Preclinical evaluation of the NF-κB pathway and β-catenin as therapeutic targets in BCR-ABL1-induced leukemia

A dissertation

submitted by

Mo-Ying Hsieh

In partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

in

Cellular & Molecular Physiology

TUFTS UNIVERSITY

Sackler School of Graduate Biomedical Sciences

Date

August, 2011

ADVISER:
Richard A. Van Etten, M.D., Ph.D.

ABSTRACT

The BCR-ABL1 oncoprotein, resulting from t(9;22) Philadelphia chromosome translocation, is the direct cause of chronic myeloid leukemia (CML) and is also found in some acute B-lymphoblastic leukemia (Ph^+ B-ALL) patients. While allogenic stem cell transplantation can cure Ph^+ leukemias, this procedure is risky, costly, and associated with graft-versus-host-disease. The standard initial therapy for CML is tyrosine kinase inhibitors (TKIs), but they are ineffective in patients with advanced stages of CML, Ph^+ B-ALL, or acquired TKI resistance. Furthermore, relapse frequently occurs after discontinuation of TKIs, likely due to residual leukemia-initiating cells (LICs). It is therefore important to identify signaling pathways critical to the pathogenesis of the Ph^+ leukemias, as they represent potential novel therapeutic targets for Ph^+ leukemia and to overcome resistance to TKIs.

To understand the functional role of NF-κB in BCR-ABL1-mediated myeloid and lymphoid leukemogenesis in an *in vivo* system, we engineered retroviruses co-expressing BCR-ABL1 with either a super-repressor mutant form of IκBα (IκBαSR) or kinase-inactive forms of IκB kinase (IKKαKM or IKKβKM) to inhibit NF-κB signaling with BCR-ABL1 using retroviral bone marrow transduction/transplantation mouse models of Ph^+ leukemias. We observed that impaired NF-κB signaling prolongs the survival of CML and B-ALL in mice suggesting that NF-κB is activated by BCR-ABL1 in part through the canonical IKK pathway, and validating NF-κB and IKKs as therapeutic targets for Ph^+ leukemias.

To interrogate the role of the nuclear, active form of β-catenin in leukemogenesis mediated by BCR-ABL1in the development and maintenance of leukemia-initiating cells (LICs) of CML in myeloid blast crisis, we co-expressed constitutively active β-catenin with BCR-ABL1 using several different strategies in the retroviral transduction/transplantation model. We found that active β-catenin impairs both myeloid and lymphoid leukemogenesis mediated by BCR-ABL1. These results show that the hematopoiesis defect resulting from expression of active β-catenin in normal progenitors cannot be overcome or rescued by BCR-ABL1.

In summary, this thesis provides better understanding of the mechanistic contributions of NF-κB and β-catenin to BCR-ABL1-mediated leukemogenesis. The data presented herein argue for NF-κB as a potential therapeutic target in the Ph^+ leukemias and provides initial insight into the role of β-catenin in BCR-ABL1-mediated leukemogenesis.

ACKNOWLEDGEMENT

This dissertation would not be completed without support from many people. Foremost, I would like to express my gratitude to my advisor Dr. Richard Van Etten. Throughout years, Rick's training has led me to become an independent scientist with critical thinking of my own research, and also have ability to cooperate with other people. I would also like to thank members in my thesis committee, Drs. Brent Cochran, Gail Sonenshien and previous committee member Amy Simon. Thank you all for your guidance, direction and ideas over the years. I would also like to thank my outside examiner, Dr. Shaoguang Li, for his thoughtful critique of this work. Of course, thanks to the support from my wonderful labmates: Drs. Nilu Jena, Crisitan Gavrilescu, Wayne Chan, Virginia Zaleskas, Rinat Yacobi, Darci Gaiotti, Christoph Walz, Yi-Fen Lu, Laurent Boissel, Rouzbeh Taghizadeh as well as Katherine Lazarides, Marcos Salazar, Elizabeth Helms, Janet Fannon and Monica Betancur. Thank you guys for creating such an enjoyable working environment.

Finally, I would like to thank my friends and family. I am very grateful to my parents who support my decision to have my graduate education in the states. I would also like to thank my roommates, Janet Chwalibog and Anglea Carcia for your moral support after work everyday. And finally, thank you Yaoyu, I had balance between life and science with your companionship in this journey.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

5-FU, 5-fluorouracil

ABL1, Abelson murine leukemia viral (v-*abl*) homolog 1

AML, acute myeloid leukemia

APC, adenomatosis polyposis coli

B-ALL, B-cell acute lymphoblastic leukemia

BCR, breakpoint cluster region

CC domain, coiled-coil domain

CK1, casein kinase 1

CML, chronic myeloid leukemia

CML-AP, CML at accelerated phase

CML-BC, CML at blast crisis phase

CML-CP, CML at chronic phase

CML-LBC, CML-lymphoid blast crisis

CML-MBC, CML myeloid blast crisis

CMP, common myeloid progenitor

ERK, extracellular signal-regulated kinase

FISH, fluorescence in situ hybridization

GAB2, GRB2-associated binding protein 2

GAP, GTPase activating protein

GEF, guanine nucleotide exchange factor

GMP, granulocyte-macrophage progenitor

Grb2, growth factor receptor-bound protein 2

GSK3β, glycogen synthase kinase 3 beta

HSC, hematopoietic stem cells

IκB, inhibitor of NF-κB

IKK, IκB kinase

IL-3, Interleukin-3

IRES, internal ribosome entry site

IκBαSR, super-repressor mutant form of IκBα

LIC, leukemia-initiating cell

MPN, myeloproliferative neoplasm

NEMO, NF-κB essential modulator

NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells

PCR, polymerase chain reaction

Ph chromosome, Philadelphia chromosome

PI3K, phosphatidylinositol 3-kinase

RT-PCR, reverse transcription polymerase chain reaction

SFKs, SRC family kinases

SH2, SRC-homology 2

SH3, SRC-homology 3

SOS, son of sevenless

STAT5, Signal transducer and activation of transcription 5

TKIs, tyrosine kinase inhibitors

WBC, white blood cell

Chapter 1.

General Introduction

1.1 THE *BCR-ABL1* ONCOGENE

1.1.1 Discovery of BCR-ABL1

BCR-ABL1 is a dysregulated tyrosine kinase resulting from fusion of the breakpoint cluster region (*BCR*) gene on chromosome 22 to Abelson murine leukemia viral (v-*abl*) homolog 1 (*ABL1*) gene on chromosome 9. Through years of studies, we now know that BCR-ABL1 is the direct cause of chronic myeloid leukemia (CML) and can be found in ~20% of patients with B-acute lymphoblastic leukemia (B-ALL). It was in1960 that Peter Nowell and David Hungerford first associated the expression of a short abnormal G-group chromosome with CML (Nowell and Hungerford, 1960). Since this discovery in the city of Philadelphia, the abnormal chromosome has been designated as the Philadelphia (Ph) chromosome, which was revealed later by Janet Rowley to be the result of a balanced translocation between chromosomes 9 and 22, t(9;22) (Rowley, 1973). In 1980, Nora Heisterkamp, John Groffen and colleagues provided molecular characterizations of the Ph chromosome, as they identified the genes involved in translocation abnormality are c-*ABL1* proto-oncogene on chromosome 9 and *BCR* gene on chromosome 22 (de Klein et al., 1982; Groffen et al., 1984; Heisterkamp et al., 1983). The fusion generated a new gene, *BCR-ABL1*, whose transcript can be found across patients with CML (Shtivelman et al., 1985; Stam et al., 1985). Further *in vivo* experiments in 1990 demonstrated that BCR-ABL1 was a protein-tyrosine kinase (Lugo et al., 1990) that is necessary and sufficient to induce CML, as lethally irradiated recipients mice transplanted with donor cells expressing BCR-ABL1 developed CML-like disease (Daley et al., 1990; Kelliher et al., 1990)

1.1.2 Variants of BCR-ABL1

Three variants of BCR-ABL1 named after the respective distinct molecular masses of the resulting polypeptides have been identified, depending on the breakpoints region within BCR gene. These variants include different transcriptional regions of BCR but they all have ABL1-derived sequences starting from exon 2 (Figure 1.1). Breakpoints within minor breakpoint cluster region (m-bcr) of BCR results in fusion of BCR exon 1 to ABL1 (e1a2 mRNA), which generates a 190 kDa (p190) form of BCR-ABL1 (also referred to as p185 in some references). Breakpoints within the Major break point cluster region (M-bcr) result in a p210 form of BCR-ABL1 (e13a2 or e14a2 fusion mRNAs, containing exons 1-13 or -14 of *BCR*), while the third break point cluster region (μ-bcr) generates a p230 isoform of BCR-ABL1 (e19a2 mRNA, derived from exons 1-19 of the *BCR* gene). The p190 form of BCR-ABL1 is commonly found in two-thirds of Ph^+ ALL cases, while p230 BCR-ABL1 can be observed in patients with chronic neutrophilic leukemia (CNL), a milder phenotypic variant of CML. This thesis will focus on studies involving the p210 form of BCR-ABL1, which is the direct cause of CML and can be found in one-third of Ph^+ B-ALL patients (Goldman and Melo, 2003; Li et al., 1999a).

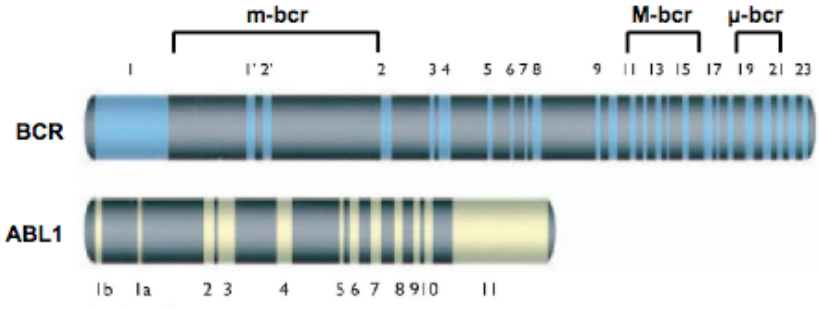

Figure 1.1 Schematic representation of *BCR* and *ABL1* genes and the breakpoint cluster regions. The *BCR* gene contains 23 exons while the *ABL*1 gene contains 11 exons. The three breakpoint cluster regions (m-bcr, M-bcr and µ-bcr) are indicated. The combination of breakpoints in *BCR* and *ABL1* generates three different forms of BCR-ABL1 polypeptides with different molecular masses. While m-bcr breakpoints generate the p190 form of BCR-ABL1, M-bcr and µ-bcr breakpoints give rise to the p210 and p230 forms of BCR-ABL1, respectively. (Figure adapted from (Quintas-Cardama and Cortes, 2009)

1.1.3 Structure of BCR-ABL1

BCR

The *BCR* (breakpoint cluster region) gene, located on chromosome 22, encodes a BCR protein with 1271 amino acids and 160 kDa molecular mass. The protein is ubiquitously expressed in the cytoplasm and does not have intrinsic oncogenic activity. The N-terminal part of BCR contains a coiled-coil (CC) domain responsible for oligomerization of BCR-ABL1 (McWhirter et al., 1993) and a unique serine–threonine kinase activity (Maru and Witte, 1991). The center part of the BCR protein contains a domain with guanine nucleotide exchange factor (GEF) activity for Rho GTPase while C-terminus of BCR has GTPase activating protein (GAP) homology domain for Rac. *BCR* null mice have been generated and show no defect in development of hematopoietic tissues or in lymphopoiesis. However, neutrophils isolated from *Bcr* null mice showed an increased respiratory burst due to deregulation of Rac expression (Diekmann et al., 1991).

c-ABL1

The ABL family of proteins belong to non-receptor tyrosine kinases, consisting of c-ABL1 (Abelson tyrosine kinase, ABL1) and its homolog, ARG (Abl-related gene, or ABL2). *c-ABL1* gene is the human homologue of the v-*abl* oncogene, a fusion oncogene between *gag* gene of M-MuLV and mouse *c-Abl*, carried by the Abelson murine leukemia virus (A-MuLV) (Abelson and Rabstein, 1970). *c-ABL1* is located on human

chromosome 9 and contains 11 exons. Due to alternative splicing of 5' exons, two isoforms of c-ABL1 are encoded with molecular weight of 145 kDa (types Ia and Ib ABL1, or types I and IV c-Abl in mice). Type Ib c-ABL1 is covalently modified by myristoyl fatty acid at its NH_2-terminal glycine residue (Jackson and Baltimore, 1989) and expressed at higher level than type Ia, which is not myristoylated (Quintas-Cardama and Cortes, 2009).

c-ABL1 shares many similarities with another non-receptor tyrosine kinase, SRC, and with its homolog ARG. They both share SRC-homology 2 (SH2), SH3, and SH1 (catalytic) domains at N-terminus, but c-ABL1 has a nuclear-localization signal (NLS), a nuclear-export signal (NES) and an actin-binding domain within its C-terminus (Van Etten et al., 1994). In response to physiological stimuli, c-ABL1 can shuttle back and forth between the nucleus (Van Etten et al., 1989) and the cytoplasm to interact with F-actin cytoskeleton (Van Etten, 1999). While cytoplasmic c-ABL1 may play a role in regulating cell adhesion and migration, several studies have pointed out the role of nuclear c-ABL1 in regulating the cell cycle (Van Etten, 1999). Like BCR, c-ABL1 is ubiquitously expressed in most tissues and does not have intrinsic oncogenic activity. Its tyrosine kinase activity is tightly regulated by phosphorylation status of serine/tyrosine residues (Brasher and Van Etten, 2000; Plattner et al., 1999), N-terminal sequences (Pluk et al., 2002) and by other cellular inhibitors interacting with its SH3 domain (Pendergast et al., 1991a; Wen and Van Etten, 1997) .

Two different strains of *c-Abl1* null mice were generated to study the physiological function of c-Abl1 (Schwartzberg et al., 1991; Tybulewicz et al., 1991). The mice exhibit

poor postnatal viability, thymic and splenic atrophy. They seems to have normal myelopoiesis but have defects in lymphopoiesis and spermatogenesis (Kharbanda et al., 1998)

BCR-ABL1

The structure of the p210 form of the BCR-ABL1 oncoprotein has been extensively studied and is illustrated in Figure 1.2. The coiled-coil (CC) domain at the N-terminal part of the BCR region is preserved from the BCR protein and is responsible for oligomerization of BCR-ABL1, which is required for autophosphorylation and interrupting autoinhibition of BCR-ABL1 mediated by interaction between SRC-homology 3 (SH3) and SH2 domain within ABL1 region (Smith et al., 2003). A critical tyrosine residue (Y177) in the BCR portion of the fusion protein is responsible for interacting with an adaptor protein, growth factor receptor-bound protein 2 (Grb2) (Pendergast et al., 1993b). Phosphoserine and phosphothreonine residues within the N-terminal part of BCR are also important in recruiting SH2 domain containing proteins (Pendergast et al., 1991b). The kinase domain within the ABL1 region, containing the ATP binding site and a Tyr1294 residue required for autophosphorylation and activation of BCR-ABL1, is the most critical part of oncoprotein (Pendergast et al., 1993a). ProXXPro motifs downstream of kinase domain function as binding sites for SH3 domain-containing proteins, such as c-CRK and CRKL (Feller et al., 1994). The C-terminal actin-binding domain is responsible for regulating cell adhesion and cell motility (Goldman and Melo, 2003; Quintas-Cardama and Cortes, 2009)

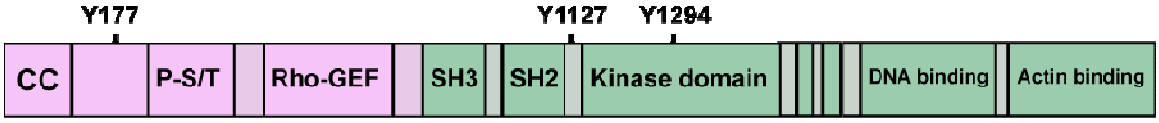

Figure 1.2. Schematic representation of the structure of BCR-ABL1 kinase. The oncoprotein results from fusion of BCR (pink) to ABL1 (green). The BCR region contains a coiled-coil domain (CC) for oligomerization, and a Rho GTP–GDP exchange-factor (GEF) domain. Tyrosine 177 (Y177) and phosphoserine and phosphotheronine (P-S/T) sequences at the N-terminal part of BCR are responsible for recruiting Grb2 and other SH2 domain-containing adapter proteins. The SH3 domain of ABL1 plays a role in maintaining kinase in its inactive state, which can be interrupted by oligomerization through CC domain followed by autophosphorylation at Tyrosines 1127 and 1294 (Y1127, Y1294). DNA and actin binding domains from original ABL1 are preserved at C-terminus of BCR-ABL1.

1.1.4 Downstream signaling of BCR-ABL1

Through interaction with its core proteins, BCR-ABL1 activates several signaling molecules and pathways in Ph^+ leukemia cells. As mentioned above, BCR-ABL1 Y177 is stoichiometrically phosphorylated in leukemic cells, probably as a consequence of autophosphorylation, and is responsible for recruiting adaptor protein GRB2-associated binding protein 2 (GAB2) via Grb2/Gab2 complex (Sattler et al., 2002), which causes activation of phosphatidylinositol 3-kinase (PI3K)/ AKT (Skorski et al., 1997; Skorski et al., 1995). Grb2 can also recruit son of sevenless (SOS), a guanine nucleotide exchange factor, resulting in activation of RAS and extracellular signal-regulated kinase (ERK) pathways (Cortez et al., 1997; Sawyers et al., 1995). On the other hand, activation of SRC family kinases (SFKs) by BCR-ABL1 contributes to the pathogenesis of Ph^+ B-ALL but not of CML (Hu et al., 2004b). Signal transducer and activation of transcription 5 (STAT5) is a latent transcription factor that is phosphorylated and activated by BCR-ABL1 (Ilaria and Van Etten, 1996). Recent studies have demonstrated that abrogation of STAT5 completely abolishes CML-like MPN in murine retroviral bone marrow transduction/transplantation model of CML (Hoelbl et al., 2010; Walz et al., 2009). The net outcome of deregulation of cellular signaling networking by BCR-ABL1 results in increased cell proliferation, reduced apoptosis and decreased adherence of Ph^+ leukemic cells to bone marrow stroma, all of which are postulated to drive leukemogenesis in Ph^+ patients.

1.2 CHRONIC MYELOID LEUKEMIA (CML) AND B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (B-ALL)

1.2.1 Pathological features of CML

CML, with overproduction of maturing neutrophils in blood, bone marrow and peripheral tissues, affects 1 to 2 people per 100,000 and counts for 7-20% cases of leukemia (Perrotti et al., 2010). In 1951, Dameshek first classified it as one of myeloproliferative neoplasms (MPNs) along with polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) (Dameshek, 1951). These disease share several common features:

- They are derived from hematopoietic stem cells or early progenitors
- They are characterized with normal differentiation of hematopoiesis but with over production of specific lineage of mature myeloid cells
- The diseases share abnormalities of hemostasis and thrombosis.
- The diseases have tendency of progressing to acute leukemia

As described earlier, the transcription product of the Ph^+ chromosome, BCR-ABL1, has been shown to be the direct cause of CML, but how the Ph^+ chromosome translocation occurs in the first place remains unclear. Previous exposure to high-dose radiation increases the risk of developing CML, suggesting that DNA damage might predispose to the Ph^+ translocation (Goldman and Melo, 2003; Preston et al., 1994). The clinical course of CML has been characterized into three phases: chronic, accelerated and blast crisis. The chronic phase (CP) can last for months or years. Besides symptoms such

as increased circulating white blood cell (WBC) count due to massive expansion of differentiated mature myeloid cells (median WBC 100,000/μL) (Savage et al., 1997), patients may present with fatigue, weight loss, unusually bleeding, sweats, anemia, splenomegaly, or platelet count above 600,000 to 700, 000/μL. The peripheral blood smear in patients with CML typically shows normal myelopoiesis from myeloblasts to mature neutrophils, with normal morphology. However, the neutrophils in CML patients have reduced leukocyte alkaline phosphatase (LAP, or NAP). The LAP score is useful in distinguishing leukocytosis resulted from infection or polycythemia vera, both of which have higher LAP scores.

The disease is manageable at the chronic phase with standard therapies (see section 1.4), but may progress to advanced stages including accelerated phase (AP) and blast crisis phases (BC), which are characterized as increasing undifferentiated leukemic blasts accumulating in peripheral blood or bone marrow. Patients at CML-AP have a median 1-2 year survival and are diagnosed with having one or more of the following symptoms and laboratory abnormalities (Cortes et al., 2006):

- 5-19% blasts in the peripheral blood or bone marrow
- Peripheral blood basophils $\geqq$ 20%
- Platelets <100,000/mL, unrelated to therapy, or >1,000,000/mL, unresponsive to therapy
- Progressive splenomegaly and increasing white cell count, unresponsive to therapy
- Cytogenetic evolution with new chromosomal abnormalities besides Philadelphia chromosome

Patients may bypass CML-AP, and progress directly to the blast crisis phase from the chronic phase. Blast crisis is the final phase in the evolution of CML, which is characterized by the following criteria:

- >20% blast cells in the bone marrow
- Extramedullary infiltration of blasts in liver and/or spleen
- Large foci or clusters of blasts found in bone marrow biopsy

Patients in blast crisis are generally refractory to treatment (Druker et al., 2001a), with a median survival of approximately 6 months (Perrotti et al., 2010). The leukemic cells in most BC patients have a myeloblastic phenotype, but approximately 25% of patients have pre-B lymphoblasts similar to B-ALL.

1.2.2 Pathological features of Ph^+ B-ALL

Patients with B-ALL or CML at the stage of lymphoid blast crisis share similar characteristics with overgrowth of immature lymphoid cells in bone marrow (>20% are blasts), blood and lymphoid organs and extrameduallary infiltration (Perrotti et al., 2010; Wong and Witte, 2004). Patients typically have other symptoms such as fever, anemia, thrombocytopenia, fatigue, spontaneous bleeding, and infections. Some patients may also exhibit hepatomegaly, splenomegaly, and lymphadenopathy. Since CML-lymphoid blast crisis (CML-LBC) is the disease transition from myeloid lineage to lymphoid disease, the presence of overproduction of myeloid cells (40%) along with significant amount of lymphoid blasts (60%) in bone marrow or peripheral blood aspirate from patients is usually used to distinguish whether patients have Ph^+ B-ALL or CML-LBC.

About 25% of B-ALL patients have the Ph chromosome. While one-third of patients express the p210 form of BCR-ABL1, the other two-third of patients have the p190 form of BCR-ABL1. Recent studies showed a high proportion of Ph^+ B-ALLs have mutation of the *IKZF1* or *Pax5* genes, both of which are transcription factors regulating normal B-cell development (Iacobucci et al., 2010; Mullighan et al., 2008).

1.2.3 Detection of the Ph chromosome

Although patients with Ph^+ leukemias can be diagnosed with a peripheral blood smear or bone marrow biopsy, advanced techniques such as cytogenetic analysis (karyotyping), fluorescence in situ hybridization (FISH) analysis, or reverse transcription polymerase chain reaction (RT-PCR) are used to further confirm the expression of *BCR-ABL1* fusion gene or transcript. These detection methods are also used to evaluate the effect of therapies (see section 1.4.1).

1.3 MOUSE MODELS TO STUDY PH^+ LEUKEMIAS

Although several *in vitro* studies have provided much knowledge regarding the molecular biology of BCR-ABL1, the complex nature of leukemia cannot be adequately modeled in cell culture systems. In order to have complete understanding of pathophysiology of BCR-ABL1-meidated leukemias, expression of the oncogene in the hematopoietic system of a living organism is required. Therefore, three types of mouse models to study BCR-ABL1-induced leukemias have been developed: BCR-ABL1 transgenic mouse models, xenotransplantation of human leukemia cells, and retroviral bone marrow transduction/transplantation mouse models.

1.3.1 Transgenic mouse models

The first transgenic mouse model of BCR-ABL1, which developed acute myeloid or lymphoid leukemia, was first generated in 1990 using the p190 form of BCR-ABL1, (Heisterkamp et al., 1990). The development of transgenic models using the p210 form of BCR-ABL1 was initially hindered by embryonic lethality caused by the strong expression of BCR-ABL1 controlled by *BCR* promoter (Heisterkamp et al., 1991). This hurdle was later overcome by the replacement of *BCR* promoter with metallothionein (MT) promoter/enhancer (Honda et al., 1995) and the development of BCR-ABL1 inducible transgenic mice using a conditional tet-off system (Huettner et al., 2000). However, both modifications result in mice that develop B-lymphoblastic leukemia but not myeloid leukemia. To develop transgenic mice with CML, Honda et al. replaced the MT promoter with the promoter of *tec* gene (cytoplasmic kinase preferentially expressed in hematopoietic precursor cells) to drive expression of BCR-ABL1 (Honda et al., 1998), while Huettner and colleagues developed BCR-ABL1–inducible transgenic mice with control of enhancer from the murine stem cell leukemia *(SCL)* gene. In both approaches, CML-like myeloproliferative disease can be observed in transgenic progenies (Koschmieder et al., 2005).

Although transgenic mice of CML are easy to produce by breeding and they express BCR-ABL1 at physiological relevant level, a few drawbacks still exist, making them less suitable for some types of studies. First, the disease takes a longer time to develop in this model, making them more cumbersome for some therapeutic evaluations. In addition, new transgenic lines need to be made in order to study the leukemogenesis of

different mutant forms of BCR-ABL1. Besides, it will be difficult to use genetic approaches to verify downstream target proteins of BCR-ABL1 and their effect on Ph^+ leukemogenesis.

1.3.2 Xenotransplantation of human leukemia cells into NOD/SCID mice

To monitor leukemogenesis of human CML cells in physiological conditions, $CD34^+$ cells were purified from the peripheral blood or bone marrow of patients with CML and transplanted into sublethally irradiated NOD/SCID mice (Holyoake et al., 1999; Wang et al., 1998). Both normal and leukemic cells were found to successfully repopulate recipient mice. Although these recipient mice did not develop lethal CML-like disease, this model allows the evaluation of the effect of pharmacological inhibitors on primitive human leukemic cells in a physiological environment (Heaney et al., 2010). It is not clear why human leukemic cells do not induce lethal disease in recipient mice, but residual immunity in recipient mice might control disease progression of human leukemic cells. NOD/SCID mice have relatively low levels of natural killer (NK) cells and are deficient in T and B cells. Other lines of immuodeficient mice have been created to reduce host immunity and improve engraftment potential of human cells. NOD/SCID/IL2Rγ^{null} mice were developed recently, lacking functional common IL-2 receptor γ chain (Shultz et al., 2005). This line of immuodeficient mouse does not have mature lymphocytes, NK cells and have severed impairments in innate immune. It would be interesting to see if using NOD/SCID/IL2Rγ^{null} mice as the recipient would improve disease development by human leukemic cells in mice.

1.3.3 Retroviral bone marrow transduction/transplantation mouse model of Ph^+ leukemia

To have a more complete understanding of BCR-ABL1–mediated leukemias, our laboratory and others have established retroviral bone marrow transduction/transplantation mouse models to faithfully study the pathogenesis of human CML or Ph^+ B-ALL in mice. The initial efforts date from 1990 (Daley et al., 1990), with subsequent several modifications to improve efficiency to induce CML-like MPN in mice, including changing the retroviral vector backbone, improving the titer of retroviral vectors, and changing conditions for bone marrow transduction (Li et al., 1999a; Pear et al., 1998).

When 5-FU-pretreated donor bone marrow is transduced with a retrovirus expressing BCR-ABL1 in the presence of myeloid cytokines, transduced cells carrying BCR-ABL1–expressing retroviral provirus can initiate CML-like MPN following transfer into irradiated recipient mice within 4-7 weeks after transplantation (Figure 1.3) (Li et al., 1999a; Van Etten, 2002). The disease that develops in mice shares many pathological features of human CML, with elevated white blood cell count, splenomegaly and massive expansion of $Gr\text{-}1^+$ and $Mac\text{-}1^+$ cells in bone marrow, spleen, liver, and peripheral blood of diseased mice. After serial transplantation, the original CML-like disease observed in primary recipients can progress into acute myeloid or lymphoid leukemia in secondary and tertiary recipients (Daley et al., 1991; Pear et al., 1998). Several studies have later provided evidence that it is BCR-ABL1 expressing HSCs, but not committed myeloid

progenitors, that initiate CML-like disease in this CML mouse model (Hu et al., 2006; Huntly et al., 2004; Naka et al., 2010).

Even though the retroviral bone marrow transduction/transplantation model induces CML-like MPN sharing several similarities with human CML-CP, there are several features of murine CML that are dissimilar to human CML. While pulmonary hemorrhage is a rare complication in human CML, it is often the cause of death in murine CML. Unlike human CML that is monoclonal, murine CML-like MPN is polyclonal disease. The difference in clonality contribute to the rapid and lethal disease progression seen in this model, which can be prolonged in recipients transplanted with fewer BCR-ABL1–transduced donor cells (Jiang et al., 2003).

We have also developed a mouse model to study leukemogenesis of Ph^+ B-ALL in mice. When bone marrow from donors who have not been treated with 5-FU is transduced with retrovirus expressing BCR-ABL1, transduced donor cells can initiate B-ALL in irradiated recipient mice within 5-7 weeks (Figure 1.4). Diseased mice with B-ALL are characterized by circulating malignant $BP1^+$ and $B220^+$ cells in peripheral blood, lymphadenopathy, moderate splenomegaly and hemorrhagic malignant pleural effusion (Roumiantsev et al., 2001; Van Etten, 2002). Hu et al. further showed that BCR-ABL1–expressing pro-B cells are the cells that initiate B-ALL in mice (Hu et al., 2006).

Both retroviral bone marrow transduction/transplantation models of CML and B-ALL provide several advantages to study Ph^+ leukemias. By replacing wild type with mutant forms of BCR-ABL1 lacking specific domains or carrying point mutations, we now have a better understanding of how individual domains/residues contribute to Ph^+

leukemogenesis (Li et al., 1999a; Million and Van Etten, 2000; Roumiantsev et al., 2001; Smith et al., 2003). In addition, the presence of *IRES* in the retroviral vector allows for simultaneous expression of BCR-ABL1 and other genes of interest to study their relationship in leukemogenesis. The pathological effects of BCR-ABL1 and its partner proteins can also be studied by using donor or recipient bone marrow from mice with targeted genetic mutations, which has been used to determine internal or external effect of specific signaling molecule (Krause et al., 2006). Finally, the relative short latency and efficient induction of Ph^+ leukemias in this mouse model provide a platform to evaluate the effect of immunological (Krause et al., 2006) or pharmacological (Chan et al., 2011) regimens in treating Ph^+ leukemias.

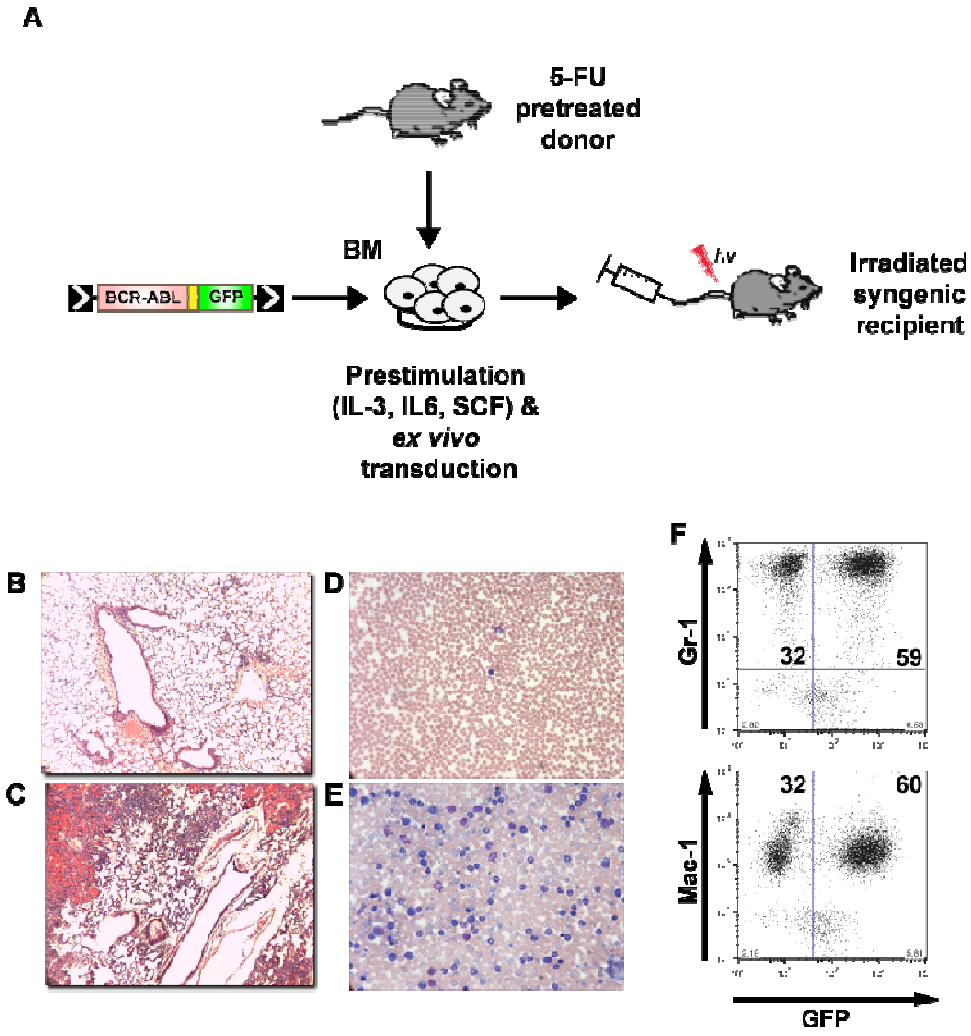

Figure 1.3. Retroviral bone marrow trasnsduction/transplantaion mouse model of CML. (A) To induce CML-like MPN in mice, bone marrow from donor mice treated with 5-FU was harvested and transduced with retrovirus expressing BCR-ABL1/GFP in the presence of myeloid cytokines, followed by transplantation into irradiated syngeneic mice. Recipients usually develop CML-like MPN within 4-7 weeks, with several symptoms such as lung hemorrhage (C) and massive expansion of myeloid (Gr-1$^+$ and Mac-1$^+$) cells in peripheral blood (E, F). Photomicrograph of H&E-stained lung section (B, C) and peripheral blood smear (D, E, Wright/Giemsa stain) of normal mouse (B, D) or recipient with CML-like MPN (C, E). **(F)** FACS analysis of leukemic cells in peripheral blood of representative mouse with CML-like MPN, showing the majority of leukemic cells (GFP$^+$) were also Gr-1$^+$ and Mac-1$^+$.

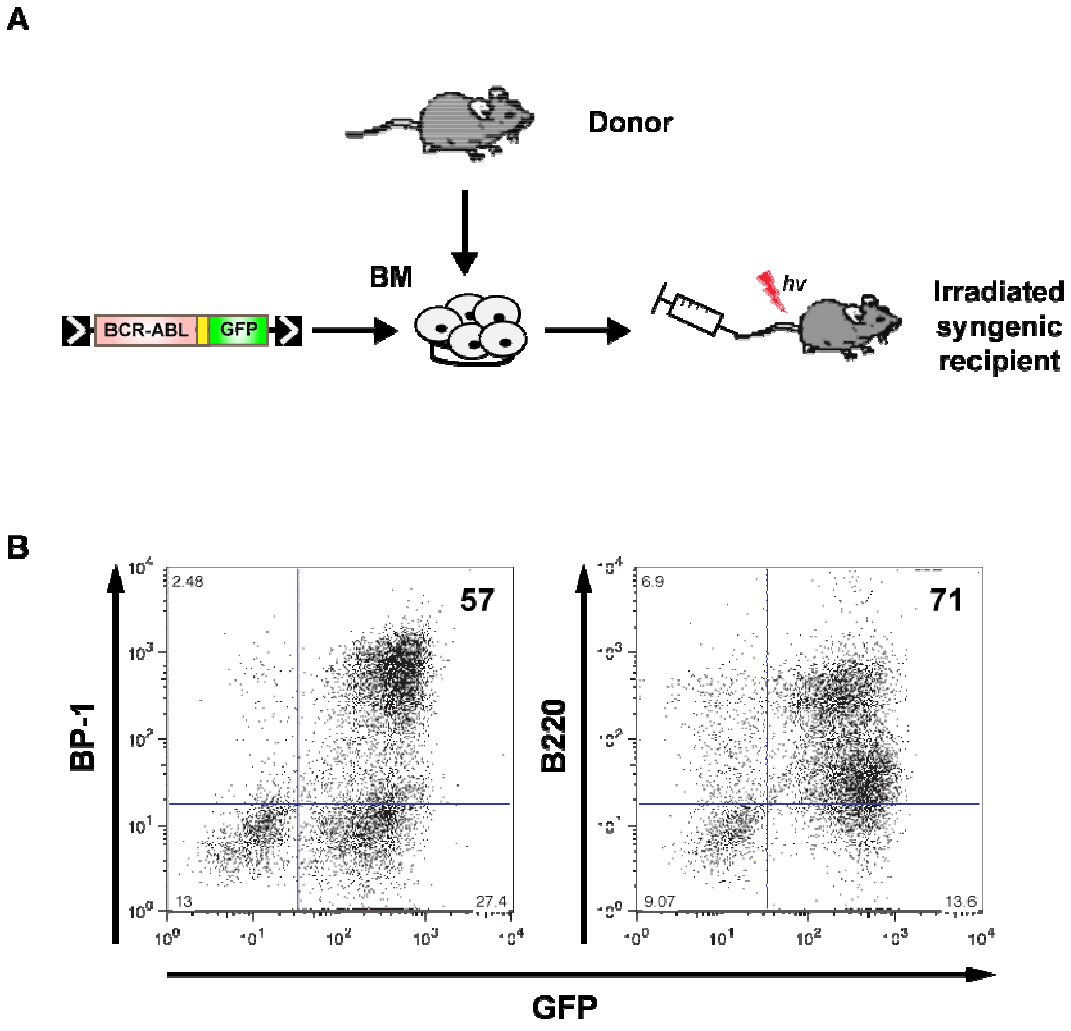

Figure 1.4. Retroviral bone marrow transduction/ transplantation mouse model of Ph$^+$ B-ALL. (A) Bone marrow from donors without 5-FU pretreatment was harvested and transduced with retrovirus expressing BCR-ABL1/GFP. Transduced marrow was transplantation into syngeneic irradiated mice. Recipients usually develop B-ALL within 5-7 weeks. **(B)** FACS analysis of leukemic cells in pleural effusion from mice that developed B-ALL, showing leukemic cells (GFP$^+$) were also BP-1$^+$ and B220$^+$.

1.4 CURRENT TREATMENT FOR BCR-ABL1$^+$ LEUKEMIA

1.4.1 Measuring response to treatment

Three criteria have been used to measure the effect of therapies in patients with Ph^+ leukemia. Complete hematologic remission (CHR), defined as normalization of the patients' peripheral white blood cell count (WBC), platelet count, and hematocrit is the earliest and easiest way to measure patients' response to therapy. Complete cytogenetic response (CCyR) is defined as the absence of Ph chromosome in cytogenetic analysis ($\geq$ 20 metaphases) from bone marrow, which is the current "gold standard" of treatment response. The third level of response measurement, complete molecular response (CMR) is based on quantitative polymerase chain reaction to measure the elimination of *BCR-ABL1* mRNA transcript. This is the most sensitive tool to assess the presence of BCR-ABL1 (Radich, 2011).

1.4.2 Treatment before development of tyrosine kinase inhibitors

The myelosuppressive chemotherapy agents busulfan and hydroxyurea, which globally inhibit both normal and leukemic cells undergoing proliferation, were the standard initial treatment for CML. Even though hydroxyurea induces hematological responses in patients in CML-CP, they were unable to inhibit the oncogenic activity of BCR-ABL1 nor did they induce cytogenic remission in patients (Sawyers, 1999) or delay the progression of the disease to blast crisis. Hence, these treatments were essentially palliative in nature.

Allogenic stem cell transplantation (alloSCT) is currently the only proven curative treatment for CML. Due to age limitation and lack of available HLA-matched donors, only ~20% of patients with CML are eligible for allogeneic transplantation. Patients at younger age (<40) with HLA-matched sibling donors do enjoy a 60-80% five-year survival rate following allografted for CML in chronic phase (Gratwohl et al., 1998; Okimoto and Van Etten, 2011), but the procedure is complicated by the risk of developing graft-versus-host disease (GvHD). To make allogenic transplantation a safer treatment, current efforts have been focused on reducing GvHD without sacrificing graft-versus-leukemia (GvL) effect. These strategies include depleting T cells from donor bone marrow and then reinfusing them at a later time or determining the minimum dose of T-cell infusion to induce GvL but not GvHD (Sawyers, 1999).

For patients who do not have matched donor for allogenic transplantation, interferon-α (INF-α) was for a long time a standard therapy for CML. Interferon is a cytokine that mediates host immune responses against viral infection. The drug may cause a wide range of side effects, such as fever, muscle pain and thrombocytopenia, but induces hematological and cytogenetic response in 10 to 30 percent of patients (Talpaz et al., 1983). It also induces higher survival rate than chemotherapy (Sawyers, 1999). The mechanism of action of IFN-α in CML is still unclear, but is possibly through its role in modulating anti-tumor immune response by activating natural killer cells or up-regulating antigen presentation to T-lymphocytes.

1.4.3 Molecularly targeted therapy of Ph^+ leukemia: Tyrosine kinase inhibitors (TKIs)

The development of tyrosine kinase inhibitors (TKIs) directed against ABL1 has ushered in a new era of targeted therapy for Ph^+ leukemia, and TKI therapy has now supplanted transplantation as the preferred initial treatment for CML. Imatinib, the first TKI approved for treating CML-CP patients, inhibits oncoprotein kinase activity by blocking ATP binding to kinase pocket of BCR-ABL1. It not only has activity against all ABL tyrosine kinases including BCR-ABL1, ABL1, v-ABL and ARG/ABL2 but also inhibits several other tyrosine kinases such as PDGFRα, PDGFRβ and c-Kit (Buchdunger et al., 2000; Sherbenou and Druker, 2007). Although generally well-tolerated, patients treated with imatinib may have mild to moderate side effects including edema, muscle cramps, diarrhea, nausea, skin rashes, and myelosuppression (Druker et al., 2006; Druker et al., 2001b). Imatinib is extraordinarily effective in most patients in CML-CP, as the IRIS study showed about 60-70% of patients achieve a CCyR (Druker et al., 2006). However, some patients fail to respond to imatinib, while a significant number of patients show an initial response but eventually develop resistance to imatinib (Druker et al., 2006; Perrotti et al., 2010). Several mechanisms of acquired resistance to imatinib have been defined, including amplification of the *BCR-ABL1* fusion oncogene, acquisition of mutations in the ABL1 kinase domain that prevent efficient binding of imatinib to BCR-ABL1 (Gorre et al., 2001) or activation of other signaling pathways that help leukemic cells escape from imatinib inhibition (Hu et al., 2004b; Hu et al., 2006).

