ROLE OF THE ADAPTER PROTEIN 3BP2
IN BCR-ABL-MEDIATED SIGNAL TRANSDUCTION AND
LEUKEMOGENESIS

By

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A Thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Medical Biophysics
University of Toronto

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Degree of Master of Science, 2012
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ABSTRACT

3BP2 was originally identified through its interaction with the ABL kinase. Fusion of ABL with the BCR gene forms the BCR-ABL onco-protein, which is causative in Chronic Myeloid Leukemia (CML) and acute lymphoid leukemia (ALL). Due to the ability of 3BP2 to regulate ABL activity in osteoblasts, we hypothesize that 3BP2 modulates BCR-ABL signalling. Overexpression of 3BP2 in the CML-T1 cell line produced a marked decrease in global tyrosine phosphorylation. 3BP2 overexpression also resulted in a significant increase in CML-T1 cell growth, accompanied by altered ERK1/2, AKT, SYK, LYN, HCK, and CBL phosphorylation and expression. A phospho-SRC family protein and a 116 kDa phospho-protein were identified as 3BP2 interaction partners in response to BCR-ABL activation. BCR-ABL bone marrow transplantation (BMT) models in 3bp2−/− mice exhibit accelerated disease compared to wild-type mice, with altered leukemic phenotype. In conclusion, 3BP2 is able to modulate signalling through BCR-ABL and affect BCR-ABL-induced disease outcome.
ACKNOWLEDGEMENTS

My graduate experience was shaped by the committed academic support and kind personal guidance from my supervisor Dwayne, without whom I could not have completed this work. Thank you for continually challenging me to strive for success and for having such faith in my abilities. Thank you to my committee members, Dr. Jane McGlade and Dr. Robert Rottapel, for your valuable advice, support, and guidance throughout my degree. And to the Barber lab, for your unwavering support, friendship, and patience, I thank you. You’ve all provided positive influence, helped me navigate through difficult times, and surely never failed to make me laugh. Last but certainly not least, I am grateful to my family for unremittingly supporting me during my years of study, and to Jonathan for being a contributor to my academic and personal growth.
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<td>3BP2-1</td>
<td>ABL SH3 binding protein 1</td>
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<td>3bp2</td>
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<td>β-mercaptoethanol</td>
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<td>Aorta-gonad-mesonephros</td>
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<td>AKT</td>
<td>AKT8 virus oncogene cellular homolog/protein kinase B</td>
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<td>ALL</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>B-ALL</td>
<td>B-cell acute lymphoblastic leukemia</td>
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<td>BCR</td>
<td>Breakpoint cluster region</td>
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<td>BM</td>
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<td>CFU</td>
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<td>CIN85</td>
<td>CBL-interacting protein of 85 kDa</td>
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<td>Chronic myeloid leukemia</td>
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<td>c-Src tyrosine kinase</td>
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<td>ddH₂O</td>
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<td>DOK-R</td>
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<td>Dulbecco’s modified Eagle’s medium</td>
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<td>DMSO</td>
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<td>DNA</td>
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<td>dNTP</td>
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<td>Embryonic day 8.5</td>
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<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>EVH1</td>
<td>Enabled/VASP homology 1</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FcεRI</td>
<td>High affinity IgE receptor</td>
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<td>A SRC family tyrosine-protein kinase</td>
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<td>Gram</td>
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<tr>
<td>GADS</td>
<td>GRB2-like adaptor protein</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GIST</td>
<td>Gastrointestinal stromal tumor</td>
</tr>
<tr>
<td>GRB2</td>
<td>Growth factor receptor-bound protein 2</td>
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<tr>
<td>GST</td>
<td>Glutathione S transferase</td>
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</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
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<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
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<td>HCK</td>
<td>A SRC family tyrosine-protein kinase</td>
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<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
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<tr>
<td>HSCT</td>
<td>Hematopoietic stem cell transplantation</td>
</tr>
<tr>
<td>IB</td>
<td>Immunoblotting</td>
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<tr>
<td>IFN-α</td>
<td>Interferon-alpha</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s media</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosomal entry site</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous(ly)</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
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<tr>
<td>Kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>kDa</td>
<td>KiloDalton</td>
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<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>LAT</td>
<td>Linker of activated T cells</td>
</tr>
<tr>
<td>Lin</td>
<td>Lineage</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>LSC</td>
<td>Leukemia stem cell</td>
</tr>
<tr>
<td>LSK</td>
<td>Lin- / Sca-1+/ c-kit+</td>
</tr>
<tr>
<td>LT-HSC</td>
<td>Long-term and reconstituting hematopoietic stem cell</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
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<tr>
<td>LYN</td>
<td>A SRC family tyrosine-protein kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>Abbr.</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of Sevenless</td>
</tr>
<tr>
<td>SRC</td>
<td>Rous sarcoma oncogene cellular homolog</td>
</tr>
<tr>
<td>SSC</td>
<td>Side-scatter</td>
</tr>
<tr>
<td>ST-HSC</td>
<td>Short-term hematopoietic stem cell</td>
</tr>
<tr>
<td>SYK</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>T315I</td>
<td>Threonine 315 to Isoleucine mutation</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween-20</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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<tr>
<td>VAV</td>
<td>The ‘onc F’ proto-oncogene</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Vesicular stomatitis virus G</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
</tr>
<tr>
<td>WHS</td>
<td>Wolf-Hirschhorn syndrome</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Y</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>ZAP70</td>
<td>z-chain-associated protein kinase</td>
</tr>
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</table>
CHAPTER 1: INTRODUCTION
1.1. Hematopoiesis

Hematopoiesis is the process by which mature blood elements are formed and developed. This process includes erythropoiesis (red blood cell development), thrombopoiesis (development of platelets), myelopoiesis (myeloid cell development) and leukopoiesis (white blood cell development; Ross and Pawlina, 2005). Blood cells have a limited life span and are continuously produced and destroyed. All blood cells originate from a common progenitor cell, the hematopoietic stem cell (HSC; Martinez-Agosto et al. 2007).

HSCs sustain blood production throughout the entire lifespan of an organism and are thus the foundation of the hematopoietic system. In addition to the self-renewal capacity that defines the HSC, these cells possess multipotency, which is the ability to proliferate and differentiate into more committed precursor and progenitor cells. These progenitors will then give rise to the cells of the mature erythroid, myeloid and lymphoid lineages (Martinez-Agosto et al. 2007).

1.1.1. Embryonic hematopoietic development

During embryonic development, both erythrocytes and leukocytes are formed in several organs before the differentiation of the bone marrow. The initial or yolk sac phase of human hematopoiesis begins in the third week of gestation and is characterized by the formation of blood islands in the wall of the yolk sac of the embryo (Ross and Pawlina, 2005). Early during gestation, an intra-embryonic region known as the aorta-gonad-mesonephros (AGM) serves as the site of generation of long-term and reconstituting HSCs (LT-HSCs). These LT-HSCs enter the circulation and migrate to the fetal liver between weeks five and six (Medvinsky and Dzierzak, 1996).

The second or hepatic phase of hematopoiesis in the human is marked by the appearance of hematopoietic centers in the liver, the major blood-forming organ in the fetus during the second trimester. This phase is marked primarily by erythropoiesis, with some leukocyte formation (Ross and Pawlina, 2005).
The third or bone marrow phase of human fetal hematopoiesis begins in the second trimester of fetal development and is characterized by blood formation in the bone marrow, as well as other lymphatic tissue. After birth and into adulthood, hematopoiesis occurs in the bone marrow and lymphatic tissues (Martinez-Agosto et al. 2007). Bone marrow is found within compact bone, in the medullary cavity of young long bones and among the spicules of spongy bone.

During mouse embryogenesis, hematopoietic development begins in the yolk sac at embryonic day 7.5 (E7.5) and the shift to definitive hematopoiesis (generation of enucleated erythrocytes, and various myeloid and lymphoid cells) starts in the embryonic para-aortic splanchnopleural region (pSp) at E8.5 (Medvinsky and Dzierzak, 1996). LT-HSCs first appear at E10.5 in the AGM region, after which hematopoiesis takes place in the fetal liver and later in the spleen and bone marrow (Johnson and Moore, 1975).

1.2. Leukemia

Leukemia is the cancer of blood cells, which results from deregulation of hematopoiesis. Malignant leukemic cells replace normal hematopoietic populations in the bone marrow, resulting in bone marrow failure (Gilliland, 2002). These leukemic cells can then circulate into the bloodstream, causing a characteristic increase in white blood cells in the peripheral blood that initially led Rudolf Virchow to coin the term ‘leukemia’, from leukos, the Greek word for white (Druker, 2008). Leukemia types are distinguished based upon the hematopoietic cell lineage affected, time to disease onset, and the extent of cell maturation. Depending on whether cells from the myeloid or lymphoid lineage are malignantly transformed, leukemias are divided into myeloid or lymphoid leukemias, respectively. Leukemias can be further classified as acute or chronic. Acute leukemias have a rapid disease onset and progression, in addition to being characterized by a block in cell differentiation. Chronic leukemias on the other hand, have a slow disease progression
and result from early progenitor cells capable of differentiation into mature hematopoietic cells with enhanced proliferative capacity (Gilliland, 2002).

1.3. **Chronic Myeloid Leukemia**

1.3.1. **Historical perspective**

Chronic Myeloid Leukemia (CML) was first described in 1845 independently by the three pathologists Dr. John Bennet, Dr. David Craigie and Dr. Rudolf Virchow (Craigie, 1845; Virchow, 1845; Wong and Witte, 2004). These accounts of CML were described prior to staining methods for blood, which was not practiced until the late 1800s. The cause of CML was later identified by Peter Nowell and David Hungerford in 1960 in their discovery of the Philadelphia (Ph) chromosome (Nowell and Hungerford, 1960). The description of the Ph chromosome marked CML as the first malignancy to be consistently linked to a genetic abnormality. Nowell and Hungerford described the Ph chromosome as a chromosomal abnormality, thought at the time to result from a chromosomal deletion. They hypothesized a causal role for the Ph chromosome in CML after examining leukemic cells from patients with chronic phase CML. Over time, chromosomal banding techniques improved and a shortening of chromosome 22 could be visualized. In 1973, Dr. Janet Rowley described a variant of chromosome 22, the Ph chromosome, to be the result of a reciprocal translocation between the long arms of chromosomes 9 and 22, t(9;22)(q34;q11) (Rowley, 1973). In 1984, Konopka *et al.* detected the 210 kDa BCR-ABL protein for the first time in the Ph-positive CML cell line K562 and described its tyrosine kinase activity, which led to our current understanding of the molecular mechanisms that drive CML.

1.3.2. **Clinical features**

We now know that CML is a malignant clonal disorder of hematopoietic stem cells and has an annual incidence of 1 to 2 cases per 100 000 per year. All age groups, including children, can be
affected with CML; however, the median age at presentation is 64 years (NCI SEER Cancer Statistics Review 2005-2009). CML accounts for 15% of all leukemia cases, with an estimated survival rate of 90% at 5 years and a 2% annual mortality rate (Hochhaus, 2011).

The most common abnormality used to diagnose CML is splenomegaly, which is found in about half of CML patients. Other symptoms of disease include fatigue, anorexia, and weight loss. Yet, approximately 40 per cent of patients are asymptomatic, in which case the CML diagnosis is based solely on an abnormal white blood cell count (Nash, 1999).

CML is characterized by 3 disease phases: chronic (initial) phase, accelerated phase, and blast crisis phase. The chronic phase has a consistent, benign presentation in patients, characterized by immature and mature myeloid cell expansion and the retention of hematopoietic differentiation (Cortes, 2004). Between 90-95% of patients will be diagnosed in this phase of the disease. In the absence of treatment, the disease will progress within a median duration of 3-4 years through an accelerated phase into blast crisis, an invariably fatal acute leukemia (Druker, 2008).

The criteria for diagnosing the transition into accelerated or blast phases are more variable than those for chronic phase diagnosis. Both accelerated and blast phases manifest in a severe reduction in cellular differentiation, with a displacement of mature cells by immature, undifferentiated cells known as blasts. Blast crisis is characterized by the accumulation of blasts in the peripheral blood or bone marrow, or the presence of extramedullary infiltrates of blasts (Jamieson et al. 2004). In blast crisis more than 50% of patients present with myeloid blasts in a disease resembling acute myeloid leukemia (AML), about 30% of patients present with a pre-B cell disease similar to acute lymphocytic leukemia (B-ALL), and 10% of patients develop erythrocytic malignancy. The development of T-cell blastic disease (T-ALL) in blast crisis is rare (Cortes, 2004).

The progression into blast crisis is likely due to the accumulation of additional genetic and/or epigenetic abnormalities, leading to the progressive loss of potential for terminal differentiation of the leukemic clone. Nevertheless, with current therapies the median survival might reach 30 years
or more, where some patients may never progress into late-stage disease and die of their leukemia
(Druker, 2008).

1.4. Acute Lymphoblastic Leukemia

Acute Lymphoblastic Leukemia (ALL) is a clonal disease in which early lymphoid progenitors
(lymphoblasts) expand rapidly and outcompete normal bone marrow populations. Lymphoblasts,
which take the form of precursor B- or T-cells, may also proliferate in other hematopoietic tissues
such as the spleen, lymph nodes and liver. A marked decrease in normal blood elements leads
individuals with ALL to present with anemia, thrombocytopenia, and neutropenia. Fever with a
lack of other signs of infection is a common symptom of ALL (Fielding, 2011).

Genetic alterations responsible for ALL include chromosomal translocations that create fusion
genes encoding activated tyrosine kinases and aberrant transcription factors, overexpression of
proto-oncogenes, and hyperdiploidy involving more than 50 chromosomes. These genetic
abnormalities result in a block in differentiation, enhanced cell proliferation, decreased apoptosis,
and altered capacity for self-renewal (Rabin and Poplack, 2011).

The Ph chromosome is the genetic alteration causative in 25% of adult and 5% of pediatric
cases of ALL. Of these Ph-positive ALL patients, about one-third express p210 BCR-ABL, and the
remainder express the p190 isoform (Fielding, 2011). Ph-positivity in ALL is associated with an
extremely poor prognosis, a markedly lower chance of complete remission (CR) than those with
Ph-negative disease, and a median survival of 8 months (Fielding, 2011). Use of BCR-ABL-
targeted tyrosine kinase inhibitors (TKIs) in therapy demonstrate promise in increasing CR rates,
but longer follow-up is required to assess their impact on remission duration and long-term
survival. In the current treatment of ALL, hematopoietic stem cell transplant (HSCT) is the most
likely curative therapy and its combination with the pre-emptive use of Imatinib (or second-
generation TKIs) appears to reduce relapse rates (Ottmann and Pfeifer, 2009; Fielding, 2011).
1.5. The BCR-ABL Tyrosine Kinase Fusion

Protein tyrosine kinases are enzymes that transfer phosphate from adenosine triphosphate (ATP) to specific tyrosine amino acid residues on substrate proteins. Phosphorylation of these tyrosine residues frequently leads to the activation of signal transduction pathways, which regulate various cellular processes, such as cell growth, differentiation, and death.

The *BCR-ABL* oncogene results from a t(9;22)(q34;q11) reciprocal translocation that fuses the Abelson murine leukemia viral oncogene homolog 1 (*ABL*) gene that is normally found on chromosome 9 with the breakpoint cluster region (*BCR*) gene on chromosome 22 (Rowley, 1973).

The *ABL* proto-oncogene encodes a 145 kDa non-receptor protein tyrosine kinase that is ubiquitously expressed (Hantschel and Superti-Furga, 2004). It is localized at several subcellular sites, including the cytoplasm, nucleus, mitochondria and the endoplasmic reticulum, and interacts with various proteins such as adapter proteins, kinases, phosphatases, transcription factors, and cytoskeletal and cell-cycle regulators (Ren, 2005). Most notably, ABL regulates cytoskeletal architecture and cell migration by mediating signal transduction from cell surface growth factor and adhesion receptors, but has also been implicated in processes of cell differentiation, growth and survival, and oxidative stress (Hantschel and Superti-Furga, 2004). *Abl*-deficient mice are osteoporotic, small with abnormal head and eye development, and have increased incidence of perinatal mortality and lymphopenia (Tybulewicz *et al.* 1991; Li *et al.* 2000).

