B Cell Acute Lymphoblastic Leukemia is Driven by Activating Janus Kinase Mutations Cooperating with Spi1 and Spib Deletions in a Murine Model

Michelle Lim, *The University of Western Ontario*

Supervisor: DeKoter, Rodney P., *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Microbiology and Immunology

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Abstract

B cell acute lymphoblastic leukemia (B-ALL) is caused by genetic lesions in developing B cells that function as drivers for accumulation of additional mutations in an evolutionary selection process. We investigated secondary drivers of leukemogenesis and their mechanism(s) of arising in a mouse model of B-ALL driven by PU.1/Spi-B deletion (Mb1-CreΔPB). Whole exome sequencing revealed recurrent mutations in \textit{Jak3} (encoding Janus Kinase 3) and \textit{Jak1}. Mutations with high variant allele frequency (VAF) were dominated by C\textgreater{}T transition mutations that were compatible with activation induced cytidine deaminase (AID), whereas the majority of mutations, with low VAF, were dominated by C\textgreater{}A transversions associated with 8-oxoguanine DNA damage caused by reactive oxygen species (ROS). The JAK inhibitor Ruxolitinib delayed leukemia onset, reduced ROS and ROS-induced gene expression signatures, and altered mutational signatures. These results indicate a reduction in ROS-induced DNA damage, revealing that JAK mutations can alter the course of leukemia clonal evolution through ROS-induced DNA damage.
Keywords

B cell acute lymphoblastic leukemia, B cells, leukemia, cancer, driver mutations, Janus kinase, mouse model, mouse, immunology, therapy, transcription factor, kinase inhibitors
Summary for Lay Audience

B cell acute lymphoblastic leukemia (B-ALL) is the second leading cause of death by cancer in children. This disease is associated with recurrent mutations in genes that encode transcription factors driving B cell development, such as SpiB and PU.1. We previously showed that deletion of both SpiB and PU.1 leads to 100% incidence of B-ALL in mice. However, the mice take 18 weeks to develop leukemia. Thus, we hypothesized that additional cooperating mutations are necessary for the development of B-ALL in mice. Using next-generation sequencing on mouse tumors, we determined that all mice had recurrent mutations in Jak genes. Analysis of the mutation patterns suggested that the mutations arose from overexpression of Aicda which led to reactive oxygen species-induced DNA damage. Treatment of mice with Ruxolitinib, a JAK inhibitor, doubled survival and delayed tumor growth. In conclusion, we have shown that Jaks are causal mutations and tumour progression can be delayed through JAK inhibition.
Co-Authorship Statement

The experiments found in the following thesis were designed by Rodney P. DeKoter and Michelle Lim, with kind input from Drs. Steven M. Kerfoot and Caroline Schild-Poulter. All experiments were performed by Michelle Lim, with the exception of the Jak3 growth advantage assay (Figure 12), which was conducted by Dr. Carolina R. Batista (DeKoter Lab); and the Gene Set Enrichment Analysis (Figure 19), which was conducted by Dr. Bruno R. de Oliviera (DeKoter Lab). The following work was written by Michelle Lim, and reviewed by Dr.’s Rodney P. DeKoter, Steven M. Kerfoot, and Caroline Schild-Poulter.
Acknowledgments

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Of course, these thanks are greatly extended to Dr. Rodney DeKoter whom I’ve had the pleasure to work with since the beginning of my fourth year of undergrad. I had no clue I would be sticking around for two more years after I graduated but undoubtedly it was the best decision I made. Thank you for all your mentorship, which has honed me into a better scientist. Thank you for also giving me the opportunity to attend many local and far-away conferences. Notably, CSI in Banff (my first ever flight!) and ICI in Beijing are experiences I will always cherish as they have exposed me to the great immunology community. I had the pleasure of listening to many informative and inspiring talks as well as explore different cities and countries.

I’d like to thank my advisors Dr. Steven Kerfoot and Dr. Caroline Schild-Poulter. Your engagement and feedback is what makes me excited for each committee meeting. I know that after the meetings I will be leaving with many great suggestions and advice. Thank you for pushing my project forward!

I will always remember the current and past lab members who have made my days in the lab so enjoyable. Dr. Carolina Batista, thank you for being such a great friend while I was transitioning as a nervous new lab member, and for teaching me nearly every lab skill I have today. Thank you Sherry Xu for letting me bug you with all of my technical questions. You are truly a lab wizard.

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AID</td>
<td>Activation induced cytidine deaminase</td>
</tr>
<tr>
<td>BAM</td>
<td>Binary alignment map</td>
</tr>
<tr>
<td>B-ALL</td>
<td>B cell acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>Blnk</td>
<td>B cell linker protein</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Btk</td>
<td>Bruton tyrosine kinase</td>
</tr>
<tr>
<td>ETS</td>
<td>E26-transformation-specific</td>
</tr>
<tr>
<td>FPKM</td>
<td>Fragments Per Kilobase of transcript per Million mapped reads</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GSEA</td>
<td>Gene set enrichment analysis</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
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<tr>
<td>i660BM</td>
<td>Inducible 660 bone marrow cell line</td>
</tr>
<tr>
<td>IgH</td>
<td>Immunoglobulin heavy chain</td>
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<tr>
<td>Igk</td>
<td>Immunoglobulin kappa chain</td>
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<tr>
<td>IgL</td>
<td>Immunoglobulin light chain</td>
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<tr>
<td>Ikzf1</td>
<td>Ikaros 1 (Ikaros)</td>
</tr>
<tr>
<td>Ikzf3</td>
<td>Ikaros 3 (Aiolos)</td>
</tr>
<tr>
<td>IL-7</td>
<td>Interleukin-7</td>
</tr>
<tr>
<td>IL-7R</td>
<td>Interleukin-7 receptor</td>
</tr>
<tr>
<td>Jak1/JAK1</td>
<td>Janus kinase 1</td>
</tr>
<tr>
<td>Jak3/JAK3</td>
<td>Janus kinase 3</td>
</tr>
<tr>
<td>p-STAT5</td>
<td>Phosphorylated STAT5</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>RAG-1/2</td>
<td>Recombination activating gene 1/2</td>
</tr>
<tr>
<td>RNA-seq</td>
<td>RNA sequencing</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>SDM</td>
<td>Site directed mutagenesis</td>
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<tr>
<td>SNV</td>
<td>Somatic nucleotide variant</td>
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</tr>
<tr>
<td>STAT5</td>
<td>Signal transducer and activator of transcription 5</td>
</tr>
<tr>
<td>VAF</td>
<td>Variant allele frequency</td>
</tr>
<tr>
<td>WES</td>
<td>Whole-exome sequencing</td>
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</table>
Chapter 1

1 Introduction

1.1 An overview of B cell development

Our immune system has the remarkable ability to recognize specific foreign antigens and combat infections. The immune system may be divided into aspects of innate immunity and adaptive immunity. The latter is comprised of diverse clonal populations of T and B lymphocytes, also called T and B cells respectively. The distinctive characteristic of B cells is that they are able to produce diverse antibodies specific to foreign antigens. Antibody diversity is generated early in B cell development whereby immunoglobulin light and immunoglobulin heavy (IgL, IgH) genes are rearranged by recombinase activating genes (RAG1/2) (Hozumi and Tonegawa 1976; Oettinger et al. 1990). Antibodies take on two forms: soluble antibodies are secreted for circulation through the lymphatic system to combat infections; whereas membrane antibodies are expressed on the B cell surface as a B cell receptor. The developmental stages that allow B cells to attain this unique antibody-producing capacity are as follows.

B cells originate from multipotent hematopoietic stem cells from the neonatal fetal liver, which migrate to the bone marrow at birth (Wang, Wei, and Liu 2012). Hematopoietic stem cells develop into lymphoid progenitor cells, which can follow pathways and respond to cytokines leading to the development of either B or T cells. Lymphoid progenitor cells that will develop into B cells first become pre-pro-B cells. However, these cells are not yet completely committed to the B cell lineage. Commitment is established when they become pro-B cells, where variable (V), diversity (D), and joining (J) recombination occurs within the IgH locus, which encodes for the IgH chain of B cell receptors (Wang et al. 2012). Cytokines such as interleukin-7 (IL-7) also bind to IL7 receptors (IL7R) to stimulate pro-B cell survival and proliferation through the JAK-STAT (Janus Kinase- Signal Transducer and Activator of Transcription) pathway (Harrison 2012). When VDJ recombination of the IgH chain is successful, pre-B cell receptors are expressed. Next, pro-B cells transition to large and small pre-B cells. Large
pre-B cells continue to respond to IL-7 to enable clonal expansion before migrating to areas of low IL-7 to become small pre-B cells (Clark et al. 2014). Next, small pre-B cells undergo VJ rearrangement within the IgL locus (which encodes for the IgL chain) to obtain antigen specificity and become immature B cells (Clark et al. 2014). Immature B cells that do not bind self-antigens leave the bone marrow and develop into mature naïve B cells in secondary lymphoid tissues (Wang et al. 2012). Naïve B cells reside in the bone marrow and circulate the lymph nodes and spleens, whereas activated B cells can be found at sites of infection. This developmental pathway is summarized in Figure 1.
Figure 1. Schematic of B cell development.

B cells begin as hematopoietic stem cells that originated from the neonatal fetal liver and migrated to the bone marrow. A series of developmental stages and gene rearrangement events allow the hematopoietic stem cells to become pre-pro-B, pro-B, then pre B cells. Finally, immature B cells exit the bone marrow and enter peripheral lymphoid tissues as naïve B cells.
1.2 Transcriptional control of B cell development by SpiB and PU.1

The stages of B cell development discussed above are undoubtedly a complicated process. Hence, an orchestra of transcription factors tightly regulate each event in order to prevent immunological disorders and cancers associated with impaired B cell development. One such family of transcription factors that regulate B cell development is the E26-transformation-specific (ETS) family which are characterized by their conserved ETS DNA-binding domain (Karim et al. 1990; Nunn et al. 1983) (Figure 2B). A subfamily of ETS is Spi, which consists of SpiB (encoding SpiB), SpiC (encoding SpiC), and Spi1 (encoding PU.1). Each Spi transcription factor plays important roles in myeloid and lymphoid differentiation and function. While SpiC is the least understood transcription factor of the three, SpiB and PU.1 are well characterized. SpiB and PU.1 are complementary transcription factors because they bind the same nucleotide sequence, GGAA (Figure 2A) (Solomon et al. 2015). These transcription factors operate at the small pre-B cell stage; they transcriptionally activate genes involved in B cell signaling and the rearrangement of Ig light chains such as Bruton tyrosine kinase and B cell linker protein (Christie et al. 2015; Iwasaki et al. 2005; Magnani, Eeckhoute, and Lupien 2011; Xu et al. 2012). Our lab has previously shown that when either SpiB or PU.1 are deleted in mice, B cell development is minimally affected due to the rescuing effect of the undeleted transcription factor (Batista et al. 2017). However, when both SpiB and PU.1 are deleted, severe impairment in B cell development was manifested as the virtual non-existence of mature follicular B cells in the spleen. Aberrations in SpiB and PU.1 are associated with the development of B cell acute lymphoblastic leukemia (B-ALL) in humans as repression of SpiB and mutations in PU.1 have been previously identified in patients (Fuka et al. 2011; Niebuhr et al. 2013; Zhang et al. 2011).
Figure 2. SpiB and PU.1 are complementary and highly related transcription factors.

A) Sequence logo depicting the common GGAA binding site of SpiB and PU.1. B) Schematics of the protein domains of SpiB and PU.1, highlighting their similarities as well as the common E26 transformation-specific (ETS) DNA binding domain. The protein domains indicated are acidic, proline-serine/threonine (P/S/T), proline/glutamic acid-serine/threonine (PEST), glutamine-rich (Gln), and DNA-binding ETS domains.
1.3 B cell acute lymphoblastic leukemia

Various B cell malignancies potentially result if there is disruption to the intricate and coordinated stages of B cell development. These neoplasms can arise from either precursor or mature B cells and can be further classified as either leukemias or lymphomas. Neoplasms mainly involving the blood and bone marrow are classified as leukemias whereas those confined to lymphatic tissue without the involvement of blood or bone marrow are called lymphomas. The malignancy of interest for this thesis is progenitor B cell acute lymphoblastic leukemia (B-ALL) as this disease is the most prevalent pediatric cancer and the leading cause of death by cancer in children (Pui, Robison, and Look 2008). B-ALL largely affects children younger than 6 years of age and accounts for more than 75% of all pediatric cancers. Remarkably, the 10-year survival rate for B-ALL has risen from less than 10% in 1970 to over 90% in 2004 (Hunger and Mullighan 2015) (Figure 3). However, there still exist patients who relapse due to drug resistance, or experience adverse side effects due to chemotherapy toxicity (Pui et al., 2008). Thus, exploration of alternative therapies is still needed for B-ALL.