To overcome mutant forms of BCR-ABL1 that fail to respond to imatinib, more potent second-generation TKIs were developed. Dasatinib, which inhibits both BCR-ABL1 and Src tyrosine kinases, binds to the phosphorylated, active form of BCR-ABL1 and is a more potent inhibitor than imatinib. It is effective in inhibiting many common imatinib resistant mutations, with exception of a particular T315I "gatekeeper" mutation (Shah et al., 2004). Nilotinib, another second-generation TKI that binds to the inactive form of BCR-ABL1, also has higher potency against BCR-ABL1 and several imatinib-resistant mutants, but again fails to inhibit the T315I mutant (Weisberg et al., 2005). Both second-generation TKIs have specific side effects in some patients. Dasatinib can be associated with pleural effusions while Nolitinib causes biochemical changes in liver and pancreatic function (Van Etten et al., 2011). Both dasatinib and nilotinib were recently approved for the initial treatment of CML patients in chronic phase.

1.5 LIMITATIONS OF CURRENT TREATMENT: RESIDUAL LEUKEMIA-INITIATING CELLS

Overall, the use of TKIs to treat patients in CML-CP is quite effective, as 60-70% of patients achieve and maintain a CCyR to imatinib, and 40-50% of those who are intolerant of or relapse on imatinib can be successfully treated with second-generation TKIs (accounting for 15-20% of total CML-CP patients). However, about 20% of patients will have no sustained response to any TKI, and disease relapse after discontinuation of TKIs happens in the majority of cases (Mahon et al., 2010). Furthermore, therapy with TKIs is not nearly as efficient in patients with advanced stages of CML or B-ALL (Druker et al., 2001a; Sawyers et al., 2002). Hence, there are still

significant numbers of Ph^+ leukemia patients that are at risk of relapsing and dying of their disease. Residual leukemia-initiating ("stem") cells (LICs) that are insensitive to TKIs have been postulated to be the fundamental cause of relapse (Graham et al., 2002). LICs are the cells with self-renewal ability that can both initiate and maintain leukemogenesis. From years of studies of human CML cells and in mouse model systems, we now understand that the LICs in CML are very primitive BCR-ABL1-expressing hematopoietic progenitors that are very similar to normal hematopoietic stem cells (HSCs).

1.5.1 Normal Hematopoiesis

HSCs are the origin of the hematopoiesis process that can self-renew and differentiate into committed progenitors, as well as subsequently into all kinds of mature blood cells (Figure 1.5). The process is hierarchical and tightly regulated by cytokines, adhesion molecules and transcription factors (Kondo et al., 2003). It starts from long-term (LT) repopulating HSCs (LT-HSC), which first differentiate into the short-term (ST) repopulating HSC (ST-HSC) and then to non-self-renewing multipotent progenitors (MPP), which can further differentiate into common myeloid progenitors (CMP) or common lymphoid progenitors (CLP). Lineage restricted CMP can give rise to granulocyte-macrophage progenitors (GMP) and megakaryocyte-erythroid progenitors (MEP) (Akashi et al., 2000). The former in turn terminally differentiate into mature granulocytes and macrophages/monocytes, while MEPs terminally differentiate into erythrocytes and platelets. CLPs are the progenitors that give rise to differentiated B-lymphoid, T-lymphoid and natural killer cells (Kondo et al., 2003). An alternative model

of hematopoiesis has been described with the existence of lymphoid primed multipotent progenitors (LMPP) that are capable of giving rise to CMPs, GMPs or CLPs, but not MEPs, which are derived directly from ST-HSCs (Adolfsson et al., 2005).

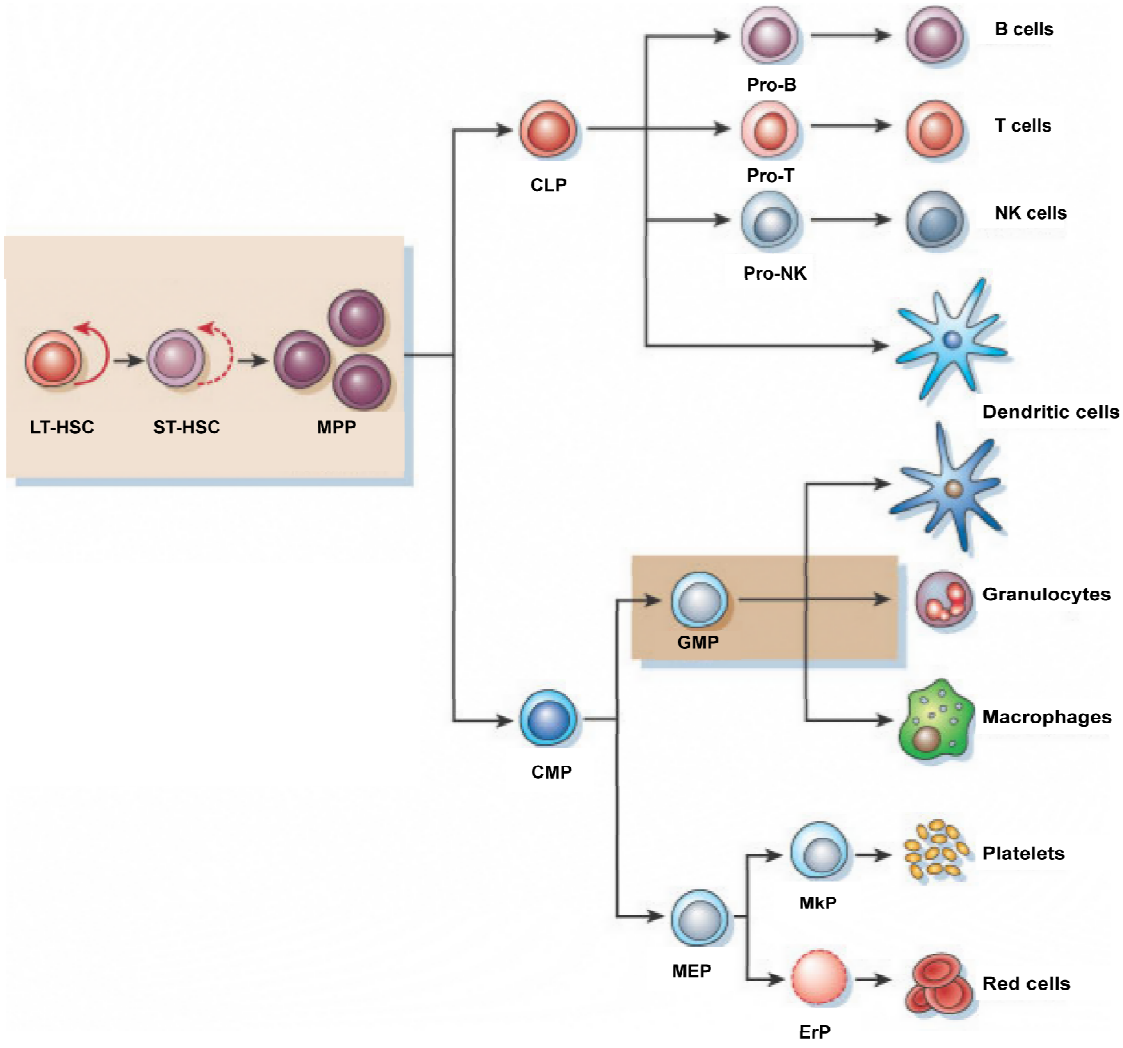

Figure 1.5. Schematic diagram of normal hematopoiesis as described in text. ErP, erythrocyte precursor; MkP, megakaryocyte precursor; NK, natural killer. (Figure adapted from (Reya et al., 2001)

1.5.2 Cancer stem cells hypothesis

It was in 1994 that Dick and colleagues, in a seminal experiment, first demonstrated that only leukemic blasts with the immature cell surface antigen expression pattern $CD34^{+}CD38^{-}$ from human acute myeloid leukemia (AML) could initiate the same disease following xenotransplantation into severe combined immunodeficiency (SCID) mice (Lapidot et al., 1994). This observation led to the formulation of the cancer stem cell hypothesis, which proposes that cancer is heterogeneous and initiated and maintained by a small fraction of cells within tumor population, which have stem-like property that could self-renew and xenograft immunodeficient mice (Reya et al., 2001). These so-called cancer stem cells could either be derived from normal stem cells transformed by oncogenes, or other cell types that gain self-renewal ability during transformation.

1.5.3 Identification of leukemia-initiating cells for Ph^{+} leukemias

It has been postulated that the cell origin to initiate CML could be HSCs or cells with multilineage repopulating potential, since CML can progress from chronic myeloid disease to lymphoid blast crisis. In addition, Ph chromosome can be found in almost all hematopoietic lineages, including granulocytes, monocytes, erythrocytes, megakaryocytes, B-lymphocytes, and occasionally T lymphocytes (Haferlach et al., 1997; Whang et al., 1963). By studying different isoforms of glucose-6-phosphate dehydrogenase in different cell lineage in CML patients, Fialkow et al. first demonstrated that CML is a clonal disease originating from HSCs (Fialkow et al., 1967). Later, studies showed only $CD34^{+}CD38^{-}$ cells from patients in CML-CP had property of LICs to engraft immuodeficient mice (Eisterer et al., 2005). In contrast to other fusion oncogenes,

MOZ-TIF2, *MLL-AF9* and *MLL-ENL* (Cozzio et al., 2003; Wang et al., 2010) that can confer on myeloid progenitors the self-renewal ability to initiate AML, *BCR-ABL1* does not provide self-renewal ability in committed hematopoietic progenitors. Only purified murine HSC ($Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{+}$) but not myeloid progenitors transduced with retrovirus expressing BCR-ABL1 could initiate CML-like MPN in mice (Huntly et al., 2004). Studies further demonstrated that BCR-ABL1 expressing HSC from mice with CML-like MPN induced by retroviral bone marrow transduction/transplantation function as CML LICs that can be serially propagated from primary recipients to secondary recipients (Hu et al., 2006; Naka et al., 2010).

On the other hand, experiments have also been carried out in identifying LICs for Ph^{+} B-ALL. As mentioned previously, the p210 form of BCR-ABL1 is involved in one-third of patients with Ph^{+} B-ALL while the p190 form of BCR-ABL1 can be found in two-third of patients of Ph^{+} B-ALL. By using FISH analysis to search primitive cells expressing Ph^{+} chromosome in Ph^{+} B-ALL patients, Castor et al. found p190 was expressed only in B cell–committed progenitor cells ($CD34^{+}CD38^{-}CD19^{+}$) but not HSCs ($CD34^{+}CD38^{-}CD19^{-}$), while the p210 form of BCR-ABL1 can be found both in populations of $CD34^{+}CD38^{-}CD19^{+}$ as well as $CD34^{+}CD38^{-}CD19^{-}$. However, only B cell–committed progenitor cells carrying BCR-ABL1 (p190 or p210) have the ability to repopulate leukemic cells in NOD/SCID mice, indicating these cells as LICs to initiate B-ALL (Castor et al., 2005). Li and colleagues later also demonstrated that BCR-ABL1-expressing pro-B cells ($CD19^{+}B220^{+}CD43^{+}$) in mice are LICs that initiate disease in the retroviral bone marrow transduction/transplantation model of B-ALL (Hu et al., 2006).

1.5.4 Leukemia-initiating cells are resistant to TKIs

Since BCR-ABL1-transformed HSCs still share many properties with normal HSCs, including their immunophenotype, several studies have isolated LICs from mouse or patients with CML and further characterize their properties. Like HSCs, LICs can reach quiescent at steady state, and are resistant to imatinib treatment (Graham et al., 2002). Recently, Druker and colleagues showed despite inhibition of BCR-ABL1 activity by imatinib in very primitive cells including the HSC population, the CML LICs could escape TKI-induced apoptosis and proliferate as well as self-renew independently of BCR-ABL1 kinase activity through support from the bone marrow microenvironment (Corbin et al., 2011). It is postulated, but not proven, that these TKI-resistant LICs are the cause of relapse and eventual transformation to blast crisis.

To develop strategies to eradicate LICs, we need to know what are the essential signaling pathways, activated either internally or provided externally by the bone marrow microenvironment, that maintain the survival and self-renewal ability of LICs (Chen et al., 2010). By using different transgenic and knockout mice as donors in the murine bone marrow transduction/transplantation model, several signaling pathways important in regulating LICs have been proposed, including Wnt/β-catenin (Hu et al., 2009; Jamieson et al., 2004; Zhao et al., 2007), Hedgehog (Dierks et al., 2008; Zhao et al., 2009), Alox5 (Chen et al., 2009), PTEN (Peng et al., 2010) and FOXO3a (Naka et al., 2010). While HSCs from donor marrow deficient of β-catenin, Smo (Smoothened), Alox5 or FOXO3a showed a relative defect in their ability to induce CML-like disease in mice following

BCR-ABL1 transduction and transplantation, deletion of PTEN, as tumor suppressor, facilitated the disease development.

Besides targeting signaling pathways regulating LICs to eradicate these cells, an alternative strategy is to block cell-surface molecule involved in the engraftment and homing of LICs but not normal HSCs with antibodies or other inhibitors. Previous studies by our lab have demonstrated the importance of CD44, an E-selectin ligand, in engraftment and homing of CML LICs. In mouse retroviral transplantation model of CML, BCR-ABL1 transduced donor marrow deficient in CD44 showed defects in their ability to induce CML-like MPN due to impaired homing of LICs. In contrast, CD44 was neither required for normal HSCs homing nor induction of Ph^+ B-ALL in mice. Further administration of antibodies specific against CD44 attenuated disease development and prolong survival (Krause et al., 2006).

1.6 LIMITATION OF CURRENT TREATMENTS: PROGRESSION TO BLAST CRISIS

CML blast crisis (CML-BC) is characterized by a profound block in hematopoietic differentiation, with overproduction of immature myeloid or lymphoid blasts in blood, bone marrow, and peripheral organs. Patients with CML-BC have a very poor prognosis, with an average survival of 6 months and only transient responses to TKI therapy (Ottmann et al., 2002; Sawyers et al., 2002). The majority of BC patients have a myeloblastic leukemia phenotype, about 25% of patients exhibit a pre-B lymphoblastic phenotype (Perrotti et al., 2010). It is not clear what mechanisms drive disease progression from chronic phase to advanced stage, but the inferior results of treating

CML-BC patients with TKIs suggest that BCR-ABL1–independent signaling activities are involved in this process.

1.6.1 Increased BCR-ABL1 expression

Enhanced BCR-ABL1 activity due to increased *BCR-ABL1* transcripts has been postulated to be a primary underlying cause of disease progression. Previous studies have shown that leukemic cells from patients in blast crisis have higher levels of BCR-ABL1 mRNA transcripts than those in chronic phase (Gaiger et al., 1995), while Jamieson et al. subsequently showed that myeloid progenitors from CML-BC patients have 5 to 6-fold more *BCR-ABL1* transcripts than those in CML-CP patients (Jamieson et al., 2004). The mechanism underlying the phenomenon of increased *BCR-ABL1* transcription is not clear, but evolutionary pressure that favors the outgrowth of clones that maintain higher BCR-ABL1 activity might mediate the selection process.

1.6.2 Genomic instability, damaged DNA repair and additional mutations

The exact consequences of increased expression of BCR-ABL1 are unclear, but might include enhanced proliferation potential in the leukemic cells. In addition, there are many studies that suggest expression of BCR-ABL1 can directly cause genome instability and result in additional mutations in the leukemic cells. Nowicki et al. and others have shown that BCR-ABL1 can increase the mitochondial production of reactive oxygen species (ROS) that cause double-strand breaks (DSBs) in BCR-ABL1 expressing cells, which cannot be faithfully repaired due to additional acquired defects in homologous recombination, non-homologous end-joining, and mismatch repair (Nowicki

et al., 2004; Slupianek et al., 2006; Stoklosa et al., 2008). Patients in late stage CML-CP or blast crisis usually develop additional mutations or deletion in tumor suppressor genes, such as *p53* (20-30% of cases) (Ahuja et al., 1989), or cyclin-dependent kinase inhibitor 2A/2B (CDKN2A/2B) (50% of cases) (Sill et al., 1995).

1.6.3 Acquisition of self-renewal ability

Additional genetic abnormalities besides BCR-ABL1 have been proposed to provide committed progenitors self-renewal ability leading them to become LICs to transform the disease to an advanced stage. In acute myeloid leukemia (AML), several leukemogenic transcription factor fusion proteins, such as MLL-ENL (Cozzio et al., 2003), MOZ-TIF2 (Huntly and Gilliland, 2005), MLL-AF9 (Wang et al., 2010) could confer self-renewal activity upon committed myeloid progenitors, which ordinarily cannot self-renew. The transformed progenitors then could initiate AML in transplantation mouse model. On the other hand, the Wnt/β-catenin pathway has been proposed to play a role in regulating function of both normal HSC and CML LICs, coinciding with disease progression from chronic phase to myeloid blast crisis (Abrahamsson et al., 2009; Jamieson et al., 2004; Minami et al., 2008).

1.6.4 Deregulation of differentiation program

During progression from chronic phase to advanced phase, abnormal expression of transcription factors regulating normal differentiation program has been proposed to be the cause of accumulation of immature myeloblasts or lymphoblasts in advanced stage. Lower expression of CCAAT/enhancer binding protein α (C/EBPα), a transcription

factor regulating normal granulopoiesis, has been associated with progression from CML-CP to CML-BC. BCR-ABL1 down-regulate C/EBPα by stabilizing the expression of an inhibitor of *C/EBPα* mRNA, the heterogeneous nuclear ribonucleoprotein-E2 (hnRNP-E2) (Perrotti et al., 2002). On the other hand, aberrant regulation of transcription factors also happens in lymphoid blast crisis transformation, as deletion of *IKZF1*, gene regulating normal pre-B differentiation has been found in 84% of Ph^+ ALL (Mullighan et al., 2008). Recent studies also reported deletion of *Pax5*, another transcription factor regulating B-cell development, in about 30% of Ph^+ B-ALL (Iacobucci et al., 2010).

1.7 OBJECTIVE OF THIS STUDY

BCR-ABL1 is the central player in the pathogenesis of Ph^+ leukemia. Through its downstream signaling network, BCR-ABL1 transform cells to become leukemogenic with increased proliferation potential, resistant to apoptosis and stromal independence. Even though current TKI therapy is effective in the majority of patients in chronic phase, resistance and relapse still occurs, in part as a consequence of acquisition of ABL1 mutations. Relapse also occurs frequently after withdrawal of TKI therapy in patients in complete molecular remission (PCR negative), due presumably to residual LICs that are insensitive to killing by TKIs. In order to achieve permanent cure of Ph^+ leukemia patients, there is a need to identify and validate additional pharmacological targets to overcome TKI resistance and eradicate LICs. Therefore, a better understanding of downstream signaling pathways that are essential for BCR-ABL1-mediated leukemogenesis and maintenance of LICs is necessary. In this dissertation, I have used the retroviral bone marrow transplantation/transduction mouse models of BCR-ABL1-

induced CML-like MPN and B-ALL to study these two topics. In the second chapter, the aim is to study the role of NF-κB as a downstream target in BCR-ABL1–induced leukemia. In the third chapter, the aim is to define the role of constitutive active β-catenin in regulating BCR-ABL1–mediated leukemogenesis.

Chapter 2.

The role of the NF-κB pathway in BCR-ABL1-induced leukemia

2.1 SUMMARY

The BCR-ABL1 tyrosine kinase activates multiple signaling pathways in leukemic cells from patients with CML and Ph^+ B-ALL. Previous studies have shown that NF-κB is activated in BCR-ABL1-expressing cell lines and primary cells, but the mechanism of activation has not been defined, and importance of NF-κB to myeloid and lymphoid leukemogenesis by BCR-ABL1 is unknown.

To interrogate the role of NF-κB in BCR-ABL1-mediated transformation, we utilized a super-repressor mutant form of IκBα (IκBαSR), which has been used to block NF-κB nuclear localization and transactivation by constitutively sequestering NF-κB in the cytoplasm. Using retrovirus co-expressing BCR-ABL1 and IκBαSR, we found that IκBαSR reduced nuclear RelA expression and inhibited the IL-3–independent growth of Ba/F3 cells and primary B-lymphoblasts transformed by BCR-ABL1. When primary bone marrow cells were transduced with BCR-ABL1 retrovirus and transplanted under conditions favoring induction of B-ALL or CML-like MPN in recipient mice, co-expression of IκBαSR significantly attenuated disease development and reduced the number of leukemic-initiating cells in mice with CML-like MPN. To investigate the mechanism through which NF-κB is activated by BCR-ABL1, we targeted two upstream kinases that negatively regulate IκBα, IKKα/IKK1 and IKKβ/IKK2. To accomplish this, we engineered retroviruses co-expressing BCR-ABL1 and kinase-inactive, dominant-negative mutants of IKKαKM and IKKβKM. Co-expression of either IKK mutant inhibited both B-lymphoid transformation and leukemogenesis by BCR-ABL1 as well as induction of CML-like MPN. Inhibition of the NF-κB pathway correlated with decreased

expression of the NF-κB targets c-Myc and Bcl-X and increased the sensitivity of BCR-ABL1-transformed lymphoblasts to ABL1 kinase inhibitors. These results demonstrate that NF-κB is activated at least in part through the canonical IKK pathway and plays distinct roles in the pathogenesis of myeloid and lymphoid leukemias induced by BCR-ABL1, validating NF-κB and IKKs as targets for therapy of Ph^+ leukemias.

2.2 INTRODUCION

Human cancer is a complicated disease with several characteristics, such as self-sufficiency in growth signal, insensitivity to growth inhibitory signals, resistance to apoptosis signals, propensity for tissue invasion, and limitless replicative potential (Hanahan and Weinberg, 2000). In addition, tumor-associated inflammation has also been linked to promote tumorigenesis and support the escape of malignant cells from destruction by immune system (Hanahan and Weinberg, 2011). Due to their role in regulating body inflammation response, cell survival, and apoptosis, members of the NF-κB family have been extensively studied to understand their roles in regulating cancer development, including Ph^+ leukemia.

2.2.1 Players in the NF-κB signaling pathway

NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) proteins were first identified as nuclear factors interacting with κB motif within the enhancer region of the immunoglobulin κ light (Igκ) chain gene in B-cells (Sen and Baltimore, 1986). Subsequently, extensive studies in the field of NF-κB signaling have identified several NF-κB target genes encoding for adhesion molecules, cytokines, chemokines, and molecules regulating cell proliferation and survival (Basseres and Baldwin, 2006). The promoters and/or enhancers of these genes usually contain κB sequences that can be recognized by NF-κB family members. The canonical κB sequence is 9-10 base pairs long with the consensus sequence 5'- GGGRNWYYCC-3' (R: A or G; N, any nucleotide; W: A or T; Y: C or T) (Hoffmann et al., 2006).

The NF-κB transcription factor family consists of five members: p50/NF-κB1, p52/NF-κB2, RelA/p65, RelB, and c-Rel. Each family member can either form homodimers or heterodimers to activate down stream genes, except for RelB that only forms heterodimers with p50 or p52 subunit (Hayden and Ghosh, 2004). Even though they exhibit similarities in protein structure, genetic studies have shown they have distinct and non-overlapping functions (Gerondakis et al., 2006). They all share a highly conserved Rel homology domain (RHD) at their N-terminus, which is responsible for DNA binding, dimerization with other NF-κB members (Figure 2.1). The RHD also contains a nuclear localization signal (NLS) for NF-κB nuclear translocation and binding site for IκBs. The NF-κB family can be further divided into two subfamilies, "Rel' and "NF-κB". The mammalian Rel subfamily includes c-Rel, RelB, and RelA/p65. They all have a transactivation domain (TAD) at their C-terminus. Members in NF-κB subfamily (p50 and p52) are smaller and only have the RHD but not the TAD.

In resting or unstimulated cells, NF-κB proteins are maintained in a transcriptionally inactive state in the cytoplasm through action of a family of inhibitor proteins, the IκB family. Several mammalian IκB proteins have been identified, including IκBα, IκBβ, IκBε, and Bcl-3. They are characterized with containing several ankyrin repeats, which are also present at C-terminal part of p105 and p100, the precursor proteins of p50/NF-κB1 and p52/NF-κB2. Like the NF-κB family, members of the IκBs family have different expression patterns and functions in different tissues. IκBs can inhibit the activity of NF-κB by blocking its NLS signal as well as preventing their interaction with partner proteins. The activity of IκBs are controlled by phosphorylation

of serine residues at their N-termini by an upstream IκB kinase (IKK) complex, which targets IκBs for ubiquitination and proteasomal degradation. The IKK complex consists of two catalytic subunits, IKKα or IKKβ, and one regulatory scaffold protein, NF-κB essential modulator (NEMO) or IKKγ. Both IKKα and IKKβ have kinase domains (KD) at their N-termini. Studies have shown that phosphorylation of two serine residues (Ser 176/Ser 180 for IKKα, and Ser 177/Ser181 for IKKβ) in the KD is required for cytokine-mediated activation of IKKs (Mercurio et al., 1997), while mutation of Lys 44 within ATP binding site in KD results in a kinase-inactive mutant of IKKs (Delhase et al., 1999; Mercurio et al., 1997). A leucine zipper (LZ) motif is responsible for dimerization of the IKKs, which is required for kinase activity. A helix-loop-helix (HLH) motif interacts with the kinase domain to regulate kinase activity (Delhase et al., 1999). A NEMO binding domain (NBD) in IKKα or IKKβ is responsible for interacting with the kinase-binding domain (KBD) in NEMO, whose polyubiquitination recruits upstream kinases to activate the catalytic subunit of IKKs.

2.2.2 Canonical and Non-canonical NF-κB pathway

Two major pathways that regulate NF-κB activity have been identified (Figure 2.2). In the canonical pathway regulating innate immunity and inflammation, the RelA/p50 heterodimers in quiescent or unstimulated cells are retained in the cytoplasm by their inhibitor protein, IκBα, through masking NLS within RelA. The binding of ligands (IL-1β or TNFα) to their specific cell surface receptors signal to activate the IKK kinase complex. Activated IKKs then phosphorylate IκBα and cause its ubiquitination

and degradation, which releases NF-κB dimers to enter the nucleus and activate transcription of target genes. This process is auto-regulated, since one of the target genes of NF-κB is IκBα, which can then be synthesized and resequester NF-κB in the cytoplasm.

The second pathway regulating the activity of NF-κB is called the non-canonical pathway. This pathway differs from canonical pathway in several aspects. It plays an important role in maintaining development of secondary lymphoid organs, as well as B cell maturation and survival. Unlike the classical pathway involving IKKα, IKKβ and IKKγ, IKKα is the main kinase player in this pathway. Binding of different class of ligands (such as Lymphotoxin B (LTβ) and B-cell activating factors (BAFF) and CD40) to their receptors leads to activation of the NF-κB-inducing kinase (NIK) and stimulation of IKKα, which then leads to proteolysis of p100 and generate p52/RelB heterodimers that can enter nucleus and regulate their target genes (Bonizzi and Karin, 2004; Gilmore, 2006; Karin, 2006).

Cross-talk between the canonical and non-canonical NF-κB pathways has also been proposed. In this hybrid pathway, the production of p52/RelA heterodimers is first controlled by the non-canonical pathway, in which IKKα regulates the processing of p100 to p52. Once the p52/RelA heterodimers is formed, its activation is subsequently regulated by IκBs in canonical pathway. p52/RelA can activate its target genes when IκBs are phosphorylated by IKKs and subsequently degraded by the proteasome (Dejardin, 2006; Dejardin et al., 1995). On the other hand, the transcription of *relb*

(encoding RelB) (Wang and Sonenshein, 2005) as well as *nfκb2* (encoding p100) (Dejardin et al., 2002) have been reported to be regulated by RelA/p50 heterodimers.

2.2.3 Using mice with targeted mutations to study the function of the NF-κB pathway in hematopoiesis

Over the years, mutant mice lacking different components in NF-κB pathway were created and have provided us with important insights regarding how these molecules regulate physiological functions in vivo. $p50^{-/-}$ mice are viable and have normal hematopoiesis. However, these mice show defects in the humoral immune response against bacterial infection (Sha et al., 1995). $RelA^{-/-}$ mice die in utero at embryonic day 15-16 due to massive TNFα–mediated apoptosis of hepatocytes (Beg et al., 1995). In addition to providing essential survival signals in hepatocytes, RelA also protects B-cell from TNFα–induced apoptosis (Prendes et al., 2003). Animals lacking both RelA and the p50 subunit ($RelA^{-/-}p50^{-/-}$) also exhibit an embryonic lethal phenotype, but their essential roles in regulating lymphopoiesis was demonstrated by using a transplantation approach. Irradiated wild-type recipient mice reconstituted with fetal liver cells from either $RelA^{-/-}$ or $RelA^{-/-}p50^{-/-}$ donors had defects in lymphopoiesis but showed increased granulocytosis. However, lymphopenia as well as granulocytosis in these recipients can be rescued by supplementing the graft with bone marrow from wild-type donors. As a result, fetal liver-derived lymphopoiesis can be detected in recipient mice, suggesting RelA and p50 control lymphopoiesis in a non-autonomous manner (Horwitz et al., 1997).

Like their downstream signaling mediators, IKKs also play a profound role in regulating immunity and hematopoiesis, as revealed by studies of knockout mice lacking IKKs. *IKKα*$^{-/-}$ mice die at birth with striking skin and skeletal abnormalities (Hu et al., 1999). Using bone marrow chimeras, Kaisho et al. showed IKKα is important in regulating peripheral B-cell survival and maturation. Lethally irradiated recipients reconstituted with IKKα–deficient fetal liver had reduction of their mature B-cell population and disruption of germinal center formation (Kaisho et al., 2001). On the other hand, *IKKβ*$^{-/-}$ mice have an embryonic lethal phenotype due to TNFα-mediated hepatic apoptosis, which phenocopies RelA-deficient mice (Li et al., 1999b; Tanaka et al., 1999). Using conditional deletion of the *IKKβ* gene in the B cell lineage, an essential role of IKKβ in regulating B-cell survival and proliferation was demonstrated (Li et al., 2003; Pasparakis et al., 2002). Interestingly, unlike *IKKβ* deletion that caused reduced lymphopoiesis, conditional deletion of *IKKβ* in myeloid lineage with Mx-Cre or LysM-Cre resulted in neutrophilia due to increased IL-1β secretion (Greten et al., 2007; Hsu et al., 2011).

2.2.4 Previous studies implicating NF-κB as downstream effector of BCR-ABL1

Several previous studies have demonstrated NF-κB activation in BCR-ABL1–transformed cell lines and primary Ph^+ leukemia cells. While the p50 subunit is constitutively active in DA-1 cells (a murine myeloid progenitor cell line), enhanced RelA DNA binding ability was observed in cells transformed by BCR-ABL1, while antisense oligonucleotides directed against RelA inhibited IL-3 independent cell growth

induced by BCR-ABL1 (Hamdane et al., 1997). Enhanced DNA-binding as well as transactivation activity of RelA/p50 dimers was also found in 32D myeloid cell lines transformed by the p185 form of BCR-ABL1 (Reuther et al., 1998), in CML blasts (Kirchner et al., 2003), and in primary Ph^+ ALL cells (Munzert et al., 2004).

In IL-3-dependent cell lines, the activation of NF-κB is dependent on Ras and kinase activity of BCR-ABL1, as expression of a kinase-inactive mutant of BCR-ABL1 (p210 and p185 forms) or a dominant-negative (N17) mutant of Ras inhibited the transactivation ability of NF-κB in BaF3 and 32D cells (Kirchner et al., 2003; Reuther et al., 1998). Other proteins as mediators of NF-κB activation by BCR-ABL1 have also been investigated. Protein kinase D2 (PKD2), a serine/threonine kinase of PKD family that regulates invasion, proliferation and survival, is tyrosine phosphorylated by BCR-ABL1 and regulates RelA and p52 activation by BCR-ABL1 in LAMA84 cells (an erythroid cell line derived from Ph^+ CML blasts) (Mihailovic et al., 2004). The expression of MEK kinase 1 (MEKK1), an upstream regulator of JNK and ERK pathway, is up-regulated in BCR-ABL1–transformed BaF3 and 32D cells, while expression of dominant negative MEKK1 inhibited NF-κB activation and induced apoptosis of BCR-ABL1–transformed cells in response to genotoxic stress (Nawata et al., 2003).

2.2.5 Rationale

Although previous *in vitro* studies have documented NF-κB activation in BCR-ABL1–expressing cells, it is not clear whether or how NF-κB plays a role in leukemogenesis mediated by BCR-ABL1. *In vivo* leukemia studies are lacking and are essential to

evaluate the NF-κB pathway as a therapeutic target in Ph^+ leukemia. In addition, it is still unclear whether the IKK complex is involved in activation of NF-κB in BCR-ABL1–expressing cells, as some studies suggest BCR-ABL1–induced NF-κB activation is mediated by IKKβ (Duncan et al., 2008; Mihailovic et al., 2004), while others suggest an IKK-independent mechanism (Kirchner et al., 2003; Munzert et al., 2004). Inhibition of IKKβ activity by expressing a kinase-inactive form of IKKβ or with a small molecule inhibitor has been shown to reduce NF-κB activation by BCR-ABL1 (Mihailovic et al., 2004) as well as inducing apoptosis in BCR-ABL1-transfromed cells (Duncan et al., 2008). However, neither Ba/F3 cells expressing p185 form of BCR-ABL1 (Kirchner et al., 2003) nor Ph^+ primary B-lymphoid blasts (Munzert et al., 2004) were found to have enhanced activity of IKKs, suggesting BCR-ABL1 activates NF-κB in an IKK-independent manner.

Here, we used the mouse retroviral bone marrow transduction/transplantation model of CML and Ph^+ B-ALL to determine the role of NF-κB and IKKs in myeloid and lymphoid leukemogenesis mediated by BCR-ABL1.

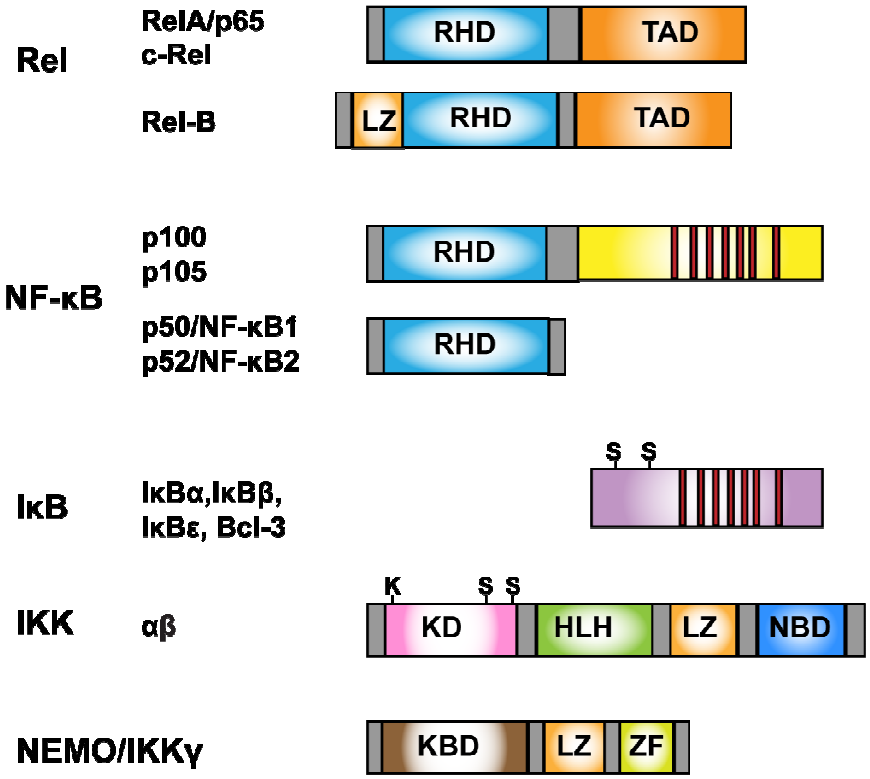

Figure 2.1. The structure of core proteins in NF-κB signaling pathway in mammalian cells. Members of the NF-κB transcription factor protein family all share a Rel homology domain (RHD). The family could be further divided into two subfamilies (Rel and NF-κB). While C-terminal of Rel subfamily has transcriptional activation domains (TAD), the NF-κB subfamily has ankyrin repeat sequences (red bars) at their C-termini, which share similar structure with IκBs. Serine residues of IκBs, which are phosphorylated by IKK kinase complex and target IκBs for proteasome degradation, are listed. IKKα and IKKβ contain a kinase domain (KD), helix-loop-helix (HLH), leucine zipper (LZ) and NEMO binding (NBD) domains, while NEMO/IKKγ has a kinase binding domain (KBD), LZ, and ZF domains. Their functions are described in text. The lysine and two serine residues in IKK kinase domain, whose phosphorylation is important for activation of kinase activity, are listed.

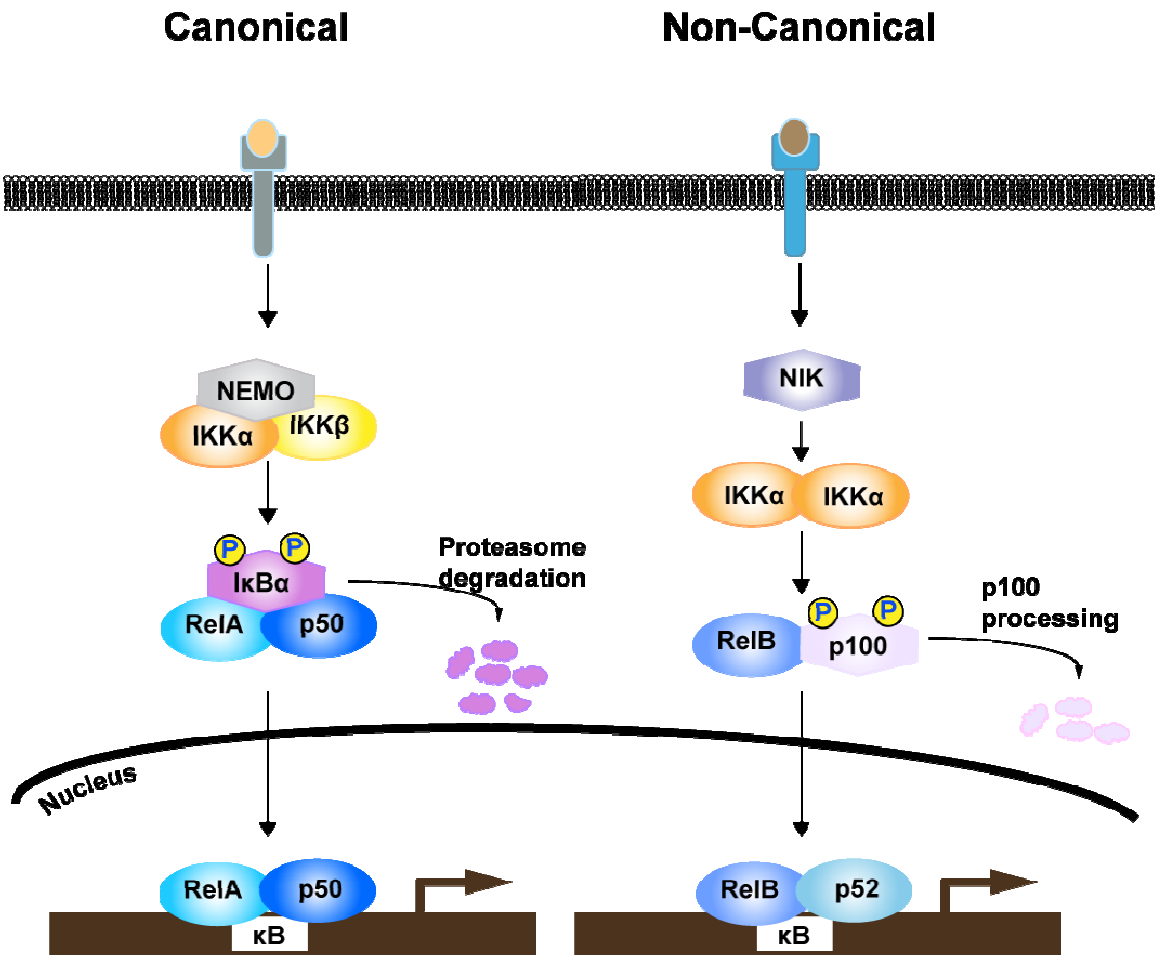
Canonical
Non-Canonical
NEMO
IKKα
IKKβ
NIK
IKKα
IKKα
P
P
IκBα
RelA
p50
Proteasome
degradation
RelB
P
P
p100
p100
processing
Nucleus
RelA
p50
κB
RelB
p52
κB

Figure 2.2

Figure 2.2. NF-κB signal transduction pathways. In the canonical (or classical) NF-κB pathway, the NF-κB dimers (such as RelA/p50) are sequestered by IκBs (often IκBα) in cytoplasm. This pathway can be activated by proinflammation cytokines, such as IL-1β or TNFα. The binding of such ligands to their receptors activates IKK kinase complex including IKKα, IKKβ and NEMO (IKKγ), which subsequently phosphorylates IκBα at two serine residues and targets IκBα for ubiquitination and proteasome degradation. NF-κB dimers then are released from cytoplasm and enter the nucleus to turn on target genes whose promoters or enhancers have the κB sequence. The non-canonical (or alternative) pathway is mainly for activation of p52/RelB complexes through activation of IKKα dimers. The binding of different class of ligands to their receptors (such as Lymphotoxin B (LTβ), B-cell activating factor (BAFF) and CD40 could activate NF-κB-inducing kinase (NIK), which could subsequently activate IKKα. Active IKKα dimers can phosphorylate p100 subunit at its serine residues, and subsequently induce proteolytic processing of p100 to p52. p52/RelB dimers then enter nucleus to target downstream genes.

2.3 RESULTS

2.3.1 NF-κB contributes to BCR-ABL1-mediated lymphoid transformation *in vitro*

To address whether NF-κB is involved in BCR-ABL1 induced leukemia, we first used a mutant "super-repressor" form of IκBα (IκBαSR) to block NF-κB nuclear translocation and transactivation in the presence of BCR-ABL1. IκBαSR contains serine to alanine mutations at position 32 and 36, which blocks phosphorylation of IκBαSR by its upstream regulatory kinases and prevents its subsequent degradation by the proteasome. To co-express both BCR-ABL1 and IκBαSR, the two genes were cloned together into a modified murine stem cell virus (MSCV) vector by replacing the GFP gene from the retrovirus BCR-ABL1/pMIGRI (Pear et al., 1998) with a cDNA encoding IκBαSR. The MSCV vector contains an internal ribosome entry site (IRES) sequence, which allows translation of two proteins from the same mRNA transcript. Thus, BCR-ABL1 is expressed from the upstream cistron while IκBαSR is expressed downstream of the IRES sequence (BCR-ABL1/IκBαSR) (Figure 2.3A).