The BCR gene product is a 160 kDa cytoplasmic phospho-protein. BCR has been shown to have serine/threonine kinase activity, GTPase-activating function (Maru and Witte, 1991) and contains multiple signalling domains. BCR-deficient mice develop normally, with the exception of elevated oxygen metabolite production following neutrophil activation (Voncken *et al.* 1995).
1.5.1. Isoforms of BCR-ABL

In comparison to normal cellular ABL, BCR-ABL is entirely cytoplasmic (Hantschel and Superti-Furga, 2004). Three different clinically relevant forms of BCR-ABL protein- p190, p210 and p230- can arise due to distinct breakpoints in the BCR gene (Figure 1.1). Fusion proteins with molecular weights of 190, 210 and 230 kDa are formed due to the translocation of either BCR exon 1 (Fainstein et al. 1997), exons 1-12/13 (Groffen et al. 1994), or exons 1-19 (Saglio et al. 1990), respectively, at the amino-terminus to ABL in the C-terminus. Almost the entire ABL gene is retained in the three isoforms of BCR-ABL and expression of the fusion gene is driven by the BCR promoter. These 3 different BCR-ABL proteins are each associated with a particular leukemia. Two-thirds of patients with Ph-positive ALL, as well as some rare cases of CML, carry the p190 protein (Fielding, 2011). The p230 protein is associated with neutrophilic CML, a disease more indolent than typical CML (Verstovsek et al. 2002). p210 is the causative agent in 95% of CML cases and is also associated with 5% of childhood and 15-30% of adult Ph-positive ALLs (Ren, 2005; Fielding, 2011). Thus, p210 is the isoform that has been, and continues to be, most widely studied in order to understand BCR-ABL-mediated pathogenesis and inform rational drug design.
Figure 1.1. Three isoforms of the BCR-ABL fusion protein are generated by reciprocal chromosomal translocations. BCR has 3 main breakpoint cluster regions (m-bcr, M-bcr, and µ-bcr) and ABL contains 2 alternative first exons (1b and 1a). Breakpoints within BCR and ABL genes result in different fusion transcript variants: e1a2 encoding the 190 kDa fusion protein (p190), e12a2 or e13a2 create the 210 kDa protein (p210) and e19a2 forms 230 kDa BCR-ABL (p230). Adapted from Quintás-Cardama and Cortes (2009) Blood.
1.5.2. BCR-ABL signal transduction and molecular mechanisms of action

Expression of BCR-ABL in the HSC results in enhanced cell proliferation, survival, and the loss of adhesion dependence (Ren, 2005). It exerts these molecular changes by modulating many different signal transduction pathways through its various domains/motifs (Figure 1.2). *In vivo* structure-function analyses of BCR-ABL domains/motifs have revealed important insights into its mechanism of action. Notably, utilization of mutant constructs of the BCR-ABL protein in bone marrow retroviral transduction and transplantation (BMT) models has led to the identification of many key signalling modules and has driven our understanding of the BCR-ABL signalling pathway. Using this method, investigators have demonstrated that in addition to the ABL kinase domain; the SRC-homology (SH) 2 domain (Zhang *et al.* 2001a), the coiled-coil (CC) domain and tyrosine 177 (Y177; Million and Van Etten, 2000; He *et al.* 2002) are all required for induction of a CML-like myeloproliferative disease (MPD). BMTs employing BCR-ABL with a deletion of the SH3 domain, the actin-binding domain, or the entire carboxy-terminal region were all still able to induce CML-like MPD in mice, demonstrating that these domain functions are either redundant or dispensable for BCR-ABL-driven MPD development (Gross *et al.* 1999).

*The kinase domain*

The oncogenicity of the BCR-ABL fusion protein results from its increased tyrosine-kinase activity through the ABL kinase domain (Hantschel and Superti-Furga, 2004). ABL kinase activity is essential for BCR-ABL-mediated transformation in cultured cells, as well as leukemogenesis *in vivo*. Inactivation of the kinase activity of ABL can be achieved by point mutation in the region encoding the ATP-binding site. In BMT models using such kinase deficient mice, leukemia did not develop (Zhang and Ren, 1998), qualifying ABL as an effective target for treating CML (discussed in detail in section 1.6).
Figure 1.2. Mechanisms of BCR-ABL signal transduction.

BCR-ABL (p210) activates several signalling pathways. BCR-ABL dimerize resulting in trans-autophosphorylation and activation of tyrosine kinase activity (as indicated by vertical arrows between the proteins). Phosphorylated Y177 binds GRB2, promoting the downstream activation of the RAS and PI3K pathways and ultimately leading to an increase in cell proliferation and survival. The ABL SH3 domain can recruit proteins involved in regulating cell adhesion and migration. Solid arrows indicate direct interactions, whereas broken arrows indicate multiple steps. ABD, actin-binding domain; CC, coiled-coil; DBD, DNA-binding domain; DH, Dbl/CDC24 guanine-nucleotide exchange factor homology; NES, nuclear exporting signal; NLS, nuclear localization signal; PP, proline-rich SH3 binding site; S/T-K, serine/threonine kinase; Y-K, tyrosine kinase. Figure was adapted from Ren (2005) Nat Reviews Cancer.
A single tyrosine-phosphorylated site in the activation loop of the ABL kinase domain (Y1294) demonstrated ability to activate the RAS signalling pathway by an unknown mechanism (Goga et al. 1995). Alteration of this residue by point mutation Y1294F abrogated the ability of BCR-ABL to induce leukemogenesis in mice, suggesting the importance of Y1294 in the activation of the BCR-ABL signalling pathway.

Nevertheless, activation of the ABL kinase alone has been shown to be insufficient in the induction of BCR-ABL-driven MPD (Gross et al. 1999). Additional regulatory domains and motifs must be recruited to ABL to facilitate BCR-ABL-driven neoplastic growth.

The CC domain

BCR-ABL proteins form dimers or tetramers and oligomerize at the N-terminal CC domain of BCR that is encoded by the first 63 amino acids (McWhirter and Wang, 1991). This oligomerization facilitates intermolecular autophosphorylation, leading to the hallmark activation of constitutive tyrosine kinase activity, as well as association of BCR-ABL with actin fibers (McWhirter et al. 1993). A mutant form of BCR-ABL with a deletion of the CC domain was unable to induce a MPD in mice, but did drive T-cell leukemia/lymphoma development with a long disease latency (Zhang et al. 2001a; He et al. 2002). However, the ability to induce a MPD was restored in most recipient mice when the CC domain deletion was combined with deletion of the SH3 domain, which reactivates ABL kinase activity (Zhang et al. 2001b). Thus, the CC domain is essential to induce MPD in mice due to its ability to activate ABL kinase activity.

Tyrosine 177

Phosphorylation of Y177 in the BCR region is another important step in BCR-ABL signal transduction. Phosphorylation at Y177 creates a high-affinity binding site for the SH2 domain of growth factor receptor-bound protein 2 (GRB2), which in turn forms a complex with the RAS
guanine nucleotide exchange factor (GEF) son of sevenless (SOS) and the adapter protein GRB2-associated binding protein 2 (GAB2) (Pendergast et al. 1993; Puil et al. 1994; Sattler et al. 2002). The formation of this complex has been shown to be responsible for activation of RAS, which specifically activates the ERK signaling pathway. In the ERK signalling pathway, RAS signals to RAF, leading to MAPK kinase 1 and 2 (MEK1/2) and downstream extracellular-regulated kinases 1 and 2 (ERK1/2) activation; ultimately leading to increased proliferative signals in the cell. Studies show that each of these MAPK proteins exhibit enhanced activity in the presence of BCR-ABL, supporting this mechanistic pathway (Cortez et al. 1997). Furthermore, GAB2 leads to recruitment of the SH2 protein tyrosine phosphatase 2 (SHP2) and phosphatidylinositol 3-kinase (PI3K), thereby mediating the activation of the PI3K/AKT signalling pathway (Sattler et al. 2002). AKT activates a range of downstream targets that promote cell survival.

Mutation of tyrosine 177 to phenylalanine (Y177F) abrogates GRB2 binding, but does not affect ABL kinase activity (Pendergast et al. 1993). BMT mouse models employing the Y177F mutant show that Y177 is required for induction of BCR-ABL-mediated myeloproliferative disease (MPD), demonstrating the importance of the interaction between BCR-ABL and GRB2 in mediating leukemogenesis (Million and Van Etten, 2000; He et al. 2002).

SH2 Domain

SH2 domains are a large family of phospho-tyrosine binding moieties that mediate intermolecular protein interactions and have particular sequence specificity (Pawson et al. 2001). In BCR-ABL-mediated signalling, the SH2 domain of ABL can activate the RAS oncoprotein by binding the adapter protein SHC, which when activated can recruit GRB2 (Goga et al. 1995). Both mutation and deletion of the SH2 domain induced a CML-like MPD in some mice only after a significant delay, and remaining recipients succumbed to B-ALL (Roumiantsev et al. 2001).
The ABL SH2 domain has been shown to activate the adjacent tyrosine kinase domain by establishing a tight interface between the SH2 domain and the N-terminal lobe of the kinase domain (Filippakopoulos et al. 2008). Disruption of the SH2-kinase domain interface completely abolished BCR-ABL kinase activity, and was thus, found to be critical in maintaining the active conformation of BCR-ABL (Greblen et al. 2011). Therefore, the SH2 domain plays an indispensable role in realizing the leukemogenic capacity of BCR-ABL.

**Functional overlap between domains**

Investigators have shown that certain domains serve redundant functions, which is suggestive of the evolutionary importance of the roles of these domains. The SH3 domain and the C-terminal proline rich region (PP, or SH3 binding site) of ABL are examples of such domains. Mouse models with single mutations in either domain were able to fully induce BCR-ABL-mediated leukemogenesis, but mice lacking both the SH3 and PP domains were not (Dai et al. 2001). Deleting both the ABL SH2 and SH3 domains of BCR-ABL also resulted in a more severe outcome than the deletion of either domain alone (Nieborowska-Skorska et al. 2001).

1.5.3. **In vivo models of BCR-ABL-mediated disease**

Studies have repeatedly demonstrated that BCR-ABL expression alone is sufficient for the induction of either chronic phase CML or ALL. Several *in vivo* models of BCR-ABL-induced leukemia have been employed to elucidate the molecular mechanisms associated with the *BCR-ABL* oncogene and to find potential therapeutic targets. These include BCR-ABL transgenic, inducible, and bone marrow retroviral transduction and transplantation models.
Transgenic and inducible models of BCR-ABL malignancy

Transgenic models of BCR-ABL proved to be challenging and for the most part did not provide effective models for the study of CML. The expression of BCR-ABL from the BCR promoter resulted in embryonic lethality due to the toxicity of the constitutively active tyrosine kinase during development (Heisterkamp et al. 1991). BCR-ABL was then expressed by metallothionein-inducible promoters, which did not result in CML-like MPD, but mice did succumb to T-ALL (Honda et al. 1995).

Expression of p210 BCR-ABL driven by the promoter of the gene of hematopoietic tyrosine kinase tec proved to be an effective mouse model for Ph-positive leukemia. These models revealed that two distinct leukemias- ALL in founder mice and CML-like MPD in founder progeny- can arise from p210 BCR-ABL expression in hematopoietic progenitor cells (Honda et al. 1998).

Inducible models with BCR-ABL expression controlled by a tetracycline-responsive promoter demonstrated that when BCR-ABL is expressed in B-cell and megakaryocytic progenitors, B-ALL and megakaryocytic malignancies will be generated, respectively (Huettner et al. 2003; Koschmieder et al. 2005). If BCR-ABL is expressed in hematopoietic stem cells, however, a chronic phase CML-like disease is induced and some mice subsequently succumb B-cell lymphoblastic disease, resembling human lymphoid blast crisis. These inducible models are useful in studying both the disease initiation and the progression to advanced disease phase, particularly blast crisis. Various other genes have been knocked in/down in inducible models to study their role in the progression toward blast crisis CML, and whether functional cooperation of BCR-ABL and other genetic abnormalities is required for blast crisis.
Bone marrow transduction and transplantation (BMT)

The murine BMT assay is an effective way to model human CML and B-ALL. The BCR-ABL oncogene is retrovirally transduced into mouse bone marrow cells (BMCs), followed by transplantation of these cells into irradiated syngeneic mice.

In order for CML to develop in these models, BCR-ABL must infect HSCs, otherwise different diseases will be induced (Huntly et al. 2004). Enrichment of HSCs in BMC populations is accomplished either by pre-treatment of donor mice with 5-fluorouracil (5-FU), or by sorting through FACS. Other progenitor BMCs are transduced when donor cells are not enriched for HSCs, in which case other forms of leukemia, such as B-ALL, T-ALL, erythroleukemia, and macrophage disease, can develop (Van Etten, 2002).

ALL will develop in mice if committed lymphoid progenitors are transduced with BCR-ABL (Chen et al. 2009). As such, pro-B cells have been shown to serve as leukemia stem cells (LSCs) for B-ALL (Hu et al. 2006).

BCR-ABL BMTs give rise to a full-blown MPD within 3-7 weeks on a C57BL/6J background (Hu et al. 2004). Common characteristics of murine CML-like disease include an increased peripheral blood leukocyte count (in CML with a predominance of granulocytes), splenomegaly, pulmonary hemorrhages due to granulocytic infiltration, and extramedullary hematopoiesis in liver. The disease is generally polyclonal and can successfully elicit disease in secondary transplanted recipient mice (Li et al. 1999). Lymphoid disease can be observed in recipients of wild-type p210 BCR-ABL-transduced BMCs on a C57BL/6J background between 4-8 weeks after transplantation. The disease is characterized by circulating malignant lymphoblasts and high peripheral blood (PB) leukocyte count, lymphadenopathy, moderate splenomegaly (with an average splenic weight of 0.25 g), and hemorrhagic pleural effusion that results in death (Hu et al. 2004).
In the production of a high titer retrovirus, bicistronic retroviral vectors are used that co-express BCR-ABL and the fluorescent marker GFP from a single mRNA via an internal ribosome entry site (IRES). The most commonly used vector is a derivative of the murine stem cell virus (MSCV), in which the expression of the internal sequences is driven by long terminal repeat (LTR) promoters (Pear et al. 1998; Li et al. 1999). Co-expression with GFP allows investigators to titer retroviral stocks and detect BCR-ABL-transduced cells in diseased mice by flow cytometric analysis of GFP fluorescence (Pear et al. 1998).

1.6. Therapies for BCR-ABL-positive leukemia

1.6.1. Early therapies

After CML was originally described in 1845, Fowler’s solution, with the active ingredient potassium arsenite, was used in CML therapy (Druker, 2008). Spleen irradiation then became a common treatment up until the 1900s, when radiotherapy and chemotherapy, specifically busulfan and hydroxyurea, became mainstay treatments (Goldman, 2009). Allogeneic HSCT was established in the late 1970s as the first curative therapy and an important landmark in the management of CML. Although survival curves with HSCTs improved dramatically (60-80% survival rate), this procedure was not an option for most patients, as only young patients with HLA-matched donors are eligible for HSCT and the median age of onset of CML is 64 years. Additionally, HSCT was associated with a 30% treatment-related mortality at 2 years, a 22% relapse rate, and potential of prolonged illness due to immune suppression and chronic graft versus host disease (Gratwohl et al. 2006).

1.6.2. Interferon-alpha

Therapy with interferon-alpha (IFN-α) was introduced in 1983, where patients were given a high daily dose of IFN-α, alone or in combination with the chemotherapeutic agent cytarabine (Guilhot
et al. 1997). The precise mechanism of the anti-leukemic effects of IFN-α are unknown, however, in vitro studies suggest that it may enhance immune regulation, have selective toxicity against the leukemic clone and alter hematopoiesis by modulating the bone marrow microenvironment (Dowding et al. 1993). Although it proved to be more effective than previous therapeutic agents, at least in a minority of patients; the range of treatment response was wide with IFN-α - from no hematologic response to complete suppression of the leukemic clone (Dowding et al. 1993; The Italian Cooperative Study Group on Chronic Myeloid Leukemia, 1994). While useful, IFN-α still did not provide an effective treatment option for most CML patients.

1.6.3. Tyrosine kinase inhibitors

An inhibitor of the BCR-ABL kinase was predicted to be an effective and selective therapeutic agent after it was established that BCR-ABL constitutive kinase activity is the cause of CML, that it is present in all CML patients, and that it is unique to leukemic cells (Druker, 2008). TKIs have been the mainstay of Ph-positive disease management since their inception.