The development of many tumors, including those of B-ALL, are subject to evolutionary processes such as natural selection (Greaves 2015). From a common precursor, multiple populations of cells arise based on genetic mutations that are naturally selected for (Ferrando and López-Otín 2017). These positively selected genetic mutations are termed drivers as they contribute to any of the hallmarks of cancer such as sustained proliferative signalling, resisting cell death, and genomic instability (Hanahan and Weinberg 2011). In contrast, genetic mutations that do not contribute to the hallmarks are termed passenger mutations for their passive role. Throughout the lifetime of the organism, additional driver mutations can develop which, together with the initiating lesions, contribute to full-blown cancer. These multiple populations of cells with varying mutations, a concept termed intratumoural heterogeneity, is a common problem in cancers as the diverse nature of tumours pose challenges to effectiveness of therapies and resistance (Hu, Sun, and Curtis 2017). As a result, heterogeneity is often a marker for poor prognosis. Given the inevitability of intratumour heterogeneity, driver mutations are important to study as they provide insight into the clonal evolutionary process(es) by which the tumor arose.
Driver mutations are validated through three methods: assessing their recurrence, detecting clusters within specific genes, and testing for functional effects (Bailey et al. 2018). The advent of next generation sequencing, particularly whole genome and whole exome sequencing (WGS, WES), has greatly aided cancer studies as the massively parallel technique reveals important mutations of many samples in a timely manner (Ma et al. 2018). Given that a large proportion of B-ALL cases arise due to congenital genetic susceptibility and additional accumulating mutations, the study of this disease through the use of WES can elucidate important driver mutations (Inaba, Greaves, and Mullighan 2013). Among the most frequently mutated genes associated as drivers for B-ALL are the *Janus kinases*, further explained in the next section.
Figure 3. Ten-year survival of pediatric B cell acute lymphoblastic leukemia patients.

Data collected from children enrolled in clinical trials under the Children’s Cancer Group and Children’s Oncology Group from 1968 to 2009. The y-axis indicates the percentage of patients from the trial that survived and the x-axis indicates the number of years that have passed since the patients’ diagnosis. Adapted from Hunger and Mullighan, 2014.
1.4 The role of *Janus kinases* in leukemia

A wide range of proteins are involved in B cell development such as the *Janus kinases* (*Jak*). The *Jak* family of kinases includes *Jak1, Jak2, Jak3*, and *Tyrosine kinase 2* (*Tyk2*) and are associated with cytokine receptors (Scott 2013). *Jak1, 2* and *3* are heavily involved in lymphoid development and cytokine-mediated signalling as they are effectors of the JAK-STAT pathway (Scott 2013). JAK proteins consist of FERM, SH2, pseudokinase, and kinase domains (*Figure 4A*) (Leonard et al. 1998). Binding of cytokines such as IL-7 to the IL-7 receptor (IL-7R) allows JAKs to autophosphorylate. JAK1 and 2 proceed to phosphorylate the alpha chain of IL-7R whereas JAK3 phosphorylates the common gamma (gc) chain. JAKs then phosphorylate tyrosine residues on STAT proteins, allowing STATs to function as transcription factors by upregulating genes responsible for immunity, proliferation, anti-apoptosis, and oncogenesis (*Figure 4B*) (Ghoreschi, Laurence, and O’Shea 2009). To prevent constitutive activation of the JAK-STAT pathway, the pseudokinase domain negatively regulates cytokine-independent activation of the kinase domain (Saharinen and Silvennoinen 2002).
Figure 4. JAK1/3 proteins and the JAK-STAT pathway activated by interleukin-7 signalling.

A) Schematic of the protein domains in JAK1 and JAK3. The protein domains indicated are 4.1 protein, ezrin, radixin, moesin (FERM), Src homology 2 (SH2), pseudokinase, and kinase. B) Overview of the JAK-STAT pathway. When cytokines such as IL-7 binds to its interleukin-7 receptor (IL-7R), JAKs begin to autophosphorylate. Next, JAK3 phosphorylates the common gamma (γc) chain of IL-7R whereas JAK1 phosphorylates the IL-7 receptor alpha (IL-7Rα chain). These phosphorylated tyrosine residues allow signal transducer and activator of transcription factor 5 (STAT5) to dock the IL-7R, allowing JAKs to phosphorylate and therefore activate STAT5. The JAK-STAT pathway is now activated and genes responsible for anti-apoptosis, proliferation, and oncogenesis are upregulated.
Mutations in \textit{Jak1} and \textit{3} have been continually implicated as primary drivers in B-ALL in humans and \textit{Mus musculus} (mouse) models. In a study conducted by Mullighan et al., pediatric B-ALL patients that had poor prognoses and were negative for \textit{BCR-ABL1} translocations were assessed for mutations (Mullighan et al. 2009). Since \textit{BCR-ABL1} is a constitutively active tyrosine kinase, exclusion of patients with this fusion protein allows for isolation of other mutations that may cause uncontrolled cell proliferation. Activating mutations in the pseudokinase and kinase domains of \textit{JAK1, 2,} and \textit{3} were found in 10.7\% of patients. Furthermore, 70\% of patients with \textit{JAK} mutations also had a deletion for the tumour suppressor \textit{IKZF1}, suggesting cooperation of different genetic lesions in leukemogenesis. Since these patients did not possess the \textit{BCR-ABL1} fusion gene, \textit{JAK} mutations were predicted to upregulate the JAK-STAT pathway, serving as drivers of B-ALL (Mullighan et al. 2009). It has also been shown that in JAK3-deficient HEK293 cells, JAK1 mutants V658F and A643D constitutively associate with IL-9R and IL-2RB to activate the JAK-STAT pathway (Hornakova et al. 2009). However, since JAK1 and 3 cross-activate during JAK-STAT activation, the absence of JAK3 resulted in inhibition of the full potential for JAK-STAT activation by JAK1 mutations alone. Hence, co-expression of the JAK1 mutants with wild type JAK3 reversed the inhibition. Recently, a new JAK1 activating mutation, S646P, has been identified in B-ALL patients (Li et al. 2017). Mutations in \textit{Jak3} alone also showed characteristics of leukemia; wild type murine hematopoietic stem cells infected with retrovirus expressing \textit{Jak3} harboring V674A mutations demonstrated a growth advantage (Losdyck et al. 2015).

Patients with pre-existing chromosomal abnormalities, such as the \textit{BCR-ABL1} or \textit{ETV6} (ETS variant 6) and \textit{RUNX1} (Runt related transcription factor 1) fusion genes, have also been found to carry \textit{Jak} mutations. Patients with either fusion showed an additional 6-8 mutations in other genes such as \textit{PAX5, JAK1, JAK2,} and \textit{JAK3} (Inaba et al. 2013; Lilljebjörn et al. 2012). Notably, the \textit{ETV6-RUNX1} fusion is the most common chromosomal defect in pediatric B-ALL (Romana, Coniat, and Berger 1994). To study the effects of \textit{ETV6/RUNX1} fusion with \textit{PAX5} mutations, whole exome sequencing was performed on \textit{ETV6/RUNX1; Pax5}\textsuperscript{+/-} mice with B-ALL (van der Weyden et al. 2015). This revealed that 35\% (6/17) of mice harbored hotspot mutations in \textit{Jak1} and 3. Specifically, the \textit{Jak1} mutations were amino acid substitutions L652F and V657F, and
the \textit{Jak3} mutations were R653H, T844M, and V670A. Except for \textit{Jak3} T844M, these mutations have corresponding human analogues. Hotspot mutations are important to study as they cluster within a certain gene with a higher frequency than expected. These results suggest that \textit{Jak 1} and 3 mutations can also serve as additional or secondary drivers. Evidently, mutations in \textit{Jaks} are recurrently observed events, suggesting their roles as drivers in B-ALL development in humans and murine models.

1.5 The role of reactive oxygen species (ROS) in leukemogenesis

Reactive oxygen species (ROS) are a group of chemical compounds that are produced when there is incomplete reduction of oxygen (D’Autréaux and Toledano 2007). Examples of ROS include superoxide anion (\(O_2^-\)), hydrogen peroxide (\(H_2O_2\)), and the hydroxyl radical (\(HO\)). This process occurs as part of normal cellular metabolic activity, such as aerobic respiration. While it is true that ROS has been shown to be toxic to cells, they also serve as important signaling molecules in crucial pathways that maintain physiological functions (Schieber and Chandel 2014). ROS homeostasis is therefore an imperative balance to maintain in order to avoid oxidative stress (i.e. high levels of cellular ROS). This balance can be achieved through upregulation of antioxidant genes which aid in oxidant scavenging. When cellular levels of ROS reach stressful levels, damage is incurred to DNA, proteins, and lipids. For this thesis, ROS-induced DNA damage, such as 8-oxoguanine, is of particular interest as this damage can result in mutations and, consequently, cancer.

Aside from ROS, there are a multitude of ways in which DNA damage can occur and lead to cancer. Backtracking the specific mutational processes leading to malignant mutations would be difficult without systematic categorization. Each cancer type displays unique patterns of mutations within specific sequence contexts. In 2013, 10,958 exomes and 1048 genomes from 40 human cancers were analyzed (Alexandrov et al. 2013). A total of 30 signatures, termed mutational signatures, were generated from cancers such as breast, lung, pancreatic, and head and neck cancers (COSMIC). This delineation of mutation patterns allows for rapid identification of the mechanistic pathway through
which the tumor developed. For example, lung tumors resulting from tobacco smoke; hepatocellular carcinomas resulting from aflatoxin B₁ exposure; or melanomas arising from UV light exposure all have distinct mutational signatures. Consequently, these signatures can influence therapeutic decisions. Unfortunately, pediatric cancers such as leukemias were underrepresented in the creation of these signatures; Signature 9 was the only one characterized from the analysis of chronic lymphocytic leukemias (CLL) and B cell lymphomas.

For this thesis, Signature 18 is of interest. Signature 18 was characterized from neuroblastomas, breast, and stomach carcinomas. Although far removed from leukemias and lymphomas, these cancers exhibit predominant C>A transversions similar to what we saw in our samples from a previous study (Batista et al. 2018). Furthermore, Signature 18 has no known etiology. There is evidence suggesting that ROS plays an important role in C>A transversions observed in cancers. It is known that the DNA base guanine has the lowest redox potential, a characteristic that allows for frequent oxidation by ROS (Kino et al. 2017). When ROS oxidizes guanine into 8-oxoguanine, the damaged form of guanine is then paired with adenine instead of cytosine. This mispairing ultimately leads to a C→A transversion after two rounds of DNA replication. Under normal circumstances, DNA repair pathways may remedy these transversions. The main DNA repair pathway in humans is base excision repair (BER), mediated by enzymes 8-oxoguanine-DNA glycosylase (OGG1) and MUTYH glycosylase (Stanczyk et al., 2011). In healthy individuals, OGG1 excises 8-oxoguanine from the DNA strand whereas MUTYH removes the wrongly paired adenine to prevent transversion (Nohmi, Kim, and Yamada 2005). However, defects in DNA repair proteins are associated with genomic instability and many leukemias and lymphomas, including B-ALL. In a study of 97 Polish patients with pediatric ALL, Ser326Cys polymorphism in OGG1 and Tyr165Cys in MUTYH were observed. These mutations were absent in healthy controls (Stanczyk et al. 2011). Similarly, 415 Chinese patients with pediatric ALL demonstrated a biased distribution of Ser326Cys compared to healthy controls (Li et al. 2011). Therefore, either an excessive level of ROS-induced 8-oxoguanine DNA damage, or a defect DNA damage repair can result in leukemogenesis. It is also possible that differentially regulated or aberrant OGG1 and/or MUTYH leads to accumulation of 8-oxoguanine.
DNA damage which contributes to genetic lesions. These genetic lesions can then serve as additional cooperative driver mutations promoting leukemogenesis.

As mentioned in previous sections, there are many cases in which the development of B-ALL is associated with Jak mutations. These mutations presumably result in an upregulated JAK-STAT pathway, which leads to excessive phosphorylated STAT5. Interestingly, it has been shown that p-STAT5 promotes ROS production by repressing antioxidant enzymes such as catalase (Bourgeais et al. 2017). Therefore, excess p-STAT5 can contribute to oxidative stress within the cell, leading to 8-oxoguanine DNA damage manifesting as Signature 18. Notably, ROS also plays a role in inactivating protein tyrosine phosphatases (PTP) (Meng, Fukada, and Tonks 2002). PTPs are enzymes critical in the dephosphorylation of JAKs, which is a process necessary for the deactivation of the JAK-STAT pathway (Rawlings 2004). As a result of ROS-mediated PTP inactivation, the JAK-STAT pathway continues to be upregulated. In summary, when Jak mutations are introduced (through unknown mechanism(s)), upregulation of the JAK-STAT pathway results in excessive ROS production, and a positive feedback loop allows for further upregulation of the JAK-STAT pathway. The mechanism by which Jak mutations occur remains unknown. In the next section, we suggest the possibility of activation-induced cytidine deaminase (AID) as a possible mechanism that causes Jak mutations.

