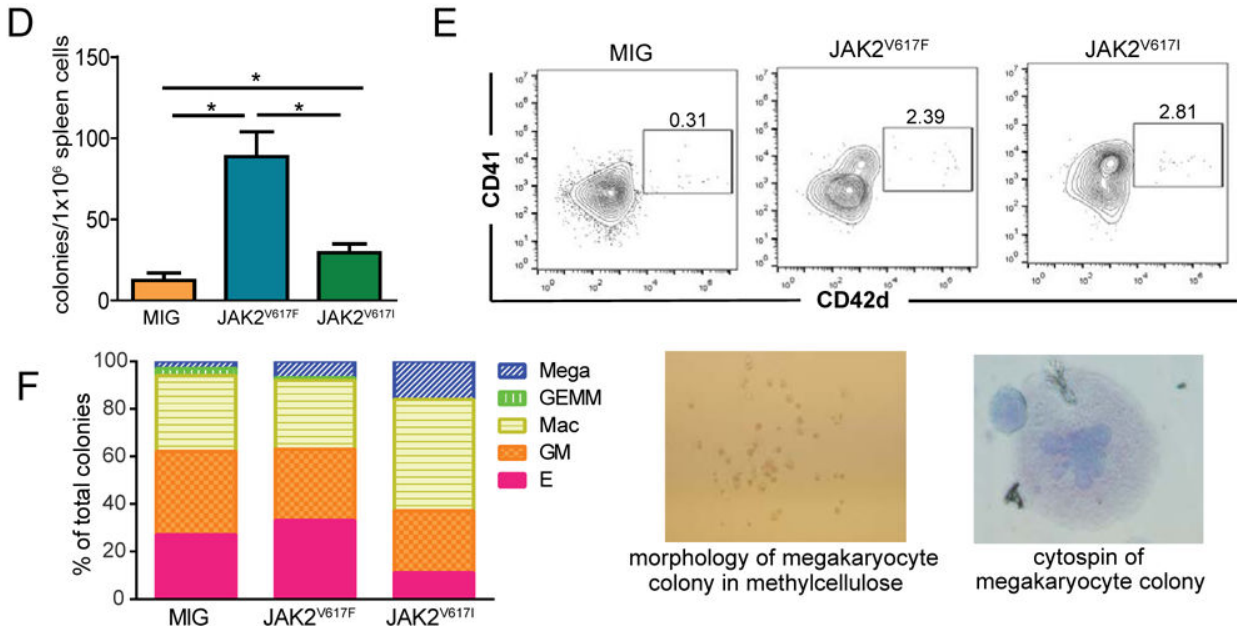



**Figure 3.2. *JAK2$^{V617I}$* expands myeloid progenitors and megakaryocytes**
**A.** Frequency of hematopoietic stem (LKS), common myeloid progenitors (CMP), granulocyte monocyte progenitors (GMP) and megakaryocyte erythroid progenitors (MEP) in the bone marrow of each mouse measured by flow cytometry. **B, C**. Frequency of granulocytes (Gr-1$^+$ CD11b$^+$), monocytes (Gr-1$^-$ CD11b$^+$), megakaryocytic lineage (CD41$^+$) and erythroid lineage cells (Ter119$^+$) in spleen (B) and bone marrow (C) measured by flow cytometry. **D.** Myeloid colony formation (CFU-GM and CFU-E combined) in methycellulose (M3231, StemCell Technologies) supplemented with mSCF, mIL-3 (peprotech) and hEpo (Procrit, Amgen). **E.** In vitro differentiation of megakaryocytes (CD41$^+$ CD42d$^+$) from LKS cells on OP9 stromal cell layers (+mTPO, mIL-11, mSCF). F. Relative frequency of myeloid colony types from JAK2 hematopoietic progenitors. Progenitors were plated in methylcellulose (M3231, StemCell Technologies)

supplemented with mSCF, mIL-3, hEpo, and mTPO. After 7 days colonies were scored morphologically and enumerated. Photos of a representative megakaryocyte colony are shown to the right. All bar graphs represent mean (+/-SEM), *denotes $p<0.05$.

To evaluate megakaryopoiesis in more detail we sorted lineage, c-kit, Sca-1 (LKS) cells ectopically expressing JAK2V617I, JAK2V617F, or MIG empty vector onto OP9 stromal cell layers (100 cells/well) in the presence of mouse thrombopoietin (mTPO) (10ng/ml), Stem Cell Factor (mSCF) (50ng/ml), and Interleukin-11 (mIL-11) (10ng/ml) to induce development of megakaryocytes. After 5 days in culture cells were harvested and analyzed by flow cytometry to identify megakaryocytes. Wells seeded with JAK2V617I and JAK2V617F cells had an increased percentage of megakaryocytes (CD41+ CD42d+) as compared to wells V617I seeded with empty vector cells (Figure 2E). We also compared the ability of JAK2V617F, JAK2V617I, and empty vector hematopoietic progenitors to form megakaryocyte colonies in methylcellulose and found a skewing toward megakaryocyte colonies in both JAK2V617I and JAK2V617F as compared empty vector (Figure 2F). Together, these data demonstrate that expression of JAK2V617I drives the expansion of myeloid progenitors and megakaryocytes despite the lack of overt leukocytosis or thrombocytosis in peripheral blood.

**3.4 JAK2V617I results in cytokine hypersensitivity without constitutive activation**

Humans with germline JAK2V617I mutations do not display constitutive activation of the kinase but they do demonstrate cytokine hyper-responsiveness as evidenced by increased phosphorylation of STATs at low concentrations of ligand. We compared phosphorylated STAT5 in peripheral blood cells taken from JAK2V617I, JAK2V617F and MIG empty vector mice following stimulation with increasing concentrations of GM-CSF. At all concentrations of GM-CSF tested JAK2V617I and JAK2V617F mice had exaggerated phosphorylation of STAT5 as

compared to MIG empty vector mice (Figure 3A). We also measured phospho-STAT3 and STAT5 in unstimulated bone marrow and spleen from each mouse at time of sacrifice, there was no difference between JAK2V617I and MIG empty vector mice. JAK2V617F mice did demonstrate phosphorylation of STAT3 and STAT5 even in the absence of cytokine stimulation, confirming the ability of JAK2V617F but not JAK2V617I to constitutively activate downstream signaling pathways (Figure 3B).

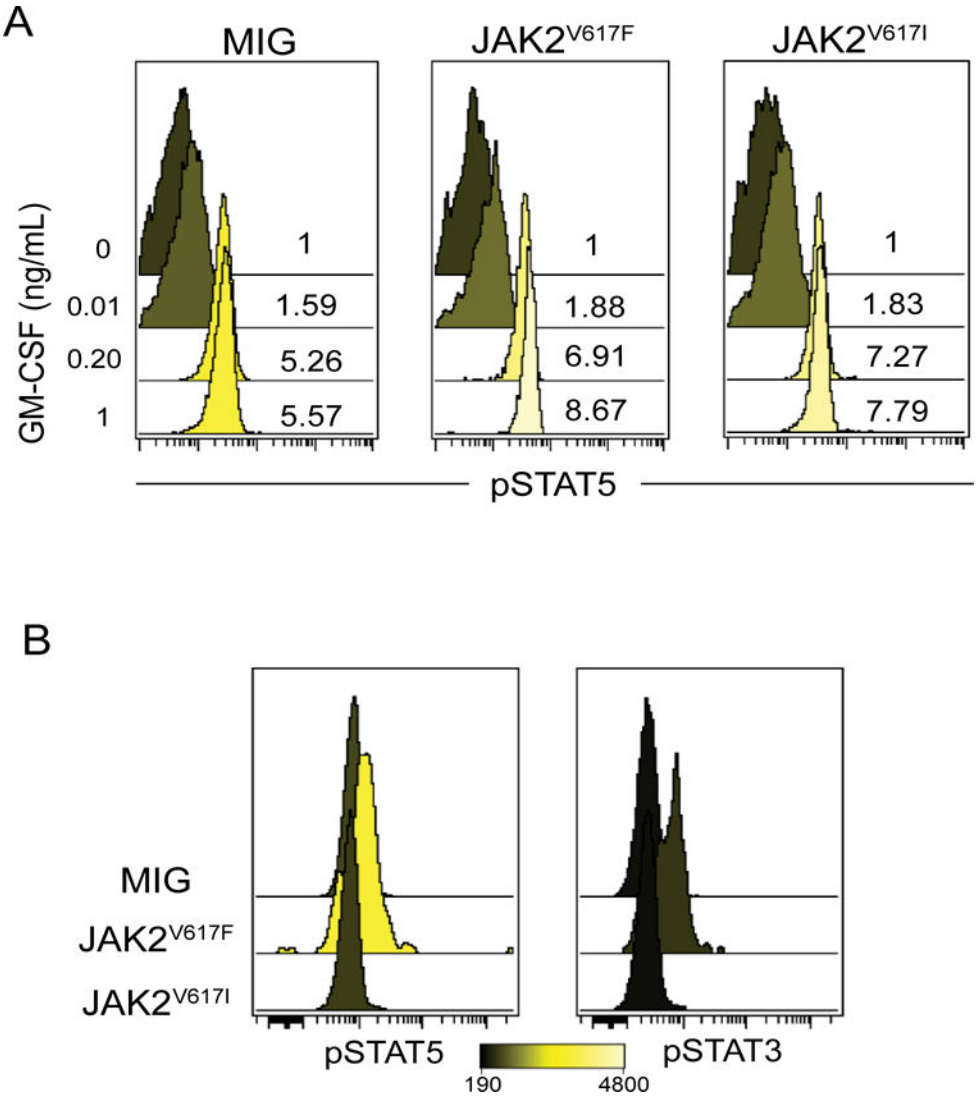



**Figure 3.3. *JAK2$^{V617I}$* results in cytokine hypersensitivity without constitutive activation**
**A.** Peripheral blood from each group of mice was pooled and stimulated with increasing concentrations of mGM-CSF and then analyzed for phospho-STAT5 (pY694) by flow cytometry. Fold increase in phospho-STAT5 over unstimulated is shown as histogram overlay (gated on GFP$^+$ cells). **B.** Unstimulated bone marrow from MIG, JAK2$^{V617F}$ and JAK2$^{V617I}$ mice were analyzed for levels of phospho-STAT5 (pY694) and phospho-STAT3 (pY705) using flow cytometry, histogram overlay represents (MFI) of a representative mouse from each group (gated on GFP$^+$ cells).

### 3.5 Discussion

The JAK2V617I mouse transduction-transplantation model has phenotypic features of acquired MPN such as expansion of megakaryocytes and mobilization of hematopoietic

progenitors to the spleen but the phenotype is not as robust as JAK2V617F. It is possible that all cells in the hematopoietic system must express JAK2V617I, as is the case for a germline mutation, in order for JAK2V617I to make a clinically relevant impact in humans. Although the JAK2V617F mutation is somatically acquired in MPN familial clustering is well described. Genome-wide analyses have revealed that JAK2 -positive MPN is strongly associated with a specific constitutional JAK2 haplotype (designated 46/1), suggesting that germline variation is an important contributor to MPN phenotype and predisposition (102-104).

### 3.6 Methods

#### Bone Marrow Transplantation

C57B/6 mice were purchased from Jackson Labs. Retroviral infection and transplantation were performed as previously described (). All mouse work was performed with approval from the Oregon Health & Science University and UC Irvine Institutional Animal Care and Use Committee.

#### Flow Cytometry

The following antibodies were used for identification of mature cell populations: CD41 PE (MWReg30, BD Biosciences), CD42d (1C2, Biolegend) CD11b APC (M1/70, BD Biosciences), Gr-1 PerCPCy5.5 (RB6-8C5, ebioscience), TER119 APC (TER119, BioLegend). For hematopoietic progenitor populations the following antibodies were used: APC lineage (Lin) markers (CD3, KT31.1; CD4, GK1.5; CD8, 53–6.7; B220, 6B2; Mac-1, M1/70; Gr-1, 8C5; and TER119, all from BD Biosciences), c-kit APC-Cy7 (2B8, BD Biosciences), CD34 PE (RAM34, BD Biosciences), CD16/32 PE-Cy7 (2.4G2, BD Biosciences), and Sca-1 Pacific Blue (D7, BioLegend). LKS, CMP, GMP, and

MEP are defined as is (100). Cells were analyzed using an Aria III flow cytometer (BD Biosciences). Data was analyzed using FlowJo software (Treestar).

**Phosphoflow**

Cells were stimulated with 0.1, 0.2, or 1 ng/ml of GM-CSF for 15 min at 37°C. Following stimulation, cells were fixed with paraformaldehyde and permeabilized in methanol. Samples were stained with PE- pSTAT3 (pY694) and A647- pSTAT5 (pY701) (BD Biosciences) along with cell surface markers. Cytobank.org was used to analyze data.

**Methylcellulose Colony Formation Assays**

For Figure 2D 1×106 spleen cells were plated in 1.1ml methylcellulose semi-solid media (M3231, StemCell Technologies) supplemented with 100ng/ml mSCF, 10ng/ml mIL-3 (peprotech) and 3U/ml hEpo (Procrit, Amgen) in triplicate. Colonies were enumerated after 12 days in culture. For Figure 2F GFP progenitors (lin , c-kit , Sca-1 ) were sorted by flow cytometry and plated at a concentration of 1000 cells per 1.1 ml of methylcellulose in triplicate (M3231, StemCell Technologies) supplemented with 100ng/ml mSCF, 10ng/ml mIL-3, 50ng/ml mTPO (peprotech), and 3U/ml hEPO. Plates were examined at 7 days of culture and scored by visual morphology. The morphology of cells in the individual colonies was confirmed by cytospin with Giemsa staining.

# Chapter 4: Calreticulin Mutations Activate MPL Signaling

## 4.1 Introduction

The unifying theme of myeloproliferative neoplasm (MPN) driver mutations, including JAK2V617F (39, 86, 87, 89), CALR (41, 105), MPL (106), and SH2B3 (107) is activation of thrombopoietin receptor signaling, highlighting the critical importance of this pathway in the pathogenesis of MPN. JAK2V617F associates with a number of homo-dimeric type I cytokine receptors, including erythropoietin receptor (EPOR), thrombopoietin receptor (MPL), and granulocyte-colony stimulating factor receptor (G-CSFR) leading to constitutive activation (108), whereas mutant CALR has been found to only associate with MPL and leads to its constitutive activation(109, 110). This selectivity for activation of MPL explains why CALR mutations are seen in essential thrombocythemia (ET) and myelofibrosis (MF) but not polycythemia vera (PV).

Ba/F3 cells are a useful tool to dissect cytokine receptor signaling pathways and to test the oncogenicity of mutations. Parental Ba/F3 require IL-3 to provide a survival signal utilizing endogenous IL-3R (111). Ectopic expression of activated tyrosine kinases or constitutively active cytokine receptors provide Ba/F3 cells with a survival signal which obviates the need for supplemental IL-3 (108, 112). We find that in vivo passage of Ba/F3 cells expressing either CALR type 1 (52 base pair deletion, $CALR^{DEL}$) or type 2 (5 base pair insertion, $CALR^{INS}$) mutations selects for the outgrowth of cells which have upregulated endogenous MPL allowing these cells to expand in mice. These findings solidify the exclusivity of MPL as the requisite cytokine receptor binding partner for CALR mediated transformation and highlight the central role for activation of thrombopoietin receptor (MPL) signaling in the pathogenesis of MPN.

## 4.2 Results

We created Ba/F3 cells stably expressing hCALR$^{DEL}$, hCALR$^{INS}$, hCALR$^{WT}$ or empty vector using an MSCV-IRES-GFP (MIG) based retrovirus. Ba/F3 cells expressing, hCALR, or empty mMPL using an MSCV-IRES-hCD4 vector were also produced. BCR-ABL p210 MIG Ba/F3 cells were used as a positive control for cytokine independent growth. Consistent with other reports (109, 110), expression of CALR$^{DEL}$ or CALR$^{INS}$ was not sufficient to render Ba/F3 cells cytokine independent in vitro (Figure 4.1A-D).