Imatinib

In 1993, Nick Lydon and his group developed a series of 2-phenylaminopyrimidine compounds, one of which was STI571 (Imatinib mesylate, Glivec, Gleevec; Novartis Pharmaceutical Corp., Basel, Switzerland) (Buchdunger et al. 1995). Druker and colleagues discovered that Imatinib specifically targets BCR-ABL, but it was also found that Imatinib inhibits multiple other protein kinases, namely ABL, platelet-derived growth factor (PDGFR; Buchdunger et al. 1995), and c-Kit (Savage and Antman, 2002). It is commonly used as a c-Kit inhibitor for the treatment of gastrointestinal stromal tumours (GIST; Tuveson et al. 2001). In the context of BCR-ABL, Imatinib acts as an ATP-competitive inhibitor that binds to the ATP-binding site or P-loop of ABL. Through this mechanism, it impairs BCR-ABL-mediated transfer of phosphate to its substrates and
therefore, signal transduction downstream of BCR-ABL (Deininger et al. 2005). Targeted BCR-ABL inhibition results in enhanced apoptosis and decreased cell proliferation of cells carrying BCR-ABL.

After in vitro studies established that Imatinib effectively killed proliferating cells containing BCR-ABL, in both Ph-positive CML and ALL, in vivo studies demonstrated that continual oral Imatinib therapy suppressed BCR-ABL-positive malignancy in mice with minimal side effects (Druker, 2008). After Imatinib successfully passed phases I (Druker et al. 2001) and II clinical trials (Kantarjian et al. 2002), the landmark Phase III IRIS trial (International Randomized Study of Interferon versus STI571) was the first to demonstrate significantly improved outcomes in CP-CML patients treated with Imatinib compared to those treated with IFN-α (O’Brien et al. 2003). Imatinib treatment in CP-CML patients was well tolerated with hematologic (normal complete blood count), cytogenetic (no Ph-positive cells) and molecular remission (negative reverse-transcriptase polymerase chain reaction (RT-PCR) result for BCR-ABL transcript), leading to its approval as first-line therapy in 2001.

Irrespective of its success, Imatinib has its limitations. In a follow-up study eight years after the IRIS trial, 45% of patients that received first-line Imatinib had discontinued therapy, either due to unsatisfactory results or toxicity (Eiring et al. 2011). Patients must remain on Imatinib treatment for their lifetime, with few exceptions. This raises the major concern of non-compliance to the treatment regimen (Hochhaus, 2011). Residual disease following treatment, that is the persistence of BCR-ABL expression, and resistance to Imatinib are the major obstacles in the treatment of Ph-positive leukemia. Additionally, patients with advanced phases of CML and those with Ph-positive ALL respond with a much lower frequency than those treated with Imatinib in CP-CML. Imatinib treatment combined with chemotherapy in Ph-positive ALL treatment is associated with progression-free survival rate of 60% after 2 years, compared to Imatinib treatment with a
progression-free survival rate of 83% after 5 years found in CP-CML patients (Fielding, 2011; Schiffer, 2007).

Relapse due to Imatinib resistance is associated with amplified BCR-ABL expression, or most frequently, mutations in BCR-ABL. Several mechanisms of resistance have been proposed, the most common of which are point mutations in the kinase domain of BCR-ABL, which reduce or preclude the ability of Imatinib to bind to the P-loop (Deininger et al. 2005). In order to overcome resistance, in some cases the Imatinib dose can be increased, however, the use of more potent “second-generation” TKIs have been effective in eliminating most Imatinib-resistant mutants (Hochhaus, 2011). The exception is the “gatekeeper” mutation at threonine 315 (T315I), which confers resistance to Imatinib, as well as second-generation inhibitors (Branford et al. 2002; Woessner et al. 2011).

Second-generation TKIs

These second-generation BCR-ABL inhibitors Dasatinib (Talpaz et al. 2007) and Nilotinib (Kantarjian et al. 2006), which were first approved for the treatment of patients who failed Imatinib treatment, are more potent inhibitors than Imatinib. These inhibitors were found to induce higher early cytogenetic and molecular response rates than imatinib, which are both major determinants of CML outcome (Jabbour et al. 2011). Both Dasatinib and Nilotinib have since also been approved as first-line therapies for CP-CML.

1.7. The ABL SH3 binding protein 2 (SH3BP2/3BP2)

The Abl SH3 binding protein 2 (3BP2) is a cytoplasmic adapter protein (Jevremovic et al. 2001). Adapter proteins are essential components of signal transduction pathways that control multiple cellular functions, such as cell proliferation, differentiation and transcriptional regulation. Although adapter proteins lack catalytic activity they can provide a physical linkage between
signaling components, including coupling receptor activation to transcriptional activation. Adapter proteins contain protein binding motifs and domains that allow for protein-protein and protein-lipid interactions.

1.7.1. **Identification and expression**

Along with 3BP1, 3BP2 was originally isolated by Ren *et al.* (1993) in a screen using a mouse λgt11 cDNA expression library with a fusion protein containing the SH3 domain of the ABL tyrosine kinase. 3BP2 was found to bind tightly to the SH3 domain of ABL. In 1998, Deckert *et al.* determined the biological function of 3BP2 as an adapter protein involved in the assembly of antigen receptor-induced signalling complexes. 3BP2 was identified as a SYK family kinase interacting protein that is highly expressed in hematopoietic cells, but is also ubiquitously expressed in other tissues (Deckert and Rottapel, 2006; Chen *et al.* 2007).

The *SH3BP2* gene, which encodes for the 3BP2 protein product, is located in humans on the chromosomal region 4p16.3. This is a region of conserved synteny with mouse chromosome 5. *SH3BP2* is composed of 13 exons, encoding a message of 2.4 kb (Bell *et al.* 1997; Deckert *et al.* 1998). The 3BP2 protein consists of 561 amino acids in human and 559 amino acids in mouse. Human and murine sequences are highly conserved with an 86% amino acid identity, suggesting similar functions in both species.

1.7.2. **Structure and function**

3BP2 contains three modular peptide recognition domains: an N-terminal pleckstrin homology (PH) domain, a central proline-rich region (PRR), and a C-terminal SH2 domain (Figure 1.3). PH domains can bind membrane phospholipids and mediate intracellular trafficking, cell signalling and cytoskeletal remodeling. The PH domain was shown to be important for 3BP2
Figure 1.3. Domain structure and binding partners of 3BP2.
3BP2 contains an N-terminal Pleckstrin-homology (PH) domain, a central proline rich region (PRR) and a C-terminal Src-homology 2 (SH2) domain. The PRR mediates the interaction of 3BP2 with SH3-domain containing proteins, such as ABL, SRC-family kinases and VAV. The SH2 domain binds proteins containing the phospho-Tyr-Glu-Asn(pYEN) motif and other tyrosine-phosphorylated proteins, several of which are listed above the SH2 domain structure. Tankyrase interacts with 3BP2 within a hexapeptide region between the PRR and the SH2 domain and is capable of mediating its degradation. 14-3-3 is capable of negatively regulating 3BP2 activity by binding to phosphorylated Ser-177 (S177). Phosphorylation at Tyr-446 (Y446) mediates the interaction of 3BP2 with LYN and LCK.
function, as its deletion suppressed signal transduction in B- and T-cells (Deckert et al. 1998; Foucault et al. 2003).

PRRs are also referred to as ‘SH3-binding domains’, which are often found as multiple tandem repeats that are involved in complex multi-protein interactions (Williamson, 1994). The PRR is over 200 amino acids in length and consists of several Proline-X-X-Proline (PxxP) motifs that bind the SH3 domains of proteins such as ABL, VAV, and the SRC-family kinases SRC, LYN and FYN (Ren et al. 1993; Deckert and Rottapel, 2006; Levaot et al. 2011a; Maeno et al. 2003; Deckert et al. 1998). The 3BP2 PRR also contains several uncharacterized motifs, which potentially serve as binding sites for WW and EVH1 protein domains (Deckert and Rottapel, 2006).

SH2 domains were originally identified within the SRC tyrosine kinase (Sadowski et al. 1986) and have a high affinity for the recognition of tyrosine-phosphorylated proteins. By these means, SH2 domains play a large role in mediating the recruitment of signalling complexes to activated receptors. SH2 domains vary among different proteins in their capability to recognize specific phosphopeptide motifs (Songyang et al., 1993). The peptide motif that the 3BP2 SH2 domain preferentially binds is phospho-Tyr-Glu-Asn (pYEN) (Songyang et al., 1993). The pYEN motif present in signalling proteins such as the receptor tyrosine kinase (RTK) FLT3, the granulocyte colony-stimulating factor receptor (GCSF-R) and the transmembrane adapter LAT, have been shown to interact with the 3BP2 SH2 domain (Wolf and Rohrschneider, 1999; Kendrick et al. 2004; Deckert et al. 1998). The SH2 domain also mediates the interaction of 3BP2 with tyrosine-phosphorylated proteins that do not contain the pYEN motif, such as the SYK kinases, SYK and ZAP70, CBL, GRB2, VAV, the SHP-1 phosphatase, and PLCγ1/2 (Figure 1.3; Deckert et al., 1998; Sada et al. 2002; Chihara et al. 2011).
Studies demonstrate a role for 3BP2 in mediating osteoclastogenic and osteoblast signalling, as well as signalling downstream of activated immune receptors. Through mostly overexpression studies, 3BP2 was found to be activated downstream of the B-cell and T-cell antigen receptors, the CD244 receptor in Natural Killer (NK) cells, and FcεRI in rat basophilic leukemia RBL-2H3 and mast cells (Deckert et al. 1998; Sada et al. 2002; Maeno et al. 2003; de la Fuente et al. 2006; Foucault et al. 2005; Chen et al. 2007). 3BP2 has also been implicated in endocytic and cytoskeletal regulation through its interaction with CIN85 and HIP-55 (Le Bras et al. 2007), as well as in the oncogenic transformation of mesenchymal stromal cells in response to hypoxia (Proulx-Bonneau et al. 2011).

Tyrosine and serine phosphorylation of 3BP2 have been reported, which are capable of modulating 3BP2 activity. 3BP2 was shown to interact with 14-3-3 in a yeast-two-hybrid screen. 14-3-3 demonstrated the ability to negatively regulate 3BP2 function and this interaction required the phosphorylation of Ser 225 and Ser 277 of 3BP2 by upstream kinases (Focault et al. 2003).

SYK phosphorylates 3BP2 on Tyr 174, Tyr 183, and Tyr 446 in vitro following the activation of the B- and T-cell antigen and Fc receptors (Maeno et al. 2003; Qu et al. 2005). Phosphorylation of both Tyr 183 and Tyr 446 seem to be required for proper 3BP2 function, as mutation of these residues led to decreased 3BP2 activity (Deckert and Rottapel, 2006). Although SYK phosphorylates 3BP2, these proteins seem to function in a reciprocal manner, as several studies have also demonstrated that 3BP2 is required for optimal SYK phosphorylation and that enhanced 3BP2 expression/expression of gain-of-function mutants results in hyperactivation of the SYK and VAV signalling pathways (Maeno et al. 2003; Chen et al. 2007; Levaot et al. 2011b). These findings suggest a role for 3BP2 as a positive regulator of immune receptor-mediated signalling by connecting SYK to downstream signalling effectors.

3BP2 was identified as a regulator of the LYN activation cycle in FcεRI signal transduction. In addition to the 3BP2 PRR mediating the interaction with the LYN SH3 domain,
phosphorylated Tyr 446 of 3BP2 mediates its interaction to the LYN SH2 domain. Moreover, 3BP2-Tyr 446 phosphorylation appears to be important for the regulation of LYN kinase activity via autophosphorylation (Maeno et al. 2003).

In addition to its ability to regulate LYN, 3BP2 can modulate SRC activity and, thereby, SRC’s association with downstream signalling effectors. Overexpression of wild-type 3BP2 and 3BP2 cherubism (gain-of-function) mutants in MC3T3-E1 and RAW264.7 cells, respectively, both enhanced SRC (Tyr 416) phosphorylation (Levaot et al. 2011a; GuezGuez et al. 2010).

3BP2 is also required for phosphorylation of ABL at Tyr 245 in vitro, a residue in the ABL kinase-SH2 linker region that is associated with high ABL kinase activity. Furthermore, the peptide region of 3BP2 that binds to the ABL SH3 domain can directly activate ABL kinase activity in osteoblasts (Levaot et al. 2011a). Songyang et al. (1994) noted that 3BP2 and ABL are also likely to have overlapping targets in vivo.

In summary, 3BP2 is involved in various signal transduction pathways, but has most notably been characterized as an important component in a multiprotein signalling complex composed of SRC family kinases, SYK, and VAV. Activation of this complex is, furthermore, crucial for B-cell antigen receptor and integrin activation (Chen et al. 2007; de la Fuente et al. 2006; Levaot et al. 2011a, b), and may also be important in regulating other hematopoietic signal transduction pathways.

1.7.3. Characterization of 3bp2<sup>−/−</sup> mouse models

3bp2<sup>−/−</sup> mouse models have illustrated the importance of 3bp2 in bone homeostasis, and B-cell maturation and function. Mice that are 3bp2-deficient have a severely osteoporotic bone phenotype, with decreased bone mineral density and bone strength. This phenotype results from defects in both the osteoclast and osteoblast lineages. Whereas in vitro studies using the mouse monocytic cell line RAW264.7 demonstrate the requirement of 3BP2 for osteoclast differentiation
(GuezGuez et al. 2010), in vivo 3bp2–/– osteoclast defects were not due to a failure to differentiate, but rather due to their inability to form podosomes and ruffled borders required for proper osteoclast function (Levaot et al. 2011a). The osteoclast defect was associated with abolished SRC activation in response to integrin stimulation. A lack of phosphorylation and activation of Abl in osteoblasts in the absence of 3bp2 was associated with maturation defects (Levaot et al. 2011a).

3bp2-deficient mice also demonstrate B-cell proliferation, cell cycle progression, calcium mobilization and survival defects following ligation of the B-cell receptor (de la Fuente et al. 2006; Chen et al. 2007). These mice have normal numbers of mature B-cells, but increased populations of pre-B cells in the bone marrow and splenic marginal-zone B-cells, a block in maturation of transitional B-cells in the spleen, reduced numbers of peritoneal B1 B-cells, and a defective thymus-independent type 2 antigen response. Separate studies showed that 3bp2–/– B-cells had severely diminished phosphorylation of SYK, as well as ERK, JNK and PLCγ2 activation in response to B-cell receptor cross-linking (Fuente et al. 2006; Chen et al. 2007), highlighting the importance of 3bp2 as a positive regulator of signal transduction.

T-cell abnormalities were not observed in the 3bp2–/– mice after examination of T-cell maturation, proliferation, cytokine activity and signalling (de la Fuente et al. 2006).

1.7.4. The role of 3BP2 in cherubism

Our understanding of 3BP2 grew after the discovery that 3BP2 mutations cause a disorder known as cherubism (Ueki et al. 2001). Cherubism is characterized by rapid bone degradation in the maxilla and/or mandible, and the accumulation of painless cysts in the jaw region, which are filled with fibrous/inflammatory tissue. This phenotype arises as a result of hyperactive osteoclast activity and infiltrating mesenchymal and inflammatory cells. Cherubism is also associated with dental abnormalities, such as early tooth loss and lack of permanent tooth eruption (Kozakiewicz et
deformation of the orbital socket leading to an upturned eye appearance that can be associated visual loss as a result of optic atrophy (Font et al. 2003); and obstructive sleep apnea and upper airway obstruction due to tongue displacement (Battaglia et al. 2000). The condition was termed cherubism due to the resemblance of those affected to the cherubs in Renaissance art. Cherubism is a rare genetic disorder with autosomal dominant inheritance, although cases of non-familial cherubism have also been reported (Jain et al. 2007). The disorder manifests in early childhood (2-5 years of age) and is not present at birth. Lesions in the jaw grow progressively, until they eventually regress after puberty. Cherubism was poorly understood until it was associated with 3BP2 mutations in families with the disorder (Ueki et al. 2001). All 3BP2 mutations associated with cherubism are missense mutations that fall within a six amino acid sequence, RSPPDG, located 31-36 amino acids upstream of the SH2 domain and 205-210 amino acids downstream of the proline-rich region (Ueki et al. 2001). Mutations are most frequently observed in proline 418 (to Leu, Arg or His), but can also be found in Gly 420 (to Glu or Arg) and Arg 415 (to Pro or Gln) (Ueki et al. 2001).