1.6 Activation-induced cytidine deaminase (AID) as a possible mechanism for introducing Jak mutations

Activation-induced cytidine deaminase (AID, encoded by Aicda) is an APOBEC enzyme unique to B cells. AID is commonly accepted to be expressed in germinal centre B cells for the purposes of somatic hypermutation and class switch recombination (Kelsoe 2014). It functions by deaminating cytosine bases, which converts cytosine to uracil. After several rounds of DNA replication, C>T transition mutations are introduced. While these point mutations allow for antibody diversity, they can also lead to B cell pathologies such as diffuse large B cell lymphoma, follicular lymphoma, chronic lymphocytic leukemia, and acute lymphoblastic leukemia (Cerutti et al. 2002; Hardianti et al. 2005; Pasqualucci et al. 2001; Smit et al. 2003). In a study by Pettersen et al., it was discovered that B cell
lymphoma cell lines contained higher uracil content that was also correlated with upregulated AID mRNA and protein expression compared to wild type lymphocytes (Pettersen et al. 2015). After AID knockdown, uracil content was reduced. In a murine model of BCR-ABL1 B cell leukemia, AID expression has been shown to contribute to genomic instability and the acceleration of clonal evolution (Gruber et al. 2010). After AID knockout, a lower frequency of gene deletions and amplifications were observed.

Interestingly, there is emerging evidence that AID is prematurely transcribed in immature and transitional B cell subsets to contribute to self-tolerance (Kelsoe 2014). These paradigm-shifting results led us to hypothesize that perhaps AID off-target activity in immature pre or pro-B cells may be inducing the C>T mutations observed in our mouse model of B-ALL. Supporting this hypothesis are the facts that 1) Signature 18 is also characterized by an abundance of C>T transition mutations, and 2) most of the Jak mutations we observed are C>T mutations. Thus, it is possible that early AID activity is the initial mechanism by which driver Jak mutations are being introduced in the leukemias.

1.7 Current therapeutic uses of Ruxolitinib

Ruxolitinib is an FDA-approved Janus kinase inhibitor sold under the trade name Jakafi® or Jakavi®. Currently, Ruxolitinib is administered to inhibit JAK2 in polycythemia vera; JAK1/2/3 in Ph-like ALL; and JAK1/2 in rheumatoid arthritis and myelofibrosis (Raedler 2015; Roberts et al. 2014; Roskoski 2016; Verstovsek et al. 2010b). Ruxolitinib functions as a competitive inhibitor as it mimics the structure of ATP, the small molecule that donates its phosphate group to allow for phosphorylation of proteins (Mesa, Yasothan, and Kirkpatrick 2012). This inhibition significantly reduces the overly active JAK-STAT signalling that is suspected to contribute to the aforementioned pathologies. Interestingly, patients with myelofibrosis taking Ruxolitinib demonstrated reduced splenomegaly regardless of whether they harbored any JAK mutations (Quintás-Cardama et al. 2010; Verstovsek et al. 2010a). These results suggest that dysregulation of the JAK-STAT pathway is fundamental to the pathogenesis of myelofibrosis. The effectiveness of Ruxolitinib for the treatment of B-ALL has not yet been established. Therefore, we hope
that our mice with *Jak* mutations will benefit from Ruxolitinib, also demonstrating that the JAK-STAT pathway is causative of B-ALL in our mouse model.

### 1.8 Rationale and central hypothesis

Our lab previously demonstrated that deletion of both SpiB and PU.1 in mice impaired pre-B cell maturation (Batista et al. 2017). Notably, these double knockout mice also exhibit a 100% incidence of B cell acute lymphoblastic leukemia requiring a median time to euthanasia of 18 weeks of age (Batista et al. 2018). This 18-week latency suggests that multiple unknown driver mutations cooperate in addition to the initial SpiB and PU.1 knockouts to drive leukemogenesis. While *Jak3* was recently identified as a driver mutation contributing selective proliferative advantages, the remaining contributors to B-ALL and their mechanisms of arrival remains unknown (Batista et al. 2018).

*We hypothesize that, in cooperation with the initial SpiB and PU.1 deletion, there exists additional driver mutations in Jak 1 and 3 necessary for the development of B-ALL in mice lacking SpiB and PU.1.*

To achieve the above hypothesis, the following aims are proposed:

I. Identify recurrent mutations that are potentially driving B-ALL development in the absence of SpiB and PU.1 through the use of whole exome sequencing and RNA sequencing.

II. Demonstrate that the identified recurrent *Jak* mutations are drivers for B-ALL using growth advantage assays.

III. Determine if 8-oxoguanine DNA damage and/or activation-induced cytidine deaminase are potential mechanisms by which *Jak* mutations arise.

IV. Assess JAK inhibition as a potential therapy for delaying or preventing B-ALL *in vivo* by using Ruxolitinib feeding experiments.
Chapter 2

2 Materials and Methods

2.1 Breeding of mice

C57BL/6 mice were purchased from Charles River Laboratories (Saint- Constant, QC, Canada). Mb1∆PB mice were generated by crossing Mb1-Cre mice with Spi1lox/loxSpib−/− mice. Experimental controls were Mb1+/− Spi1+/− Spib−/− (ΔB). All mice were housed in a 12-hour light/dark cycle and fed regular chow and water ad libidum. Upon signs of severe illness, mice were euthanized in fulfilment of ethical standards provided by the Western University Council on Animal Care.

2.2 Whole exome sequencing

DNA was isolated from the thymus of five sick Mb1∆PB mice using Wizard Genomic DNA Purification Kit (Promega, Madison, WI). DNA from the tails of the same mice were also isolated for use as matched controls. Sequencing was performed by Genome Quebec Innovation Centre using SureSelectXT Mouse All Exon Kit (Agilent Technologies, Mississauga, Canada) for exome target capture, SureSelectXT Target Enrichment System to produce the paired-end DNA library, and Illumina HiSeq4000 (Illumina, San Diego, CA) for exome sequencing. Generated BAM files had adaptor sequences trimmed using TrimGalore! and aligned to the reference mm10 genome using Bowtie packages found on usegalaxy.org.

2.3 Somatic variant calling

To identify single nucleotide variants (SNVs) in the sequenced thymic tumor DNA, the processed files were subjected to mutation callers Strelka, FreeBayes, and VarScan2. Each variant caller was performed using standard settings. Only the generated VCF files denoted as “passed” proceeded in the pipeline. Annotation was performed on these files using SnpEffect. From these results, the frequency of variants (termed variant allele frequencies, VAF) were calculated.
2.4 RNA sequencing

RNA was isolated from the thymus of the same five mice using RNeasy Mini Kit (Qiagen, Hilden, Germany). Samples were sequenced by Genome Quebec Innovation Centre using Truseq Stranded Total RNA Library Prep Kit and Illumina HiSeq4000 PE100 (Illumina, San Diego, CA). Generated BAM files were trimmed using TrimGalore!, aligned to mm10 using TopHat2, and tested for differential expression using Cufflinks packages. All packages are found on usegalaxy.org. Differential gene expressions identified by Cufflinks are represented by fragments per kilobase of transcript per million mapped reads (FPKM).

2.5 Identifying genes with high impact mutations

Using the VAF and FPKM obtained from whole exome sequencing and RNA sequencing, respectively, RStudio was used to generate a VAF vs. FPKM plot for each leukemia. This plot allowed identification of genes that were both frequently mutated (high VAF) and highly expressed (high FPKM). Focusing on genes that have a high VAF and FPKM allowed us to exclusively study high impact mutations.

2.6 Mutational signatures

Since it has been shown that cancers possess 1 of 30 mutational signatures, we defined our tumors’ signatures using deconstructSigs on RStudio (Alexandrov et al. 2013; Gehring et al. 2015; Saunders et al. 2012). Defining the “fingerprint” of our tumors allowed us to begin elucidating the mechanisms by which these spontaneous genetic lesions arise after deletion of SpiB and PU.1.

2.7 Plasmid generation

The MSCV-Jak1 (WT) and MSCV-Jak1V657F(V657F) retroviral vector plasmids were a generous gift from Dr. Charles G. Mullighan (St. Jude’s Children Research Hospital, Memphis, TN). Plasmids were transformed into DH5α E. coli cells and recovered with a mini-prep kit (Geneaid Biotech, New Taipei City, Taiwan). Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA) and primer pairs were used to introduce the V655L mutation into wild type Jak1 (Table 1). The mutated plasmids were
then recovered with a maxi-prep kit (Geneaid Biotech, New Taipei City, Taiwan) after confirmation of the correct mutations by Sanger sequencing and alignment with MacVector 14.0 (Table 2). The maxi-prepped plasmids were confirmed to be MSCV-\textit{Jak1} by BglII digestion which followed the protocol provided by New England BioLabs (Ipswich, MA).
**Table 1.** Primer pair used for site-directed mutagenesis. Red lowercase letters indicate the introduced mutation.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>DNA sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>V655L SDM</strong></td>
<td>F  CCTCTACGG[ct]GTGTGTCCG</td>
</tr>
<tr>
<td></td>
<td>R  TACACTATATGTTTGTGGAAACC</td>
</tr>
</tbody>
</table>

**Table 2.** Primer pair used for Sanger sequencing of the mutated plasmid.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>DNA sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Jak1 Sanger</strong></td>
<td>F  TATTCTGGGACCCTGCTGGA</td>
</tr>
<tr>
<td></td>
<td>R  CGGGCCAGAAGGAGGTTTTT</td>
</tr>
</tbody>
</table>
2.8 Retroviral transfection

Mutated plasmids were transfected into Platinum-E (Plat-E) cells to produce retroviruses. To prepare for transfection, Plat-E cells were cultured in low glucose Dulbecco’s Modified Eagle’s Medium (DMEM) with blasticidin (1 μg/mL) and puromycin (1 μg/mL). The presence of antibiotics ensures that only Plat-E cells with packaging plasmids are cultured as they encode for blasticidin and puromycin resistance. Upon near-confluent growth, the cells were passaged in antibiotic-free DMEM. The cells were transfected with 5 μg each of wild type or mutated plasmids using PolyJet (SignaGen Laboratories, Rockville, MD). Fluorescence-activated cell sorting (FACS) analysis of green fluorescent protein (GFP) expression measured transfection efficiency (BD Biosciences, San Jose, CA). FACS results were analyzed with FlowJo (FlowJo LLC, Ashland, OR). Ultracentrifugation with Amicon Ultra Centrifugal Filters (Sigma Aldrich, St. Louis, MO) were used to concentrate the viral supernatant. The supernatant was then collected and frozen. NIH3T3 fibroblasts were infected to measure viral titre.

2.9 Retroviral infection

To infect 38B9 pro-B cells, 1 million cells were spin-infected with 1 million particles of the appropriate retroviruses and 4 μg/mL of polybrene for 3 hours at 3000 rpm and 32°C. The addition of polybrene reduces repulsive charges between the retroviruses and cell membrane. The cells were cultured for 48 hours in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 1X penicillin/streptomycin/L-glutamine, and 5 x 10^-5 M b-mercaptoethanol to allow optimal proliferation and infection. The cells were then sorted for GFP+ cells in the Robarts Flow Cytometry Facility.

2.10 Western blot for Imatinib-treated cells

To inhibit Abl kinase in 38B9 cells, 10 μM of Imatinib (TCI America, Portland, OR) was added to the cell culture for 16 hours. Whole cell lysates were created by suspending cells in lysis buffer treated with 1x protease inhibitor cocktail at a concentration of 10 uL/10^6 cells (Thermo Fisher, Waltham, MA). Lysates were then denatured on a 100°C heat block for 15 minutes. Laemmlli buffer containing 10% β-mercaptoethanol was added. Proteins were separated by electrophoresis and transferred to a nitrocellulose membrane for 1 hour
at 15V on a semi-dry transfer cell (Bio-Rad, Hercules, CA). The nitrocellulose membranes were blocked with 5% skimmed milk in 1× TBST for 1 hour at room temperature. Nitrocellulose membranes were then incubated with 1:1000 anti-STAT5 or 1:1000 anti-p-STAT5 and 1:5000 anti-actin antibodies overnight at 4°C (Cell Signalling Technology, Danvers, MA). After washing with 1× TBST, the membranes were incubated for 1 h at room temperature with a 1:20000 dilution of goat anti-rabbit IgG (Thermo Fisher). Imaging was performed on the ChemiDoc XRS+ imager in Dr. Shawn Li’s lab (BioRad). Quantification of bands was performed using Image Lab (BioRad).

### 2.11 Heatmap generation

The eligibility of pro-oxidant and antioxidant genes for heatmap analysis was determined based on genes that were expressed to an appreciable degree in all three cell types (FPKM > 0.5) and were differing in expression greater than 1.3 fold (highest FPKM / lowest FPKM). Heat maps were generated using Morpheus (https://software.broadinstitute.org/morpheus/).