To verify that IκBαSR is functional and has an effect on BCR-ABL1–mediated transformation, we first transduced Ba/F3 and 32D cells with either BCR-ABL1/pMIGRI or BCR-ABL1/IκBαSR retrovirus and assessed the growth of cells in the absence of IL-3 shortly after transduction. Ba/F3 or 32D parental cells cannot survive in culture media depleted with IL-3, while BCR-ABL1 can transform both cell lines to grow independently of IL-3 (Daley and Baltimore, 1988). In agreement with previous findings by Reuther et al. (Reuther et al., 1998) that IκBαSR had no effect on the viability of 32D

cells expressing BCR-ABL1 in the absence of IL-3, we did not observe increased apoptosis in 32D cells expressing BCR-ABL1/IκBαSR upon IL-3 deprivation (Figure 2.4). Unlike BaF3 parental cells, which cannot survive without IL-3, cells expressing both BCR-ABL1 and IκBαSR can grow independently in the absence of IL-3 (Figure 2.3B). However, we did observe that co-expression of IκBαSR inhibited IL-3-independent cell growth mediated by BCR-ABL1, as the cells grew slower than cells expressing BCR-ABL1/GFP. We did not observe increased apoptosis in BaF3 cells co-expressing IκBαSR (Figure 2.4), suggesting the inhibitory effect of IκBαSR on BCR-ABL1-mediated IL-3 independent cell growth is predominantly on proliferation rather than on survival.

We further verified the expression of BCR-ABL1 and IκBαSR in cells cultured in the absence of IL-3 by immunoblot analysis. Whereas the level of BCR-ABL1 expression was similar between cells transduced with BCR-ABL1/GFP or BCR-ABL1/IκBαSR, we observed lower endogenous IκBα expression in BCR-ABL1/IκBαSR transduced cells (Figure 2.3C). This is a good indication of IκBαSR activity, since the expression of IκBα is regulated by NF-κB (Reuther et al., 1998). We also observed another IκB species with higher mobility, which was recognized by anti-IκBα antibody but not anti-phospho-IκBα antibody (suggesting this form of IκBαSR is resistant to phosphorylation). Consistent with previous findings by Kirchner et al. (Kirchner et al., 2003), there was increased phospho-IκBα in cells expressing BCR-ABL1 alone relative to parental cells. Co-expression of IκBαSR reduced phospho-IκBα compared to cells expressing BCR-ABL1 alone (Figure 2.3C)

To determine whether the defect in cell growth correlated with decreased NF-κB activation, we examined nuclear RelA expression in transformed cells by immunofluorescence staining in combination with confocal microscopy. Expression of BCR-ABL1 enhanced the expression of nuclear RelA to a similar extent as in Ba/F3 parental cells stimulated with NF-κB agonist TNFα, while cells co-expressing BCR-ABL1 and IκBαSR showed significantly reduced nuclear RelA nuclear expression (Figure 2.5). Together, these results suggest that NF-κB acts downstream of BCR-ABL1 in promoting the proliferation of this immortalized pro-B lymphoid cell line.

We next asked if NF-κB contributes to BCR-ABL1–mediated transformation of primary B-lymphoid progenitors. BCR-ABL1 can stimulate the *in vitro* outgrowth of immature B-lymphoid cells that are stroma-dependent and not highly leukemogenic in mice (McLaughlin et al., 1989). A modified assay by plating serial dilutions of BCR-ABL1 transduced bone marrow cells was described earlier, which allows more quantification assessment of oncogene transformation (Smith et al., 2003). As shown in Figure 2.6A, as few as 3000 BCR-ABL1/GFP-transduced bone marrow cells can reach confluence of outgrowth of primary B-lymphoid progenitors but at least 10-fold more BCR-ABL1/IκBαSR–transduced cells were required to achieve the same outcome. BCR-ABL1/IκBαSR–transduced bone marrow cells exhibited slower outgrowth than cells transformed by BCR-ABL1/GFP. Like in BaF3 cells transformed by BCR-ABL1/IκBαSR, co-expression of IκBαSR primarily inhibited proliferation (Figure 2.6B, $P<0.0001$, t-test) but not survival (data not shown) of primary B-lymphoid progenitors transformed BCR-ABL1. Further analyzing nuclear RelA expression, we found BCR-

ABL1/IκBαSR transformed primary B-lymphoid progenitors have significantly lower nuclear RelA expression than cells transformed by BCR-ABL1/GFP (Figure 2.6C, *P*=0.018). These results indicate that NF-κB contributes to transformation of B-lymphoid progenitors by BCR-ABL1.

2.3.2 NF-κB is required for efficient induction of lymphoid and myeloid leukemias by BCR-ABL1

We next assessed whether inhibition of NF-κB through co-expression of IκBαSR might cause a defect in BCR-ABL1–mediated leukemogenesis *in vivo*. We first tested whether IκBαSR impaired BCR-ABL1–induced B-ALL development (Roumiantsev et al., 2001). To induce B-ALL in mice, bone marrow from non-5-FU-treated donors was transduced with matched-titer retroviruses expressing BCR-ABL1/GFP or BCR-ABL1/IκBαSR. Transduced cells were then transplanted into irradiated syngeneic mice. Consistent with the previous study (Roumiantsev et al., 2001), recipients transplanted with BCR-ABL1/GFP transduced marrow developed B-ALL and died within 4 weeks after transplantation. Diseased mice were characterized by circulating malignant $BP1^+$ and $B220^+$ cells in peripheral blood, lymphadenopathy, moderate splenomegaly (average weight 0.3 g), and a hemorrhagic malignant pleural effusion. Mice transplanted with BCR-ABL1/IκBαSR–transduced marrow also developed B-ALL with similar pathological features but had a modest but significant increase in their survival (Figure 2.7A, *P*=0.0096, Mantel-Cox test). Nuclear RelA expression was further evaluated in primary pleural effusion lymphoblasts or cultured B-lymphoid cells derived from mice with B-ALL induced by BCR-ABL1/GFP or BCR-ABL1/IκBαSR. Leukemic cells

expressing BCR-ABL1/IκBαSR had decreased nuclear RelA expression relative to the cells expressing BCR-ABL1/GFP (Figure 2.8).

To determine whether inhibiting NF-κB has an effect on the frequency of LICs in mice with BCR-ABL1 induced B-ALL, we quantified the number of unique proviral integration events in leukemic cells from individual recipients from two cohorts. Genomic DNA from malignant tissue of B-ALL mice induced by BCR-ABL1/GFP or BCR-ABL1/IκBαSR was extracted and subjected to Southern blot analysis with an *IRES* probe. B-lymphoid leukemias induced by BCR-ABL1/GFP showed an oligoclonal to polyclonal pattern of LICs, with an average of 8.1±1.1 independent proviral clones per recipient. By contrast, the recipients transplanted with BCR-ABL1/IκBαSR transduced marrow had a lower average number of proviral clones (5.8±0.6), which was of borderline statistical significance (Figure 2.7B, P=0.0697, t-test). These results demonstrate that inhibition of NF-κB impairs B-lymphoid leukemogenesis by BCR-ABL1.

We next tested if IκBαSR had an effect on pathogenesis of the CML-like myeloproliferative neoplasm (MPN) induced by BCR-ABL1 in retroviral bone marrow transduction/transplantation mouse model. To induce CML-like MPN in mice, bone marrow was harvested from donors pre-treated with 5-fluorouacil (5-FU). After incubation and transduction with matched-titer retroviruses expressing BCR-ABL1/GFP or BCR-ABL1/IκBαSR in the presence of myeloid cytokines (IL-3, IL-6 and SCF), transduced marrow was transplanted into irradiated recipient mice. All mice transplanted with BCR-ABL1/GFP transduced marrow developed and died of CML-like MPN around

3 weeks after transplant (Figure 2.9A). Consistent with previous studies from our laboratory (Li et al., 1999a), the disease was characterized by a greatly elevated leukocyte count in peripheral blood (about 1-2x10^5/μl), splenomegaly (mean spleen weight 1.2 g), lung hemorrhages and massive expansion of Gr-1^+ Mac-1^+/CD11b^+ progenitors in bone marrow, spleen, liver, and peripheral blood. Mice transplanted with BCR-ABL1/IκBαSR transduced marrow showed a similar pathological phenotype but had significant longer survival (Figure 2.9A, median survival 25 days vs. 19 days for BCR-ABL1/GFP recipients; $P<0.0001$, Mantel-Cox test).

To determine whether inhibition of NF-κB affected the number of LICs that induced CML-like MPN, we assessed the number of independent proviral clones in individual disease mice. Genomic DNA from spleen of diseased mice from each arm was extracted and subjected to Southern blot analysis with the *IRES* probe. Mice transplanted with BCR-ABL1/GFP–transduced marrow revealed significant higher number of independent clones (9.9±0.8) in their leukemic cells than mice transplanted with BCR-ABL1/IκBαSR–transduced marrow (Figure 2.9B, 6.8±0.5 independent clones, $P=0.0096$, t-test). This result suggests that NF-κB inhibition impairs BCR-ABL1-induced CML-like MPN in part through attenuation of LICs in this model.

To determine which downstream signaling pathways of BCR-ABL1 were affected by the expression of IκBαSR, protein lysates from spleens of mice with CML-like MPN induced by BCR-ABL1/GFP or BCR-ABL1/IκBαSR were subjected to immunoblot analysis. Previous studies indicated that *c-Myc* gene and the prosurvival gene *Bcl-X* both contain κB binding sites and are induced by BCR-ABL1 expression (Chen et al., 1999;

Duyao et al., 1990; Goldman and Melo, 2003). Consistent with these studies, expression of both c-Myc and Bcl-X was induced by BCR-ABL1, whereas co-expression of IκBαSR reduced the expression of c-Myc but not Bcl-X (Figure 2.10). As in Ba/F3 cells, myeloid leukemia cells from disease mice induced by BCR-ABL1/IκBαSR transduced marrow had reduced expression of IκBα, indicative of suppression of NF-κB signaling.

2.3.3 Inhibition of IKKα and IKKβ impairs BCR-ABL1-mediated B-lymphoid transformation *in vitro*

To investigate the mechanism of activation of NF-κB in BCR-ABL1-expressing cells, we further tested whether two upstream kinases that negatively regulate IκBα, IKKα/IKK1 and IKKβ/IKK2, were involved. To accomplish this, we engineered retroviruses co-expressing BCR-ABL1 and kinase-inactive IKKα (IKKαKM) or IKKβ (IKKβKM) mutants, created by substitution of alanine for lysine within the ATP binding site. The ability of these IKKαKM and IKKβ KM mutants to block NF-κB activation has been demonstrated previously (Nakano et al., 1998). The *GFP* gene from BCR-ABL1/GFP retrovirus was replaced with either IKK mutant to create BCR-ABL1/IKKαKM or BCR-ABL1/IKKβKM retroviruses (Figure 2.11A).

We first tested the effect of co-expression of IKKαKM or IKKβKM on BCR-ABL1–mediated B-lymphoid transformation in the stromal growth assay (Smith et al., 2003). Expression of either kinase-inactive mutant significantly impaired BCR-ABL1-mediated transformation, as marrow cultures transduced with BCR-ABL1/IKKαKM or BCR-ABL1/IKKβKM retroviruses not only took longer to reach confluence than BCR-

ABL1/GFP-transduced marrow, but also required more cells to initiate the same outgrowth. BCR-ABL1/GFP–transduced marrow cultures could efficiently reach confluence when ≥ 3000 cells were seeded, but a >30-fold increase in the number of BCR-ABL1/IKKαKM– or BCR-ABL1/IKKβKM–transduced cells were required to achieve maximal growth in all wells (Figure 2.11B). In a complementary transformation assay, pre-B cell colony formation in agarose (Rosenberg and Baltimore, 1976; Warren et al., 2003), bone marrow transduced with BCR-ABL1/IκBαSR, or either BCR-ABL1/IKK-KM formed significantly fewer colonies (Figure 2.11C) that were much smaller in size (Figure 2.11D) than those generated by BCR-ABL1/GFP–transduced marrow. Primary B-lymphoblasts transformed by either BCR-ABL1/IKK-KM retrovirus also had significantly reduced nuclear RelA expression relative to BCR-ABL1/GFP expressing cells (Figure 2.12, $P<0.0001$, t-test). Together, these results demonstrate that inhibition of IKKs impairs BCR-ABL1-mediated B-lymphoid transformation by interfering with NF-κB activation.

2.3.4 Kinase inactive IKK mutants attenuate BCR-ABL1-mediate B-ALL

To test whether IKK inhibition affects B-lymphoid leukemogenesis by BCR-ABL1, bone marrow from donors not treated with 5-FU was transduced with matched-titer retroviruses expressing BCR-ABL1/GFP, BCR-ABL1/IKKαKM, or BCR-ABL1/IKKβKM without myeloid cytokines. Transduced marrow was subsequently transplanted into irradiated recipients. As before, recipients of BCR-ABL1/GFP–transduced bone marrow succumbed to B-ALL within four weeks of transplantation (Figure 2.13A). By contrast, recipients of marrow transduced with either BCR-

ABL1/IKK-KM retrovirus also developed B-ALL, but had significantly longer survival than BCR-ABL1/GFP recipients (P<0.0001, Mantel-Cox test) and displayed a milder disease phenotype, with smaller malignant pleural effusions (Figure 2.13B) and lymph nodes (data not shown) at the time of morbidity or death. Interestingly, inhibition of IKKα appeared to have a greater effect on lymphoid leukemogenesis than IKKβ. Recipients of BCR-ABL1/IKKαKM–transduced marrow survived significantly longer than those with BCR-ABL1/IKKβKM–transduced marrow (Figure 2.13A, median survival 67 days vs. 41 days for BCR-ABL1/IKKβKM recipients, P=0.0007, Mantel-Cox test). These results suggest that IKKα might affect IKKβ–independent signaling pathway(s) downstream of BCR-ABL1.

Since both IKK-KM mutants were FLAG-tagged, we could confirm the expression of FLAG-tagged kinase-inactive IKK mutants in transformed lymphoblasts from diseased mice (Figure 2.13C). Analysis of RelA nuclear expression in pleural effusion lymphoblasts from diseased mice (Figure 2.13, D and E) demonstrated that leukemic cells from B-ALL induced by BCR-ABL1/GFP have higher RelA nuclear expression than those transformed by BCR-ABL1/IKKαKM (P= 0.0003, t-test) or BCR-ABL1/IKKβKM (P<0.0001, t-test). Southern blot analysis of the number of leukemic clones in mice with B-ALL demonstrated that co-expression of either IKK-KM mutant with BCR-ABL1 significantly reduced the number of LICs that initiated the B-ALL. While recipients of BCR-ABL1/IKKαKM or BCR-ABL1/IKKβKM transduced marrow had an average of 2.0 ± 0.4 and 2.7 ± 0.6 independent proviral clones (Figure 2.14),

respectively, the average clonality of BCR-ABL1/GFP induced leukemia was 6.2 ± 0.8 (P=0.001, and P=0.0057, respectively, t-test).

2.3.5 Kinase-inactive IKK mutants impair myeloid transformation and leukemogenesis by BCR-ABL1

Since we observed that IKK mutants had an effect on BCR-ABL1–mediated lymphoid transformation, we further investigated whether IKK mutants also affected primary myeloid progenitor transformation by BCR-ABL1. Bone marrow cells from donors pretreated with 5-FU were transduced with BCR-ABL1/GFP or either BCR-ABL1/IKK-KM retrovirus in the presence of myeloid cytokines. Transduced cells were subsequently either plated in methylcellulose culture *in vitro* without any cytokines or transplanted into syngeneic irradiated recipients to study the development of CML-like MPN *in vivo*. In accordance to previous studies (Gishizky and Witte, 1992; Zhao et al., 2009), BCR-ABL1–transformed myeloid progenitors can form cytokine-independent myeloid colonies in methylcellulose culture (Figure 2.15A). Bone marrow progenitors transduced by either BCR-ABL1/IKK-KM formed fewer colonies in methylcellulose culture, demonstrating that both IKK mutants impaired BCR-ABL1–mediated myeloid transformation *in vitro*.

In the *in vivo* study to test the effect of IKK mutants on BCR-ABL1 induced CML-like MPN, we observed that all recipients with BCR-ABL1/GFP–transduced marrow developed CML-like MPN within 25 days of transplantation (Figure 2.15B). By contrast, mice receiving bone marrow transduced with either BCR-ABL1/IKK-KM retrovirus survived significantly longer (P<0.0001, Mantel-Cox test). While about half of recipients

with BCR-ABL1/IKK-KM–transduced marrow developed CML-like MPN, the remaining recipients developed other hematopoietic malignancies such as B-ALL or histiocytic sarcoma, sometimes in combination with MPN. Mice that develop mixed CML-like MPN and B-ALL are frequently observed in recipients of BCR-ABL1–transduced bone marrow under conditions when CML-like disease is attenuated (Roumiantsev et al., 2001). Using immunoblot analysis, we confirmed the expression of the mutant IKKs and BCR-ABL1 in leukemic myeloid cells from diseased mice (Figure 2.15C). Analysis of the number of leukemic clones that initiated the MPN in these recipients showed a striking reduction in the frequency of leukemia-initiating cells in recipients of either BCR-ABL1/IKK-KM–transduced bone marrow. While CML-like MPN induced by BCR-ABL1/GFP was polyclonal (7.6± 0.6 independent clones), the disease induced by BCR-ABL1/IKKαKM and BCR-ABL1/IKKβKM had 1.4 ± 0.3 and 3.3 ± 0.5 independent clones, respectively (Figure 2.15D, $P<0.0001$ and $P=0.0003$, t-test). These results suggest that both IKKα and IKKβ are involved in establishing and maintaining the population of B-ALL and CML leukemia-initiating or leukemic stem cells.

To determine how the kinase-inactive mutants affected the signaling pathways downstream of BCR-ABL1 to impair its transformation ability, we analyzed protein lysates from leukemic tissues of mice with CML-like MPN (Figure 2.16) by immunoblot analysis. Compared to lysates from mice with CML-like MPN induced by BCR-ABL1/GFP, those induced by either BCR-ABL1/IKK-KM retrovirus showed a consistent reduction in the level of anti-apoptotic protein, Bcl-X. On the other hand, reduced expression of c-Myc, which regulates proliferation in BCR-ABL1-transformed cells

(Sawyers et al., 1992), was more variable but was found in some MPNs induced by BCR-ABL1/IKKβKM. The expression of IκBα was lower in cells expressing either IKK mutant, suggesting inhibition of NF-κB activity. These results are in accord with our previous findings with IκBαSR, and indicate that the canonical IKKβ-IκBα pathway may regulate proliferation as well as survival in CML.

2.3.6 IκBαSR as well as kinase-inactive IKK mutants increase the sensitivity of BCR-ABL1-expressing leukemic cells to TKIs

Our results so far suggest that inhibition of NF-κB through co-expression of IκBαSR or either kinase-inactive IKK mutant attenuates lymphoid and myeloid leukemogenesis by BCR-ABL1, validating components in NF-κB pathway as potential therapeutic targets in Ph^+ leukemia. Therefore, we further tested if co-expression of these mutants could enhance sensitivity of BCR-ABL1-epxressing leukemic cells to TKIs, such as imatinib or dasatinib. Primary B-lymphoid progenitors transformed by BCR-ABL1/GFP, BCR-ABL1/IκBαSR, BCR-ABL1/IKKαKM, or BCR-ABL1/IKKβKM were subjected to cell growth analysis in the presence of increasing concentration of TKIs. Co-expression of IκBαSR sensitized leukemic cells to imatinib (Figure 2.17A). While cells expressing BCR-ABL1/GFP had an IC_{50} for imatinib of 348 nM, cells expressing BCR-ABL1/IκBαSR had modest but significantly decreased IC_{50} at 127 nM (P<0.0001, t-test).

Similarly, co-expression of IKKαKM or IKKβKM increased the sensitivity of BCR-ABL1 expressing lymphoblasts to imatinib, with IC_{50} falling from 720 nM to

171nM and 146 nM, respectively (Figure 2.17B, $P<0.0001$ in both cases, t-test). Co-expression of either IKK mutant also sensitized leukemic cells to a more potent second-generation TKI, dasatinib. While growth of BCR-ABL1/GFP–transformed cells were inhibited by dasatinib with an IC_{50} of 0.76 nM, cells transformed by BCR-ABL1/IKKαKM or BCR-ABL1/IKKβKM were significantly more sensitive to dasatinib, with IC_{50} at 0.255 nM and 0.439 nM, respectively (Figure 2.17C, $P<0.0001$ and $P=0.0099$, t-test). Co-expression of IKK mutant did not affect the sensitivity of BCR-ABL1-transformed B-lymphoblasts to corticosteroids (dexamethasone, Figure 2.18). Our results show that co-expression of either kinase-inactive IKK mutant sensitize BCR-ABL1-transformed leukemic cells to imatinib and dasatinib, suggesting that IKK inhibition in combination with TKIs may be a rational strategy for treatment of Ph^+ leukemias.

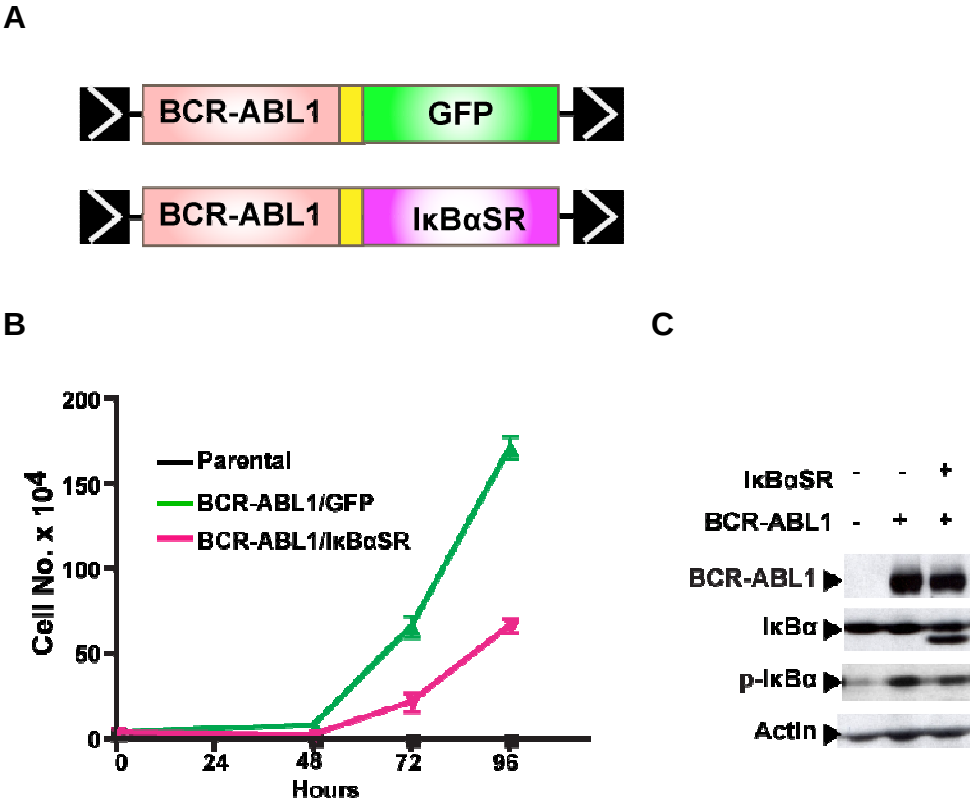

Figure 2.3. IκBαSR inhibits BCR-ABL1 transformation ability. (A) Structure of BCR-ABL1/GFP and BCR-ABL1/IκBαSR retroviruses. Both viruses had BCR-ABL1 expressed at upstream of IRES (yellow box), and either GFP or IκBαSR expressed downstream of the IRES. **(B)** $4x10^4$ Ba/F3 parental or cells transduced with retrovirus expressing BCR-ABL1/GFP or BCR-ABL1/IκBαSR were plated in triplicate in the absence of IL-3 at day 0. Viable cells were determined by trypan blue staining every 24 hrs. The difference between cells expressing BCR-ABL1/GFP and BCR-ABL1/IκBαSR at 96 hours was significant (P<0.0001, t-test). **(C)** Immunoblot of protein extracts from the cell lines in (B), demonstrating expression of IκBαSR and BCR-ABL1.

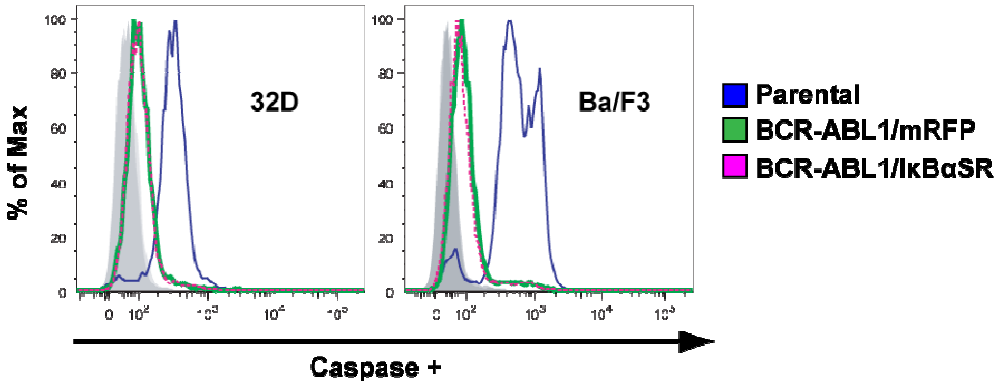

Figure 2.4. IκBαSR does not have effect on survival of cells expressing BCR-ABL1 after IL-3 depletion. 32D or Ba/F3 cells transformed by BCR-ABL1/mRFP, or BCR-ABL1/IκBαSR were cultured in the absence of IL-3 for 72 hrs. Apoptotic cells were detected by caspase marker that binds to most of caspases, and analyzed with FACS. Shaded area: negative control.

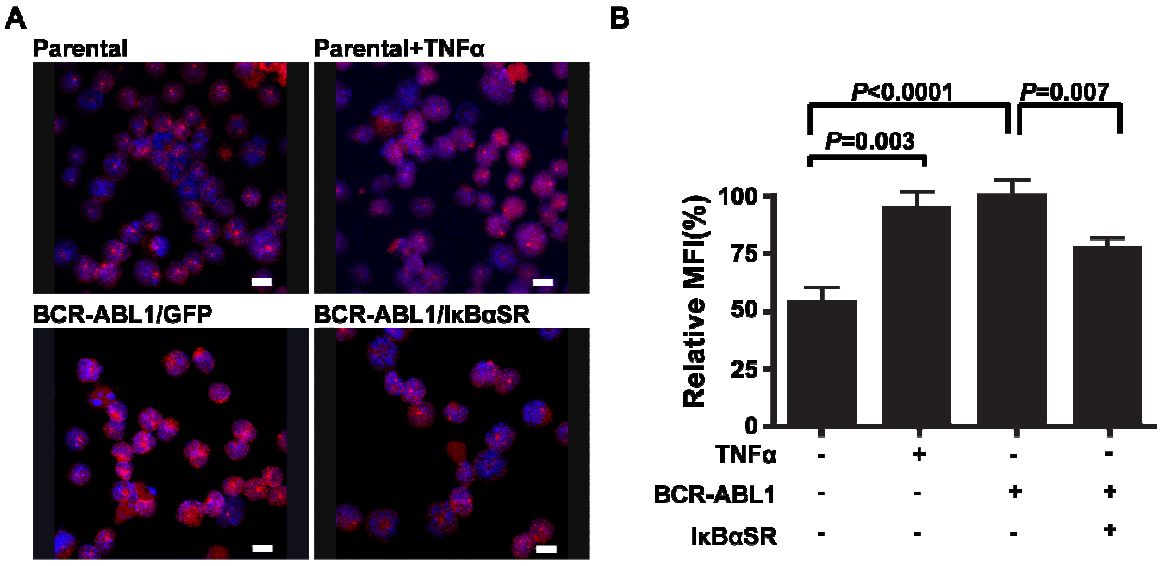

Figure 2.5. Ba/F3 cells transformed by BCR-ABL1/IκBαSR have reduced nuclear RelA expression relative to cells transformed by BCR-ABL1/GFP. (A) Representative confocal photomicrographs of nuclear RelA expression in Ba/F3 parental cells unstimulated or treated with TNFα (20 ng/ml) for 15 min, and of Ba/F3 cells expressing BCR-ABL1/GFP or BCR-ABL1/IκBαSR. Cells were stained with antibody against RelA (red) and counterstained with Hoechst dye (blue). Scale bar=10 μm. **(B)** Quantification of nuclear RelA fluorescence intensity per cell in cells shown in (A). Data are presented as mean of fluorescence intensity (MFI) of RelA staining relative to cells expressing BCR-ABL1/GFP. P values are indicated (t-tests).

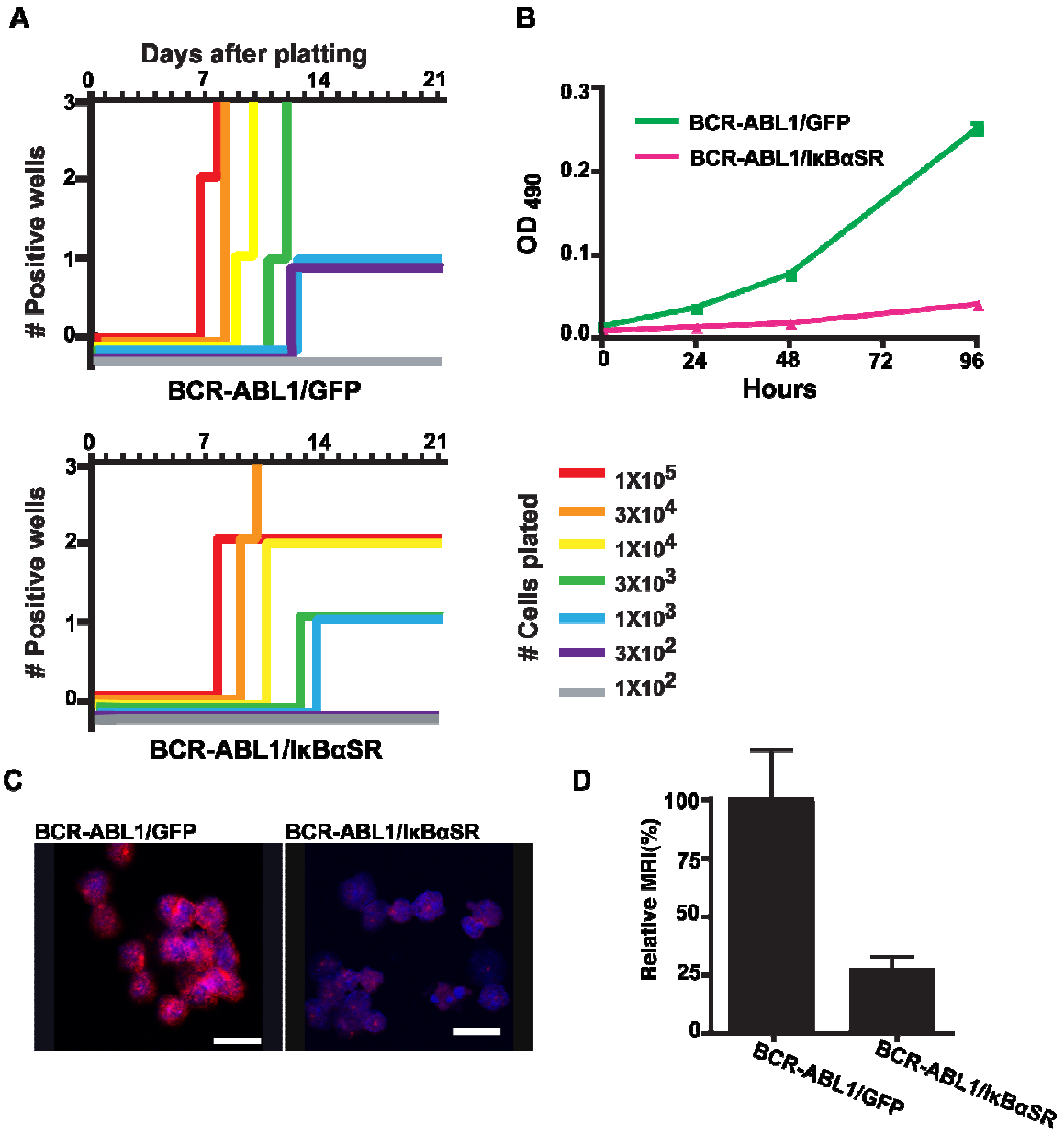
A
Days after platting
0
7
14
21
Positive wells
BCR-ABL1/GFP
BCR-ABL1/IκBαSR
B
OD 490
BCR-ABL1/GFP
BCR-ABL1/IκBαSR
Hours
Cells plated
1X10^5
3X10^4
1X10^4
3X10^3
1X10^3
3X10^2
1X10^2
C
BCR-ABL1/GFP
BCR-ABL1/IκBαSR
D
Relative MRI(%)
BCR-ABL1/GFP
BCR-ABL1/IκBαSR

Figure 2.6

Figure 2.6. IκBαSR inhibits primary B-lymphoid transformation by BCR-ABL1. **(A)** Bone marrow cells from Balb/c mice were transduced with retroviruses expressing BCR-ABL1/GFP or BCR-ABL1/IκBαSR and plated in triplicate at decreasing numbers of cells per well, as indicated by the colored lines. Nontransduced cells were added to 10^6 total cells per well to provide stromal support. Positive wells were scored vs. time when the viable non-adherent cell number reached 10^6/well. **(B)** Proliferation of primary B-lymphoid progenitors transformed by BCR-ABL1/GFP or BCR-ABL1/IκBαSR. Equal number of cells were plated in triplicate at day 0. Viable cells were determined by MTS assay. The difference in viable cell number at 96 hours was significant ($P<0.0001$, t-test). **(C)** Representative confocal photomicrographs of nuclear RelA (red) expression in B-lymphoblasts expressing BCR-ABL1/GFP or BCR-ABL1/IκBαSR. Cells were counterstained Hoechst dye (blue). Scale bar=10 μm. **(D)** Quantification of nuclear RelA fluorescence expression of cells shown in (C). Data are presented as mean nuclear RelA fluorescence intensity per cell relative to cells expressing BCR-ABL1/GFP. The average nuclear RelA expression of BCR-ABL1/IκBαSR-transformed B-lymphoid cells was significant reduced ($P=0.0026$, t-test).

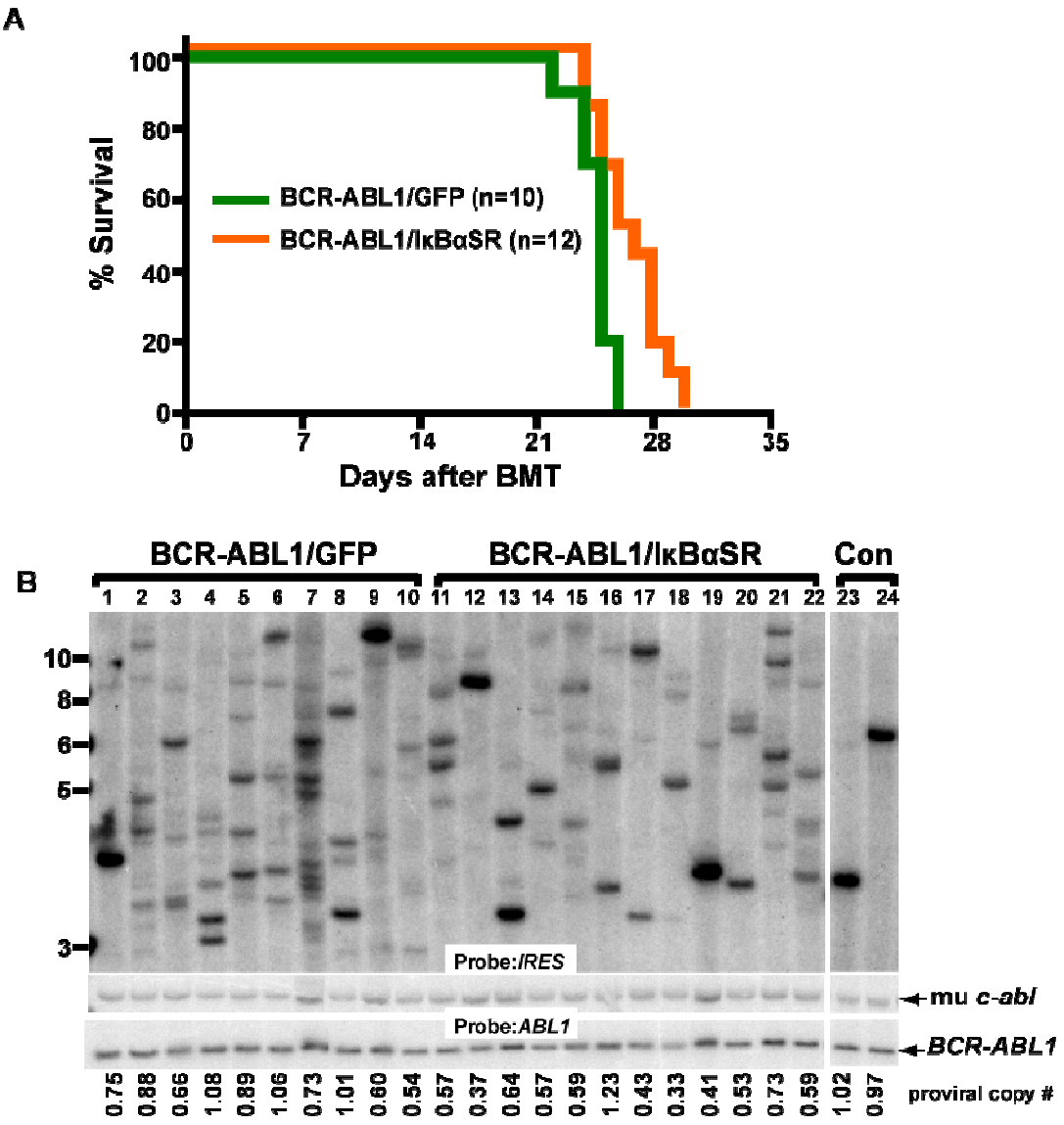
A
100
80
60
40
20
0
% Survival
BCR-ABL1/GFP (n=10)
BCR-ABL1/IκBαSR (n=12)
0
7
14
21
28
35
Days after BMT
B
BCR-ABL1/GFP
BCR-ABL1/IκBαSR
Con
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
10
8
6
5
3
Probe:IRES
mu c-abl
Probe:ABL1
BCR-ABL1
0.75 0.88 0.66 1.08 0.89 1.06 0.73 1.01 0.60 0.54 0.57 0.37 0.64 0.57 0.59 1.23 0.43 0.33 0.41 0.53 0.73 0.59 1.02 0.97
proviral copy #

Figure 2.7

Figure 2.7. IκBαSR expression prolongs survival of mice with BCR-ABL1–induced B-ALL. (A) Kaplan-Meier survival curve for recipients of BCR-ABL1/GFP or BCR-ABL1/ IκBαSR transduced marrow. The number of individual mice in each arm is indicated. All mice developed B-ALL as described. Mice with B-ALL induced by BCR-ABL1/ IκBαSR survived significant longer than control (P=0.0096, Mantel-Cox test). **(B)** Genomic DNA from pleural effusion lymphoblasts of B-ALL mice was subjected to Southern blot analysis to quantify the number of proviral clones with *IRES* probe. The difference between B-ALL mice induced by BCR-ABL1/GFP (lane 1-10, 8.1±1.1 independent clones) and BCR-ABL1/ IκBαSR (lane 11-22, 5.8±0.6 independent clones) was of borderline significance (P=0.0697, t-test). Two control DNAs (Con, lane 23-24) were from cell lines that each contained a single BCR-ABL1 provirus. The same blot was later probed with a human *ABL1* probe to calculate total proviral copy number per genome, as indicated.

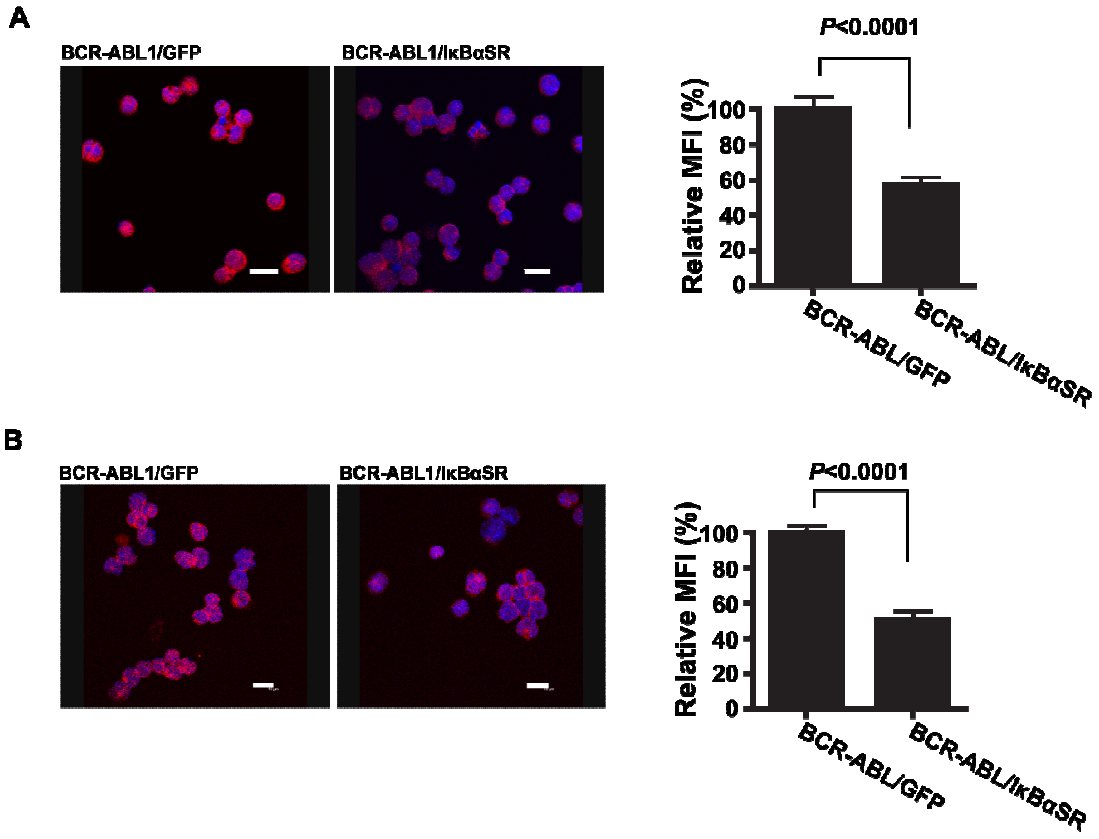

Figure 2.8. IκBαSR reduces nuclear RelA expression in BCR-ABL1-induced B-ALL. Representative confocal micrographs of nuclear RelA expression in pleural effusion lymphoblasts (A) or cultured B-lymphoid cells (B) derived from mice with B-ALL induced by BCR-ABL1/GFP or BCR-ABL1/IκBαSR. Cells were stained with antibody against RelA (red) and counterstained with Hoechst dye (blue). Images were taken under confocal microscope. Quantification of nuclear RelA fluorescence intensity per cell is shown next to images. Data are presented as mean of fluorescent intensity (MFI) of nuclear RelA expression relative to cells expressing BCR-ABL1/GFP.