It was noted that 3BP2 is located in a chromosomal region that is often deleted in individuals with Wolf-Hirschhorn Syndrome (WHS; Zollino et al. 2000). WHS patients, however, do not exhibit cherubism or related symptoms, which provided the initial support for a gain-of-function explanation causative in cherubism. Further support to the gain-of-function hypothesis was provided by Ueki et al. (2007), who created mice with the most common 3bp2 cherubism mutation, a homozygous proline-to-arginine (P418R in humans, P416R in mice) substitution, which displayed the major characteristics of cherubism (systemic inflammation, bone loss, increased osteoclast differentiation) and shared the same pattern of activation of signalling molecules as found in overexpression models of wild-type 3bp2. These signalling characteristics included increased ERK1/2-mediated TNF-α expression in macrophages and elevated SYK activation in osteoclasts in vitro.
The molecular mechanism driving cherubism pathogenesis was then described as enhanced 3BP2 protein stability resulting from 3BP2 cherubism mutations, which furthermore increase the ability of 3BP2 to promote osteoclast differentiation through signal transduction (Levaot et al. 2011b). ADP-ribosylation of 3BP2 by the poly (ADP-ribose) polymerase (PARP) family member Tankyrase controls 3BP2 protein levels, as the ribosylation is coupled to ubiquitylation of 3BP2 by the E3 ubiquitin ligase RNF146. Cherubism mutations in the hexapeptide sequence disrupt the binding of Tankyrase functional ankyrin repeat clusters (ARCs) to 3BP2 (Guettler et al. 2011), thereby abolishing Tankyrase-mediated degradation. The increased 3BP2 protein stability furthermore leads to enhanced activation of SRC, SYK and VAV signalling.

Therefore, 3BP2 has the capacity to mediate crucial signal transduction pathways and the deregulation of its activity can lead to a disease state.
1.8. **Study Rationale**

A detailed understanding of BCR-ABL signal transduction is crucial, as it may lead to novel therapeutic approaches in CML and Ph-chromosome positive ALL by illuminating key targets in the signalling pathway. 3BP2 is an adapter protein that has many shared interaction partners with BCR-ABL, such as the SRC-family kinases, CBL and GRB2 (Deckert *et al.* 1998). More importantly, 3BP2 is capable of directly activating ABL kinase activity (Levaot *et al.* 2011). Aberrant ABL kinase activity in BCR-ABL represents the foundation of its oncogenic potential, and therefore, 3BP2 is an attractive candidate to analyze in BCR-ABL-mediated signal transduction and leukemia.

**Hypothesis:**

3BP2 plays a critical role in BCR-ABL signal transduction and leukemia.

1.9. **Aims of Study**

**Aim 1:** Examine effects of 3BP2 overexpression and identify 3BP2-associated proteins in Chronic Myeloid Leukemia (CML) cell lines to analyze the role of 3BP2 in BCR-ABL signal transduction.

**Aim 2:** Analyze the contribution of 3BP2 in a retroviral transduction and bone marrow transplantation model expressing BCR-ABL.
CHAPTER 2: MATERIALS AND METHODS
2.1. DNA Constructs

The p3xFLAG CMV10 construct expressing full-length murine 3bp2 with a neo selection marker was kindly provided by R. Rottapel (Toronto, ON). MSCV-IRES-GFP (MIEV) BCR-ABL\textsuperscript{p210} was courteously supplied by M. Carroll (Philadelphia, PA). The vesicular stomatitis virus G (VSV-G) expression plasmid was a gift from G. Sauvageau (Montreal, QC). The vector expressing Gag and Pol sequences (SV-\psi−env−) was kindly provided by J. Dick (Toronto, ON).

2.2. Cell Culture

The CML-T1, EM2, LAMA84, MC3, MEG01 and K562 CML cell lines, as well as the mouse monocytic cell line RAW264.7, were grown in RPMI Complete media (RPMI 1640 with antibiotics, containing 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS; GIBCO, Grand Island, New York).

CML-T1-3BP2 cells were generated by electroporation of 1 x 10\textsuperscript{7} CML-T1 cells with 20 µg of p3xFLAG (CMV10)-3BP2 plasmid at 350 mV and 950 µF. Control CML-T1 cells were electroporated without plasmid. CML-T1-3BP2 cells were grown in RPMI Complete media for one week to allow for recovery from electroporation, after which they were only grown in selection medium (RPMI Complete media supplemented with 1 mg/mL Geneticin (GIBCO, Grand Island, New York)).

Ba/F3 and Ba/F3 BCR-ABL-expressing cells were maintained in RPMI Complete media supplemented with 50 µM β-mercaptoethanol (β-ME; Fisher Scientific, Waltham, MA) and 100 pg/mL of recombinant mouse (rm) Interleukin-3 (IL-3; Cedarlane Labs, Burlington, ON). For experimentation all Ba/F3 cell lines were expanded in WEHI conditioned media as a source of IL-3 (RPMI Complete media supplemented with 10-15% culture supernatant from WEHI-3 cells).
293T cells were expanded in Iscove’s Modified Dulbecco’s Media (IMDM) plus antibiotics, supplemented with 10% FBS for production of the virus used in BMT.

2.3. Animals

Studies were approved by the Animal Care Committee at the Ontario Cancer Institute (OCI), University Health Network, Toronto, ON. 3bp2−/− mice were generously provided by R. Rottapel (OCI, Toronto). The animals used for BMTs were back-crossed on to a C57BL/6J background to a minimum of 9 generations (F9). All animals were housed in the OCI Animal Research Centre in micro-isolator cages and maintained with autoclaved chow and acidified water. C57BL/6J recipient mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

Genomic DNA was isolated from tail clippings using Proteinase K digestion and phenol/chloroform extraction. DNA was dissolved in double-distilled water (ddH2O) and used in the Polymerase Chain Reaction (PCR) amplification of 3bp2 wild-type (WT) or knock-out (KO) sequences to determine mouse genotype. Primer sequences are as follows: WT (5’ – GAG CTG GAT GGC CTT GCT ACG CAG GGA A- 3’), Common (5’ – GGA ATA GGC ACA TAC GAG CCC CAC ACA GG- 3’) and Neo/KO (5’- AAG CGC ATG CTC CAG ACT GCC TTG GGA A- 3’). WT reactions consisted of 300 nM of each the WT and common primers, whereas KO reactions consisted of 300 nM of each the neo and common primers. Reactions contained 200 µM dNTPs (Invitrogen), 300 nM of each oligo, 1X PCR buffer (Invitrogen), 1.5 mM MgCl2 (Invitrogen), 3% dimethyl sulfoxide (DMSO; Fisher), 2.5 units of Taq DNA polymerase (Invitrogen) and 5 µl of DNA. The thermocycler profile includes an initial denaturation at 94°C for 2 minutes, followed by 30 cycles of: denaturation at 94°C for 30 seconds, annealing at 61°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step was run for 7 minutes at 72°C, and the product was preserved at 4°C. Both WT and KO products were 400-500 bp.
2.4. Antibodies

Mouse 4G10 phospho-tyrosine antibody was kindly provided by R. Rottapel (Toronto, ON). SH3BP2 mouse monoclonal antibody (M01, clone 1E9) was purchased from Abnova (Jhongli City, Taiwan). Phospho-AKT (S473), total AKT, phospho-CBL (Y774), phospho-LYN (Y507), total LYN rabbit (2732), phospho-SRC family kinase (Y416), phospho-SYK (Y525/526), and GAPDH antibodies were all obtained from Cell Signaling Technology (Danvers, MA). Anti-FLAG monoclonal mouse (M2) antibody was purchased from Sigma (St. Louis, MO). Anti-phospho-STAT5 was procured from Invitrogen (Carlsbad, CA) and total STAT5 antibody from BD Biosciences (Franklin Lakes, NJ). The monoclonal phospho-ERK1/2 (E-4), goat polyclonal HCK, rabbit polyclonal SYK (C-20), rabbit polyclonal CBL (C-15), and rabbit polyclonal GST (Z-5) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Flow cytometry antibodies that were obtained from BD Biosciences include Rat anti-mouse CD16/CD32 Fc Block (2.4G2); FITC-labeled Streptavidin; Phycoerythrin (PE)-conjugated anti-mouse B220 (RA3-6B2), CD4 (H129.19), Gr-1 (RB6-8C5), TER-119, Thy-1.2 (53-2.1), CD41 (MWReg30); and Peridinin-chlorophyll proteins (PerCP)-Cy5.5-conjugated rat monoclonal anti-CD19 (1D3), CD8a (53-6.7) and CD11b/Mac-1 (M1/70).

Signalling lymphocyte activation molecule (SLAM) marker flow cytometry anti-mouse CD48 Allophycocyanin (APC)-conjugated and CD150 PE-conjugated antibodies were purchased from eBioscience (San Diego, CA).

2.5. Immunoblotting

CML-T1, CML-T1-3BP2, EM2, K562, LAMA84, MC3, MEG01, and RAW264.7 cells were collected and washed once with 10 mM HEPES (pH 7.4)/ Hank’s Balanced Salt Solution (HBSS) plus protease inhibitors (10 mM sodium pyrophosphate (Na₄P₂O₇), 10 mM sodium fluoride (NaF), 10 mM EDTA and 1 mM sodium orthovanadate (Na₃VO₄)).
Ba/F3 and Ba/F3-BCR-ABL cells were cytokine-starved by washing three times with 10 mM HEPES (pH 7.4)/HBSS, followed by incubation in RPMI Complete media for four hours at 37°C. Cells were stimulated with warm RPMI Complete media either in the presence or absence of 100 pg/mL rm IL-3 for 10 minutes, and subsequently washed once with 10 mM HEPES (pH 7.4)/HBSS supplemented with protease inhibitors.

Cell pellets were then collected and resuspended in 1 mL of ice-cold Lysis Buffer (1% (v/v) Triton X-100, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM Na$_4$P$_2$O$_7$, 10 mM NaF, 10 mM EDTA, 1 mM Na$_3$VO$_4$, 1 mM phenylmethyl sulfonyl fluoride (PMSF) and 1 Complete Mini Protease Inhibitor tablet (Roche, Mannheim, Germany)). After a 20 minute incubation period on ice, cell membranes were isolated by centrifugation of lysates at 10,000 g for 10 minutes at 4°C. Lysate protein concentrations were determined by Bradford Protein Assay (Bio-Rad, Hercules, CA) with spectroscopic analysis at 595 nm.

Cell lysates (200 µg) were mixed with an equal volume of 2x sample buffer (69 mM Tris-HCl (pH 6.8), 11% (v/v) glycerol, 2.2% (w/v) sodium dodecyl sulfate (SDS), 0.02% (w/v) bromophenol blue and 50mM dithiothreitol (DTT)) boiled for 5 minutes at 95°C to denature the protein, and resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were then transferred overnight to polyvinylidene fluoride (PVDF) membranes for immunoblotting.

For the 4G10 phospho-tyrosine immunoblot and all immunoblots using Santa Cruz antibodies, membranes were blocked for one hour with blocking buffer (2.5% (w/v) bovine serum albumin (BSA; Sigma-Aldrich) in Tris-buffered saline supplemented with 0.1% (v/v) Tween-20 (TBST; 50mM Tris-HCl (pH 8.0), 150 mM NaCl) and sodium azide). Membranes were incubated for one hour with primary antibody diluted 1:1000 in TBST, after which they were washed three times for 5 minutes with TBST. HRP-conjugated secondary antibodies corresponding to the primary used were then applied at a dilution of 1:5000 in TBST for at least 30 minutes. Membranes were then washed again for 5 minutes in triplicate with TBST, after which proteins
were visualized using Western Lightning™ Chemiluminescence Reagent Plus (ECL; Perkin Elmer, Waltham, MA) and autoradiographic Hyperfilm (GE Healthcare). Membranes were stripped for 30 minutes at 50°C in 63.5 mM Tris-HCl (pH 6.8), 2% (v/v) SDS and 0.1 M β-ME and washed four times with TBST prior to reprobing for other antibodies.

Detection of 3BP2 protein and immunoblots using Cell Signaling Technology antibodies involved blocking for one hour with 5% (w/v) skim milk powder dissolved in TBST (milk). A dilution of 1:1000 of primary antibody in blocking buffer for Cell Signaling antibodies, and in 5% milk for 3BP2 antibody, was applied to the membrane overnight with light agitation at 4°C. Membranes were washed three times 5 minutes and incubated for one hour with the appropriate HRP-conjugated secondary antibody at a 1:5000 dilution in TBST. Three washes were then performed, followed by ECL visualization.

Reprobes for total ERK1/2 involved blocking for 30 minutes with 3% milk and incubation with rabbit polyclonal antibody (0.25 µg/mL in 3% milk) overnight at 4°C. After two washes, HRP-conjugated Protein A secondary antibody was added (1:5000 dilution in TBST) for one hour, and blots were washed four times and developed with ECL.

To probe for FLAG expression, membranes were blocked with 3% milk and mouse monoclonal antibody was added (1:1000 in 2.5% BSA) for one hour. Membranes were washed three times, incubated with HRP-linked anti-mouse secondary antibody (1:5000 in TBST), washed another three times, and visualized with ECL.

For GAPDH reprobing, membranes were blocked with 5% milk for one hour and incubated with rabbit monoclonal antibody at a 1:5000 dilution in 5% milk for one hour, followed by three washes, a 30 minute incubation with HRP-linked anti-rabbit IgG diluted 1:2000 in 5% milk, three washes and detection using ECL.
2.6. **GST Pull-down Assay**

GST fusion proteins (GST control protein and GST fused to 3BP2-SH2 domain) were expressed in BL21 bacteria with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 hours with shaking at 37 °C. Fusion proteins were affinity purified using glutathione-Sepharose beads (GE Healthcare, Cooksville, ON). Purified proteins were resolved by SDS-PAGE and analyzed by Coomassie staining. For GST pull-downs, clarified lysates (see lysate preparation above) were incubated with 4 µg of the indicated GST fusion protein and glutathione-Sepharose beads overnight with mixing at 4 °C. After washing three times with IP wash buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% (v/v) Triton X-100, 10 mM Na₃P₂O₇, 10 mM NaF, 10 mM EDTA and 1 mM Na₃VO₄), the bound proteins were detected by immunoblotting as indicated above.

2.7. **Trypan Blue Exclusion Cell Growth Assay**

CML-T1 and CML-T1-3BP2 cells were each seeded at a density of 1 x 10⁵ cells per 1.9 cm² (one well of 24-well cell culture plate) in RPMI complete media or RPMI complete media supplemented with 1 mg/mL Geneticin, respectively. Cell proliferation was monitored over 7 days. An aliquot of cells was diluted with 0.4% Trypan blue (Sigma) and unstained (viable) cells were counted manually using a hemacytometer. Cell growth assays were repeated in triplicate.

2.8. **Isolation of Stem and Progenitor Cells**

Bone marrow cells were flushed from the long bones (femurs and tibias) with PBS without calcium or magnesium (from here on referred to as ‘PBS’), supplemented with 2% FBS (PBS+), and filtered through the 35 µm nylon mesh screen on cell strainer capped FACS tubes (BD Falcon, San Jose, CA) to create bone marrow cell suspensions. Cells were washed and resuspended in ice-cold degassed MACS Buffer (PBS, 0.5% BSA, 2 mM EDTA). Bone marrow was enriched for lineage negative cells using magnetic cell sorting and the MACS lineage cell depletion kit
(Miltenyi Biotec, Bergisch Gladbach, Germany). Biotin-antibody cocktail (biotin-conjugated monoclonal antibodies for CD5, B220, Mac-1, Gr-1 and Ter119) was added to $1 \times 10^7$ cells, after which cells were incubated with anti-biotin microbeads, washed, and subjected to magnetic separation using MACS LS columns (Miltenyi Biotec). Once cells were lineage depleted, they were incubated with anti-mouse PE-conjugated CD150 antibody (eBioscience), anti-mouse APC-conjugated CD48 antibody, and FITC-labeled streptavidin antibody (to detect residual biotin-labeled lineage-positive cells; BD Biosciences), after which they were analyzed via flow cytometry as described below (Section 2.10).

2.9. Immunophenotypic Analysis of Spleen and Bone Marrow

Spleens were triturated using a wire mesh and run through the cell strainer on a FACS tube to create a cell suspension in PBS+. Bone marrow cell suspensions were prepared as mentioned above. Erythrocytes were lysed in both spleen and bone marrow cell suspensions using 1x ACK (10x ACK: pH 7.3, 155 mM ammonium chloride, 100 µM disodium ethylene diamine tetraacetic acid (EDTA) and 10 mM potassium bicarbonate), after which cells were promptly washed with PBS. Cells were resuspended in FACS blocking buffer (1:1000 dilution of anti-mouse CD16/CD32 Fc Block (2.4G2) in PBS+) and incubated with the following antibodies: PE-conjugated Ter119, PE-conjugated B220 and PerCP-Cy5.5-conjugated CD19, PE- Thy1.2, PE-Gr-1 and PerCP-Cy5.5-Mac-1, PE-CD4 and PerCP-Cy5.5-CD8a, or PE-CD41 antibody. After a 30 minute incubation with antibodies, cells were washed with PBS, fixed, and analyzed via flow cytometry.