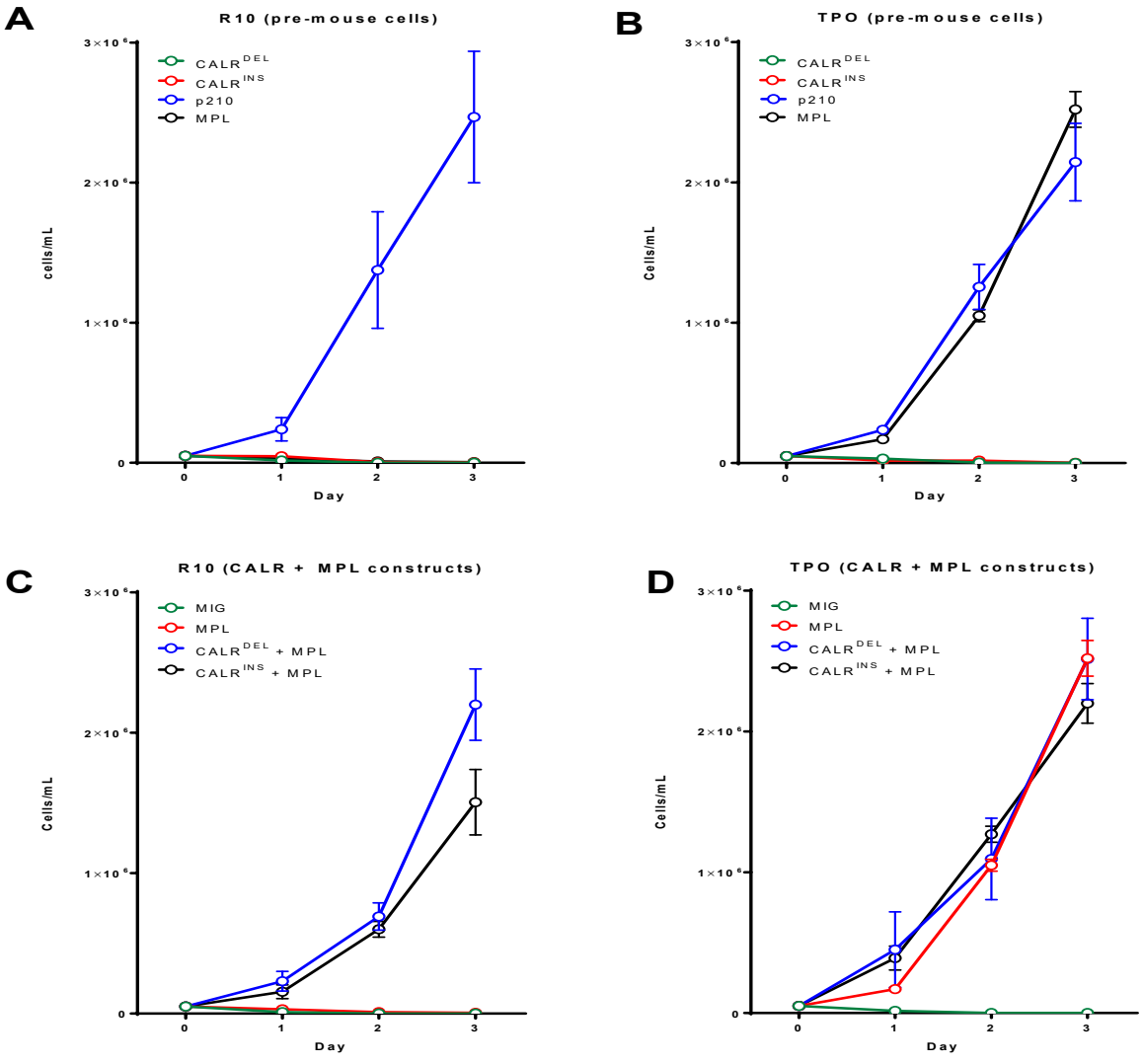
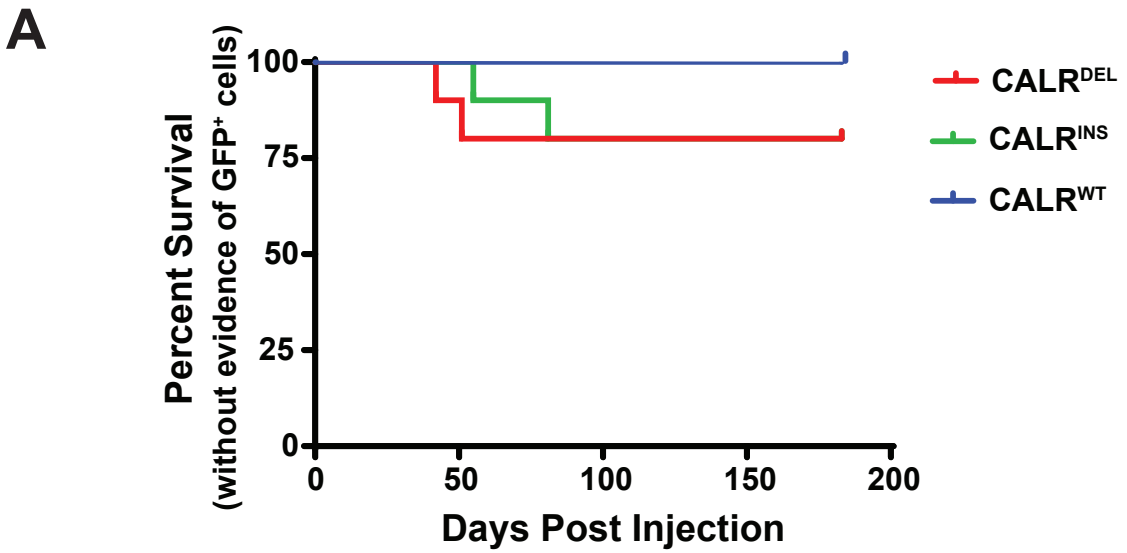


**Figure 4.1.** ***CALR mutant Ba/F3 cells are not cytokine independent without MPL.*** Ba/F3 cells were created expressing empty vector, $CALR^{DEL}$, or $CALR^{INS}$, $CALR^{DEL}$ + MPL, $CALR^{INS}$ + MPL, or p210. 5 x $10^4$ cells were placed in either RPMI + 10% FBS (R10) media without cytokines (A and C) or R10 + 10ng/mL TPO (B and D) and cells were counted daily.

To further evaluate the oncogenic potential of Ba/F3 cells expressing mutant calreticulin we injected $CALR^{DEL}$, $CALR^{WT}$ Ba/F3 cells into BALB/c mice (2.5x10 cells, and CALR each) and monitored for expansion of GFP+ cells in the peripheral blood. GFP+ cells emerged in the peripheral blood in two out of ten mice each injected with $CALR^{DEL}$ or $CALR^{INS}$ cells, but not in mice injected with $CALR^{WT}$ cells. Expansion of GFP+ cells caused mice to become moribund and require sacrifice (Figure 4.2A).




We retrieved GFP+ cells by FACS sorting from the bone marrow of moribund mice and tested their ability to grow in vitro in the absence of cytokines. GFP+ cells retrieved from mice (referred to hereafter as post-mouse) injected with $CALR^{DEL}$ Ba/F3 cells were able to grow in the absence of cytokines, however GFP+ cells retrieved from mice injected with $CALR^{INS}$ Ba/F3 cells did not grow in the absence of cytokines (Figure 4.2B) but grew well with supplemental IL-3. We tested the ability of multiple cytokines, including thrombopoietin (TPO), erythropoietin

(EPO), granulocyte- macrophage colony-stimulating factor (G-CSF), interleukin-11 (IL-11), interleukin-7 (IL- 7), interleukin-11 (IL-11), Stem Cell Factor (SCF), and Interferon-gamma (IFNγ) to support growth of post-mouse Ba/F3 GFP$^+$ CALR$^{INS}$. However, only TPO was able to support growth of post-mouse Ba/F3 GFP$^+$ CALR$^{INS}$ cells (Figure 4.2D)

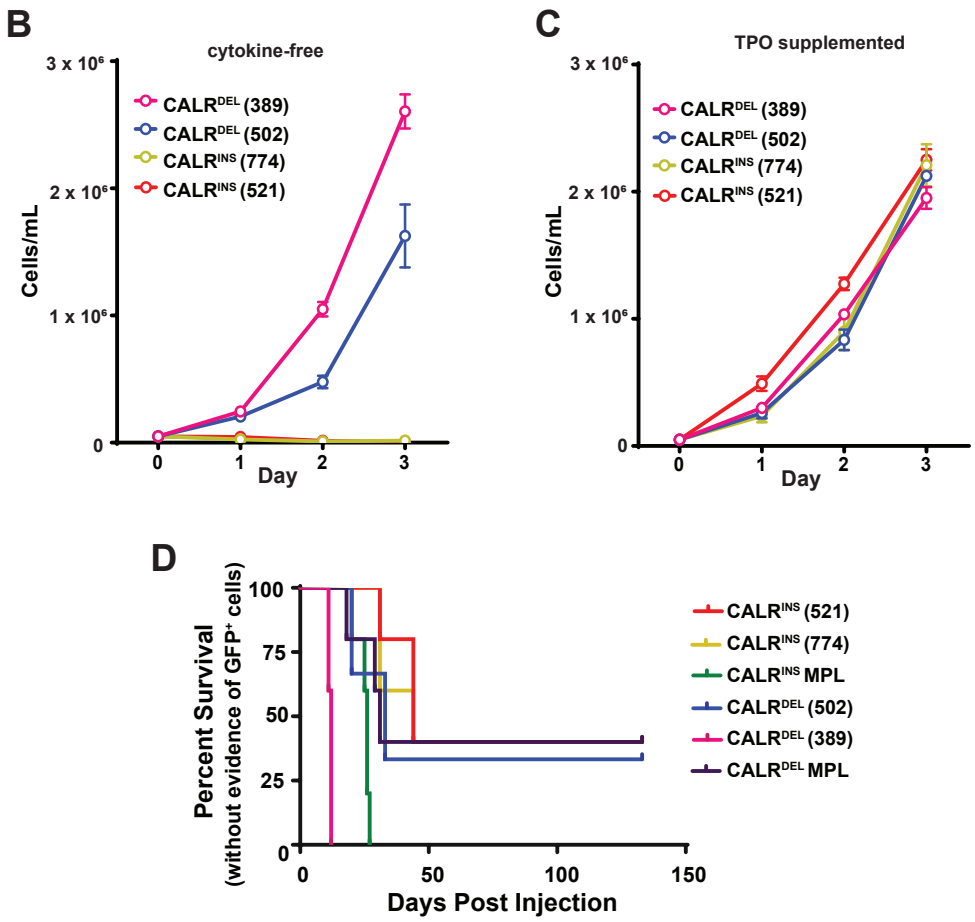
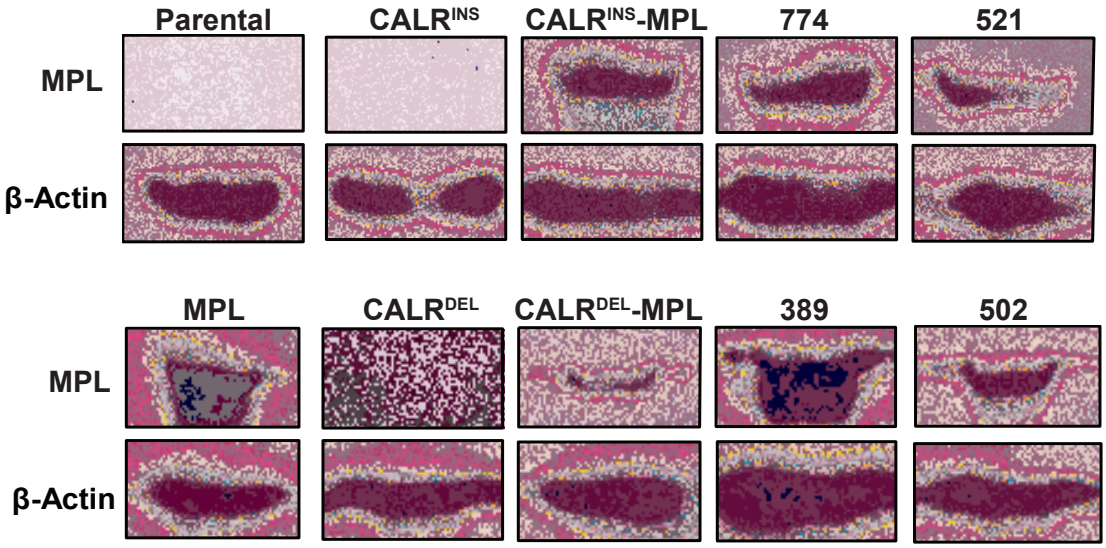
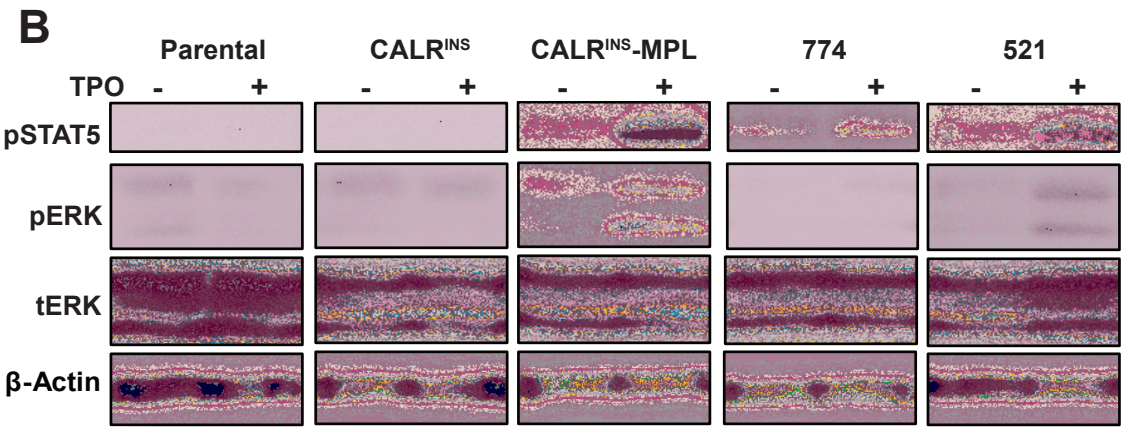



**Figure 4.2. Ba/F3 cells expressing CALR$^{DEL}$ and CALR$^{INS}$ mutations expand in a fraction of mice and gain cytokine independence.** (A) Survival of mice injected with 2.5 x $10^6$ CALR$^{WT}$, CALR$^{DEL}$, or CALR$^{INS}$ Ba/F3 cells (n=10 mice each). All mice remaining mice were sacrificed at day 183 post-transplant, no GFP$^+$ cells were detected in the peripheral blood, bone marrow, or spleen of any of these mice. (B) Growth of post-mouse cell lines in cytokine free media. (C) Growth of post-mouse cells lines in media supplemented with 10ng/mL TPO. (n=5 each).

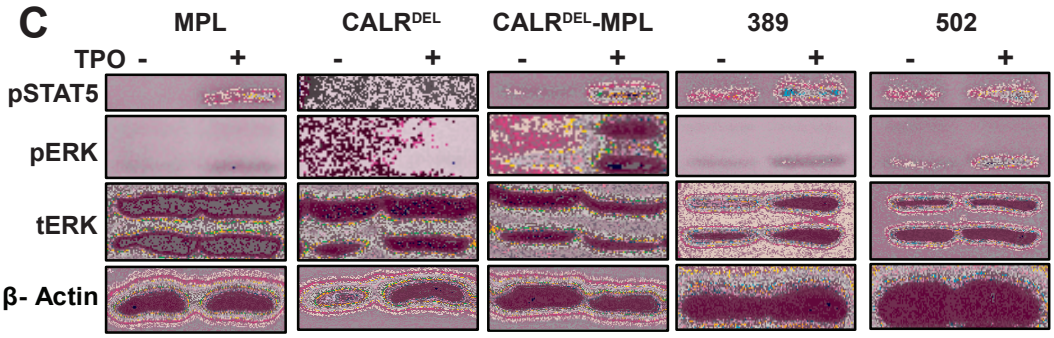
This suggested that the Ba/F3 cells which expanded in vivo expressed endogenous MPL. Indeed, we found that all post-mouse cell lines expressed MPL (Figure 4.3A) on Western Blot.




Next, we stimulated the cell lines with TPO and measured activation of ERK and STAT5 by phosphorylation of ERK and STAT5 (Figure 4.3B). All post-mouse cells activated ERK and STAT5 upon TPO stimulation, providing further evidence for presence of MPL on the cell surface. Activation of ERK and STAT5 was present without stimulation in $CALR^{DEL}$ post-mouse cells as well as $CALR^{DEL}$ cells with ectopic MPL, consistent with constitutive activation of MPL signaling. $CALR^{INS}$ post-mouse cells also had activation of STAT5 without TPO, suggesting constitutive activation of MPL.



After expansion, in vitro post-mouse cell lines were injected back into BALB/c mice to compare the ability of pre- versus post-mouse cells to expand in vivo.




Ba/F3 CALR$^{DEL}$ and CALR$^{INS}$ cells with ectopic expression of MPL (mMPL-MSCV-hCD4 vector) were also used as a comparator. We found that all post-mouse cell lines expanded and caused death with similar kinetics as Ba/F3 CALR$^{DEL}$ and CALR$^{INS}$ with ectopic expression of MPL (Figure 4.3D).