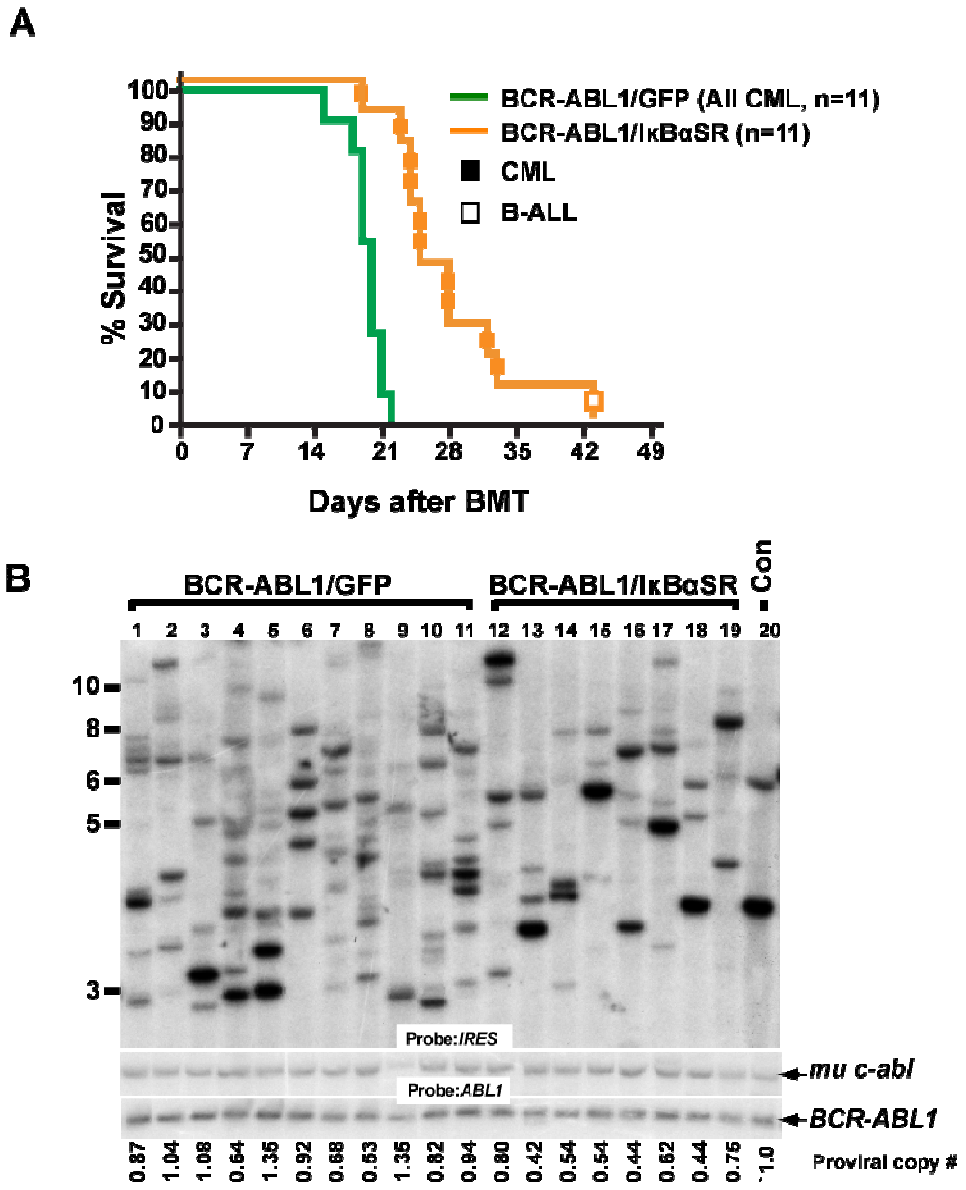
A
100
90
80
70
60
50
40
30
20
10
0
% Survival
0
7
14
21
28
35
42
49
Days after BMT
BCR-ABL1/GFP (All CML, n=11)
BCR-ABL1/IκBαSR (n=11)
CML
B-ALL
B
BCR-ABL1/GFP
BCR-ABL1/IκBαSR
Con
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
10
8
6
5
3
Probe:IRES
Probe:ABL1
mu c-abl
BCR-ABL1
0.87 1.04 1.08 0.64 1.35 0.92 0.88 0.53 1.35 0.82 0.94 0.80 0.42 0.54 0.54 0.44 0.62 0.44 0.75
Proviral copy #

Figure 2.9

Figure 2.9. Expression of IκBαSR attenuates CML-like MPN induced by BCR-ABL1. (A) Kaplan-Meier survival curve for CML-like MPN induced by BCR-ABL1/GFP or BCR-ABL1/IκBαSR. The number of individual mice in each arm is indicated. All mice receiving BCR-ABL1/GFP transduced donor marrow developed CML-like MPN; recipients of BCR-ABL1/IκBαSR transduced marrow developed CML-like MPN (closed square), except for one recipient who developed B-ALL (open square). Recipients of marrow transduced with BCR-ABL1/IκBαSR had significant longer survival than recipients of BCR-ABL1/GFP-transduced bone marrow ($P<0.0001$, Mantel-Cox test). **(B)** Analysis of number of proviral clones in recipients from the two cohorts in (A) by Southern blotting with *IRES* probe. Mice receiving BCR-ABL1/GFP-transduced marrow (lane 1-11) had a significant higher number of independent clones (9.9±0.8) than mice receiving BCR-ABL1/IκBαSR-transduced marrow (lane 12-19, 6.8±0.5 clones, $P=0.0096$, t-test). The control DNA (Con, lane 20) was from a cell line that contained a single *BCR-ABL1* provirus. The blot was reprobed with human *ABL1* probe to determine total proviral copy number per genome.

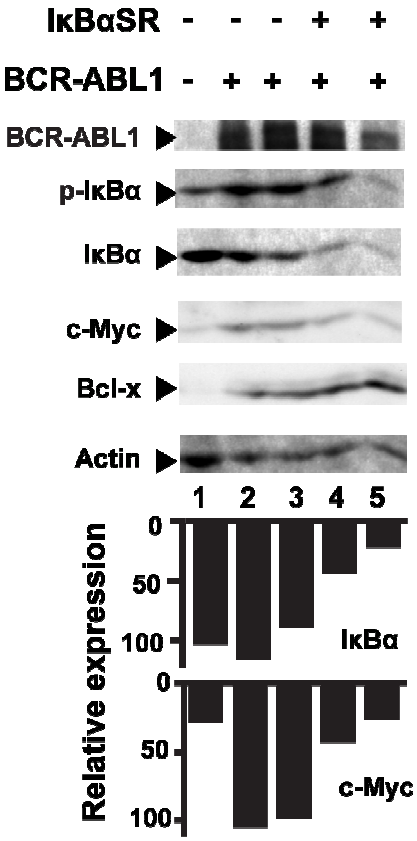

Figure 2.10. IκBαSR inhibits c-Myc expression in BCR-ABL1-induced CML-like MPN. Immunoblot of protein lysates from spleens of mice with CML-like MPN induced by BCR-ABL1/GFP (lane 2 and 3) or BCR-ABL1/IκBαSR (lane 4 and 5), probed with the indicated antibodies. Lysate from untransduced Balb/c mouse (lane 1) serves as control. The expression of c-Myc was quantified and normalized to actin. Relative c-Myc and IκBα expression are shown in the bar graph below.

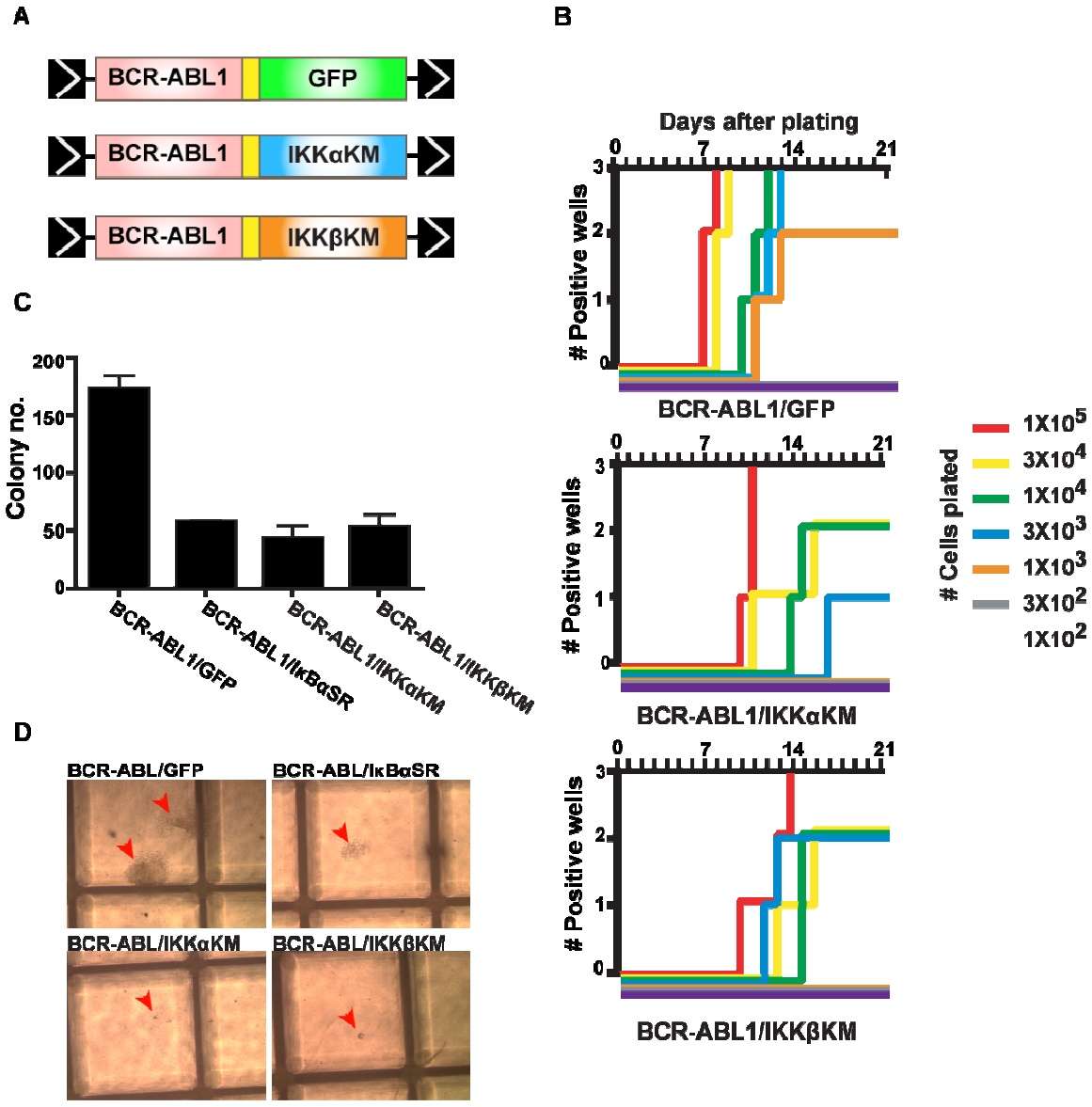
A
BCR-ABL1
GFP
BCR-ABL1
IKKαKM
BCR-ABL1
IKKβKM
B
Days after plating
0
7
14
21
Positive wells
BCR-ABL1/GFP
BCR-ABL1/IKKαKM
BCR-ABL1/IKKβKM
Cells plated
1X10^5
3X10^4
1X10^4
3X10^3
1X10^3
3X10^2
1X10^2
C
Colony no.
200
150
100
50
0
BCR-ABL1/GFP
BCR-ABL1/IκBαSR
BCR-ABL1/IKKαKM
BCR-ABL1/IKKβKM
D
BCR-ABL/GFP
BCR-ABL/IκBαSR
BCR-ABL/IKKαKM
BCR-ABL/IKKβKM

Figure 2.11

Figure 2.11. Kinase inactive IKK mutants inhibit B-lymphoid transformation by BCR-ABL1. (A) Structure of retroviruses expressing BCR-ABL1/GFP, BCR-ABL1/IKKαKM, or BCR-ABL1/IKKβKM. All viruses had BCR-ABL1 expressed upstream of the IRES (yellow box), while GFP or either kinase-inactive mutant expressed downstream of the IRES. Bone marrow from non-5-FU treated Balb/c mice was harvested and transduced with retroviruses expressing BCR-ABL1/GFP, BCR-ABL1/IKKαKM or BCR-ABL1/IKKβKM. Transduced bone marrow was evaluated for the ability to initiate stroma dependent B-lymphoid outgrowth (B) or to form colonies in agarose (C, D). **(B)** Transduced cells were plated in triplicate at decreasing cell number, as indicated by colored lines. Nontransduced cells were added to 10^6 total cells per well to provide stromal support. Positive wells were scored vs. time when the viable non-adherent cell number reached 10^6/well. **(C)** Transduced cells ($2x10^6$ per plate, in duplicate) were seeded in agarose as described in Methods. Colony formation was assessed at day 14 post-seeding. Co-expression of IκBαSR (*P*=0.0063, t-test), IKKαKM (*P*=0.0082, t-test) and IKKβKM (*P*=0.0101, t-test) significantly reduced B-lymphoid colony formation mediated by BCR-ABL1. (D) Representative photomicrographs of the relative sizes of the colonies indicated by arrowheads. Grid size = 2 mm.

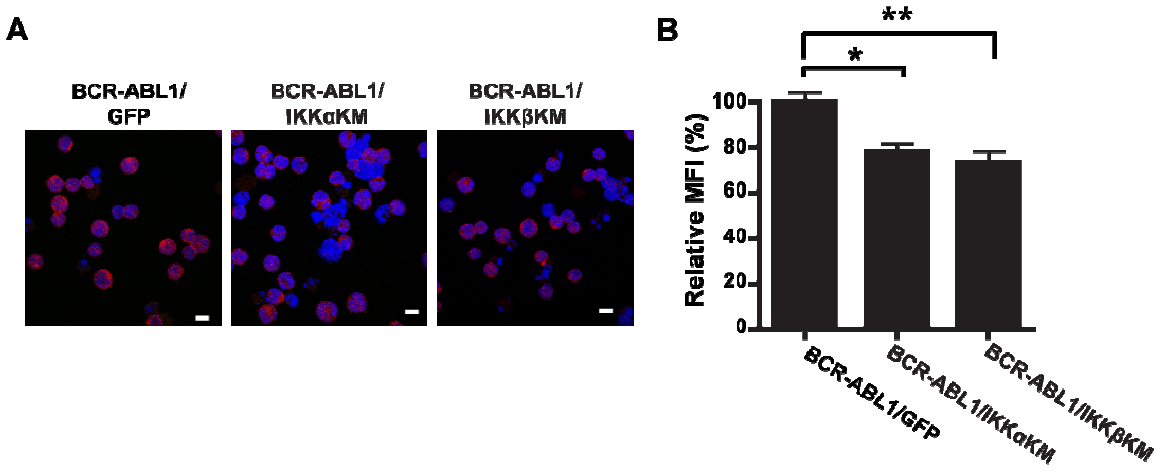

Figure 2.12. Co-expression of either kinase inactive IKK mutant reduces nuclear RelA expression induced by BCR-ABL1. (A) Representative confocal micrographs of nuclear RelA (red) expression in primary B-lymphoblasts transformed by BCR-ABL1/GFP, BCR-ABL1/IKKαKM, or BCR-ABL1/IKKβKM. Cells were counterstained with Hoechst dye (blue). Scale bar=10 μm. **(B)** Quantification of nuclear RelA fluorescence expression of cells shown in (A). Data are presented as mean of fluorescent intensity (MFI) of nuclear RelA per cell relative to cells expressing BCR-ABL1/GFP. The average nuclear RelA expression in cells transformed by BCR-ABL1/IKKαKM (*) or BCR-ABL1/IKKβKM (**) was significant reduced ($P<0.0001$, t-test).

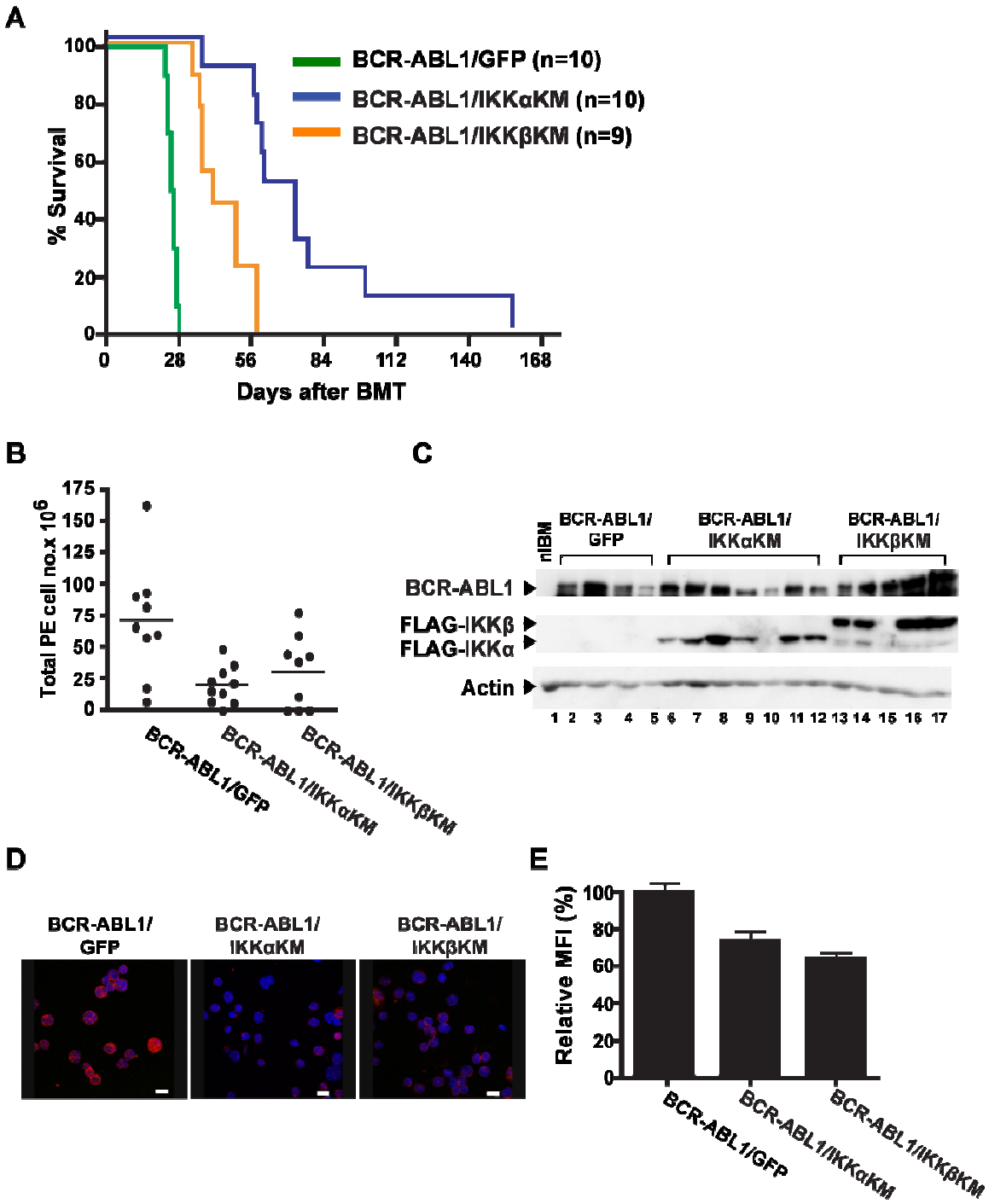
A
100
80
60
40
20
0
% Survival
0
28
56
84
112
140
168
Days after BMT
BCR-ABL1/GFP (n=10)
BCR-ABL1/IKKαKM (n=10)
BCR-ABL1/IKKβKM (n=9)
B
175
150
125
100
75
50
25
0
Total PE cell no.x 10^6
BCR-ABL1/GFP
BCR-ABL1/IKKαKM
BCR-ABL1/IKKβKM
C
nlBM
BCR-ABL1/ GFP
BCR-ABL1/ IKKαKM
BCR-ABL1/ IKKβKM
BCR-ABL1
FLAG-IKKβ
FLAG-IKKα
Actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
D
BCR-ABL1/ GFP
BCR-ABL1/ IKKαKM
BCR-ABL1/ IKKβKM
E
100
80
60
40
20
0
Relative MFI (%)
BCR-ABL1/GFP
BCR-ABL1/IKKαKM
BCR-ABL1/IKKβKM

Figure 2.13

Figure 2.13. Kinase inactive IKK mutants attenuate B-lymphoid leukemogenesis by BCR-ABL1 in mice. (A) Kaplan-Meier survival curve for B-ALL mice induced by BCR-ABL1/GFP, BCR-ABL1/IKKαKM or BCR-ABL1/IKKβKM. The number of individual mice in each arm is indicated. All recipients developed B-ALL. Co-expression of either IKKαKM or IKKβKM significantly prolonged the survival of mice with BCR-ABL1-induced B-ALL (P<0.0001, Mantel-Cox test). In addition, mice with B-ALL induced by BCR-ABL1/IKKαKM survived significantly longer than those induced by BCR-ABL1/IKKβKM (P=0.0007, Mantel-Cox test). **(B)** Total cell number collected from malignant pleural effusions in leukemic mice from the cohorts in (A). Compared to B-ALL induced by BCR-ABL1/GFP, recipients with B-ALL induced by BCR-ABL1/IKKαKM (P=0.0039, t-test) or BCR-ABL1/IKKβKM (P=0.0393, t-test) had significantly fewer leukemic cells in malignant pleural effusions. **(C)** Immunoblot analysis of protein lysates from pleural effusion lymphoblasts from mice with B-ALL induced by BCR-ABL1/GFP (lane 2-5), BCR-ABL1/IKKαKM (lane 6-12) or BCR-ABL1/IKKβKM (lane 13-17). Bone marrow (nlBM) from untransplanted mouse was loaded as a control (lane 1). Blots were analyzed with antibodies against c-Abl, FLAG and actin. **(D)** Representative confocal photomicrographs of nuclear RelA (red) expression in B-lymphoid leukemic cells from recipient of bone marrow transduced with BCR-ABL1/GFP, BCR-ABL1/IKKαKM, or BCR-ABL1/IKKβKM. Cells were counterstained with Hoechst dye (blue). Scale bar=10 μm. **(E)** Quantification of nuclear RelA fluorescence expression of cells shown in (D). Data are presented as mean fluorescence intensity (MFI) of nuclear RelA per cell relative to cells expressing BCR-ABL1/GFP. Leukemic cells expressing BCR-ABL1/GFP showed significant higher nuclear RelA expression than cells expressing BCR-ABL1/IKKαKM (P= 0.0003, t-test) or BCR-ABL1/IKKβKM (P<0.0001, t-test).

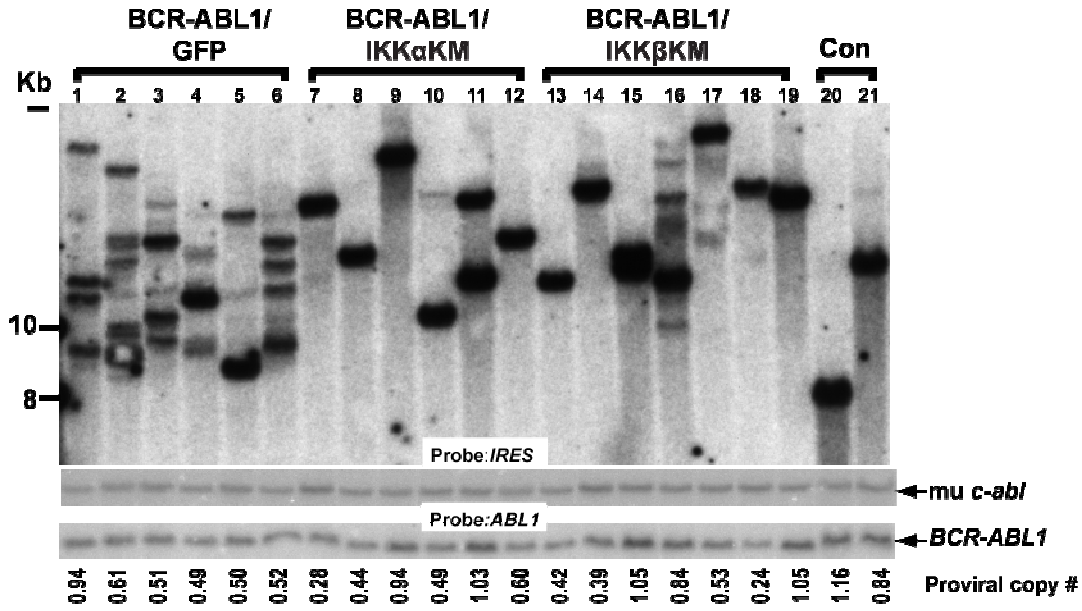

Figure 2.14. Co-expression of kinase inactive IKK mutants reduce the number of leukemic clones in mice with B-ALL induced by BCR-ABL1. Clonality analysis of genomic DNA from leukemic tissues of mice with B-ALL induced by BCR-ABL1/GFP (lane 1-6), BCR-ABL1/IKKαKM (lane 7-12) or BCR-ABL1/IKKβKM (lane 13-19) by Southern blot with *IRES* probe to detect distinct proviral integration events. Two control DNAs (Con, lanes 20-21) were from cell lines that each contains a single *BCR-ABL1* provirus. B-ALL induced by BCR-ABL1/IKKαKM (P=0.001, t-test) and BCR-ABL1/IKKβKM (P=0.0057, t-test) showed significantly decreased frequency of proviral clones as compared to leukemias induced by BCR-ABL1/GFP. To determine proviral copy number, the genomic DNAs were digested with *Bgl*II and analyzed with a human *ABL1* probe.

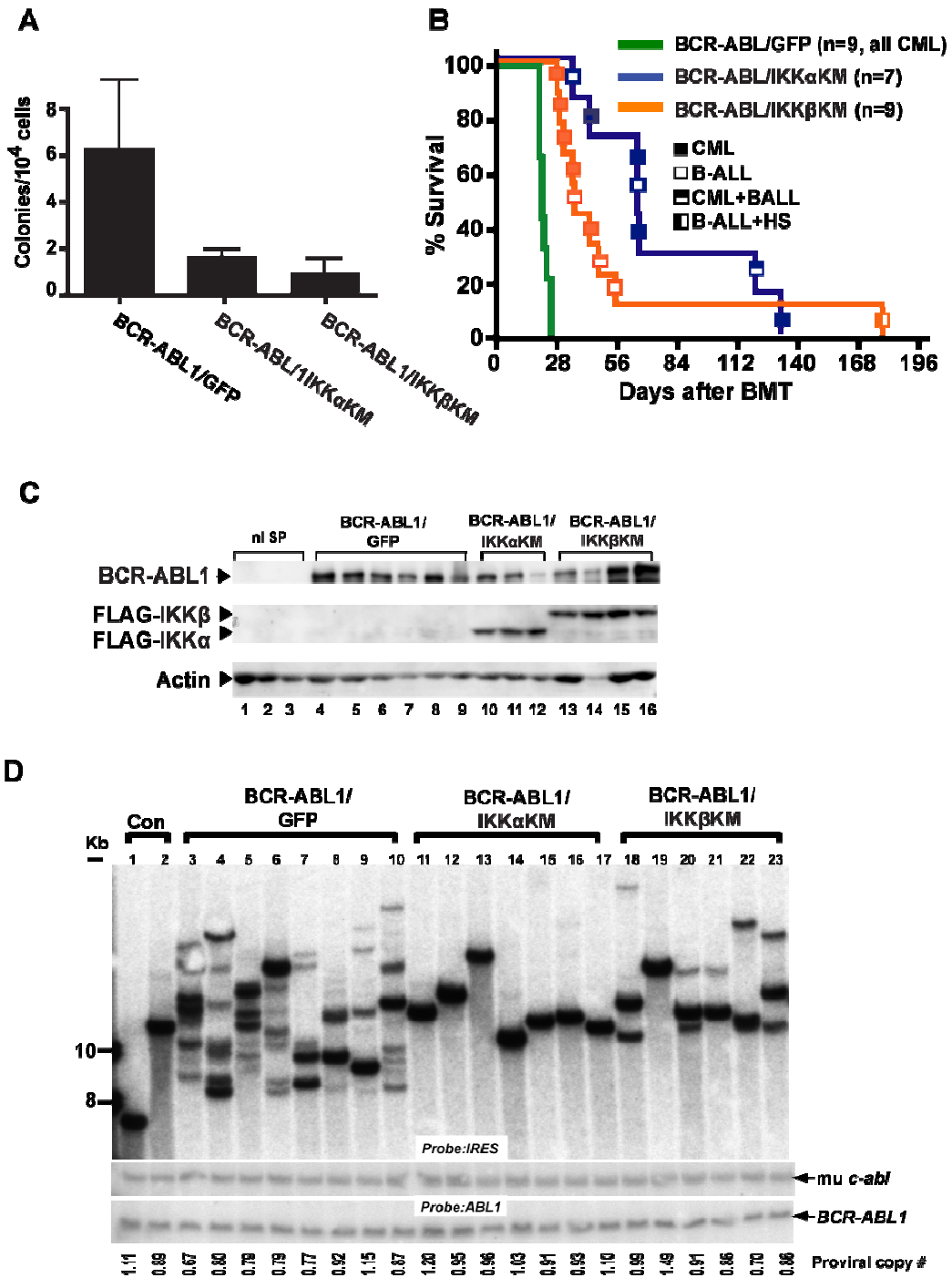
A
Colonies/10^4 cells
0
2
4
6
8
BCR-ABL1/GFP
BCR-ABL/1IKKαKM
BCR-ABL1/IKKβKM
B
% Survival
0
20
40
60
80
100
0
28
56
84
112
140
168
196
Days after BMT
BCR-ABL/GFP (n=9, all CML)
BCR-ABL/IKKαKM (n=7)
BCR-ABL/IKKβKM (n=9)
CML
B-ALL
CML+BALL
B-ALL+HS
C
nl SP
BCR-ABL1/ GFP
BCR-ABL1/ IKKαKM
BCR-ABL1/ IKKβKM
BCR-ABL1
FLAG-IKKβ
FLAG-IKKα
Actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
D
Con
BCR-ABL1/ GFP
BCR-ABL1/ IKKαKM
BCR-ABL1/ IKKβKM
Kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
10
8
Probe:IRES
mu c-abl
Probe:ABL1
BCR-ABL1
1.11 0.89 0.67 0.80 0.79 0.79 0.77 0.92 1.15 0.87 1.20 0.95 0.96 1.03 0.91 0.93 1.10 0.99 1.49 0.91 0.86 0.70 0.96
Proviral copy #

Figure 2.15

Figure 2.15. Kinase-inactive IKK mutants attenuate CML-like MPN induced by BCR-ABL1. Bone marrow from 5-FU-treated donors was transduced with retroviruses expressing BCR-ABL1/GFP, BCR-ABL1/IKKαKM or BCR-ABL1/IKKβKM. Transduced cells were plated in methylcellulose culture (A) or transplanted into irradiated recipients to induce CML-like MPN (B). **(A)** Myeloid colony assay. $1x10^4$ transduced bone marrow cells were seeded per plate in triplicate. Colony number was determined at day 14^{th} post-seeding. **(B)** Kaplan-Meier survival curve for recipients of BCR-ABL1 transduced bone marrow in CML model. The numbers of mice from different arms as well as phenotype of disease are indicated. Recipients of bone marrow transduced by BCR-ABL1/IKKαKM (P<0.0001, Mantel-Cox test) or BCR-ABL1/IKKβKM (P<0.0001, Mantel-Cox test) survived significantly longer than BCR-ABL1/GFP recipients. **(C)** Immunoblot analysis of protein lysate from spleen of mice with CML-like MPN induced by BCR-ABL1/GFP (lane 4-9), BCR-ABL1/IKKαKM (lane10-12), and BCR-ABL1/IKKβKM (lane13-16). Lysates from spleens of three untransplanted mice (nl SP, lane 1-3) were loaded as controls. The blot was analyzed with antibodies against c-Abl, FLAG and actin. **(D)** Analysis of genomic DNA from leukemic tissues of mice with CML-like MPN induced by BCR-ABL1/GFP (lane 3-10), BCR-ABL1/IKKαKM (lane 11-17) or BCR-ABL1/IKKβKM (lane 18-23) by Southern blot with *IRES* probe to detect distinct proviral integration events. Two control DNAs (Con, lanes 1-2) were from cell lines that each contained single BCR-ABL1 provirus. CML-like MPN induced by BCR-ABL1/IKKαKM (P<0.0001, t-test) and BCR-ABL1/IKKβKM (P=0.0003, t-test) showed a significant reduction in clone number of leukemia-initiating cells as compared to BCR-ABL1/GFP. The genomic DNA was digested with *Bgl*II and hybridized with a human *ABL1* probe to determine the proviral copy number, as indicated.

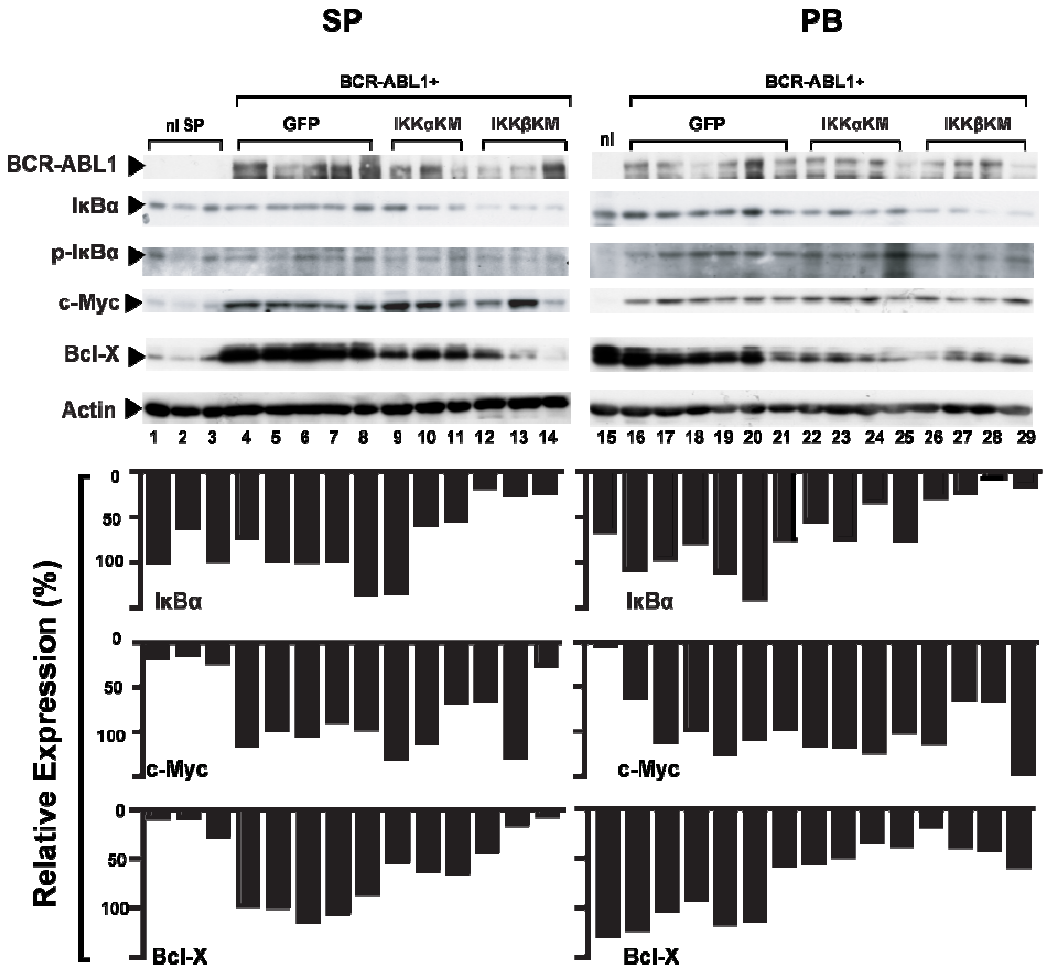

Figure 2.16. Kinase-inactive IKK mutants inhibit Bcl-X expression induced by BCR-ABL1 in myeloid leukemia cells. Lysates from leukemic cells in spleen (SP) or peripheral blood (PB) from mice with CML-like MPN induced by BCR-ABL1/GFP (lane 4-8, 16-12), BCR-ABL1/IKKαKM (lane 9-11, 22-25) or BCR-ABL1/IKKβKM (lane 12-14, 26-29) were analyzed by immunoblot for expression of BCR-ABL1, IκBα, c-Myc, and Bcl-X. Lysate from normal spleen (nl SP, lane 1-3) and peripheral blood (nl, lane 15) were loaded as controls. Blots were analyzed with different antibodies as indicated, including antibody against actin as a loading control.

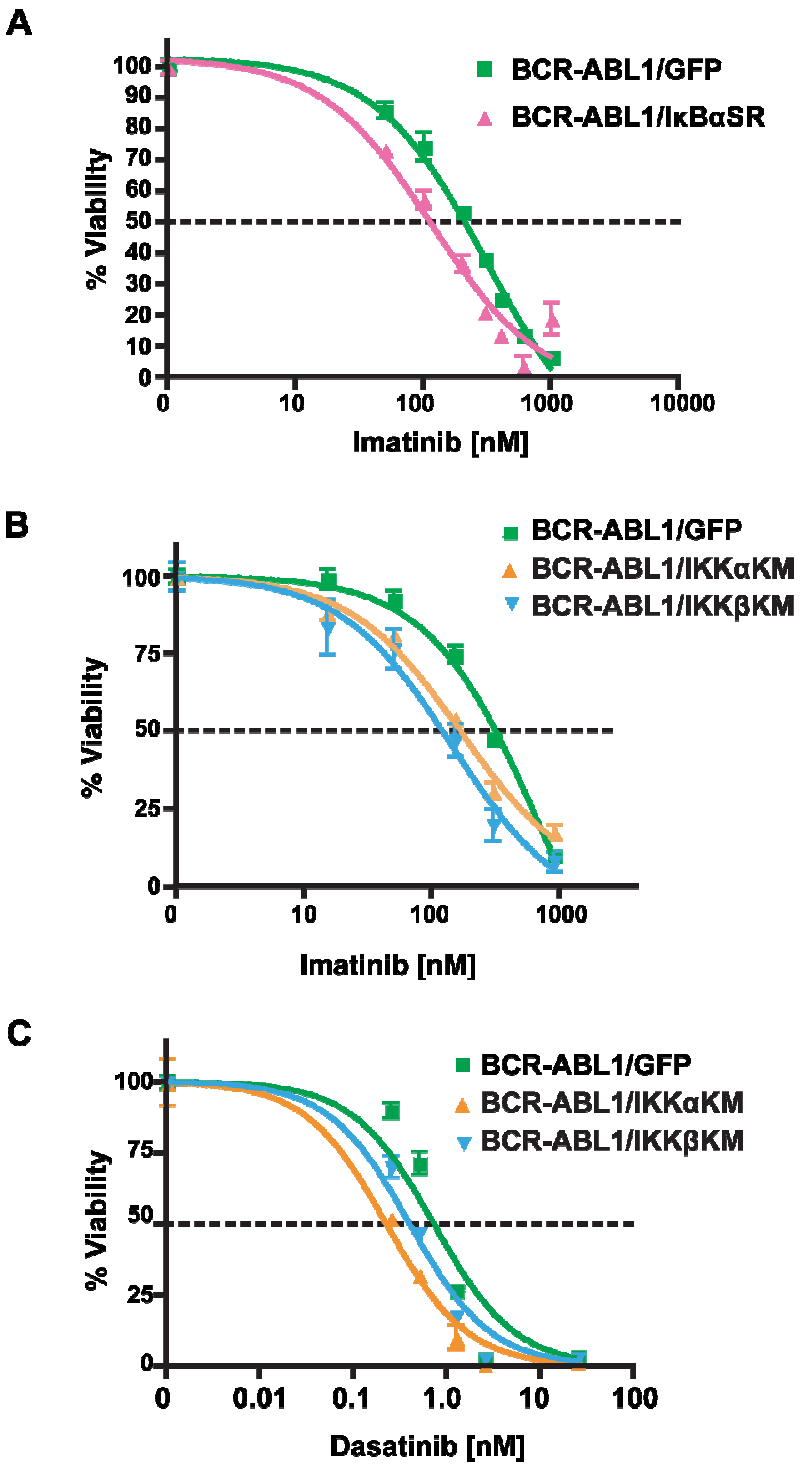
A
BCR-ABL1/GFP
BCR-ABL1/IκBαSR
% Viability
Imatinib [nM]
B
BCR-ABL1/GFP
BCR-ABL1/IKKαKM
BCR-ABL1/IKKβKM
% Viability
Imatinib [nM]
C
BCR-ABL1/GFP
BCR-ABL1/IKKαKM
BCR-ABL1/IKKβKM
% Viability
Dasatinib [nM]

Figure 2.17

Figure 2.17. IκBαSR and kinase-inactive IKK mutants sensitize BCR-ABL1- transformed B-lymphoid progenitors to TKIs. (A) Equal numbers of BCR-ABL1/GFP or BCR-ABL1/IκBαSR-transformed primary B-lymphoid progenitors were incubated with different concentrations of imatinib as indicated. Cell viability was determined by MTS assay after 96 hours of incubation. **(B)** Equal numbers of primary B-lymphoid progenitors transformed by BCR-ABL1/GFP, BCR-ABL1/IKKαKM or BCR-ABL1/IKKβKM were incubated with different concentrations of imatinib (B) or dasatinib (C) as indicated. Cell viability was determined by MTS assay after 96 hours of incubation. Sigmoidal curves were fitted and IC_{50} values were calculated with Prism software. The pairwise differences in IC_{50} values between BCR-ABL1/GFP-transformed primary B-lymphoblasts and leukemic cells expressing IκBαSR, IKKαKM or IKKβKM for imatinib were in all cases highly significant ($P<0.0001$, extra sum-of squares F test). The pairwise differences in IC_{50} values between BCR-ABL1/GFP-transformed primary B-lymphoblasts and leukemic cells expressing IKKαKM or IKKβKM for dasatinib were also significant ($P<0.0001$ and $P=0.0099$, extra sum-of squares F test).

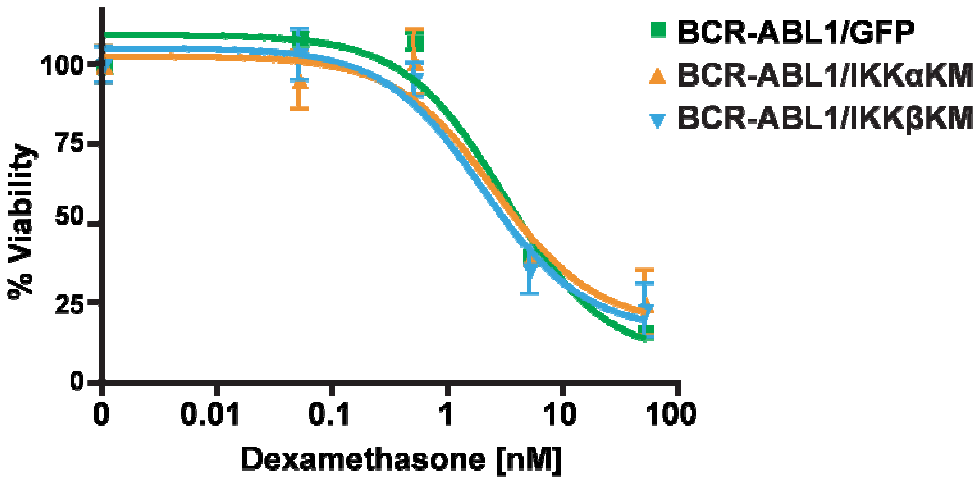

Figure 2.18. Kinase-inactive IKK mutants do not sensitize BCR-ABL1-transformed B-lymphoid progenitors to dexamethasone. Equal numbers of primary B-lymphoid progenitors transformed by BCR-ABL1/GFP, BCR-ABL1/IKKαKM or BCR-ABL1/IKKβKM were incubated with different concentrations of dexamethasone as indicated. Cell viability was determined by MTS assay after 96 hours of incubation. Sigmoidal curves were fitted and IC_{50} values were calculated with Prism software.

2.4 DISCUSSION

Despite the current success of TKIs in treating the majority of patients with CML-CP, limitations of TKIs still exist, which reduce their therapeutic efficacy. Long-term (>2 years) complete molecular remission can only be achieved in less than 10% of patients (Okimoto and Van Etten, 2011), while relapse occurs in majority of patients after discontinuation of imatinib (Mahon et al., 2010). In addition, currently available TKIs are not effective in treating patients with advanced stages of CML or with Ph^+ ALL (Druker et al., 2001a; Sawyers et al., 2002); neither do they have an effect on patients acquiring TKI resistance. It is likely that TKIs alone may control disease development, but will not be curative. To achieve permanent cure for Ph^+ leukemia and overcome TKIs resistance, identification of critical signaling pathways as new therapeutic targets in the Ph^+ leukemias is necessary (Van Etten, 2004).

In this regard, the NF-κB signaling pathway is an attractive candidate, and has been described to play roles in regulating development of several different types of tumors and hematological malignancies (Basseres and Baldwin, 2006). In acute myeloid leukemia (AML), NF-κB activation can be found in myeloid blasts (Baumgartner et al., 2002) and primitive leukemia-initiating cells (Guzman et al., 2001). In Ph^+ leukemia, NF-κB activation is also observed in BCR-ABL1 transformed cell lines as well as blasts from CML-BC or Ph^+ B-ALL (Kirchner et al., 2003; Munzert et al., 2004; Reuther et al., 1998). Using biochemical or pharmacological approaches, several *in vitro* studies have demonstrated that inhibition of NF-κB impairs transformation ability of BCR-ABL1, and induces apoptosis of leukemic cells (Cilloni et al., 2006; Duncan et al., 2008; Lounnas et

al., 2009; Reuther et al., 1998). However, experiments carried out in cell lines often do not correlate well with therapeutic response in primary leukemias *in vivo*. Therefore, we have used two well- characterized genetic approaches and performed a series of *in vitro* and *in vivo* studies in physiologically accurate and quantitative mouse model of CML and Ph^+ ALL and for the first time we have demonstrated that NF-κB as direct mediator in the pathogenesis of lymphoid and myeloid leukemias induced by BCR-ABL1.

The first approach we used is to co-express BCR-ABL1 with a super-repressor form of IκBα, IκBαSR, which is resistant to phosphorylation by IKKs and inhibits NF-κB signaling by sequestering NF-κB complex in cytoplasm (Feuerhake et al., 2005). In the second part of the study, we used kinase-inactive forms of IKKα and IKKβ, which have been shown to impair NF-κB activation by inhibiting the serine phosphorylation and kinase activity of the IKK complex (Nakano et al., 1998). We have shown that both strategies reduce RelA nuclear expression in primary BCR-ABL1-induced leukemias as demonstrated by immunofluorescence staining. While a previous report indicated that the p185 form of BCR-ABL1 increased the transactivation ability of NF-κB without increasing its nuclear translocation in Ba/F3 cells (Reuther et al., 1998), we did find that the p210 form of BCR-ABL1 can enhance RelA nuclear expression in Ba/F3 cells while co-expression IκBαSR decreased nuclear RelA expression to the levels observed in unstimulated parental cells. Co-expression of either IKKαKM or IKKβKM individually with BCR-ABL1 was slightly less effective at suppressing relative levels of nuclear RelA, possibly because of compensation from the other IKK subunit.

In agreement with previous findings by Reuther et al., who demonstrated IκBαSR has no effect on the viability of 32D cells expressing p185 form of BCR-ABL1 after IL-3 depletion (Reuther et al., 1998), we also found IκBαSR has no effect on survival of Ba/F3 or 32D cells expressing the p210 form of BCR-ABL1 in the absence of IL-3. Interestingly, we did find co-expression of IκBαSR inhibits IL-3-independent Ba/F3 cell growth as well as primary lymphoid transformation by the p210 form of BCR-ABL1, assessed by a more quantitative and dynamic B-lymphoid transformation assay. The mechanism of activation of NF-κB by BCR-ABL1 is controversial, with studies in different cell lines suggesting either IKK-dependent (Duncan et al., 2008; Mihailovic et al., 2004) or independent mechanisms (Kirchner et al., 2003; Munzert et al., 2004). Our results demonstrate that co-expression of either IKKαKM or IKKβKM attenuated transformation and leukemogenesis ability of BCR-ABL1, linking IKK to the activation of NF-κB in BCR-ABL1^{+} leukemias and validating IKK as a therapeutic target in these diseases.

It is interesting that attenuation of both lymphoid and myeloid leukemogenesis by the IKK mutants (Figures 2.13 and 2.15) was more profound than that mediated by IκBαSR (Figures 2.7 and 2.9), despite less effective inhibition of nuclear RelA. These results suggest IKKs might affect BCR-ABL1- mediated leukemogenesis partially through IκBα independent mechanisms. Indeed, substrates of IKK other than IκBα have been implicated in regulating pro-inflammatory, survival, proliferative and tumor-promoting function of IKKs (Perkins, 2007). While IKKβ was shown to support anti-apoptosis of breast cancer cell lines through phosphorylating and inhibiting FOXO3a (Hu

et al., 2004a), it was also shown to stabilize mRNA of cytokine, chemokines and growth factor through phosphorylating 14-3-3β to inhibit tristetraprolin/14-3-3β mRNA-binding complex (Gringhuis et al., 2005). Both IKKα and IKKβ can directly phosphorylate RelA, which enhances nuclear translocation and transcactivation ability of RelA independent of IκBα degradation (Adli et al., 2010; Perkins, 2007). Recent study by Baldwin and colleagues also demonstrated that IKKs are involved in regulating the expression of autophagic genes in a NF-κB independent manner (Comb et al., 2011).

Another interesting finding in our current study is IKKαKM mutant acts more effectively in attenuating BCR-ABL1-meidated leukemogenesis than IKKβKM, although IKKβ is generally considered to be the principal player in IKK mediating activation of NF-κB in the canonical pathway (Li et al., 1999b). The function of IKKα in the alternative pathway regulating mature B-cell development (Kaisho et al., 2001) might be relevant to its role in the pathogenesis of BCR-ABL1-induced B-ALL. On the other hand, p100 was aslo shown to sequester p50/RelA heterodimers in the cytoplasm while IKKα-mediated phosphorylation and proteolytic processing of p100 allow p50/RelA to enter the nucleuus and regulate target genes (Dejardin et al., 1995). Recently, IKKα was implicated as direct mediator regulating TNFα-induced NF-κB activation in canonical pathway (Adli et al., 2010).

The precise mechanism of the inhibition of BCR-ABL1 leukemogenesis by NF-κB blockade will require further studies, but our present data suggest the mechanism might differ between lymphoid and myeloid leukemias. In BCR-ABL1-expressing B-

lymphoblasts, the effect of NF-κB inhibition seems to be on cell proliferation rather than survival, with no observation of increase in apoptosis. On the other hand, NF-κB inhibition seems to effect survival pathways in myeloid cells, as we found decreased expression of Bcl-X in CML-like MPN induced by either BCR-ABL/IKK-KM, with a variable effect on c-Myc, consistent with a previous study in the Ph^+ myeloid cell line K562 (Morotti et al., 2006).

By using IκBαSR or either IKK-KM mutant to inhibit NF-κB activation, our results for the first time have implicated NF-κB in the maintenance of BCR-ABL1 leukemia-initiating cells, defined here as distinct provirally marked clones that contribute to a fatal leukemia following transplantation (Krause et al., 2006). Co-expression of these mutants significantly attenuated the frequency of distinct leukemia-initiating cells in bone marrow to initiate B-ALL or CML-like MPN in mice (Li et al., 1999a). Studies by our group have demonstrated that mice transplanted with 5-FU pretreated marrow transduced with BCR-ABL1 developed exclusively CML-like disease within 3-5 weeks (Li et al., 1999a). On the other hand, we found recipients developed mixture of CML-like disease, B-ALL, or histiocytic sarcoma when downstream signaling of BCR-ABL1 is impaired, as when BCR-ABL1 mutants lacking the Grb2 binding site (Million and Van Etten, 2000) or the Src homology (SH2) domain (Roumiantsev et al., 2001) are used to induce disease. Further analysis of proviral integration and use of isolated cell populations to induce Ph^+ leukemia in mice have demonstrated that the target cells for these three diseases are distinct. While the hematopoietic stem cell is the target cell for induction of CML-like disease, committed B-lymphoid and monocyte progenitors are responsible for inducing B-ALL or macrophage disease, respectively (Hu et al., 2006; Huntly and Gilliland, 2005;

Li et al., 1999a). Our results showed that co-expression of mutants proteins in NF-κB pathway, especially IKKαKM or IKKβKM, significantly reduced the frequency of cells that initiate CML-like MPN, but do not completely eliminate cells initiating B-ALL or macrophage disease. As a result, some recipients with BCR-ABL/IKK-KM–transduced marrow eventually developed mixed disease with CML-like MPN and either B-lymphoid leukemia or histiocytic sarcoma in the mouse model of CML. In line with previous findings in AML, in which NF-κB inhibition induce apoptosis of leukemic primitive AML cells (Guzman et al., 2001), our results suggest the NF-κB pathway also plays a significant role in maintaining leukemia-initiating cells that induce B-ALL or CML-like MPN. It would be interesting to see if NF-κB inhibition causes a defect in the initial homing or engraftment of these leukemia-initiating cells.

2.5 METHODS

Retroviral constructs

To co-express both BCR-ABL1 and IκBαSR in a retroviral vector, cDNA of IκBαSR (Feuerhake et al., 2005; Suh et al., 2002), IKKαKM and IKKβKM (Nakano et al., 1998) were cloned into pMIGR1 (Pear et al., 1998) vector by replacing GFP. An IκBαSR cDNA without the five prime and three prime untranslated region was amplified with polymerase chain reaction (PCR) from plasmid MSCV-eGFP-SR-IκBα (kind gift of Dr. M. A. Shipp (Feuerhake et al., 2005)), using a 5' primer containing *BamH*I site and Kozak sequence and 3' primer containing *BamH*I site (5' primer, 5'-CGC GGA TCC ACC ATG TTC CA-3', 3' primer, 5'-CGC GGA TCC TCA TAA CGT CAG A-3'). *BamH*I linker was previously introduced into 3' of IRES sequence in pMIGR1 and cDNA of IκBαSR was subcloned into pMIGR1 with *BamH*I site. *BCR-ABL1* was subsequently cloned into *EcoR*I site at 5' end of IRES to make retrovirus expressing plasmid BCR-ABL1/IκBαSR.

IKKαKM, IKKβKM cDNA was amplified with PCR from pCR-FLAG-IKKα-KM or pCR-FLAG-IKKβ-KM (Addgene), and *Sal*I was introduced at both end of cDNAs during PCR reaction. To amplify IKKαKM, 5'primer containing *Sal*I site and Kozak sequence and 3' primer containing *Sal*I site was used (5' primer, 5'-GCG TCG ACA CCA TGG ACT ACA AG-3', 3' primer, 5'-CGG TCG ACT CAT TCT GCT AAC CAA). The same 5' primer was used to amplify cDNA of IKKβKM, but different 3' primer was used (3' primer, 5'-CGG TCG ACT CAG TCA CAG GC-3'). *Sal*I site was

used to subsequently cloned cDNA of IKKαKM or IKKβKM into pMIGR1. BCR-ABL1 was cloned into pMIGR1 at *Hpa*I site to make BCR-ABL1/IKKαKM, or BCR-ABL1/IKKβKM.

Generation of retrovirus stocks

All DNAs for making retrovirus stock were prepared by two rounds of buoyant density centrifugation with CsCl. High-titer, replication-defective ecotropic retroviral stocks were made by transient transfection of 293T cells with retroviral vector DNA and *kat* packaging construct (Finer et al., 1994; Gavrilescu and Van Etten, 2008). Medium was changed at 24 hours post-transfection and virus supernatant was collected at 48 hours post-transfection. Virus supernatant was frozen immediately at -80 °C after harvesting. To titer virus, an aliquot was thawed and used to transduce NIH3T3 cells. At 48 hours post-transduction, transduced cells were harvested. Genomic DNA from transduced cells was extracted and subject to Southern blot analysis to determine proviral copy number per cell. Matched tittered retroviruses were used in the same experiment.

Transformation of cytokine-dependent hematopoietic cell lines

32D cl3 cells and Ba/F3 cells were maintained at 37°C with 10% CO_2 in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum, 200 μM L-glutamine, 5% (vol/vol) WEHI-3B cell-conditioned medium (as a source of Interleukin-3), penicillin/ streptomycin. Cells were later transduced with retrovirus co-expressing BCR-ABL1 and mutant proteins. At 48 hours post-transduction, culture was replaced with fresh medium without supplement of IL-3. To test the proliferation of transduced cells in

the absence of IL-3, $4x10^4$ cells were seeded in 24 well plates at day 0 in 1 ml culture medium in the absence of IL-3. Viable cells were determined by trypan blue staining every 24 hrs. Each experiment was done in triplicate.

Myeloid transformation and leukemogenesis studies in mice

For myeloid transformation and leukemogenesis, bone marrow was collected from 6-10 weeks old Balb/c donor mice treated 4 days previously with 200 mg/kg 5-fluorouracil (5-FU). Cells were collected by flushing the femur and tibia with a syringe and 27-gauge needle. Marrow cells were then cultured *ex vivo* with high glucose Dulbecco's Modification of Eagle's Medium (DMEM) (cellgro) containing 15% (vol/vol) heat-inactivated FBS (Gibco), penicillin/ streptomycin (cellgro), 1.0g/ml ciprofloxicin (Sigma), 200 μM L-glutamin (cellgro), 5% (vol/vol) WEHI-3B cell-conditioned medium, 6 ng/mL IL-3, 10 ng/mL IL-6, and 50 ng/mL SCF (all from Peprotech) overnight. After cytokine prestimulation, marrow cells were subjected to cosedimentation in medium with same condition but supplemented with retroviral stocks, 4 μg/mL polybrene (Sigma) and 10mM HEPES (Cellgro) at 1,000 g for 90 min in a Sorvall RT-6000 centrifuge. Marrow cells were cultured again in fresh prestimulation medium overnight. The next day, second round of transduction and cosedimentation was performed. Cells were collected and washed with HBSS 2 hours later. For assessment of myeloid leukemogenesis, $2\text{-}5x10^5$ transduced BM cells were injected intravenously into lethally irradiated (621-777 cGy) recipients.

For assessment of myeloid colony formation, $1x10^4$ transduced cells/35mm dish were plated in triplicate in cytokine-free methylcellulose (M3234, Stem Cell Technologies) and culture in 37°C, 5% CO_2. Number of colonies was scored on day 14 post-seeding.

Lymphoid transformation and leukemogenesis study in mice

For lymphoid transformation and leukemogenesis, bone marrow was harvested from 6-10 weeks old donors. Harvested marrow was immediately subjected to one round of cosedimentation with retroviral stocks at 1,000 g for 90 min in a Sorvall RT-6000 centrifuge in the presence of 4 μg/mL polybrene (Sigma), 15% (vol/vol) heat-inactivated FBS (Gibco), penicillin/ streptomycin (cellgro), 1.0g/ml ciprofloxicin (Sigma), 10mM HEPES (Cellgro), 5% (vol/vol) WEHI-3B cell-conditioned medium, and 50 μM 2-mercaptoethanol (EMD). Transduced cells were washed and collected 2-4 hours after cosedimentation. For assessment of lymphoid leukemogenesis, $1x10^6$ transduced donor cells per recipient were injected intravenously into irradiated female Balb/c recipients (621 cGy-777 cGy).

Assessment of BCR-ABL1 B-lymphoid transformation by pre-B cell colony formation in agarose (Warren et al., 2003) and stromal dependent growth (Smith et al., 2003) was performed as described. To assess pre-B colony formation in agarose, transduced cells were mixed with RPMI-1640 in 0.3% agarose (Fisher) supplemented with 20% FBS, 50 μM 2-mercaptoethanol (EMD), and penicillin/ streptomycin (cellgro). $2x10^6$ cells were plated in 35-mm plastic Petri dishes (Nunclon™Δ Surface) on top of

0.6% medium containing agarose base. B-lymphoid colonies were scored at day 14 post-seeding.

To assess stromal-dependent growth of transformed pre-B cells, transduced cells were plated in triplicate in 24-well plates at $1x10^5$, $3x10^4$, $1x10^4$, $3x10^3$, $1x10^3$, $3x10^2$ and $1\ x10^2$ per well density in RPMI 1640 medium (cellgro) supplemented with 20% fetal bovine serum (Gibco), 200 μM L-glutamine (cellgro), 50 μM 2-mercaptoethanol (EMD) 1.0g/ml ciprofloxicin (Sigma), and penicillin/ streptomycin (Cellgro). All wells were supplemented with untransduced bone marrow to make final total cell number equal to $1x10^6$. Half volume of medium were removed and replaced with equal volume of fresh medium without agitation twice a week. Start at five days post-plating, nonadherent cells were counted daily. A cell density of $1x10^6$ cells per well was scored as positive growth.

Analysis of diseased mice

Recipient mice were evaluated for their disease development daily after transplant. Animals showing signs of morbidity, failure to thrive, and splenomegaly were euthanized by CO_2 asphyxiation. Peripheral blood was obtained by submandibular venous plexus puncture. Hematopoietic tissues were harvested and used for analyzing clinical disease features by cytospin, histopathologic analysis or lineage analysis by FACS with antibodies against Ter119, CD90 (Thy1.2), CD45RA (B220), BP-1, CD11b (Mac1) and Gr-1 (BD Pharmingen). Based on animal pathological features, we diagnosed CML-like leukemia, B-ALL or histiocytic sarcoma as described previously (Li et al., 1999a; Roumiantsev et al., 2001). Hematopoietic tissues were also subjected to genomic DNA

preparation, protein lysate preparation and intracellular staining for further analysis of molecular features of disease mice. All mouse experiments were approved by the Institutional Animal Use and Care Committee of Tufts Medical Center.

Southern blot analysis

To determine the frequency of leukemia-initiating cells, distinct proviral integration events were quantified by Southern blot analysis of genomic DNA from leukemia tissues, as described (Krause et al., 2006). To analyze distinct proviral integration events between disease mice induced by BCR-ABL1/GFP or BCR-ABL1/IκBαSR, genomic DNA from disease mice was digested with *Bgl*II, transferred to nylon membranes and hybridized with a radioactive probe derived from the *IRES*. To analyze proviral integration events between disease mice induced by BCR-ABL1/GFP, BCR-ABL1/IKKαKM, or BCR-ABL1/IKKβKM, genomic DNA was digested with *Nsi*I and analyzed with IRES probe. To determine proviral copy number per cell, genomic DNA was digested with *Bgl*II, and subjected to Southern blot analysis with a radioactive probe derived from human *c-ABL1* gene, which detects a 2.2-kb fragment from each *BCR-ABL1* provirus, as described (Li et al., 1999a). Intensity of hybridization was determined by PhosphorImager analysis (Molecular Dynamics). Intensity of hybridization to endogenous murine *c-Abl* was used as internal control to normalize differences during DNA loading. Copy number was determined by comparing ratio between intensity of hybridization of *BCR-ABL1* and endogenous c-*Abl* to that of genomic DNA of cell lines containing a single provirus.

Immunoblot analysis

Protein lysates were prepared from primary tumor cell suspensions from bone marrow, spleen or peripheral blood by direct boiling. 10^7 cells were resuspended in 100 μl of cold PBS, followed by immediate addition of equal volume of 2X sample buffer and boiling for 10 min. To determine protein concentration, lysates were either standardized by SDS-PAGE and Commassie blue staining or analyzed by 660 nm Protein Assay (Thermo Scientific Pierce) in the presence of Ionic Detergent Compatibility Reagent (Thermo Scientific Pierce). Equal amount of protein were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies against ABL1 (BD Pharmingen), IκBα (Cell Signaling), phospho-IκBα (Cell Siganling), Bcl-X (BD Transduction Laboratories), c-Myc (Santa Cruz) and anti-Actin (Sigma) antibodies.

Immunofluorescence analysis of nuclear RelA

Transformed cells were washed with cold PBS, fixed with 2% paraformaldehyde (Electron Microscopy Sciences) on ice for 20 min and permeabilized with 100% methanol (Fisher) for 20 min on ice. Cells were then washed and blocked with cold PBS buffer containing 0.1% Triton X-100, 5% FBS and mouse Fc block (BD Pharmingen) on ice for 10 min. After blocking, cells were incubated with 1:50 anti-p65/RelA (Santa Cruz) antibody at room temperature for an hour, followed by incubation with 1:200 secondary Alexa Fluor 555 conjugated goat anti-rabbit antibody (Invitrogen) at room temperature for 30 min. Cells were then washed PBS with 0.05% Triton X-100 and 2.5% FBS, then placed on coverslips by using a Cytospin (Shandon). Cells were imaged using a Leica TCS SP2 confocal microscope. Leica LCS software was used to quantify RelA nuclear expression.

Cell viability assay with TKIs

Primary B-lymphoid cells were plated in triplicate in 96-well plates in RPMI 1640 medium (Cellgro) supplemented with 20% fetal bovine serum (Gibco), 200 μM L-glutamine (Cellgro), 50 μM 2-mercaptoethanol (EMD), 1.0g/ml ciprofloxicin (Sigma). Imatinib (Novartis Pharmaceuticals) or dasatinib (Bristol-Myers Squibb) were included in media with increasing concentrations. At 96 hours post-incubation, cell viability was determined by MTS assay (Promega), following manufacture's instruction.

Chapter 3.

The role of β-catenin in

BCR-ABL1-induced leukemia

3.1 INTRODUCTION

As mentioned in Chapter 1, residual LICs in Ph^+ leukemia patients after treatment with TKIs are postulated to be the fundamental cause of relapse and progression to blast crisis. In order to develop better therapeutic strategies to eradicate LICs to achieve permanent cure for Ph^+ leukemia, we need a better understanding of the mechanisms maintaining these LICs. The role of β-catenin in regulating the development of normal stem cells has been studied but remains controversial, and it is not clear how the nuclear, active form of β-catenin regulates the development of Ph^+ leukemia. Hence, in this chapter we used the retroviral bone marrow transduction/transplantation mouse model to test the role of constitutively active β-catenin in BCR-ABL1–mediated leukemia.

3.1.1 β-catenin signaling pathway

β-catenin is a central molecule in the canonical Wnt signaling pathway. In unstimulated cells, β-catenin resides in the cytoplasm, and is targeted for degradation through phosphorylation by the cytoplasmic GSK protein complex, consisting of glycogen synthase kinase 3 beta (GSK3β), casein kinase 1 (CK1), axin and adenomatous polyposis coli (APC) (Figure 3.1). The binding of a Wnt ligand to its receptor, Frizzled, will simulate Dishevelled (DSH) to inactivate the GSK3β complex, which stabilizes β-catenin and allows it to enter the nucleus and function as a coactivator for the transcription factor LEF/TCF to activate target genes (Malhotra and Kincade, 2009; Pardal et al., 2003).

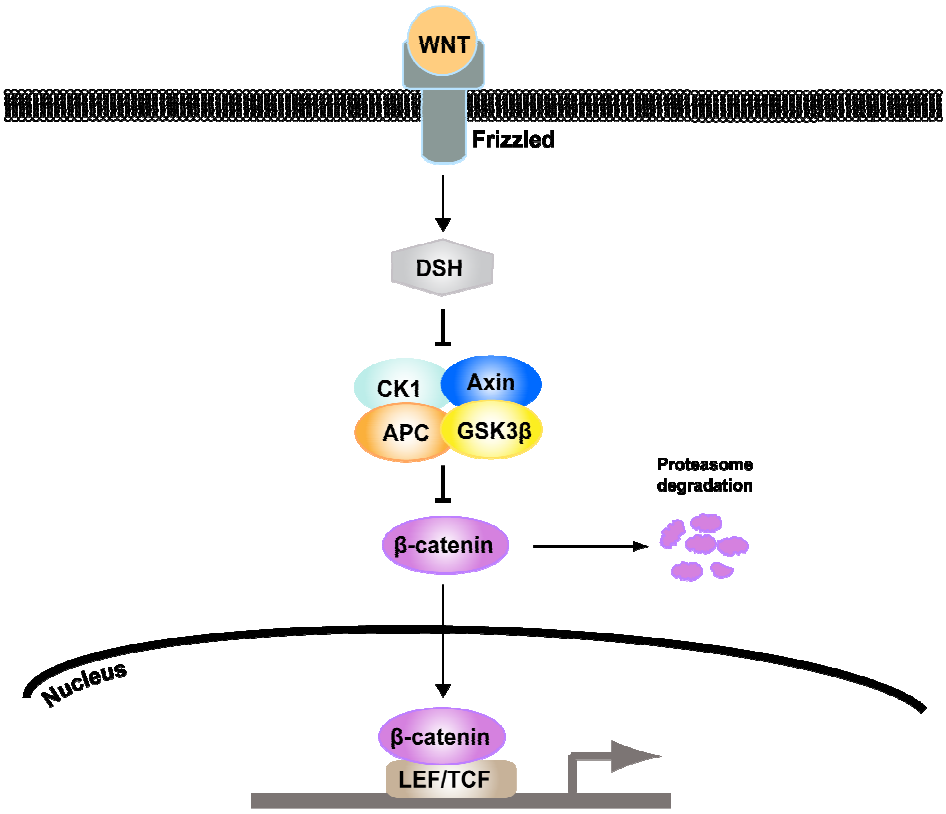

Figure 3.1. An overview of Wnt/β-catenin pathway. β-catenin normally resides in the cytoplasm and is regulated by a disruption complex containing glycogen synthase kinase 3 beta (GSK3β), casein kinase 1 (CK1), axin and adenomatous polyposis coli (APC). GSK3β and CK1 phosphorylate β-catenin in the cytoplasm, which causes β-catenin to be targeted by ubiquitin ligase and subsequently to be degraded in the proteasome. The binding of WNT ligand to its receptor, Frizzled, can activate Dishevelled (DSH), which disrupts the GSK3 complex and allows β-catenin to enter the nucleus. Nuclear active β-catenin can then act as a coactivator for the transcription factor LEF/TCF, to activate target genes encoding proteins that regulate cell proliferation and survival.

3.1.2 Previous studies of β-catenin in regulating normal hematopoietic stem cells

Using gain- or loss-of-function approaches, the role of β-catenin in normal hematopoiesis has been studied. Conditional deletion of β-catenin in adult murine hematopoietic stem cells did not impair their ability to initiate hematopoiesis or repopulate lethally irradiated recipient mice (Cobas et al., 2004; Koch et al., 2008); by contrast, conditional deletion of β-catenin from fetal liver-derived HSCs impaired their ability to self-renew and repopulate recipient mice competitively (Zhao et al., 2007). These results suggest that β-catenin is essential in early HSC development but might be dispensable in adult hematopoiesis.

Several different gain-of-function approaches have been utilized to understand the role of β-catenin in regulating hematopoiesis. Using retroviral transduction, Reya et al. showed that expression of a constitutively active mutant of β-catenin in HSCs from Bcl-2 transgenic mice expanded the pool of HSCs in culture, which could functionally repopulate lethally irradiated recipients (Reya et al., 2003). However, Baba et al. showed expressing active β-catenin in HSCs from wild type mice can promote expansion of HSCs in culture but not their ability to reconstitute irradiated recipients (Baba et al., 2006), suggesting that Bcl-2 expression might play a role in the observation of Reya et al. Two other independent studies using transgenic mice simultaneously reported that conditional activation of β-catenin in adult HSCs can block their differentiation ability and cause failure of hematopoiesis (Kirstetter et al., 2006; Scheller et al., 2006). Several

genes important in regulating function of HSCs, such as *Cdkn1a*, *Hoxb4*, *Bmi1*, and *Sfpi1*, were shown to be down-regulated in the presence of constitutively active β-catenin. Together, these studies indicate a complicated role for active β-catenin in regulating hematopoiesis, suggesting the expression of β-catenin is required to be finely regulated, probably through interaction with other signaling molecules, in order to balance the self-renewal and differentiation ability of HSCs (Malhotra and Kincade, 2009).

3.1.3 Previous studies of β-catenin in regulating leukemia-initiating cells

Previous *in vitro* studies showed that BCR-ABL1 could stabilize β-catenin by phosphorylating β-catenin at Tyr86, and Tyr 654, which interferes with its binding with Axin (Coluccia et al., 2007). A few *in vivo* studies evaluating the role of β-catenin in regulating LICs have also been carried out. Using a loss-of-function strategy, Reya and colleagues showed conditional deletion of β-catenin using HSC from Vav-Cre transgenic mice impaired the frequency of development of CML-like MPN in recipient mice in the bone marrow transplantation/transduction model, but still allowed development of B-ALL in the majority of recipients (Zhao et al., 2007). This study as well as a study by Li and colleagues showed that β-catenin deficiency prevented propagation of disease from primary CML to secondary recipients (Hu et al., 2009; Zhao et al., 2007), suggesting an essential role of β-catenin for maintenance of BCR-ABL1-expressing stem cells.

On the other hand, studies with gain-of-function approaches have also illustrated an important role of active β-catenin in development of acute myeloid leukemia and CML

myeloid blast crisis. Recent work by Armstrong and colleagues demonstrated that granulocyte-macrophage progenitors (GMP) transformed by the transcription factors HoxA9 and Meis1 could initiate acute myeloid leukemia (AML) only when a constitutively active mutant form of β-catenin was co-expressed. They further showed inhibition of β-catenin through pharmacological or genetic approaches impaired AML development initiated from GMP expressing the leukemic fusion protein MLL-AF9 (Wang et al., 2010). On the other hand, elevated expression of β-catenin has been found in GMPs from CML patients in myeloid blast crisis (CML-MBC) but not in chronic phase, which can be propagated in a serial replating assay, suggesting β-catenin provides self-renewal ability in BCR-ABL1–expressing GMP (Jamieson et al., 2004). The same group subsequently identified an in-frame misspliced variant of GSK3β expressed exclusively in GMP from CML-MBC, which might contribute to enhanced β-catenin expression and transformation of GMP to become LICs (Abrahamsson et al., 2009). However, direct evidence that β-catenin itself could provide self-renewal ability on BCR-ABL1 expressing myeloid progenitors in physiological condition is lacking. We also need to understand if active β-catenin plays a role in the development of lymphoid blast crisis.

3.1.4 Mouse model with constitutively active β-catenin generated by using a Cre-loxP system

Here, we proposed to study the role of active β-catenin in BCR-ABL1–mediated leukemogenesis with the *Ctnnb1*$^{Fl/+}$ mouse strain that carries a conditionally activated β-

catenin allele (Harada et al., 1999). The exon 3 of the β-catenin allele in this transgenic mouse is flanked by loxP sites. The exon 3 contains the serine and threonine residues that can be phosphorylated by GSK3β, which subsequently targets β-catenin for proteasome degradation. Cre recombinase-mediated in-frame deletion of exon 3 generates a constitutively active form of β-catenin (Figure 3.2) that is stably expressed in the nucleus (Gounari et al., 2001).

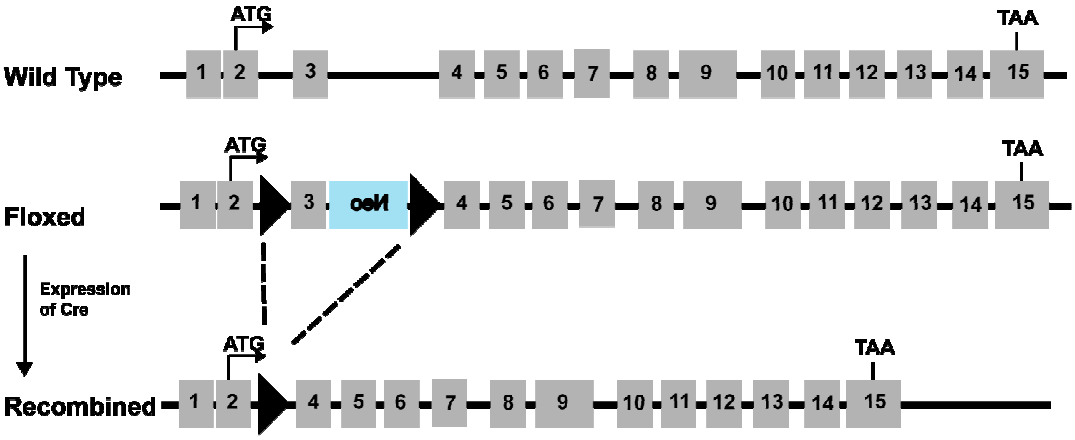

Figure 3.2. Diagram of conditional active β-catenin allele. The wild type β-catenin gene has 15 exons. To engineer a conditional active β-catenin allele, exon 3 (containing the phosphorylation sites recognized by the GSK destruction complex) was flanked with loxP sites (black arrows). Tissue-specific expression of Cre recombinase leads to excision of exon 3 and in frame splicing of exons 2 and 4, which results in recombined allele encoding a stable form of β-catenin. The location of start and stop codons are listed.

3.1.5 Rationale

Although deregulated active β-catenin has been shown to be required for maintaining LICs in AML and is associated with progression of CML to myeloid blast crisis (Jamieson et al., 2004; Wang et al., 2010), direct proof from *in vivo* studies is still required in order to evaluate whether Wnt/β-catenin is an essential target for treating Ph^+ leukemia and for the development of therapeutic strategies. Based on previous studies, we hypothesized that the activation of β-catenin alone may be sufficient to confer self-renewal ability on BCR-ABL1-expressing myeloid progenitors, which might allow disease induction from committed myeloid progenitors, and contribute to disease progression from chronic phase to myeloid blast crisis. We further hypothesized that activated β-catenin might be involved in the development of Ph^+ B-ALL or lymphoid blast crisis. By using *Ctnnb1*$^{fl/+}$ mice as donors or a constitutively active β-catenin retroviral allele (Reya et al., 2003) in combination with the retroviral bone marrow transduction/transplantation mouse model, we carried out several *in vitro* and *in vivo* experiments to examine the role of active β-catenin in BCR-ABL1–mediated leukemia.

3.2 RESULTS

3.2.1 Activated β-catenin inhibits BCR-ABL1–mediated primary lymphoid transformation

By using the *Ctnnb1*$^{fl/+}$ mouse strain and retrovirus co-expressing BCR-ABL1 and a GFP-CRE fusion protein (Heinrich et al., 2004) (BCR-ABL1/GFP-CRE), we first tested the effect of activated β-catenin in primary B-lymphoid transformation mediated by BCR-ABL1 (McLaughlin et al., 1989; Smith et al., 2003). Bone marrow from wild type (*Ctnnb1*$^{+/+}$) or *Ctnnb1*$^{Fl/+}$ mice was harvested and transduced with BCR-ABL1/GFP-CRE to activate the *Ctnnb1*^{Fl} allele. Transduced cells were plated in serial dilutions in stromal cultures suitable for growth of B-lymphoid cells. The outgrowth of transformed B-lymphoid progenitors as function of time is shown in Figure 3.3. We found that BCR-ABL1/GFP-CRE transformed *Ctnnb1*$^{+/+}$ progenitors initiated outgrowth faster than transformed *Ctnnb1*$^{Fl/+}$ cells. As few as 30,000 transduced *Ctnnb1*$^{+/+}$ cells initiated outgrowth of B-lymphoid progenitors, but more than 100,000 transduced *Ctnnb1*$^{Fl/+}$ cells were required to initiate any outgrowth. This initial result indicates that activated β-catenin inhibits the B-lymphoid transformation ability of BCR-ABL1 *in vitro*, which suggests that activated β-catenin might also interfere with B-ALL development mediated by BCR-ABL1.

3.2.2 Activated β-catenin is associated with loss of GFP-Cre expression and alters BCR-ABL1-induced B-lymphoid leukemogenesis

To test if activated β-catenin affects the development of B-ALL mediated by BCR-ABL1, we induced B-ALL in recipient mice with donor marrow from *Ctnnb1*$^{+/+}$ or *Ctnnb1*$^{Fl/+}$ mice. Bone marrow from donors without 5-FU pretreatment was transduced with BCR-ABL1/GFP-CRE retrovirus and transplanted into irradiated syngeneic recipients (Figure 3.4). All mice receiving BCR-ABL1/GFP-CRE transduced *Ctnnb1*$^{+/+}$ marrow developed B-ALL, with a latency around 5-10 weeks (Figure 3.5A). Some mice receiving BCR-ABL1/GFP-CRE–transduced *Ctnnb1*$^{Fl/+}$marrow developed B-ALL (5 out of 9) while a few mice (4 out of 9) died of mixed disease with B-ALL and CML-like MPN. The development of CML-like disease in some recipients in this cohort was unexpected, as this leukemia is ordinarily initiated from transduction of HSCs (Huntly et al., 2004), which are very poorly transduced under these conditions (ie, no 5-FU treatment of donors and a single transduction without cytokines). The CML-like MPN was characterized by an elevated white blood cell count, severe splenomegaly (Figure 3.5B), lung hemorrhage, and expansion of Gr-1^{+} and Mac-1/CD11b^{+} myeloid cells in bone marrow, spleen, liver, and peripheral blood. No statistically significant difference was found in survival between two arms. The absolute number of distinct proviral clones was similar between the two cohorts (Figure 3.6, *Ctnnb1*$^{+/+}$, 1.88 ± 0.35 clones; *Ctnnb1*$^{Fl/+}$, 1.8 ± 0.2 clones, *P*=0.8777).

Although marrow from both *Ctnnb1*$^{+/+}$ and *Ctnnb1*$^{Fl/+}$ donors was transduced with the same retrovirus expressing BCR-ABL1/GFP-CRE, only two mice from the cohort

receiving transduced *Ctnnb1*$^{Fl/+}$ marrow had GFP-positive cells in malignant tissue, while leukemic cells from all recipients of transduced *Ctnnb1*$^{+/+}$ marrow expressed GFP. We hypothesized that deleterious effects of activated β-catenin might select for gene rearrangement and/or deletions in the retroviral provirus, which caused loss of GFP expression. To test this hypothesis, we analyzed the proviral integrity in leukemic cells from diseased mice using Southern blotting with *ABL1* and *GFP* probes (Figure 3.7A). While all mice transplanted with transduced *Ctnnb1*$^{+/+}$ marrow had intact proviral *GFP-CRE* and *BCR-ABL1* gene structures (Figure 3.7B), most recipients of transduced *Ctnnb1*$^{Fl/+}$ marrow had rearrangement or deletion of the *GFP-CRE* gene (5 out of 9), and a few mice (3 out of 9) also had evidence of *BCR-ABL1* gene rearrangement, as assessed by a 3' *ABL1* probe. Although these mice had the *BCR-ABL1* gene rearranged, we observed intact BCR-ABL1 protein expression in lysates from leukemic cells of diseased mice with transduced *Ctnnb1*$^{Fl/+}$ marrow (Figure 3.8), suggesting the expression of functional BCR-ABL1 was preserved to induce disease in these recipients. Although constitutive expression of Cre recombinase in mammalian cells has been associated with genomic instability (Silver and Livingston, 2001), we did not observe proviral rearrangements in *Ctnnb1*$^{+/+}$ cells (Figure 3.7B, left panel). Hence, these results suggest that the presence of a conditional active β-catenin allele is associated with selection for proviral rearrangement/deletion and loss of GFP-CRE expression in transduced cells.

As mentioned above, the appearance of CML-like MPN in recipients of marrow transduced with BCR-ABL1 retrovirus under the conditions used in the B-ALL model is highly unusual, and suggested the possibility that committed myeloid progenitors, which are abundant and readily transduced under these conditions, might be the source of the

myeloid leukemia. Because committed myeloid progenitors from wild-type mice cannot initiate CML-like MPN following BCR-ABL1 retroviral transduction (Huntly et al., 2004), this in turn led us to hypothesize that activated β-catenin might confer self-renewal to myeloid progenitors, allowing development of CML-like disease in this B-ALL mouse model. To investigate the efficiency of recombination of the *Ctnnb1*^{Fl} allele mediated by Cre recombinase, genomic DNA from diseased mice was subjected to analysis by PCR (Figure 3.9) or by Southern blot (Figure 3.10) with a probe that simultaneously detects the wild-type, floxed and recombined alleles. As shown in Figure 3.10B, all recipients transplanted with transduced *WT*$^{+/+}$ marrow had only the wild-type allele detected, as expected. The recombined *Ctnnb1* allele can be observed in control DNA from thymocytes of CD4Cre–*Ctnnb1*$^{Fl/+}$ transgenic mice (Guo et al., 2007); (Figure 3.10B, lane 19), but unexpectedly it was not detected in diseased mice that received BCR-ABL1/GFP-CRE–transduced *Ctnnb1*$^{Fl/+}$ marrow. Interestingly, we did not observe significant recombination of the floxed *Ctnnb1* allele even in myeloid cells from mice with mixed MPN and B-ALL (lane 4-9). These results suggest a possibility that cells expressing active β-catenin encoded by the recombined allele were outcompeted by the cells retaining the unrecombined floxed allele, which has been reported previously in other studies (Scheller et al., 2006). The appearance of CML-like MPN in recipients of BCR-ABL1/GFP-CRE–transduced *Ctnnb1*$^{Fl/+}$ marrow therefore cannot be ascribed to active β-catenin, and remains unexplained.

3.2.3 BCR-ABL1 cannot compensate for the hematopoiesis defect caused by Mx-Cre–mediated activation of β-catenin

To rule out the possibility that remaining unrecombined floxed allele we observed in diseased mice transplanted with transduced *Ctnnb1*$^{Fl/+}$ marrow was due to inefficient recombination mediated by GFP-CRE, we chose to use a different method of Cre recombinase expression to test if activation of β-catenin by Mx-Cre–mediated recombination has an effect on development of BCR-ABL1–induced CML-like MPN. The expression of Cre recombinase in Mx-Cre transgenic mice is under the control of type I interferon-inducible Mx promoter (Kuhn et al., 1995), and can be induced by administration of polyinosinic-polycytidylic acid (poly(I:C)), a synthetic double-stranded RNA. Previous studies have shown that 100% recombination of β-catenin floxed allele can be achieved in bone marrow of Mx-Cre;*Ctnnb1*$^{Fl/+}$ mice 12 days after poly(I:C) injection (Scheller et al., 2006).

Hence, *Ctnnb1*$^{Fl/+}$ mice were crossed with *Mx-Cre* transgenic mice to generate *Mx-Cre*;*Ctnnb1*$^{Fl/+}$ donor mice. To test the role of active β-catenin in CML development mediated by BCR-ABL1, bone marrow from 5-FU-pretreated *Ctnnb1*$^{Fl/+}$ or *Mx-Cre*;*Ctnnb1*$^{Fl/+}$ mice was transduced with retrovirus co-expressing BCR-ABL1 and GFP (BCR-ABL1/GFP) in the presence of myeloid cytokines. Transduced bone marrow cells were transplanted into irradiated recipient mice at day 0. Recipient mice were subsequently treated with poly(I:C) at day 10, 12 and 14 post-transplant to induce expression of Cre recombinase under control of the Mx promoter, or not poly(I:C) treated (Figure 3.11). Mice transplanted with BCR-ABL1/GFP–transduced *Ctnnb1*$^{Fl/+}$ marrow

treated with poly(I:C) or untreated, as well as recipients of transduced *Mx-Cre*;*Ctnnb1*$^{Fl/+}$ marrow that were not treated with poly(I:C) (predicted to not express activated β-catenin) all succumbed to CML-like disease around 3-4 weeks following transplantation (Figure 3.12A). However, mice receiving BCR-ABL1-transduced *Mx-Cre*;*Ctnnb1*$^{Fl/+}$ marrow that were treated with poly(I:C), which should express activated β-catenin upon recombination of the floxed *Ctnnb1* allele [Mx-Cre;Ctnnb1$^{Fl/+}$, Poly(I:C)], survived longer than the control group (P<0.0005, Mantel-Cox test, Figure 3.12A). Whereas control mice gradually developed CML-like disease with high peripheral blood leukocyte counts (PBL) after bone marrow transplantation, mice from [Mx-Cre; Ctnnb1$^{Fl/+}$, Poly(I:C)] group had moderate PBL counts around day 18 post-transplant, but dropped to less than 5000/μl PBL counts around day 24 (Figure 3.12B). All the mice in this group eventually died of hematopoiesis failure, with pancytopenia (Figure 3.12C). Only the last two surviving mice from this group developed symptoms of CML-like disease (2 out of 8).

To confirm that the prolonged survival and pancytopenia in diseased mice from the *Mx-Cre*; *Ctnnb1*$^{Fl/+}$, Poly(I:C) cohort were due to active β-catenin, the recombination efficiency mediated by Mx-Cre was evaluated. Diseased mice from control arm, Ctnnb1$^{Fl/+}$, Poly(I:C), retained their floxed *Ctnnb1* allele as expected (Figure 3.13A). In contrast, the floxed allele disappeared in splenocytes from mice in the Mx-Cre; Ctnnb1$^{Fl/+}$, Poly(I:C) cohort while the recombined allele was detected (with average recombination efficiency 91.8 ± 4.2 %), but was not present at the level of the recombined allele in control CD4Cre-Ctnnb1$^{Fl/+}$ thymocytes (Figure 3.13A, lane 14). This suggests BCR-ABL1–expressing donor cells with active β-catenin induced by Mx-Cre

were not the predominant population in these hematopoietic organs, and may have been diluted by radioresistant recipient hematopoietic cells. This result was further confirmed by immunofluorescence staining of β-catenin in malignant tissue from the diseased mice. As shown in Figure 3.13B, very few peripheral blood leukocytes expressed detectable nuclear β-catenin in a representative mouse from the Mx-Cre; Ctnnb1$^{Fl/+}$, Poly(I:C) cohort, while nearly 100% of CD4Cre-Ctnnb1$^{Fl/+}$ thymocytes expressed active β-catenin. Notably, we did not observe significant nuclear active β-catenin in malignant tissue from the last two mice which developed symptoms with CML-like MPN in the cohort of Mx-Cre; Ctnnb1$^{Fl/+}$, Poly(I:C) (data not shown).

The prolonged survival of diseased mice from the Mx-Cre; Ctnnb1$^{Fl/+}$, Poly(I:C) cohort was not due to reduced numbers of engrafting LICs, as there was no difference in the number of proviral clones in hematopoietic cells from control mice (Ctnnb1$^{Fl/+}$, Poly(I:C); 9±1.0 independent clones) and the Mx-Cre; Ctnnb1$^{Fl/+}$, Poly(I:C) cohort (6.6±1.3 independent clones, P=0.169, t-test; Figure 3.13C). Previously, Scheller et al. have shown that hematopoietic stem cells from poly(I:C)–treated *Mx-Cre;Ctnnb1*$^{Fl/+}$ donor mice have a defect in differentiation and fail to repopulate irradiated recipients (Scheller et al., 2006). Our results further suggest that the differentiation defect resulting from activated β-catenin in HSCs cannot be rescued or overcome by BCR-ABL1 expression. As a corollary, it appears that in BCR-ABL1-expressing HSC, constitutively active β-catenin generated by Cre-mediated recombination from the *Ctnnb1*$^{Fl/+}$ allele also results in exhaustion and a differentiation block of LICs, which causes the mice to ultimately succumb to pancytopenia.

3.2.4 A constitutively active retroviral allele of β-catenin blocks development of Ph^+ B-ALL and CML-like MPN mediated by BCR-ABL1

Unlike the very strong active β-catenin allele generated by Cre recombination of the *Ctnnb1*$^{Fl/+}$ allele described earlier, the constitutively active retroviral allele of β-catenin does not appear to adversely affect LIC maintenance, at least in the AML model (Wang et al., 2010). Therefore, we extended our previous findings by using a retrovirus expressing a constitutively active form of β-catenin (ΔCatnb/GFP) (Barth et al., 1999) to evaluate whether activated β-catenin plays a positive or negative regulatory role in BCR-ABL1–mediated leukemogenesis. This mutant form of β-catenin has alanine substitutions for the NH_2-terminal serine and threonine residues, which prevent its phosphorylation by GSK3β and subsequent degradation by the proteasome.

We first used the *in vitro* B-lymphoid transformation assay as described earlier to test the effects of retroviral expression of mutant β-catenin. Bone marrow from donors not treated with 5-FU was transduced with a cocktail of two retroviruses expressing BCR-ABL1/mRFP and ΔCatnb/GFP, respectively. As control, a portion of the donor marrow was transduced with retroviruses expressing BCR-ABL1/mRFP and empty vector only expressing GFP (MIG). Transduced cells expressing both mRFP and GFP were enriched by flow sorting and subjected to the primary B-lymphoid transformation assay. Whereas marrow co-transduced with BCR-ABL1/mRFP and MIG could initiate lymphoid outgrowth with as low as 3000 cells plated per well, marrow co-transduced with BCR-ABL1/mRFP and ΔCatnb/GFP was absolutely defective in this assay and could not initiate outgrowth at this plating density (Figure 3.14A). This suggests that the

constitutively active retroviral allele of β-catenin also interferes with B-lymphoid transformation mediated by BCR-ABL1.

The ability of co-transduced marrow to initiate B-ALL in mice was subsequently tested by transplanting the cells into irradiated syngeneic recipients over a range of $1x10^5$ to $1x10^4$ co-transduced marrow cells per recipient (Figure 3.14B). For the cohorts transplanted with BCR-ABL1/mRFP and MIG co-transduced marrow, recipients of ≥ $3x10^4$ cells succumbed to B-ALL with latency between 26 and 69 days. One out of two recipients transplanted with $1x10^4$ co-transduced marrows cells also developed B-ALL. In contrast, none of the mice transplanted with BCR-ABL1/mRFP and ΔCatnb/GFP co-transduced marrow showed evidence of disease upon sacrifice at day 120 post-transplantation. These results demonstrate that constitutively active retroviral allele of β-catenin not only inhibits transformation of B-lymphoid progenitors by BCR-ABL1 *in vitro* but also blocked its ability to initiate B-ALL in mice.

We further investigated whether activated β-catenin has a negative effect on development of BCR-ABL1-induced CML-like disease in mice. Bone marrow from 5-FU-treated C57BL/6 donor mice was harvested and transduced with retroviruses expressing BCR-ABL1/mRFP and ΔCatnb/GFP after myeloid cytokine prestimulation. Co-transduced cells expressing both mRFP and GFP were enriched by cell sorting and transplanted at different cell doses into irradiated syngeneic recipient mice. As a control, some recipients were transplanted with BCR-ABL1/mRFP and MIG co-transduced donor marrow. Recipients in the control arm transplanted with ≥ $1.5\ x10^4$ co-transduced marrow succumbed to CML-like MPN around 7 weeks after transplantation, but none of

recipients of marrow co-transduced with BCR-ABL1/mRFP and ΔCatnb/GFP, expected to express activated β-catenin, showed any disease symptoms upon sacrifice (Figure 3.15). These results are in agreement with our previous findings, and suggest that this constitutively active retroviral allele of β-catenin also has a profound negative effect on BCR-ABL1–induced CML-like MPN as well as on B-lymphoid leukemogenesis.

Figures- Results

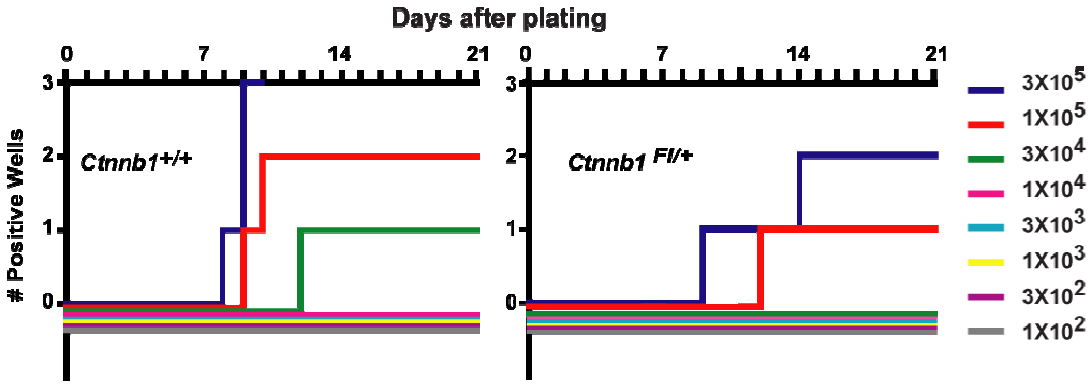

Figure 3.3. Active β-catenin inhibits primary B-lymphoid transformation mediated by BCR-ABL1. Bone marrow cells from *Ctnnb1*$^{+/+}$ or *Ctnnb1*$^{Fl/+}$ mice were harvested and transduced with retrovirus expressing BCR-ABL1/GFP-CRE. The transduced cells were plated at indicated cell numbers per well in triplicate, starting with as high as $3x10^5$ cells/well to 100 cells/well, as indicated by colored lines. Nontransduced cells were added to 10^6 total cells per well to provide stromal support. Positive wells were scored vs. time when the viable non-adherent cell number reached 10^6/well.

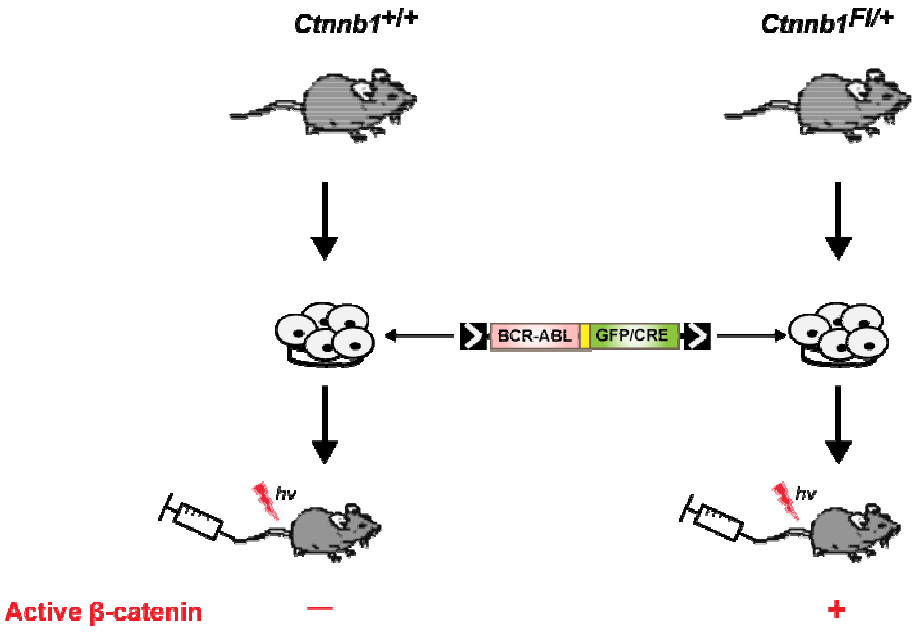

Figure 3.4. Scheme of experiment to test if active β-catenin affects BCR-ABL1-mediated B-ALL in mice. Bone marrow from *Ctnnb1*$^{+/+}$ or *Ctnnb1*$^{Fl/+}$ donors was transduced with BCR-ABL1/GFP-CRE retrovirus. Transduced marrow was subsequently transplanted into syngeneic irradiated recipients. Due to Cre-mediated recombination of the floxed *Ctnnb1* allele, the cohorts expected to have active β-catenin are indicated.

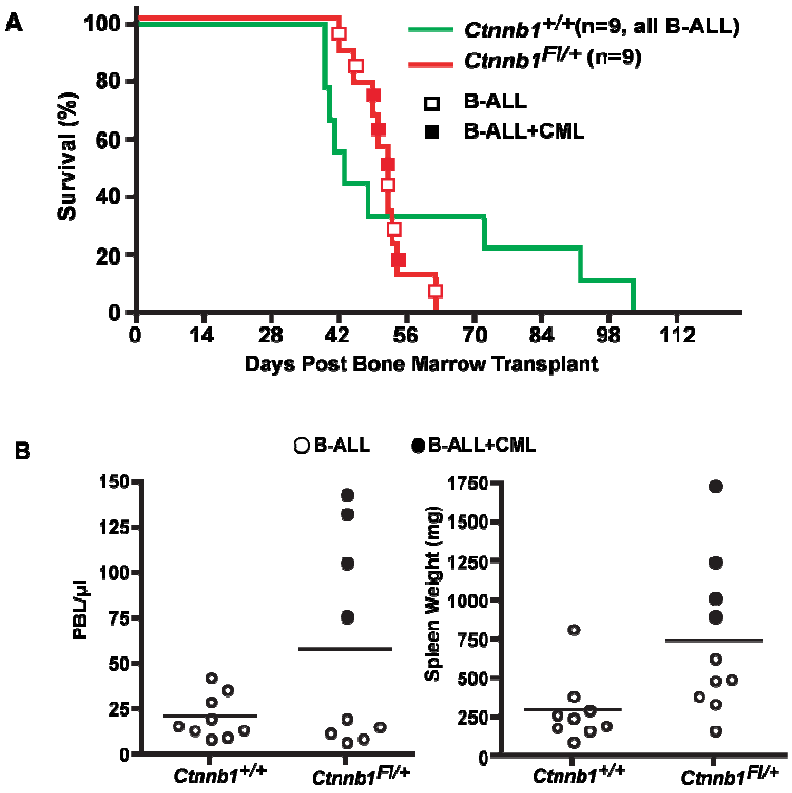

Figure 3.5. Active β-catenin alters the development of B-ALL mediated by BCR-ABL1. (A) Kaplan-Meier survival curve for recipient mice transplanted with *Ctnnb1*$^{+/+}$ or *Ctnnb1*$^{Fl/+}$ donor marrow transduced with retrovirus expressing BCR-ABL1/GFP-CRE. The number of individual mice in each arm is indicated. All mice receiving transduced *Ctnnb1*$^{+/+}$ marrow developed B-ALL. Among mice receiving transduced *Ctnnb1*$^{Fl/+}$ marrow, some developed B-ALL (open squares) while few developed B-ALL and CML-like disease simultaneously (closed squares). No significant difference in survival between two arms of recipients was observed (P=0.7819, Mantel-Cox test). **(B)** Peripheral blood leukocyte (PBL) counts and spleen weight of diseased mice from each cohort at the time of morbidity or death. The cause of death of individual disease mice is indicated (open circles, B-ALL; closed circles, B-ALL/CML mixed disease). The difference in spleen weight between two arms was significant (P<0.05, t-test).

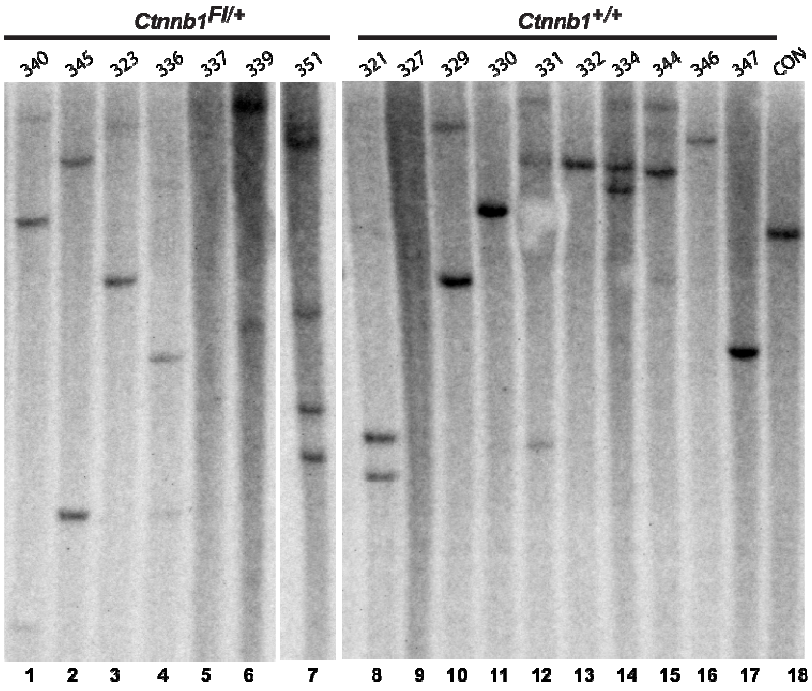

Figure 3.6. B-ALL mice carrying BCR-ABL1/GFP-CRE-transduced *Ctnnb1*$^{+/+}$ or *Ctnnb1*$^{Fl/+}$ marrow have similar number of distinct proviral clones. Genomic DNA from malignant tissue of B-ALL mice was subjected to Southern blot analysis to quantify the number of proviral clones with *IRES* probe. No significant difference in proviral clone number was found between B-ALL induced by BCR-ABL1/GFP-CRE –transduced *Ctnnb1*$^{+/+}$ (lane 8-17, 1.88 ± 0.35 clones) or *Ctnnb1*$^{Fl/+}$ marrow (lane 1-7, 1.8 ± 0.2 clones, *P*=0.8777, t-test). Control DNA (Con, lane 18) was from a cell line that contained a single *BCR-ABL1* provirus.

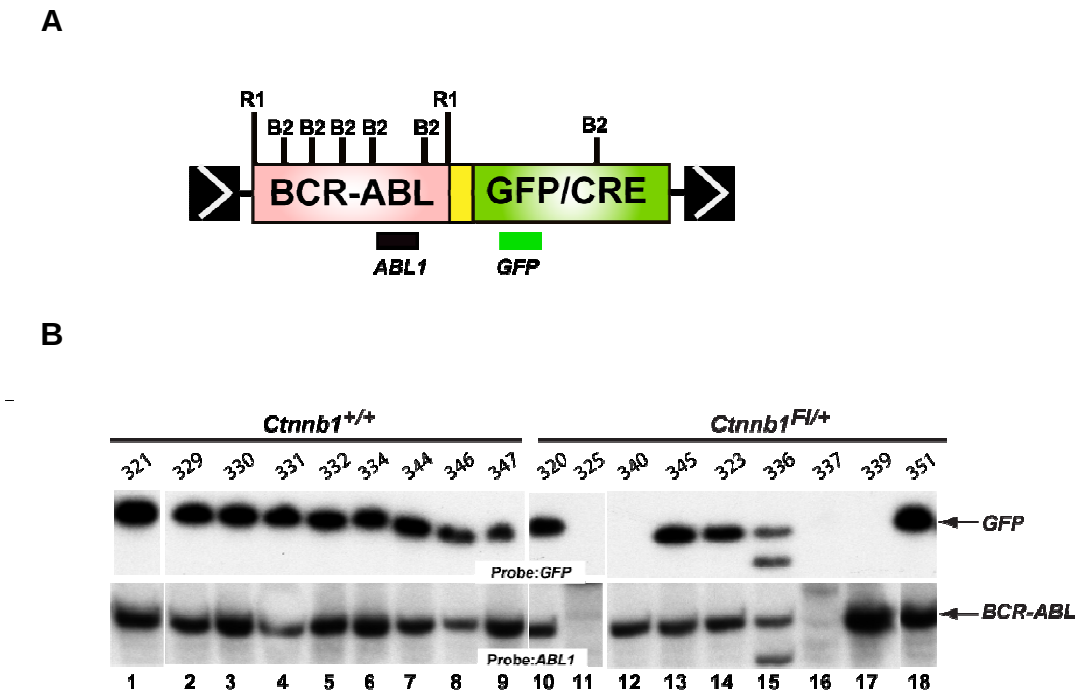

Figure 3.7. Active β-catenin is associated with loss of GFP-CRE expression in B-ALL induced by BCR-ABL1. (A) Structure of retrovirus expressing BCR-ABL1/GFP-CRE. The location of *ABL1* and *GFP* probes as well as restriction enzymes used in panel (B) are indicated: *Bgl*II (B2) and *EcoR*I (R1). (B) Integrity of proviral *GFP-CRE* and *BCR-ABL1* genes was assessed by subjecting genomic DNA from disease mice receiving BCR-ABL1/GFP-CRE transduced *Ctnnb1*$^{+/+}$ (lane 1-9) or *Ctnnb1*$^{Fl/+}$ (lane 10-18) donor marrow to Southern blot analysis. For analyzing *GFP-CRE* integrity, the DNA was digested with *Bgl*II and analyzed with a *GFP* probe. To assess *BCR-ABL1* integrity, DNA was digested with *EcoR*I and analyzed with a human *ABL1* probe.

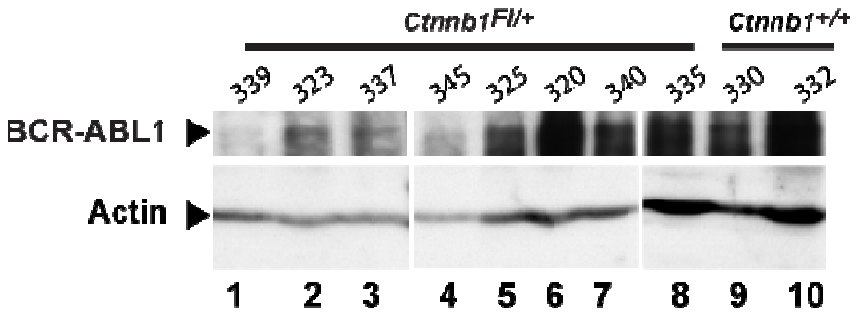

Figure 3.8. BCR-ABL1 expression in B-ALL mice carrying BCR-ABL1/GFP-CRE-transduced *Ctnnb1*$^{+/+}$ or *Ctnnb1*$^{Fl/+}$ marrow. Immunoblot of protein lysates from malignant tissue of B-ALL mice induced by BCR-ABL1/GFP-CRE-transduced *Ctnnb1*$^{+/+}$ (lanes 9-10) or *Ctnnb1*$^{Fl/+}$ marrow (lanes 1-8), probed with the indicated antibodies. Note mice # 325 and 337 had rearrangement of *BCR-ABL1* in Figure 3.6.

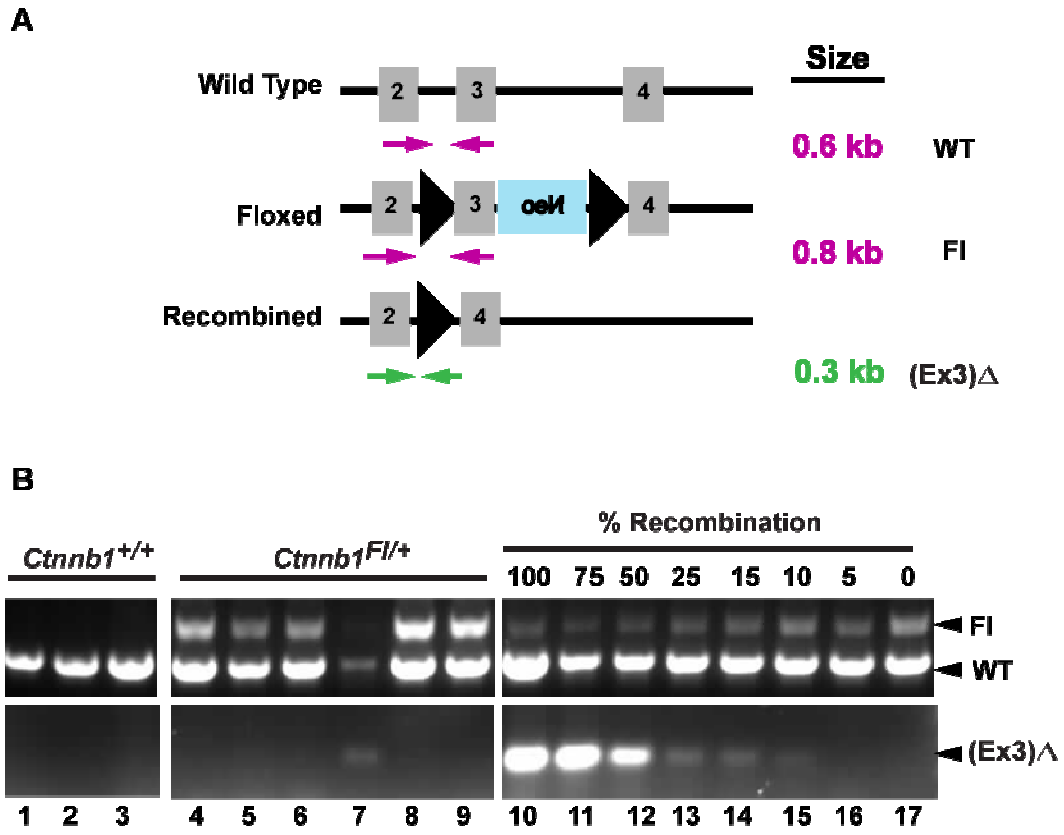

Figure 3.9. Using PCR to determine recombination efficiency of floxed Ctnnb1 allele mediated by BCR-ABL1/GFP-CRE. (A) Locations of primer sets used to detect Cre-mediated recombination efficiency of *Ctnnb1*^{Fl} allele. The first primer set (pink) was used to detect WT and floxed alleles. The second primer set (green) was used to detect the recombined allele. **(B)** Genomic DNA from malignant tissue of disease mice receiving BCR-ABL1/GFP-CRE-transduced *Ctnnb1*$^{+/+}$ (lane 1-3) or *Ctnnb1*$^{Fl/+}$ (lane 4-9) marrow was subjected to PCR analysis to determine recombination efficiency by using two primer set described in (A). Genomic DNA from thymocytes of *LckCre- Ctnnb1*$^{Fl/+}$ was mixed with genomic DNA from *Ctnnb1*$^{Fl/+}$ bone marrow at different ratios to establish a standard of different percentages of recombination efficiency.

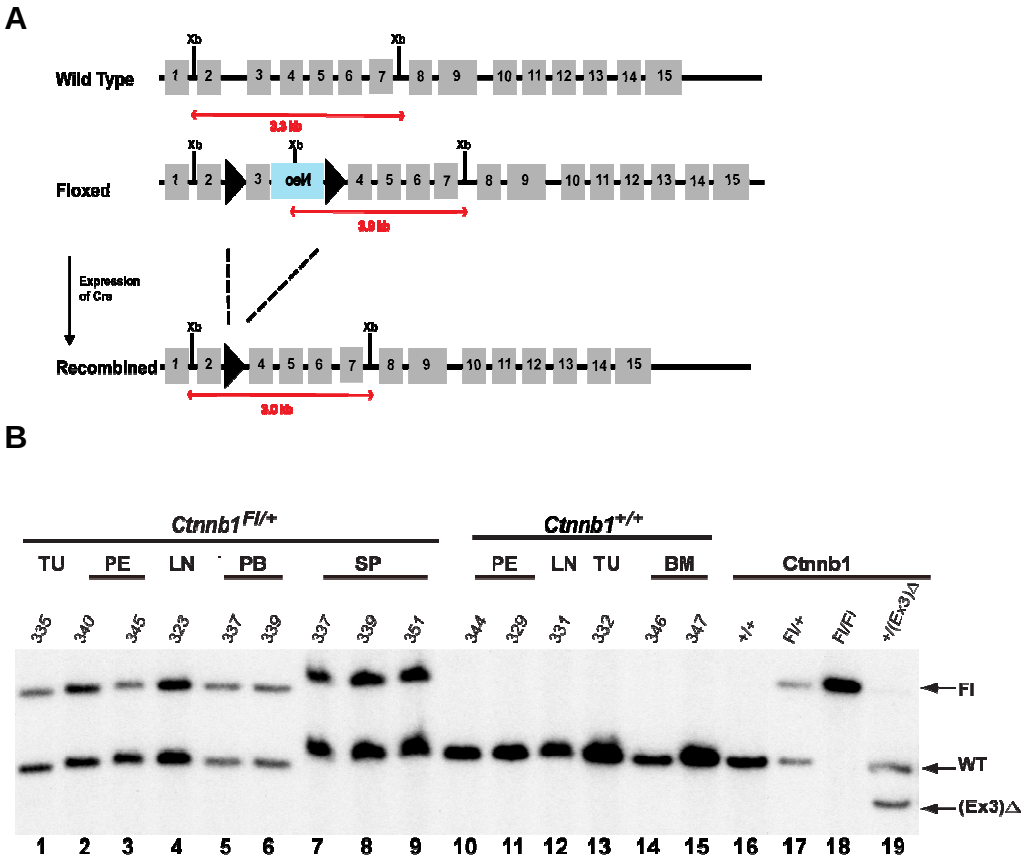

Figure 3.10. Recombination efficiency of floxed *Ctnnb1* allele in mice with leukemia induced by BCR-ABL1/GFP-CRE retrovirus. (A) Schematic map demonstrating the location of the *Xba*I (Xb) site used in the Southern blot in (B) to analyze recombination efficiency. The probe, located within exon 4, was used to detect the wild type (3.3 kb), floxed (3.9 kb), and recombined (3.0 kb) alleles. **(B)** Genomic DNA from malignant tissues from diseased mice receiving BCR-ABL1/GFP-CRE–transduced *Ctnnb1*$^{+/+}$ (lane 10-15) or *Ctnnb1*$^{Fl/+}$ (lane 1-9) marrow was subjected to Southern blot analysis to assess recombination efficiency of the floxed allele mediated by Cre recombinase. DNA was digested with *Xba*I and analyzed with a probe from exon 4 of *Ctnnb1*. Genomic DNA of thymocytes from of CD4Cre-Ctnnb1$^{Fl/+}$ (lane 19), which have 100% recombination of the floxed allele, served as a control. BM: bone marrow, LN: lymph node, PB: peripheral blood, PE: pleural effusion, SP: spleen, TU: tumor. Fl: floxed allele, WT: wild-type allele, (Ex3)Δ: recombined allele.

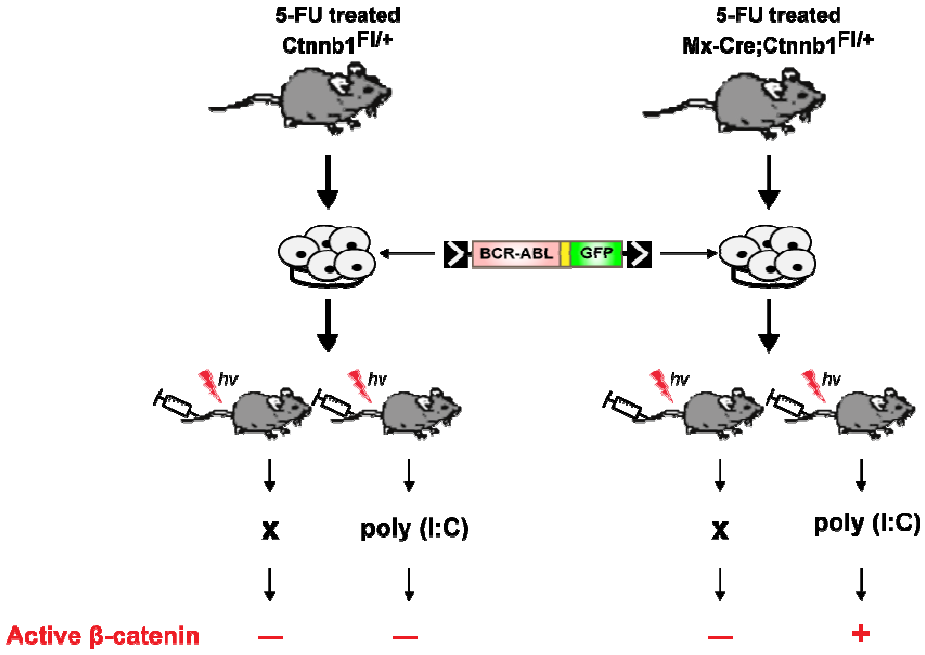

Figure 3.11. Scheme of experiment to test if active β-catenin affects BCR-ABL1-mediated CML-like MPN in mice. Bone marrow from *Ctnnb1*$^{Fl/+}$ or *Mx-Cre*;*Ctnnb1*$^{Fl/+}$ donors pretreated with 5-FU was transduced with retrovirus expressing BCR-ABL1/GFP in the presence of myeloid cytokines. Transduced bone marrow cells were subsequently transplanted into syngeneic irradiated recipient mice at day 0. Recipients were treated with or without poly(I:C) at day 10, 12 and 14 to induce the expression of Cre recombinase. The cohort expected to have expression of active β-catenin is indicated.

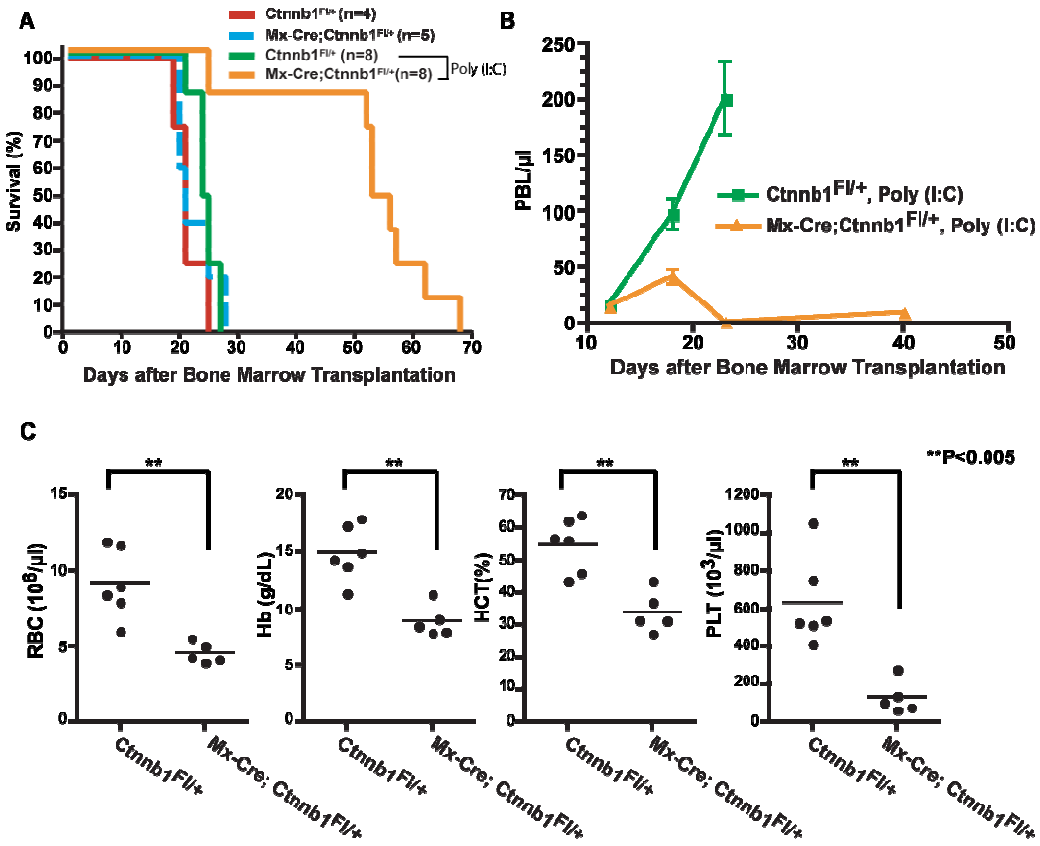

Figure 3.12. BCR-ABL1 cannot rescue the hematopoiesis defect caused by activation of β-catenin mediated by Mx-Cre. (A) Kaplan-Meier survival curve for recipient mice transplanted with bone marrow transduced with retrovirus expressing BCR-ABL1/GFP, as described in Figure 3.8. Donor marrow was either from control (*Ctnnb1*$^{Fl/+}$) or *Mx-Cre*;*Ctnnb1*$^{Fl/+}$ mice. The number of individual mice in each arm is indicated. The survival between *Ctnnb1*$^{Fl/+}$ and *Mx-Cre*;*Ctnnb1*$^{Fl/+}$ mice treated with poly(I:C) was significant (*P*=0.0002, Mantel-Cox test). (B) Plot of Peripheral blood leukocyte (PBL) counts as a function of time after bone marrow transplant between recipients transplanted with *Ctnnb1*$^{Fl/+}$ or *Mx-Cre*;*Ctnnb1*$^{Fl/+}$ marrow. Mice from both arms were treated with poly(I:C). (C) Red blood cells (RBC) counts, hemoglobin (Hb), hematocrit (HCT) and platelet (PLT) counts in diseased mice that were recipients of Ctnnb1$^{Fl/+}$ or Mx-Cre;Ctnnb1$^{Fl/+}$ BCR-ABL1/GFP–transduced marrow. Both arms were treated with poly(I:C).

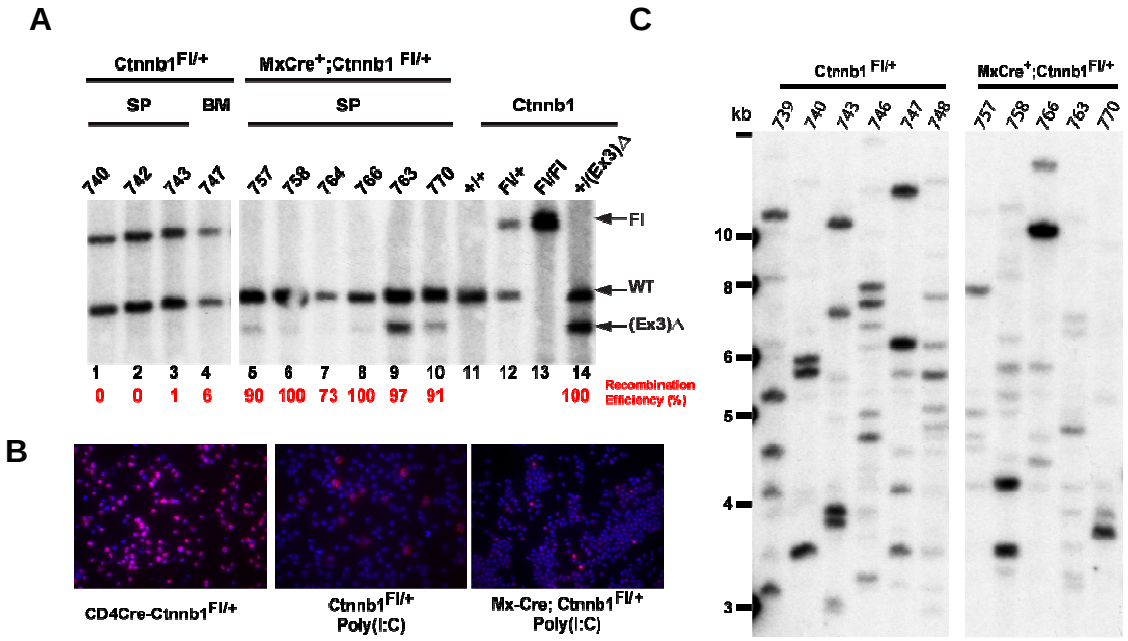

Figure. 3.13. BCR-ABL1-transformed donor cells with expression of active β-catenin induced by Mx-Cre do not predominate in hematopoietic tissues of recipient mice. (A) Efficiency of recombination of the floxed *Ctnnb1* allele in poly(I:C)–treated recipient mice carrying BCR-ABL1-transduced marrow from control (*Ctnnb1*$^{Fl/+}$) and *Mx-Cre*;*Ctnnb1*$^{Fl/+}$ donor mice. Fl: floxed allele, WT: wild-type allele, (Ex3)Δ: recombined allele. Genomic DNA from disease mice were digested with *Xba*I and subjected to Southern blot analysis with probe located within exon 4 of *Ctnnb1*. **(B)** Immunofluorescence staining of β-catenin (red) in peripheral blood leukocytes of recipients of BCR-ABL1-transduced *Ctnnb1*$^{Fl/+}$ or *Mx-Cre*; *Ctnnb1*$^{Fl/+}$ marrow. Both mice were treated with poly(I:C). Thymocytes from CD4Cre-*Ctnnb1*$^{Fl/+}$ were stained as positive control (left panel). Cells were counterstained with Hoechst dye (blue). **(C)** To determine proviral clone number (LIC frequency) in poly(I:C)–treated recipient mice of transduced marrow from control (Ctnnb1$^{Fl/+}$) or *Mx-Cre*;*Ctnnb1*$^{Fl/+}$ donor mice, genomic DNA from hematopoietic tissues of diseased mice was digested with *Bgl*II and analyzed with Southern with a *GFP* probe. Note mouse # 763 and 770 were the last two surviving mice that developed symptoms of CML-like MPN.

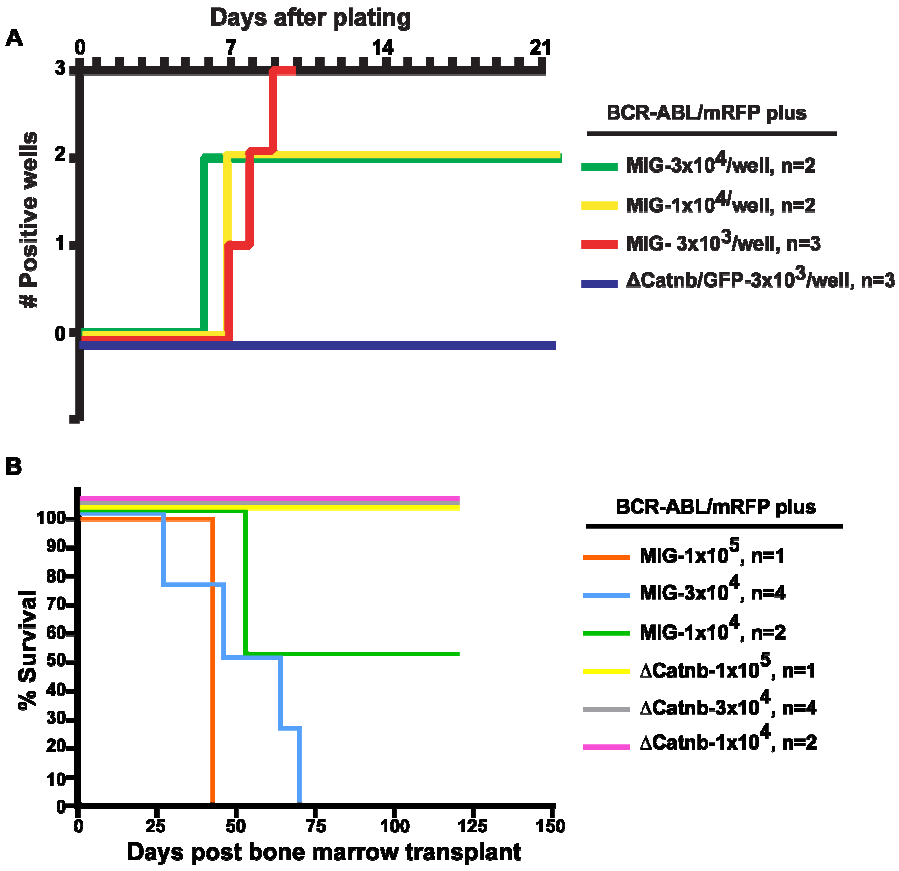

Figure. 3.14. A constitutively active retroviral allele of β-catenin inhibits BCR-ABL1–mediated B-lymphoid transformation in vitro and B-ALL development in mice. (A) Bone marrow from donors not treated with 5-FU was transduced with retroviruses expressing BCR-ABL1/mRFP and either ΔCatnb/GFP or a control virus, MIG. Co-transduced cells expressing both mRFP and GFP were purified by cell sorting and seeded in triplicate at indicated cell number per well. Nontransduced cells were added to 10^6 total cells per well to provide stromal support. Positive wells were scored vs. time when the viable non-adherent cell number reached 10^6/well. **(B)** Kaplan-Meier survival curve for B-ALL mice receiving BCR-ABL1/mRFP and MIG or ΔCatnb/GFP co-transduced marrow from Balb/c donors. Co-transduced cells expressing mRFP and GFP enriched by cell sorting were transplanted into sublethally irradiated recipients at different cell dosages, as indicated by the colored lines. The number of individual mice in each arm is indicated.

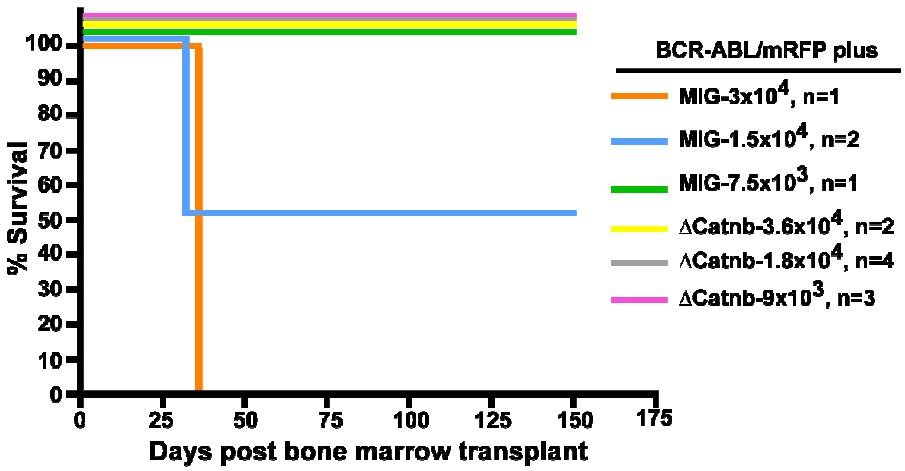

Figure. 3.15. The constitutively active retroviral allele of β-catenin prevents the development of BCR-ABL1-induced CML-like MPN in mice. Survival curve for recipients of marrow from 5-FU pretreated C57BL/6 donor mice co-transduced with BCR-ABL1/mRFP and either MIG or ΔCatnb/GFP. Co-transduced cells expressing mRFP and GFP were enriched by cell sorting and transplanted into irradiated syngeneic recipients at different cell dosages, as indicated by the colored lines. The number of individual mice in each arm is listed. Control mice receiving ≥ 1.5 x10^4 cotransduced marrows succumbed to CML-like disease while none of the recipients of BCR-ABL1/mRFP and ΔCatnb/GFP co-transduced marrow showed any signs of disease upon sacrifice.

3.3 DISCUSSION

Although imatinib or second generation TKIs are currently front-line treatment for patients with CML in chronic phase, they are less effective in CML patients with accelerated or blast-crisis phase. Disease relapse or progression during imatinib treatment occurs and may be due to existence of residual LICs, which seem to have some level of intrinsic resistance to TKIs. Therefore, it is important to understand the mechanisms that these LICs depend on for their maintenance and survival in order to develop effective therapy to eradicate them. One of attractive candidate pathways is the Wnt/β-catenin pathway, due to its documented role in regulating normal HSCs. Several studies have used gain- or loss-of-function approaches to investigate the role of β-catenin in regulating functions of normal HSC and LICs. By using loss-of-function strategies to study normal HSC and LICs, previous studies suggest that β-catenin may be dispensable during adult normal hematopoiesis but is essential for the maintenance of BCR-ABL1 expressing LICs to initiate CML-like disease in mice (Cobas et al., 2004; Hu et al., 2009; Koch et al., 2008; Zhao et al., 2007).

Results from gain-of-function approaches to study how β-catenin regulates HSCs function are interesting but remain controversial. While Reya et al. showed that Wnt pathway stimulation can enhance self-renewal potential of HSCs from a Bcl-2 transgenic background (Reya et al., 2003), two independent studies using the Cre-loxP system to induce active β-catenin in HSCs from wild type mice demonstrated that active β-catenin actually caused HSC exhaustion and failure to maintain normal hematopoiesis (Kirstetter et al., 2006; Scheller et al., 2006). Although deregulated active β-catenin has been shown

to be required for maintaining LICs in AML and is associated with progression of CML to myeloid blast crisis (Jamieson et al., 2004; Wang et al., 2010), direct proof from *in vivo* studies is still required in order to evaluate whether Wnt/β-catenin is an essential target for treating Ph^+ leukemia and for the development of therapeutic strategies.

Based on previous studies, we hypothesized that the activation of β-catenin alone would be sufficient to confer self-renewal ability on BCR-ABL1-expressing myeloid progenitors, and might accelerate disease transformation from chronic phase to myeloid blast crisis. We further hypothesized that activated β-catenin may be involved in the development of Ph^+ B-ALL or lymphoid blast crisis. To study the role of β-catenin in BCR-ABL1–mediated leukemogenesis, we conducted several *in vitro* and *in vivo* experiments using the retroviral bone marrow transduction/transplantation mouse model in combination with conditional activated β-catenin allele in transgenic mice (Harada et al., 1999) or a constitutively active β-catenin retroviral allele (Reya et al., 2003). We first found that activated β-catenin inhibited B-lymphoid transformation ability of BCR-ABL1 *in vitro*, when a retrovirus expressing BCR-ABL1/GFP-CRE was used to delete exon 3 of β-catenin in B-lymphoid progenitors from *Ctnnb1*$^{Fl/+}$ mice. Similar results were subsequently observed when activated β-catenin was introduced by transducing B-lymphoid progenitors with retrovirus expressing ΔCatnb/GFP. As we extended these *in vitro* studies to the B-ALL mouse model, we found that activated β-catenin also had a negative effect on the development of acute lymphoid leukemia mediated by BCR-ABL1 *in vivo*. Activation of β-catenin by BCR-ABL1/GFP-CRE interfered with the

development of B-ALL in recipient mice, causing some recipients to develop B-ALL and CML-like disease simultaneously.

It is highly unusual to have the appearance of CML-like MPN in recipients of BCR-ABL1-transduced marrow in retroviral trasnsduction/transplantaion model of B-ALL. While it is possible that the expression of activated β-catenin in B-lymphoid progenitors may alter their potential to transdifferentiate into myeloid cells to allow these Ph^+ lymphoid progenitors to initiate myeloid leukemia (Baba et al., 2005), it is also possible that activated β-catenin might confer self-renewal to myeloid progenitors, allowing development of CML-like disease in this B-ALL mouse model (Huntly et al., 2004). Pilot experiments were carried out to determine if committed myeloid progenitors, which are abundant and readily transduced under these conditions, might be the source of the myeloid leukemia (see Appendix). However, due to technical difficulties, the question remains unanswered and will be addressed in future experiments (see below).

Even though the previous two possibilities are plausible explanations for the mice that developed myeloid leukemia in the B-ALL model, we were unable to detect significant recombination of the floxed *Ctnnb1* allele even in myeloid cells from mice with mixed MPN and B-ALL. These results indicate the possibilities that cells expressing active β-catenin encoded by the recombined allele were outcompeted by the cells retaining the unrecombined floxed allele, which has been reported previously in other studies (Scheller et al., 2006). In addition, we later observed that a constitutively active retroviral allele of β-catenin also inhibited B-ALL development mediated by BCR-ABL1. Previous studies analyzing proviral integration sites in different hematopoietic

lineages and testing isolated stem/progenitor populations for the ability to induce Ph^+ leukemia in mice showed that the LICs for CML-like disease are HSCs, while B-lymphoid progenitors are responsible for inducing B-ALL (Hu et al., 2006; Huntly et al., 2004; Li et al., 1999a; Million and Van Etten, 2000; Roumiantsev et al., 2001). Analyzing proviral integrity and recombination efficiency mediated by GFP-CRE showed extensive proviral gene rearrangements in recipients of BCR-ABL1/GFP-CRE transduced $Ctnnb1^{Fl}$ marrow. As a result, only clones with the unrecombined form of the *Ctnnb1*^{Fl} allele gave rise to leukemias. Therefore, it is also possible that activation of β-catenin by BCR-ABL1/GFP-CRE caused the exhaustion of early B-lymphoid progenitors, so that rare BCR-ABL1-transformed HSCs, which are ordinarily out-competed by B-ALL, have a chance to give rise to a myeloproliferative neoplasm.

On the other hand, we found that BCR-ABL1 expressing HSCs cannot tolerate activated β-catenin and failed to initiate and maintain CML-like disease in mice. When we used BCR-ABL1–transduced marrow from 5-FU pretreated Mx-Cre;$Ctnnb1^{Fl/+}$ donor to initiate CML-like disease, activation of β-catenin upon poly(I:C) treatment resulted in severe pancytopenia in all recipients. Expression of active β-catenin did not reduce the number of LIC clones, but nonetheless still appeared to cause differentiation defects in LICs, which could not be overcome or rescued by BCR-ABL1. Consistent with this, we observed very few cells containing active β-catenin in the leukocytes from these mice by using Southern blot analysis (Figure 3.13A) or immunofluorescence staining (Figure 3.13B). This observation was further confirmed in recipients transplanted with BCR-ABL1/mRFP and ΔCatnb/GFP cotransduced marrows from 5-FU pretreated donors. No

recipients showing any sign of disease development upon sacrifice. Unlike the AML disease model involving overexpression of HoxA9 and Meis1 (Wang et al., 2010), in which expression of constitutively active β-catenin through retroviral transduction was able to confer self-renewal on GMPs, our results demonstrated that myeloid and lymphoid leukemias induced by BCR-ABL1 cannot tolerate overexpression of active β-catenin. Even though a previous study in primary leukemic cells from patients showed aberrant expression of activated β-catenin in GMPs that was associated with acquisition of self-renewal and progression of CML from chronic phase to myeloid blast crisis (Jamieson et al., 2004), our data suggest that too much β-catenin can cause a defect in leukemogenesis mediated by BCR-ABL1. The positive effect of β-catenin on the self-renewal ability of normal HSCs or leukemic cells might depend on its expression level at a specific range. It would be interesting to further investigate how much active β-catenin could transform GMP to initiate CML-like disease in mice with a pharmacological regimen.

There is also a possibility that additional mutations provide survival signals in acute myeloid leukemia or CML in advanced stage that allow hematopoietic progenitors to tolerate the expression of activated β-catenin. Under the protection of survival signals through these unknown mutations, LICs could not only escape from exhaustion caused by activated β-catenin but also gain more self-renewal ability and transform the disease to more aggressive phenotype (Jamieson et al., 2004). It would be interesting to see if activated β-catenin is expressed in the mouse model of CML myeloid blast crisis induced by cooperation between BCR-ABL1 and NUP98/HOXA9 (Dash et al., 2002). On the

other hand, earlier work by Reya et al. (Reya et al., 2003) showed that introducing a constitutively active retroviral allele of β-catenin into HSCs from Bcl-2 transgenic mice could enhance HSC self-renewal ability as measured by repopulation. In contrast to the findings from Reya et al., who observed 15-60% donor derived chimerism in lethally irradiated recipients, Baba et al. only observed <3% donor derived chimerism in NOD/SCID mice transplanted with wild type HSCs transduced with the same constitutively active retroviral allele (Baba et al., 2006). This study suggests that Bcl-2 might provide survival signals to protect HSCs from the exhaustion observed in other studies (Kirstetter et al., 2006; Scheller et al., 2006). Even though BCR-ABL1 itself has been reported to up-regulate several pro-survival proteins including Bcl-2 (Sanchez-Garcia and Grutz, 1995; Skorski et al., 1997) and Bcl-X (Gesbert and Griffin, 2000), it will be important to investigate whether activated β-catenin has a positive effect on BCR-ABL1-mediated leukemia when Bcl-2 is overexpressed.

3.4 METHODS

Mice and genotyping

Ctnnb1$^{Fl/+}$ mice (kind gift from Dr. Fotini Gounari, The University of Chicago) and *Mx-Cre* mice (kind gift from Dr. Paul Ney, St. Jude Children's Research Hospital) have been described previously (Harada et al., 1999; Kuhn et al., 1995). *Mx-Cre* mice were crossed with *Ctnnb1*$^{Fl/+}$ mice to generate (*Mx-Cre*;*Ctnnb1*$^{Fl/+}$) mice. To genotype *Mx-Cre* mice, primers (5'-AGGCGTTTTCTGAGCATACC-3', 5'-TAGCTGGCTGGTGGCAGATG-3') were used while primers (5'-AGAATCACGGTGACCTGGGTTAAA-3', 5'-CATTCATAAAGGACTTGGGAGGT-3') were used to genotype *Ctnnb1*$^{Fl/+}$ mice.

Retroviral constructs and generation of retrovirus stocks

The retroviral vector expressing BCR-ABL1/GFP was described previously (Krause et al., 2006). ΔCatnb/GFP (Reya et al., 2003) was obtained from Addgene. To engineer a retroviral vector expressing BCR-ABL1/mRFP, BCR-ABL1 cDNA (p210 isoform) was cloned into 5' end of IRES in plasmid MSCV-IRES-mRFP (kind gift of Dr. P.N. Tsichlis, Tufts Medial Center). Generation of retrovirus stocks was described in the Methods section of Chapter 2.

Bone marrow transduction, transformation, and transplantation

Detailed methods regarding transformation and leukemogenesis studies in mice were provided in the Methods section of Chapter 2. To induce excision of *Ctnnb1*^{Fl} by *Mx-Cre*, each transplanted recipient was injected with 250 μg per mouse of (poly(I:C)) (InvivoGen) in sterile water intraperitoneally at day 10, 12, and 14 post- transplantation.

Southern blot analysis

Distinct proviral integration events were counted as described in Methods section of Chapter 2, to determine the number of leukemia-initiating cells. To analyze distinct proviral integration events in mice with disease induced by BCR-ABL1/GFP-CRE, genomic DNA from malignant tissues of diseased mice was digested with *Eco*RI and analyzed with a probe derived from *IRES*. To analyze proviral integration events in mice with disease induced by BCR-ABL1/GFP, DNA was digested with *Bgl*II and analyzed with *GFP* probe.

To analyze recombination efficiency of Ctnnb1$^{Fl/+}$ allele mediated by Cre recombinase, genomic DNA from diseased mice was digested with *Xba*I, and analyzed with probe derived from exon 4 of *Ctnnb1*. The digestion generated 3.9-kilobase floxed allele, 3.3-kilobase wild-type allele, and 3-kilobase recombined allele. The blot was quantified with PhosphorImager (Molecular Dynamics) to determine recombination efficiency.

To assess the integrity of *GFP-CRE* in mice with diseases induced by BCR-ABL1/GFP-CRE, genomic DNA was digested with *Bgl*II and analyzed with a *GFP* probe. To study the integrity of proviral *BCR-ABL1*, DNA was digested with *Eco*RI and analyzed with a human *ABL1* probe.

Immunofluorescence staining of β-catenin

Cells were washed with cold PBS, fixed with 2% paraformaldehyde (Electron Microscopy Sciences) on ice for 15 mins and permeabilized with PBS buffer containing 2% serum and 0.1% saponin (Sigma) buffer for 10 min at room temperature. Cells were then incubated with 1:100 rabbit anti-β-catnein (Invitrogen) antibody at room temperature for an hour, followed by incubation with 1:200 secondary Alexa Fluor 555 conjugated goat anti-rabbit antibody (Invitrogen) at room temperature for half hour. After washing with saponin buffer, cells were placed on coverslips by using a Cytospin (Shandon), and imaged using Leica TCS SP2 confocal microscope.

Chapter 4.

Conclusions and Future directions

Although TKI treatment can induce cytogenetic remissions in the majority of patients with CML in chronic phase, acquired resistance to TKI therapy and relapse of leukemia occurs quiet frequently. Hence, TKI therapy alone might not be able to achieve permanent cure for majority of patients with Ph^+ leukemia. Other targeted therapies are needed to overcome TKIs resistance and eradicate the remaining LICs that are responsible for relapse. To verify if two attractive signaling pathways, NF-κB and β-catenin, could be potential therapeutic targets in treating Ph^+ leukemia, in this thesis we used genetic approaches and conducted several *in vitro* and *in vivo* experiments to define their roles in BCR-ABL1-induced leukemias.

In Chapter 2, by expressing different mutant forms of regulatory proteins in the NF-κB pathway, we demonstrated a role of this pathway in BCR-ABL1-induced myeloid and lymphoid leukemias. We found that NF-κB is activated predominantly through the canonical IKK pathway and is important in maintaining LICs that initiate the disease. We further showed that expression of IκBαSR, IKKαKM or IKKβKM sensitize BCR-ABL1–expressing B-lymphoid blasts to TKIs such as imatinib and dasatinib. This result suggests combination therapy with TKIs plus an IKK inhibitor might be a more specific and effective targeted therapy for Ph^+ CML or B-ALL patients, and might also reduce the side effects associated with traditional cytotoxic therapies, including TKIs and chemotherapy or corticosteroid treatment (Ohno, 2010; Vignetti et al., 2007). Our results also suggest that dual inhibition of both IKKα and IKKβ might augment the efficacy of inhibiting leukemogenesis by BCR-ABL1, supporting the development of small molecule inhibitors against IKKα or IKKα/β in addition to the available IKKβ inhibitors that are currently in clinical trials. Depending on safety and anti-leukemic effect of these

inhibitors in preclinical studies, targeting NF-κB pathway might be a promising therapy in Ph^+ ALL and CML.

In Chapter 3, we used different genetic approaches to activate β-catenin in BCR-$ABL1^+$ leukemic cells to study its function in the development of Ph^+ leukemia. The results demonstrated that constitutive expression of an active β-catenin can impair the development of BCR-ABL1-induced myeloid and lymphoid leukemias despite the documented involvement of deregulated active Wnt/β-catenin pathway in oncogenesis and tumor progression in several malignancies. Is active β-catenin a friend or foe in fighting Ph^+ B-ALL and CML? There are two potential possibilities. First, its effect might depend on the specific and narrow range expression level of active β-catenin in leukemic cells. A second possibility might be the existence of other mutations (eg, Bcl-2) to counteract hematopoiesis defect resulting from too much β-catenin activity.

To test the second hypothesis, we will transduce donor cells from H2K-Bcl-2 mice (Domen et al., 1998) with BCR-ABL1/mRFP and MIG or ΔCatnb/GFP and observe disease development in irradiated recipients of cells expressing both mRFP and GFP. If we do see that disease development is possible from HSCs co-expressing BCR-ABL1 and activated β-catenin, we will further isolate HSCs and myeloid progenitors, perform co-transduction with BCR-ABL1/mRFP and ΔCatnb/GFP retroviruses, and test by serial transplantation if active β-catenin provides self-renewal ability in BCR-ABL1-expressing myeloid progenitors (Figure 4.1). We will also test whether active β-catenin enhances the development of B-ALL induced by BCR-ABL1 when Bcl-2 is overexpressed. These

experiments should provide important new knowledge about the cooperative roles of Wnt signaling and pro-survival pathways in the pathogenesis of Ph^+ leukemias.

Given the fact that both the NF-κB and β-catenin share common target genes (such as cyclin D1 and c-Myc) and have both been implicated in regulating cellular proliferation and cancer progression, is there potential crosstalk between these two pathways in regulating BCR-ABL1-induced leukemias? Previous studies have shown that two upstream catalytic kinases in NF-κB pathway, IKKα and IKKβ, can also phosphorylate β-catenin. While IKKβ decreased transcription activity of β-catenin, IKKα increased β-catenin-dependent transcription activation (Albanese et al., 2003; Lamberti et al., 2001). Interestingly, β-catenin has been shown to form a complex with both RelA and p50 and inhibit the DNA binding as well as transactivation activity of RelA/p50 heterodimers in colon cancer cell lines. Using siRNA to knock down the expression of β-catenin further restored the DNA binding ability of RelA/p50 heterodimers (Deng et al., 2002; Kim et al., 2005). Whether the same observations can be found in Ph^+ leukemias is unclear. On the other hand, CK1α and GSK3-β, two upstream kinases that phosphorylate and negatively regulate β-catenin, have also been shown to play a role in regulating NF-κB pathway. While CK1α acts bifunctionally to first turn on and then terminate antigen-receptor-induced NF-κB activation in human lymphoma cell lines (Bidere et al., 2009), inhibition of GSK3-β reduces the transactivation activity of NF-κB in murine embryonic fibroblasts (Hoeflich et al., 2000). Together, these results suggest the NF-κB and β-catenin pathways might have different roles in regulating the same target genes. Since activated β-catenin was not found in patients with CML-CP but was associated with

development of AML (Wang et al., 2010) and CML-MBC (Jamieson et al., 2004), it will be interesting in the future to see if the crosstalk between these two pathways regulates disease progression of Ph^+ leukemias.

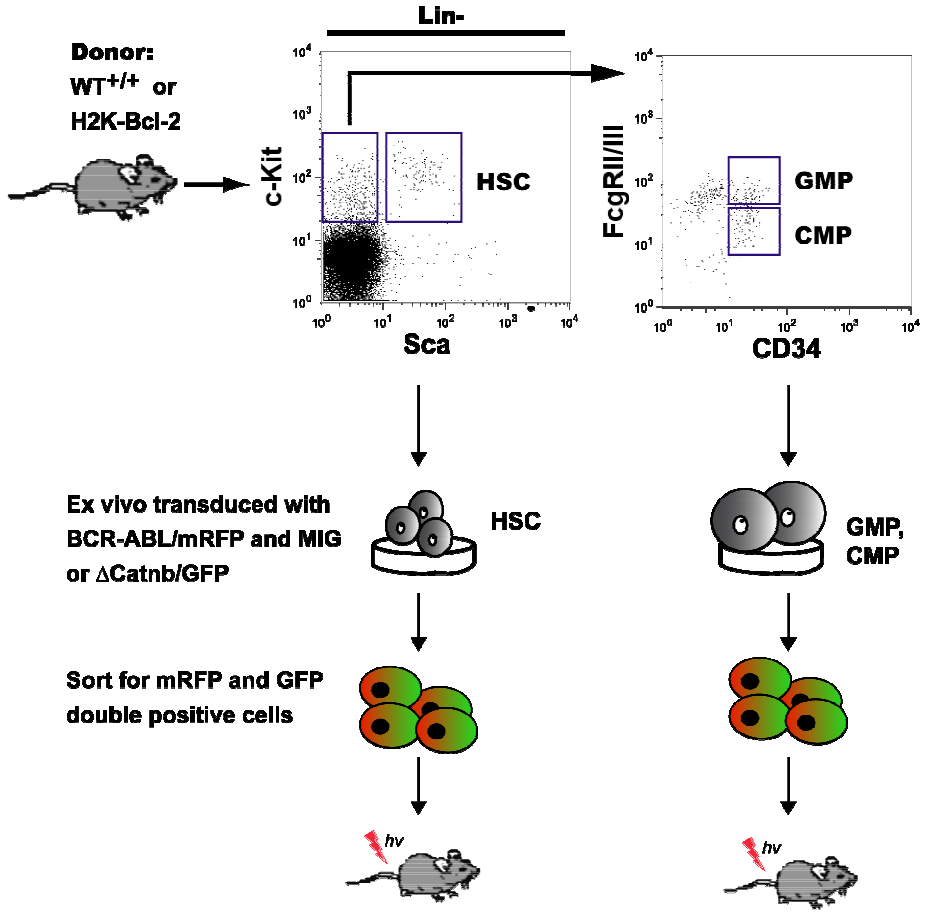

Figure 4.1. Scheme for using HSCs or myeloid progenitors from H2K-Bcl-2 transgenic mice to study if β-catenin provides progenitors self-renewal ability to initiate CML- or AML-like disease in mice. Sorted HSCs ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$), CMP ($Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^+$ $Fc\gamma RII/III^{low}$), and GMP ($Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^+Fc\gamma RII/III^{high}$) will transduced with BCR-ABL1/mRFP and MIG or ΔCatnb/GFP. Transduced cells will be transplanted into irradiated recipients.

Appendix

INDUCING PH$^+$ LEUKEMIA WITH ISOLATED HSCS OR MYELOID PROGENITORS (C/GMP) EXPRESSING BCR-ABL1 AND ACTIVE β-CATENIN

Because some mice that received BCR-ABL1/GFP-CRE–transduced *Ctnnb1*$^{Fl/+}$ marrow developed mixed CML/B-ALL disease (Figure 3.5), it is possible that active β-catenin confers myeloid development potential to B-lymphoid progenitors (Baba et al., 2005) or increases the self-renewal capacity of committed myeloid progenitors allowing them to initiate myeloid leukemia (Jamieson et al., 2004). To test the latter possibility, we induced diseased from lineage-specific hematopoietic progenitor populations purified by known cell surface markers. HSC and myeloid progenitors including common myeloid progenitors (CMP) and GMP were isolated by using the combination of cell-surface markers described before (Huntly et al., 2004; Na Nakorn et al., 2002). Due to poor expression of Sca-1 in Balb/c background (Spangrude and Brooks, 1993), CD150 was used to replace Sca-1 (Kiel et al., 2005). Accordingly, purified HSCs (Lin$^-$c-Kit$^+$CD150$^+$) as well as myeloid progenitors (Lin$^-$c-Kit$^+$FcγRII/III$^+$CD34$^+$) were purified from C*tnnb1*$^{Fl/+}$ donor marrow. Isolated cells were pre-stimulated with myeloid cytokines and transduced with retroviruses expressing BCR-ABL1/GFP-CRE or BCR-ABL1/GFP as control. Transduced cells were transplanted into irradiated recipient mice. Due to contamination of B-lymphoid progenitors during purification process, mice receiving transduced myeloid progenitors either developed B-ALL or showed no sign of disease upon sacrifice at day 120 post-transplantation. One mouse from the cohort that received HSCs transduced with BCR-ABL/GFP died of CML (1 out of 5) at day 27 while the rest of mice in the same group developed B-ALL (4 out of 5). In the cohort that received HSCs transduced with BCR-ABL1/GFP-CRE, one mouse died of CML at day 105 (1 out

of 5), two mice died of B-ALL (2 out of 5) and two mice (2 out of 5) did not develop disease. Overall, the development of Ph^+ leukemias from transduced cells expected to have active β-catenin (ie. cells transduced with retrovirus expressing BCR-ABL1/GFP-CRE) is longer than that from transduced control cells. In agreement with our previous findings, these results suggest activated β-catenin generated by Cre-loxP system inhibits Ph^+ leukemogenesis. However, we were not able to address our original hypothesis due to technical problems with contamination with lymphoid progenitors. We will further test if activated β-catenin provides Ph^+ myeloid progenitors self-renewal ability in the presence of overexpressing Bcl-2. Future experiments will be carried out by using donor mice in an H2K-Bcl-2 background, as described in Figure 4.1s.

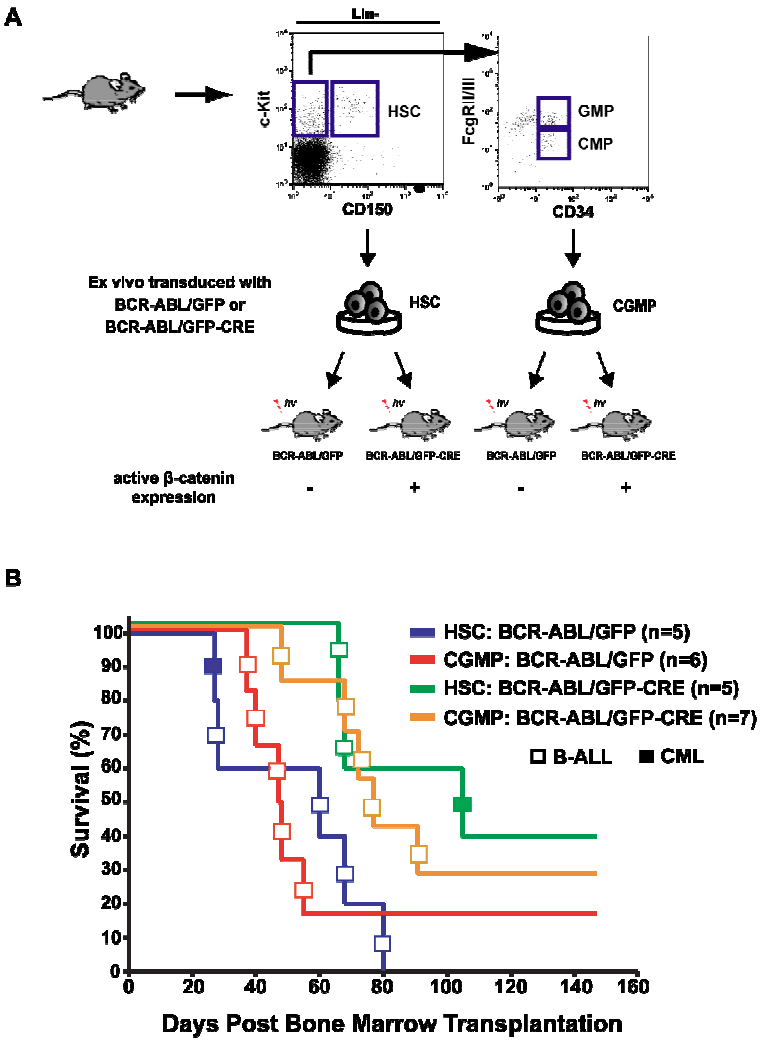

Figure A1. Inducing Ph$^+$ leukemia with isolated HSCs or myeloid progenitors (C/GMP) expressing BCR-ABL1 and active β-catenin. (A) Scheme of the experiment. Purified cells from Balb/c mice were transduced with retroviruses BCR-ABL/GFP or BCR-ABL/GFP-CRE, followed by transplantation into syngeneic recipients. Each mouse either received 5000 transduced myeloid progenitors or 2500 transduced HSCs. **(B)** Kaplan-Meier survival curve for recipients transplanted with BCR-ABL/GFP or BCR-ABL/GFP-CRE transduced HSCs or myeloid progenitors from Ctnnb1$^{Fl/+}$ donor, described in (A). Number of animals in each arm is listed. Most of animals developed B-ALL, except two mice developed CML as indicated.

REFERENCES

Abelson, H.T., and Rabstein, L.S. (1970). Lymphosarcoma: virus-induced thymic-independent disease in mice. Cancer Res *30*, 2213-2222.

Abrahamsson, A.E., Geron, I., Gotlib, J., Dao, K.H., Barroga, C.F., Newton, I.G., Giles, F.J., Durocher, J., Creusot, R.S., Karimi, M., *et al.* (2009). Glycogen synthase kinase 3beta missplicing contributes to leukemia stem cell generation. Proc Natl Acad Sci U S A *106*, 3925-3929.

Adli, M., Merkhofer, E., Cogswell, P., and Baldwin, A.S. (2010). IKKalpha and IKKbeta each function to regulate NF-kappaB activation in the TNF-induced/canonical pathway. PLoS One *5*, e9428.

Adolfsson, J., Mansson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C.T., Bryder, D., Yang, L., Borge, O.J., Thoren, L.A., *et al.* (2005). Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell *121*, 295-306.

Ahuja, H., Bar-Eli, M., Advani, S.H., Benchimol, S., and Cline, M.J. (1989). Alterations in the p53 gene and the clonal evolution of the blast crisis of chronic myelocytic leukemia. Proc Natl Acad Sci U S A *86*, 6783-6787.

Akashi, K., Traver, D., Miyamoto, T., and Weissman, I.L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature *404*, 193-197.

Albanese, C., Wu, K., D'Amico, M., Jarrett, C., Joyce, D., Hughes, J., Hulit, J., Sakamaki, T., Fu, M., Ben-Ze'ev, A., *et al.* (2003). IKKalpha regulates mitogenic signaling through transcriptional induction of cyclin D1 via Tcf. Mol Biol Cell *14*, 585-599.

Baba, Y., Garrett, K.P., and Kincade, P.W. (2005). Constitutively active beta-catenin confers multilineage differentiation potential on lymphoid and myeloid progenitors. Immunity *23*, 599-609.

Baba, Y., Yokota, T., Spits, H., Garrett, K.P., Hayashi, S., and Kincade, P.W. (2006). Constitutively active beta-catenin promotes expansion of multipotent hematopoietic progenitors in culture. J Immunol *177*, 2294-2303.

Barth, A.I., Stewart, D.B., and Nelson, W.J. (1999). T cell factor-activated transcription is not sufficient to induce anchorage-independent growth of epithelial cells expressing mutant beta-catenin. Proc Natl Acad Sci U S A *96*, 4947-4952.

Basseres, D.S., and Baldwin, A.S. (2006). Nuclear factor-kappaB and inhibitor of kappaB kinase pathways in oncogenic initiation and progression. Oncogene *25*, 6817-6830.

Baumgartner, B., Weber, M., Quirling, M., Fischer, C., Page, S., Adam, M., Von Schilling, C., Waterhouse, C., Schmid, C., Neumeier, D., *et al.* (2002). Increased IkappaB kinase activity is associated with activated NF-kappaB in acute myeloid blasts. Leukemia *16*, 2062-2071.

Beg, A.A., Sha, W.C., Bronson, R.T., Ghosh, S., and Baltimore, D. (1995). Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. Nature *376*, 167-170.

Bidere, N., Ngo, V.N., Lee, J., Collins, C., Zheng, L., Wan, F., Davis, R.E., Lenz, G., Anderson, D.E., Arnoult, D., *et al.* (2009). Casein kinase 1alpha governs antigen-receptor-induced NF-kappaB activation and human lymphoma cell survival. Nature *458*, 92-96.

Bonizzi, G., and Karin, M. (2004). The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol *25*, 280-288.

Brasher, B.B., and Van Etten, R.A. (2000). c-Abl has high intrinsic tyrosine kinase activity that is stimulated by mutation of the Src homology 3 domain and by autophosphorylation at two distinct regulatory tyrosines. J Biol Chem *275*, 35631-35637.

Buchdunger, E., Cioffi, C.L., Law, N., Stover, D., Ohno-Jones, S., Druker, B.J., and Lydon, N.B. (2000). Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-derived growth factor receptors. J Pharmacol Exp Ther *295*, 139-145.

Castor, A., Nilsson, L., Astrand-Grundstrom, I., Buitenhuis, M., Ramirez, C., Anderson, K., Strombeck, B., Garwicz, S., Bekassy, A.N., Schmiegelow, K., *et al.* (2005). Distinct patterns of hematopoietic stem cell involvement in acute lymphoblastic leukemia. Nat Med *11*, 630-637.

Chan, W.W., Wise, S.C., Kaufman, M.D., Ahn, Y.M., Ensinger, C.L., Haack, T., Hood, M.M., Jones, J., Lord, J.W., Lu, W.P., *et al.* (2011). Conformational control inhibition of the BCR-ABL1 tyrosine kinase, including the gatekeeper T315I mutant, by the switch-control inhibitor DCC-2036. Cancer Cell *19*, 556-568.

Chen, F., Demers, L.M., Vallyathan, V., Lu, Y., Castranova, V., and Shi, X. (1999). Involvement of 5'-flanking kappaB-like sites within bcl-x gene in silica-induced Bcl-x expression. J Biol Chem *274*, 35591-35595.

Chen, Y., Hu, Y., Zhang, H., Peng, C., and Li, S. (2009). Loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia. Nat Genet *41*, 783-792.

Chen, Y., Peng, C., Sullivan, C., Li, D., and Li, S. (2010). Critical molecular pathways in cancer stem cells of chronic myeloid leukemia. Leukemia *24*, 1545-1554.

Cilloni, D., Messa, F., Arruga, F., Defilippi, I., Morotti, A., Messa, E., Carturan, S., Giugliano, E., Pautasso, M., Bracco, E., *et al.* (2006). The NF-kappaB pathway blockade by the IKK inhibitor PS1145 can overcome imatinib resistance. Leukemia *20*, 61-67.

Cobas, M., Wilson, A., Ernst, B., Mancini, S.J., MacDonald, H.R., Kemler, R., and Radtke, F. (2004). Beta-catenin is dispensable for hematopoiesis and lymphopoiesis. J Exp Med *199*, 221-229.

Coluccia, A.M., Vacca, A., Dunach, M., Mologni, L., Redaelli, S., Bustos, V.H., Benati, D., Pinna, L.A., and Gambacorti-Passerini, C. (2007). Bcr-Abl stabilizes beta-catenin in chronic myeloid leukemia through its tyrosine phosphorylation. Embo J *26*, 1456-1466.

Comb, W.C., Cogswell, P., Sitcheran, R., and Baldwin, A.S. (2011). IKK-dependent, NF-kappaB-independent control of autophagic gene expression. Oncogene *30*, 1727-1732.

Corbin, A.S., Agarwal, A., Loriaux, M., Cortes, J., Deininger, M.W., and Druker, B.J. (2011). Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. J Clin Invest *121*, 396-409.

Cortes, J.E., Talpaz, M., O'Brien, S., Faderl, S., Garcia-Manero, G., Ferrajoli, A., Verstovsek, S., Rios, M.B., Shan, J., and Kantarjian, H.M. (2006). Staging of chronic myeloid leukemia in the imatinib era: an evaluation of the World Health Organization proposal. Cancer *106*, 1306-1315.

Cortez, D., Reuther, G., and Pendergast, A.M. (1997). The Bcr-Abl tyrosine kinase activates mitogenic signaling pathways and stimulates G1-to-S phase transition in hematopoietic cells. Oncogene *15*, 2333-2342.

Cozzio, A., Passegue, E., Ayton, P.M., Karsunky, H., Cleary, M.L., and Weissman, I.L. (2003). Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. Genes Dev *17*, 3029-3035.

Daley, G.Q., and Baltimore, D. (1988). Transformation of an interleukin 3-dependent hematopoietic cell line by the chronic myelogenous leukemia-specific P210bcr/abl protein. Proc Natl Acad Sci U S A *85*, 9312-9316.

Daley, G.Q., Van Etten, R.A., and Baltimore, D. (1990). Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. Science *247*, 824-830.

Daley, G.Q., Van Etten, R.A., and Baltimore, D. (1991). Blast crisis in a murine model of chronic myelogenous leukemia. Proc Natl Acad Sci U S A *88*, 11335-11338.

Dameshek, W. (1951). Some speculations on the myeloproliferative syndromes. Blood *6*, 372-375.

Dash, A.B., Williams, I.R., Kutok, J.L., Tomasson, M.H., Anastasiadou, E., Lindahl, K., Li, S., Van Etten, R.A., Borrow, J., Housman, D., *et al.* (2002). A murine model of CML blast crisis induced by cooperation between BCR/ABL and NUP98/HOXA9. Proc Natl Acad Sci U S A *99*, 7622-7627.

de Klein, A., van Kessel, A.G., Grosveld, G., Bartram, C.R., Hagemeijer, A., Bootsma, D., Spurr, N.K., Heisterkamp, N., Groffen, J., and Stephenson, J.R. (1982). A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. Nature *300*, 765-767.

Dejardin, E. (2006). The alternative NF-kappaB pathway from biochemistry to biology: pitfalls and promises for future drug development. Biochem Pharmacol *72*, 1161-1179.

Dejardin, E., Bonizzi, G., Bellahcene, A., Castronovo, V., Merville, M.P., and Bours, V. (1995). Highly-expressed p100/p52 (NFKB2) sequesters other NF-kappa B-related proteins in the cytoplasm of human breast cancer cells. Oncogene *11*, 1835-1841.

Dejardin, E., Droin, N.M., Delhase, M., Haas, E., Cao, Y., Makris, C., Li, Z.W., Karin, M., Ware, C.F., and Green, D.R. (2002). The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways. Immunity *17*, 525-535.

Delhase, M., Hayakawa, M., Chen, Y., and Karin, M. (1999). Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation. Science *284*, 309-313.

Deng, J., Miller, S.A., Wang, H.Y., Xia, W., Wen, Y., Zhou, B.P., Li, Y., Lin, S.Y., and Hung, M.C. (2002). beta-catenin interacts with and inhibits NF-kappa B in human colon and breast cancer. Cancer Cell *2*, 323-334.

Diekmann, D., Brill, S., Garrett, M.D., Totty, N., Hsuan, J., Monfries, C., Hall, C., Lim, L., and Hall, A. (1991). Bcr encodes a GTPase-activating protein for p21rac. Nature *351*, 400-402.

Dierks, C., Beigi, R., Guo, G.R., Zirlik, K., Stegert, M.R., Manley, P., Trussell, C., Schmitt-Graeff, A., Landwerlin, K., Veelken, H., *et al.* (2008). Expansion of Bcr-Abl-positive leukemic stem cells is dependent on Hedgehog pathway activation. Cancer Cell *14*, 238-249.

Domen, J., Gandy, K.L., and Weissman, I.L. (1998). Systemic overexpression of BCL-2 in the hematopoietic system protects transgenic mice from the consequences of lethal irradiation. Blood *91*, 2272-2282.

Druker, B.J., Guilhot, F., O'Brien, S.G., Gathmann, I., Kantarjian, H., Gattermann, N., Deininger, M.W., Silver, R.T., Goldman, J.M., Stone, R.M., *et al.* (2006). Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. N Engl J Med *355*, 2408-2417.

Druker, B.J., Sawyers, C.L., Kantarjian, H., Resta, D.J., Reese, S.F., Ford, J.M., Capdeville, R., and Talpaz, M. (2001a). Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. N Engl J Med *344*, 1038-1042.

Druker, B.J., Talpaz, M., Resta, D.J., Peng, B., Buchdunger, E., Ford, J.M., Lydon, N.B., Kantarjian, H., Capdeville, R., Ohno-Jones, S., *et al.* (2001b). Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med *344*, 1031-1037.

Duncan, E.A., Goetz, C.A., Stein, S.J., Mayo, K.J., Skaggs, B.J., Ziegelbauer, K., Sawyers, C.L., and Baldwin, A.S. (2008). IkappaB kinase beta inhibition induces cell death in Imatinib-resistant and T315I Dasatinib-resistant BCR-ABL+ cells. Mol Cancer Ther *7*, 391-397.

Duyao, M.P., Buckler, A.J., and Sonenshein, G.E. (1990). Interaction of an NF-kappa B-like factor with a site upstream of the c-myc promoter. Proc Natl Acad Sci U S A *87*, 4727-4731.

Eisterer, W., Jiang, X., Christ, O., Glimm, H., Lee, K.H., Pang, E., Lambie, K., Shaw, G., Holyoake, T.L., Petzer, A.L., *et al.* (2005). Different subsets of primary chronic myeloid leukemia stem cells engraft immunodeficient mice and produce a model of the human disease. Leukemia *19*, 435-441.

Feller, S.M., Knudsen, B., and Hanafusa, H. (1994). c-Abl kinase regulates the protein binding activity of c-Crk. EMBO J *13*, 2341-2351.

Feuerhake, F., Kutok, J.L., Monti, S., Chen, W., LaCasce, A.S., Cattoretti, G., Kurtin, P., Pinkus, G.S., de Leval, L., Harris, N.L., *et al.* (2005). NFkappaB activity, function, and target-gene signatures in primary mediastinal large B-cell lymphoma and diffuse large B-cell lymphoma subtypes. Blood *106*, 1392-1399.

Fialkow, P.J., Gartler, S.M., and Yoshida, A. (1967). Clonal origin of chronic myelocytic leukemia in man. Proc Natl Acad Sci U S A *58*, 1468-1471.

Finer, M.H., Dull, T.J., Qin, L., Farson, D., and Roberts, M.R. (1994). kat: a high-efficiency retroviral transduction system for primary human T lymphocytes. Blood *83*, 43-50.

Gaiger, A., Henn, T., Horth, E., Geissler, K., Mitterbauer, G., Maier-Dobersberger, T., Greinix, H., Mannhalter, C., Haas, O.A., Lechner, K., *et al.* (1995). Increase of bcr-abl chimeric mRNA expression in tumor cells of patients with chronic myeloid leukemia precedes disease progression. Blood *86*, 2371-2378.

Gavrilescu, L., and Van Etten, R. (2008). Applications of Murine Retroviral Bone Marrow Transplantation Models for the Study of Human Myeloproliferative Disorders. . Current Protocols in Pharmacology *14*.

Gerondakis, S., Grumont, R., Gugasyan, R., Wong, L., Isomura, I., Ho, W., and Banerjee, A. (2006). Unravelling the complexities of the NF-kappaB signalling pathway using mouse knockout and transgenic models. Oncogene *25*, 6781-6799.

Gesbert, F., and Griffin, J.D. (2000). Bcr/Abl activates transcription of the Bcl-X gene through STAT5. Blood *96*, 2269-2276.

Gilmore, T.D. (2006). Introduction to NF-kappaB: players, pathways, perspectives. Oncogene *25*, 6680-6684.

Gishizky, M.L., and Witte, O.N. (1992). BCR/ABL enhances growth of multipotent progenitor cells but does not block their differentiation potential in vitro. Curr Top Microbiol Immunol *182*, 65-72.

Goldman, J.M., and Melo, J.V. (2003). Chronic myeloid leukemia--advances in biology and new approaches to treatment. N Engl J Med *349*, 1451-1464.

Gorre, M.E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P.N., and Sawyers, C.L. (2001). Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science *293*, 876-880.

Gounari, F., Aifantis, I., Khazaie, K., Hoeflinger, S., Harada, N., Taketo, M.M., and von Boehmer, H. (2001). Somatic activation of beta-catenin bypasses pre-TCR signaling and TCR selection in thymocyte development. Nat Immunol *2*, 863-869.

Graham, S.M., Jorgensen, H.G., Allan, E., Pearson, C., Alcorn, M.J., Richmond, L., and Holyoake, T.L. (2002). Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood *99*, 319-325.

Gratwohl, A., Hermans, J., Goldman, J.M., Arcese, W., Carreras, E., Devergie, A., Frassoni, F., Gahrton, G., Kolb, H.J., Niederwieser, D., *et al.* (1998). Risk assessment for patients with chronic myeloid leukaemia before allogeneic blood or marrow transplantation. Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation. Lancet *352*, 1087-1092.

Greten, F.R., Arkan, M.C., Bollrath, J., Hsu, L.C., Goode, J., Miething, C., Goktuna, S.I., Neuenhahn, M., Fierer, J., Paxian, S., *et al.* (2007). NF-kappaB is a negative regulator of IL-1beta secretion as revealed by genetic and pharmacological inhibition of IKKbeta. Cell *130*, 918-931.

Gringhuis, S.I., Garcia-Vallejo, J.J., van Het Hof, B., and van Dijk, W. (2005). Convergent actions of I kappa B kinase beta and protein kinase C delta modulate mRNA stability through phosphorylation of 14-3-3 beta complexed with tristetraprolin. Mol Cell Biol *25*, 6454-6463.

Groffen, J., Stephenson, J.R., Heisterkamp, N., de Klein, A., Bartram, C.R., and Grosveld, G. (1984). Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. Cell *36*, 93-99.

Guo, Z., Dose, M., Kovalovsky, D., Chang, R., O'Neil, J., Look, A.T., von Boehmer, H., Khazaie, K., and Gounari, F. (2007). Beta-catenin stabilization stalls the transition from double-positive to single-positive stage and predisposes thymocytes to malignant transformation. Blood *109*, 5463-5472.

Guzman, M.L., Neering, S.J., Upchurch, D., Grimes, B., Howard, D.S., Rizzieri, D.A., Luger, S.M., and Jordan, C.T. (2001). Nuclear factor-kappaB is constitutively activated in primitive human acute myelogenous leukemia cells. Blood *98*, 2301-2307.

Haferlach, T., Winkemann, M., Nickenig, C., Meeder, M., Ramm-Petersen, L., Schoch, R., Nickelsen, M., Weber-Matthiesen, K., Schlegelberger, B., Schoch, C., *et al.* (1997). Which compartments are involved in Philadelphia-chromosome positive chronic myeloid leukaemia? An answer at the single cell level by combining May-Grunwald-Giemsa staining and fluorescence in situ hybridization techniques. Br J Haematol *97*, 99-106.

Hamdane, M., David-Cordonnier, M.H., and D'Halluin, J.C. (1997). Activation of p65 NF-kappaB protein by p210BCR-ABL in a myeloid cell line (P210BCR-ABL activates p65 NF-kappaB). Oncogene *15*, 2267-2275.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Harada, N., Tamai, Y., Ishikawa, T., Sauer, B., Takaku, K., Oshima, M., and Taketo, M.M. (1999). Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. Embo J *18*, 5931-5942.

Hayden, M.S., and Ghosh, S. (2004). Signaling to NF-kappaB. Genes Dev *18*, 2195-2224.

Heaney, N.B., Pellicano, F., Zhang, B., Crawford, L., Chu, S., Kazmi, S.M., Allan, E.K., Jorgensen, H.G., Irvine, A.E., Bhatia, R., *et al.* (2010). Bortezomib induces apoptosis in primitive chronic myeloid leukemia cells including LTC-IC and NOD/SCID repopulating cells. Blood *115*, 2241-2250.

Heinrich, A.C., Pelanda, R., and Klingmuller, U. (2004). A mouse model for visualization and conditional mutations in the erythroid lineage. Blood *104*, 659-666.

Heisterkamp, N., Jenster, G., Kioussis, D., Pattengale, P.K., and Groffen, J. (1991). Human bcr-abl gene has a lethal effect on embryogenesis. Transgenic Res *1*, 45-53.

Heisterkamp, N., Jenster, G., ten Hoeve, J., Zovich, D., Pattengale, P.K., and Groffen, J. (1990). Acute leukaemia in bcr/abl transgenic mice. Nature *344*, 251-253.

Heisterkamp, N., Stephenson, J.R., Groffen, J., Hansen, P.F., de Klein, A., Bartram, C.R., and Grosveld, G. (1983). Localization of the c-ab1 oncogene adjacent to a translocation break point in chronic myelocytic leukaemia. Nature *306*, 239-242.

Hoeflich, K.P., Luo, J., Rubie, E.A., Tsao, M.S., Jin, O., and Woodgett, J.R. (2000). Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature *406*, 86-90.

Hoelbl, A., Schuster, C., Kovacic, B., Zhu, B., Wickre, M., Hoelzl, M.A., Fajmann, S., Grebien, F., Warsch, W., Stengl, G., *et al.* (2010). Stat5 is indispensable for the maintenance of bcr/abl-positive leukaemia. EMBO Mol Med *2*, 98-110.

Hoffmann, A., Natoli, G., and Ghosh, G. (2006). Transcriptional regulation via the NF-kappaB signaling module. Oncogene *25*, 6706-6716.

Holyoake, T., Jiang, X., Eaves, C., and Eaves, A. (1999). Isolation of a highly quiescent subpopulation of primitive leukemic cells in chronic myeloid leukemia. Blood *94*, 2056-2064.

Honda, H., Fujii, T., Takatoku, M., Mano, H., Witte, O.N., Yazaki, Y., and Hirai, H. (1995). Expression of p210bcr/abl by metallothionein promoter induced T-cell leukemia in transgenic mice. Blood *85*, 2853-2861.

Honda, H., Oda, H., Suzuki, T., Takahashi, T., Witte, O.N., Ozawa, K., Ishikawa, T., Yazaki, Y., and Hirai, H. (1998). Development of acute lymphoblastic leukemia and myeloproliferative disorder in transgenic mice expressing p210bcr/abl: a novel transgenic model for human Ph1-positive leukemias. Blood *91*, 2067-2075.

Horwitz, B.H., Scott, M.L., Cherry, S.R., Bronson, R.T., and Baltimore, D. (1997). Failure of lymphopoiesis after adoptive transfer of NF-kappaB-deficient fetal liver cells. Immunity *6*, 765-772.

Hsu, L.C., Enzler, T., Seita, J., Timmer, A.M., Lee, C.Y., Lai, T.Y., Yu, G.Y., Lai, L.C., Temkin, V., Sinzig, U., *et al.* (2011). IL-1beta-driven neutrophilia preserves antibacterial defense in the absence of the kinase IKKbeta. Nat Immunol *12*, 144-150.

Hu, M.C., Lee, D.F., Xia, W., Golfman, L.S., Ou-Yang, F., Yang, J.Y., Zou, Y., Bao, S., Hanada, N., Saso, H., *et al.* (2004a). IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell *117*, 225-237.

Hu, Y., Baud, V., Delhase, M., Zhang, P., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999). Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. Science *284*, 316-320.

Hu, Y., Chen, Y., Douglas, L., and Li, S. (2009). beta-Catenin is essential for survival of leukemic stem cells insensitive to kinase inhibition in mice with BCR-ABL-induced chronic myeloid leukemia. Leukemia *23*, 109-116.

Hu, Y., Liu, Y., Pelletier, S., Buchdunger, E., Warmuth, M., Fabbro, D., Hallek, M., Van Etten, R.A., and Li, S. (2004b). Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukemia but not chronic myeloid leukemia. Nat Genet *36*, 453-461.

Hu, Y., Swerdlow, S., Duffy, T.M., Weinmann, R., Lee, F.Y., and Li, S. (2006). Targeting multiple kinase pathways in leukemic progenitors and stem cells is essential for

improved treatment of Ph+ leukemia in mice. Proc Natl Acad Sci U S A *103*, 16870-16875.

Huettner, C.S., Zhang, P., Van Etten, R.A., and Tenen, D.G. (2000). Reversibility of acute B-cell leukaemia induced by BCR-ABL1. Nat Genet *24*, 57-60.

Huntly, B., and Gilliland, D. (2005). Leukaemia stem cells and the evolution of cancer-stem-cell research. Nature reivew of cancer *5*, 311.

Huntly, B.J., Shigematsu, H., Deguchi, K., Lee, B.H., Mizuno, S., Duclos, N., Rowan, R., Amaral, S., Curley, D., Williams, I.R., *et al.* (2004). MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. Cancer Cell *6*, 587-596.

Iacobucci, I., Lonetti, A., Paoloni, F., Papayannidis, C., Ferrari, A., Storlazzi, C.T., Vignetti, M., Cilloni, D., Messa, F., Guadagnuolo, V., *et al.* (2010). The PAX5 gene is frequently rearranged in BCR-ABL1-positive acute lymphoblastic leukemia but is not associated with outcome. A report on behalf of the GIMEMA Acute Leukemia Working Party. Haematologica *95*, 1683-1690.

Ilaria, R.L., Jr., and Van Etten, R.A. (1996). P210 and P190(BCR/ABL) induce the tyrosine phosphorylation and DNA binding activity of multiple specific STAT family members. J Biol Chem *271*, 31704-31710.

Jackson, P., and Baltimore, D. (1989). N-terminal mutations activate the leukemogenic potential of the myristoylated form of c-abl. EMBO J *8*, 449-456.

Jamieson, C., Ailles, L., Dylla, S., Muijtjens, M., Jones, C., Zehnder, J., Gotlib, J., Li, K., Manz, M., Keating, A., *et al.* (2004). Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. N Engl J Med *351*, 657-657.

Jiang, X., Stuible, M., Chalandon, Y., Li, A., Chan, W.Y., Eisterer, W., Krystal, G., Eaves, A., and Eaves, C. (2003). Evidence for a positive role of SHIP in the BCR-ABL-mediated transformation of primitive murine hematopoietic cells and in human chronic myeloid leukemia. Blood *102*, 2976-2984.

Kaisho, T., Takeda, K., Tsujimura, T., Kawai, T., Nomura, F., Terada, N., and Akira, S. (2001). IkappaB kinase alpha is essential for mature B cell development and function. J Exp Med *193*, 417-426.

Karin, M. (2006). Nuclear factor-kappaB in cancer development and progression. Nature *441*, 431-436.

Kelliher, M.A., McLaughlin, J., Witte, O.N., and Rosenberg, N. (1990). Induction of a chronic myelogenous leukemia-like syndrome in mice with v-abl and BCR/ABL. Proc Natl Acad Sci U S A *87*, 6649-6653.

Kharbanda, S., Pandey, P., Morris, P.L., Whang, Y., Xu, Y., Sawant, S., Zhu, L.J., Kumar, N., Yuan, Z.M., Weichselbaum, R., *et al.* (1998). Functional role for the c-Abl tyrosine kinase in meiosis I. Oncogene *16*, 1773-1777.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell *121*, 1109-1121.

Kim, J.H., Kim, B., Cai, L., Choi, H.J., Ohgi, K.A., Tran, C., Chen, C., Chung, C.H., Huber, O., Rose, D.W., *et al.* (2005). Transcriptional regulation of a metastasis suppressor gene by Tip60 and beta-catenin complexes. Nature *434*, 921-926.

Kirchner, D., Duyster, J., Ottmann, O., Schmid, R., Bergmann, L., and Munzert, G. (2003). Mechanisms of Bcr-Abl-mediated NF-kappaB/Rel activation. Exp Hematol *31*, 504-511.

Kirstetter, P., Anderson, K., Porse, B.T., Jacobsen, S.E., and Nerlov, C. (2006). Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. Nat Immunol *7*, 1048-1056.

Koch, U., Wilson, A., Cobas, M., Kemler, R., Macdonald, H.R., and Radtke, F. (2008). Simultaneous loss of beta- and gamma-catenin does not perturb hematopoiesis or lymphopoiesis. Blood *111*, 160-164.

Kondo, M., Wagers, A.J., Manz, M.G., Prohaska, S.S., Scherer, D.C., Beilhack, G.F., Shizuru, J.A., and Weissman, I.L. (2003). Biology of hematopoietic stem cells and progenitors: implications for clinical application. Annu Rev Immunol *21*, 759-806.

Koschmieder, S., Gottgens, B., Zhang, P., Iwasaki-Arai, J., Akashi, K., Kutok, J.L., Dayaram, T., Geary, K., Green, A.R., Tenen, D.G., *et al.* (2005). Inducible chronic phase of myeloid leukemia with expansion of hematopoietic stem cells in a transgenic model of BCR-ABL leukemogenesis. Blood *105*, 324-334.

Krause, D.S., Lazarides, K., von Andrian, U.H., and Van Etten, R.A. (2006). Requirement for CD44 in homing and engraftment of BCR-ABL-expressing leukemic stem cells. Nat Med *12*, 1175-1180.

Kuhn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995). Inducible gene targeting in mice. Science *269*, 1427-1429.

Lamberti, C., Lin, K.M., Yamamoto, Y., Verma, U., Verma, I.M., Byers, S., and Gaynor, R.B. (2001). Regulation of beta-catenin function by the IkappaB kinases. J Biol Chem *276*, 42276-42286.

Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M.A., and Dick, J.E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature *367*, 645-648.

Li, S., Ilaria, R.L., Jr., Million, R.P., Daley, G.Q., and Van Etten, R.A. (1999a). The P190, P210, and P230 forms of the BCR/ABL oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have different lymphoid leukemogenic activity. J Exp Med *189*, 1399-1412.

Li, Z.W., Chu, W., Hu, Y., Delhase, M., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999b). The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. J Exp Med *189*, 1839-1845.

Li, Z.W., Omori, S.A., Labuda, T., Karin, M., and Rickert, R.C. (2003). IKK beta is required for peripheral B cell survival and proliferation. J Immunol *170*, 4630-4637.

Lounnas, N., Frelin, C., Gonthier, N., Colosetti, P., Sirvent, A., Cassuto, J.P., Berthier, F., Sirvent, N., Rousselot, P., Dreano, M., *et al.* (2009). NF-kappaB inhibition triggers death of imatinib-sensitive and imatinib-resistant chronic myeloid leukemia cells including T315I Bcr-Abl mutants. Int J Cancer *125*, 308-317.

Lugo, T.G., Pendergast, A.M., Muller, A.J., and Witte, O.N. (1990). Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. Science *247*, 1079-1082.

Mahon, F.X., Rea, D., Guilhot, J., Guilhot, F., Huguet, F., Nicolini, F., Legros, L., Charbonnier, A., Guerci, A., Varet, B., *et al.* (2010). Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. Lancet Oncol *11*, 1029-1035.

Malhotra, S., and Kincade, P.W. (2009). Wnt-related molecules and signaling pathway equilibrium in hematopoiesis. Cell Stem Cell *4*, 27-36.

Maru, Y., and Witte, O.N. (1991). The BCR gene encodes a novel serine/threonine kinase activity within a single exon. Cell *67*, 459-468.

McLaughlin, J., Chianese, E., and Witte, O.N. (1989). Alternative forms of the BCR-ABL oncogene have quantitatively different potencies for stimulation of immature lymphoid cells. Mol Cell Biol *9*, 1866-1874.

McWhirter, J.R., Galasso, D.L., and Wang, J.Y. (1993). A coiled-coil oligomerization domain of Bcr is essential for the transforming function of Bcr-Abl oncoproteins. Mol Cell Biol *13*, 7587-7595.

Mercurio, F., Zhu, H., Murray, B.W., Shevchenko, A., Bennett, B.L., Li, J., Young, D.B., Barbosa, M., Mann, M., Manning, A., *et al.* (1997). IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. Science *278*, 860-866.

Mihailovic, T., Marx, M., Auer, A., Van Lint, J., Schmid, M., Weber, C., and Seufferlein, T. (2004). Protein kinase D2 mediates activation of nuclear factor kappaB by Bcr-Abl in Bcr-Abl+ human myeloid leukemia cells. Cancer Res *64*, 8939-8944.

Million, R.P., and Van Etten, R.A. (2000). The Grb2 binding site is required for the induction of chronic myeloid leukemia-like disease in mice by the Bcr/Abl tyrosine kinase. Blood *96*, 664-670.

Minami, Y., Stuart, S.A., Ikawa, T., Jiang, Y., Banno, A., Hunton, I.C., Young, D.J., Naoe, T., Murre, C., Jamieson, C.H., *et al.* (2008). BCR-ABL-transformed GMP as myeloid leukemic stem cells. Proc Natl Acad Sci U S A *105*, 17967-17972.

Morotti, A., Cilloni, D., Pautasso, M., Messa, F., Arruga, F., Defilippi, I., Carturan, S., Catalano, R., Rosso, V., Chiarenza, A., *et al.* (2006). NF-kB inhibition as a strategy to enhance etoposide-induced apoptosis in K562 cell line. Am J Hematol *81*, 938-945.

Mullighan, C.G., Miller, C.B., Radtke, I., Phillips, L.A., Dalton, J., Ma, J., White, D., Hughes, T.P., Le Beau, M.M., Pui, C.H., *et al.* (2008). BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. Nature *453*, 110-114.

Munzert, G., Kirchner, D., Ottmann, O., Bergmann, L., and Schmid, R.M. (2004). Constitutive NF-kappab/Rel activation in philadelphia chromosome positive (Ph+) acute lymphoblastic leukemia (ALL). Leuk Lymphoma *45*, 1181-1184.

Na Nakorn, T., Traver, D., Weissman, I.L., and Akashi, K. (2002). Myeloerythroid-restricted progenitors are sufficient to confer radioprotection and provide the majority of day 8 CFU-S. J Clin Invest *109*, 1579-1585.

Naka, K., Hoshii, T., Muraguchi, T., Tadokoro, Y., Ooshio, T., Kondo, Y., Nakao, S., Motoyama, N., and Hirao, A. (2010). TGF-beta-FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. Nature *463*, 676-680.

Nakano, H., Shindo, M., Sakon, S., Nishinaka, S., Mihara, M., Yagita, H., and Okumura, K. (1998). Differential regulation of IkappaB kinase alpha and beta by two upstream kinases, NF-kappaB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. Proc Natl Acad Sci U S A *95*, 3537-3542.

Nawata, R., Yujiri, T., Nakamura, Y., Ariyoshi, K., Takahashi, T., Sato, Y., Oka, Y., and Tanizawa, Y. (2003). MEK kinase 1 mediates the antiapoptotic effect of the Bcr-Abl oncogene through NF-kappaB activation. Oncogene *22*, 7774-7780.

Nowell, P.C., and Hungerford, D.A. (1960). Chromosome studies on normal and leukemic human leukocytes. J Natl Cancer Inst *25*, 85-109.

Nowicki, M.O., Falinski, R., Koptyra, M., Slupianek, A., Stoklosa, T., Gloc, E., Nieborowska-Skorska, M., Blasiak, J., and Skorski, T. (2004). BCR/ABL oncogenic kinase promotes unfaithful repair of the reactive oxygen species-dependent DNA double-strand breaks. Blood *104*, 3746-3753.

Ohno, R. (2010). Changing paradigm of the treatment of Philadelphia chromosome-positive acute lymphoblastic leukemia. Curr Hematol Malig Rep *5*, 213-221.

Okimoto, R.A., and Van Etten, R.A. (2011). Navigating the road toward optimal initial therapy for chronic myeloid leukemia. Curr Opin Hematol *18*, 89-97.

Ottmann, O.G., Druker, B.J., Sawyers, C.L., Goldman, J.M., Reiffers, J., Silver, R.T., Tura, S., Fischer, T., Deininger, M.W., Schiffer, C.A., *et al.* (2002). A phase 2 study of imatinib in patients with relapsed or refractory Philadelphia chromosome-positive acute lymphoid leukemias. Blood *100*, 1965-1971.

Pardal, R., Clarke, M.F., and Morrison, S.J. (2003). Applying the principles of stem-cell biology to cancer. Nature reivew of cancer *3*, 895-902.

Pasparakis, M., Schmidt-Supprian, M., and Rajewsky, K. (2002). IkappaB kinase signaling is essential for maintenance of mature B cells. J Exp Med *196*, 743-752.

Pear, W.S., Miller, J.P., Xu, L., Pui, J.C., Soffer, B., Quackenbush, R.C., Pendergast, A.M., Bronson, R., Aster, J.C., Scott, M.L., *et al.* (1998). Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. Blood *92*, 3780-3792.

Pendergast, A.M., Gishizky, M.L., Havlik, M.H., and Witte, O.N. (1993a). SH1 domain autophosphorylation of P210 BCR/ABL is required for transformation but not growth factor independence. Mol Cell Biol *13*, 1728-1736.

Pendergast, A.M., Muller, A.J., Havlik, M.H., Clark, R., McCormick, F., and Witte, O.N. (1991a). Evidence for regulation of the human ABL tyrosine kinase by a cellular inhibitor. Proc Natl Acad Sci U S A *88*, 5927-5931.

Pendergast, A.M., Muller, A.J., Havlik, M.H., Maru, Y., and Witte, O.N. (1991b). BCR sequences essential for transformation by the BCR-ABL oncogene bind to the ABL SH2 regulatory domain in a non-phosphotyrosine-dependent manner. Cell *66*, 161-171.

Pendergast, A.M., Quilliam, L.A., Cripe, L.D., Bassing, C.H., Dai, Z., Li, N., Batzer, A., Rabun, K.M., Der, C.J., Schlessinger, J., *et al.* (1993b). BCR-ABL-induced oncogenesis is mediated by direct interaction with the SH2 domain of the GRB-2 adaptor protein. Cell *75*, 175-185.

Peng, C., Chen, Y., Yang, Z., Zhang, H., Osterby, L., Rosmarin, A.G., and Li, S. (2010). PTEN is a tumor suppressor in CML stem cells and BCR-ABL-induced leukemias in mice. Blood *115*, 626-635.

Perkins, N.D. (2007). Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat Rev Mol Cell Biol *8*, 49-62.

Perrotti, D., Cesi, V., Trotta, R., Guerzoni, C., Santilli, G., Campbell, K., Iervolino, A., Condorelli, F., Gambacorti-Passerini, C., Caligiuri, M.A., *et al.* (2002). BCR-ABL suppresses C/EBPalpha expression through inhibitory action of hnRNP E2. Nat Genet *30*, 48-58.

Perrotti, D., Jamieson, C., Goldman, J., and Skorski, T. (2010). Chronic myeloid leukemia: mechanisms of blastic transformation. J Clin Invest *120*, 2254-2264.

Plattner, R., Kadlec, L., DeMali, K.A., Kazlauskas, A., and Pendergast, A.M. (1999). c-Abl is activated by growth factors and Src family kinases and has a role in the cellular response to PDGF. Genes Dev *13*, 2400-2411.

Pluk, H., Dorey, K., and Superti-Furga, G. (2002). Autoinhibition of c-Abl. Cell *108*, 247-259.

Prendes, M., Zheng, Y., and Beg, A.A. (2003). Regulation of developing B cell survival by RelA-containing NF-kappa B complexes. J Immunol *171*, 3963-3969.

Preston, D.L., Kusumi, S., Tomonaga, M., Izumi, S., Ron, E., Kuramoto, A., Kamada, N., Dohy, H., Matsuo, T., Matsui, T., *et al.* (1994). Cancer incidence in atomic bomb survivors. Part III. Leukemia, lymphoma and multiple myeloma, 1950-1987. Radiat Res *137*, S68-97.

Quintas-Cardama, A., and Cortes, J. (2009). Molecular biology of bcr-abl1-positive chronic myeloid leukemia. Blood *113*, 1619-1630.

Radich, J.P. (2011). Measuring response to BCR-ABL inhibitors in chronic myeloid leukemia. Cancer.

Reuther, J.Y., Reuther, G.W., Cortez, D., Pendergast, A.M., and Jr., A.S.B. (1998). A requirement for NF-kabaB activation in BCR-ABL-mediated transformation. Genes and Dev *12*, 968-981.

Reya, T., Duncan, A., Ailles, L., Domen, J., Scherer, D., Willert, K., Hintz, L., Nusse, R., and IL., W. (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature *423*, 409-414.

Reya, T., Morrison, S.J., Clarke, M.F., and Weissman, I.L. (2001). Stem cells, cancer, and cancer stem cells. Nature *414*, 105-111.

Rosenberg, N., and Baltimore, D. (1976). A quantitative assay for transformation of bone marrow cells by Abelson murine leukemia virus. J Exp Med *143*, 1453-1463.

Roumiantsev, S., de Aos, I.E., Varticovski, L., Ilaria, R.L., and Van Etten, R.A. (2001). The src homology 2 domain of Bcr/Abl is required for efficient induction of chronic myeloid leukemia-like disease in mice but not for lymphoid leukemogenesis or activation of phosphatidylinositol 3-kinase. Blood *97*, 4-13.

Rowley, J.D. (1973). Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature *243*, 290-293.

Sanchez-Garcia, I., and Grutz, G. (1995). Tumorigenic activity of the BCR-ABL oncogenes is mediated by BCL2. Proc Natl Acad Sci U S A *92*, 5287-5291.

Sattler, M., Mohi, M.G., Pride, Y.B., Quinnan, L.R., Malouf, N.A., Podar, K., Gesbert, F., Iwasaki, H., Li, S., Van Etten, R.A., *et al.* (2002). Critical role for Gab2 in transformation by BCR/ABL. Cancer Cell *1*, 479-492.

Savage, D.G., Szydlo, R.M., and Goldman, J.M. (1997). Clinical features at diagnosis in 430 patients with chronic myeloid leukaemia seen at a referral centre over a 16-year period. Br J Haematol *96*, 111-116.

Sawyers, C.L. (1999). Chronic myeloid leukemia. N Engl J Med *340*, 1330-1340.

Sawyers, C.L., Callahan, W., and Witte, O.N. (1992). Dominant negative MYC blocks transformation by ABL oncogenes. Cell *70*, 901-910.

Sawyers, C.L., Hochhaus, A., Feldman, E., Goldman, J.M., Miller, C.B., Ottmann, O.G., Schiffer, C.A., Talpaz, M., Guilhot, F., Deininger, M.W., *et al.* (2002). Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. Blood *99*, 3530-3539.

Sawyers, C.L., McLaughlin, J., and Witte, O.N. (1995). Genetic requirement for Ras in the transformation of fibroblasts and hematopoietic cells by the Bcr-Abl oncogene. J Exp Med *181*, 307-313.

Scheller, M., Huelsken, J., Rosenbauer, F., Taketo, M.M., Birchmeier, W., Tenen, D.G., and Leutz, A. (2006). Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. Nat Immunol *7*, 1037-1047.

Schwartzberg, P.L., Stall, A.M., Hardin, J.D., Bowdish, K.S., Humaran, T., Boast, S., Harbison, M.L., Robertson, E.J., and Goff, S.P. (1991). Mice homozygous for the ablm1 mutation show poor viability and depletion of selected B and T cell populations. Cell *65*, 1165-1175.

Sen, R., and Baltimore, D. (1986). Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell *46*, 705-716.

Sha, W.C., Liou, H.C., Tuomanen, E.I., and Baltimore, D. (1995). Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. Cell *80*, 321-330.

Shah, N.P., Tran, C., Lee, F.Y., Chen, P., Norris, D., and Sawyers, C.L. (2004). Overriding imatinib resistance with a novel ABL kinase inhibitor. Science *305*, 399-401.

Sherbenou, D.W., and Druker, B.J. (2007). Applying the discovery of the Philadelphia chromosome. J Clin Invest *117*, 2067-2074.

Shtivelman, E., Lifshitz, B., Gale, R.P., and Canaani, E. (1985). Fused transcript of abl and bcr genes in chronic myelogenous leukaemia. Nature *315*, 550-554.

Shultz, L.D., Lyons, B.L., Burzenski, L.M., Gott, B., Chen, X., Chaleff, S., Kotb, M., Gillies, S.D., King, M., Mangada, J., *et al.* (2005). Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J Immunol *174*, 6477-6489.

Sill, H., Goldman, J.M., and Cross, N.C. (1995). Homozygous deletions of the p16 tumor-suppressor gene are associated with lymphoid transformation of chronic myeloid leukemia. Blood *85*, 2013-2016.

Silver, D.P., and Livingston, D.M. (2001). Self-excising retroviral vectors encoding the Cre recombinase overcome Cre-mediated cellular toxicity. Mol Cell *8*, 233-243.

Skorski, T., Bellacosa, A., Nieborowska-Skorska, M., Majewski, M., Martinez, R., Choi, J.K., Trotta, R., Wlodarski, P., Perrotti, D., Chan, T.O., *et al.* (1997). Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt-dependent pathway. EMBO J *16*, 6151-6161.

Skorski, T., Kanakaraj, P., Nieborowska-Skorska, M., Ratajczak, M.Z., Wen, S.C., Zon, G., Gewirtz, A.M., Perussia, B., and Calabretta, B. (1995). Phosphatidylinositol-3 kinase activity is regulated by BCR/ABL and is required for the growth of Philadelphia chromosome-positive cells. Blood *86*, 726-736.

Slupianek, A., Nowicki, M.O., Koptyra, M., and Skorski, T. (2006). BCR/ABL modifies the kinetics and fidelity of DNA double-strand breaks repair in hematopoietic cells. DNA Repair (Amst) *5*, 243-250.

Smith, K.M., Yacobi, R., and Van Etten, R.A. (2003). Autoinhibition of BCR-ABL through its SH3 domain. Mol Cell *12*, 27-37.

Spangrude, G.J., and Brooks, D.M. (1993). Mouse strain variability in the expression of the hematopoietic stem cell antigen Ly-6A/E by bone marrow cells. Blood *82*, 3327-3332.

Stam, K., Heisterkamp, N., Grosveld, G., de Klein, A., Verma, R.S., Coleman, M., Dosik, H., and Groffen, J. (1985). Evidence of a new chimeric bcr/c-abl mRNA in patients with chronic myelocytic leukemia and the Philadelphia chromosome. N Engl J Med *313*, 1429-1433.

Stoklosa, T., Poplawski, T., Koptyra, M., Nieborowska-Skorska, M., Basak, G., Slupianek, A., Rayevskaya, M., Seferynska, I., Herrera, L., Blasiak, J., *et al.* (2008). BCR/ABL inhibits mismatch repair to protect from apoptosis and induce point mutations. Cancer Res *68*, 2576-2580.

Suh, J., Payvandi, F., Edelstein, L.C., Amenta, P.S., Zong, W.X., Gelinas, C., and Rabson, A.B. (2002). Mechanisms of constitutive NF-kappaB activation in human prostate cancer cells. Prostate *52*, 183-200.

Talpaz, M., McCredie, K.B., Mavligit, G.M., and Gutterman, J.U. (1983). Leukocyte interferon-induced myeloid cytoreduction in chronic myelogenous leukemia. Blood *62*, 689-692.

Tanaka, M., Fuentes, M.E., Yamaguchi, K., Durnin, M.H., Dalrymple, S.A., Hardy, K.L., and Goeddel, D.V. (1999). Embryonic lethality, liver degeneration, and impaired NF-kappa B activation in IKK-beta-deficient mice. Immunity *10*, 421-429.

Tybulewicz, V.L., Crawford, C.E., Jackson, P.K., Bronson, R.T., and Mulligan, R.C. (1991). Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. Cell *65*, 1153-1163.

Van Etten, R.A. (1999). Cycling, stressed-out and nervous: cellular functions of c-Abl. Trends Cell Biol *9*, 179-186.

Van Etten, R.A. (2002). Studying the pathogensisof BCR-ABL+ leukemia in mice. Oncogene *21*, 8643-8651.

Van Etten, R.A. (2004). Mechanisms of transformation by the BCR-ABL oncogene: new perspectives in the post-imatinib era. Leuk Res *28 Suppl 1*, S21-28.

Van Etten, R.A., Jackson, P., and Baltimore, D. (1989). The mouse type IV c-abl gene product is a nuclear protein, and activation of transforming ability is associated with cytoplasmic localization. Cell *58*, 669-678.

Van Etten, R.A., Jackson, P.K., Baltimore, D., Sanders, M.C., Matsudaira, P.T., and Janmey, P.A. (1994). The COOH terminus of the c-Abl tyrosine kinase contains distinct F- and G-actin binding domains with bundling activity. J Cell Biol *124*, 325-340.

Van Etten, R.A., Koschmieder, S., Delhommeau, F., Perrotti, D., Holyoake, T., Pardanani, A., Mesa, R., Green, T., Ibrahim, A.R., Mughal, T., *et al.* (2011). The Ph-positive and Ph-negative myeloproliferative neoplasms: some topical pre-clinical and clinical issues. Haematologica *96*, 590-601.

Vignetti, M., Fazi, P., Cimino, G., Martinelli, G., Di Raimondo, F., Ferrara, F., Meloni, G., Ambrosetti, A., Quarta, G., Pagano, L., *et al.* (2007). Imatinib plus steroids induces complete remissions and prolonged survival in elderly Philadelphia chromosome-positive patients with acute lymphoblastic leukemia without additional chemotherapy: results of the Gruppo Italiano Malattie Ematologiche dell'Adulto (GIMEMA) LAL0201-B protocol. Blood *109*, 3676-3678.

Walz, C., Lazarides, K., Patel, N., Hennighausen, L., Zaleskas, V.M., and Van Etten, R.A. (2009). Essential Role for Stat5a/b in Myeloproliferative Neoplasms Induced by BCR-ABL1 and Jak2 V617F. BLood *114*, 312a.

Wang, J.C., Lapidot, T., Cashman, J.D., Doedens, M., Addy, L., Sutherland, D.R., Nayar, R., Laraya, P., Minden, M., Keating, A., *et al.* (1998). High level engraftment of NOD/SCID mice by primitive normal and leukemic hematopoietic cells from patients with chronic myeloid leukemia in chronic phase. Blood *91*, 2406-2414.

Wang, X., and Sonenshein, G.E. (2005). Induction of the RelB NF-kappaB subunit by the cytomegalovirus IE1 protein is mediated via Jun kinase and c-Jun/Fra-2 AP-1 complexes. J Virol *79*, 95-105.

Wang, Y., Krivtsov, A.V., Sinha, A.U., North, T.E., Goessling, W., Feng, Z., Zon, L.I., and Armstrong, S.A. (2010). The Wnt/beta-catenin pathway is required for the development of leukemia stem cells in AML. Science *327*, 1650-1653.

Warren, D., Griffin, D.S., Mainville, C., and Rosenberg, N. (2003). The extreme carboxyl terminus of v-Abl is required for lymphoid cell transformation by Abelson virus. J Virol *77*, 4617-4625.

Weisberg, E., Manley, P.W., Breitenstein, W., Bruggen, J., Cowan-Jacob, S.W., Ray, A., Huntly, B., Fabbro, D., Fendrich, G., Hall-Meyers, E., *et al.* (2005). Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. Cancer Cell *7*, 129-141.

Wen, S.T., and Van Etten, R.A. (1997). The PAG gene product, a stress-induced protein with antioxidant properties, is an Abl SH3-binding protein and a physiological inhibitor of c-Abl tyrosine kinase activity. Genes Dev *11*, 2456-2467.

Whang, J., Frei, E., 3rd, Tjio, J.H., Carbone, P.P., and Brecher, G. (1963). The Distribution of the Philadelphia Chromosome in Patients with Chronic Myelogenous Leukemia. Blood *22*, 664-673.

Wong, S., and Witte, O.N. (2004). The BCR-ABL story: bench to bedside and back. Annu Rev Immunol *22*, 247-306.

Zhao, C., Blum, J., Chen, A., Kwon, H.Y., Jung, S.H., Cook, J.M., Lagoo, A., and Reya, T. (2007). Loss of beta-catenin impairs the renewal of normal and CML stem cells in vivo. Cancer Cell *12*, 528-541.

Zhao, C., Chen, A., Jamieson, C.H., Fereshteh, M., Abrahamsson, A., Blum, J., Kwon, H.Y., Kim, J., Chute, J.P., Rizzieri, D., *et al.* (2009). Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. Nature *458*, 776-779.