2.10. Flow Cytometry

Cells were incubated with FACS blocking buffer and stained with the above-described combinations of fluorescently labeled primary antibodies. All cells were fixed using 2% methanol-free formaldehyde for 10 minutes, washed with PBS, resuspended in 500 µL PBS+ and filtered
through cell strainers. Analysis was done on a FACSCalibur flow cytometer (BD), whereby data were acquired using CellQuest (BD) and analyzed with FlowJo software (Ashland, OR).

2.11. Generation of Retroviral Stocks

Human embryonic kidney 293T cells (hereafter referred to as ‘293T cells’) were thawed 5-7 days prior to transfection for expansion and grown in IMDM media without antibiotics, supplemented with 10% FBS. Cells were harvested via trypsinization, counted using Trypan blue, and plated at 2x10^6 cells per 10 cm culture plate (100 plates). Calcium phosphate transient co-transfections (CalPhos Mammalian Transfection Kit, Clontech, Mountain View, CA) of 293T cells were performed with 10 µg of retrovirus, 10 µg of SV-ψ^-env^- packaging construct and 3.5 µg of VSV-G envelope vector per plate. Cells were washed with PBS and medium was changed 18 hours post-transfection, followed by supernatant collection at 48 and 72 hours. Harvested viral supernatant was immediately filtered (0.4 µM filter) and concentrated by ultracentrifugation at 53 000 g for 2 hours at 4°C. Viral pellets were each resuspended in 100 µL of DMEM and pooled to ensure even distribution of viral particles. The viral stock was then distributed at either 100 or 200 µL into cryovials and frozen on dry ice before transferring the stocks to storage at -80°C.

To determine retroviral titer, 1x10^6 Ba/F3 cells were transduced in a single well of a 6-well suspension dish with 125 µL retroviral supernatant in a total volume of 1 mL WEHI IL-3 conditioned media supplemented with 3 µg/mL Polybrene® (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide, hexadimethrine bromide; Sigma-Aldrich, St. Lois, MO). Infection media was replaced with 2 mL fresh WEHI after 24 hours. At 48 hours after infection cells were collected, fixed using 2% methanol-free formaldehyde and analyzed by flow cytometry for the expression of GFP. The relative viral titer in colony forming units (CFU)/mL was calculated as the percentage of GFP-positive (GFP^+) cells multiplied by the number of cells.
infected \((1\times 10^6)\), all divided by the volume of supernatant applied to the cells in milliliters \((0.125 \text{ mL})\). All retroviral stocks were used at titers of \(2\times 10^5\) CFU/mL for BMC transduction.

### 2.12. Bone Marrow Transduction and Transplantation

Donor and recipient mice were used at six to twelve weeks of age. Donor mice were sacrificed by carbon dioxide (CO\(_2\)) asphyxiation followed by cervical dislocation. BMCs were collected by flushing femurs and tibias with PBS+ . BMCs were counted using Turk’s and plated without erythrocyte lysis into adherent cell culture dishes at a concentration of \(1\times 10^6\) cells/mL in pre-stimulation cocktail. Pre-stimulation cocktail was made by adding 10% FBS, 5% WEHI IL-3 conditioned media, 6 ng/mL rm IL-3, 10 ng/mL rm IL-6, 10 ng/mL rm IL-7 and 50 ng/mL rm stem cell factor (SCF) to High Glucose (H21) DMEM plus antibiotics. All cytokines were purchased from Cedarlane (Burlington, ON). Twenty-four hours after seeding, BMCs were trypsinized, viable cells were counted and cells were transduced with retroviral stocks. BMCs were transduced with pre-stimulation cocktail containing 3 µg/mL of polybrene and retroviral stock at a final infectious dose of \(2\times 10^5\) CFU/mL. At 48 hours, a second round of transduction was performed. At 72 hours transduced BMCs were collected, washed once in PBS and \(5\times 10^6\) WBC/mL were resuspended in PBS.

Recipient male mice were used which were age-matched to donor mice. These recipients were lethally irradiated (9 Gy) prior to receiving an intravenous (IV) tail-vein injection with \(1\times 10^6\) WBCs each.

### 2.13. Analysis of Diseased Mice

After the transplant, recipient mice were monitored daily for signs of morbidity as evidenced by cachexia, weight loss, failure to thrive and splenomegaly. Peripheral blood (PB) of mice was evaluated weekly or after signs of morbidity for WBC count and BCR-ABL/GFP-positive WBCs.
PB was obtained through saphenous vein bleeds and PB smears were made. PB was exposed to erythrocyte-lysis and washed with PBS, after which cells were fixed using 2% formaldehyde and analyzed via flow cytometry to determine the percentage of BCR-ABL/GFP-positive WBCs in the PB.

Premorbid mice were sacrificed by CO₂ asphyxiation, after which spleen, liver, lung, lymph nodes, and bone marrow (flushed as described in Section 2.8) were harvested. The type of disease was diagnosed for each mouse based on immunophenotypic and histopathologic analysis, and PB differential counts. Clinicopathologic features such as splenomegaly, enlarged lymph nodes (LNs), pulmonary hemorrhages, or other abnormalities were also noted. Spleen and bone marrow were subjected to immunophenotypic analysis as described above. For histopathology, the remaining sections of tissues were fixed in 10% neutral buffered formalin (NBF) for 24 hours, after which they were transferred to a 95% ethanol solution. Hematoxylin and Eosin (H&E) sections and slides were prepared by the Pathology Core Centre for Modeling Human Disease (Toronto Centre for Phenogenomics, Toronto, ON). Bone marrow cytospins were prepared. Cytospin and PB smear slides were fixed in methanol and stained with May-Gruenwald and Giemsa (both from EMD Chemicals, Gibbstown, NJ).

2.14. Statistical Analysis

A 2-way ANOVA was used to determine whether cell growth curves were significantly different in Fig. 2.3. The Kaplan-Meier survival curve survival analysis was performed using the log-rank test. For all other data, statistical significance was determined by Student’s t test. For all tests a p-value < 0.05 was considered statistically significant.
CHAPTER 3: RESULTS
3.1. 3BP2 is expressed in CML cell lines

To investigate whether 3BP2 is involved in the BCR-ABL signalling pathway we began by determining whether 3BP2 was expressed in CML cell lines. We examined a panel of CML cell lines for 3BP2 protein expression, using the mouse monocytic cell line RAW364.7 as a positive control due to its high levels of endogenous 3BP2 expression (GuezGuez et al. 2010). All CML cell lines (CML-T1, EM2, LAMA84, MC3, MEG01 and K562) displayed a pronounced protein tyrosine phosphorylation profile, compared to RAW264.7 cells that do not express BCR-ABL (Figure 2.1A). Expression of 3BP2 was observed in all cell lines using an anti-3BP2 antibody that detects 3BP2 as a 75 kDa protein (Figure 2.1A). Three 3BP2 bands with different SDS-PAGE migration patterns were detected that varied in level of expression in each cell line. The unique bands either represent different 3BP2 isoforms or alterations in post-translational modification. Densitometric quantification of the immunoblot confirmed that 3BP2 was most highly expressed in EM2 and LAMA84 cells, at approximately equal intermediate levels in MC3, MEG01 and CML-T1 cells, and was detected least in K562 cells (Figure 2.1B).

3.2. Global tyrosine phosphorylation driven by BCR-ABL is attenuated upon overexpression of 3BP2

To determine whether 3BP2 overexpression affected CML cell line growth and downstream signalling, CML-T1 cells were generated to stably overexpress FLAG-tagged full-length 3BP2. This cell line was selected because of the detection of unique 3BP2 associated complexes (see Section 3.5 below). Upon expression of 3BP2, global tyrosine phosphorylation was markedly decreased in CML-T1 cells (Figure 2.2A). Notably, BCR-ABL phosphorylation was attenuated, suggesting that a lack of BCR-ABL phosphorylation may be resulting in diminished tyrosine phosphorylation of other proteins. CML-T1-3BP2 cells exhibit increased phosphorylation of 3BP2 and an unknown 116 kDa protein. These results were found in 2 biological replicates (separate
Figure 2.1. 3BP2 is expressed at various levels in CML cell lines. (A) Lysates from CML cell lines CML-T1, EM2, LAMA84, MC3, MEG01, and K562, as well as murine monocytic RAW264.7 cells were probed by immunoblotting (IB) for tyrosine-phosphorylated proteins with the 4G10 phospho-tyrosine (pTyr) antibody. RAW264.7 cells serve as a positive control due to their high levels of endogenous 3BP2 expression. Lysates were probed with an anti-3BP2 antibody to examine endogenous protein expression, and probed with anti-GAPDH antibody to control for loading. (B) The immunoblot was used to quantify expression of total 3BP2 in each cell line using Image J software.
Figure 2.2. 3BP2 overexpression results in attenuation of global tyrosine phosphorylation in CML-T1 cells. CML-T1 and CML-T1-3BP2 cell lysates were probed by immunoblotting (IB) for tyrosine-phosphorylated proteins with the 4G10 phospho-tyrosine (pTyr) antibody. Lysates were also probed for total FLAG, 3BP2 and GAPDH. Results represent one of six repeats, three in one biological replicate and three in a second electroporated cell population.
populations electroporated with the FLAG-3BP2 construct, data not shown), as well as three independent clones (see Figure 2.3).

Three independent cell clones were isolated from the CML-T1-3BP2 cell population: CML-T1-3BP2 (B7), CML-T1-3BP2 (B8) and CML-T1-3BP2 (D5). As seen with the CML-T1-3BP2 population, 3BP2 expression in each of the clones resulted in a striking decrease in global tyrosine phosphorylation compared to CML-T1 cells (Figure 2.3A). FLAG-3BP2 was expressed at different levels in each clone (as shown by both anti-3BP2 and anti-FLAG immunoblots), with the highest expression in CML-T1-3BP2 (B7) cells and lowest expression level in CML-T1-3BP2 (D5) cells (Figure 2.3B). The decreased level of 3BP2 expression in CML-T1-3BP2 (D5) cells was associated with more enhanced global tyrosine phosphorylation, compared to the tyrosine phosphorylation levels observed in the higher expressing CML-T1-3BP2 (B7) and CML-T1-3BP2 (B8) cells (Figure 2.3A).

3.3. Expression of 3BP2 significantly enhances cell growth in CML-T1 cells

CML-T1-3BP2 cells exhibited significantly greater cell growth than CML-T1 cells, as measured via Trypan Blue exclusion assay over 7 days (Figure 2.4; p<0.0001 for days 3, 5 and 7). 3BP2 expression resulted in a 4.6-fold increase in the number of cells counted at day 7. This demonstrates for the first time that 3BP2 is capable of markedly altering cell growth in BCR-ABL-transformed cells. In addition to increased growth, CML-T1-3BP2 cells also displayed more adherence to the cell culture flask during expansion of cultures (data not shown).

Cell growth was further examined in a biological replicate of CML-T1-3BP2 (denoted as CML-T1-3BP2 (2)), as well as CML-T1-3BP2 (B7), CML-T1-3BP2 (B8) and CML-T1-3BP2 (D5) cells. In all 3BP2-overexpressing cells, cell growth was significantly greater than that of CML-T1 cells (Figure 2.5; 2-way ANOVA, p<0.0001). CML-T1-3BP2 (B7) cells, which have the
Figure 2.3. Higher expression of 3BP2 results in greater attenuation of global tyrosine phosphorylation in CML-T1 cells. (A) CML-T1 and CML-T1-3BP2 cell lysates were probed by immunoblotting (IB) for tyrosine-phosphorylated proteins with the 4G10 phospho-tyrosine (pTyr) antibody. Lysates were also probed for total FLAG, 3BP2 and GAPDH. CML-T1-3BP2 B7, B8 and D5 are each different cell clones with varied levels of 3BP2 expression, compared to CML-T1 cells (1-4). (C) The 3BP2 expression level was quantified for each lane using Image J densitometric analysis.
Figure 2.4. Overexpression of 3BP2 in the human CML cell line CML-T1 significantly enhances cell growth. CML-T1 cells were electroporated with a FLAG-tagged 3BP2 construct, selected for stable expression of the construct and cell growth was evaluated via Trypan Blue exclusion assays from day 0 to 7. Results are the mean ± SEM of 2 independent cultures (n=9) and a third replicate mirrored the displayed results. The asterisks indicates a significant increase in cell growth in the presence of 3BP2 (p<0.0001).
Figure 2.5. Enhanced cell growth in CML-T1 cells correlates with greater 3BP2 protein expression. CML-T1 cells were electroporated with a FLAG-tagged 3BP2 construct, selected for stable expression of the construct and cell growth was evaluated via Trypan Blue exclusion assays from day 0 to 7. CML-T1-3BP2 B7, B8 and D5 are each different cell clones with varying levels of 3BP2 expression, compared to CML-T1 cells. Results are the mean ± SD of one representative of 3 replicate experiments. The asterisks indicates a significant increase in cell growth in the presence of 3BP2 (p<0.0001).
highest level of 3BP2 expression (Figure 2.3), also have the most pronounced enhancement of cell growth. CML-T1-3BP2 (D5) cells have the lowest level of 3BP2 expression in relation to the other clones (Figure 2.3), which correlates to a lesser increase in cell growth (Figure 2.5). CML-T1-3BP2 (B8) cells fall into the middle in terms of both 3BP2 expression levels and enhancement of cell growth.

3.4. 3BP2 alters the expression and activation of effector proteins in the BCR-ABL signalling pathway

3BP2 stimulates ERK1/2 and AKT signalling

CML-T1-3BP2 cells have much higher levels of phosphorylated ERK1/2, particularly ERK2 (Figure 2.6A). In the total ERK1/2 immunoblot, we observed a slight upward shift in the migration of ERK1 compared to the band observed in in CML-T1 cells. The discrepancy in ERK1 between the two cell lines may be attributable to differences in phosphorylation in response to increased 3BP2 expression, or could represent another type of post-translational modification. After quantification of these blots, a slight increase in total ERK1/2 was observed in CML-T1-3BP2 cells (Figure 2.6B). The notable increase in phospho-ERK1/2 is was also evident in Fig. 2.6B after densitometric quantification.

Similarly, a striking increase in both the phosphorylation and total protein expression of AKT were observed upon 3BP2 overexpression in CML-T1 cells (Figure 2.6C and D). Collectively, these data show that 3BP2 promotes signal transduction pathways leading to enhanced phosphorylation and expression of two principal cell signalling effectors, ERK1/2 and AKT. Furthermore, these results suggest that activation of ERK1/2 and AKT signal transduction, may be responsible for the significant increase in cell growth in CML-T1 cell lines overexpressing 3BP2 (Figures 2.4 and 2.5).
Figure 2.6. Enhanced 3BP2 expression elicits increased ERK1/2 phosphorylation and elevated AKT protein expression and phosphorylation. CML-T1 and CML-T1-3BP2 cell lysates were probed by immunoblotting (IB) for phosphorylated (*upper panel*) and total (*second panel*) (A) ERK1/2 (44/42 kDa) and (C) AKT (60 kDa) with respective antibodies, followed by IB with 3BP2 (62 kDa) antibody and IB with GAPDH (37 kDa) antibody as a loading control. Blots were quantified via densitometry analysis (Image J software) for (B) phospho- and total ERK1/2, and for (D) phospho- and total AKT. These data are represented as a ratio of protein/ GAPDH. Results represent one of six repeats, three in one biological replicate and three in a second electroporated cell population.
3BP2 modulates expression and phosphorylation of SRC-family kinases LYN and HCK

We further investigated whether expression and/or activity of certain kinases is altered by 3BP2, since these may also directly play a role in modulating ERK1/2 and AKT activity. In addition to its capacity to regulate SRC kinase activity (Levaot et al. 2011b), 3BP2 is a potential regulator of the activity of another SRC-family kinase, LYN (Maeno et al. 2003). In this study, overexpression of 3BP2 resulted in a marked increase in total LYN protein levels, as well as an increase in the phosphorylation of LYN at tyrosine 507 (Y507; Figure 2.7A). Y507 is a negative regulatory residue of LYN that is phosphorylated when the enzyme is in its inactive state, where phospho-Y507 creates a binding site for its own SH2 domain. Therefore, these results suggest that in CML-T1 cells, 3BP2 enhances expression of LYN by more than 100-fold (as quantified via densitometry, Figure 2.7A), but suppresses its activity by affecting phosphorylation of Y507.