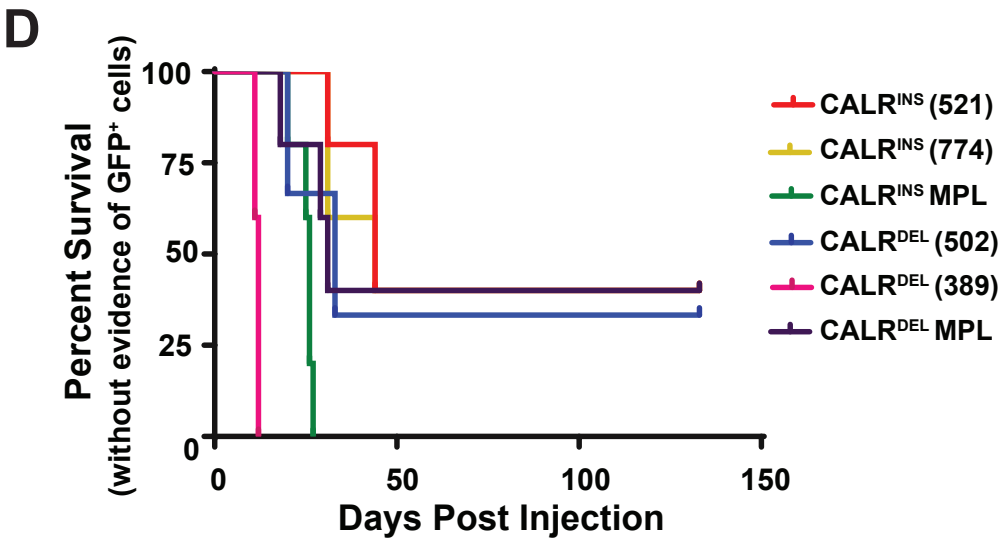



**Figure 4.3. Post-mouse cell lines have upregulated endogenous MPL.** (A) Western blot demonstrating MPL expression in post-mouse cell lines. (B-C) Western blot of cells stimulated with TPO (50ng/mL) or PBS and harvested 15 minutes later to assess activation of downstream signaling pathways. (D) Survival of mice injected with 2.5 x $10^6$ post-mouse cell lines (389, 502, 521, 774), Ba/F3 CALR$^{DEL}$ MPL, or Ba/F3 CALR$^{INS}$ MPL (n=5).

### 4.3 Methods

**Cell lines:** Ba/F3 cells were transduced with GFP- or human CD4-tagged MSCV retrovirus expressing murine MPL or human CALR wild-type ($CALR^{WT}$), 52bp deletion ($CALR^{DEL}$), or 5bp insertion ($CALR^{INS}$). Cells were sorted for GFP or hCD4 positivity (FACSAria Fusion, BD Biosciences). For double-transduced cell lines, Ba/F3 cells expressing MPL were clone-sorted and expanded prior to transduction with CALR retroviruses. Cells were maintained in RPMI-1640 supplemented with 10% heat- inactivated fetal bovine serum (FBS), penicillin, streptomycin, and L-glutamine (PSL), and 5% WEHI-3 conditioned medium as a source of murine IL-3.

**Mice:** BALB/c mice were retro-orbitally injected with Ba/F3 cells expressing $CALR^{WT}$ , $CALR^{DEL}$ (n=10), $CALR^{INS}$ (n=10), post-mouse cell lines (389, 502, 521, 774) (n=5 each), $CALR^{DEL}$ MPL (n=5) or $CALR^{INS}$ MPL (n=5). Mice were monitored for presence of Ba/F3 cells ($GFP^+$ cells) in the peripheral blood every 1-2 weeks post-transplant. Mice with expanding $GFP^+$ cells in the peripheral blood (>10%) were monitored twice daily and sacrificed once they appeared moribund at which time $GFP^+$ cells were sorted from the bone marrow or spleen (FACSAria Fusion, BD Biosciences).

**Cytokine independence assay:** Ba/F3 cells were washed four times with RPMI-1640 and plated at a concentration of 50,000 cells/ml in duplicate in either cytokine-free R10 medium (RPMI-1640 + 10% FBS + Pen/Strep/L-glutamine) or R10 supplemented with

5ng/ml murine TPO (BioLegend). Cells were counted daily by flow cytometry (Accuri C6, BD Biosiences) and dead cells were excluded by gating via FSC vs. SSC.

**Western blotting:** Ba/F3 cells were plated in RPMI-1640 medium without FBS for 4 hours prior to stimulation with murine thrombopoietin (TPO) for 15 minutes. Cells were washed with PBS then lysed in RIPA buffer containing phosphatase and protease inhibitors (Sigma-Aldrich). Protein concentration was measured via BCA assay (Pierce). 30µg protein was run on 12% polyacrylamide gels then transferred onto nitrocellulose membranes. Membranes were blocked with 5% BSA and probed with antibodies detecting phospho-STAT5 (BD Biosciences), phospho-ERK1/2, total ERK1/2 (Cell Signaling Technologies), MPL, β-actin, and calreticulin (Abcam). Proteins were detected using HRP-conjugated secondary antibodies (Abcam) and chemiluminescence (Pierce) and visualized by a CCD imager (G Box, Syngene).

### 4.4 Conclusion

Here we demonstrate that in vivo passage of Ba/F3 cells expressing mutant CALR leads to the selection of cells which have upregulated endogenous MPL thus allowing for their subsequent expansion in mice. This work highlights the exclusivity of MPL as the requisite scaffold cytokine receptor for mutant calreticulin and solidifies the central importance of activated MPL signaling in the pathogenesis of MPN.

# Chapter 5: Calreticulin Expression Can Modulate MPL Signaling

## 5.1 Introduction to Calreticulin's role in Hematopoiesis

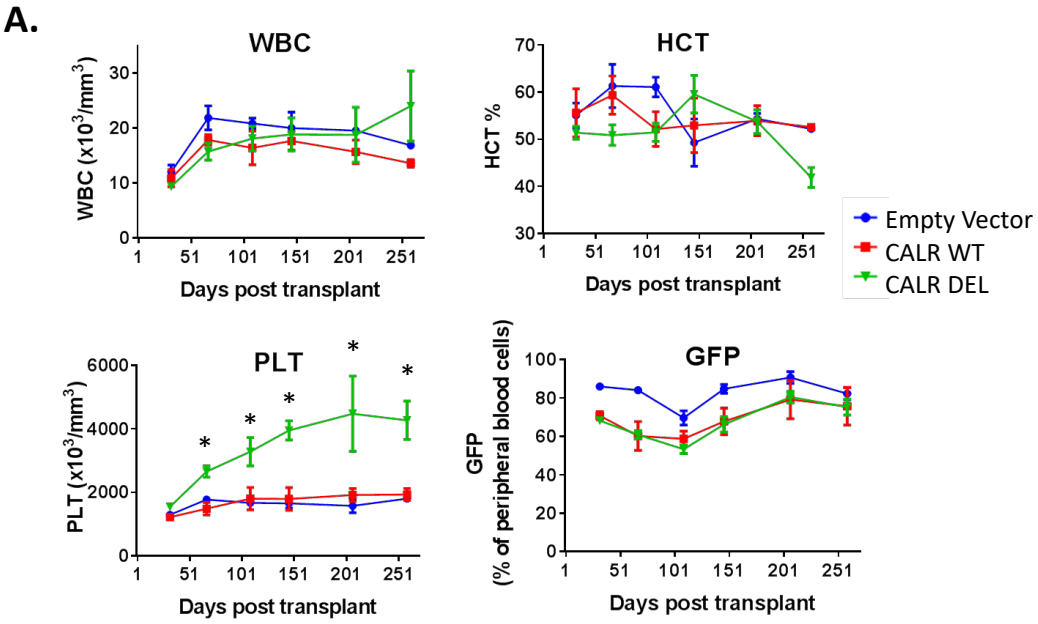
As mutations in calreticulin, somewhat surprisingly, have been demonstrated to activate the JAK/STAT pathway and lead to an increase in proliferation of myeloid cells (41), it still has not yet been uncovered whether wild type calreticulin plays any role in hematopoietic stem cell function or differentiation. However, it has been demonstrated that calreticulin helps contribute in T cell receptor activation through its role in sequestering calcium ions, which are important for T cell activation (113). This suggests that calreticulin does play some role in hematopoiesis through its role in the cell of binding calcium. It has not yet been demonstrated if calreticulin's role as a chaperone protein, specifically for glycosylated cell surface receptors for signaling pathways, could have any effect on hematopoiesis. This works seeks to demonstrate that this may be the case.

*In vivo* primary mouse models can be a useful tool to examine exactly how expression of genes in certain populations in the bone marrow affect hematopoiesis. Through secondary and competitive transplants, it can be determined whether certain genes have a positive or negative effect on HSC differentiation or self-renewal. We find that overexpression of calreticulin seems to dampen MPL signaling *in* vitro and *in vivo.* Congruently, absence of calreticulin leads to an increase in colony formation *in vitro*, an increase in LKS frequency and a proliferation advantage *in vivo,* suggesting an increase in MPL signaling. These data suggest that calreticulin may play a role in hematopoiesis through dampening MPL signaling.

### 5.2 Results

**CALR$^{DEL}$ leads to ET Phenotype *in vivo* and CALR$^{DEL}$ but not CALR$^{WT}$ can secondarily transplant**

To investigate the mechanism of disease for mutated calreticulin in MPN pathogenesis, we developed a transduction transplantation mouse model in which wild-type bone marrow cells are infected with Green Fluorescent Protein (GFP) tagged retrovirus encoding either empty vector (EV), an overexpression of wild-type calreticulin (CALR$^{WT}$), or calreticulin containing the 52 base pair deletion (CALR$^{DEL}$) and transplanted into lethally irradiate recipient mice (C57B/6). All mice in the three cohorts demonstrated robust stable contribution of GFP+ cells to peripheral blood leukocytes (Figure 5.1A). All mice in the CALR$^{DEL}$ cohort developed thrombocytosis without leukocytosis or erythrocytosis, akin to human MPN Essential Thrombocythemia (Figure 5.1A).




We performed secondary transplants into lethally irradiated recipient mice (Figure 5.1B). CALR$^{DEL}$ mice developed thrombocytosis as in the primary transplant. Surprisingly, GFP+

cells were not seen in the secondary transplant from mice overexpressing wild type calreticulin ($CALR^{WT}$), although cells from the original donor (CD45.2) were detected in these secondary transplants (CD45.2).

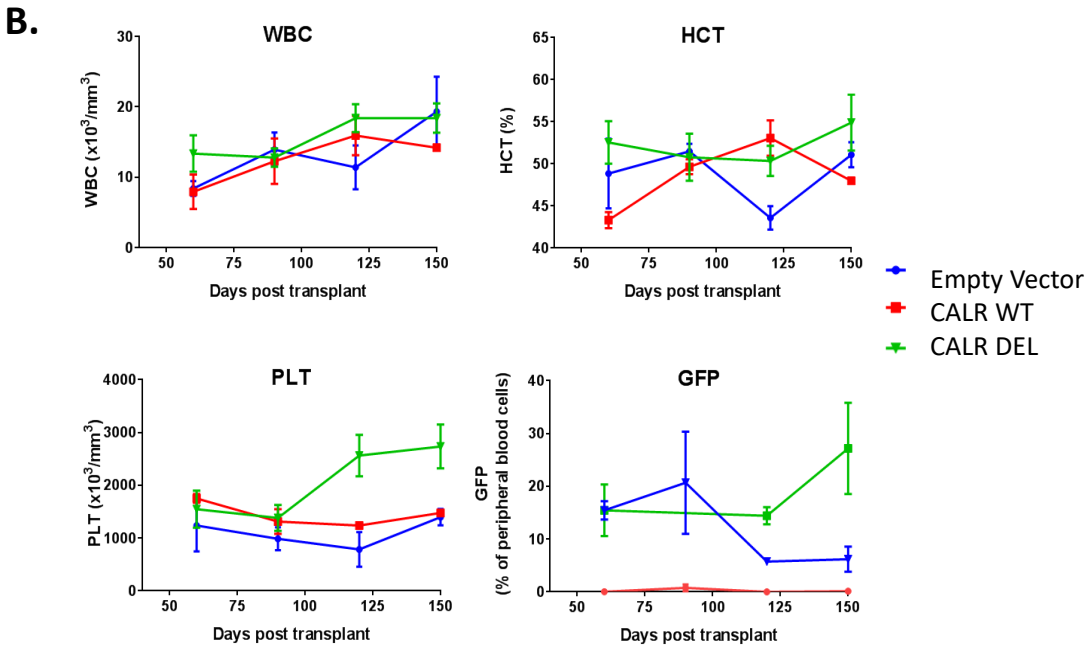
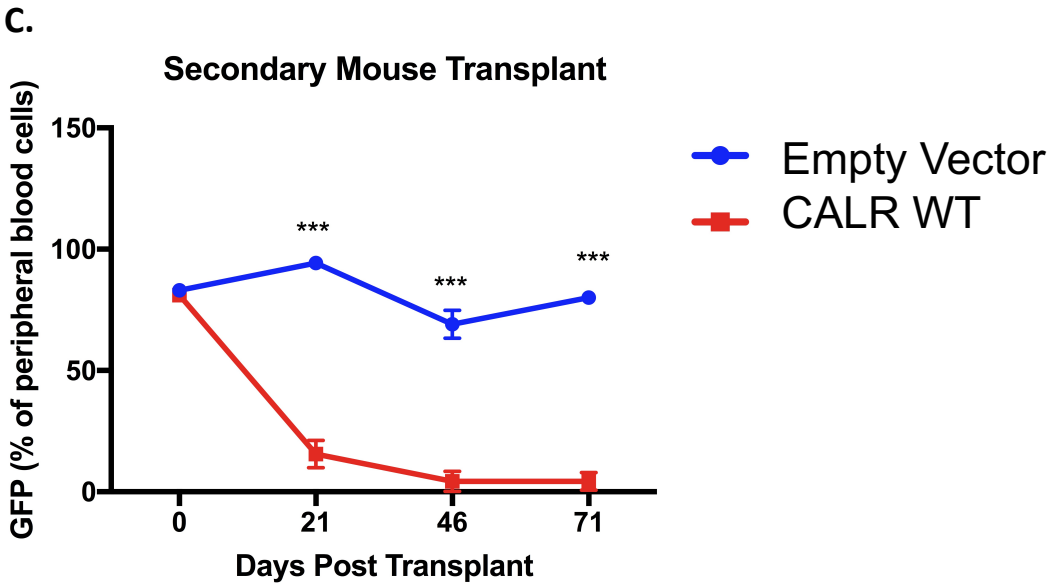



**Figure 5.1: $CALR^{DEL}$ develop ET like phenotype and $CALR^{DEL}$ but not $CALR^{WT}$ cells can secondarily transplant.** (A) Primary Transplant of bone marrow cells transduced with $GFP^+$ retrovirus of empty vector (blue), overexpression of calreticulin (red) or overexpression of 52 base pair deletion in exon 9 of calreticulin (green) into lethally irradiated recipient mice. Graphs display white blood cell count, hematocrit, platelet count and chimerism ($GFP^+$ cells in peripheral blood). (B) Secondary Transplant of bone marrow cells isolated from primary transplant mice of empty vector (blue), overexpression of calreticulin (red) or overexpression of 52 base pair deletion in exon 9 of calreticulin (green) into lethally irradiated recipient mice. Graphs display white blood cell count, hematocrit, platelet count and chimerism. (C) A second independent secondary transplant of bone marrow cells isolated from primary transplanted mice. N=5 $p < 0.05$ two way ANOVA.

The inability of $CALR^{WT}$ cells to contribute to hematopoiesis in secondary transplants was confirmed with a second independent experiment (Figure 5.1C).




**Inability for CALR$^{WT}$ cells to secondarily transplant is not due to lack of proper homing or an activated immune system**

Because no GFP+ cells were seen in secondary transplants even at the initial peripheral blood assessment at four weeks post-transplant, we investigated whether CALR$^{WT}$ cells were defective in their ability to home to the bone marrow in secondary transplants. We injected GFP$^{+}$ Lin$^{-}$, c Kit$^{+}$ Sca-1$^{+}$ (LKS) cells sorted from primary transplanted mice into secondary recipients and harvested bone marrow and spleen from transplanted mice 24 hours later (Figure 5.2A-B).