### 2.12 Staining for 8-oxoguanine DNA damage

Previously established leukemic cell lines obtained from thymuses of Mb1ΔPB mice were cytospun for five minutes at 720 rpm onto slides (200,000 cells per slide). Cells were fixed for 30 minutes using 4% paraformaldehyde and rinsed with 0.3% Tripton X-100. After blocking for 1 hour, RNA was depleted using 0.2 mg/mL RNase solution. Nuclear DNA was denatured using 2M hydrochloric acid for 10 minutes. The cell spots were immersed in 200 μL blocking solution containing 5% goat serum (Southern Biotech, Birmingham, AL, USA), 0.5% Fc block (BD Biosciences) and 0.1% Triton X-100. A 1:50 dilution of anti-oxoguanine-8 antibody (Abcam, Cambridge, United Kingdom) was used to stain the cells overnight at 4°C. A 1:500 dilution of Alexa Fluor 647-conjugated IgG anti-biotin antibody (Jackson ImmunoResearch Laboratories, West Grovem PA) was used to incubate cells for 1 hour at room temperature in the dark. Slides were mounted with Fluoromount-G with DAPI (ThermoFisher Scientific, Waltham, MA) and imaged using the Leica Widefield Microscope (Leica, Wetzlar, Germany).
2.13 Sorting the frequency of C>A and C>T mutations according to VAF bins

The VAF from all eight leukemia samples were binned according to VAF: 0-0.1, 0.1-0.2, 0.2-0.3, 0.3-0.4, 0.4-0.5, >0.5. Percentage of C>A and C>T mutations in each bin were calculated and plotted.

2.14 Analysis of trinucleotide motifs of C>T mutations

A text file containing all C>T mutations were uploaded to usegalaxy.org. Using the Get Flanks program and its default settings, 15 bases preceding the C>T mutation was extracted. These files were converted to FASTA format using the bedtools GetFastaBed program. Microsoft Excel was used to calculate the frequency of each possible trinucleotide motif ending with C.

2.15 Fractionating bone marrow cells for quantitative PCR (qPCR)

Fresh bone marrow cells were harvested from mice that were WT, ΔB, or ΔPB using a previously described method (Amend, Valkenburg, and Pienta 2016). The cells were then stained with V450-B220 (BD Biosciences, Franklin Lake, NJ), Biotin-CD43 (BD Biosciences), Streptavidin-FITC (eBiosciences, San Diego, CA), PE-BP1 (BD Biosciences), APC-CD24 (BioLegend, San Diego, CA), and PE-Cy7-IgM (BioLegend) at a concentration of 1 µL/10⁶ cells in 100 µL stain volume for 20 minutes on ice. Stained cells were then sorted for Fractions A, B, C, and D at the London Regional Flow Cytometry Facility. Total RNA was extracted from fractionated bone marrow cells using RNeasy Mini Kit (Qiagen). cDNA synthesis was performed using iScript cDNA Synthesis Kit (BioRad, Missisauga, ON) followed by qPCR analysis using QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific, Rochester, NY). All qPCR reactions were completed in triplicates using SensiFAST SYBR No-ROX Kit (Bioline, Singapore). To analyze qPCR results, expression of genes of interest were normalized to expression of Tata-binding protein (Tbp). These relative expressions were then calculated as a fold change using the 2^(-ΔΔCT) method (Pfaffl 2004). Primers used for qPCR are indicated in Table 3.
### Table 3. Primer sequences used for qPCR.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>DNA sequence (5’ ⇒ 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aicda Mm.PT.58.42247522</em></td>
<td>F  CTCCTGCTCACTGGACTTC</td>
</tr>
<tr>
<td></td>
<td>R  GTCTGAGATGTAGCGTAGGAAC</td>
</tr>
<tr>
<td><em>Tbp qPCR</em></td>
<td>F  ACCGTGAATCTTTGGCTGTAAAC</td>
</tr>
<tr>
<td></td>
<td>R  GCAGCAAATCGCTTGGGATTA</td>
</tr>
</tbody>
</table>
2.16 Western blot of Ruxolitinib-treated cells

Wild-type, bone marrow, and untreated 973 cells were cultured in Iscove’s Modified Dulbecco’s Media (IMDM) with 10% fetal bovine serum (FBS), 1X penicillin/streptomycin/L-glutamine, 5 x 10^{-5} M b-mercaptoethanol, and 5% of IL-7 conditioned media produced from J558-IL-7 cell line (Winkler, Melchers, and Rolink 1995). To treat 973 cells, 75 nM of Ruxolitinib was added to the cell culture and incubated for 3 days. Whole cell lysates were created using Laemmli buffer as described in Section 2.10. Lysates were blotted and probed with anti-STAT5 and anti-p-STAT5 antibodies (Cell Signalling Technology, Danvers, MA). Imaging was performed on the ChemiDoc XRS+ imager in Dr. Shawn Li’s lab (BioRad, Hercules, CA).

2.17 Feeding schedule

Novartis/Incyte provided us with 1 kg each of Ruxolitinib (2000 mg/kg) and drug-free vehicle chow (Incyte, Wilmington, DE). After weaning, 4-week old Mb1ΔPB littermates were individually housed and randomly assigned to the control or experimental group. Each mouse was provided with 10 g of Ruxolitinib or vehicle chow every two days. Every other day, the remaining chow’s weight was recorded and topped up again to 10 g per mouse. After 30 days, all mice were fed regular chow.

2.18 Monitoring health

Mice were weighed every other day and monitored for significant changes. In addition to weight loss, mice were monitored for signs of sickness including lethargy, labored breathing, and/or piloerection. Mice displaying signs of illness were euthanized in fulfillment of ethical standards provided by the Western University Council on Animal Care.

2.19 Post-mortem experiments

After euthanasia, thymic leukemias were harvested and cells were isolated. Similarly to 2.2-4, DNA from leukemias and matched tails were extracted using Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin). RNA was extracted from the
same leukemias using QIAGEN RNeasy (Qiagen, Hilden, Germany). Samples were sent to Genome Quebec for whole exome sequencing and RNA sequencing.

2.20 Statistical analysis

All data reported in this thesis were graphed as mean ± SEM. Statistical analyses were conducted on Prism 7.0 (Graphpad Software, La Jolla, CA) using Student’s t-test, ANOVA, Kruskal-Wallis test, or Log-rank (Mantel-Cox) test. P values ≤ 0.05 were considered statistically significant. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****).
Chapter 3

3 Results

3.1 *Janus kinase 1* and 3 have a high mutation frequency and are also highly expressed

In our recent publication, we identified *Jak1* and 3 mutations in three leukemias that were subjected to WES (Batista et al. 2018). Since the publication, we have sequenced five additional leukemias, totalling to eight samples. All DNA datasets were processed through our pipeline, shown in Figure 5. We chose to compare the tumor DNA to its matched tail DNA control instead of a standard wild-type mouse tail DNA to standardize for any genetic variability between the mice. Although the genetic background of all our mice is C57BL/6, we have unexpectedly identified single nucleotide variants (SNVs) among our mice by comparing tail DNA from two different mice (Figure 6). This result was likely because our mice are not fully inbred. Variants were identified using the stringent mutation caller Strelka.

With the exception of sample 932, the same samples were also subjected to RNA-sequencing. Sample 932 failed quality control necessary for RNA-sequencing and was therefore replaced by sample 933. RNA isolated from the eight leukemias were sequenced by Genome Quebec and the resulting BAM files were processed on usegalaxy.org in the following pipeline: convert file to FASTQ using BAM to FASTQ, trim adaptors using TrimGalore!, align to the reference genome mm10 using TopHat2, identify differential gene expression using Cufflinks. The result are measures of gene expression in the units of Fragments Per Kilobase of transcript per Million mapped reads (FPKM). Using RStudio, dot plots of gene expression (FPKM) vs. mutation frequency (VAF) were generated for each leukemia to visually identify highly frequent SNVs (Figure 8). Genes that were frequently mutated (high VAF) were labelled. We observed that 5/8 and 2/8 leukemias harboured high-frequency SNVs in *Jak3* and *Jak1*, respectively. We were curious to determine if the number of mutations identified in our mouse model were similar to other leukemia/lymphoma models. We observed 1479 and
960 variants from data in Dang, et al. and Dudgeon, et al., respectively (Figure 7). Additionally, both data exhibit a similar trend of increasing variants at low VAF.

To identify mutations that would be expected to confer high-impact functional effects on the leukemias, we filtered the list of SNVs for missense, splice region, stop gain, and start loss amino acid mutations (MSSS) as well as a VAF > 0.1. Genes with VAF <0.1 are most likely the result of sequencing artefacts (Cai et al. 2016). The 5/8 and 2/8 leukemias that harbored Jak3/1 mutations persisted after MSSS and VAF >0.1 filters (Table 4). Given that no other gene was mutated to this frequency, the Jaks became the focus of this Master’s degree.
Figure 5. Pipeline used for the processing and analysis of whole exome sequencing data.

Flowchart showing each processing step performed for whole exome sequencing analysis. **Processing:** The pipeline begins with raw binary alignment map (BAM) files produced from the whole exome sequencing reaction. Raw BAM files are converted to a text file called FASTQ. The adaptor sequences attached to the FASTQ files are trimmed. Trimmed files are then ready to be mapped to the reference genome, *Mus musculus* GRCm38 (mm10), after which they become sequence alignment/map (SAM) files. Aligned files are finally converted back to BAM format before use in variant caller programs. **Variant calling:** To execute Strelka and FreeBayes, paths to leukemic and control BAM files are indicated by command line. VarScan requires an additional step of converting BAM files to a pileup (mpileup) format before executing VarScan. Finally, output files are annotated and extracted into a text file format (.txt). Specific programs used for this thesis are listed beside arrows. Somatic variant callers are highlighted in peach.
Raw BAM files
  ↓ Bedtools' BAM to FASTQ
  BAM -->
  FASTQ
  ↓ TrimGalore!
  Trim adaptors
  ↓ BowTie for Illumina
  Map to mm10 reference genome
  ↓ SamTools' SAM to BAM
  SAM -->
  BAM
  ↓ SamTools' mpileup
  Strelka
  (stringent)
  ↓ BAM -->
  mpileup
  FreeBayes
  (sensitive)
  ↓ VarScan
  (sensitive)
  ↓ SnpEff
  Annotate variants (.vcf)
  ↓ SnpSift
  Extract fields (.txt)
Figure 6. Number of SNVs identified for different Variant Allele Frequencies (VAF) when comparing tail-tail or tail-leukemia exomes.

The number of genes identified at a continuum of variant allele frequencies was counted and plotted. The y-axis indicates the variant allele frequency (VAF) and the x-axis indicates the number of genes found with a particular VAF (Count). Full counts of variants (Unfiltered) as well as variants filtered for missense, splice region, stop loss, and start gain mutations (MSSS) are shown. A) Gene variants identified by comparing exome data from the tail of mouse 853 to the exome data from the tail of mouse 857 (top). Gene variants identified by comparing exome data from the leukemia and tail of mouse 853 (bottom). B) Gene variants identified by comparing exome data from the tail of mouse 856 to the exome data from the tail of mouse 406 (top). Gene variants identified by comparing exome data from the leukemia and tail of mouse 856 (bottom).
Figure 7. Number of variants identified with different Variant Allele Frequencies (VAF) when comparing control-leukemia exomes from literature.

Whole exome data from two datasets obtained from literature was processed using our pipeline shown in Figure 5. From the processed files, the number of genes identified at a continuum of variant allele frequencies was counted and plotted. The y-axis indicates the variant allele frequency (VAF) and the x-axis indicates the number of genes found with a particular VAF (Count). Full counts of variants (Unfiltered) as well as variants filtered for missense, splice region, stop loss, and start gain mutations (MSSS) are shown. Gene variants identified by comparing exome data from the tail and tumour of mice with A) AMKL (Dang et al., 2017) and B) p53 knockouts (Dudgeon et al., 2014).
Figure 8. Integration of data from whole exome sequencing and RNA-sequencing for eight leukemias.