Interestingly, phosphorylation of SRC-family kinase tyrosine 416 (Y416), a residue phosphorylated during the activation of various SRC-family kinases, was markedly greater in CML-T1 cells than in CML-T1-3BP2 cells (Figure 2.7B). The phosphorylated doublet was identified as HCK, suggesting that HCK phosphorylation was markedly decreased with enhanced 3BP2 expression. However, total HCK levels were enhanced 2.6-fold in the presence of 3BP2 (Figure 2.7B). This leads to the conclusion that 3BP2 overexpression in CML-T1 cells leads to an increase in the expression of both LYN and HCK, yet results suggest a decrease in their activity.

SYK and CBL proteins are regulated by 3BP2

Since 3BP2 is known to closely interact with SYK in an interaction characterized by reciprocal regulation (Maeno et al. 2003; Levaot et al. 2011), the expression and phosphorylation status of SYK was examined in this study. CML-T1-3BP2 cells exhibited a marked 26-fold increase in total SYK expression and a 2.8-fold increase in SYK phosphorylation (Figure 2.7C).
Figure 2.7. 3BP2 expression results in elevated expression, but decreased activity of LYN and HCK, increased SYK expression and phosphorylation, and diminished CBL phosphorylation in CML-T1 cells. CML-T1 and CML-T1-3BP2 cell lysates were probed by immunoblotting (IB) for phosphorylated (upper panel) and total (second panel) (A) LYN, (B) SRC family kinase (Y416) / HCK, (C) SYK, and (D) CBL with respective antibodies, followed by IB with GAPDH antibody as a loading control. All blots were quantified using densitometry analysis (Image J software), which is displayed as the amount of protein/GAPDH. All results represent one of six repeats, three in one biological replicate and three in a second electroporated cell population.
On the other hand, expression of the E3 ubiquitin ligase CBL was approximately equal in both CML-T1 and CML-T1-3BP2 cells, yet phosphorylation of CBL was 100-fold greater in the absence of 3BP2 overexpression (Figure 2.7D). Furthermore, CBL can negatively regulate SYK expression and activity (Lupher et al. 1998), and therefore, decreased CBL levels in CML-T1-3BP2 cells may be responsible for the observed increase in SYK expression and phosphorylation.

3.5. The 3BP2-SH2 domain interacts with tyrosine phosphorylated proteins in a BCR-ABL-dependent manner

To identify tyrosine-phosphorylated proteins that interact with 3BP2 in CML cell lines and a Ba/F3 cell line engineered to express BCR-ABL, glutathione-S-transferase (GST) pull-down assays were performed with the SH2 domain of 3BP2. Untagged GST protein served as a control, for which no tyrosine-phosphorylated proteins were observed. In murine Ba/F3 BCR-ABL cells and in the CML cell line CML-T1, the SH2 domain of 3BP2 bound to a 116 kDa phospho-protein and a phospho-protein of approximately 50 kDa (Figure 2.8, lanes 8-9). These proteins were not identified with the 3BP2-SH2 domain pull-down in the Ba/F3 cell line (Figure 2.8, lanes 6-7), indicating that these interactions are uniquely present when BCR-ABL is expressed. The approximately 50 kDa phospho-protein was identified further as a SRC-family kinase, based on its detection with a phosphorylation-specific antibody that recognizes pY416.

3.6. 3BP2-deficiency results in altered hematopoietic progenitors

Bone marrow hematopoietic progenitor populations were examined in 3bp2−/− mice using SLAM cell surface markers in flow cytometry analysis. SLAM markers can be used to distinguish HSCs (CD150+/CD48−) from multipotent progenitor (MPPs, CD150−/CD48−) and lineage-restricted
Figure 2.8. The 3BP2 SH2 domain interacts with tyrosine phosphorylated Src family kinase and a 116 kDa phospho-protein in a BCR-ABL-dependent manner. Ba/F3, Ba/F3 BCR-ABL, and CML-T1 cells were depleted of cytokine and then stimulated in the presence (+) or absence (-) of IL-3. GST in vitro mixes were performed with GST (lanes 1-5) or GST fused to the SH2 domain of 3BP2 (lanes 6-10). Tyrosine phosphorylation was assayed by immunoblotting (IB) with anti-phospho-tyrosine (4G10) antibody, followed by IB with anti-phospho-Src family kinase antibody (Y416) and IB for total GST.
Figure 2.9. 3bp2<sup>−/−</sup> mice have altered numbers of hematopoietic progenitor cells in the bone marrow. 3BP2-deficient mice display a trend of lower numbers of HSCs and possess significantly more MPPs in the bone marrow, as indicated using SLAM markers. Wild-type (WT) and 3bp2<sup>−/−</sup> bone marrow cells were collected from femurs and tibias of mice, lineage-negative stem and progenitor cells were enriched by depletion of magnetically labeled mature hematopoietic cells, and cells were incubated with anti-CD48 and anti-CD150 antibodies prior to flow cytometric analysis. The asterisk indicates a statistically significant difference (p<0.05) between WT (n=5) and 3bp2<sup>−/−</sup> mice (n=5).
progenitor populations (CD150+/CD48−; Kiel et al. 2005). In moving forward with BMT experiments, differences in the number of HSCs and MPPs were of interest to us in order to understand the composition of 3bp2+− versus WT bone marrow, since differences in these important progenitors following transduction with BCR-ABL could translate into different disease outcomes.

Bone marrow from 3bp2−/− mice had fewer HSCs, but this difference was not statistically significant (Figure 2.9; p=0.2). However, a significantly greater number of MPPs, was found in 3bp2−/− bone marrow (p<0.05). These results suggest that 3bp2 may be playing a role in the maintenance of HSC self-renewal capacity or in negatively regulating the differentiation of hematopoietic progenitor populations.

3.7. Analysis of hematopoietic cell populations in spleen and bone marrow of 3bp2-deficient mice

A panel of antibodies against specific hematopoietic cell markers (erythrocyte marker Ter119, B-cell markers B220 and CD19, the HSC, thymocyte and peripheral T-cell marker Thy1.2, granulocyte marker Gr-1, the neutrophil, NK cell and macrophage marker Mac-1, the T-cell and dendritic cell markers CD4 and CD8, and the megakaryocyte marker CD41) was utilized to investigate whether 3bp2−/− mice had altered hematopoietic cell populations in the spleen and/or bone marrow.

We observed a significant increase in the number of CD19+ cells in the spleens of 3bp2-deficient mice (Figure 2.10), which suggests that these mice have increased numbers of splenic B-cells (Scheuermann et al. 1995). Interestingly, 3bp2−/− mice also displayed a significant decrease in the number of double positive B220+ and CD19+ cells, indicating that certain B-cell populations are more rare in these mice (Figure 2.10).
Figure 2.10. 3bp2-deficient mice have altered numbers of CD19\(^+\), B220\(^+\)/CD19\(^+\) and Gr-1\(^+\)/Mac-1\(^+\) cells in the spleen. Spleens were obtained from wild-type (WT) and 3bp2\(^-\) mice, cell suspensions were prepared and cells were incubated with either anti-Ter119, -B220 and -CD19, -Thy1.2, -Gr-1 and –Mac-1, -CD4 and –CD8, or -CD41 antibodies. All populations were analyzed using flow cytometry. The asterisks imply a statistically significant difference (*p<0.05, **p<0.01) between wild type (WT) (n=5) and 3bp2\(^-\) mice (n=5).
A lack of 3bp2 expression in mice was also associated with a significant decrease in Gr-1\(^+\)/Mac-1\(^+\) cells (Figure 2.10), suggesting that these mice harbour fewer myeloid cells in the spleen than their WT littermates. Other hematopoietic markers were expressed at equivalent levels in the spleens of both 3bp2\(^{-/-}\) and WT mice.

Further analysis revealed that 3bp2-deficiency does not significantly alter hematopoietic cell populations in the bone marrow, with the exception of an increase in CD4\(^+\) cells (Figure 2.11; \(p<0.05\)). This result suggests that in comparison to 3bp2-expressing mice, larger populations of T helper cells, monocytes, macrophages, or dendritic cells may be found in 3bp2\(^{-/-}\) mice.

3.8. 3bp2-deficiency results in shorter latency of leukemogenesis and altered disease manifestation in a BCR-ABL retroviral transduction and bone marrow transplantation model

Since we found that 3BP2 could markedly alter BCR-ABL signalling \textit{in vitro}, murine BMT experiments were conducted to further investigate the role of 3BP2 in the development of BCR-ABL-induced leukemia. A high titer VSV-G-pseudotyped retrovirus (at least 2 x 10\(^5\) CFU/mL) was generated using the MSCV-IRES-GFP (MIEV) vector carrying BCR-ABL\(^{p210}\). Viral titer was determined by GFP expression of transduced Ba/F3 cells, where GFP served as a surrogate marker for BCR-ABL since both transcripts are encoded in a bicistronic message. Infection of 3bp2\(^{-/-}\) donor bone marrow was found to be comparable to that of WT donors (11% vs. 13.5% infection of primary cells, respectively) upon flow cytometry analysis for GFP expression (Figure 2.12A). After transplantation of BCR-ABL-transduced bone marrow, recipient mice were evaluated daily for signs of morbidity and weekly for elevated peripheral blood GFP expression and leukocyte counts. Moribund animals were sacrificed and immunophenotypic and histopathologic traits were analyzed.
Figure 2.11. 3bp2-deficient mice have an enhanced number of CD4+ cells, but otherwise no significant differences in bone marrow cell populations compared to wild-type (WT) mice. BM was collected from the femurs and tibias of WT and 3bp2−/− mice, and BMCs were incubated with either anti-Ter119, -B220 and -CD19, -Thy1.2, -Gr-1 and −Mac-1, -CD4 and −CD8, or -CD41 antibodies. Bone marrow populations were analyzed via flow cytometry. The asterisk implies a statistically significant difference (p<0.05) between wild type (WT) (n=5) and 3bp2−/− mice (n=5).
Figure 2.12. 3bp2−/− mice succumb to BCR-ABL-induced lymphoid or mixed myeloid and lymphoid leukemia after a shorter disease latency than their WT littermates.

(A) Transduction efficiency was compared between wild-type (WT 3bp2+/+) and 3bp2−/− bone marrow cells. Cells were pre-stimulated overnight with growth factors, followed by 2 rounds of infection with MIEV BCR-ABLp210 retrovirus. (B) Kaplan-Meier survival analysis of BMT recipients of BCR-ABL-transduced bone marrow. The number of recipients are indicated in parentheses and the disease phenotype is indicated by shape and shading: CML-like MPD (closed squares), ALL-like lymphoid disease (closed circles), and mixed disease (half-closed circles). Undetermined (open squares) indicate mice succumbed to a leukemia of which the analysis was not able to be completed. Although 3bp2−/− differ from WT recipients in the stage at which they acquire certain diseases, the overall survival difference between 3bp2−/− and WT recipients is not statistically significant (Log Rank test, p=0.095).
We observed that all mice transplanted with 3bp2\textsuperscript{-/-} bone marrow succumbed to either B-lymphoid or mixed (lymphoid and myeloid) leukemia \( (n=5) \) after 2.5-3 weeks and to a MPD \( (n=1) \) after 8 weeks, compared to WT recipients, which died of B-lymphoid \( (n=2) \) or T-lymphoid \( (n=1) \) leukemia after 7-9 weeks, and MPD \( (n=1) \) after 3 weeks (Figure 2.12B). A statistical comparison of the 2 survival curves using the log-rank test did not indicate an overall significant difference in survival \( (p=0.095) \). Nevertheless, the mean survival in days of the 3bp2\textsuperscript{-/-} recipients was shorter than that of the WT recipients (Table 1).

Mice transplanted with 3bp2-deficient bone marrow had distinct pathologic features when compared to WT, such as a significantly lower peripheral blood leukocyte count \( (p<0.01) \), distinct differential counts (supporting the diagnosis of mixed disease that is unique to 3bp2\textsuperscript{-/-} recipients), fewer pulmonary hemorrhages (probable cause of death), and differences in spleen appearance (Table 1).

Immunophenotyping of spleen (Figure 2.13A) and bone marrow (data not shown) was employed to analyze leukemia phenotype. A representative WT recipient with B-lymphoid leukemia had 88.8% GFP\textsuperscript{+} spleen cells, which were highly B220\textsuperscript{+}/CD19\textsuperscript{+}. 3bp2\textsuperscript{-/-} recipients with mixed disease were characterized by a large population of GFP\textsuperscript{+} cells in the spleen that were positive for both B220 and Gr-1/Mac-1 expression. Myeloid disease (or MPD) was diagnosed based on GFP\textsuperscript{+} cells in the spleen with an expansion of Gr-1\textsuperscript{+}/Mac-1\textsuperscript{+} cells and the absence of lymphoid expansion.

Peripheral blood analysis of lymphoid disease confirmed the presence of immature blasts (red arrow) and an expansion of lymphocytes (black arrow), whereas myeloid disease was characterized by the marked expansion of neutrophils (arrowhead) and some monocytes (curved arrow), and mixed disease showed both elevated numbers of neutrophils, lymphocytes and blasts (Figure 2.13B). The low blood leukocyte count of 3bp2\textsuperscript{-/-} recipients observed via manual counting
Table 2.1. Characteristics of recipients of wild-type or 3bp2\textsuperscript{−/−} p210 BCR-ABL – transduced bone marrow

<table>
<thead>
<tr>
<th>Donor genotype</th>
<th>Latency*</th>
<th>Type of disease*</th>
<th>Mean survival in days</th>
<th>Mean WBC count ($10^9$/mL) ± SEM</th>
<th>Differentials</th>
<th>Pulmonary hemorrhages</th>
<th>Clinicopathologic features</th>
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<td><strong>WT (n=5)</strong></td>
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<tr>
<td>21</td>
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<td>34.25 ± 9.59</td>
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<td>ND</td>
<td>▶️</td>
<td></td>
<td></td>
<td>Splenomegaly</td>
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<tr>
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<td>Lymphoid</td>
<td></td>
<td></td>
<td>ND</td>
<td></td>
<td></td>
<td>Splenomegaly, hindlimb paralysis</td>
</tr>
<tr>
<td>54</td>
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<td></td>
<td>64% blasts 21% lymphocytes</td>
<td></td>
<td></td>
<td></td>
<td>Splenomegaly*, Enlarged LNs, pleural effusion</td>
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<tr>
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<td></td>
<td>49% blasts 37% lymphocytes</td>
<td></td>
<td></td>
<td>▶️</td>
<td>Splenomegaly, Enlarged LNs, hindlimb paralysis</td>
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<tr>
<td><strong>3BP2\textsuperscript{−/−} (n=6)</strong></td>
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<tr>
<td>18</td>
<td>Mixed</td>
<td>25</td>
<td>3.94 ± 1.03**</td>
<td>1/6</td>
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<tr>
<td>18</td>
<td>Undetermined</td>
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<td>56% blasts 32% lymphocytes 12% neutrophils 27% neutrophils</td>
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<td></td>
<td></td>
<td>Irregular spleen, enlarged LNs</td>
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<td>9% blasts 46% lymphocytes 15% neutrophils 28% eosinophils</td>
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<td></td>
<td>▶️</td>
<td>Splenomegaly/irregular spleen</td>
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<td>56% blasts 33% lymphocytes</td>
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<td>Irregular spleen, enlarged LNs</td>
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<tr>
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<td></td>
<td></td>
<td>Splenomegaly</td>
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</table>

\*Time in days to premorbidity or death after transplantation of transduced bone marrow.
† Determination of the type of disease was based on immunophenotypic flow cytometry analysis of spleen and bone marrow, the peripheral blood differentials and histopathology.
* Marked splenomegaly with spleen weight above 0.4 g.
• See Figure 2.13C.
** Indicates a statistically significant difference in the mean WBC count between WT and 3bp2\textsuperscript{−/−} recipients (p<0.01).
Note: Differentials are a % of total WBCs. Those displayed represent the most prevalent cell types. Lymphocytes, neutrophils, basophils, eosinophils, monocytes and blasts were counted.
Figure 2.13. 3bp2<sup>−/−</sup> recipients succumbed to lymphoid, myeloid, or mixed disease with decreased leukemic infiltration of secondary organs.