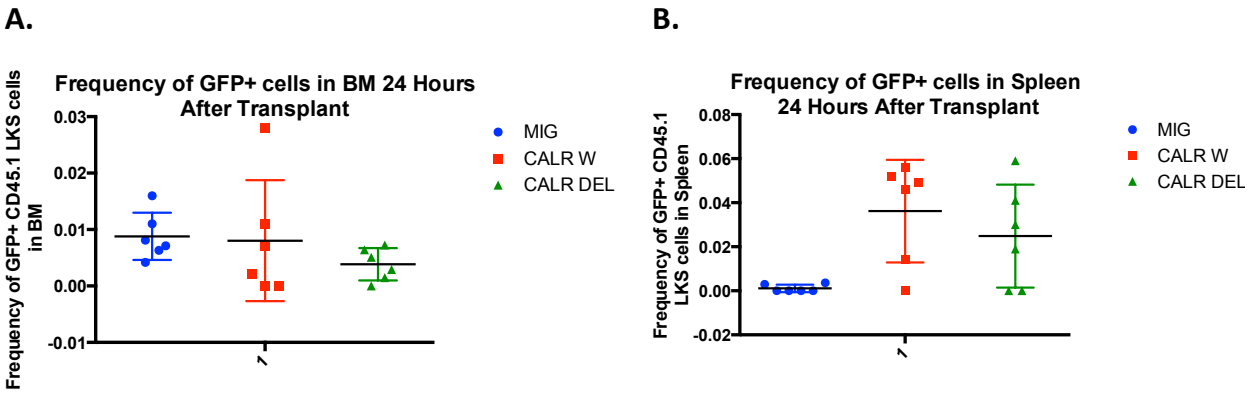
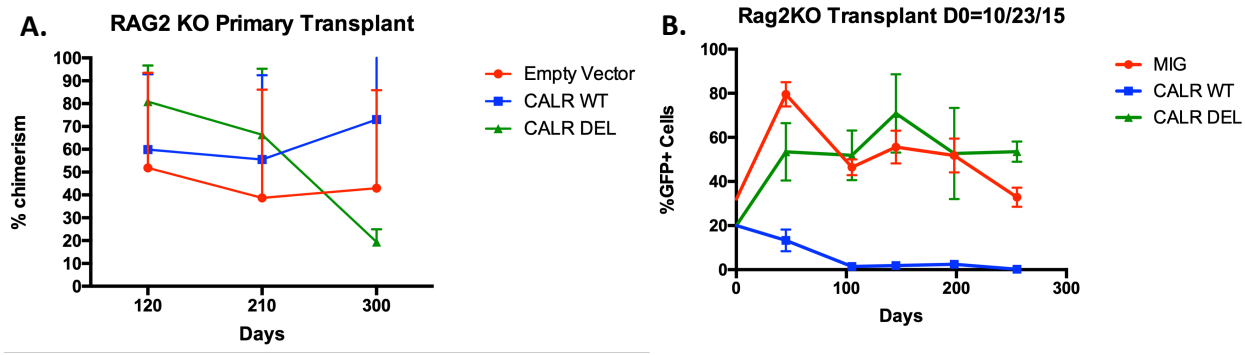



**Figure 5.2 No difference of Empty Vector, CALR$^{WT}$ or CALR$^{DEL}$ cells in homing to bone marrow and spleen 36 Hours post-transplant.** (A)Frequency of GFP$^{+}$ cells in the bone marrow 24 hours after transplant (B) Frequency of GFP$^{+}$ cells in spleen 24 hours after transplant. Lin$^{-}$, c Kit$^{+}$, Sca-1$^{+}$ GFP$^{+}$ CD 45.1 cells were FACS sorted from primary transplanted mice and transplanted into lethally irradiated recipient mice. After 24 hours, mice were sacrificed, cells from bone marrow were harvested, stained and analyzed on Novocyte. N=3 $p > 0.05$, unpaired t test.

We found no difference in the frequency of GFP$^{+}$ LKS cell in the bone marrow of mice injected with CALR$^{WT}$ cells compared to CALR$^{DEL}$ or empty vector cells, demonstrating that the inability of CALR$^{WT}$ cells to contribute to hematopoiesis in secondary transplants is not due to a defect in their ability to correctly home to sites of hematopoiesis in these animals.

As demonstrated in Chapter 2, cells undergoing apoptosis can express calreticulin on their cell surface as a signal to immune cells for destruction. To rule out the possibility that CALR$^{WT}$ cells were being removed by the adaptive immune system, we performed primary and secondary transplants in a RAG2-/- background which lack T or B cells (114). Even in the absence of mature T and B cells, CALR$^{WT}$ bone marrow cells failed to contribute to hematopoiesis in secondary transplants, as demonstrated by a lack of GFP$^{+}$ cells in the peripheral blood of these mice over time (Figure 5.3B). This suggested that the inability of CALR$^{WT}$ cells to contribute to hematopoiesis in secondary transplants was not due to an activated immune response by T or B cells.




To test this *in vitro*, we performed a phagocytosis assay by mixing CALR$^{WT}$, CALR$^{DEL}$ or empty vector Ba/F3 cells with bone marrow derived macrophages activated by LPS. CALR$^{WT}$ cells were not more vulnerable to phagocytosis than CALR$^{DEL}$ or empty vector cells (Figure 5.3C).

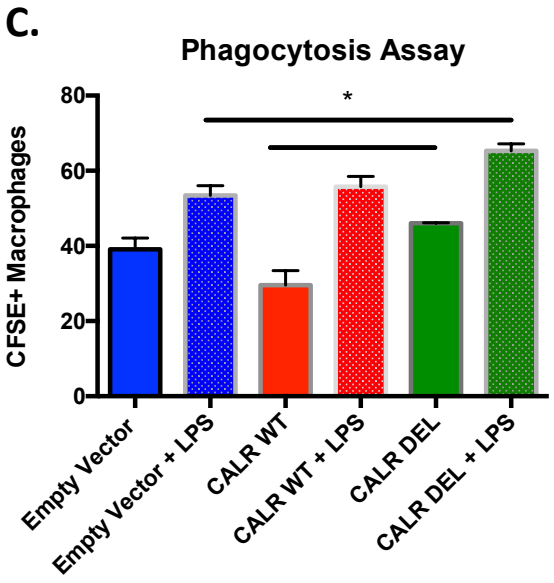
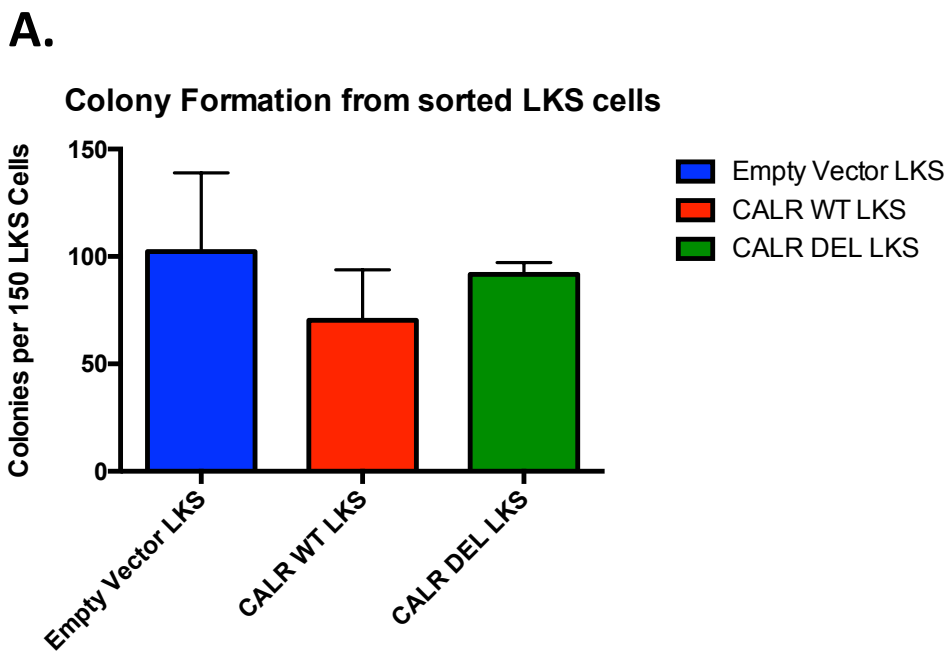



**Figure 5.3 Absence of Mature T or B cells has no impact on inability of CALR$^{WT}$ bone marrow. cells to secondarily transplant and CALR$^{WT}$ cells do not demonstrate an increased ability to be phagocytosed by activated bone marrow derived macrophages.** (A)Chimerism of lethally irradiated recipient mice transplanted with RAG2KO$^{KO}$ bone marrow cells transduced with retrovirus expressing GFP$^{+}$ empty vector (red), overexpression of calreticulin (blue) or overexpression of 52 base pair deletion in exon 9 of calreticulin (green) into lethally irradiated recipient RAG2$^{KO}$mice. (B) Chimerism of lethally irradiated RAG2$^{KO}$ recipient mice transplanted with bone marrow cells harvested from primary transplant mice. (C) CFSE$^{+}$ Bone Marrow Derived Activated Macrophages after mixing with Ba/F3 cells transduced with empty vector, CALR$^{WT}$ or CALR$^{DEL}$. P< 0.05, unpaired t test.

This suggests that CALR$^{WT}$ cells are not trafficking calreticulin to the cell surface to be phagocytosed by activated macrophages.

**Overexpression of calreticulin leads to significant decrease in colony formation, blunts TPO dependent growth and physically interacts with MPL in Ba/F3 cells**

This observation, that CALR$^{WT}$ cells are able to contribute to hematopoiesis in primary transplants but not secondary transplants, suggests that overexpression of CALR may actually impair the self-renewal of HSC. To further investigate the self-renewal capacity of hematopoietic stem and progenitor cells overexpressing calreticulin, we sorted GFP$^{+}$ LKS cells from primary transplants and performed methylcellulose colony formation assays. On the initial plating, we found no significant difference between empty vector, CALR$^{WT}$, and CALR$^{DEL}$ (Figure 5.4A).




However, on serial re-plating CALR$^{WT}$ cells were inferior in their ability to form colonies as compared to empty vector and CALR$^{DEL}$ (Figure 5.4B).

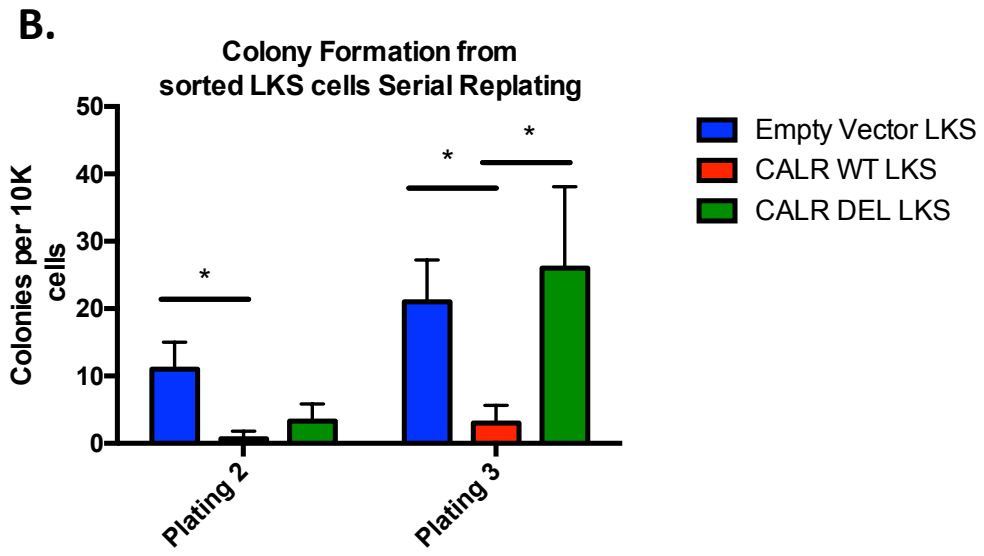


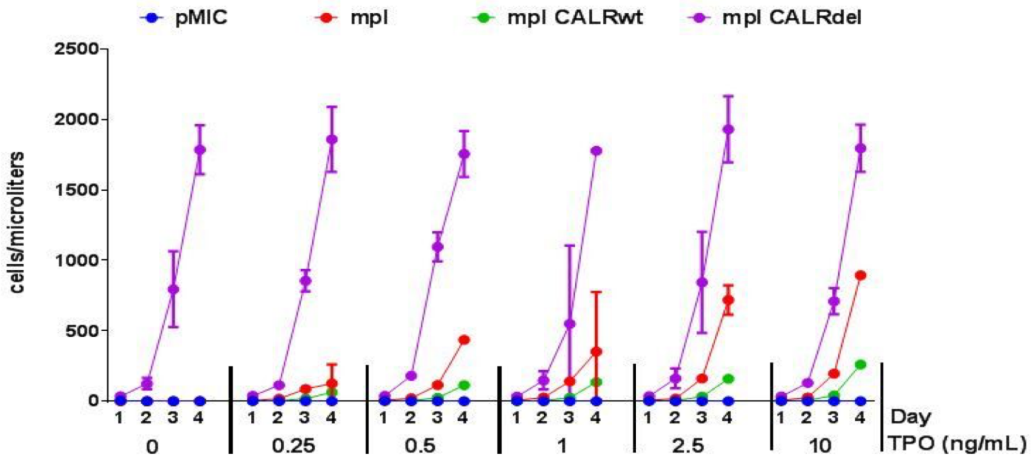

**Figure 5.4 CALR$^{WT}$ LKS cells form significantly fewer colonies in serial re-plating assay.** (A)Colony Formation for Sorted LKS GFP+ Empty Vector, CALRWT or CALRDEL CD 45.1 cells for first plating. (B) Colony Formation for Second and Third Plating. Lin-, c Kit+, Sca-1+ GFP+ Empty Vector, CALRWT and CALRDEL CD 45.1 cells were FACS sorted from primary transplanted mice and placed in (M3231, StemCell Technologies) supplemented with mSCF, mTPO, mIL-3 (Peprotech) and hEpo (Procrit, Amgen). Colonies were counted every 7 days. For re-plating, cells were washed of methylcellulose and cytokines, counted for a total of 5,000 and re-plated in (M3231, StemCell Technologies) supplemented with mSCF, mIL-3 (peprotech) and hEpo (Procrit, Amgen). N=3 P< 0.01, multiple t tests

This suggests that the overexpression of calreticulin may negatively impacts the self-renewal of LKS cells.

We observed a phenotype of defective self-renewal in CALR$^{WT}$ LKS and thought this was reminiscent to the phenotype of loss of thrombopoietin receptor (MPL) (29). We hypothesized that overexpression of wild type calreticulin may dampen MPL signaling since MPL signaling is necessary for LKS self-renewal. As demonstrated in Chapter 1 and 2, we now know that mutations in calreticulin interact with and activate MPL (42). There is some evidence that suggests that wild type calreticulin may also bind MPL (85). We hypothesized that overexpression of wild type calreticulin may bind to MPL and may inhibit its role in MPL signaling. To test this, we performed growth assays in Ba/F3 cells co-expressing MPL and CALR

with increasing amounts of TPO with TPO being the only growth factor allowing for survival in culture. At all concentrations of TPO, $CALR^{WT}$ cells grow slower than cells transfected with $CALR^{DEL}$ and MPL or MPL alone (Figure 5.5A).

**A.**




Next, we performed a coimmunoprecipitation assay with Ba/F3 cells that had been transduced with c-MPL and calreticulin and found that in both lysates that contain endogenous calreticulin and c-MPL or overexpressed calreticulin and c-MPL, there is an interaction that takes place (Figure 5.5B).

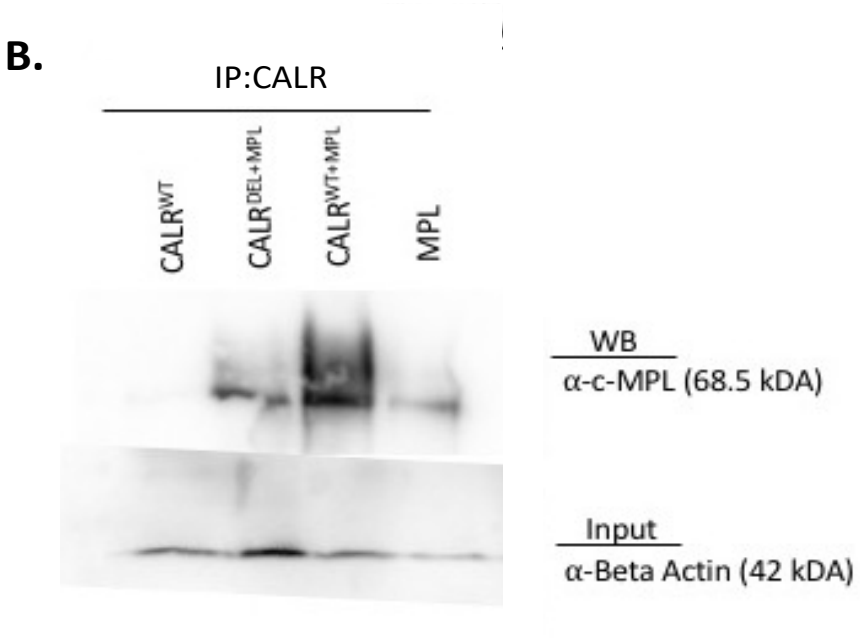
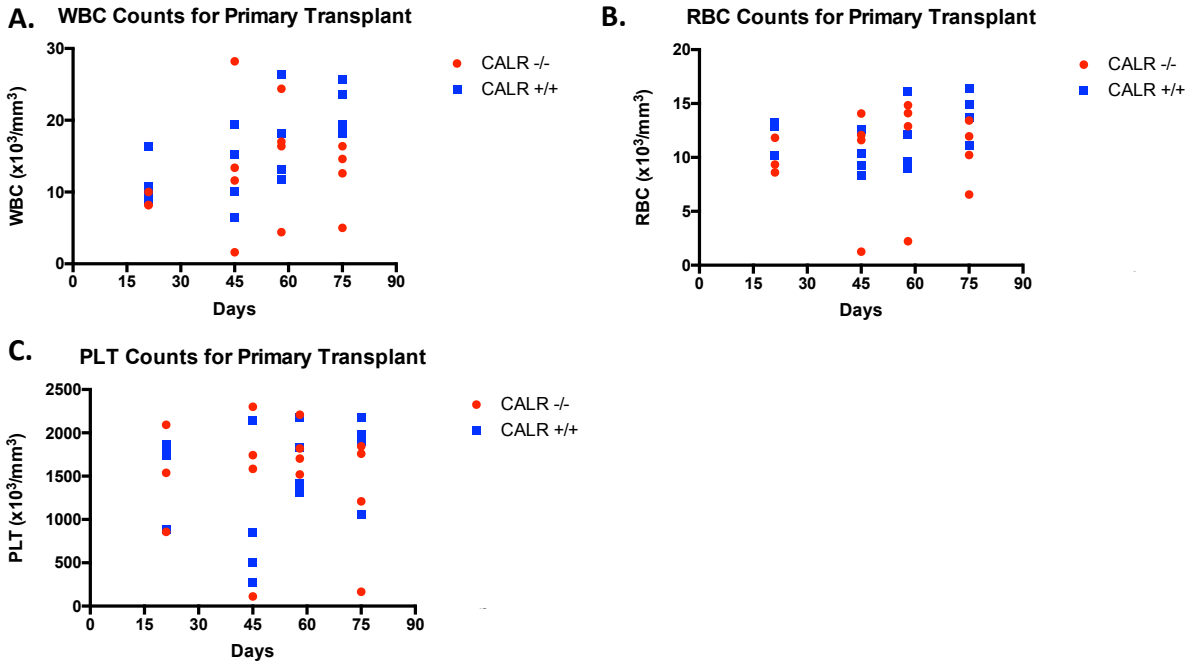



**Figure 5.5 Overexpression of calreticulin suppresses TPO dependent growth and physically interacts with MPL in Ba/F3 cells.** (A) Growth Assay of Ba/F3 cells transduced with empty vector (hCD4), MPL($GFP^{+)}$, MPL ($GFP^{+}$) $CALR^{WT}$ (hCD4), or MPL (GFP) $CALR^{DEL}$ (hCD4) cultured with increasing concentration of TPO. Cells were counted every day for 4 days on Accuri Flow Cytometer. $p < 0.05$ t test (B) Western Blot demonstrating that calreticulin physically interacts with MPL.