Variant allele frequencies as well as expression levels of genes from eight leukemias were intersected and visualized onto a dot plot. The y-axis indicates the expression level measured as fragments per kilobase of transcripts per million mapped reads (FPKM, obtained from RNA-sequencing) and the x-axis indicates the variant allele frequency (VAF, obtained from whole exome sequencing). Genes with somatic nucleotide variants that are high in frequency (high VAF) are labelled.
Table 4. SNVs identified in *Jak3* and *Jak1* that remained after filtration for mutations that were high impact.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mouse</th>
<th>VAF</th>
<th>FPKM</th>
<th>Position</th>
<th>Base change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Jak3</em></td>
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High impact mutations are defined as those that have a VAF >0.10 and are either missense, splice region, start loss, or stop gain mutations (MSSS).
3.2 Additional variant callers VarScan and FreeBayes agree with Strelka

There are a wide variety of variant callers available with different sensitivities and positive predictive values (Sandmann et al. 2017). To confirm that bona fide mutations were identified by Strelka, we reanalyzed our whole exome data using two more distinct callers: VarScan and FreeBayes. For all variant callers, the results were filtered for MSSS mutations and a VAF >0.1. Strelka, VarScan, and FreeBayes identified 75, 281, and 889 SNVs per exome on average, respectively. Evidently, Strelka demonstrated the greatest stringency for identifying mutations whereas FreeBayes demonstrated the highest sensitivity. VarScan was able to identify all Jak3 and Jak1 SNVs identified by Strelka. However, FreeBayes could not identify one Jak3 mutation in leukemia 857: V670A (Table 5). Given that the high sensitivity of FreeBayes should be able to call high-frequency SNVs, we excluded that mutation from our study. These results confirm that all mutations we are studying in Table 4, save for one, are bona fide. A summary of the commonly mutated genes identified by the three variant callers are shown in Figure 9. The location of these mutations in the context of protein domains are illustrated in Figure 10.

Although VAF is one of the most common metrics of mutation frequencies, it does not account for tumour purity. Consequently, the VAF measurement may be confounded by other non-cancerous cell types within the tumour. To circumvent this issue, we recalculated VAF as a cancer cell fraction, which is defined by the equation

$$CCF = VAF \times \frac{1}{purity} \times (CN \times purity + 2(1-purity))$$

CCF takes into account copy number variants as well as the fraction of cancer cells within the sample. This ensures that the VAFs reported are true to the percentage of cancerous cells found in the thymic leukemias.
Gene variants in each leukemia sample were obtained from the variant callers Strelka, VarScan, and FreeBayes and then intersected to find variants called by all three algorithms. The resulting variants from each leukemia were then intersected to determine common gene variants among the samples. A) \textit{Jak3} is the only gene that intersects five leukemias whereas B) \textit{Jak1} is the only gene intersecting two leukemias. The numbers outside of colored shapes indicate the leukemia sample. The numbers inside colored shapes indicate the number of variants called by all three variant callers.
Table 5. *Jak3* and *Jak1* mutations identified by Strelka, VarScan, and FreeBayes.

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<th>CCF (VarScan)</th>
<th>CCF (FreeBayes)</th>
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<th>Location</th>
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</table>

The one mutation failed to be recognized by FreeBayes is highlighted in red.
Figure 10. Schematic of the JAK1 and JAK3 protein domains with the identified mutations labelled.

Locations of the mutations identified by all three variant callers are displayed as amino acid substitutions. The protein domains indicated are 4.1 protein, ezrin, radixin, moesin (FERM), Src homology 2 (SH2), pseudokinase, and kinase.
3.3 Mutational signatures

Using DeconstructSigs, mutational signature analyses were conducted on the mutations identified by Strelka, VarScan, and FreeBayes. Both Strelka and VarScan identified mutations that represent signatures 24 and 18 (Figure 11). Signatures 24 and 18 are defined by a majority of C>A mutations as well as the presence of C>T mutations. These agreements between Strelka and VarScan indicate that the mutations in our genes of interest and the reoccurrence of C>A mutations are not artefacts. On the other hand, FreeBayes identified mutations that represent signature 3. Signature 3 is characterized by an even distribution of mutations across all trinucleotide contexts. This non-specific distribution of mutations is likely due to the high sensitivity of FreeBayes identifying every type of mutation across all contexts.
The DeconstructSigs package was used on RStudio to determine the mutational signature pattern of each leukemia sample. The y-axis indicates the fraction of mutations and the x-axis indicates each of 96 trinucleotide contexts. The weighted combination of signatures are shown below each graph. **A)** Mutational signature analysis on SNVs identified by Strelka. **B)** Mutational signature analysis was repeated on each leukemia’s SNVs identified by Strelka, VarScan, and FreeBayes (data not shown). The signatures identified are summarized in pie charts along with their proposed or known aetiologies. ROS, reactive oxygen species; NER, nucleotide excision repair; DNA pol η, DNA polymerase η; BRCA1/2, breast cancer gene 1/2.
3.4 Identified Jak3 mutants confer a growth advantage to progenitor B cells

Our lab has previously shown that pro-B cells infected with mutated Jak3 demonstrated a clear growth advantage over a period of 14 days in vitro (Figure 12) (Batista et al. 2018). We then sought to demonstrate a growth advantage conferred by Jak1 mutants in a similar manner. The MSCV-Jak1 WT and V657F plasmids were a generous gift from Dr. Charles Mullighan. Using the MSCV-Jak1 WT plasmids, site-directed mutagenesis was performed to create the remaining Jak1 mutant, V655L. Restriction enzyme digestion followed by gel electrophoresis demonstrated that each plasmid was of the expected size (Figure 13A). Additionally, the Jak1 mutations were confirmed by Sanger sequencing (Figure 13B).

Our attempts to produce Jak1 retroviruses were unsuccessful due to low viral titres. To address this issue, ten transfections were performed per plasmid. Ultracentrifugation with Amicon Ultra Centrifugal Filters were used to concentrate the viruses to less than 4 mL each. The resulting viral titres for Jak1 WT, V655L, and V657F, respectively, were (in viral particles/mL): 808,000; 2,448,000; and 968,000. We attempted to infect WT pro-B cells using these high-titre solutions, but the infection efficiency of the Jak1 viruses remained less than 1%. Since retroviruses have poor infection frequencies unless cells are rapidly proliferating, we circumvented this issue by infecting a rapidly proliferating pro-B cell line called 38B9 cells. However, the infection efficiency was low again (Figure 13C). Since 38B9 cells grow very rapidly, a growth curve-based assay would not properly demonstrate proliferative advantages. Thus, we sorted the GFP+ cells and expanded them in culture. We decided to perform Western blots to detect phosphorylated STAT5 in cells cultured in varying concentrations of IL-7 to determine if the Jak1 mutants upregulated the JAK-STAT pathway (Figure 14A). However, strong signals for p-STAT5 occurred even for the uninfected cells. This indicated that STAT5 is highly phosphorylated in 38B9 cells. To eliminate these background p-STAT5 levels, the same cells were cultured under the same conditions but with the addition of 10 µM of Imatinib, an abl kinase inhibitor (Figure 14B). We were able to demonstrate that cells cultured
without IL-7 failed to show p-STAT5. In this way, the use of Imatinib ensures that any p-STAT5 detected are due to JAKs which would indirectly demonstrate growth advantages. Since 0.5% and 5% IL-7 conditioned medium did not lead to differences in p-STAT5 between the uninfected and infected cells, we could not conclude that Jak1 mutants confer an increase in p-STAT5 in pro-B cells.
Figure 12. Growth advantage assays of pro-B cells infected with R653H, T844M, and V670A.

Uninfected pro-B cells and cells infected with the empty MSCV backbone (MSCV-empty), WT Jak3 (MSCV-Jak3), Jak3 R653H (MSCV-R653H), Jak3 T844M (MSCV-T844M), or Jak3 V670A (MSCV-V670A) were cultured in 0.5% IL-7 conditioned media over a period of 14 days. The percentage of GFP+ cells were measured every two days. The y-axis indicates percentage of GFP+ cells and the x-axis indicates time in days. Data represents mean ± SEM. n = 3, repeated measures ANOVA.
Figure 13. Preparation of Jak1 retroviruses.

A) Gel electrophoresis of MSCV, Jak1 WT, V655L, and V657F plasmids digested with BglII (lanes 1-4) or undigested (lanes 6-9) on a 0.5% agarose gel. B) Alignment of wild type and mutated (red) MSCV-Jak1 sequences confirming the induced mutation, V655L, with its chromatogram. The valine codon is highlighted and the nucleotide targeted for mutation is bolded in red. C) Flow cytometry of GFP expressed in 38B9 pro-B cells infected with the indicated viruses. The y-axis indicates forward scatter and the x-axis indicates the GFP emission using the 530/30 emission filter.
Figure 14. Establishing a growth advantage assay for Jak1 using Western blotting and c-Abl kinase inhibition.

Expression of STAT5 and p-STAT5 in 38B9 cells infected with WT Jak1 (WT), Jak1 V655L (V655L or VL), or Jak1 V657F (V657F or VF) retrovirus. A) STAT5 and p-STAT5 expression in 38B9 cells that were uninfected or infected with WT Jak1, Jak1 V655L, or Jak1 V657F retrovirus. All cells were cultured in media without IL-7. Actin is used as a loading control. B) STAT5 and p-STAT5 expression in 38B9 cells uninfected or infected with the indicated retroviruses and cultured in 10 uM of Imatinib. The cells were cultured in 0.5% and 5% of IL-7. Actin is used as a loading control.
3.5 Cultured leukemic pro-B cells have greater levels of 8-oxoguanine DNA damage than cultured wild type pro-B cells as a result of reduced antioxidant response

Mutational signature analyses revealed that our tumor samples have a mutational pattern compatible with Signature 18, characterized by a predominance of C>A transversions. This transversion commonly arises as a result of ROS-induced 8-oxoguanine DNA damage (Ma et al. 2018). Recognizing this, fourth year honours thesis student Jacob Ferguson, under my supervision, analyzed the gene expression levels of several pro-oxidant and antioxidant genes in cultured leukemia cells to identify imbalances in redox homeostasis (Appendix A, Tables A1, A2). The cell lines used were Mb1ΔPB cells derived from eight leukemic mice as described above (numbered 1-8), as well as the following previously described preleukemic cell lines (Christie et al. 2015): three Mb1ΔPB cell lines induced for Spi1 expression by doxycycline (Spi-1 induced) and three Mb1ΔPB cell lines not induced with doxycycline (uninduced). The cell lines were subjected to RNA-sequencing as described in Chapter 2: Methods. Briefly, the RNA-sequencing BAM files were processed on usegalaxy.org using TrimGalore! to trim adaptors, TopHat2 for aligning to mm10 reference genome, and Cufflinks to identify differential gene expression. The result are measures of gene expression in the units of Fragments Per Kilobase of transcript per Million mapped reads (FPKM). While no pro-oxidant genes were eligible for heatmap analysis according to criteria described in Methods, 29 antioxidant genes remained eligible (Appendix A, Tables A1, A2). Many (16/29) selected antioxidant genes were expressed at lower levels in leukemic cells compared to pre-leukemic bone marrow cells that were either uninduced or induced for PU.1 expression (Appendix A, Figure A1).

Due to the abundance of C>A mutations as well as a reduction in antioxidant gene expression, we speculated that ROS-mediated DNA damage may be a mechanism by which these C>A mutations arise. To test this hypothesis, we used an anti-8-oxoguanine antibody to stain wild type and leukemic pro-B cells isolated from various Mb1ΔPB mice. Leukemic pro-B cells exhibited 3.4 times greater amounts of 8-oxoguanine-induced...
fluorescence compared to wild type pro-B cells (leukemic, n = 57; wild type, n = 69; p = 0.0002) (Appendix A, Figure A2).

3.6 Irregular activity of AID may be initiating secondary driver mutations in Jaks

While ROS seem to play a significant role in our leukemia model and the observed C>A transversions, further analysis of the mutations in the thymic leukemias revealed a shift in dominating mutations as a function of variant allele frequencies (VAF). Mutations with a low VAF are predominantly C>A transversions whereas mutations with a high VAF are mainly C>T transitions (Figure 15A). This shift in mutation type occurs at a VAF of 0.3-0.4. To explore this interesting trend of dominating mutation types, the trinucleotide context of every C>T mutation (XXC) observed in all eight thymic tumors were extracted and counted as a fraction. This was repeated for C>T mutations with VAF >0.01, >0.1, >0.2, and >0.3. Remarkably, we found that C>T mutations at higher VAF (i.e. >0.2 and 0.3) were compatible with motifs recognized by AID whereas those at lower VAF were not (Figure 15B). Specifically, the motifs preferentially targeted by AID were trinucleotides WRC, where W can be adenine or thymine, and R can be adenine or guanine.

Next, we sought to determine the level of Aicda expression in fractionated bone marrow of mice that were wild type (WT), lacking expression of SpiB (∆B), and lacking expression of both SpiB and Spi1 (∆PB) using RT-qPCR. This experiment would determine whether the knockout of SpiB and PU.1 affected AID expression at specific immature B cell subsets. We found that ∆PB mice highly expressed Aicda across all fractions of B cell development compared to WT or ∆B mice, significantly for Fraction B cells (Figure 15C). This suggests that AID activity plays a role in clonal evolution of leukemia in our mouse model.
Figure 15. Emerging evidence that activation-induced cytidine deaminases may be responsible for the mutations observed in thymic leukemias.