(A) Immunophenotypic analysis of spleen cell suspensions was performed using flow cytometry. Unstained spleen cells were analyzed for the expression of green fluorescent protein (GFP) as a measure of BCR-ABL expression (left panel). Cells were stained with PE-labelled Gr-1 and PerCP-Cy5.5-labelled Mac-1, or with PE-labelled B220 and PerCP-Cy5.5-labelled CD19. Representatives are shown of each lymphoid, mixed and myeloid disease. Lymphoid disease of a wild-type (WT) 3bp2<sup>+/−</sup> recipient and mixed and myeloid disease of a 3bp2<sup>−/−</sup> recipient are displayed. Percentages indicate the percent of GFP<sup>+</sup>, B220<sup>+</sup>/CD19<sup>+</sup>, B220<sup>+</sup>, Gr-1+/Mac-1<sup>+</sup> or Mac-1<sup>+</sup> spleen cells. (B) Histopathological analysis of peripheral blood was evaluated by microscopic evaluation of peripheral blood smear slides stained with May-Gruenwald and Giemsa. (C) Gross spleen images and histopathological characterization of disease of a representative WT 3bp2<sup>+/−</sup> recipient with lymphoid disease and 3bp2<sup>−/−</sup> recipients that succumbed to lymphoid and mixed disease. Histopathological analysis using H&E staining reveals low to no infiltration of leukemic cells into spleen (ii, iii), liver (v, vi) and lung (viii) of 3bp2<sup>−/−</sup> BCR-ABL-transduced bone marrow recipient mice, compared to the marked infiltration into spleen (i), liver (iv) and lung (vii) in WT 3bp2<sup>+/−</sup> BCR-ABL-transduced BM recipients, upon microscopic evaluation. Bone marrow (ix, x) was analyzed by cytospin with bone marrow suspension cells and slides were stained with May-Gruenwald and Giemsa.
B

Peripheral Blood

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<th>WT Myeloid</th>
<th>3bp2⁻⁻⁻ Mixed</th>
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</thead>
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C

<table>
<thead>
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<th>Lung</th>
<th>Bone Marrow</th>
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<td><img src="image16" alt="Bone Marrow 3bp2⁻⁻⁻ Mixed Mixed 63x" /></td>
</tr>
</tbody>
</table>

Legend:
- Lymphoid
- Myeloid
- Mixed
using Turk’s, was also evident upon examination of peripheral blood smears for mice that succumbed to lymphoid or mixed disease (Figure 2.13B). Histopathological disease characterization revealed leukemic blast infiltration surrounding splenic venules (Figure 2.13C (i)), extramedullary hematopoiesis and disruption of splenic architecture in WT recipients, compared to 3bp2−/− recipients where no splenic infiltration was observed (ii and iii). 3bp2−/− recipients displayed some areas of necrotic spleen tissue (iii), associated with a lumpy, irregular spleen shape. In WT recipients, periportal infiltration of leukemic blasts was observed surrounding hepatic portal systems in the liver, whereas some sinusoidal infiltration (v) or none (vi) was seen in 3bp2−/− recipients. Nodules consisting of leukemic blasts were present in WT (vii), but no pulmonary infiltration was observed in 3bp2−/− (viii). Bone marrow of 3bp2−/− recipients was hypercellular due to the expansion of blasts (x), whereas WT recipients displayed a greater population of lymphocytes and maturing neutrophils (ix).

A few mice died before immunophenotyping could be performed on spleen or bone marrow. The disease latency and splenomegaly of the WT ‘undetermined’ mouse point toward the diagnosis of MPD, whereas the short latency and similarity in clinicopathologic features (and in one case peripheral differential counts) to other 3bp2−/− recipient mice with lymphoid or mixed disease indicate a similar fate of the ‘undetermined’ 3bp2−/− recipients.
CHAPTER 4: DISCUSSION
Aberrant ABL kinase activity is the hallmark of BCR-ABL-driven leukemia, and as such, strict regulation of the kinase activity of ABL is crucial. 3BP2 is a unique ABL-SH3-binding protein that has been shown to be indispensable for the activation of ABL in vivo (Levaot et al. 2011), unlike similar proteins of its class, such as 3BP-1 (Cicchetti et al. 1992), CBL (Miyoshi-Akiyama et al. 2001), and DOK-R (Master et al. 2003). Our investigation demonstrates for the first time that 3BP2 is capable of modulating BCR-ABL-induced signalling and disease outcome.

Expression of 3BP2 was observed at different levels in all CML cell lines that were examined, suggesting a potential role for 3BP2 in BCR-ABL signalling. 3BP2 expression was previously found ubiquitously in three distinct isoforms in a panel of various cell lines from different tissues, including the CML cell line K562 (Jevremovic et al. 2001). As in the aforementioned study, we observed 3BP2 either as a singlet, doublet or triplet band, depending on the cell line (Figure 2.1A). Although the study by Jevremovic et al. (2001) suggests that these proteins arise due to alternative mRNA splicing, it remains elusive whether the doublet/triplet bands we observed in several CML cell lines are products of differential post-translational modification, such as phosphorylation, or if they represent different protein isoforms.

When the effect of 3BP2 overexpression was examined in the CML cell line CML-T1, which was established from a patient with T-lymphoid blast crisis CML (Kuriyama et al. 1989), a striking decrease in phospho-tyrosine levels was consistently observed in both separate cell populations and in independent cell clones (Figures 2.2 and 2.3). We demonstrated that the attenuation of global tyrosine phosphorylation was correlated with an increase in 3BP2 expression (Figure 2.3). Similarly, when CML-T1 cells were treated with Imatinib, Wetzel et al. (2005) observed a 4.11-fold increase in the inhibition of global tyrosine phosphorylation. Like Imatinib, it is possible that 3BP2 may be suppressing phospho-tyrosine levels by affecting BCR-ABL activity, since BCR-ABL phosphorylation was greatly attenuated upon 3BP2 expression (Figures 2.2 and 2.3).
Paradoxically, the attenuation of tyrosine phosphorylation in the presence of 3BP2 was accompanied by a significant increase in cell growth that was correlated with increased 3BP2 expression (Figures 2.4 and 2.5). These results support the previously suggested role for 3BP2 in the regulation of cell growth, as 3bp2-deficient mice have B-cell proliferation and survival defects (Chen et al. 2007), and lethality of homozygous cherubism mice is caused by systemic myelomonocytic infiltration into various organs (Ueki et al. 2007).

Although global tyrosine phosphorylation was markedly reduced in CML-T1-3BP2 cells, expression and phosphorylation of SYK, ERK1/2 and AKT were greatly enhanced (Figures 2.6 and 2.7). SYK is a protein tyrosine kinase that is most widely studied in its capacity to alter cell proliferation and survival by coupling B-cell receptor activation with downstream signalling pathways (Kurosaki et al. 1995; Wossning et al. 2006). Constitutive tyrosine phosphorylation of SYK at activating residues has been observed in B-lineage ALL (Guillaume et al. 2005), Acute Myeloid Leukemia (AML; Hahn et al. 2009), Chronic Lymphocytic Leukemia (Buchner et al. 2009) and various different types of lymphomas (Chen et al. 2008b; Gururajan et al. 2006; Leseux et al. 2006). A role for SYK in CML had not been investigated until recently, when Gioia et al. (2011) highlighted the necessity for SYK and LYN activation in Nilotinib resistance. Furthermore, SYK has been shown to positively regulate the PI3K/AKT and the MAPK/ERK pathways (Leseux et al. 2006; Slack et al. 2007). Therefore, it is possible that the increase in SYK expression and phosphorylation due to enhanced 3BP2 is upregulating expression and phosphorylation of ERK1/2 and AKT, and thereby promoting an increase in cell growth.

The expression levels of LYN and HCK were also increased upon 3BP2 expression, yet phosphorylation of the enzyme-inactivating Y507 residue of LYN was enhanced and phosphorylation of HCK at Y416 was suppressed (Figure 2.7), indicating defective activation of both SRC-family kinases. Phosphorylation of LYN Y507 in the C-terminal regulatory domain leads to the inactivation of LYN through intramolecular binding of Y507 to the SH2 domain
In addition to the interaction of Y446 of 3BP2 with the SH2 domain of LYN, the PRR region of 3BP2 associates with the SH3 domain of LYN and this interaction was found to occur during the LYN resting state (Maeno et al. 2003). Thus, 3BP2 may be capable of negatively regulating LYN activity, in addition to the positive regulatory role that has previously been demonstrated with LYN, as well as SRC (Maeno et al. 2003; Levaot et al. 2011). A mechanism by which it might do so is by direct interaction or by modulating CSK activity. CSK is a membrane-bound PTK that inactivates LYN by mediating phosphorylation of Y507 (Nada et al. 1991; Bergman et al. 1992), and thus, 3BP2 may enhance CSK activity, leading to LYN inactivation.

The similar profiles of increased expression but decreased activation upon 3BP2 expression that were found for both LYN and HCK (Figure 2.7) suggest that 3BP2 may regulate these closely related SRC-family kinases by the same or a similar mechanism. To our knowledge, this is the first study to demonstrate that 3BP2 can modulate HCK expression and phosphorylation. Although 3BP2 has been shown to interact with other members of the SRC-family (Ren et al. 1993; Deckert et al. 1998; Maeno et al. 2003; Levaot et al. 2011b), an association with HCK has not been described.

Overexpression of 3BP2 was furthermore associated with a 100-fold decrease in the phosphorylation of CBL. CBL is a proto-oncogene that encodes an E3 ubiquitin ligase that is capable of mediating proteasomal protein degradation of various tyrosine kinases, including LYN, HCK and SYK (Andoniou et al. 2000; Kaabeche et al. 2004; Howlett and Robbins, 2002; Lupher et al. 1998; Sohn et al. 2003). Therefore, a lack of CBL activity may be contributing to the increased levels of SYK, LYN and HCK that were observed in CML-T1-3BP2 cells.

CBL is prominently phosphorylated by BCR-ABL and participates in signal transduction downstream of BCR-ABL (Johnson et al. 2009), but was not found to be required for BCR-ABL-mediated MPD in a BMT model (Dinulescu et al. 2003). The downregulation of CBL
phosphorylation that we have observed could, therefore, either be a result of BCR-ABL inactivation, down-regulation of SRC or other tyrosine kinase activity, or 3BP2 may directly regulate CBL activity. Interestingly, a previous study shows that the expression of kinase deficient SRC results in the inhibition of ABL to tyrosine phosphorylate CBL (Shishido et al. 2000). Therefore, a lack of LYN and HCK activity with 3BP2 overexpression may be responsible for the attenuated CBL phosphorylation.

An interaction between CBL and the SH2 domain of 3BP2 was previously demonstrated in activated T-cells (Deckert et al. 1998). In our GST pull-down study, we found that the SH2 domain of 3BP2 associates with a 116 kDa phospho-protein in a BCR-ABL-specific manner (Figure 2.8). This phospho-protein could potentially be CBL, however, future work must be done to verify the protein identity and whether 3BP2 is capable of regulating CBL activity.

3BP2-SH2 domain appears to interact in a complex with a phosphorylated SRC-family kinase and a 116 kDa phospho-protein uniquely in BCR-ABL-expressing cells, suggesting that 3BP2 recruits binding partners in a BCR-ABL-specific manner. To identify which SRC-family kinase was detected, immunoblots were performed for phospho-LYN (Y507) and total LYN, LCK, HCK, FGR and SRC, because the interaction was in Ba/F3-BCR-ABL cells and from previous microarray data we know that these SRC-family kinases are present in Ba/F3 cells (data not shown). No proteins were observed in any of the aforementioned immunoblots (data not shown), suggesting that the phospho-SRC member we isolated is not LYN, LCK, HCK, FGR or SRC, but may be a different SRC-family kinase that is upregulated when BCR-ABL is expressed in Ba/F3 cells. Alternatively, our inability to identify which SRC kinase(s) associate with the 3BP2 SH2 domain may be due to differences in sensitivity between anti-phospho-tyrosine and total SRC kinase antibodies.

In summary, we propose a model of the effect of 3BP2 on BCR-ABL signal transduction whereby 3BP2 promotes dephosphorylation of BCR-ABL and increases the expression and
Figure 3.1. Proposed model of the effect of 3BP2 on BCR-ABL signal transduction. 3BP2 expression results in downregulation of BCR-ABL phosphorylation, and increased expression of SYK protein levels and phosphorylation. CBL is an E3 ubiquitin ligase capable of targeting SYK for degradation, and its phosphorylation has been shown to be regulated by BCR-ABL. Diminished CBL phosphorylation can lead to decreased degradation of SYK, leading to its high expression in the presence of 3BP2. SYK may furthermore be promoting the increase in ERK1/2 activation, and AKT expression and activation that is observed in the presence of 3BP2. The increases in ERK1/2 and AKT activity can promote increased cell proliferation and growth. Both LYN and HCK have increased expression, but down-regulated activity, which may further contribute to the decrease in BCR-ABL phosphorylation.
activation of SYK, leading to ERK1/2- and AKT-mediated enhancement of cell growth (Figure 3.1). The increase in SYK expression could potentially be explained by decreased CBL activity and a lack of CBL-mediated degradation of SYK protein. Future experiments should address whether E3 ubiquitin ligase activity is altered by 3BP2 expression and whether this affects SYK degradation.

Our results suggest that LYN and HCK activation is suppressed by 3BP2. This may be an effect of the attenuation of BCR-ABL phosphorylation, where BCR-ABL can no longer activate LYN and HCK (Danhauser-Riedl et al. 1996). The combined suppression of BCR-ABL, LYN, and HCK of kinase activity may explain the marked downregulation of global phospho-tyrosine in the presence of 3BP2. Alternatively, 3BP2 may be directly inactivating these SRC-family members. In turn, LYN and HCK, which are also capable of modulating BCR-ABL signalling by directly phosphorylating BCR-ABL (Meyn et al. 2006), may be contributing to the decrease in BCR-ABL phosphorylation.

3BP2 markedly alters BCR-ABL signalling in CML-T1 cells, which begs the question of whether it can also modulate BCR-ABL-mediated disease outcome. Upon investigating the role of 3bp2 in mouse BMT models, our results demonstrate that 3bp2 was not required for the induction of BCR-ABL-mediated ALL-like lymphoid disease or CML-like MPD in mice. Nevertheless, recipients of 3bp2-deficient BCR-ABL-transduced bone marrow had altered disease latency for both lymphoid and myeloid leukemia, accompanied by different pathologic characteristics (Figures 2.12 and 2.13, Table 1).

Several 3bp2−/− recipients succumb to leukemia with a mixed myeloid and lymphoid phenotype, whereas mixed disease was not observed in the WT recipients. Further experiments are required to investigate why 3bp2-deficiency results in a greater mixed disease outcome, however, as our in vitro studies suggest, it is plausible that 3bp2 may negatively regulate BCR-ABL activity, and therefore, the absence of 3bp2 may result in more potent BCR-ABL activity in both myeloid
and lymphoid progenitors. If 3bp2 is indeed capable of negative BCR-ABL regulation and can do so via its interaction with the ABL SH3 domain, this may also explain why BCR-ABL SH3 deletion mutants have constitutive ABL kinase activity (Zhang et al. 2001b). Although the interaction of 3bp2 and the ABL SH3 domain has been described to promote ABL kinase activity (Levaot et al. 2011a), it is possible that the regulation is altered in the BCR-ABL fusion protein, or that 3bp2 is capable of both positive and negative regulation of ABL kinase activity. Further examination of the effect of 3bp2 on BCR-ABL function and the mechanism by which it may alter BCR-ABL activity is necessary.

The accelerated manifestation of lymphoid and mixed leukemia in 3bp2−/− recipients (WT 7-9 weeks vs. 3bp2−/− under 3 weeks) further points to the negative regulation of BCR-ABL activity by 3bp2. 3bp2−/− recipients succumbed to B-cell lymphoid leukemia and mixed leukemia with B-lymphoid expansion. Hu et al. (2004) showed that SRC-family kinases LYN, HCK and FGR are required for BCR-ABL-induced B-cell ALL and that mutation of any two of these kinases significantly extends survival of BCR-ABL BMT mice. Since our in vitro work suggests that 3BP2 suppresses LYN and HCK activity, 3bp2-deficient mice may succumb to short-latency leukemia with a B-cell phenotype as a result of aberrant LYN and HCK activity.