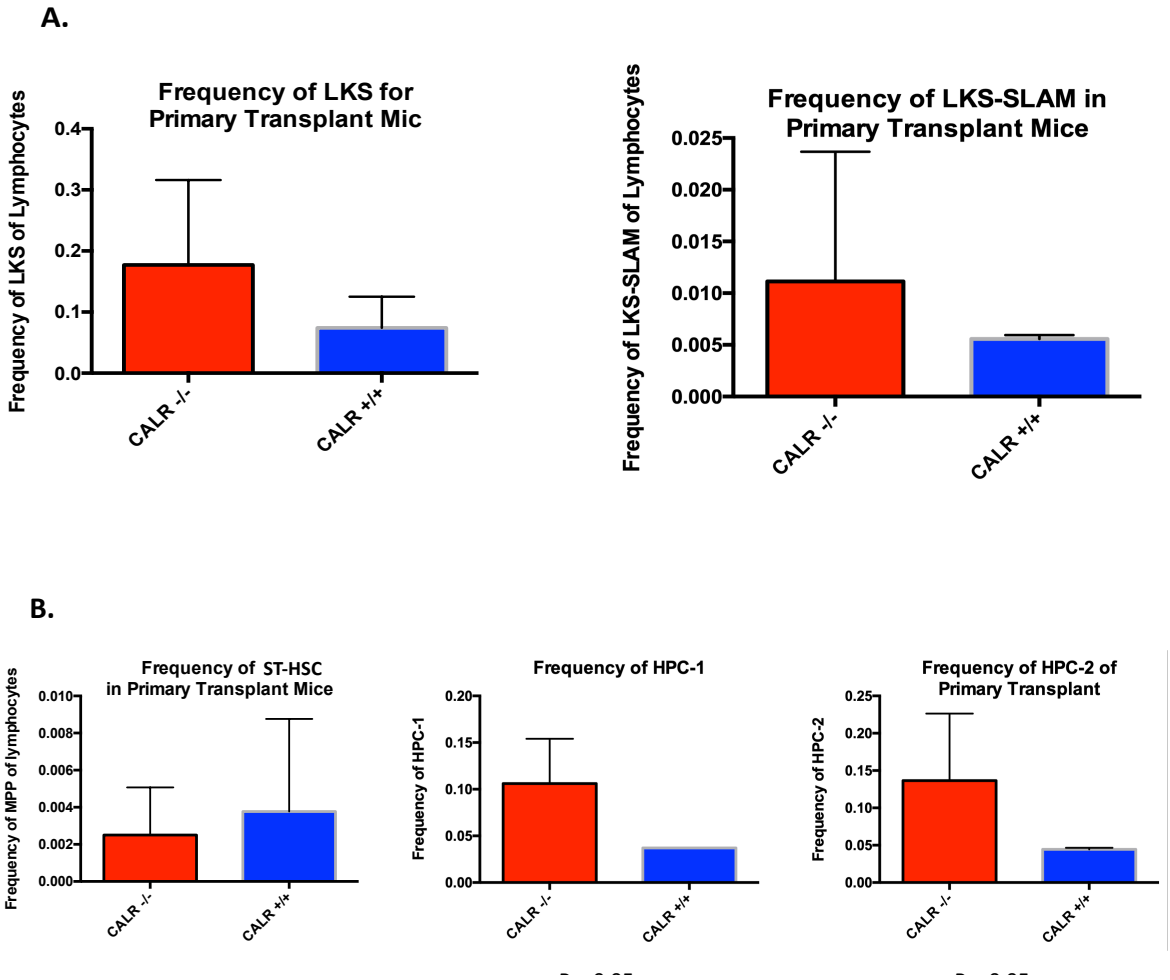
**$CALR^{-/-}$ mice have a trend of an increase in frequency of LKS, LKS-SLAM and MEPs** With the overexpression of calreticulin in LKS cells demonstrating an impairment in self renewal and suppression of MPL signaling, we sought to investigate if the loss of calreticulin would have any effect hematopoietic stem and progenitor cell function. We hypothesized the absence of calreticulin would result in increased MPL signaling. As demonstrated in Chapter 2, $CALR^{-/-}$ knockout mice are embryonically lethal at day 14.5, and therefore to study the effect of loss of calreticulin on hematopoiesis *in vivo*, we had to find a work around. As demonstrated in Chapter 1, mice embryos have a fully formed fetal liver at day 14.5. The fetal liver contains hematopoietic stem and progenitor cells. (10). The Michalak lab, who have contributed

immensely to understanding calreticulin's role in the ER, had developed a $CALR^{+/-}$ mouse line which we requested (52). The Michalak lab generously provided several mice for us to use. We backcrossed them into a C57B6 CD 45.2 background for 6-10 generations. To generate $CALR^{-/-}$ embryos, we mated a male $CALR^{+/-}$ and female $CALR^{+/-}$, waiting 14 days and harvested the fetal livers. After genotyping, we transplanted $CALR^{-/-}$ fetal liver cells into lethally irradiated recipient mice. Mice were bled weekly for peripheral blood cell counts. We determined that the absence of calreticulin in the bone marrow did not lead to any distinguishing phenotype of increased WBCs, RBCs or platelet counts compared to mice transplanted with cells containing calreticulin (Figure 5.6A-C).




(A) **Figure 5.6 There is no difference in white blood cell, red blood cell or platelet count in mice transplanted with $CALR^{+/+}$ or $CALR^{-/-}$ fetal liver cells.** (A)White blood cell count for Primary Transplant of fetal liver cells from $CALR^{+/+}$ and $CALR^{-/-}$ embryos. (B) Red blood cell count for primary transplant of fetal liver cells from $CALR^{+/+}$ and $CALR^{-/-}$ embryos. (C) Platelet count for primary transplant of fetal liver cells from $CALR^{+/+}$ and $CALR^{-/-}$ embryos. $P > 0.05$ t test.

We investigated if the absence of calreticulin caused any changes in the makeup of the bone marrow. Interestingly, it appears that in CALR$^{-/-}$ bone marrow, there was a tread of increase in LKS frequency. Although it was not significant, we feel as though with an increase in the sample size it may be. Furthermore, there was not a significant increase in LKS-SLAM (CD 150$^{+,}$ CD48$^{-}$), a population that is defined as HSCs, compared to bone marrow containing calreticulin (Figure 5.7A). There did seem to be a significant increase in the HPC1/2 (LKS-CD 150$^{-}$ CD48$^{+}$) but the significance of this was not something we pursued further (Figure 5.7B).




As demonstrated in Chapter 1, HSC cells differentiate into more committed progenitor cells. These progenitor cells lack self-renewal potential and are lineage committed. In the bone

marrow of mice without calreticulin, we do see a trend of an increase in the megakaryocyte-erythrocyte (MEP) population but no difference in the common myeloid (CMP) or granulocyte-macrophage (GMP) population compared to cells that contain calreticulin (Figure 5.7C).

**C.**

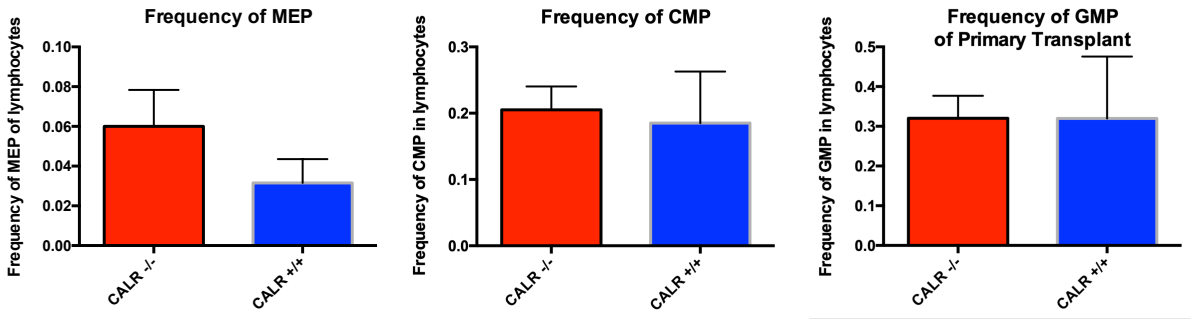
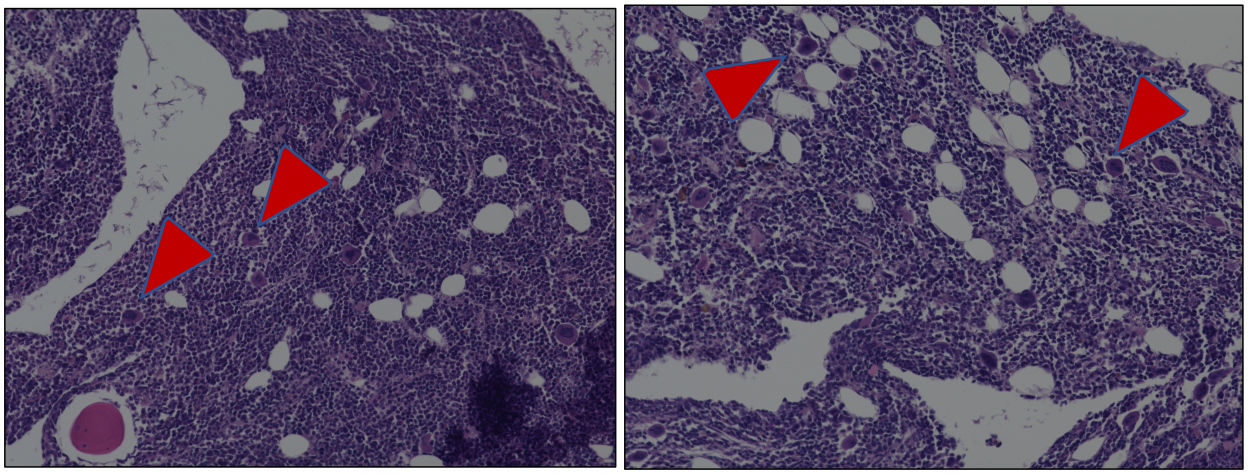



**Figure 5.7 There is a trend of an increase in frequency of LKS, LKS-SLAM and MEPs cells in CALR$^{-/-}$ bone marrow. (A)** Frequency of hematopoietic stem (LKS) and long term hematopoietic stem cells (LKS-SLAM) in the bone marrow , (B) Frequency of short term hematopoietic stem (ST-HSC), HPC-1 and HPC-2 in the bone marrow (C) Frequency of common myeloid progenitors (CMP), granulocyte monocyte progenitors (GMP) and megakaryocyte erythroid progenitors (MEP) in the bone marrow of each mouse measured by flow cytometry. N=3 $p<0.05$ t test

**CALR$^{-/-}$ mice have increase in megakaryocytes in bone marrow and LKS cells have increase in colony formation**

We also took bone marrow histology sections from these mice and counted megakaryocytes per field of view. We saw a significant increase in megakaryocytes in CALR$^{-/-}$ mice bone marrow slides as compared to CALR$^{+/+}$ mice bone marrow slides (Figure 5.8A-B).

**A.**



**CALR$^{+/+}$ H&E Stain 20x** **CALR$^{-/-}$ H&E Stain 20x**

**B.**

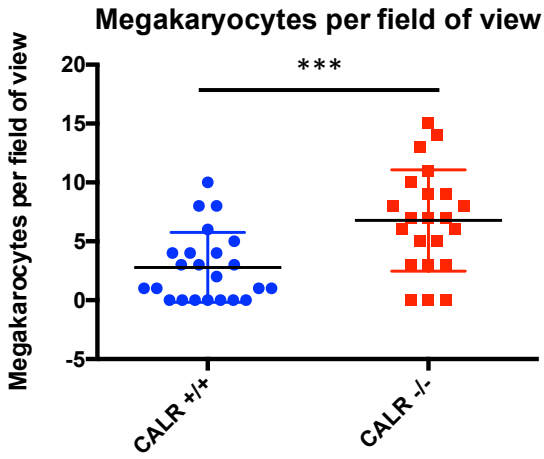


**Figure 5.8 There is a significant increase in megakaryocytes in CALR$^{-/-}$ bone marrow**
(A)Representative sections of H&E staining of bone marrow sections from CALR+/+ and CALR-/- mice. (20X magnification shown, Keyence microscope running Keyence software) (B) Quantification of megakaryocytes per field of view. P <0.001 t test.

To determine if the absence of calreticulin gives the LKS cell any advantage *in vitro*, we sorted LKS cells from calreticulin$^{-/-}$ and calreticulin$^{+/+}$ bone marrow and plated equal numbers in methylcellulose supplemented with mIL, mSCF, hEPO and mTPO to assess colony formation. We saw a significant increase in the number of colonies that were formed from CALR$^{-/-}$ LKS cells compared to CALR$^{+/+}$ LKS cells (Figure 5.9A). To determine if the absence of calreticulin affected LKS self-renewal, we performed a serial re-plating assay and saw a significant increase in colonies formed from CALR$^{-/-}$ LKS cells compared to CALR$^{+/+}$ cells (Figure 5.9A).