A) A shifting dominance of C>A to C>T mutations as VAF increases. The y-axis indicates frequency of SNVs and the x-axis indicates each of six VAF bins. B) As VAF increases, the dinucleotides preceding the C>T SNV are enriched for the WRC motif recognized by activation-induced cytidine deaminase (AID). The y-axis indicates the frequency of motifs and the x-axis indicates all possible trinucleotide motifs ending with C. Repeated measures one-way ANOVA, n = 16, * p < 0.05, ** p < 0.01, **** p < 0.0001. C) RT-qPCR analysis was performed for Aicda expression using whole RNA extracted from the bone marrows of 6-8 week old WT, ΔB, and ΔPB mice. The y-axis indicates relative fold change and the x-axis indicates the Hardy fractions of B cell development. The Hardy fractions divide B cell precursors into the following subpopulations: Fraction A includes pre-pro B cells; Fraction B includes pro-BI cells; Fraction C includes pro-BII, pre-B cell, and large pre-B cells; and Fraction D includes small pre-B cells. Relative gene expression was analyzed using the 2^(-ΔΔCT) method and normalized to Tbp expression. Fold change in expression is relative to WT Fraction A. Data shown as mean ±SEM. Two-way ANOVA, n = 3, *p < 0.05.
3.7 Ruxolitinib reduces proliferation of cultured leukemic pro-B cells

Since it was previously shown that our mouse model harbours activating \(\text{Jak1}\) and \(\text{Jak3}\) mutations, we believed that globally inhibiting the activated JAK proteins can potentially inhibit malignant growth of pro-B cells. We previously demonstrated a reduction in cell counts when leukemic cell lines were cultured in 75 or 100 nM of Ruxolitinib (Lim et al., \textit{under revision}). This result served as stepping stone evidence that Ruxolitinib has inhibitory effects on mouse pro-B cells. To further demonstrate that the decrease in proliferation was due to inhibition of the JAK-STAT pathway, Western blots were performed staining for STAT5 and phosphorylated STAT5 (p-STAT5) in wild-type pro-B cells derived from fetal liver cells of wild type mice, preleukemic bone marrow cells derived from Mb1\(\Delta\)PB mice, and the 973 cell line either untreated or treated with Ruxolitinib (\textbf{Figure 16}). The Western blot shows that untreated 973 leukemic cells have an increased amount of p-STAT5 compared to the wild-type cell line. Treatment with Ruxolitinib reduces p-STAT5 to levels comparable to the wild-type. Furthermore, pre-leukemic bone marrow cells do not yet exhibit increased levels of p-STAT5 as they are early in clonal evolution.
Figure 16. Increased p-STAT5 demonstrating upregulated JAK-STAT pathways in leukemic cell line 973 cultured without Ruxolitinib.

Expression of STAT5 and p-STAT5 in 973 cells cultured with (+RUX) or without (-RUX) 75 nM Ruxolitinib for 24 hours. Treatment of 973 cells with Ruxolitinib in culture reduces levels of p-STAT5 similar to that of the wild-type pro-B cells (WT) or pre-leukemic bone marrow cells (BM). Actin used as a loading control.
3.8 Mice fed Ruxolitinib-infused chow doubled in survival time on average than mice fed control chow

Since cultured leukemic pro-B cells demonstrated a reduction in proliferation in vitro as a result of Ruxolitinib, we hypothesized that mice fed with Ruxolitinib chow will develop B-ALL later than the median 18 week latency observed in Batista et al., 2018. A total of three cohorts underwent this experiment, totaling to 5 control mice and 8 experimental mice. (Table 6). The control mice consumed 7.09 g of chow on average. Comparably, the experimental mice consumed 6.86 g of Ruxolitinib chow on average. All control mice developed leukemia at a median age of 17.26 weeks. One mouse from Cohorts 1 and 2 and two mice from Cohort 3 did not display symptoms of leukemia past 40 weeks of age. Given that historically, Mb1ΔPB mice do not survive past 30 weeks of age (Batista et al. 2018), we euthanized all mice when they reached 40 weeks of age. Notably, the thymuses of mice on Ruxolitinib were significantly smaller than those of mice on the control chow (Figure 17A). However, the spleens were not significantly different between mice that were fed Ruxolitinib or the control chow (Figure 17B). Overall, mice fed Ruxolitinib for four weeks following weaning doubled in survival in comparison to their control littermates (Figure 18A). This increased survival remained significant when comparing within female and male mice alone whereas females and males do not significantly differ in this increased survival (Figure 18B).

The thymus and matched tails of mice in this experiment were extracted and DNA and RNA were sent to Genome Quebec for WES and RNA-Seq, as described Chapter 2: Materials and Methods. RNA-seq was performed on RNA extracted from four mice fed vehicle chow and five mice fed Ruxolitinib. After processing raw RNA data and determining normalized gene expression as described above, differential gene expression was determined with DESeq2 (Love, Huber, and Anders 2014). Notably, most genes in the Ruxolitinib samples were downregulated as opposed to upregulated. Of those downregulated genes, many were pro-B and pre-B-specific genes such as VpreB1, Igll1, and Cd79b (Figure 19A). Genes for antioxidant defense and the electron transport chain were also downregulated in Ruxolitinib samples compared to the control samples.

Upregulated genes include Jun and Myc, which are heavily involved in cellular
proliferation. These genes are possibly upregulated to compensate for the loss of JAK activity. To determine enriched classes of genes dominant in the RNA seq data, GSEA was performed. It was revealed that there is significant downregulation of antioxidant gene expression in the Ruxolitinib samples (Figure 19B).

WES was performed on DNA extracted from four mice fed vehicle chow and seven mice fed Ruxolitinib. Raw WES files were processed as described above, after which SNVs were identified using variant callers Strelka, VarScan2, and FreeBayes. Although there were no SNVs in common within or between the Ruxolitinib and control samples, there was an overall reduction of Jak variants in the Ruxolitinib samples compared to the control samples (Figure 20A). Mutational signature analysis using deconstructSigs was then performed on variants obtained from Strelka and VarScan2. This analysis was not conducted on FreeBayes variants as we have previously shown that the sensitivity of FreeBayes did not allow for deconstructSigs to yield meaningful results. As expected, Signatures 18 and 24 were dominant in the control samples (Figure 20B-D). In contrast, the prevalence of Signatures 18 and 24 in the Ruxolitinib samples varied, and there was also an increase in the weight of other signatures such as 10, 15, 20, and 29 for Strelka and 3, 5, 14, 20, and 29 for Varscan2. Finally, variants identified by VarScan2 were divided into two groups: those with VAF <0.3 and those with VAF >0.3. This was done to determine the mutational signatures of genes that arose late or early in clonal evolution. We found that variants with VAF >0.3 (early in clonal evolution) demonstrated greater variance in mutational signatures compared to those with VAF <0.3 (Figure 20E-F).
Table 6. Information of mice used in the Ruxolitinib chow study.

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Each cohort consisted of littermates. *Mice euthanized not due to illness, but due to the assumption they would never develop B-ALL as their survival were surpassing the historical maximum age.
Figure 17. Mice fed Ruxolitinib chow exhibited delayed thymic tumour progression compared to mice fed vehicle chow.

Relative weights of thymuses and spleens harvested from Mb1ΔPB mice that were fed either control (n = 5) or Ruxolitinib chow (n = 8) between 4-8 weeks of age. The y-axis indicates relative weights of organs and the x-axis indicates the treatment. Error bars represent mean ± standard deviation. Unpaired t-test, **p < 0.01.
Figure 18. Mice fed Ruxolitinib chow doubled in survival compared to mice fed vehicle chow, independent of sex.

A) Survival of Mb1ΔPB mice fed either control vehicle chow or Ruxolitinib chow between 4-8 weeks of age. Mice fed control chow were euthanized due to illness at a median age of 17.26 weeks. Four of eight mice fed Ruxolitinib chow were surviving past 40 weeks of age and were euthanized to allow for analysis of their thymuses. Log-rank (Mantel-Cox) test, control n = 5, Ruxolitinib n = 8, p = 0.0002. B) Survival of the same Mb1ΔPB mice separated by sex. Log-rank (Mantel-Cox) test, female control n = 3, female Ruxolitinib n = 6, male control n = 2, male Ruxolitinib n = 2, p = 0.1642. For both curves, the y-axis indicates the percentage of mice surviving and the x-axis indicates age in weeks.
Figure 19. Effect of Ruxolitinib on gene expression in Mb1-Cre\DeltaPB mice.

A) Heat map of gene expression in Mb1-Cre\DeltaPB mice fed either Control chow (229, 258, 262, 484) or Ruxolitinib chow (226, 261, 307, 310, 486) at 4-8 weeks of age. Increasing intensity of blue cells indicates greater gene expression as determined by FPKM. B) Gene set enrichment analysis of the RNA-seq data using the same antioxidant gene set as shown in Figure A1 in Appendix.
Figure 20. Effect of Ruxolitinib on genes in Mb1-CreAPB mice.

(A) Variants in *Jak1*, *Jak2*, and *Jak3* are reduced in mice fed Ruxolitinib compared to mice fed vehicle chow, regardless of the variant caller used. Shown is a stacked bar graph showing the added frequencies of mice with *Jak* variants detected by three independent variant callers. The y-axis indicates the frequency of *Jak* variants and the x-axis indicates the treatment group. (B, C) Heat maps of mutational signatures (numbers on right panel) in leukemias from Mb1-APB mice fed with control or Ruxolitinib chow. The colors indicate weights of mutational signatures called by Strelka (B) or Varscan (C). (D) Weights expressed as fractions (x-axis) of mutational signatures called using variants identified by Strelka for mice fed with control or Ruxolitinib chow (y-axis) (E, F) Heat maps of mutational signatures in leukemias from mice fed control or Ruxolitinib chow. The colors indicate weights of mutational signatures called by Varscan for variants separated into VAF <0.3 (E) or VAF > 0.3 (F).
Chapter 4

4 Discussion

4.1 General discussion

4.1.1 Mb1ΔPB mice harbour \( \text{Jak3} \) and \( \text{Jak1} \) mutations similar to those found in pediatric patients

The tumors of the Mb1ΔPB mice are most apparent in their enlarged thymuses and spleens. Strikingly, flow cytometry revealed that more than 90% of the cells in the thymic tumours are \( \text{CD19}^+ \) B cells (Batista et al. 2018). Given that B cells do not naturally home to the thymus, this high level of tumor purity made the thymic tumors the focus of our study.

We first demonstrated that mutational caller Strelka was able to identify SNVs between the tail DNA from two different mice. This suggests there is germline variability between our Mb1ΔPB mice, most likely due to incomplete inbreeding. These results highlight the importance of comparing leukemia exome data to a matched tail control instead of a standard wild type tail control. This method ensured that all SNVs identified between the leukemia and tail DNA are somatic. Comparison of our variant caller results to two different literature data also confirms that our mouse model is similar to other established models, and is not exhibiting an excessive nor limited amount of mutations (Dang et al. 2017; Dudgeon et al. 2014).

With the controls and pipeline justified, we continued processing the data to generate dot plots that would enable us to visually identify high-VAF mutations. Mutations that occur at a high VAF are frequent within the tumour sample; this is an indication that those particular cells or clones experience a positive selection pressure due to, for example, growth advantages that allows them to persist at high frequencies in the tumour. In pediatric patients of B-ALL, pivotal driver genes contributing to their diseases occur at a high VAF. Thus, we focused on genes that are on the far right side of the dot plots. The plots demonstrated that \( \text{Jak3} \) and \( \text{Jak1} \) were high-VAF mutations in 5/8 and 2/8 leukemias. No other gene except \( \text{Ikzf3} \), which is currently being studied by Dr. Bruno
Rodrigues de Oliveira in the lab, were as frequently mutated at a high VAF as the Jaks. Remarkably, every mutation identified from our mouse model in Table 4, except T844M, have human equivalents that were previously identified in pediatric ALL patients. Specifically, the human equivalents Jak3 R657Q and V674F were identified as activating mutations in ALL; Jak3 A572V was identified in T cell leukemia; Jak1 V658F was also identified as an activating mutation in ALL; and Jak1 F838V was found in T cell lymphoma (Elliott et al. 2011; Hornakova et al. 2009; Jain et al. 2017; Losdyck et al. 2015; Roberti et al. 2016). Since each mouse independently developed leukemia through the acquirement of secondary drivers similar to those in human pediatric patients, our Mb1∆PB mice are a suitable and reproducible model for B-ALL.