Additionally, our studies showed that 3bp2−/− mice have a greater population of multipotent progenitors (MPPs; Figure 2.9), which may provide an alternative explanation to the observed leukemia latency and phenotype in 3bp2−/− recipient mice. BCR-ABL-positive MPPs have very high proliferation rates (Reynaud et al. 2011), and thus, if more MPPs are available for BCR-ABL transduction, it could result in the acceleration of disease onset that we have observed in 3bp2−/− recipients. Reynaud et al. (2011) also demonstrated that BCR-ABL-positive MPPs promote B-cell overproduction, but that these progenitors can be reprogrammed toward a myeloid fate during CML development. Therefore, the increased number of MPPs in 3bp2−/− may also be resulting in
the phenotypic disease outcome (of lymphoid or mixed) in 3bp2<sup>-/-</sup> recipients, by an unknown mechanism.

A third potential reason for the decreased lymphoid and mixed leukemia latency in 3bp2<sup>-/-</sup> recipients may be accelerated death from infiltration of leukemic cells into the central nervous system (CNS). We did not examine the central nervous system in the BMT mice, but we did observe low or no infiltration of leukemic cells into spleen, lung and liver tissues (Figure 2.13), suggesting that leukemic cells may be infiltrating other tissues, such as the brain. No pleural effusions or pulmonary infiltration were found in these mice, which are characteristics previously noted as the main causes of death in BMT recipients (Li et al. 1999; Million and Van Etten, 2000). Several endocrine organs, pancreas and thyroid, as well as exocrine organs, salivary glands and gall bladder, were also examined and found to be negative for the presence of leukemic infiltrates (data not shown). Although not observed in 3bp2<sup>-/-</sup> recipients, a few WT recipients that succumbed to lymphoid leukemia presented with hindlimb paralysis (Table 1), suggesting the spread of leukemic cells to the CNS. Therefore, it is possible that lymphoid and mixed leukemias in 3bp2<sup>-/-</sup> recipients are more aggressive because they spread to the CNS. However, further BMT experiments are required to investigate this hypothesis.

Although future experiments are needed to fully understand the role of 3bp2 in BCR-ABL-mediated MPD, our results show that only one (of 6) 3bp2<sup>-/-</sup> recipient succumbs to MPD after an extended disease latency (WT after 3 weeks vs. 3bp2<sup>-/-</sup> after 8 weeks). These results could be explained in two ways: (a) 3bp2 may play an important role in the induction of MPD through BCR-ABL by mediating signal transduction downstream of BCR-ABL, or (b) delayed onset of MPD in 3bp2<sup>-/-</sup> recipients is a result of fewer HSCs in 3bp2<sup>-/-</sup> bone marrow (Figure 2.9). In order to determine whether the former is true, further BMT experiments involving donor 5-FU treatment are necessary to evaluate the characteristics of MPD in recipients of transduced 3bp2<sup>-/-</sup> bone marrow. In terms of the latter, CML is considered a clonal stem cell disorder, since expression of
the BCR-ABL fusion transcript in the HSC is sufficient to cause CML (Sirard et al. 1996). Thus, our findings that 3bp2⁻/⁻ mice have fewer HSCs may result in a delayed MPD due to less clonal CML stem cell expansion.

Lastly, recipients of 3bp2⁻/⁻ BCR-ABL-transduced bone marrow demonstrate a significantly lower mean peripheral blood leukocyte count than those of WT (Table 1). Upon examination of the bone marrow, it does not appear aplastic (Figure 2.13C). Leukocytopenia in the 3bp2⁻/⁻ recipients therefore may be a result of a failure of white blood cells to migrate from the bone marrow to the bloodstream, and presents another possible cause of death in these mice.
CHAPTER 5: FUTURE DIRECTIONS
5.1. **In vitro** characterization of the interaction of 3BP2 with BCR-ABL and BCR-ABL-specific 3BP2 binding partners

Our study demonstrates that CML cell lines express 3BP2, however, an interaction between 3BP2 and BCR-ABL requires further characterization. 3BP2 interacts directly with the ABL SH3 domain and through this interaction is capable of inducing ABL kinase activity in osteoblasts (Levoat *et al.* 2011a). To determine whether 3BP2 is retained as a direct binding partner for BCR-ABL, far western-blotting will be employed to confirm the interaction is in fact direct. Far western blots will be conducted by transiently overexpressing BCR-ABL structural and functional mutants, such as deletions of the ABL kinase, coiled-coil, SH3, SH2, and actin-binding domains, in 293T cells and probing for full-length, FLAG-tagged 3BP2. By utilizing these functional domains and mutated inactive forms of these domains, we hope to characterize the mechanism by which 3BP2 interacts with BCR-ABL.

Additionally, interactions partners of 3BP2 in response to BCR-ABL activation will be further explored. Since important binding partners often have several mechanisms of interacting with each other, a panel of 3BP2 mutant GST fusion proteins will be employed to assess the impact of deleting certain 3BP2 domains on the interaction of 3BP2 with BCR-ABL, and BCR-ABL signalling. Pull-down experiments will be performed, such as we did using the 3BP2 SH2 domain where we found that 3BP2 uniquely forms a complex with a phospho-SRC family member and a 116 kDa phospho-protein in BCR-ABL-expressing cells (Figure 2.8). Further pull-down assays will involve GST-3BP2 PRR and GST-3BP2 PH domain. To characterize proteins of unknown identity, including the 116 kDa protein we identified as a 3BP2 SH2 domain binding partner in this study, mass spectrometry-based identification will be employed. Sample preparation will involve specialized gel staining of binding partners (isolated in pull-down assays), band excision and in-gel tryptic digestion (Brymora *et al.* 2004).
5.2. Characterizing the effect of 3BP2 on BCR-ABL signal transduction

To better understand how 3BP2 is affecting global tyrosine phosphorylation, 3BP2 should be silenced in CML-T1 and CML-T1-3BP2 cells. We hypothesize that the knock-down of 3BP2 will potentiate tyrosine phosphorylation, restoring it to the levels observed in CML-T1 cells. This experiment will confirm that 3BP2 is indeed causing the suppression of phospho-tyrosine levels (Figure 2.2). Furthermore, silencing of 3BP2 in CML-T1 cells will allow us to validate the changes in expression and/or phosphorylation in ERK1/2, AKT, SYK, LYN, HCK and CBL proteins that we observed when 3BP2 was overexpressed (Figures 2.6 and 2.7), and to investigate whether cell growth is suppressed by 3BP2 silencing, as we would expect from the results in this study. We propose knock-downs of 3BP2 with shRNA using a lentiviral expression vector. Alternatively, CML-T1 and CML-T1-3BP2 cells will be transfected with 3BP2-targeted siRNA using Amaxa Nucleofector™ Solutions (Lonza; Basel, Switzerland).

We will identify the 3BP2 domain(s) responsible for modulating the activities of ERK1/2, AKT, SYK, LYN, HCK and CBL by transfecting CML-T1 cells with vectors containing structural and functional mutants. Our collaborator R. Rottapel has the R486K SH2 domain mutant that will be utilized. Additionally, previous studies employed 3BP2 PH and SH2 deletion mutants, and a PRR mutant in which 14 prolines were changed to alanine (Jevremovic et al. 2001). Utilizing these mutants in CML-T1 cells will guide our mechanistic understanding of the ability of 3BP2 to alter crucial cell signalling pathways.

Since we hypothesize that ERK1/2 and AKT pathways are driving the increase in cell growth, the MEK inhibitor U0126 (Yu et al. 2005) and/or ERK inhibitor PD98059, as well as the PI3K inhibitor wortmannin and/or AKT inhibitor A6730 (Hu et al. 2012), will be used to treat CML-T1-3BP2 cells. These inhibitors will reveal the pathway(s) instrumental in increasing CML-T1-3BP2 cell growth and we hypothesize that treatment with either both MEK/ERK and PI3K/AKT inhibitors, or the inhibition of one pathway, will suppress CML-T1-3BP2 cell growth.
The experimental caveat in this case is, however, that we could not be certain of whether the inhibitors are reducing the activity of BCR-ABL or other signalling effectors, in addition to their primary target.

Further characterization of the interaction of 3BP2 with SYK, LYN, HCK and CBL are required to validate the proposed model of the effect of 3BP2 on BCR-ABL signal transduction (Figure 3.1). Since an interaction between 3BP2 and HCK has not been previously demonstrated, and the interaction of 3BP2 with SYK and LYN has not been confirmed in BCR-ABL-expressing cells, co-immunoprecipitation experiments will confirm these interactions. We and others (Foucault et al. 2003; Qu et al. 2005) have experienced difficulties in detecting 3BP2 binding partners endogenously, and therefore, 293T cells will be transfected to transiently overexpress 3BP2 alone, 3BP2 and SYK, 3BP2 and LYN, 3BP2 and HCK, each aforementioned 3BP2 domain mutant with each kinase, and each kinase alone.

An important question that remains is whether 3BP2 can alter the activity of its downstream targets. A kinase assay utilizing CRKL as an in vitro substrate could be used to examine BCR-ABL dependent kinase activity. Similarly, SYK (Histone 2B or Raytide as substrate), LYN (enolase as substrate) and HCK (enolase as substrate) should be assessed to determine whether 3BP2 alters the activity of these proteins, as the 3BP2-mediated changes in their phosphorylation suggests it may.

We have demonstrated that 3BP2 expression results in a marked decrease in CBL phosphorylation at Y774. To further determine whether phosphorylation of other CBL tyrosines is altered upon 3BP2 expression, phosphorylation of CBL at Y700 and Y731 will be examined in CML-T1 and CML-T1-3BP2 cells using available phospho-antibodies. Since SRC kinases can bind to CBL via Y731 (Hunter et al. 1999), monitoring CBL phosphorylation at this residue is an important priority.
In our model, we hypothesized that SYK, LYN and HCK expression are enhanced due to suppression of CBL E3 ubiquitin ligase activity. To investigate this hypothesis, we will treat CML-T1 and CML-T1-3BP2 cells with proteasomal and lysosomal inhibitors, perform immunoprecipitations of SYK, LYN and HCK and examine whether ubiquitylation of these proteins is altered upon 3BP2 expression by probing for ubiquitin.

5.3. **Investigate the role of 3BP2 in the maintenance and functional control of hematopoietic progenitors**

Using SLAM cell surface markers, we found that 3bp2−/− mice have fewer HSCs and more MPPs (Figure 2.9). In addition to CD150+ expression, mouse HSCs express stem cell antigen 1 (Sca1+) and c-Kit (Kit+, CD117) and are denoted as lineage-negative (Lin−). Lin−Sca1+c-Kit+ (LSK) enrichment is another standard way to identify HSCs and LSK enrichment should be combined with SLAM marker analysis of 3bp2−/− bone marrow to further refine the HSC and MPP populations and to verify that these populations are altered in 3bp2−/− mice (Chen et al. 2008a).

Altered numbers of HSCs and progenitor cells could result from changes in the ability to self-renew, a loss of quiescence, and changes in cell proliferation and survival. Proliferation of HSCs and MPPs in 3bp2−/− mice will be assessed by examining cell-cycle parameters in LSK and LSK/CD150+ cells.

Since altered numbers of progenitors do not necessarily translate to changes in progenitor function, functional stem cell assays must be conducted. Colony assays and limiting dilution assays will be performed to investigate whether 3bp2-deficiency results in altered HSC and progenitor cell function.

The expression of 3bp2 in various progenitors should also be investigated to gain a better understanding of the role 3bp2 may play in the regulation of hematopoietic progenitors. Quantitative RT-PCR analysis will reveal 3bp2 expression levels in long-term HSCs, short-term
HSCs, MPPs, common myeloid progenitors, granulocyte-macrophage progenitors, megakaryocytic-erythroid progenitors, and common lymphoid progenitors. Understanding how progenitor populations are altered by 3bp2-deficiency will also inform our BMT experiments, by providing information on the type and function of cells available for BCR-ABL infection.

5.4. **Determine the role of 3BP2 in BCR-ABL-induced lymphoid and mixed leukemia**

BMT experiments without treatment of donors with 5-FU will be repeated with a greater number of mice in order to better understand the role of 3bp2 in BCR-ABL-induced ALL. A more detailed analysis of the cause behind the shorter disease latency of 3bp2−/− recipients will involve the examination of recipients for leukemic CNS infiltration. Additionally, investigating expression of SYK, LYN and HCK in leukemic cells will reveal whether the loss of 3bp2 results in their aberrant activity, which could further explain the acceleration of lymphoid disease onset.

It is necessary to assess whether changes in the hematopoietic progenitor populations of 3bp2-deficient mice are responsible for the altered disease latency and phenotype in our study. We will harvest HSCs and MPPs from recipients of BCR-ABL-transduced WT and 3bp2−/− bone marrow using SLAM markers and examine the expression of BCR-ABL in these cells. Additionally, transplantation of leukemic cells into WT secondary recipients would reveal whether 3bp2−/− LSCs have altered capacity to induce leukemia (Li et al. 1999). Based on our BMT results and the greater MPP population we observed in 3bp2−/− mice, we expect that secondary transplantation of 3bp2−/− lymphoid or mixed disease would result in shorter disease latency than WT lymphoid.

5.5. **Determine the role of 3BP2 in BCR-ABL-induced MPD**

CML is effectively modeled by BMT experiments where donors are treated with 5-FU prior to harvesting their BMCs (Li et al. 1999). After WT and 3bp2−/− donor mice are treated with 5-FU, the
same BMT protocol will be followed that was described in this study. Previous studies show that treatment of donors with 5-FU results in the enrichment of progenitor cells in the bone marrow (Li et al. 1999; Hu et al. 2004). Thus, the availability of different cell populations in comparison to whole bone marrow during retroviral transduction results in different disease outcomes. For example, our laboratory has shown that mice receiving 5-FU primed, BCR-ABL- transduced bone marrow from mice deficient of the adapter protein GADS succumb to MPD that is indistinguishable from WT; however, when whole bone marrow (no 5-FU) is used, GADS-deficient recipients die more rapidly of BCR-ABL-induced leukemia (Gillis et al. unpublished). Results from the current study suggest that 3bp2 may play an important role in the leukemogenesis of BCR-ABL-induced CML-like MPD (Figure 2.12).

If HSCs in 3bp2−/− have lower functional capacity, in addition to their low numbers, we would expect to see a delay in the onset of MPD. To ensure that similar populations are transduced in both 3bp2−/− and WT and that disease outcome is not based on cell differences, we will transduce sorted populations of bone marrow cells isolated from wild type and 3bp2−/− animals. For example, we will transduce Common Lymphoid Progenitor cell populations isolated from both wild type and 3bp2−/− bone marrow with BCR-ABL to determine whether disease phenotype and latency is distinct as we have observed when whole bone marrow is transduced with BCR-ABL. Comparable experiments will be performed with Multipotent Progenitor Cells (lin−CD34+,SCA-1+, Thy1.1−, c-kit+, CD135hi,CD150−CD11bloCD4lo), Common Myeloid Progenitors (lin−SCA-1−c-kit−CD34+CD16/32mid) and Granulocyte Macrophage Progenitors (lin−SCA-1−c-kit−CD34+CD16/32hi) and if possible with long term (lin−CD34+,CD38−,SCA-1+, Thy1.1+lox, c-kit+, CD135−,CD150lo), short term (lin−CD34+,CD38+,SCA-1+, Thy1.1+lox, c-kit+, CD135−,CD150−,CD11blo) hematopoietic stem cells and multipotent progenitor cells (lin−CD34−,CD38+,SCA-1+, Thy1.1+lox, c-kit+ CD135−,CD150−,CD11blo).
Lastly, BMT models do have inherent limitations due to the use of irradiated recipients and the constraints of retroviral transduction. Reynaud et al. (2011) developed a model of CML whereby BCR-ABL expression is induced in HSCs 5 weeks after birth with doxycycline withdrawal in Scl/Tal1-tTA and TRE-BCR-ABL transgenic mice (BA mice). Mice become moribund 6 weeks after induction, with severe myeloid expansion, splenomegaly, lymphoid reduction in spleen and bone marrow, and development of myelofibrosis (as described in 30% of CML patients; Buesche et al. 2007). Crossing BA mice with 3bp2−/− mice will allow us to further evaluate the importance of 3bp2 in CML pathogenesis without the limitations of BMT models.

5.6. Conclusion

3BP2 appears to have a profound effect on BCR-ABL signalling in vitro and demonstrates the ability to modulate BCR-ABL-mediated disease outcome in vivo. The mechanisms involved in BCR-ABL signal transduction are extremely complex and interconnected. Although treatment options for Ph-chromosome-positive leukemias are available, they currently do not provide a cure. We hope that further investigation of the role of 3BP2 in BCR-ABL-mediated disease will provide novel insight into the regulation of BCR-ABL signalling and will highlight new, more effective therapeutic targets.
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