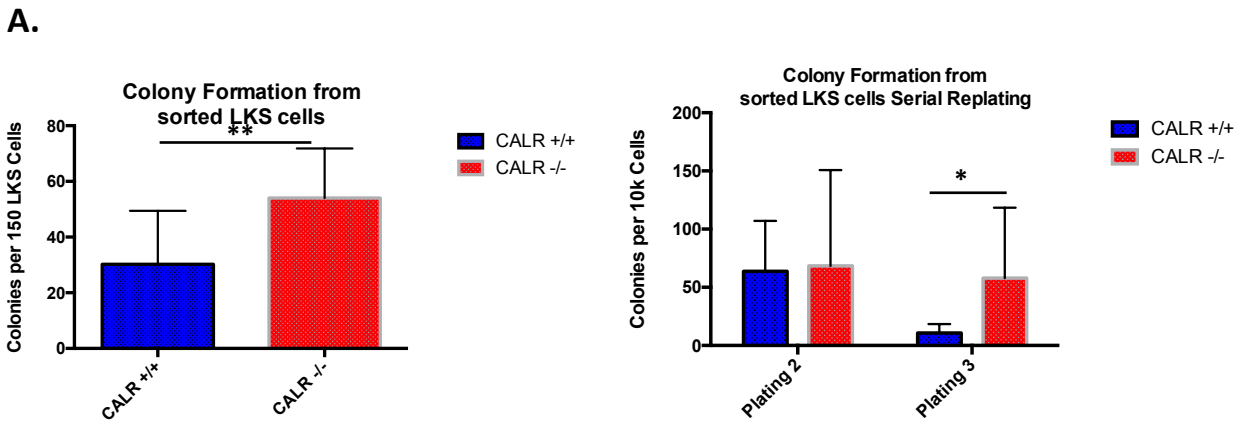



**Figure 5.9 There is a significant increase in colony formation from CALR$^{-/-}$ LKS cells in a serial re-plating assay.** (A)Colony Formation for Sorted LKS CALR$^{-/-}$ or CALR$^{+/+}$ CD 45.1 cells for first plating. (B) Colony Formation for Second and Third Plating. Lin$^{-}$ , c Kit$^{+}$ , Sca-1$^{+}$ CALR$^{-/-}$ and CALR$^{+/+}$ CD 45.1 cells were FACS sorted from primary transplanted mice and placed in (M3231, StemCell Technologies) supplemented with mSCF, mTPO, mIL-3 (Peprotech) and hEpo (Procrit, Amgen). Colonies were counted every 7 days. For re-plating, cells were washed of methylcellulose and cytokines, counted for a total of 5,000 and re-plated in (M3231, StemCell Technologies) supplemented with mSCF, mIL-3 (peprotech) and hEpo (Procrit, Amgen). N=3 P< 0.01, multiple t tests

With LKS cells being one of two populations in the bone marrow that have c-MPL expressed, we sought to determine if absence of calreticulin has any observable changes in megakaryocytes and megakaryocyte progenitors. To test this, we isolated cells from $CALR^{-/-}$ and $CALR^{+/+}$ fetal livers and plated them in Mega-Cult supplemented with cytokines. After 14 days, we stained for acetylcholinesterase and counted colonies. We did see a slight increase in the number of megakaryocyte progenitor colonies formed on Mega Cult from $CALR^{-/-}$ cells compared to colonies formed from $CALR^{+/+}$ (Data not shown). To further investigate if absence of calreticulin had any observable changes in megakaryocytes, we cultured $CALR^{-/-}$ and $CALR^{+/+}$ fetal liver cells in media containing TPO to differentiate them into megakaryocytes. After 14 days, we harvested cells, cytospun them, and stained with Wright Giemsa stain to look at megakaryocyte ploidy. We saw no significant difference in the ploidy of megakaryocytes with or without calreticulin (Data not shown).

To determine if the absence of calreticulin gives HSCs a competitive advantage over wild-type HSCs *in vivo*, we FACS sorted LKS cells from $CALR^{-/-}$ (CD45.1) or $CALR^{+/+}$(CD45.1/2) donor mice, then transplanted equal numbers of $CALR^{-/-}$ CD45.1 and $CALR^{+/+}$ CD45.1/2 LKS cells along with lineage selected rescue bone marrow cells (CD45.2) into lethally irradiated recipients mice. We found that starting from 4 weeks post-transplant, there was consistently a higher percentage of $CALR^{-/-}$ CD45.1 cells than $CALR^{+/+}$ CD45.1/2 cells making up the donor derived cells in the peripheral blood (Figure 5.10A).

**CALR$^{-/-}$ LKS cells seem to have a competitive advantage *in vivo***

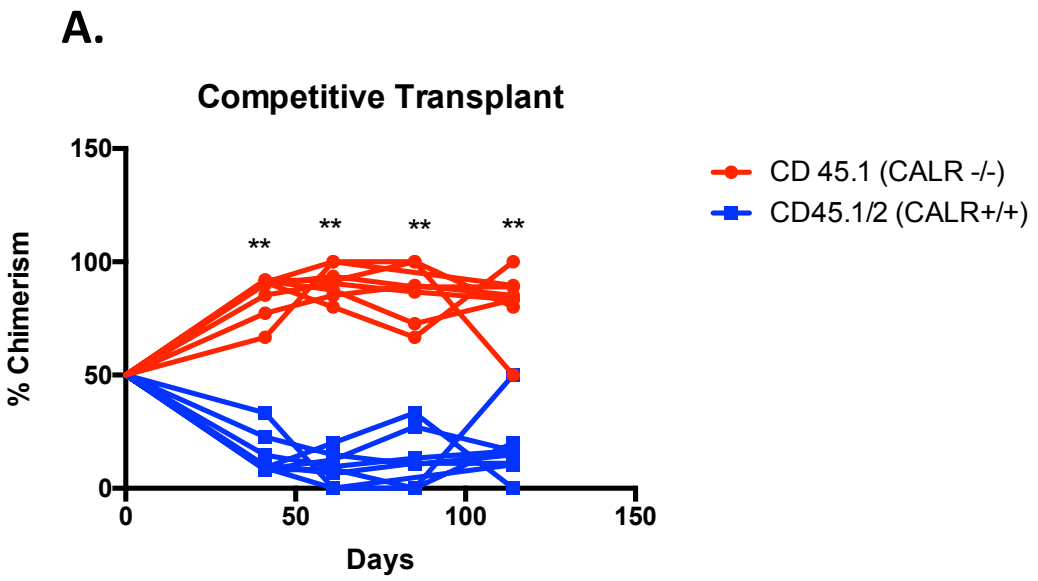



**Figure 5.10 CALR$^{-/-}$ LKS cells seem to have a competitive advantage *in vivo***
Chimerism of lethally irradiated recipient mice transplanted with 200 sorted CD 45.1 CALR-/- and 200 sorted CD 45.1/2 CALR+/+. $p > 0.05$ t test

This demonstrates that the absence of calreticulin does give LKS cells a competitive advantage over cells that do have calreticulin expressed. All these data taken together suggest that the absence of calreticulin leads to an increase in frequency of LKS cells in the bone marrow, an increase in colony formation, a competitive advantage *in vivo*, an increase of megakaryocyte colonies, all processes that involve MPL signaling.

**5.3 Discussion**

Calreticulin is involved in many different pathways in the cell including cell adhesion, phagocytosis and calcium signaling, as demonstrated in Chapter 2. Its role in hematopoiesis has not been determined yet. However, these data suggest that calreticulin may play a role in

hematopoiesis through its interaction with MPL. In the overexpression of calreticulin, there is a dampening of MPL signaling. In its absence, there is an increase in MPL signaling. To us, these data further suggests that calreticulin is involved in the self-renewal pathway in LKS cells. We hypothesize that the mechanism for this is potentially through its role as a chaperone protein for glycosylated proteins. It could be possible that calreticulin is sequestering MPL in the ER to be properly glycosylated. In the absence of calreticulin, MPL may trafficked to the cell surface even without proper glycosylation. Further work is needed to demonstrate that in the absence of calreticulin, mature and immature forms of MPL are present at the cell surface, that immature forms of MPL can activate the JAK/STAT pathway and to discover where this interaction between calreticulin and MPL takes place in the cell. Since calreticulin has many roles inside and outside a cell, its interaction with MPL in the self-renewal pathway may be playing a small but significant role in the context of hematopoiesis.

### 5.4 Materials and Methods

**Transduction-transplantation model of CALR overexpression.** Transduction-transplantation mouse model overexpressing WT calreticulin was performed as described previously (TK Nguyen et al., 2016). Briefly, donor mice were treated with 5-flurouracil (5-FU, 150mg/kg). Whole bone marrow cells were isolated and transduced with retrovirus encoding $CALR^{WT}$, $CALR^{Del}$ or empty vector (MIG) with GFP marker. Post-infection, cells were transplanted into lethally irradiated C57Bl/6J hosts.

Peripheral blood cell counts. Peripheral blood was obtained from the saphenous vein and hematologic parameters were analyzed by an automated cell counter machine (ABCVet Analyzer).

**Isolation of bone marrow LKS cells.** LKS cells were purified from whole BM cells by staining cells with antibodies against lineage cocktail, c-Kit and Sca-1 ($Lin^{neg}$, $c\text{-}kit^{+}$ $Sca\text{-}1^{+}$) followed by fluorescence-activated cell sorting (FACS) on the FACSAria (BD Bioscienes).

**Colony forming unit (CFU) assay.** CFU assays were performed on sorted bone marrow LKS cells from the transduction-transplantation model. Sorted LKS cells (200 cells) were isolated as described above and plated in 3ml of Methocult (Stemcell Technologies) containing murine IL-3 10 ng/ml, murine SCF 50ng/ml and human EPO 20ng/ml. Colonies were counted after 7 days of culture.

**Western blotting:** Ba/F3 cells were plated in RPMI-1640 medium with FBS. Cells were washed with PBS then lysed in RIPA buffer containing phosphatase and protease inhibitors (Sigma-Aldrich). Protein concentration was measured via BCA assay (Pierce). 30µg protein were added to 2 µg of calreticulin antibody (Abcam) and mixed over night at 4 degrees C. Lysates mixtures was then added to washed A/G Magnetic Agarose Beads (Pierce, Thermo Fischer) and mixed at room temperature for an hour. Lysate mixtures were added to a magnet and flow through was collected for analysis. Agarose Beads were washing 3 times, beads were then added to Laemeli Buffer with BME for 30 minutes at room temperature while mixing to separate beads and proteins lysates. Beads were added to magnet to elute protein lysates and protein lysates were run on 12% polyacrylamide gels then transferred onto nitrocellulose membranes. Membranes were blocked with 5% BSA and probed with antibodies detecting MPL (EMD Millipore) β-actin (Abcam), and calreticulin (Abcam). Proteins were detected using HRP-conjugated secondary antibodies (Abcam) and chemiluminescence (Pierce) and visualized by a CCD imager (G Box, Syngene).

**Mice.** CALR$^{+/-}$ mice genotyped were obtained from the Michalak lab, University of Alberta, and back crossed 7 generations into a C57BL/6 CD 45.2 or CD 45.1 background. Mice were bred and maintained in the animal facility at the University of California, Irvine. Food and water were provided ad libitum. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

**Fetal liver harvest and transplant.** We paired female and male mice together and weighed the female mice daily. We found that between 9-10 grams of weight gain correlated strongly to day 14 of the pregnancy cycle. On day 14 we sacrificed the pregnant females and harvested the fetal liver tissue from the embryos. We used tissue harvest from the heads of the embryos for genotypic analysis by PCR for primers specific to calreticulin. Fetal livers were placed in PBS and ran through an 18, 21- and 23-gauge needle with a 3 mL syringe. Cells were spun down, lysed for red blood cells and counted.

**Isolation of HSCs from fetal livers.** Calr heterozygous (Calr$^{+/-}$) mice were paired to obtain embryonic fetal liver tissues. Fetal livers were harvested from embryos dissected at embryonic day E12.5 to E13.5 (based on weight gain of pregnant female). Single cell suspensions were prepared by serially aspirating and expelling the tissue through 18, 21 and 23-guage needles in DMEM medium containing 10% FBS. Genomic DNA for genotyping was isolated from embryo head tissue.

**Flow cytometry analysis of bone marrow and fetal liver HSCs.** Fetal liver cell suspensions were obtained as above. Whole bone marrow (BM) was harvested from both femurs and tibiae, RBCs were lysed with ACK buffer and cells stained for 30 minutes on ice. Pacific blue conjugated antibodies against Ter119, Mac1, Gr1, B220, CD3, CD4 and CD8 were used to detect

mature cells (Lineage cocktail). HSC populations were detected by staining with mouse specific antibodies against Lineage markers, c-Kit, Sca-1 (LKS), CD48 and CD150 (LKS-SLAM). Progenitor subsets (CMP/GMP/MEP) were detected using antibodies against CD34 and CD16/32. Flow cytometry was performed on the Novocyte (ACEA Biosciences) at the UCI Immunology Core facility. Data were analyzed using FlowJo software (Tree Star Inc.).

**Fetal liver transplant and peripheral blood chimerism.** Fetal liver cells were obtained as described above. $CALR^{+/+}$ or $CALR^{-/-}$ cells ($1x10^6$) were injected (via retro orbital) into lethally irradiated recipient mice. Peripheral blood cell counts were obtained monthly. For competitive transplants, sorted LKS cells from $CALR^{-/-}$ (CD45.1) and $CALR^{+/+}$ (CD45.1/2) FL donors, (2000 cells each) were injected into lethally irradiated recipients (CD45.2). Flow cytometry for chimerism of mature cells in peripheral blood was performed using CD45.2 APC and CD45.1 FITC antibodies along with CD11b and Gr-1 to detect myeloid cells. Data was acquired on the BD Accuri C6 (BD Biosciences).

**Megakaryocyte colony forming assay**. MK colony forming assays were performed on $CALR^{+/+}$ and $CALR^{-/-}$ fetal liver cells using the MegaCult™ (Stemcell Technologies), following manufacturer's instructions. Colonies were stained for acetylcholinesterase activity after 6 to 8 days. Intensity of acetylcholinesterase stain was quantified using ImageJ.

**In vitro differentiation of fetal-liver derived megakaryocytes.** Homogenized fetal liver cells were cultured in DMEM medium containing 10% FCS, 0.5% penicillin/streptomycin and 50 ng/ml recombinant murine TPO at 37°C and 5% $CO_2$. Between 3 and 5 days of cultures, cells were imaged for proplatelet formation.

**Determination of MK ploidy.** For ploidy measurements, cultured cells were washed with PBS/BSA 0.5 % and stained with APC conjugated anti-CD41 for 30 minutes at 4°C. Cells were then incubated in staining buffer containing propidium iodide (50 μg/ml), sodium citrate (4mM), RNase A (0.2 mg/ml) and Triton-X 100 (0.1%) for 30 minutes before flow cytometry.

**Statistical Analysis.** Data are presented as mean ± standard error of the mean. Data were analyzed using unpaired or paired (competitive transplants) Student's t-test, and p-value<0.05 was considered statistically significant.

# Chapter 6 Conclusions and Future Work

## 6.1 Conclusion

Calreticulin's role in MPN has been well characterized. We know that mutations in exon 9 of calreticulin interact with c-MPL protein to activate the JAK/STAT pathway. It has been demonstrated, by us and other labs, that in Ba/F3 cells that simultaneously express c-MPL and mutations in exon 9 of calreticulin are cytokine independent. Interestingly, mutations in calreticulin do not seem to lead to a selective advantage at the HSC level in a mouse model which is quite different from activating mutations in JAK2 and MPL in mouse models. Furthermore, there is some evidence that mutant calreticulin can be secreted from the cell and activate JAK/STAT signaling in a paracrine manner (84). It has also been hypothesized that mutations in calreticulin lead to either the mRNA transcript that is not stable and gets degraded or the protein expression is not stable and gets degraded (81). Most of the phenotypic effects of mutant calreticulin's role in MPN are linked to its interaction with MPL. But with these mutations in calreticulin leading to a novel C terminus that abrogates its native ability to bind calcium ions, it has been suggested that mutations in calreticulin that cause MPN pathogenesis alone cannot cause all the MPN phenotypes that we see in mouse models and in humans. Some published reviews have speculated that the disruption of calcium signaling within HSCs and progenitor cells with mutant calreticulin could be contributing to some of the disease phenotypes. This work did not dive into that possibility.

This work started off looking at mutations in JAK2 that lead to a mild MPN phenotype, then drifted to helping to determine the mechanism for how mutations in calreticulin lead to MPN pathogenesis then finally sought to determine what is calreticulin's role in hematopoiesis in general. Since calreticulin has many functions inside a cell, most MPN patients are treated

with a JAK1/2 non-selective inhibitor and specific targeted therapies like Gleevac are the desired therapeutic target, it is essential to determine what calreticulin's role in hematopoiesis is. This work provides evidence that calreticulin can suppress MPL signaling, an important signaling pathway for self-renewal of HSCs and differentiation in hematopoiesis. We had several follow up experiments planned that would help us further unlock calreticulin's role in the cell in hematopoiesis but due to the COVID-19 epidemic restrictions, those could not be completed in time for this dissertation. We thought a critical experiment would be to evaluate SOCS3 expression in $CALR^{WT}$ and $CALR^{-/-}$ cells. We know SOCS3 is a key negative regulator of JAK2 signaling and have demonstrated that in JAK2 mutant patients, there is some dysregulation of SOCS3 in response to IL-10 activation. If overexpression of calreticulin is suppressing MPL signaling through JAK2, possibly there may be a synergistic effect with SOCS3 to keep this pathway suppressed. Furthermore, if absence of calreticulin is leading to an increase of MPL signaling through JAK2, perhaps SOCS3 is suppressed in some way to keep this pathway active longer than it should be. To further determine if MPL signaling in the presence and absence of calreticulin is increased or decreased, we wanted to look at phosphorylation of STAT3 and STAT5 after serum starvation and TPO stimulation in $CALR^{WT}$ and $CALR^{-/-}$ cells. A couple years ago, we sent stimulated and fixed bone marrow cells from one $CALR^{DEL}$, one $CALR^{WT}$ and one empty vector mouse to Steve Oh's lab at Washington University where they perform CyTOF analysis at these samples. They determined that there was definitely a difference in phosphorylation of STAT3/5, with $CALR^{WT}$ have a decrease in phosphorylation of STAT3/5. At that point, we were not focused on this project and didn't pursue it further. However, given what we know now, we would like to evaluate pSTAT3/5 status after serum

starvation and TPO stimulation in these cells through Western Blot of Ba/F3 and fetal liver cells, Phosphoflow and CyTOF to confirm in HSC and progenitor populations. This would give us the clearest picture of MPL signaling status through the JAK/STAT pathway. Finally, we would like to evaluate the cell cycle status of $CALR^{WT}$ and $CALR^{-/-}$ HSCs. As mentioned in Chapter 1, most HSCs exist in quiescence exiting only to go through the cell cycle when necessary. If overexpression of calreticulin was suppressing MPL signaling and thus self-renewal of HSCs, then these HSCs would be existing in the $G^0$ phase for a longer period of time compared to control. Likewise, if the absence of calreticulin was leading to an upregulation of MPL signaling in HSCs, then potentially these HSCs would be going through all stages of the cell cycle at a faster rate than control cells.

### 6.2 Future Directions

If given more time and unlimited resources, I would love to dive more into the mechanism for this interaction between calreticulin and MPL. I would love to try to determine which domains of calreticulin are necessary and sufficient for interaction with MPL. We can already hypothesize that it may be the N or P domains since those domains are the same in wild type calreticulin and mutant calreticulin. I would further seek to determine where in the cell this interaction takes place, whether it is solely in the E.R. or if calreticulin travels with MPL to the cell surface in vesicles to ensure it stays properly folded. I would also seek to determine if calreticulin is an integral chaperone for MPL and other cell surface receptors or if other chaperone proteins can compensate for calreticulin loss. All together these future directions would allow us to determine if a mutant calreticulin specific inhibitor would potentially demonstrate some clinical benefit to MPN patients with calreticulin mutations or if modulating

calreticulin expression through enhancing wild type calreticulin expression or calreticulin peptide treatment could have some clinical benefit. As I mentioned in Chapter 2, Ross Levine's lab has demonstrated some benefit of treating primary cells from calreticulin mutant patients with a wild type c terminal calreticulin peptide mimetic (85).

The evidence presented here seems to hint at a bigger question: are chaperone proteins important for other roles in the cell, not solely just folding proteins? This question asked another way might be can you modulate the availability of certain cell surface receptors, including those responsible for activating pathways associated with cancers simply by modulating the availability of chaperone proteins? We know one of the basic principles of biology is that form follows function and that correct form or folding of a protein is necessary for its proper function in the cell. Since most proteins make a stop in the E.R. to ensure they are correctly folded and post translationally modified, the E.R. may play a key role in stopping cell surface receptors with activating or inactivating mutations from ever reaching the cell surface. If we could figure out a way to modulate the availability of certain cell surface receptors by way of chaperone proteins, that may unlock a potential therapeutic treatment for certain cancers that have been linked to activating mutation in cell surface receptors.

# Bibliography


1. Challen, Grant A., et al. “Mouse Hematopoietic Stem Cell Identification and Analysis.” *Cytometry Part A*, vol. 75A, no. 1, 2009, pp. 14–24., doi:10.1002/cyto.a.20674.
2. Grinenko, Tatyana, et al. “Hematopoietic Stem Cells Can Differentiate into Restricted Myeloid Progenitors before Cell Division in Mice.” *Nature Communications*, vol. 9, no. 1, 2018, doi:10.1038/s41467-018-04188-7.
3. Vadakke-Madathil, Sangeetha, et al. “Flow Cytometry and Cell Sorting Using Hematopoietic Progenitor Cells.” *Methods in Molecular Biology Progenitor Cells*, 2019, pp. 235–246., doi:10.1007/978-1-4939-9631-5_18.
4. Larsson, Jonas. “Do HSCs Divide Asymmetrically?” *Blood*, vol. 119, no. 11, 2012, pp. 2431–2432., doi:10.1182/blood-2012-01-400713.
5. Kubota, Yasushi, and Shinya Kimur. “Regulation of Hematopoietic Stem Cell Fate: Self-Renewal, Quiescence and Survival.” *Advances in Hematopoietic Stem Cell Research*, 2012, doi:10.5772/31367.
6. Singh, Satyendra K., et al. “Id1 Ablation Protects Hematopoietic Stem Cells from Stress-Induced Exhaustion and Aging.” *Cell Stem Cell*, vol. 23, no. 2, 2018, doi:10.1016/j.stem.2018.06.001.
7. Claudinon, Julie, et al. “Interfering with Interferon Receptor Sorting and Trafficking: Impact on Signaling.” *Biochimie*, vol. 89, no. 6-7, 2007, pp. 735–743., doi:10.1016/j.biochi.2007.03.014.
8. Muench, David E., and H. Leighton Grimes. “Transcriptional Control of Stem and Progenitor Potential.” *Current Stem Cell Reports*, vol. 1, no. 3, 2015, pp. 139–150., doi:10.1007/s40778-015-0019-z.
9. Sugiyama, Tatsuki, and Takashi Nagasawa. “Bone Marrow Niches for Hematopoietic Stem Cells and Immune Cells.” *Inflammation & Allergy-Drug Targets*, vol. 11, no. 3, 2012, pp. 201–206., doi:10.2174/187152812800392689.
10. Sugiyama, Daisuke, et al. “Embryonic Regulation of the Mouse Hematopoietic Niche.” *The Scientific World JOURNAL*, vol. 11, 2011, pp. 1770–1780., doi:10.1100/2011/598097.
11. Szade, Krzysztof, et al. “Where Hematopoietic Stem Cells Live: The Bone Marrow Niche.” *Antioxidants & Redox Signaling*, vol. 29, no. 2, 2018, pp. 191–204., doi:10.1089/ars.2017.7419.
12. Kim, Chang. “Homeostatic and Pathogenic Extramedullary Hematopoiesis.” *Journal of Blood Medicine*, 2010, p. 13., doi:10.2147/jbm.s7224.
13. Pang, W. W., et al. “Human Bone Marrow Hematopoietic Stem Cells Are Increased in Frequency and Myeloid-Biased with Age.” *Proceedings of the National Academy of Sciences*, vol. 108, no. 50, 2011, pp. 20012–20017., doi:10.1073/pnas.1116110108.
14. Luis, T C, et al. “Signal Transduction Pathways Regulating Hematopoietic Stem Cell Biology: Introduction to a Series of Spotlight Reviews.” *Leukemia*, vol. 26, no. 1, 2012, pp. 86–90., doi:10.1038/leu.2011.260.
15. Jatiani, S. S., et al. “JAK/STAT Pathways in Cytokine Signaling and Myeloproliferative Disorders: Approaches for Targeted Therapies.” *Genes & Cancer*, vol. 1, no. 10, 2010, pp. 979–993., doi:10.1177/1947601910397187.

16. Park, Hyun Jung, et al. “Cytokine-Induced Megakaryocytic Differentiation Is Regulated by Genome-Wide Loss of a USTAT Transcriptional Program.” *The EMBO Journal*, vol. 35, no. 6, 2015, pp. 580–594., doi:10.15252/embj.201592383.
17. Hubbard, Stevan R. “Mechanistic Insights into Regulation of JAK2 Tyrosine Kinase.” *Frontiers in Endocrinology*, vol. 8, 2018, doi:10.3389/fendo.2017.00361.
18. Kershaw, Nadia J, et al. “SOCS3 Binds Specific Receptor–JAK Complexes to Control Cytokine Signaling by Direct Kinase Inhibition.” *Nature Structural & Molecular Biology*, vol. 20, no. 4, 2013, pp. 469–476., doi:10.1038/nsmb.2519.
19. Mcmullin, Mary Frances, and Holger Cario. “LNK Mutations and Myeloproliferative Disorders.” *American Journal of Hematology*, vol. 91, no. 2, 2016, pp. 248–251., doi:10.1002/ajh.24259.
20. Kleppe, Maria, et al. “Jak1 Integrates Cytokine Sensing to Regulate Hematopoietic Stem Cell Function and Stress Hematopoiesis.” *Cell Stem Cell*, vol. 22, no. 2, 2018, p. 277., doi:10.1016/j.stem.2017.12.018.
21. Park, Sung O., et al. “Conditional Deletion of Jak2 Reveals an Essential Role in Hematopoiesis throughout Mouse Ontogeny: Implications for Jak2 Inhibition in Humans.” *PLoS ONE*, vol. 8, no. 3, 2013, doi:10.1371/journal.pone.0059675.
22. Sakamoto, Kazuhito, et al. “Generation of Janus Kinase 1 (JAK1) Conditional Knockout Mice.” *Genesis*, vol. 54, no. 11, 2016, pp. 582–588., doi:10.1002/dvg.22982.
23. Baird, Allison M., et al. “T Cell Development and Activation in Jak3-Deficient Mice.” *Journal of Leukocyte Biology*, vol. 63, no. 6, 1998, pp. 669–677., doi:10.1002/jlb.63.6.669.
24. Karaghiosoff, Marina, et al. “Partial Impairment of Cytokine Responses in Tyk2-Deficient Mice.” *Immunity*, vol. 13, no. 4, 2000, pp. 549–560., doi:10.1016/s1074-7613(00)00054-6.
25. Takeda, K., et al. “Targeted Disruption of the Mouse Stat3 Gene Leads to Early Embryonic Lethality.” *Proceedings of the National Academy of Sciences*, vol. 94, no. 8, 1997, pp. 3801–3804., doi:10.1073/pnas.94.8.3801.
26. Ohkubo, Nobutaka, et al. “Accelerated Destruction of Erythrocytes in Tie2 Promoter-Driven STAT3 Conditional Knockout Mice.” *Life Sciences*, vol. 93, no. 9-11, 2013, pp. 380–387., doi:10.1016/j.lfs.2013.07.025.
27. Wang, Zhengqi, and Kevin D Bunting. “STAT5 In Hematopoietic Stem Cell Biology and Transplantation.” *Jak-Stat*, vol. 2, no. 4, 2013, doi:10.4161/jkst.27159.
28. Bunting, Kevin D., et al. “Reduced Lymphomyeloid Repopulating Activity from Adult Bone Marrow and Fetal Liver of Mice Lacking Expression of STAT5.” *Blood*, vol. 99, no. 2, 2002, pp. 479–487., doi:10.1182/blood.v99.2.479.
29. Murone, Maximilien, et al. “Hematopoietic Deficiencies in c-Mpl and TPO Knockout Mice.” *Stem Cells*, vol. 16, no. 1, 1998, pp. 1–6., doi:10.1002/stem.160001.
30. Alexander, Ws, et al. “Deficiencies in Progenitor Cells of Multiple Hematopoietic Lineages and Defective Megakaryocytopoiesis in Mice Lacking the Thrombopoietic Receptor c-Mpl.” *Blood*, vol. 87, no. 6, 1996, pp. 2162–2170., doi:10.1182/blood.v87.6.2162.bloodjournal8762162.
31. Soverini, Simona, et al. “Treatment and Monitoring of Philadelphia Chromosome-Positive Leukemia Patients: Recent Advances and Remaining Challenges.” *Journal of Hematology & Oncology*, vol. 12, no. 1, 2019, doi:10.1186/s13045-019-0729-2.

32. Bittencourt, Rosane Isabel, et al. “Philadelphia-Negative Chronic Myeloproliferative Neoplasms.” *Revista Brasileira De Hematologia e Hemoterapia*, vol. 34, no. 2, 2012, pp. 140–149., doi:10.5581/1516-8484.20120034.
33. Berlin, Nathanial I. “Polycythemia Vera.” *Hematology/Oncology Clinics of North America*, vol. 17, no. 5, 2003, pp. 1191–1210., doi:10.1016/s0889-8588(03)00083-2.
34. Pearson, T. “The Risk of Thrombosis in Essential Thrombocythemia and Polycythemia Vera.” *Seminars in Oncology*, vol. 29, no. 3, 2002, pp. 16–21., doi:10.1016/s0093-7754(02)70148-6.
35. Mudireddy, Mythri, et al. “Prefibrotic versus Overtly Fibrotic Primary Myelofibrosis: Clinical, Cytogenetic, Molecular and Prognostic Comparisons.” *British Journal of Haematology*, vol. 182, no. 4, 2017, pp. 594–597., doi:10.1111/bjh.14838.
36. Reiter, Andreas, and Jason Gotlib. “Myeloid Neoplasms with Eosinophilia.” *Blood*, vol. 129, no. 6, 2017, pp. 704–714., doi:10.1182/blood-2016-10-695973.
37. Elliott, Michelle A., and Ayalew Tefferi. “Chronic Neutrophilic Leukemia: 2018 Update on Diagnosis, Molecular Genetics and Management.” *American Journal of Hematology*, vol. 93, no. 4, 2018, pp. 578–587., doi:10.1002/ajh.24983.
38. Carter, Melody C., et al. “Mastocytosis.” *Immunology and Allergy Clinics of North America*, vol. 34, no. 1, 2014, pp. 181–196., doi:10.1016/j.iac.2013.09.001.
39. Kralovics, Robert, et al. “A Gain-of-Function Mutation ofJAK2in Myeloproliferative Disorders.” *New England Journal of Medicine*, vol. 352, no. 17, 2005, pp. 1779–1790., doi:10.1056/nejmoa051113.
40. Akpınar, Timur Selçuk, et al. “MPL W515L/K Mutations in Chronic Myeloproliferative Neoplasms.” *Turkish Journal of Hematology*, vol. 30, no. 1, 2013, pp. 8–12., doi:10.4274/tjh.65807.
41. Klampfl, Thorsten, et al. “Somatic Mutations of Calreticulin in Myeloproliferative Neoplasms.” *New England Journal of Medicine*, vol. 369, no. 25, 2013, pp. 2379–2390., doi:10.1056/nejmoa1311347.
42. Elf, Shannon, et al. “Defining the Requirements for the Pathogenic Interaction between Mutant Calreticulin and MPL in MPN.” *Blood*, vol. 131, no. 7, 2018, pp. 782–786., doi:10.1182/blood-2017-08-800896.
43. Maxson, Julia E., et al. “Oncogenic CSF3R Mutations in Chronic Neutrophilic Leukemia and Atypical CML.” *New England Journal of Medicine*, vol. 368, no. 19, 2013, pp. 1781–1790., doi:10.1056/nejmoa1214514.
44. Dwivedi, Pankaj, and Kenneth D. Greis. “Granulocyte Colony-Stimulating Factor Receptor Signaling in Severe Congenital Neutropenia, Chronic Neutrophilic Leukemia, and Related Malignancies.” *Experimental Hematology*, vol. 46, 2017, pp. 9–20., doi:10.1016/j.exphem.2016.10.008.
45. Hochhaus, Andreas, et al. “Long-Term Outcomes of Imatinib Treatment for Chronic Myeloid Leukemia.” *New England Journal of Medicine*, vol. 376, no. 10, 2017, pp. 917–927., doi:10.1056/nejmoa1609324.
46. Plosker, Greg L. “Ruxolitinib: A Review of Its Use in Patients with Myelofibrosis.” *Drugs*, vol. 75, no. 3, 2015, pp. 297–308., doi:10.1007/s40265-015-0351-8.
47. Verger, Emmanuelle, et al. “Ropeginterferon Alpha-2b Targets JAK2V617F-Positive Polycythemia Vera Cells in Vitro and in Vivo.” *Blood Cancer Journal*, vol. 8, no. 10, 2018, doi:10.1038/s41408-018-0133-0.

48. Kiladjian, J-J, et al. “Interferon-Alpha for the Therapy of Myeloproliferative Neoplasms: Targeting the Malignant Clone.” *Leukemia*, vol. 30, no. 4, 2015, pp. 776–781., doi:10.1038/leu.2015.326.
49. Jia, Xiao-Yun, et al. “Calreticulin: Conserved Protein and Diverse Functions in Plants.” *Physiologia Plantarum*, 2009, doi:10.1111/j.1399-3054.2009.1223.x.
50. Shivarov, V, et al. “Mutated Calreticulin Retains Structurally Disordered C Terminus That Cannot Bind Ca2+: Some Mechanistic and Therapeutic Implications.” *Blood Cancer Journal*, vol. 4, no. 2, 2014, doi:10.1038/bcj.2014.7.
51. Gong, Fang-Yuan, et al. “Aberrant Glycosylation Augments the Immuno-Stimulatory Activities of Soluble Calreticulin.” *Molecules*, vol. 23, no. 3, 2018, p. 523., doi:10.3390/molecules23030523.
52. Mesaeli, Nasrin, et al. “Calreticulin Is Essential for Cardiac Development.” *Journal of Cell Biology*, vol. 144, no. 5, 1999, pp. 857–868., doi:10.1083/jcb.144.5.857.
53. Wang, Wen-An, et al. “Loss of Calreticulin Uncovers a Critical Role for Calcium in Regulating Cellular Lipid Homeostasis.” *Scientific Reports*, vol. 7, no. 1, 2017, doi:10.1038/s41598-017-05734-x.
54. Han, Arum, et al. “Calreticulin Is a Critical Cell Survival Factor in Malignant Neoplasms.” *PLOS Biology*, vol. 17, no. 9, 2019, doi:10.1371/journal.pbio.3000402.
55. Hacken, Elisa Ten, et al. “Calreticulin as a Novel B-Cell Receptor Antigen in Chronic Lymphocytic Leukemia.” *Haematologica*, vol. 102, no. 10, 2017, doi:10.3324/haematol.2017.169102.
56. Molica, Stefano, et al. “Serum Levels of Soluble Calreticulin Predict for Time to First Treatment in Early Chronic Lymphocytic Leukaemia.” *British Journal of Haematology*, vol. 175, no. 5, 2016, pp. 983–985., doi:10.1111/bjh.13907.
57. Zamanian, Mohammadreza, et al. “Calreticulin Mediates an Invasive Breast Cancer Phenotype through the Transcriptional Dysregulation of p53 and MAPK Pathways.” *Cancer Cell International*, vol. 16, no. 1, 2016, doi:10.1186/s12935-016-0329-y.
58. Harada, Koji, et al. “Calreticulin Is a Novel Independent Prognostic Factor for Oral Squamous Cell Carcinoma.” *Oncology Letters*, vol. 13, no. 6, 2017, pp. 4857–4862., doi:10.3892/ol.2017.6062.
59. Xu, Qin, et al. “Significance of Calreticulin as a Prognostic Factor in Endometrial Cancer.” *Oncology Letters*, 2018, doi:10.3892/ol.2018.8495.
60. Sheng, Weiwei, et al. “Calreticulin Promotes EGF-Induced EMT in Pancreatic Cancer Cells via Integrin/EGFR-ERK/MAPK Signaling Pathway.” *Cell Death & Disease*, vol. 8, no. 10, 2017, doi:10.1038/cddis.2017.547.
61. Rizvi, Syed Monem, et al. “A Polypeptide Binding Conformation of Calreticulin Is Induced by Heat Shock, Calcium Depletion, or by Deletion of the C-Terminal Acidic Region.” *Molecular Cell*, vol. 15, no. 6, 2004, pp. 913–923., doi:10.1016/j.molcel.2004.09.001.
62. Peters, Larry Robert, and Malini Raghavan. “Endoplasmic Reticulum Calcium Depletion Impacts Chaperone Secretion, Innate Immunity, and Phagocytic Uptake of Cells.” *The Journal of Immunology*, vol. 187, no. 2, 2011, pp. 919–931., doi:10.4049/jimmunol.1100690.
63. Yang, Yunzhi, et al. “The ER-Localized Ca2+-Binding Protein Calreticulin Couples ER Stress to Autophagy by Associating with Microtubule-Associated Protein 1A/1B Light

Chain 3." *Journal of Biological Chemistry*, vol. 294, no. 3, 2018, pp. 772–782., doi:10.1074/jbc.ra118.005166.
64. Nakamura, Kimitoshi, et al. "Functional Specialization of Calreticulin Domains." *Journal of Cell Biology*, vol. 154, no. 5, 2001, pp. 961–972., doi:10.1083/jcb.200102073.
65. Kozlov, Guennadi, et al. "Structural Basis of Carbohydrate Recognition by Calreticulin." *Journal of Biological Chemistry*, vol. 285, no. 49, 2010, pp. 38612–38620., doi:10.1074/jbc.m110.168294.
66. Lu, Yi-Chien, et al. "Functional Roles of Calreticulin in Cancer Biology." *BioMed Research International*, vol. 2015, 2015, pp. 1–9., doi:10.1155/2015/526524.
67. Pike, Sandra E., et al. "Vasostatin, a Calreticulin Fragment, Inhibits Angiogenesis and Suppresses Tumor Growth." *Journal of Experimental Medicine*, vol. 188, no. 12, 1998, pp. 2349–2356., doi:10.1084/jem.188.12.2349.
68. Raj, Hanumantharao G., et al. "Novel Function of Calreticulin: Characterization of Calreticulin as a Transacetylase-Mediating Protein Acetylator Independent of Acetyl CoA Using Polyphenolic Acetates." *Pure and Applied Chemistry*, vol. 78, no. 5, 2006, pp. 985–992., doi:10.1351/pac200678050985.
69. Goicoechea, Silvia, et al. "The Anti-Adhesive Activity of Thrombospondin Is Mediated by the N-Terminal Domain of Cell Surface Calreticulin." *Journal of Biological Chemistry*, vol. 277, no. 40, 2002, pp. 37219–37228., doi:10.1074/jbc.m202200200.
70. Vance, Barbara A., et al. "Human CD69 Associates with an N-Terminal Fragment of Calreticulin at the Cell Surface." *Archives of Biochemistry and Biophysics*, vol. 438, no. 1, 2005, pp. 11–20., doi:10.1016/j.abb.2005.04.009.
71. Kozlov, Guennadi, et al. "Mapping the ER Interactome: The P Domains of Calnexin and Calreticulin as Plurivalent Adapters for Foldases and Chaperones." *Structure*, vol. 25, no. 9, 2017, doi:10.1016/j.str.2017.07.010.
72. Chouquet, Anne, et al. "X-Ray Structure of the Human Calreticulin Globular Domain Reveals a Peptide-Binding Area and Suggests a Multi-Molecular Mechanism." *PLoS ONE*, vol. 6, no. 3, 2011, doi:10.1371/journal.pone.0017886.
73. Ellgaard, Lars, et al. "Three-Dimensional Structure Topology of the Calreticulin P-Domain Based on NMR Assignment." *FEBS Letters*, vol. 488, no. 1-2, 2001, pp. 69–73., doi:10.1016/s0014-5793(00)02382-6.
74. Frickel, E.-M., et al. "TROSY-NMR Reveals Interaction between ERp57 and the Tip of the Calreticulin P-Domain." *Proceedings of the National Academy of Sciences*, vol. 99, no. 4, 2002, pp. 1954–1959., doi:10.1073/pnas.042699099.
75. Liu, Changzhen, et al. "Suppression of MHC Class I Surface Expression by Calreticulin's P-Domain in a Calreticulin Deficient Cell Line." *Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research*, vol. 1803, no. 5, 2010, pp. 544–552., doi:10.1016/j.bbamcr.2010.03.001.
76. Giraldo, Ana María Villamil, et al. "The Structure of Calreticulin C-Terminal Domain Is Modulated by Physiological Variations of Calcium Concentration." *Journal of Biological Chemistry*, vol. 285, no. 7, 2009, pp. 4544–4553., doi:10.1074/jbc.m109.034512.
77. Afshar, N., et al. "Retrotranslocation of the Chaperone Calreticulin from the Endoplasmic Reticulum Lumen to the Cytosol." *Molecular and Cellular Biology*, vol. 25, no. 20, 2005, pp. 8844–8853., doi:10.1128/mcb.25.20.8844-8853.2005.
78. Migliaccio, Anna Rita, and Vladimir N. Uversky. "Dissecting Physical Structure of Calreticulin, an Intrinsically Disordered Ca2+-Buffering Chaperone from Endoplasmic

Reticulum." *Journal of Biomolecular Structure and Dynamics*, vol. 36, no. 6, 2017, pp. 1617–1636., doi:10.1080/07391102.2017.1330224.
79. Rizvi, Syed Monem, et al. "A Polypeptide Binding Conformation of Calreticulin Is Induced by Heat Shock, Calcium Depletion, or by Deletion of the C-Terminal Acidic Region." *Molecular Cell*, vol. 15, no. 6, 2004, pp. 913–923., doi:10.1016/j.molcel.2004.09.001.
80. Wijeyesakere, Sanjeeva Joseph, et al. "The C-Terminal Acidic Region of Calreticulin Mediates Phosphatidylserine Binding and Apoptotic Cell Phagocytosis." *The Journal of Immunology*, vol. 196, no. 9, 2016, pp. 3896–3909., doi:10.4049/jimmunol.1502122.
81. Li, Juan, et al. "Mutant Calreticulin Knockin Mice Develop Thrombocytosis and Myelofibrosis without a Stem Cell Self-Renewal Advantage." *Blood*, vol. 131, no. 6, 2018, pp. 649–661., doi:10.1182/blood-2017-09-806356.
82. Takei, Hiraku, et al. "Skewed Megakaryopoiesis in Human Induced Pluripotent Stem Cell-Derived Haematopoietic Progenitor Cells Harbouring Calreticulin Mutations." *British Journal of Haematology*, vol. 181, no. 6, 2018, pp. 791–802., doi:10.1111/bjh.15266.
83. Liu, Peng, et al. "Secreted Calreticulin Mutants Subvert Anticancer Immunosurveillance." *OncoImmunology*, vol. 9, no. 1, 2019, p. 1708126., doi:10.1080/2162402x.2019.1708126.
84. Garbati, Michael R., et al. "Mutant Calreticulin-Expressing Cells Induce Monocyte Hyperreactivity through a Paracrine Mechanism." *American Journal of Hematology*, vol. 91, no. 2, 2016, pp. 211–219., doi:10.1002/ajh.24245.
85. Pronier, Elodie, et al. "Targeting the CALR Interactome in Myeloproliferative Neoplasms." *JCI Insight*, vol. 3, no. 22, 2018, doi:10.1172/jci.insight.122703.
86. Baxter, E Joanna, et al. "Acquired Mutation of the Tyrosine Kinase JAK2 in Human Myeloproliferative Disorders." *The Lancet*, vol. 365, no. 9464, 2005, pp. 1054–1061., doi:10.1016/s0140-6736(05)71142-9.
87. James, Chloé, et al. "A Unique Clonal JAK2 Mutation Leading to Constitutive Signalling Causes Polycythaemia Vera." *Nature*, vol. 434, no. 7037, 2005, pp. 1144–1148., doi:10.1038/nature03546.
88. Kralovics, Robert, et al. "A Gain-of-Function Mutation ofJAK2in Myeloproliferative Disorders." *New England Journal of Medicine*, vol. 352, no. 17, 2005, pp. 1779–1790., doi:10.1056/nejmoa051113.
89. Levine, Ross L., et al. "Activating Mutation in the Tyrosine Kinase JAK2 in Polycythemia Vera, Essential Thrombocythemia, and Myeloid Metaplasia with Myelofibrosis." *Cancer Cell*, vol. 7, no. 4, 2005, pp. 387–397., doi:10.1016/j.ccr.2005.03.023.
90. Zhao, Runxiang, et al. "Identification of an Acquired JAK2 Mutation in Polycythemia Vera." *Journal of Biological Chemistry*, vol. 280, no. 24, 2005, pp. 22788–22792., doi:10.1074/jbc.c500138200.
91. Skoda, Radek C., et al. "Pathogenesis of Myeloproliferative Neoplasms." *Experimental Hematology*, vol. 43, no. 8, 2015, pp. 599–608., doi:10.1016/j.exphem.2015.06.007.
92. Rumi, Elisa, et al. "Familial Chronic Myeloproliferative Disorders: Clinical Phenotype and Evidence of Disease Anticipation." *Journal of Clinical Oncology*, vol. 25, no. 35, 2007, pp. 5630–5635., doi:10.1200/jco.2007.12.6896.

93. Tapper, William, et al. “Genetic Variation at MECOM, TERT, JAK2 and HBS1L-MYB Predisposes to Myeloproliferative Neoplasms.” *Nature Communications*, vol. 6, no. 1, 2015, doi:10.1038/ncomms7691.
94. Jones, Amy V., and Nicholas C. P. Cross. “Inherited Predisposition to Myeloproliferative Neoplasms.” *Therapeutic Advances in Hematology*, vol. 4, no. 4, 2013, pp. 237–253., doi:10.1177/2040620713489144.
95. Landgren, Ola, et al. “Increased Risks of Polycythemia Vera, Essential Thrombocythemia, and Myelofibrosis among 24 577 First-Degree Relatives of 11 039 Patients with Myeloproliferative Neoplasms in Sweden.” *Blood*, vol. 112, no. 6, 2008, pp. 2199–2204., doi:10.1182/blood-2008-03-143602.
96. Mead, Adam J., et al. “GermlineJAK2Mutation In a Family with Hereditary Thrombocytosis.” *New England Journal of Medicine*, vol. 366, no. 10, 2012, pp. 967–969., doi:10.1056/nejmc1200349.
97. Mead, Adam J., et al. “Impact of Isolated Germline JAK2V617I Mutation on Human Hematopoiesis.” *Blood*, vol. 121, no. 20, 2013, pp. 4156–4165., doi:10.1182/blood-2012-05-430926.
98. Dusa, Alexandra, et al. “Substitution of Pseudokinase Domain Residue Val-617 by Large Non-Polar Amino Acids Causes Activation of JAK2.” *Journal of Biological Chemistry*, vol. 283, no. 19, 2008, pp. 12941–12948., doi:10.1074/jbc.m709302200.
99. Bumm, T. G.p., et al. “Characterization of Murine JAK2V617F-Positive Myeloproliferative Disease.” *Cancer Research*, vol. 66, no. 23, 2006, pp. 11156–11165., doi:10.1158/0008-5472.can-06-2210.
100. Akashi, Koichi, et al. “A Clonogenic Common Myeloid Progenitor That Gives Rise to All Myeloid Lineages.” *Nature*, vol. 404, no. 6774, 2000, pp. 193–197., doi:10.1038/35004599.
101. Fleischman, Angela G., et al. “TNFα Facilitates Clonal Expansion of JAK2V617F Positive Cells in Myeloproliferative Neoplasms.” *Blood*, vol. 118, no. 24, 2011, pp. 6392–6398., doi:10.1182/blood-2011-04-348144.
102. Kilpivaara, Outi, et al. “A Germline JAK2 SNP Is Associated with Predisposition to the Development of JAK2V617F-Positive Myeloproliferative Neoplasms.” *Nature Genetics*, vol. 41, no. 4, 2009, pp. 455–459., doi:10.1038/ng.342.
103. Jones, Amy V, et al. “JAK2 Haplotype Is a Major Risk Factor for the Development of Myeloproliferative Neoplasms.” *Nature Genetics*, vol. 41, no. 4, 2009, pp. 446–449., doi:10.1038/ng.334.
104. Olcaydu, Damla, et al. “A Common JAK2 Haplotype Confers Susceptibility to Myeloproliferative Neoplasms.” *Nature Genetics*, vol. 41, no. 4, 2009, pp. 450–454., doi:10.1038/ng.341.
105. Nangalia, J., et al. “Somatic CALRMutations in Myeloproliferative Neoplasms with NonmutatedJAK2.” *New England Journal of Medicine*, vol. 369, no. 25, 2013, pp. 2391–2405., doi:10.1056/nejmoa1312542.
106. Pikman, Yana, et al. “MPLW515L Is a Novel Somatic Activating Mutation in Myelofibrosis with Myeloid Metaplasia.” *PLoS Medicine*, vol. 3, no. 7, 2006, doi:10.1371/journal.pmed.0030270.
107. Oh, Stephen T., et al. “Novel Mutations in the Inhibitory Adaptor Protein LNK Drive JAK-STAT Signaling in Patients with Myeloproliferative Neoplasms.” *Blood*, vol. 116, no. 6, 2010, pp. 988–992., doi:10.1182/blood-2010-02-270108.

108. Lu, X., et al. “Expression of a Homodimeric Type I Cytokine Receptor Is Required for JAK2V617F-Mediated Transformation.” *Proceedings of the National Academy of Sciences*, vol. 102, no. 52, 2005, pp. 18962–18967., doi:10.1073/pnas.0509714102.
109. Balligand, T, et al. “Pathologic Activation of Thrombopoietin Receptor and JAK2-STAT5 Pathway by Frameshift Mutants of Mouse Calreticulin.” *Leukemia*, vol. 30, no. 8, 2016, pp. 1775–1778., doi:10.1038/leu.2016.47.
110. Chachoua, Ilyas, et al. “Thrombopoietin Receptor Activation by Myeloproliferative Neoplasm Associated Calreticulin Mutants.” *Blood*, vol. 127, no. 10, 2016, pp. 1325–1335., doi:10.1182/blood-2015-11-681932.
111. Palacios, Ronald, and Michael Steinmetz. “IL3-Dependent Mouse Clones That Express B-220 Surface Antigen, Contain Ig Genes in Germ-Line Configuration, and Generate B Lymphocytes in Vivo.” *Cell*, vol. 41, no. 3, 1985, pp. 727–734., doi:10.1016/s0092-8674(85)80053-2.
112. Daley, G. Q., and D. Baltimore. “Transformation of an Interleukin 3-Dependent Hematopoietic Cell Line by the Chronic Myelogenous Leukemia-Specific P210bcr/Abl Protein.” *Proceedings of the National Academy of Sciences*, vol. 85, no. 23, 1988, pp. 9312–9316., doi:10.1073/pnas.85.23.9312.
113. Porcellini, Simona, et al. “Regulation of Peripheral T Cell Activation by Calreticulin.” *Journal of Experimental Medicine*, vol. 203, no. 2, 2006, pp. 461–471., doi:10.1084/jem.20051519.
114. Shinkai, Y. “RAG-2-Deficient Mice Lack Mature Lymphocytes Owing to Inability to Initiate V(D)J Rearrangement.” *Cell*, vol. 68, no. 5, 1992, pp. 855–867., doi:10.1016/0092-8674(92)90029-c.