Acknowledging that the genes identified in the dot plots can be a result of sequencing artefacts, we applied several filters to reduce the probability of studying artefacts. SNVs that introduce stop codons, abrogate start codons, alter codons to missense mutations, and reside in splice regions wreak the most havoc on the protein and therefore cellular functions. Additionally, since bona fide mutations often exist at a high frequency due to their increased fitness during clonal evolution, SNVs with a VAF of less than 0.10 are more likely to be the result of sequencing errors or artefacts. Given these facts, we filtered the SNVs for VAF > 0.10, and missense, splice region, stop, and start (MSSS) variants. This refined list still returned the same Jak mutations observed in the dot plots, confirming that they are not artefacts. An important limitation to consider for this project is that every mutation caller differs in their algorithm, filtering strategy, and setting defaults (Sandmann et al. 2017). Thus, it is inevitable that depending on the mutation caller used, the identified mutated genes will differ. To increase our confidence that the identified genes are truly mutated, we re-analyzed our whole exome data using two more distinct callers in addition to Strelka: VarScan and FreeBayes. VarScan was chosen because it was shown to have a high sensitivity and positive predictive value compared to seven other mutation callers, whereas FreeBayes had the highest sensitivity (Sandmann et al. 2017). For all variant callers, the results were filtered for MSSS mutations and a VAF >0.1. Strelka, VarScan, and FreeBayes identified 75, 281, and 889 gene variants per exome on average, respectively. Evidently, Strelka demonstrated the greatest stringency for identifying mutations whereas FreeBayes demonstrated the highest sensitivity.
Intersection of these variant callers resulted in 20 gene variants per exome on average. Of those 20 variants, \textit{Jak1} and \textit{Jak3} were among the most recurrently mutated genes. All three variant callers were able to identify five \textit{Jak3} mutations and two \textit{Jak1} mutations. These results confirm that all mutations except for V670A in leukemia 857 are \textit{bona fide}.

The majority of the identified \textit{Jak} mutations reside within the pseudokinase domain. This enzymatically-dead domain functions to negatively regulate the kinase activity of the protein (Ghoreschi et al. 2009). Human analogues of R653H and V670A in \textit{Jak3}, R657Q and V674A, have been reported to disrupt the pseudokinase domain’s activation loop (Bergmann et al. 2014). This disruption abolishes the pseudokinase domain’s ability to bind the kinase domain to exert its regulatory function. It is possible that the \textit{Jak3/1} mutations we have identified are activating by upregulating the JAK-STAT pathway in this manner as well.

Mutation \textit{Jak3} T844M is the only one that resides in the kinase domain. Although human analogues of T844M have not yet been reported, it was previously established that proline at position 933 (P933) in human JAK2 provides rigidity to a hinge in the kinase domain (Lucet et al. 2006). As this rigid hinge provides substrate specificity, P933 mutations enable lenient phosphorylation by the kinase domain. Since JAK2 and JAK3 are closely related and T848 (human analogue of T844) is relatively close to P933, we speculate that T844M also abolishes rigidity of the hinge, therefore increasing kinase activity. It is also known that JAK3 autophosphorylates Y980 and Y981 within its kinase activation loop (Cornejo, Boggon, and Mercher 2009). Since tyrosine and methionine are hydrophobic amino acids, we also speculate that substituting threonine for methionine encourages favorable interactions between the kinase domain and Y980 or Y981 to increase kinase activity. Future studies on the JAK3 protein structure can elucidate conformational consequences of the \textit{Jak3} T844M mutation.

Pan-cancer mutational signature analyses revealed that a large number of pediatric B-ALL cases harbor a high frequency of C>A transversions (Ma et al. 2018). These transversions are thought to be the result of 8-oxoguanine DNA damage. All eight of our leukemia samples also demonstrate similar mutational patterns, categorized as Signature
These results are indicative of a mechanism of mutagenesis that may arise from 8-oxoguanine DNA damage, a hypothesis we later explored in 4.1.3.

4.1.2 The identified Jak3 mutations confer growth advantages to progenitor B cells

Infection of wild type pro-B cells with mutated MSCV-Jak3 plasmids demonstrated positive results; all control cell lines (pro-B cells, MSCV empty, and WT Jak3) maintained the same relative ratio of GFP+ to GFP- cells over a period of 14 days, indicating that only mutated Jak3 cell lines outgrew uninfected wild type cells. Human analogues of R653H and V670A in Jak3, R657Q and V674A, have been reported to disrupt the pseudokinase domain’s activation loop (Bergmann et al. 2014). This disruption abolishes the pseudokinase domain’s ability to bind the kinase domain to exert its regulatory function. It is possible that R653H and V670A upregulate the JAK-STAT pathway in a similar manner. Human analogues of T844M have not been studied in such a way.

This growth advantage assay was reattempted using MSCV-Jak1 plasmids to also test the gain-of-function status of Jak1 V655L and V657F. Unfortunately, while construction of the plasmids was successful, retroviral infection of wild type pro-B cells proved difficult. This difficulty is most likely due to the large size of the MSCV-Jak1 plasmid, which was approximately 11kb. Thus, we infected a rapidly proliferating pro-B cell line called 38B9 so that the scarce GFP+ cells could be sorted and rapidly expanded in culture. After culturing in Imatinib, Western blots for STAT5 and p-STAT5 demonstrated that MSCV-Jak1 infections conferred JAK-STAT pathway activation. However, culturing infected 38B9s in low (0.5%) IL-7 conditions could not demonstrate differences in levels of p-STAT5 between the WT and mutated Jak1-infected cell lines. We predict that p-STAT5 levels are similar between the control and infected groups because 0.5% IL-7 may be considered excessive for this model. This abundance in IL-7 may therefore be causing STAT5 phosphorylation levels that are too high to allow for discernment between the two groups. In future, a range of lower concentrations of IL-7 (ex. 0.05, 0.10, 0.20) can be used to culture the infected 38B9 cells followed by Western blots to determine
differences in p-STAT5. Since the *Jak1* mutations (V655L and V657F) reside in the pseudokinase domain, we expect them to be gain-of-function. In addition to this, the human equivalent of V655L, which is V658F, has been previously shown to be a frequent activating driver mutation in human ALL (Hornakova et al., 2009). In summary, these experiments demonstrated that three of the *Jak3* mutations we identified are activating whereas the effect of the *Jak1* mutations we identified are yet to be determined.

4.1.3 AID and ROS are two distinct mechanisms of leukemogenesis in Mb1ΔPB mice

We have discovered two possible distinct mechanisms of mutagenesis contributing to B-ALL in our Mb1ΔPB mice. The first is AID off-target activity introducing activating mutations within *Jak3* and/or *Jak1*. The second mechanism is a consequence of the first, whereby upregulation of the JAK-STAT pathway contributes to ROS-mediated 8-oxoguanine DNA damage manifested as C>A transversions.

Previous studies have shown that mice doubly deleted for PU.1 and IRF8, both transcription factors that regulate a variety of genes involved in B cell development and function, exhibit increased expression of AID (Wang et al. 2019). Furthermore, AID was also previously shown to be upregulated in mice doubly deleted for SpiB and PU.1 (Willis et al. 2017). These studies in combination with our findings suggest that, through an unknown mechanism, SpiB and/or PU.1 play pivotal roles in regulating the expression of AID in immature B cells during development. Without either or both transcription factors, AID is upregulated and exerts off-target deamination as a result.

In our mouse model, AID is possibly introducing the activating *Jak* mutations we observed. Notably, B-ALL has been previously shown to exhibit reduced antioxidant gene expression in cell lines as well as pediatric patients (Prieto-Bermejo et al. 2018). This inability to scavenge ROS in the cell is a characteristic in our leukemia model as well; our results show that leukemic pro-B cells have more 8-oxoguanine DNA damage than wild type cells, likely facilitated through impaired antioxidant activity. While ROS at normal levels play important roles in cellular signaling and gene expression (Sardina et al. 2012), an excess can contribute to JAK-STAT signaling through the inactivation of
protein tyrosine phosphatases (PTP) and phosphatase and tensin homolog (PTEN) (Irwin, Rivera-Del Valle, and Chandra 2013; Ray, Huang, and Tsuji 2012). Furthermore, STAT5 directly represses the expression of antioxidant genes such as catalase and glutaredoxin-1 (Bourgeais et al. 2017). This combination of excessive ROS inactivating PTP and STAT5-mediated antioxidant gene repression causes the JAK-STAT signaling to enter a positive feedback loop, further exacerbating ROS levels within the cell. Importantly, excessive ROS oxidizes guanine in the cell, resulting in C>A transversion mutations. This may explain the genomic instability observed in the whole exome data. In conclusion, excessive ROS may be initiated by AID-induced Jak mutations upregulating the JAK-STAT pathway.

In the next section, we show that this hypothesis is corroborated by the evidence that treatment of leukemia cells and Mb1∆PB mice with Ruxolitinib, an FDA-approved JAK inhibitor, reduces cellular proliferation in vitro and delays tumor progression in vivo.

4.1.4 Global inhibition of JAK proteins delays tumour progression and improves survival of Mb1∆PB mice

The JAK-STAT pathway is implicated in many pathologies, notably leukemias. Human B-ALL patients have been previously documented to harbour varying JAK mutations as well as IL7R mutations (Jain et al. 2017; Roberts et al. 2017; Shochat et al. 2011). Additionally, ALLs frequently demonstrate arrest of developing B cells at the pre-B cell stage (Buchner et al. 2015; Müschen 2015). These malignant pre-B cells also express IL-7R and exhibit upregulated JAK-STAT signalling implying a dependency on this pathway for the cells’ proliferation and survival. The frequency of mutations within genes associated with the JAK-STAT pathway, such as those that directly activate cytokine signalling or JAKs, suggest that they function as driver mutations in B-ALL. We have so far observed recurrent high-frequency Jak3 and Jak1 mutations in our Mb1∆PB mice, many of which were already proven to be activating/gain-of-function mutations. Furthermore, all Jak mutations we have identified except one are found in human pediatric ALL patients (Elliott et al. 2011; Hornakova et al. 2009; Jain et al. 2017; Losdyck et al. 2015; Roberti et al. 2016). These findings strongly suggest that dysregulation of JAKs and/or upregulation of the JAK-STAT pathway through an
unknown mechanism are central pathogenic components of B-ALL. The next goal of this research was to delay or prevent tumour development in our mouse model using a JAK inhibitor. Therefore, we hoped that our mice without Jak mutations will benefit from Ruxolitinib, also demonstrating that the JAK-STAT pathway is causative of B-ALL in our mouse model.

We explored the effects of inhibiting JAKs both in vitro and in vivo using Ruxolitinib, a selective JAK inhibitor that docks ATP-binding pockets to prevent activation of the JAK-STAT pathway and reduce cellular proliferation (Duan et al. 2014). We cultured leukemic cell line 973 in varying concentrations of Ruxolitinib to demonstrate its effectiveness against mouse pro-B cells before pursuing in vivo experiments (Lim et al., under revision). We also confirmed Ruxolitinib’s ability to inhibit JAK-mediated phosphorylation as treated 973 cells demonstrated decreased amounts of p-STAT5.

To further the in vitro results, we explored the effects of JAK inhibition by Ruxolitinib in mice. This part of the study was most limited by the fact that Novartis only provided us with 1 kg each of Ruxolitinib and vehicle chow. As these chow formulas are not commercially available, promising preliminary data must be generated before more chow can be requested. We also recognize that intravenous administration of Ruxolitinib would better control dosing. However, Ruxolitinib is orally administered in humans leading to better translatability of results; and C57BL/6 mice have previously been shown to regulate their appetite and consume approximately 4 g of chow per day, eliminating the concern for over-or-under administration based on appetite (Bachmanov et al. 2002). It was for these reasons that we were unable to keep the mice on Ruxolitinib long term until they develop B-ALL. With this in consideration, we decided to feed the mice from 4 to 8 weeks of age; at 4 weeks, mice are able to consume solid food and are pre-leukemic, while at 8 weeks mice begin entering early leukemic stages. Inhibition of JAKs in the pre-leukemic/early leukemic stages would sufficiently demonstrate any beneficial effects since the inhibition would remove the positive selection pressure on cells with JAK mutations. The doubling survival of mice fed Ruxolitinib suggests that the inhibition of JAK proteins at a preliminary stage of leukemia confers increased survival via reduction of the JAK-STAT pathway in developing tumors. Half of the Ruxolitinib-treated mice
eventually developed leukemia, suggesting resistance to the drug or development of alternative secondary driver mutations utilizing different mechanisms of pathogenesis. Given that mice within the control and experimental group consumed very similar amounts of chow, our observed effects were not confounded by differing levels of chow consumed. Most remarkably, thymic leukemias in Ruxolitinib-treated mice were significantly delayed compared to their control littermates. Interestingly, spleen sizes did not significantly differ between the control and Ruxolitinib-treated mice. These outcomes suggest that inhibition of JAKs for four months in mice sufficiently impose a negative selection pressure on cells with excessive JAK-STAT signalling. Perhaps these cells are undergoing apoptosis similar to myelofibrosis patients being treated with Jakafi®; or the cells no longer have a growth advantage in high IL-7 organs like the thymus and instead develop niches in the spleen (Quintás-Cardama et al. 2010). It is important to note that human patients with myeloproliferative neoplasms undergoing JAK1/2 inhibition therapy (Ruxolitinib or Fedratinib) demonstrated a 15-16 fold increased risk of developing B cell non-Hodgkin lymphoma compared to conventional chemotherapy (Porpaczy et al. 2018). This severe side effect of JAK inhibition suggests that while JAK activity contributes to B cell leukemias, it may play important roles in suppressing B cell lymphomas.

Finally, RNA sequencing and whole exome sequencing analysis of the thymic tumor samples allowed us to examine how clonal evolution of the leukemias is affected by the presence of Ruxolitinib. The reduction in expression of antioxidant genes suggests that Ruxolitinib reduces ROS in leukemia cells. The downregulation of pro/pre-B cell genes also suggests that the number of pro/pre-B cells within the thymic leukemias are reduced when treated with Ruxolitinib. This reduction in cellularity suggest that Ruxolitinib is imposing a negative selection for pro/pre-B cell migration to the thymus, which is what we almost always observe in untreated Mb1ΔPB mice. These RNA-seq results are corroborated by the WES results; the dominance of ROS-associated mutational signatures 18 and 24 decreased in Ruxolitinib-treated samples with the introduction of varied signatures not seen in the control samples. Assuming that Jak mutations arise from off-target AID activity, the observed reduction in ROS may be a direct result of a lower Jak variant frequency and JAK-STAT pathway activity. We believe the lower frequency of Jak mutations resulted from Ruxolitinib’s negative selection of cancerous cells harboring
Overall, feeding Mb1ΔPB mice with Ruxolitinib for just four weeks results in a reduction in ROS and a corresponding shift in mutational signatures. As mentioned in Chapter 1, our mice spontaneously develop mutations in Jak3 much like pediatric B-ALL. Thus, inhibition of JAK proteins in our mouse model provides valuable information on Ruxolitinib’s effects on the clonal evolution of leukemias, potential causes for resistance, and insight on its ability to be used as treatment for human patients.

4.2 Conclusion

The aim of this thesis was to identify driver mutations contributing to B cell acute lymphoblastic leukemia and elucidate the mechanisms responsible for these mutations in a mouse model. Our hypothesis was that mutations in \textit{Janus kinase} genes were secondary drivers in cooperation with SpiB and PU.1 deletions in mice. Our results supported this hypothesis as we were able to demonstrate that \textit{Jak3} and \textit{Jak1} are the only recurrent mutated genes in the leukemias. Furthermore, every \textit{Jak3} mutation identified conferred growth advantages to progenitor B cells both \textit{in vitro} and \textit{in vivo}. We have evidence suggesting that the mechanisms by which these \textit{Jak} mutations arise may be via off-target activity by AID; and that the abundant C>A mutations observed in various other genes may have resulted from excessive ROS within the cell caused by upregulated JAK-STAT signaling. Finally, inhibition of JAKs in mice demonstrated effectiveness in delaying tumour progression and doubling survival time. A working model summarizing these results can be found in Figure 21. Together, these results highlight the prognostic relevance of \textit{Jaks} to B-ALL as well as the importance of targeting the JAK-STAT pathway with molecular therapies. This thesis also demonstrates the power of high-throughput sequencing in uncovering mutations associated with disease in an accurate yet timely manner.

4.3 Future directions

We are still in the process of demonstrating that the \textit{Jak1} mutations we discovered are driver mutations. With the new ImPaKt Facility, we propose to take advantage of the PET imaging equipment available by transplanting \textit{Jak1}-expressing cells into lightly irradiated SCID mice that can be visualized using firefly luciferase. To do this, bone
marrow cells will be infected with a lentiviral vector expressing firefly luciferase as described in Nyström et al., 2019 (Nyström et al. 2019). This pool of bone marrow cells will then be divided into two groups. The first group will be secondarily infected with an empty vector as a control whereas the second group will be infected with the vector encoding the gene of interest. In our case, given that MSCV-Jak1 is proving difficult to create viruses from, we propose to clone Jak1 into a lentiviral vector termed pLVX-IRES-ZsGreen1. Thus, the first group of cells will be infected with pLVX-IRES-ZsGreen1 whereas the second group with pLVX-Jak1-IRES-ZsGreen1. Transplanting these doubly-infected cells into mice followed by PET imaging will allow us to visualize the migration of leukemia cells in vivo. Additionally, this technique will demonstrate the ability of Jak1 mutants to cause leukemia if the mice transplanted with mutated Jak1 decrease in survival compared to the empty vector control.

The discovery that the trinucleotide context of high-VAF C>T transition mutations are compatible with the WRC motif recognized by AID opened a new avenue of research in our lab. We have yet to gather conclusive evidence on the role of AID in leukemogenesis. Perhaps the most direct way to achieve this is to breed AID knockout mice with our Mb1ΔPB mice to obtain a genotype of AID\(^{\text{-/-}}\) Mb1\(^{+/\text{Cre}}\) Spi1\(^{\text{lox/lox}}\) Spib\(^{\text{-/-}}\). We expect that the complete absence of AID in SpiB and PU.1 knockout mice will result in a reduced frequency of C>T mutations, particularly within the Jak genes. These results would provide evidence that SpiB and PU.1 play pivotal roles in constraining the expression of Aicda during B cell development through an unknown mechanism.

We would also like to further investigate the effectiveness of Ruxolitinib in the treatment of B-ALL. If the pilot experiment shown in this thesis warrants additional supply of Ruxolitinib and vehicle chow from Novartis, we would no longer be required to restrain the feeding of the mice to four weeks. We propose to feed the mice at two different schedules: 1) ad libidum at weaning; or 2) ad libidum at 10 weeks of age when the mice begin to develop B-ALL. These two different feeding schedules will allow us to discern the effectiveness of Ruxolitinib as a preventative (schedule 1) as well as a treatment (schedule 2). We believe that this additional Ruxolitinib experiment will provide valuable evidence supporting the use of JAK inhibitors in clinical trials. In addition to Ruxolitinib,
we also hypothesize that combination therapy with N-acetylcysteine will further assist in delaying tumour progression. N-acetylcysteine is an exogenous antioxidant that scavenges reactive oxygen species. This compound is available as a solution, which can be easily administered to mice as part of their water supply. We believe that inhibiting JAKs as well as providing additional support for scavenging intracellular ROS will be a therapy superior to either Ruxolitinib or N-acetylcysteine alone.
Figure 21. A working model for accumulation of mutations leading to B cell acute lymphoblastic leukemia in Mb1ΔPB mice.

The absence of SpiB and PU.1 may be allowing activation-induced cytidine deaminase (AID) to be prematurely expressed in developing B cells. This can introduce gain of function/activating mutations in Jak1/3, consequently upregulating the JAK-STAT pathway. As a result, there is increased intracellular reactive oxygen species (ROS) leading to 8-oxoguanine (8-oxoG) DNA damage (C>A) and the inhibition of tyrosine phosphatases. This leads to the JAK-STAT pathway entering a positive feedback loop. JAK inhibition via Ruxolitinib may mitigate the positive feedback loop.
References


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Li, Qian, Botao Li, Liangding Hu, Hongmei Ning, Min Jiang, Danhong Wang, Tingting Liu, Bin Zhang, Hu Chen, Qian Li, Botao Li, Liangding Hu, Hongmei Ning, Min


Appendices

Appendix A: Figures and Tables generated by Jacob Ferguson under the supervision of Michelle Lim.

Table A1. List of pro-oxidant genes gathered from literature considered for heat map analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Included or Excluded</th>
<th>Reason for Exclusion (if Applicable)</th>
<th>Pro-oxidant Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nox5***</td>
<td>E</td>
<td>Low abundancy* and lack of variable expression**</td>
<td>NADPH oxidase/dual oxidase: directly generates O$_2^-$ and indirectly generates H$_2$O$_2$ (Bokoch and Knaus 2003; Donkó et al. 2005; Lambeth 2004)</td>
</tr>
<tr>
<td>Nox4***</td>
<td>E</td>
<td>Low abundancy and lack of variable expression</td>
<td></td>
</tr>
<tr>
<td>Nox3***</td>
<td>E</td>
<td>Low abundancy and lack of variable expression</td>
<td></td>
</tr>
<tr>
<td>Nox1***</td>
<td>E</td>
<td>Low abundancy and lack of variable expression</td>
<td></td>
</tr>
<tr>
<td>Duox2*</td>
<td>E</td>
<td>Low abundancy only</td>
<td></td>
</tr>
<tr>
<td>Duox1*</td>
<td>E</td>
<td>Low abundancy only</td>
<td></td>
</tr>
<tr>
<td>Alox5*</td>
<td>E</td>
<td>Low abundancy only</td>
<td>Lipoxygenase: catabolizes arachidonic acid into ROS-stimulating metabolites (Cho, Seo, and Kim 2011; Woo et al. 2002)</td>
</tr>
<tr>
<td>Alox12*</td>
<td>E</td>
<td>Low abundancy only</td>
<td></td>
</tr>
</tbody>
</table>

*Low abundancy is defined as average FPKM < 0.5 in all three cell types

**Lack of variable expression across defined as: average FPKM $\frac{\text{highest expression cell type}}{\text{lowest expression cell type}}$ <1.3)
Table A2. List of antioxidant genes gathered from literature considered for heat map analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Included or Excluded</th>
<th>Reason for Exclusion (if Applicable)</th>
<th>Antioxidant Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nfe2l2</td>
<td>I</td>
<td></td>
<td>Transcriptional control of antioxidant enzymes (Ma 2013; Mullighan 2012; Sakamoto et al. 2009)</td>
</tr>
<tr>
<td>Pten</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cat</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gpx8*</td>
<td>E</td>
<td>Low abundancy* only</td>
<td></td>
</tr>
<tr>
<td>Gpx7***</td>
<td>E</td>
<td>Low abundancy and lack of variable expression**</td>
<td>Peroxidase: degrades H₂O₂ (Gelain et al. 2009; Ma 2013; Mathew, Ranganna, and Milton 2014; Rasool et al. 2015)</td>
</tr>
<tr>
<td>Gpx6***</td>
<td>E</td>
<td>Low abundancy and lack of variable expression</td>
<td></td>
</tr>
<tr>
<td>Gpx5***</td>
<td>E</td>
<td>Low abundancy and lack of variable expression</td>
<td></td>
</tr>
<tr>
<td>Gpx4</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gpx3</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gpx2***</td>
<td>E</td>
<td>Low abundancy and lack of variable expression</td>
<td></td>
</tr>
<tr>
<td>Gpx1</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gss**</td>
<td>E</td>
<td>Lack of variable expression only</td>
<td></td>
</tr>
<tr>
<td>Lpo***</td>
<td>E</td>
<td>Low abundancy and lack of variable expression</td>
<td></td>
</tr>
<tr>
<td>Mpo</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prdx6</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prdx5</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
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**Superoxide dismutase:** converts O$_2^-$ to O$_2$ or H$_2$O$_2$ (Gelain et al. 2009; Ma 2013; Rasool et al. 2015)

**Thiol redox:** impacts the reduction/oxidation state of sulphhydryl groups, present in cysteine side chains (Gelain et al. 2009; Ma 2013)
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*Low abundance is defined as average FPKM < 0.5 in all three cell types

**Lack of variable expression across defined as:

\[
\frac{\text{average FPKM}_{\text{highest expression cell type}}}{\text{average FPKM}_{\text{lowest expression cell type}}} < 1.3
\]
**Figure A1.** Heat map of antioxidant gene expression in Spi1-induced, uninduced, and leukemic (1-8) cell lines. Red cells indicate higher relative expression whereas blue cells indicate lower relative expression. Gene categories 1 to 6 were designated based on patterns of gene expression in the three cell lines. 1: lowest expression in leukemic, highest expression in Spi1-induced; 2: lowest expression in leukemic, highest expression in uninduced; 3: lowest expression in uninduced, highest expression in Spi1 induced; 4: lowest expression in Spi1-induced, highest expression in uninduced; 5: lowest expression in uninduced, highest expression in leukemic; 6: lowest expression in Spi1-induced, highest expression in leukemic.
Figure A2. Levels of 8-oxoguanine DNA damage as indicated by fluorescence in leukemic pro-B cells (n = 57) and wild type pro-B cells (n = 69). Background fluorescence (controls) in wild type (n = 58) and leukemic cells (n = 56) were minimal. Kruskal-Wallis test, ***p < 0.001, *** p < 0.001.
Curriculum Vitae

Name: Michelle Lim

Post-secondary Education and Degrees
Western University
London, Ontario, Canada
2014-2018 BSc. *Honours Specialization in Genetics*

Western University
London, Ontario, Canada
2018-2020 MSc. Candidate, *Microbiology and Immunology*

Honours and Awards
RGE Murray Seminar Award
$500
2020

Canadian Graduate Scholarship- Master’s (CGS-M)
$17,500
2019-2020

Ontario Graduate Scholarship (OGS)
$15,000 (Declined)
2018-2019

International Union of Immunological Sciences
*Travel Award, $1500*
2019

Microbiology and Immunology Department
*Travel Award, $1000*
2019

Ontario Graduate Scholarship (OGS)
$15,000
2018-2019

Frederick W. Luney Entry Scholarship
$2000
2018-2019

Western Gold Medal in Genetics
2018

Workshops
Canadian Bioinformatics Workshop
*Bioinformatics Applied to Cancer Genomics*
2019
Publications:

