“Cooperation of *Setbp1* with *BCR/ABL* in Development of CML Myeloid Blast Crisis”

By

[Vijay Negi]

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Name of Candidate: Vijay Negi
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DISSERTATION AND ABSTRACT APPROVED:

DATE:

[redacted] 9-16-16
Dr. Chen-Zen Giam
DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY
Committee Chairperson

[redacted] 9-16-16
Dr. Yang Du
DEPARTMENT OF PEDIATRICS
Dissertation Advisor

[redacted] 9-9-2016
Dr. Mary Lou Cutler
DEPARTMENT OF PATHOLOGY
Committee Member

[redacted] 9-9-2016
Dr. Saibal Dey
DEPARTMENT OF BIOCHEMISTRY & MOLECULAR BIOLOGY
Committee Member

[redacted] 9-9-2016
Dr. Jonathan R. Keller
NATIONAL CANCER INSTITUTE
Committee Member

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DEDICATION

I dedicate this dissertation to my entire family in India, and my wife, Mamta, for being a source of inspiration throughout my life.

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Vijay Negi
Molecular and Cell Biology (MCB) Graduate Program Department of Pediatrics
Uniformed Services University Date 05-09-2017

ABSTRACT

Mechanisms underlying the progression of Chronic Myeloid Leukemia (CML), primarily induced by *BCR/ABL* translocation, from chronic phase to blast crisis are poorly understood. Our laboratory has previously shown that *SETBP1* activation contributes to this progression by conferring unlimited self-renewal capability to granulocyte macrophage progenitors (GMPs). Here I show that overexpression of *Hoxa9* or *Hoxa10* alone, both transcriptional target of Setbp1 is able to promote self-renewal of primary myeloid progenitors *in vitro* in the presence of stem cell factor (SCF) and interleukin-3 (IL-3). In addition, similar to *Setbp1*, *Hoxa9* or *Hoxa10* is able to cooperate with *BCR/ABL* to induce development of aggressive leukemias which resemble CML myeloid blast crisis from GMPs. I further identify *Myb* as a critical downstream target of Hoxa9 and Hoxa10, as *Myb* knockdown significantly reduced colony-forming potential of myeloid progenitors immortalized by *Hoxa9* and *Hoxa10*. Interestingly, ectopic expression of *Myb* efficiently immortalized primary myeloid progenitors in culture and was also capable of cooperating with BCR/ABL to induce leukemic transformation of GMPs in mice. These results suggest that activation of *Myb* is a critical downstream event of *Setbp1/Hoxa9/Hoxa10* activation in their cooperation with *BCR/ABL* to induce CML blast crisis transformation. Therefore, *Myb* may represent a promising therapeutic target for treating CML blast crisis.

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LIST OF ABBREVIATIONS

4-OHT: 4-hydroxytamoxifen

5-FU: 5-fluorouracil

5'RACE: 5' rapid amplification of cDNA ends

ALL: Acute Lymphocytic Leukemia

AML: Acute Myelogenous Leukemia

APC: Adenomatous Polyposis Coli Gene Product

Arhgap24: Rho GTPase Activating Protein 24

C57BL/6: C57 Black 6

CHIP: Chromatin Immunoprecipitation

CK1: Casein Kinase 1

CLL: Chronic Lymphocytic Leukemia

CLPs: Common Lymphoid Progenitors

CML: Chronic Myeloid Leukemia

CMPs: Common Myeloid Progenitors

DBD: DNA Binding Domain

GMPs: Granulocyte Macrophage Progenitors

GSK3: Glycogen Synthase Kinase 3

HOX: Homeobox

Hoxa9: Homeobox protein Hox-A9

Hoxa10: Homeobox protein Hox-A10

HSCs: Hematopoietic Stem Cells

HTH: Helix-Turn-Helix

LEF: Lymphoid Enhancer Factor

LMPP: Lymphoid Primed Multi Potent Progenitors

IL3: Interleukin 3

IL6: Interleukin 6

LICs: Leukemia-initiating Cells

Ltb4r1: Leukotriene B4 receptor 1

LSC: Leukemic Stem Cells

MEF2: Monocyte Enhancer Factor

MEPs: Megakaryocyte/Erythrocyte Progenitors

MLL: Mixed Lineage Leukemia

MPPs: Multi Potent Progenitors

MSCV: Murine Stem Cell Virus

MSI2: Musashi 2

NEO: Neomycin

NRD: Negative Regulatory Domain

PGK: Phosphoglycerate Kinase

PP2A: Protein Phosphatase Type 2A

PURO: Puromycin

RBCs: Red Blood Cells

SCF: Stem Cell Factor

SET: SET Nuclear Proto-Oncogene

Setbp1: SET Binding Protein 1

TAD: Trans-Activation Domain

TCF: T-Cell Factor

TKI: Tyrosine Kinase Inhibitor

UCSC: University of California Santa Cruz

WBCs: White Blood Cells

CHPATER 1: INTRODUCTION, HYPOTHEISIS AND AIMS:

Chronic myeloid leukemia (CML) is a cancer of blood. In 95% of patients this disease is characterized by reciprocal translocation of chromosomes 9 and 22 generating an abnormal chromosome called Philadelphia Chromosome. This results in a fusion of *BCR* and *ABL* gene to produce a constitutively active fusion kinase *BCR/ABL*. CML can be divided into three phases: chronic phase, accelerated phase and blast crisis. It is thought that *BCR/ABL* translocation within a hematopoietic stem cell (HSC) initiates the disease and marks the beginning of the chronic phase. Accumulation of additional mutations promoting self-renewal of myeloid progenitor cells in chronic phase is thought to induce progression to more severe myeloid blast crisis which is lethal without effective treatment. Identification and characterization of such mutations will be critical to finding effective therapeutic strategies for CML myeloid blast crisis. SETBP1 is predominantly nuclear localized protein. Results from our lab have also showed that ectopic expression of *Setbp1* promotes self-renewal of myeloid progenitors causing their immortalization in culture [1]. Furthermore, while *BCR/ABL* (p210) alone is unable to transform mouse myeloid progenitors, co-expression of *Setbp1* and *BCR/ABL* (p210) in mouse granulocyte macrophage progenitors (GMPs) induces myeloid leukemia resembling human CML blast crisis suggesting cooperation between *BCR/ABL* and *Setbp1* in inducing CML blast crisis. This idea is further supported by the finding of SETBP1 overexpression in a subset of accelerated phase/blast crisis patients.

By performing Chromatin Immunoprecipitation (ChIP) assay in Setbp1-immortalized mouse myeloid progenitors our laboratory found that SETBP1 binds to the promoter region of known oncogenic transcription factors genes *Hoxa9* and *Hoxa10*. A role of Setbp1 in the activation of *Hoxa9* and *Hoxa10* promoters has been further confirmed by luciferase assays [1]. These data therefore suggest that *Hoxa9* and *Hoxa10* could mediate the cooperation of *SETBP1* with

BCR/ABL in inducing CML myeloid blast crisis development. In addition our preliminary study has shown decrease in colony forming potential of *Setbp1* immortalized myeloid progenitors after Ctnnb1 (β-catenin) knockout. As activated Wnt/β-catenin pathway is associated with CML progression, therefore it is also possible that Setbp1 may activate Wnt/ β-catenin pathway.

Based on these findings I hypothesize that:

***SETBP1* cooperates with *BCR/ABL* to transform myeloid progenitors into leukemic cells in CML blast crisis by activating multiple downstream pathways.**

To test this hypothesis, I have proposed following specific aims:

AIM 1: To examine the cooperation between Hoxa9 and BCR/ABL to promote CML myeloid blast crisis.

Primary myeloid progenitors will be infected with *Hoxa9*-expressing retrovirus to examine if this Setbp1 transcriptional target can promote the self-renewal of myeloid progenitors in culture. Further, in order to examine whether *Hoxa9* is sufficient to cooperate with BCR/ABL to induce myeloid blast crisis *in vivo* I will transplant GMPs (granulocyte macrophage progenitors) co-expressing *Hoxa9* and *BCR/ABL* cDNA into irradiated recipient mice.

AIM 2: To examine the cooperation between Hoxa10 and BCR/ABL to promote CML myeloid blast crisis.

Primary myeloid progenitors will be infected with *Hoxa10*-expressing retrovirus to examine if it can promote the self-renewal of myeloid progenitors in culture. Further, in order to examine

whether *Hoxa10* is sufficient to cooperate with *BCR/ABL* to induce myeloid blast crisis *in vivo* I will transplant GMPs co-expressing *Hoxa10* and *BCR/ABL* cDNA into irradiated recipient mice.

AIM 3: To examine the role of Myb in promoting development of CML myeloid blast crisis.

As Myb has been suggested to be a Hoxa9 target for transcriptional activation during hematopoiesis as well as leukemic transformation, we will examine the role of *Myb* in promoting self-renewal of myeloid progenitors in vitro and its cooperation with *BCR/ABL* to induce CML blast crisis in vivo.

AIM 4: To examine whether β-catenin is critical for maintaining *Setbp1* induced self-renewal in myeloid progenitors.

Abnormal activation of Wnt/β-catenin pathway has been implicated in conferring self-renewal capability to GMPs during chronic myeloid leukemia (CML) disease progression. We will investigate any possible interaction between *Setbp1* and Wnt/β-catenin pathway and explore a possible role of *Setbp1* in modulating Wnt/β-catenin pathway.

By establishing the role of these potential downstream effectors of *SETBP1* in CML progression we hope to gain insights into the molecular mechanism underlying the development of CML blast crisis which could help to develop novel therapeutic strategy to treat patients in which CML has progressed to blast crisis.

CHAPTER 2: BACKGROUND INFORMATION

Hematopoiesis

Hematopoiesis is the process of formation of blood cells. Mammalian hematopoietic system consists of more than 10 distinct mature blood cell types, all of which originate from hematopoietic stem cells (HSCs) that reside in adult bone marrow [2]. HSCs were previously thought to be the only cells within the hematopoietic system that are both multi-potent (i.e. have the capacity to differentiate into different cells types) and capable of extensive self-renewal (i.e. have the capacity to give rise to identical daughter cells) [3]. However, recent studies have shown that the multipotent progenitors (MPPs) that are immediately down stream of HSCs are also capable of extensive self-renewal [4](Figure 1). These MPPs differentiate into either lymphoid-primed multipotent progenitor (LMPPs) or common myeloid progenitors (CMPs). LMPPs can differentiate into either oligopotent granulocyte/macrophage progenitors (GMPs) or common lymphoid progenitors (CLPs). CMPs differentiate into either GMPs or megakaryocyte/erythroid progenitors (MEPs), which then terminally mature into red blood cells (RBCs), platelets and white blood cells (WBCs) of myeloid origin including granulocytes and macrophages. On the other hand CLPs differentiate into white blood cells of lymphoid origin including B-cells, T-cells and NK cells [5, 6].

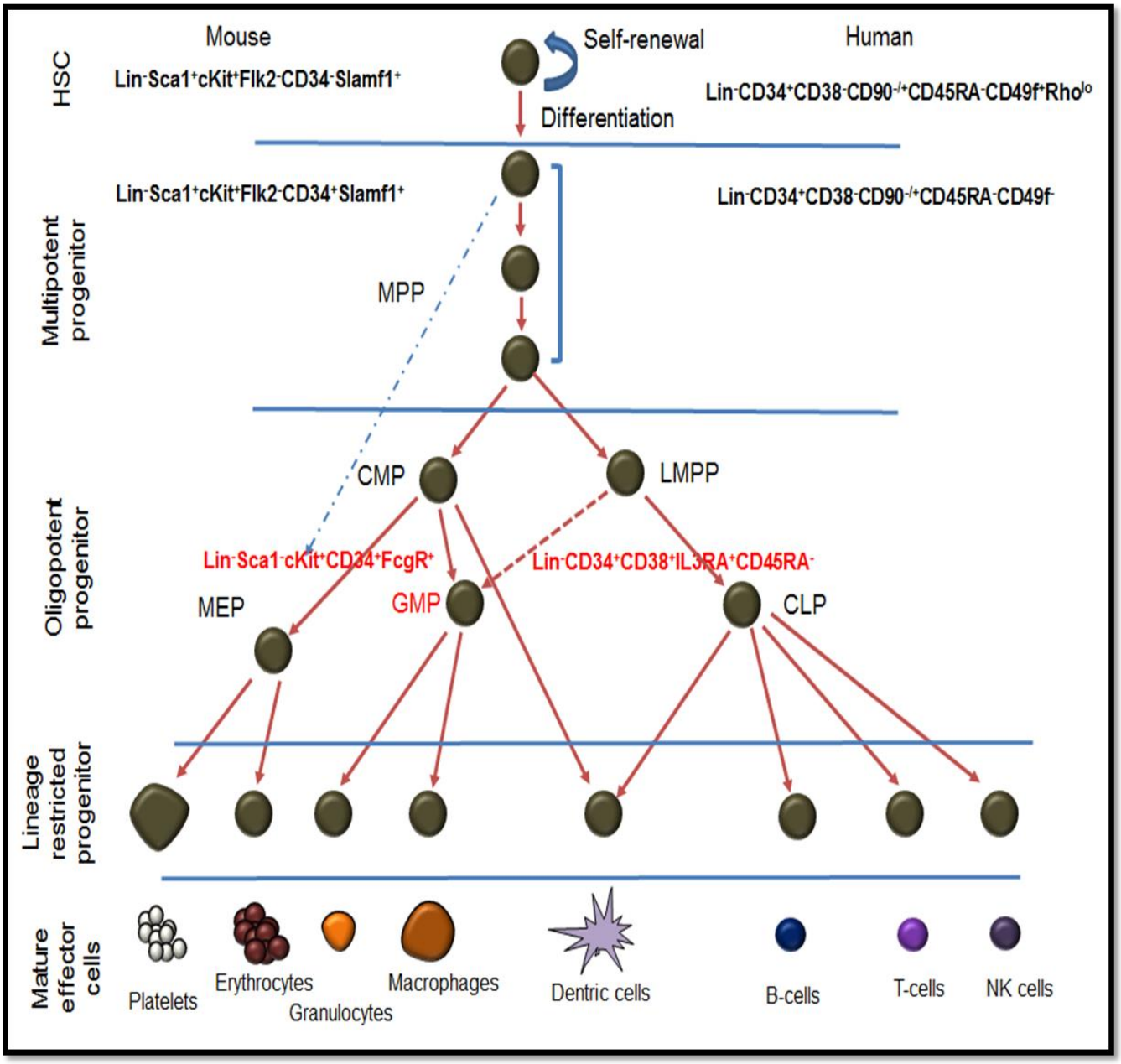


Figure 1:Schematic diagram of Hematopoiesis in Mouse and Human

The process of formation of all terminally differentiated hematopoietic cells from a common source called HSCs. The diagram represents the process of HSC self-renewal and differentiation into non-self-renewing multipotent, oligopotent, and lineage restricted progenitors and their maturation into terminally differentiated cell. Figure modified from Jun Seita and Weissman, 2010 [7].

Leukemia

Leukemia is the cancer of blood. Leukemia is generally believed to be caused by clonal expansion of mutant stem or progenitor cells that have acquired growth and survival advantages over normal hematopoietic cells [8].Based on the hematopoietic cell type involved and on the latency of the disease, leukemia is mainly classified into four major types, namely:

- Chronic Myelogenous Leukemia (CML),
- Acute Myelogenous Leukemia (AML),
- Chronic Lymphocytic Leukemia (CLL),
- Acute Lymphocytic Leukemia (ALL).

Leukemia is caused due to genetic mutations that activate proto-oncogenes and inactivate tumor suppressor genes [8-10]. Such mutational events cause deregulation of number of molecular pathways involved in proliferation, self-renewal, differentiation and cell survival which eventually leads to leukemic transformation of hematopoietic cells. Our focus in this study is on CML which is a form of leukemia that affects cells belonging to the myeloid lineage of hematopoietic system.

Chronic myeloid leukemia

CML also known as Chronic Myelogenous Leukemia, accounts for about 20% of adult leukemias. In 95% of the CML patients there is a reciprocal translocation of chromosomes 9 and 22, t (9; 22). This translocation give rise to an abnormal chromosome known as the Philadelphia Chromosome. This translocation event also brings together two genes called *BCR* and *ABL*, leading to the production of a constitutively active fusion tyrosine kinase *BCR/ABL* [11-13]. BCR/ABL protein is cytoplasmic and can phosphorylate substrate that lead to the deregulation of several intracellular

pathways promoting cell survival and proliferation. These include *RAS-RAF*, *STAT*, *JUN Kinase*, *MYC*, phosphatidylinositol-3 kinase, *AKT* pathway [14, 15].

CML is a slow progressing disease which mostly affects people in their mid-50's and rarely in childhood. Clinical progression of CML can be divided into three phases: chronic, accelerated and blast crisis. This characterization is based on the presence of immature abnormal white blood cells — myeloblasts (blast cells) in the blood and bone marrow of patients. Chronic phase is characterized by the *BCR/ABL* mutation originating in the HSCs which mainly affects the myeloid cell compartment. Patients in this phase typically have less than 10% blast cells and show mild symptoms of granulocyte and macrophage expansion. Patients in accelerated phase may have more than 10% but fewer than 20% blast cells and may have symptoms such as fever, poor appetite, and weight loss. Blast crisis phase is associated with greater than 20% blast cells with tissue infiltration (other than bone marrow) of the blast cells. Although *BCR/ABL* translocation in HSCs is considered the initial genetic abnormality that results in the initiation of the chronic phase of CML, its progression to accelerated phase and blast crisis is due to additional events (mutations) that occur in progenitors. For myeloid blast crisis these additional mutations can confer GMPs with self-renewal capabilities increasing the leukemic burden with undifferentiated blast cells. Progression of CML from chronic to blast crisis phase is associated with adverse prognosis of the disease.

Current drug therapies for the treatment of CML involving small molecule inhibitors of BCR-ABL such as imatinib or second generation tyrosine kinase inhibitors (TKIs) such as dasatinib or nilotinib [16, 17] target the disease in chronic phase. Although effective in bringing remission of disease in chronic stage patients, 10-20% of patients show relapse during or after the treatment. In blast crisis patients, response to current therapy is very limited and often limited to bone marrow

transplantation. Studies have identified that genomic instability of CML cells in advanced stage can lead to loss of *BCR/ABL* oncogene addiction which makes complete remission of CML impossible [18, 19]. These characteristics of CML blast crisis are attributed to the persistence of leukemia-initiating cells (LICs). Further, it has been shown that patients that do not respond or develop resistance to TKI therapy have the deregulation of the pathways involved in DNA replication, recombination and repair as well as cellular growth and proliferation[20].

Leukemia-Initiating Cells

LICs are slow cycling subpopulation of leukemia cells with unlimited self-renewal capability, which are responsible for the initiation and maintenance of the disease [21]. The concept of LICs first emerged in early 1970. Initially, HSCs were identified as the source of LICs. LICs were purified as $Thy1^-$, $CD34^+$, $CD8^-$ cell population from human myeloid leukemia and were the only cells from the leukemic bulk population to be consistently clonogenic and to have HSCs like characteristics[21, 22]. Xenograft studies involving limiting dilution transplantation experiment as well as leukemia studies using syngeneic mouse model suggested that LICs frequencies usually vary between leukemias of different molecular subtypes [23]. Studies thereafter have shown that LICs can arise from HSCs or from more restricted progenitors that acquire self-renewal characteristics due to mutations[24, 25].

The molecular mechanisms responsible for LIC self-renewal in myeloid leukemia have been poorly understood with limited knowledge about the genetic and epigenetic events involved. In the case of AML; these include protein translocation mutations involving the mixed lineage leukemia (*MLL*) gene as well as *MOZ/TIF2* mutation that give rise to chimeric transcription factors that have been shown to transform GMPs into LICs in AML mouse models[26-28]. Expression

profiling comparing LICs in MLL-AF9-induced leukemia and their normal counterpart has revealed that overexpression of *HOXA9* cluster genes and *MEF2C* may contribute to the self-renewal of these LICs. In case of CML, it is believed that during chronic phase of the disease, LICs are directly derived from a HSC which acquires *BCR/ABL* mutation, whereas during CML blast crisis phase, GMPs acquire self-renewal capability through additional mutations and transform into LICs. Jameison *et al* showed that abnormal activation of β-catenin in GMPs confer them with self-renewal capability and transform them to LICs [24]. Later studies further confirmed the role of GMP transformation to LIC during CML blast crisis progression [29]. *Nup98/Hoxa9*, *Hes1*, *Setbp1* are examples of mutations apart from β-catenin deregulation that have been identified to confer self-renewal to GMPs [1].

SETBP1

SETBP1 is located on human chromosome 18q21.1. It encodes a predominantly nuclear localized large protein of 1542 amino acids with unclear function [30]. Mouse and human SETBP1 proteins share over 90% identity in their amino acid sequence and therefore suggested to have conserved functions. It is likely that *Setbp1* possess chromatin remodeling functions as our sequence analysis confirmed 3 conserved AT-hook DNA-binding motifs, and presence of multiple copies of AT-hook motifs in a protein is thought to cause bending of DNA through which chromatin access can be regulated (Figure2) [31]. *SETBP1* has been suggested to bind to SET protein [32], which is a small protein inhibitor for the tumor suppressors *PP2A* and *NM23-H1* [33, 34], through its set binding domain. *SET* has been involved in regulating many different cellular processes including transcription [35], histone acetylation [36], apoptosis [32], and cell migration [37]. *SET* has also been implicated in leukemia development by its fusion with another gene *CAN* in acute

undifferentiated leukemia [38, 39], its inhibition of *PP2A* during progression of CML [40] and its potential complex formation with *MLL* and *PP2A* in leukemia induced by *MLL* translocations [41].

Our laboratory has shown that ectopic expression of *Setbp1* promotes self-renewal of myeloid progenitors, causing their immortalization in culture [1]. Furthermore, while *BCR/ABL* alone is unable to transform myeloid progenitors, co-expression of *Setbp1* and *BCR/ABL* (p210) in myeloid progenitors induces myeloid leukemia resembling CML blast crisis in recipient mice providing evidence to support cooperation between *BCR/ABL* and *Setbp1* in inducing CML blast crisis. Our laboratory has also shown high *SETBP1* expression in a subset of myeloid CML accelerated phase and blast crisis patients, thus supporting that *SETBP1* overexpression contributes to human CML progression[1].

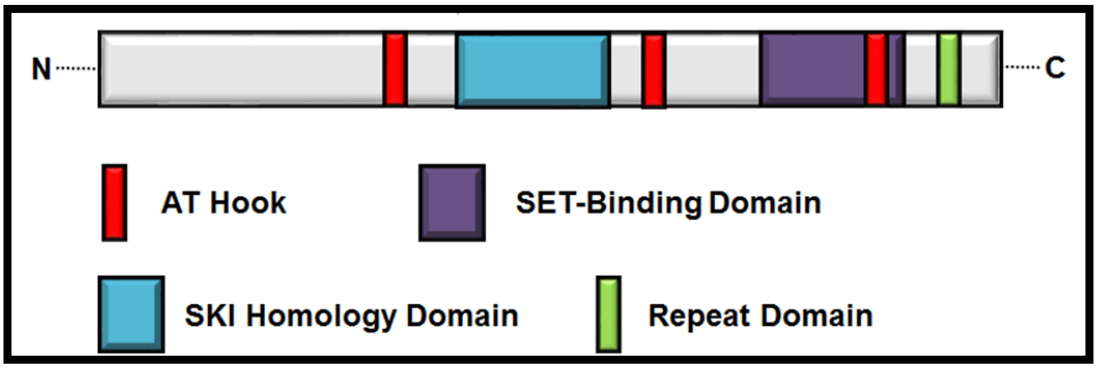


Figure 2:Schematic diagram of SETBP1 protein

Three AT Hook domain, one SKI Homology domain, one SET-Binding domain and one Repeat domain are indicated. Figure modified from Inoue et al, 2014 *[42].*

More recently, whole-exome sequencing studies on various human leukemic samples including atypical chronic myeloid leukemia, chronic myelomonocytic leukemia, secondary AML and juvenile myelomonocytic leukemia, have identified recurrent somatic *SETBP1* mutations along

with a subset of CML blast phase patients with activating *SETBP1* mutations, further suggesting its role in CML progression [43]. All of these somatic mutations are located within the portion of the gene that codes for Ski-Homologous domain of *SETBP1* and presumably result in gain of function. Further, it is also observed that these *SETBP1* mutations are usually associated with myeloid leukemia transformations and poor prognosis [42-46]. Identification of somatic *SETBP1* mutation in various leukemic malignancies has further suggested a significant role of *SETBP1* in human leukemic transformation. More recently, studies in our laboratory have shown that *Setbp1* overexpression is capable of initiating myeloid leukemia development in mice by significantly enhancing self-renewal potential in HSC and GMP population, demonstrating that *Setbp1* is a potent oncogene [47].

By performing *Setbp1* functional studies we have previously identified *Hoxa9* and *Hoxa10* genes as transcriptional targets of Setbp1 which are essential for its self-renewal promoting function [1]. Our laboratory has shown that for myeloid progenitors immortalized by ectopic expression of *Setbp1,* there is activation of both *Hoxa9* and *Hoxa10* expression. By performing ChIP assay in these cells our laboratory have shown that Setbp1 binds to the promoter region of these oncogenic transcription factors. Furthermore, using luciferase promoter assay we have shown that both *Hoxa9* and *Hoxa10* promoters can be activated by Setbp1. Furthermore, this activation of *HOXA9* and *HOXA10* was also observed in leukemias harboring *SETBP1* mutations [1, 43].

Hoxa9* and *Hoxa10

Both *HOXA9* and *HOXA10* belong to a class of transcription factors called Homeobox (*HOX*) genes found in clusters named A, B, C, and D on four separate chromosomes. *HOXA9* and *HOXA10* are part of the A cluster on chromosome 7. HOX proteins are important regulators of

development [48]. HOX proteins contain a highly conserved DNA-binding domain called homeodomain. This homeodomain, consisting of 60 highly conserved amino acids, binds DNA through a helix-turn-helix (HTH) structure. HOX proteins have variable sequences flanking the homeodomain that influence its DNA-binding specificity. Both *HOXA9* and *HOXA10* have been found to be involved in regulation of hematopoiesis and are validated oncogenes in acute leukemia and myelodysplastic syndrome [49, 50]. *HOXA9* and *HOXA10* are preferentially expressed in primitive hematopoietic cell compartment. In case of *HOXA9*, it's over expression is associated with HSC expansion and decrease in *HOXA9* expression level is associated with differentiation of HSCs[48]. Further it has been shown that HSCs deficient for *Hoxa9* exhibit a substantial loss of proliferative capacity[48]. For *HOXA10,* expansion of HSCs is more dependent on the concentration of *HOXA10*; where low-to-intermediate levels of *HOXA10* promote proliferation of primitive progenitors (Lin^- c-Kit^+ Sca-1^+) cells and high levels of *HOXA10* has no effect on HSC proliferation but blocks erythroid and megakaryocyte development [51]. Deregulation of *HOXA9* or *HOXA10* has been observed in many leukemic malignancies [48].

Myb

MYB is a basic helix-loop-helix transcription factor. MYB proteins has three distinct functional domains: A highly conserved DNA binding domain (DBD) near the N-terminus with three tandem repeats R1, R2 and R3 [52, 53], trans-activation domain (TAD) and Carboxyl terminal negative regulatory domain (NRD). DBD and TAD can interact with co-regulators such as CBP, P300, C/EBPbeta, Menin etc. [53-55]. NRD comprising of EVES domain is involved in intra-molecular interactions and negative regulation [52]. Early work with *MYB* identified differential expression of *MYB* in the human hematopoietic system [56]. High *MYB* expression was observed in all early

progenitors which decreased with maturation or terminal differentiation of hematopoietic cells suggesting that *MYB* is involved in hematopoietic proliferation and differentiation. Studies have shown that ectopic expression of *Myb* is associated with differentiation block effecting the myeloid compartment of the hematopoietic system [57-59]. Also conditional knockout of *Myb* is associated with loss of self-renewal and accelerating differentiation in hematopoietic stem cells [60]. Consistent with these studies there are more than 80 targets of MYB and many of these downstream targets like *CD34*, *c-Kit*, *c-Myc*,*Flt-3*, *Bcl-2* have important role in the proliferation and survival of hematopoietic cells [61].

Myb null mice studies further highlighted the role of functional *Myb* for normal murine fetal hepatic hematopoiesis [62]. Here it was observed that absence of functional *Myb* resulted in prenatal lethality due to defect in murine fetal hepatic hematopoiesis. The mouse fetuses from day 15 gestation period were severely anemic (with 10-fold decrease in hematocrit) and small for the *Myb* null mice as compared to wild type mice. All other organs were normal. There was also decrease in the myeloid precursor and mature cells in *Myb* null mice along with MPP.

Myb role in leukemic transformation was first discovered in avian leukemia [63, 64]. Since then studies have identified *MYB* as an important human oncogene which is a target of somatic mutations and translocations in human cancers [65]. These include translocation and duplication of *MYB* locus in lymphoblastic leukemia and *MYB-GATA1* fusion mutation in acute myelomonocytic and basophilic leukemia [65-68]. Overexpression of *MYB* has also been observed in a number of human leukemia subtypes [69]. *In vitro* study involving the knockdown of *MYB* expression using *MYB* anti sense oligonucleotide in human blast crisis samples have shown decrease in the colony forming potential of these leukemic cells. Similarly, knockdown studies in *MLL-ENL* leukemia have shown that *MYB* is an essential part of the signal transduction pathway

involved in leukemic transformation [70]. Interestingly, study involving conditional activation of *Hoxa9* and *Meis1* gene, has identified *Myb* as an essential downstream target for *Hoxa9* [70].
It was observed that leukemic cells are more sensitive to the effect of *MYB* knockdown than normal hematopoietic stem and progenitor cells, suggesting that *Myb* could be promising target for leukemia therapy [71, 72].

Wnt/β-catenin Pathway in Hematopoietic System

Wnt/β-catenin canonical signaling pathway plays an important role in normal hematopoiesis and hematopoietic malignancies. During embryonic stage of mouse development, transient activity of Wnt/β-catenin has been shown to be important for generation of HSCs [73]. Here activation of β-catenin has been shown to induce hematopoietic differentiation from endothelial like cell population residing within the aorta-gonad-mesonephros region of developing embryo and also contributes towards adult hematopoiesis [74]. Further, ectopic expression of active β-catenin has been shown to promote the growth of HSCs *in vitro* and their engraftment into lethally irradiated mice [75], suggesting that Wnt/β-Catenin pathway also regulates the self-renewal of adult HSCs.

The activation of Wnt/β-catenin canonical pathway initiates with the binding of a Wnt ligand, a secreted glycoprotein, to a frizzled receptor on the cell surface [76, 77]. In the absence of Wnt ligand, cytoplasmic β-catenin protein is acted upon by Axin complex, composed of the scaffolding protein Axin, adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3), and degraded constantly. In the presence of Wnt ligand and co-receptor LRP5/6, a complex is formed with Wnt-bound Frizzled that leads to the dissociation of Axin complex. Thus cytoplasmic β-catenin now can escape degradation and is available for translocation of to the nucleus, where it interacts with lymphoid enhancer factor/T-cell factor

(LEF/TCF) transcription factors and regulates the transcription of its target genes including *Myc* and *Cyclin D1*[78].

Wnt/β-catenin signaling has also been found to be associated with hematopoietic malignancies. LICs that are derived from certain oncogene dependent leukemic transformation of HSCs or GMPs depend on the activation of β-catenin for maintenance of their self-renewal function. Examples include MLL-AF9 mediated transformation of GMPs or Hoxa9 mediated transformation of HSCs [79]. In case of CML, progression of disease to blast crisis stage is characterized by activation of Wnt/ β-catenin pathway in granulocyte-macrophage progenitors (GMPs) [24]. Hematopoietic malignancies involving abnormal activation of Wnt/β-catenin pathway highlight the role of β-catenin in leukemic transformation and also identify β-catenin as an important target for disease treatment. With the knowledge from studies that identify β-catenin to be dispensable for the maintenance and function of adult HSCs, targeting β-catenin could be an attractive therapeutic intervention for the treatment of CML and other leukemic malignancies [80-82].

CHAPTER 3: TO EXAMINE THE COOPERATION BETWEEN *HOXA9* AND *BCR/ABL* TO PROMOTE CML BLAST CRISIS

Ectopic expression of *Hoxa9* efficiently immortalizes primary myeloid progenitors in culture

Our laboratory has previously shown that overexpression of *Setbp1* promotes self-renewal of primary myeloid progenitors *in vitro* in the presence of stem cell factor (SCF) and interleukin-3 (IL-3). Using chromatin immunoprecipitation (CHIP) and luciferase reporter assay our lab has also confirmed that *Hoxa9* and *Hoxa10* genes are transcriptional targets of Setbp1 in myeloid progenitors. Further, our lab has shown that continuous expression of these genes is important for the self-renewal of *Setbp1* immortalized cells as knockdown of *Hoxa9* and *Hoxa10* decreases the colony forming potential of *Setbp1* immortalized myeloid progenitors[1].

To test whether *Hoxa9* alone is sufficient to induce self-renewal of myeloid progenitors we tested if ectopic expression of *Hoxa9* would induce immortalization of primary myeloid progenitors in culture in the presence of SCF and IL3. For this study, we first harvested BM from C57BL/6 mice. BM cells were then expanded under two different cytokine condition. First these cells were cultured in serum free medium containing cocktail of four cytokines; SCF, TPO, FGF-1 and IGF-2 for a period of 6 days to expand HSCs. These cells were then induced to differentiate into myeloid progenitors by culturing the cells in SCF and IL-3 medium for a period of 4 days. This was done to enrich the harvested BM cells for primary myeloid progenitors. We transduced primary myeloid progenitors with MSCV retrovirus expressing *Hoxa9* cDNA (MSCV-Hoxa9-PGK-Neo). Cells infected by empty virus were included as negative control. Infections were carried out using low titers of virus to reduce the chance of insertional mutagenesis (1 virus: 4 cells). Infected cells were passaged for 4 weeks to test whether they were immortalized (Figure 5).

4 weeks after infection there were still proliferating myeloid progenitor cells in all cultures transduced by *Hoxa9* virus, suggesting that *Hoxa9* could induce immortalization of myeloid progenitors. To confirm that these cells were truly immortalized we continuously passaged these cells for 6 months until the experiment was terminated (Figure 6A). In contrast, primary myeloid progenitors that were infected with empty MSCV virus did not become immortalized and differentiated terminally into mature macrophages (Figure 5B). Flow cytometric analysis of cells after establishing immortalization showed that the cells were positive for myeloid lineage markers (Mac-1 & Gr-1) and negative for lymphoid and erythroid markers (Figure 36).

We also investigated the ability of *Hoxa9* to immortalize myeloid progenitors from GMPs and 5-FU-treated hematopoietic progenitors (using 5FU treated C57BL/6 mice BM cells). We observed that transducing these cell populations with *Hoxa9* virus also led to immortalization of myeloid progenitors (Figure 6B-C).

Hoxa9 cooperates with *BCR/ABL* to transform GMPs *in vivo*

The ability of *Hoxa9* to immortalize myeloid progenitors including GMPs *in vitro* suggested that *Hoxa9* may also help confer limitless self-renewal potential to LICs originating from GMPs. To test whether Hoxa9 can also cooperate with BCR/ABL *in vivo* to transform GMPs into LICs, we co-transduced GMPs (sorted from C57BL/6 mice), with pMSCV retrovirus expressing *BCR/ABL* (pMSCV-BCR/ABL-IRES-GFP) and pMSCV retrovirus expressing *Hoxa9* (pMSCV-Hoxa9-PGK-Neo). Co-transduced GMPs were then transplanted into lethally irradiated congenic recipient mice along with supporting bone marrow. *BCR/ABL* alone and *Hoxa9* alone transduced GMPs were transplanted into mice as controls (Figure 7A). Mice were then aged and monitored closely for possible leukemia development. It was observed that for all mice receiving GMPs co-

transduced with *BCR/ABL* and *Hoxa9* retrovirus became sick in the first 4 week of transplantation (Figure 7B). These moribund mice were euthanized and examined for possible leukemia development. We performed cytospin analysis for the bone marrow and spleen cells of the moribund mice and observed that majority of the cells resemble myeloid progenitor, supporting the development of CML myeloid blast crisis (Figure 8A). To further investigate the lineage of these cells we performed flow cytometry analysis. We observed that more than 95% of the leukemic cells in spleen were positive for Gr1 and Mac1, which are myeloid lineage markers, and negative for CD3, CD19 and Ter119, which are markers for T cells, B cells and erythroid cells respectively (Figure 8B). We also examined the presence of immature markers on these cells including c-Kit and Sca-1(Figure 8C). We observed that between 5% to 11% of the cells in the bone marrow and spleen were positive for Sca-1 and less than 1% of the cells were positive for c-Kit. Pathological examination of these mice also showed that they had developed leukemias with leukemic infiltrations into spleen and liver (Figure 9). Mice transplanted with GMP cells that were singly transduced with either *BCR/ABL* or *Hoxa9* virus did not develop leukemia and remained healthy for 4 months (Figure 7B). We also wanted to confirm that the leukemia development required both *Hoxa9* and *BCR/ABL* expression within the same cell. To test this we performed colony assay using cells isolated from the bone marrow of moribund mice. Cells were plated in colony assay media along with neomycin. After 8 days, colonies were observed under ultra-violet microscope to check for GFP expression. We observed that the colonies growing under neomycin selection were positive for expression of GFP, thus confirming that co-expression of *Hoxa9* and *BCR/ABL* resulted in the transformation of GMPs into LICs in mice. We also confirmed the expression of *Hox9* and *BCR/ABL* in *BCR/ABL + Hoxa9* leukemias by QRT-PCR analysis (Figure 33). Here we also confirmed the overexpression of *Myb* in both *BCR/ABL + Hoxa9* and *BCR/ABL*

+ *Hoxa10* leukemias using Western Blotting (Figure 35).We also tried to establish cell lines form *BCR/ABL+Hoxa9* leukemic cells but were unable to do so.

We performed secondary transplantation experiment for *BCR/ABL + Hoxa9* leukemias. We observed that all secondary mice developed leukemias similar to the primary leukemias in 4 weeks when $1x10^6$ spleen cells from the primary recipient were transplanted, suggesting that these leukemias are transplantable (Figure 7B).

In combination, above *in vitro* and *in vivo* data demonstrate that *Hoxa9* can promote the self-renewal of myeloid progenitor in culture and cooperate with *BCR/ABL* to transform GMPs and induce CML blast crisis in mice.

CHAPTER 4: TO EXAMINE THE COOPERATION BETWEEN *HOXA10* AND *BCR/ABL* TO PROMOTE CML BLAST CRISIS

Ectopic expression of *Hoxa10* efficiently immortalizes primary myeloid progenitors in culture

Next, in order to examine whether *Hoxa10* is sufficient to induce self-renewal of myeloid progenitors we tested if ectopic expression of *Hoxa10* would immortalize primary myeloid progenitors in culture in presence of SCF and IL3. Here again we transduced primary myeloid progenitors (harvested from C57BL/6 mice) with pMSCV retrovirus expressing *Hoxa10* (pMSCV-Hoxa10-PGK-Puro) cDNA. Cells infected by empty virus were included as negative control. Infection was performed using low viral titers (1 virus: 4 cells). Infected cells were passaged for 4 weeks to test for immortalization.

We found that *Hoxa10* was also able to immortalize primary myeloid progenitors in culture in the presence of SCF and IL3 (Figure 10A). Flow cytometric analysis of cells after establishing immortalization showed that the cells were positive for myeloid lineage markers (Mac-1 & Gr-1) and negative for lymphoid and erythroid markers (Figure 36).

We also investigated the ability of *Hoxa10* to immortalize myeloid progenitors from GMPs and 5-FU-treated bone marrow progenitor population (using 5FU treated C57BL/6 mice BM cells). In both cases, we were able to generate immortalized myeloid progenitor cells (Figure 10B-C).

Hoxa10 cooperates with *BCR/ABL* to transform GMPs and cause Leukemia *in vivo*

Next we examined if *Hoxa10* can also cooperate with *BCR/ABL in vivo* to transform GMPs and cause CML blast crisis leukemia. To test this, we co-transduced GMPs (sorted from C57BL/6 mice), with pMSCV-BCR/ABL-IRES-GFP and pMSCV retrovirus expressing *Hoxa10* (pMSCV-Hoxa10-PGK-Puro). Co-transduced GMPs were then transplanted into lethally irradiated congenic recipient mice along with supporting bone marrow ($1.5x10^5$ GMPs and $7.5x10^5$ Supporting Bone Marrow cells per mouse). *BCR/ABL* alone and *Hoxa10* alone transduced GMPs were transplanted into mice as control (Figure 11A). Mice were then aged and monitored for possible leukemia development.

We observed that all mice transplanted with GMPs co-transduced with *BCR/ABL* and *Hoxa10* expressing retrovirus became sick in time period between 50 to 100 days after transplantation (Figure 11B). These moribund mice were euthanized and examined for possible leukemia development. Cytospin analysis from the bone marrow and spleen cells of the moribund mice showed that majority of the cells resemble myeloid progenitor, supporting the development of CML myeloid blast crisis (Figure 12A). To determine the lineage of these cells we performed flow cytometry analysis. We observed that more than 95% of the leukemic cells were positive Gr1 and Mac1, which are myeloid lineage markers, and negative for CD3, CD19 and Ter119, which are markers for T cells, B cells and erythroid cells respectively (Figure 12B). Less than 1% cells were also positive for immature markers including c-Kit and Sca-1(Figure 12C). We also observed that less than 1% of the leukemic cells expressed c-Kit and between 6% and 10% were positive for Sca-1 expression. Pathological examination of these mice also showed that they had developed leukemias with leukemic infiltrations into spleen and liver (Figure 13). Mice transplanted with GMP cells that were singly transduced with either *BCR/ABL* or *Hoxa10* expressing retrovirus did

not develop leukemia and remained healthy for next 4 months after which the experiment was terminated (Figure 11B). We confirmed the expression of *Hoxa10* and *BCR/ABL* in *BCR/ABL + Hoxa10* leukemias by QRT-PCR analysis (Figure 33). Here we also confirmed the overexpression of *Myb* in both *BCR/ABL + Hoxa9* and *BCR/ABL + Hoxa10* leukemias using Western Blotting (Figure 35).We also tried to establish cell lines form *BCR/ABL+Hoxa10* leukemic cells but were unable to do so.

Next, we performed secondary transplantation experiment for *BCR/ABL + Hoxa10* leukemias. We observed that all secondary mice developed leukemia in less than 50 days (Figure 11B).

In combination, above *in vitro* and *in vivo* data demonstrate that *Hoxa10* can also promote self-renewal of myeloid progenitor in culture and cooperate with *BCR/ABL* to transform GMPs and induce CML blast crisis in mice.

Expression level of *Hoxa9* and *Hoxa10* downstream targets

Microarray analysis have identified overlap between *Hoxa9* and *Hoxa10* target genes [51, 83-85]. Within this overlap, there are genes that are similarly or differentially regulated by these two *HOX* genes. Apart from overlapping targets there are genes that are specific targets for either *Hoxa9* or *Hoxa10* [85-87]. In our transplantation experiments we have observed that leukemias induced by *BCR/ABL+Hoxa10* have longer latencies than leukemias induced by *BCR/ABL+Hoxa9*. We reasoned that this difference in latency for disease development could be due to the difference in the regulation of downstream targets of *Hoxa9* and *Hoxa10* genes. So to investigate the expression levels of Hoxa9 and Hoxa10 downstream targets, we used 5-FU-treated mouse bone marrow progenitors which were freshly transduced with *Hoxa9* (MSCV-Hoxa9-puro) or *Hoxa10* (MSCV-Hoxa10-puro) expressing retrovirus. Based on previous studies, we decided to examine if there

was any difference in the expression levels of several downstream targets of *Hoxa9* and *Hoxa10* genes which are known to be involved in leukemia development. Targets examined include *Erg, Junb, Sox4, Myc, Pim1, Msi2* and *Flt3* [48, 85, 87-89]. *Erg, Junb, Sox4* are targets of both *Hoxa9* and *Hoxa10* while *Pim1, Flt3, Myc* and *Msi2* are targets of *Hoxa9.*

In QRT-PCR analysis we observed that the mRNA levels of *Erg* and *Myc* were significantly higher in *Hoxa9* transduced cells than *Hoxa10* transduced cells (48 hrs. after transduction) (Figure 14A). For other targets we did not observe significant difference (Figure 14B). *Erg* deregulation or overexpression has been associated with poor clinical outcome in AML. Also, MYC is shown to be occasionally amplified and overexpressed in CML-BC patients.These results suggest that the ability of *Hoxa9* to activate higher levels of transcription at *Myc* and *Erg* than *Hoxa10* may be responsible for the shorter latency of *BCR/ABL+Hoxa9* leukemias.

CHAPTER 5: TO EXAMINE THE ROLE OF *MYB* IN PROMOTING DEVELOPMENT OF CML MYELOID BLAST CRISIS

Myb is a critical target of *Hoxa9* and *Hoxa10* in their induction of immortalization of myeloid progenitors

In order to confirm that *Hoxa9* and *Hoxa10* are overexpressed in *BCR/ABL + Hoxa9* and *BCR/ABL + Hoxa10* leukemias respectively, we performed Western blotting analysis for Hoxa9 and Hoxa10 in whole cell lysate prepared from the spleen of these leukemic mice. As expected, overexpression of *Hoxa9* and *Hoxa10* were detected in these leukemic samples. We also examined the expression of Myb since it has been suggested to be an essential downstream target for Hoxa9. Western blotting analysis confirmed the expression of *Myb* in *BCR/ABL + Hoxa9* leukemic samples. Interestingly we also observed similar levels of *Myb* expression in *BCR/ABL + Hoxa10* leukemic samples. This result suggests that *Myb* could be a common critical target downstream of *Hoxa9* and *Hoxa10* (Figure 15).

Next we examined if continuous expression of *Myb* is required for the self-renewal of myeloid progenitors immortalized by *Hoxa9* and *Hoxa10*. We knocked down *Myb* in these cells using *Myb*-specific lentiviral shRNAs. Cells infected with a non-targeting shRNA were included as a control. We observed dramatic decrease in colony-forming potential for *Myb* knockdown cells in comparison to control cells (Figure 16-17). Cytospin analysis revealed significant differentiation, suggesting these cells failed to self-renew after *Myb* knockdown (Figure 18). Therefore, *Myb* is essential for *Hoxa9* or *Hoxa10*-induced self-renewal of myeloid progenitors.

To further test the possibility that *Myb* could be a direct target of *Hoxa9* and *Hoxa10* we transduced early hematopoietic progenitors obtained from 5- Fluorouracil (5-FU) treated C57BL/6 mice with

Hoxa9 (MSCV-Hoxa9-puro) or *Hoxa10* (MSCV-Hoxa10-puro) expressing retrovirus and examined *Myb* expression at 48hrs after infection. Significant increase in *Myb* mRNA levels was observed in both *Hoxa9* and *Hoxa10* infected cells as early as 48hrs after infection when compared to control cells infected with empty virus (Figure 19A). Western blotting analysis also detected increase in *Myb* protein levels in both *Hoxa9* and *Hoxa10* infected cells as compared to control cells (Figure 19A-B). Increased Myb protein levels were also detected in the colonies generated by the *Hoxa9* and *Hoxa10* transduced cells 7 days after infection (Figure 19B). Quick activation of *Myb* expression upon ectopic expression of *Hoxa9* and *Hoxa10* in primary progenitor cells suggest *Myb* could be a direct target of both transcription factors.

We further did quantification of differentiation after *Myb* knock down in myeloid progenitors immortalized by *Hoxa9* and *Hoxa10*. Differentiation markers were analysed in knock down cells and compared to control cells using QRT-PCR analysis. We observed significant increase in differentiation markers after *Myb* knockdown in both *Hoxa9* and *Hoxa10* immortalized myeloid progenitors (Figure 31-32).

In combination, above data demonstrate that *Myb* expression is critical for the continuous proliferation/self-renewal of myeloid progenitors induced by *Hoxa9* and *Hoxa10* and suggest that *Myb* is likely a direct transcriptional target of both Hoxa9 and Hoxa10 in myeloid progenitors.

Ectopic expression of *Myb* short isoform efficiently immortalizes primary myeloid progenitors in culture

Our *Myb* knockdown studies suggest that *Myb* is an important target downstream of both *Hoxa9* and *Hoxa10*. Since both *Hoxa9* and *Hoxa10* can induce immortalization of myeloid progenitors, we decided to examine if ectopic expression of *Myb* could also induce self-renewal of primary

myeloid progenitors causing their immortalization *in vitro*. Flow cytometric analysis of cells after establishing immortalization showed that the cells were positive for myeloid lineage marker and negative for lymphoid and erythroid markers (Figure 36).

MYB proto-oncogene is located on chromosome 6q in humans. Alternative splicing of *MYB* transcript results in the production of two isoforms: a predominantly expressed 75kda form and a less expressed 89kda full length form [90]. The shorter *MYB* isoform is also predominantly expressed in *Hoxa9* and *Hoxa10* immortalized myeloid progenitors and *BCR/ABL+Hoxa9/Hoxa10* leukemia cells (Figure 15). Therefore, we decided to test the ability of the shorter *MYB* isoform to induce self-renewal of myeloid progenitors.

We cloned the cDNA encoding the shorter *Myb* isoform (75kda isoform: MSCV-Myb-IRES-GFP) into MSCV retroviral expression vector. We transduced primary myeloid progenitors with *Myb*-expressing MSCV retrovirus and passaged them for a period of 4 weeks to assess whether they can be immortalized.

Four weeks after infection we observed that cultures transduced by *Myb* short isoform are still composed of proliferating myeloid progenitors (Figure 20).These cells are truly immortalized as they could be continuously passaged for 6 months until the experiment was terminated. This result suggest strongly that *Myb* can promote self-renewal of primary myeloid progenitors.

Myb cooperates with *BCR/ABL* to transform GMPs and cause CML Blast Crisis *in vivo*

As we have now observed that *Myb* can immortalize primary myeloid progenitors *in vitro*, suggesting that *Myb* could confer self-renewal capabilities to myeloid progenitors, we next wanted to examine if *Myb* could cooperate with *BCR/ABL in vivo* to cause leukemic transformation of

GMPs. To test this, we co-transduced GMPs (sorted from C57BL/6 mice donor mice), with MSCV retrovirus expressing *BCR/ABL* (MIGR1-BCR/ABL-GFP) and MSCV retrovirus expressing *Myb* short isoform (MSCV-MybSI-IRES-GFP). Co-transduced GMPs were then transplanted into lethally irradiated congenic recipient mice along with supporting bone marrow. GMPs transduced by *BCR/ABL* alone and *Myb* short isoform alone were transplanted as controls. Mice were then aged and monitored for leukemia development. Six out of eight mice receiving GMPs co-transduced with *BCR/ABL* and *Myb* short isoform expressing retrovirus became moribund in 6 months after transplantation (Figure 21B). Moribund mice were euthanized and examined for leukemia development. We performed cytospin analysis for the bone marrow and spleen cells of the moribund mice and observed that majority of the cells resembled myeloid progenitors, supporting the development of CML blast crisis (Figure 22A). In confirming the myeloid origin of these cells we observed that more than 95% of the leukemic cells were positive for Gr1 and Mac1 myeloid lineage markers and negative for CD4, CD19 and Ter119 markers (Figure 22B). We also observed that less than 4% of the leukemic cells were c-Kit positive and less than 2% were positive for Sca-1(Figure 22C). Pathologic examination of these mice also showed that they had developed leukemias with leukemic infiltrations into spleen and liver (Figure 23). Mice transplanted with GMP cells that were singly transduced with either *BCR/ABL* or *Myb* expressing retrovirus did not develop leukemia and remained healthy for the 6 months period (Figure 21B). We confirmed the expression of *Myb* and *BCR/ABL* in *BCR/ABL* + *Myb* leukemias by QRT-PCR analysis (Figure 34).We also tried to establish cell lines form *BCR/ABL+Myb* leukemic cells but were unable to do so.

We also performed secondary transplantation experiment for *BCR/ABL + Myb* short isoform leukemias. We observed that all secondary transplanted mice developed leukemia within 4 weeks of transplantation (Figure 21B).

In combination, these data demonstrate that *Myb* can cooperates with BCR/ABL to induce CML myeloid blast crisis *in vivo*, possibly by conferring self-renewal capabilities to GMPs.

Increased *Myb* expression is detected in CML Blast Crisis Patients

The ability of *Myb* to cooperate with *BCR/ABL in vivo* to transform GMPs into LICs suggests that its activation in LICs may play a critical role in the progression of human CML myeloid blast crisis. To test this idea, we collaborated with Dr. Ravi Bhatia at University of Alabama to examine *MYB* mRNA levels in the BM aspirates of normal and CML patients at different stages of CML progression. Relatively low levels of *MYB* mRNA were observed in the BM of normal human volunteers, chronic phase CML patients and accelerated phase patients. In contrast, significantly higher levels of *MYB* mRNA were detected in blast crisis CML patients (Figure 24). This result supports the notion that *MYB* could be a critical regulator of LICs self-renewal in human myeloid CML blast crisis.

CHAPTER 6: TO EXAMINE WHETER *β-CATENIN* IS CRITICAL FOR MAINTAINING *SETBP1* SELF-RENEWAL IN MYELOID PROGENITORS

Targeting genes enabling unlimited self-renewal of leukemic stem cells or leukemia-initiating cells (LSCs/LICs) represents a promising therapeutic strategy for treating myeloid leukemias. Previously, our laboratory found that overexpression of *Setbp1* promotes the self-renewal of granulocyte-macrophage progenitors (GMPs) in vitro and in vivo, suggesting that *Setbp1* could be involved in the regulation of LSC/LIC self-renewal. Abnormal activation of Wnt/β-catenin pathway has also been implicated in conferring self-renewal capability to GMPs during chronic myeloid leukemia (CML) disease progression [24]. To investigate possible interaction between *Setbp1* and Wnt/β-catenin pathway and to explore a possible role of *Setbp1* in modulating Wnt/β-catenin pathway we examined the colony forming potential of *Setbp1* immortalized myeloid progenitors after β-catenin (*Ctnnb1*) deletion.

We first generated a β-catenin conditional *Setbp1*-immortalized myeloid progenitor cell line. For this purpose, bone marrow cells were isolated from β-catenin (*Ctnnb1*) conditional mice (Figure 3) [91]. These BM cells were then expanded under two different cytokine condition (as described in chapter 3). Expanded myeloid progenitor cell culture was then retrovirally transduced with *Setbp1* cDNA. After transduction cells were passaged for 2-3 weeks in the presence of SCF and IL-3 to establish immortalization. The immortalized cells were then transduced again with retrovirus expressing Cre-ERT2 (pMSCV-Neo-Cre-ERT2) which is a tamoxifen-inducible cre-recombinase. Neomycin (G418) treatment was used to select Cre-ERT2 virus infected cells after 48 hour of infection. Cells were then maintained in the presence of neomycin (Figure 25A).

Once we had made the β-catenin conditional *Setbp1*-immortalized myeloid progenitor cell line (2176-cre), we tested the cells for the efficiency of Cre-ERT2 mediated β-catenin deletion by treatment with 4-hydroxytamoxifen (4-OHT). For this experiment $5X10^5$ cells were cultured in 24 well plate with 2ml media containing SCF and IL3 and treated with 1mM 4-OTH. Cells treated with ethanol were included as control group. Genotyping analysis was done 48hrs after treatment to test for the deletion of β-catenin in the 4-OHT treated experimental group using PCR. We used a combination of 3 primers RM41/42/43 for our genotyping assay [91]. For β-catenin deletion the combination of these 3 primers generates a 500bp PCR product, for the undeleted allele a 324bp PCR product is generated. Only deletion bands were detected in 2 of the cell lines after 4-OHT treatment suggesting β-catenin deletion occurring efficiently in these cells (Figure 25B).

Next we tested whether β-catenin knockout in these cells would affect their self-renewal potential. To test this, 2176 cre cells ($1X10^4$) were plated per plate in methylcellulose media supplemented with SCF and IL3. Cells were divided into two groups with one group treated with 4-OHT and the other group treated with ethanol. Colonies were counted 8-10 days after plating. We observed that there was a significant decrease in the numbers of colonies in 4-OHT treated plates as compared to control plates. Colony assay cells were isolated and analyzed for deletion of β-catenin by genotyping assay (Figure 25C).

Our above data suggested to us that continuous expression of β-catenin is required by *Setbp1* immortalized cells to maintain their self-renewal capability.

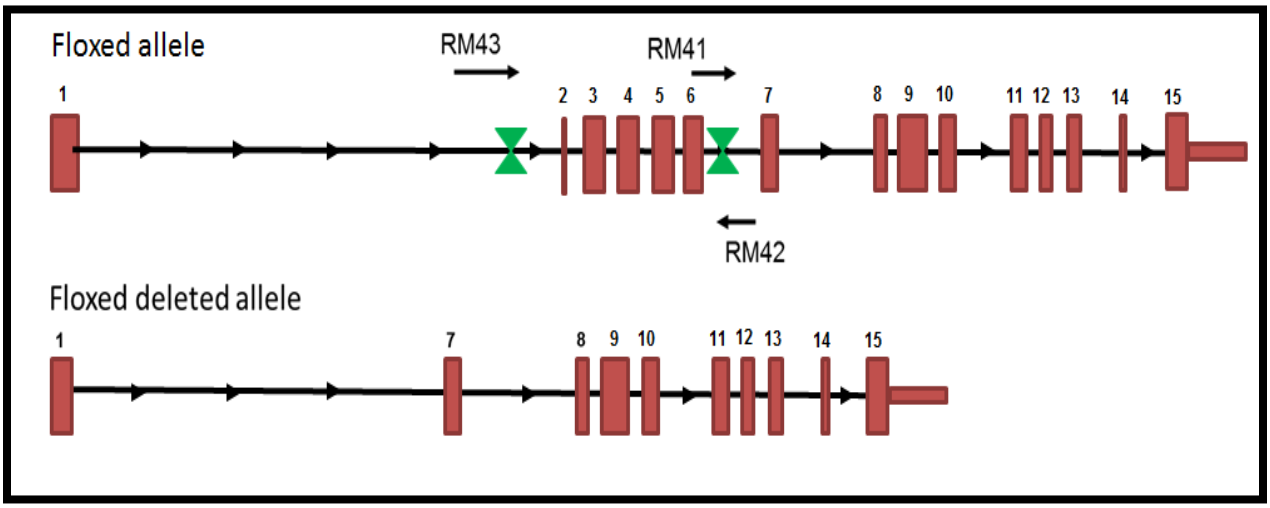


Figure 3:Schematic diagram of β-catenin conditional floxed allele and floxed deleted allele after 4-OHT induced CRE-ERT2 recombination

Individual exons are numbered from 1 to 15 and represented by brown boxes. Intermittent solid lines between the exons represent the intronic region. Green triangles represent the loxP sites on the floxed allele. 4-OHT induced CRE-ERT2 mediated recombination deletes region consisting of exon 2 to exon 6 and generates truncated floxed deleted allele.

In order to rule out that the concentration of 4-OHT used in this experiment was toxic to cells, we treated *Setbp1* immortalized wild-type myeloid progenitor cell lines with same 4-OHT concentration. We did not observe any decrease in colony formation in these cell lines, suggesting that 4-OHT is not toxic to these cells at this concentration.

For experiments involving cre-mediated gene deletion there has been some concern regarding cre-toxicity, where activity of cre can induce cell death [92]. This phenotype is unrelated to the cre activity on target gene. To address this problem and to confirm the results of β-Catenin knockout experiment, we decided to test lower concentrations of 4-OHT for deletion experiment. We found

that we were able to achieve complete deletion of β-catenin at 100nM of 4-OHT. Next we decided to repeat the β-Catenin deletion and colony assay experiment using 100nM 4-OHT. However under this condition we did not observe significant decrease in the colony forming capability of β-Catenin deleted cells. This suggested that the decrease in colony forming capability of β-Catenin deleted cells that we observed using 1mM 4-OHT was likely due to cre toxicity.

Because a truncated β-Catenin is still produced after cre recombination of this β-Catenin conditional allele, which could be sufficient to support the self-renewal of *Setbp1*-immortalized cells we decided to change our approach and use a shRNA knockdown strategy. We knocked down β-catenin in *Setbp1* immortalized cells using two different β-catenin-specific lentiviral shRNAs. Lentivirus carrying a non-targeting shRNA was used as control. We observed dramatic decrease in colony-forming potential for β-catenin knockdown cells in comparison to control cells (Figure 26A). We confirmed the knockdown of β-catenin by performing QRT-PCR and Western blotting analysis (Figure 26A). To confirm functional knockdown of β-Catenin we investigated the effect of β-catenin knockdown in these cells on a known β-catenin downstream target *Myc* (Figure 26B). For sh1 shRNA mediated knockdown, we observed significant decrease of *Myc* mRNA. This was opposite to the *Myc* mRNA levels observed for sh2 shRNA, where there was significant increase of *Myc* mRNA levels after β-catenin knockdown. We used UCSC genome browser blat search to identify what region of β-catenin was targeted by these two shRNAs. We observed that sh1 shRNA targets the 3'UTR region of β-catenin and sh2 shRNA targets region encoded by both 3rd and 4th exon of β-catenin (Figure 4). UCSC genome browser suggested a potential shorter isoform of β-catenin starting form exon 10. If the mRNA encoding this potential β-catenin isoform was generated in our cells, then it would be targeted by sh1 shRNA but not by sh2 shRNA. Continued

expression of this shorter isoform may explain the lack of reduction in *Myc* expression in sh2 shRNA transduced cells.

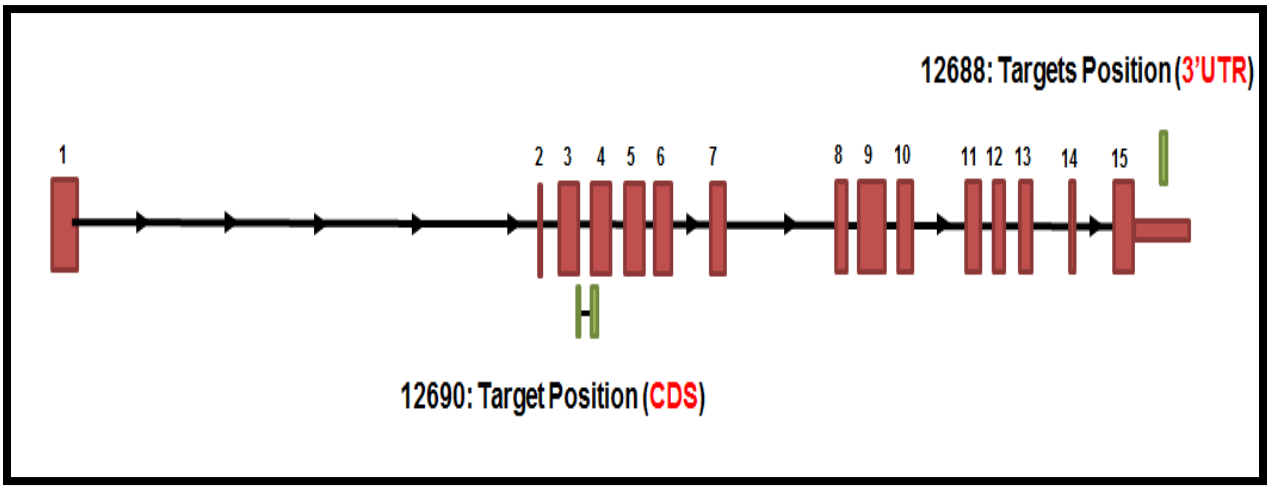


Figure 4:Schematic diagram of β-catenin specific shRNA targeting regions

12688 shRNA (sh#1) targets the 3'UTR region of β-catenin. 12690 shRNA (sh#2) targets the region encoded by both 3rd and 4th exon of β-catenin. Target region of both the shRNA are represented by green lines. Individual exons are numbered from 1 to 15 and represented by brown boxes. Intermittent solid lines between the exons represent the intronic region.

Since sh1 shRNA targets both full-length and shorter isoform, to test the importance of this potential shorter isoform, we investigated whether expression of constitutively active full-length β-catenin could rescue the effect of sh1 shRNA in *Setbp1*-immortalized myeloid progenitor cells. *Setbp1* immortalized myeloid progenitors (23923 cells) were infected with sh1 shRNA. Lentivirus carrying a non-targeting shRNA was used as control. 24 hrs. after infection cells were infected with retrovirus expressing constitutively active β-catenin cDNA (MSCV- ΔGSKβ-catenin-IRES-GFP) or empty MSCV virus. Infected cells were selected in media containing puromycin for next 24hrs and analyzed by colony assay forming assay. As expected we observed significant decrease

in the colony forming capability of sh2 shRNA transduced knockdown cells when compared to control. However ectopic expression of constitutively active β-catenin was not able to rescue the decrease in colony forming capability of 23923 cells (Figure 27).

Our above results point towards the possibility of the presence of a potential shorter isoform of β-catenin that is required by *Setbp1* immortalized myeloid progenitors to maintain self-renewal capability. The existence of such an isoform also help explain the lack of effects on colony formation by β-catenin deletion in *Setbp1*-immortalized cells as exons encoding this short isoform are not affected by the deletion using this conditional allele [91].

To further test whether a smaller putative isoform of β-catenin is made in our *Setbp1* immortalized progenitor cells, we decided to perform 5' rapid amplification of cDNA ends (5'RACE). For this experiment total RNA was isolated form Setbp1-immortalized cells. From this total RNA, first-strand cDNA synthesis was done using the SMARTer RACE 5'/3' Kit (Clonetech). Next, amplification of cDNA ends was performed using nested PCR with gene specific primer and products were resolved on agarose gel (Figure 28). This was followed by isolation and characterization of the amplified RACE product. By this method we were able to isolate a major 5'RACE fragment and characterize it by sequencing. By performing BLAT search on UCSC genome browser we found that this fragment aligns with β-catenin from the end region of exon 7 and the translational start site for this short isoform is in exon 8.

We subsequently cloned the coding region of this short isoform in MSCV-IRES-GFP retroviral vector and investigated whether expression of short isoform of β-catenin could rescue the effect of β-catenin knockdown in *Setbp1*-immortalized hematopoietic cells (BM70 cells). BM70 cells were infected with sh1 shRNA. Lentivirus carrying a non-targeting shRNA was used as control.

24 hrs. after infection cells were infected with retrovirus expressing constitutively active β-catenin cDNA (MSCV- ΔGSKβ-catenin-IRES-GFP) or short β-catenin isoform cDNA (MSCV-short isoform β-catenin-IRES GFP) or empty MSCV-IRES-GFP virus. Infected cells were grown in media containing puromycin for next 24hrs. 48hrs after knockdown GFP positive cells were sorted from each sample and colony assay was performed using $1X10^4$ cells per plate. However, we observed that ectopic expression of neither constitutively active β-catenin nor β-catenin short isoform was able to rescue the decrease in colony forming capability of BM70 cells after sh1 shRNA infection (Figure 29).

Although β-catenin short isoform was unable to rescue the decrease in colony forming capability of BM70 cells induced by sh1 shRNA transduction, we observed that ectopic expression of this β-catenin short isoform was able to increase the colony forming potential of BM70 cells in control samples. We did not observe a similar phenotype for constitutively active β-catenin control samples.

These above data suggest that while both full-length and the shorter isoform of β-catenin are likely required, the shorter isoform of β-catenin is more potent in driving the self-renewal of *Setbp1*-immortalized cells than the full-length β-catenin.

CHAPTER 7: *MEF2C* AND *MS12* CANNOT CONFER SELF-RENEWAL CAPABILITY TO COMMITTED MYELOID PROGENITORS

Monocyte Enhancer Factor 2C (*MEF2C*)

ChIP assay previously performed in our laboratory using Setbp1-immortalized myeloid progenitors also identified binding of Setbp1 to the promoter region of another known oncogenic transcription factor *Mef2c*. *Mef2c* belongs to the MADS-box containing monocyte enhancer factor (MEF2) family of transcription factors [93]. *Mef2c* was originally identified as a transcription factor that functions in skeletal muscle formation [94]. Within the hematopoietic system, *Mef2c* is highly expressed in HSCs and CMPs [95]. Studies have later revealed the role of *Mef2c* in leukemic transformation where aberrant *Mef2c* expression was associated with hematological malignancies. Studies involving deregulation of *Mef2c* expression by retrovirus-induced insertional mutagenesis has shown to accelerate myeloid leukemia in mice [96, 97]. Leukemic studies involving Nup98-Hoxa10 and MLL translocations have identified activation of *Mef2c* expression in microarray analysis in these translocations [28, 98]. More recently, studies have also identified *MEF2C* to be significantly up regulated in CML, where increase in its expression level is associated with CML progression[99, 100]. Study by Krivtisov *et al*. has shown decrease in colony formation in leukemic MLL-AF9 GMP after *Mef2c* knockdown [28] suggesting that *MEF2C* may play an important role in myeloid differentiation arrest. These studies indicate that *Mef2c* plays an important role in cooperating with other mutations during leukemic transformation but whether it also plays a critical role in mediating self-renewal of LICs from committed progenitors is not known.

So we wanted to examine the requirement of *Mef2c* in the maintenance of the self-renewal of cells immortalized by *Setbp1* overexpression. To test this we used a knockdown approach in BM70 cells (bone marrow cells immortalized by retroviral insertional activation of *Setbp1*). We knocked down *Mef2c* in these cells using *Mef2c*-specific lentiviral shRNAs. Lentivirus carrying a non-targeting shRNA was used as control. 48 hours after lentiviral shRNAs infection, $2X10^4$ puromycin resistant cells were plated per plate for colony formation assay. We observed a significant decrease in colony-forming potential for *Mef2c* knockdown cells in comparison to control cells (Figure 30). This data suggested that continuous expression of *Mef2c* is essential for *Setbp1* immortalized cells to maintain their self-renewal capability.

Next we tested if ectopic expression of *Mef2c,* like *Hoxa9* and *Hoxa10,* would induce immortalization of primary myeloid progenitors in culture in the presence of SCF and IL3. For this study, we transduced myeloid progenitors (harvested from C57BL/6 mice) with MSCV retrovirus expressing *Mef2c* cDNA (MSCV-Mef2c-PGK-Puro). Cells infected by empty virus were included as negative control. Infections were carried out using low titers of virus to reduce the chance of insertional mutagenesis (1 virus: 4 cells). Infected cells were passaged for 4 weeks to test whether they were immortalized. 4 weeks after infection there were no proliferating myeloid progenitor cells present in any of the cultures transduced by *Mef2c* virus. Similar results were also obtained when we tested purified GMPs. These results suggest to us that ectopic expression of *Mef2c* is unable to immortalize primary myeloid progenitors *in vitro.*

We also investigated whether ectopic expression of *Mef2c* is able to cooperate with *BCR/ABL in vivo* to transform GMPs, causing CML myeloid blast crisis. To test this, we co-transduced GMPs (sorted from C57BL/6 mice), with pMSCV-BCR/ABL-IRES-GFP and a pMSCV retrovirus expressing *Mef2c* (pMSCV-Mef2c-PGK-Puro). Co-transduced GMPs were then transplanted into

lethally irradiated congenic recipient mice along with supporting bone marrow ($1.5x10^5$ GMPs and $7.5x10^5$ Supporting Bone Marrow cells per mice). Mice were then aged for 6 months for possible development. None of the mice that were transplanted with GMPs co-expressing both *Mef2c* and *BCR/ABL* developed leukemia suggesting that *Mef2c* is unable to cooperate with *BCR/ABL* in transforming GMPs into LICs to promote CML myeloid blast crisis *in vivo*.

In combination, these results suggest that *Mef2c* is essential for *Setbp1* induced self-renewal but not sufficient by itself to confer self-renewal capability to myeloid progenitors.

Musashi 2 (*MSI2*)

Musashi 2 (*MSI2*) is a RNA-binding protein of Musashi family. *MSI2* is preferentially expressed in HSCs where it acts as a critical regulator of hematopoiesis and HSC maintenance [101]. Studies have also shown that *MSI2* is important to maintain the self-renewal program in LICs in AML [102]. Studies have also identified involvement of *MSI2* in CML. Translocation mutations of MSI2 with *HOXA9* has been associated with MSI2 deregulation and CML progression [103, 104]. Aberrant activation of *MSI2* is also observed during blast crisis progression for CML having *NUP98-HOXA9* mutation, where expression of *MSI2* is shown to be regulated by *HOXA9* [103]. *In vivo* experiments have further shown that in doxycycline inducible *Msi2* BM cells (not GMPs), *Msi2* can cooperate with *BCR/ABL* and result in aggressive myeloid leukemia in mice resembling myeloid blast crisis of CML [101].

Previous studies in our laboratory have also found overexpression of *Msi2* in *Setbp1*-immortalized cells, suggesting that *Msi2* could play a role in promoting self-renewal of these cells. So we decided to investigate the role of *Msi2* in conferring self-renewal capabilities to primary myeloid progenitors *in vitro*. To investigate whether ectopic expression of *Msi2* can confer self-renewal

capability to myeloid progenitors we transduced myeloid progenitors (generated as described in Figure 5A) with MSCV retrovirus expressing *Msi2* cDNAs (MSCV-Msi2-PGK-Puro) for two different *Msi2* isoforms reported previously [105]. Cells infected by MSCV retrovirus expressing *Hoxa9* cDNA virus were included as positive control. Infected cells were passaged for 4 weeks to test whether they were immortalized. 4 weeks after infection there were no proliferating myeloid progenitor cells present in any of the cultures transduced by either *Msi2* isoform expressing retrovirus, suggesting that neither of the two *Msi2* isoforms could induce immortalization of myeloid progenitors. In contrast *Hoxa9* virus was able to immortalize primary myeloid progenitors in culture in the presence of SCF and IL3 as described previously. This data suggest that ectopic expression of *Msi2* alone is unable to confer self-renewal capability to primary myeloid progenitors.

CHAPTER 8: DISCUSSION

Hoxa9 and *Hoxa10*

Mechanisms underlying the progression of CML from chronic phase to blast crisis are not well understood. It is believed that there is a shift of LIC population during this transition. It has been suggested that in chronic phase HSCs with *BCR/ABL* mutation are the source of LICs. Whereas in the case of blast crisis, myeloid progenitors (GMPs) that have acquired self-renewal capabilities due to additional mutations become the source of LICs[24]. Our lab has previously shown that *SETBP1* activation contributes to this progression by conferring unlimited self-renewal capability to GMPs [1]. In this study both *Hoxa9* and *Hoxa10* were identified as critical *Setbp1* downstream regulators of self-renewal during leukemic transformation of myeloid progenitors. We showed here that overexpression of *Hoxa9* or *Hoxa10* alone is sufficient to promote self-renewal of myeloid progenitors *in vitro* in the presence of stem cell factor and IL-3. In supporting the role of *Hoxa9* and *Hoxa10* in conferring self-renewal capability to LICs *in vivo*, we demonstrated that *Hoxa9* and *Hoxa10* are able to cooperate with *BCR/ABL* in transforming GMPs inducing development of aggressive leukemia which resembles CML myeloid blast crisis. Flow cytometric analysis for the leukemic cells isolated from both spleen and BM cells of moribund mice confirmed that majority of the leukemic cells were negative for c-Kit marker. We are sure that the antibody used for c-Kit detection is very effective during flow cytometric analysis as we were able detect c-Kit expression in recipient mice bone marrow and spleen cells. This is similar to the cooperation of Setbp1 with *BCR/ABL* during induction of CML blast crisis in mice [1]. Therefore, we have identified two new pathways that can confer limitless self-renewal potential to GMPs which normally lack the self-renewal capabilities. Although *Hoxa9* and *Hoxa10* are known to have

overlapping targets, our results further showed that *BCR/ABL + Hoxa9* mice developed leukemia with similar latency as *BCR/ABL + Setbp1* mice, suggesting that *Hoxa9* is the major downstream mediator of *Setbp1*-induced self-renewal and its cooperation with *BCR/ABL* during leukemic transformation. High degree of homology for 60 amino acid DNA-binding HOX homeodomain among HOX proteins is very likely responsible for the overlap in their targets [83]. It is known that HOX proteins interact with cis regulatory elements (enhancers and promoters) of its target in combination with HOX cofactors and lineage specific transcriptional factors [85]. Overexpression of HOX proteins can result in enhanced interaction with cofactors and transcriptional factors. This may cause stronger (stable) binding at the target cis elements and result in more up-regulation or down-regulation of target genes [87]. There is very little homology between these two HOX proteins outside the 60 amino acid DNA-binding-HOX homeodomain, suggesting that they are likely to have different interacting partners [83]. It was also suggested that *Hoxa9* and *Hoxa10* can identify different cis-regulatory elements for the same target gene [88]. Different specificity for downstream targets between *Hoxa9* and *Hoxa10* and their respective cofactors can probably cause the observed difference in the latencies of respective leukemias in our study [51, 85, 87, 88].

Myb

It has been proposed that *Myb* is a critical target of *Hoxa9* in myeloid progenitors [70]. This notion is supported by our detection of high level of *Myb* expression in *BCR/ABL + Hoxa9* leukemic samples. Interestingly, our data also identify similar *Myb* expression in *BCR/ABL + Hoxa10* leukemic samples. This suggests that *Myb* could be a common target of *Hoxa9* and *Hoxa10*. It is unclear how *Hoxa10* activate *Myb* expression. We further showed that continuous expression of *Myb* is important for self-renewal induced by *Hoxa9* and *Hoxa10* as *Myb* knockdown significantly reduced colony forming potential of myeloid progenitors immortalized by *Hoxa9* and *Hoxa10*.

Results from this experiment suggest that *Hoxa9* and *Hoxa10* infection confer self-renewal capabilities to early hematopoietic progenitor cells by activating *Myb* expression as early as 48hrs of infection. On the other hand cells infected with empty virus do not show *Myb* activation and therefore tend to undergo differentiation. As *Myb* is normally expressed at high levels in early hematopoietic progenitor cells, it is also possible that maintaining this levels of *Myb* by *Hoxa9* or *Hoxa10* may be sufficient to block differentiation. In our experiment we see that differentiation is correlated to the Myb protein levels. Control cells show low to undetectable Myb protein level in 48hr samples (less differentiated) and colony assay samples (more differentiated) respectively. Whereas *Myb* activation in *Hoxa9* and *Hoxa10* infected cells present proliferating progenitor phenotype. Our data also indicated that there is more significant increase in the *Myb* protein levels as compared to Myb mRNA level between control and infected cells, suggesting that *Myb* expression could be regulated post transcriptionally in *Hoxa9* and *Hoxa10* infected progenitor cells. Interestingly, we also found *Myb* can confer self-renewal capability to myeloid progenitor as ectopic expression of *Myb* efficiently immortalized primary myeloid progenitors in culture and was also capable of cooperating with *BCR/ABL* to induce leukemic transformation of GMPs in mice.

Myb has been shown to be direct target of *Hoxa9-Tale* complex involving *Meis1* and *Pbx* protein in cell line studies [106]. From our study, activation of *Myb* mRNA (in early hematopoietic progenitors) within 48hrs of infection of *Hoxa9* or *Hoxa10* suggested to us that *Myb* could be a direct transcriptional target of *Hoxa9* and *Hoxa10*. We wanted to further confirm the direct binding of *Hoxa9* and *Hoxa10* at *Myb* promoter region by ChIP analysis. But anti-Hoxa9 and anti-Hoxa10 antibodies have been a problem for ChIP experiment and do not work well. Also, we do not have FLAG-tagged Hoxa9 or Hoxa10 protein construct, so cannot use anti FLAG antibody for our ChIP

experiment instead of anti-Hoxa9 and anti-Hoxa10 antibody. *Myb* activity is primarily regulated at transcriptional level [107] and mainly through transcriptional attenuation, which involves a block in transcriptional elongation within the first intron [107]. It is also suggested that during leukemic transformation, cell can overcome this *Myb* transcription attenuation. This is achieved when Hoxa9-Tale complex identifies a different promoter in intron 1 of *Myb*, switching the RNA Polymerase II occupancy and thus overcome the transcriptional block[106]. It is unclear how *Hoxa10* could activate *Myb* transcription but it is possible that utilization of a different promoter might be involved. *Myb* intrinsically does not have transforming abilities [70] which could explain the longer latency and incomplete penetrance for CML blast crisis induced by *BCR/ABL+ Myb* as additional mutations may be required. Our results suggest that activation of *Myb* is a critical downstream event of *Setbp1/Hoxa9/Hoxa10* activation in their cooperation with *BCR/ABL* to induce CML blast crisis transformation.

Our examination of CML patient samples identifies significant activation/overexpression of *Myb* in CML blast crisis patients in comparison to non-leukemic, chronic phase and accelerated phase samples. Current drug therapies for the treatment of CML use small molecule tyrosine kinase inhibitors (TKIs) such as imatinib or second generation compounds such as dasatinib or nilotinib [16, 17] to target the disease in chronic phase. Although these TKIs are effective in bringing remission of disease in chronic stage patients, 10-20% of patients showed relapse during the treatment. In blast crisis patients, response to TKIs is very limited. Recent studies have identified that leukemic cells with increased *Myb* levels tend to be more sensitive than normal cells to the levels of *Myb* and can be specifically targeted using *Myb* inhibitors such as celastrol [108]. Our results suggest that *Myb* may represent promising therapeutic target for treating CML myeloid blast crisis.

MYB protein has three major functional domains. These are, DNA binding domain (DBD), transactivation domain (TAD) and negative regulatory domain (NRD)[69]. As a transcription factor MYB function is facilitated by a number of co-regulators which can interact with these domains. These include *CBP/P300, P100, Menin*, *FLASH* and *MI-2α* as co-activators and *N-CoR*, *c-Ski* and *TIF1β* as co-repressors[69]. Many of the MYB transcritional targets like *CD34, c-Kit, c-Myc, Flt-3, Bcl-2, Gstm1, Smyd2, and Bmi1* have important role in the proliferation and survival of hematopoietic cells. Studies have highlighted the role of MYB transcriptional targets in leukemia and leukemia initiating cells survival. For example, BMI-1 expression has been shown to be significantly higher in the blast and accelerated phase patients than in the chronic phase CML patients[109]. Similarly MYC is shown to be occasionally amplified and overexpressed in CML-BC patients[110]. FLT-3 mutations have been identified in a subset CML patients[111]. Inhibition of BCL-2 has been shown to be effective in targeting LICs and CML progenitors[112, 113]. For therapeutic purpose targeting MYB interaction with P300 and other co-regulators represent an effective strategy. For example, as mentioned above a small-molecule inhibitor of the *Myb/p300* interaction called celastrol has been shown to inhibit proliferation of AML cells in vitro and enhance survival of mice with aggressive AML in vivo[108]. Targeting downstream effectors of *Myb* like *Bcl-2* and *Myc* (with known inhibitors) can also be a good strategy[113, 114]. Finally, our leukemic mice model offer excellent *in vivo* setup for testing the role of these inhibitors in reducing leukemic burden and providing survival advantage to leukemic mice.

Wnt/β-Catenin

Abnormal activation of Wnt/β-Catenin pathway has been implicated in conferring self-renewal capability to committed myeloid progenitors during chronic myeloid leukemia (CML) disease progression. We tested this oncogenic role of β-catenin activation in *Setbp1*-immortalized β-

catenin conditional cell line where deletion of β-catenin could be induced after 4-OHT treatment. From colony assay experiment we observed decrease in the self-renewal capability after β-catenin deletion, although this decrease was likely due to cre toxicity. Following a shRNA knockdown approach, targeting β-catenin, we observed dramatic decrease in colony-forming potential for β-catenin knockdown cells confirming that β-catenin is required by *Setbp1*-immortalized myeloid progenitors to maintain self-renewal capability. By performing 5' rapid amplification of cDNA ends (5'RACE) we were able to isolate and clone a potential shorter isoform of β-catenin. We were unable to detect the short isoform of β-catenin in the Western Blotting analysis of whole cell lysate from *Setbp1*-immortalized cells. This could be partly due the presence of short isoform of β-catenin protein at very low levels in these cells or due to the inability of c-terminal anti-β-catenin antibody to pick up the shorter isoform. Still, we were able to confirm the presence of short isoform of *β-catenin* by identifying the endogenous shorter isoform's mRNA by 5' RACE experiment in *Setbp1*-immortalized cells. Ectopic expression of this β-catenin short isoform was able to increase the colony forming potential of *Setbp1*-immortalized cells in control samples. We did not observe a similar phenotype for constitutively active β-catenin control samples. In our rescue experiment, although we saw decrease in β-catenin mRNA and protein levels, neither full-length β-catenin nor its shorter isoform was able to rescue the β-catenin knockdown effect in *Setbp1*-immortalized cells. This suggest to the possibility that decrease in self-renewal capability of *Setbp1*-immortalized after β-catenin knockdown could be due to non-specific targeting of shRNA. Our data also suggested that while both full-length and the shorter isoform of β-catenin are likely required, the shorter isoform of β-catenin is more potent in driving the self-renewal of *Setbp1*-immortalized cells than the full-length β-catenin.

Cytoplasmic β-catenin protein is acted upon APC-Axin complex and phosphorylated by glycogen synthase kinase 3β (*Gsk3β)* near the NH2-terminal residues [115].The potential shorter isoform of β-catenin identified and cloned by us lacks the putative *Gsk3β* phosphorylation site, but still maintains lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factors and thus can potentially regulate the transcription of its target genes. Studies have shown that an artificially generated, N-terminally truncated β-catenin protein was stable and accumulated in the nucleus [116]. This might explain why the shorter isoform of β-catenin is more potent in driving the self-renewal of *Setbp1*-immortalized cells than the full-length β-Catenin. Further studies will be needed to further clarify the role of this shorter isoform of β-catenin in CML blast crisis development.

Table 1:Cell types used in vitro and in vivo experiments

Gene	*in vitro* Immortalization, Cell Type			Growth Factors	*in vivo* Leukemia Formation from GMPs
	MPs	5FUPs	GMPs		
Setbp1	+	NT	+	SCF+IL-3	-
BCR/ABL	-	NT	-	NA	-
Hoxa9	+	+	+	SCF+IL-3	-
Hoxa10	+	+	+	SCF+IL-3	-
Myb	+	NT	+	SCF+IL-3	-
Setbp1+BCR/ABL	NT	NT	NT	NA	+
Hoxa9+BCR/ABL	NT	NT	NT	NA	+
Hoxa10+BCR/ABL	NT	NT	NT	NA	+
Myb+BCR/ABL	NT	NT	NT	NA	+

MPs=Myeloid Progenitors, 5FUPs=5FU treated BM Progenitors,
GMPs=Granulocyte Macrophage Progenitors, NT=Not Tested,
NA=Not Applicable, **(+)** sign indicate immortalization or leukemia formation, **(-)** sign indicate inability to immortalize or induce leukemia.

METHODS

Mice

7-12 weeks old C57BL/6 and C57BL/6-Ly5.2 mice were purchased from Charles River, Frederick, MD. These mice were maintained in the animal facility of Laboratory of Animal Medicine at Uniformed Services University of the Health Sciences (USUHS). All mouse experiments were carried out according to protocols approved by the USUHS Institutional Animal Care and Use Committee.

Retrovirus Generation

Hoxa9 and *Hoxa10* cDNA were cloned into the MSCV retroviral expression vector for the generation of pMSCV-*Hoxa9*-PGK-Neo and pMSCV-*Hoxa10*-PGK-Puro virus. *BCR/ABL* cDNA was cloned into MSCV retrovirus for the generation of MSCV-*BCR/ABL*-IRES-GFP virus. *Myb short and long isoform* cDNA were cloned into MSCV retrovirus for the generation of MSCV-*Myb*-IRES-GFP virus. Virus were produced by the transfection of *Hoxa9, Hoxa10, BCR/ABL*, *Myb* short & long isoform and β-catenin long & short isoform cloned retroviral plasmid into Plat-E virus packaging cell line using Fugene. Titer for the virus was determined by infecting NIH3T3 cells using serial dilution method.

***Msi2* retrovirus generation**: Total RNA isolated from 2176 cells was used to generate 1st strand cDNA using superscript III commercial kit from ThermoFisher Scientific. Primers designed for PCR amplification of *Msi2* coding sequence (CDS) were used to generate amplified PCR product. When we ran the amplified PCR product on agarose gel, we observed that there were two PCR products. These two bands represent the two isoforms of *Msi2*: a longer canonical isoform and a

shorter spliced variant [105]. DNA was then isolated from both the bands and cloned into MSCV-Puro expression vector. Transformation of MSCV-Msi2-Puro cDNA gave us colonies on LB agar plates from both bands. Individual colonies were grown and plasmid was isolated for sequencing. After confirming the positive clones for both isoforms of *Msi2*, we performed plasmid maxi prep for both bands of *Msi2*. Next virus was made using PlatE virus packaging cells from both the *Msi2* expression plasmids.

Retroviral transduction and Transplantation

For myeloid progenitors transduction bone marrow cells were harvested from femur of C57BL/6 mice. These cells were cultured in serum free medium containing cocktail of four cytokines; SCF, TPO, FGF-1 and IGF-2 for a period of 6 days to expand HSCs. These cells were then induced to differentiate into myeloid progenitors by culturing the cells in SCF and IL-3 medium for a period of 4 days. This was done to enrich the harvested BM cells for primary myeloid progenitors. We then transduced these myeloid progenitors with *Hoxa9*, *Hoxa10*, *Myb* short isoform and *Myb* long isoform expressing retrovirus using retronectin coated plates and passaged them for period of 4 weeks to assess immortalization of transduced myeloid progenitors**.**

For 5FU cell transduction, C57BL/6 mice were administered 5 Fluorouracil (5FU) (150mg/kg of body weight) intraperitonially. Four days after drug treatment, bone marrow cells were harvested from treated mice. After LSM treatment cells were washed with cold sterile PBS and re-suspended in culture media consisting of 15%FBS, DMEM, 1%PS, SCF(100ng/ml), IL3(6ng/ml) and IL6(10ng/ml). Cells were maintained under this culture condition for 5 days and then transduced by *Hoxa9* or *Hoxa10* expressing retrovirus virus. Empty virus was used as control. Cells were infected twice over 2 consecutive days. $2.8X10^6$cfu viruses were used to infect $7X10^5$ cells in 24

well plate treated with retronectin. Puromycin selection was done on third day of first infection. 24 hrs. after puromycin selection, colony assay was performed. 48hrs after puromycin selection protein and RNA samples were prepared. Cells were passaged for 4 weeks to establish immortalization.

For transplantation experiments, we transduced GMPs with 4 times of retrovirus using retronectin coated plates. We performed the infection process twice in two days. On third day the cells were isolated from the retronectin coated plate and (1.5×10^5 cells per mice) transplanted into lethally irradiated recipient mice (B6-Ly5.2) along with supporting bone marrow cells (7.5×10^5 cells per mice). Transplanted mice were aged and monitored for signs of leukemia development.

For secondary transplantation, 1×10^6 spleen cells from primary recipients with leukemia were injected into lethally irradiated secondary recipients (B6-Ly5.2) along with 7.5×10^5 supporting bone marrow cells. Mice were aged and monitored for leukemia development.

Lentiviral Production and Infection

pLKO.1 lentiviral constructs containing shRNA were used to generate virus in 293T cells by tranfecting them with lentivirus envelope and packaging plamids. We used LipoD293 as DNA transfection agent. For Infection, cells were infected with virus (1cell : 4virus) by centrifugation at 37^0C, 2000g for 90 minutes. Next, cells were incubated for 30 minutes at 37^0C and then pelleted and resuspended in SCF and IL-3 media and incubated at 37^0C. 24 hours after incubation antibiotic selection was performed.

Flow Cytometry

For GMP sorting, bone marrow cells were harvested from femur and tibia of donor mice (B6-Ly5.1). Harvested bone marrow cells were filtered and washed with ice cold PBS. Mononuclear cells were isolated from the bone marrow cells by density centrifugation using lymphocyte separation medium. Cells were then incubated with a cocktail of purified rat anti-mouse antibodies specific to Gr-1, Mac-1, CD4, CD8, B220, CD127, and Ter-119. Lineage positive cells were subsequently removed by magnetic separation using sheep anti-rat IgG conjugated magnetic beads (Invitrogen). The isolated lineage negative cells were then stained with anti-Sca-1-APC, anti-CD34-Alexa fluor-700, anti-c-Kit-PE, and anti-FcR-II/III-PE-Cy7. GMPs were sorted as population IL-7Rα^{-}Sca-1^{-}c-Kit^{+}Fc-γR-II/IIIhighCD34^{+} using FACSAria cell sorter.

For lineage determination of leukemic cells, bone marrow and spleen cells were harvested from sick euthanized mice. ACK lyses was done to deplete RBCs. Cells were subsequently blocked with anti-FcγR-II/III and incubated with antibodies against markers for myeloid (Gr-1, Mac-1), erythroid (Ter-119), lymphoid B (CD19) and T (CD4) lineage cells. Cells were also stained with anti CD45.2 antibody. Leukemic cell population was identified as cells positive for both GFP and CD45.2. Dead cells were excluded by staining with Sytox Blue (Invitrogen). Flow cytometry was performed using BD LSRII flow cytometer.

For engraftment, peripheral blood was obtained by retro-orbital bleeding of transplanted mice followed by similar process as mentioned above.

Colony Formation Assay

24hrs after infection cells were selected using blasticidin (14ug/ml).Colony assay was performed at 48 hrs. after *Myb* shRNA mediated knockdown. $1X10^4$ cells were plated per plate in methylcellulose medium supplemented with 15% Fetal Bovine Serum, mouse SCF (50ng/ml) and IL-3 (6ng/ml) and Blasticidin. Colony numbers were counted after 7days.

For β-catenin knockout colony assay we plated 2x104 cells per well in colony assay media (Methyl Cellulose, M3134, IMDM, FBS, SCF, IL3 and Penicillin/Streptomycin). Cells were divided into two groups with one group (Experimental) treated with G418 (1mg/ml) and Tamoxifen (1X) and the other group (Control) treated with G418 and Ethanol (1X). Colony assay plates were incubated at 37^0C. Colony numbers were counted after 7days.

Western blotting analysis

$1x10^6$ cells were washed in PBS and whole cell lysate were prepared by direct lysis of cell pellets in heated 2 x SDS sample buffer. SDS sample buffer treated cells were vortexed and boiled in water bath for 5 min and then cooled for 5 min on ice. Samples were then directly loaded on the gel or stored at -80^0 for future use. 4-12% tris-glycine gels from Life Technologies was used for resolving the protein samples and then transferred onto nitrocellulose membranes from Bio-Rad**.**

For *Myb* knockdown assay infected cells were selected using blasticidin (14ug/ml) 24hrs after infection cells. Protein samples were made in 2 x SDS sample buffer 72 hrs. after infection.

Following antibodies were used for western blot protein detection:

Primary antibodies: anti-Setbp1 (16841-1AP, Protein-tech), anti-Hoxa9 (07-178, Millipore), anti-Myb (05-175 Millipore), β-actin (MAB1501R, Millipore) and anti-β-catenin (9587, Cell Signalling).

Secondary antibodies: Goat anti rabbit IgG-HRP (sc-2004, Santa Cruz Biotechnology) and anti-mouse IgG-HRP (a-9044, Sigma Aldrich).

Protein bands were visualized by incubation with SuperSignal West chemi-luminescent substrate (Pierce) and quantified using Quantity One data analysis software (Bio-Rad).

Real-time RT-PCR

RNA preparation was done at 72 hrs. after infection with retrovirus or lentivirus. RNAeasy Plus mini kit from QIAGEN was used for isolation of Total RNA from cells. Superscript III from Invitrogen was used to prepare oligo-dT-primed cDNA from Total RNA. Next, real-time PCR analysis was performed in triplicates using SYBR green detection reagents from Invitrogen on a 7500 real time PCR system (Applied Biosystems). Relative changes in expression of *Hoxa9, Hoxa10, and cMyb* were calculated according to the ΔCt method. The cycling conditions are 50°C for 2 minutes, followed by 95°C for 2 minutes, and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute**.**

Splinkerette PCR

Genomic DNA was isolated from leukemic mice spleen cells. This genomic DNA was digested with 20 units of either NLAIII or MseI (NEB) for 4 hours at 37^0C. Enzyme was inactivated at 65^0C

for 20 minutes and purified using Qiaquick PCR purification kit (Qiagen). Digested genomic DNA was then ligated to either Splinkerette-NlaIII or Splinkerette-MseI linker overnight using T4 DNA ligase. Next we setup primary PCR reaction using ligation product with MuLV-LTR1 primer and Splinkerette Primer I. PCR product was diluted using 1:50 dilution and then secondary nested PCR was performed with MuLV-LTR2 primer and Splinkerette Primer 2. For our primary PCR we used Expand Long Template PCR system (Roche Life Science) and for secondary nested PCR we used Platinum Taq Green Hot Start DNA Polymerase (Thermo Fisher). PCR product was resolved on a 2% agarose gel. Distinct bands mostly under 1KB size were eluted from the gel and sequencing was performed using MuLV-LTR2 primer.

RACE

For 5' RACE reaction we first isolated total RNA from Setbp1 immortalized cells (2525IM). First strand cDNA synthesis was done from the total RNA using 5'-CDS Primer A, SMARTer II an Oligonucleotide and SMARTScribe Reverse Transcriptase provided in the kit. This resulted in the formation of 5' RACE ready cDNA. This 5' RACE ready cDNA was next used as a template for the rapid amplification of cDNA ends (RACE) reaction by performing PCR with universal primer A mix (UPM) provided in the kit and β-catenin gene specific primer 1 (GSP1). The PCR product generated was then used for nested PCR reaction using universal primer short and nested β-catenin gene specific primer 2 (GSP2). Nested PCR product was resolved on a 1.2% agarose gel. Distinct prominent band is isolated from the gel using gel extraction method.

The RACE product was cloned into pRACE vector using In-Fusion HD cloning Kit (Clonetech) and transformed into Steller Competent Cells. Individual colonies were picked, grown and digested with HindIII and EcoRI to select for clones with RACE product. Clones with RACE

product were then sequenced using M13F/R primers and used for characterization of β-catenin isoform. After identifying the putative short isoform of β-catenin we amplified the short isoform using β-catenin specific primers and cloned it into pMYs-Neo retroviral expression vector and MSCY-GFP expression vector.

Table 2:List of primers used

Primers	Sequence
Erg	Forward: CCGATGACGTTGATAAGGCTTT Reverse: GCTGCACCCCCTGTGTTT
Junb	Forward: CTGTGTCCCCCATCAACATG Reverse: TTCCGCTTCCGGCACTT
Msi2	Forward: GACCTGTCGCCGATCTCTAC Reverse: GCGCTTATGTAATTCCCCACTC
Sox4	Forward: CGGCGCAAGATCATGGA Reverse: TTTGCCTAGCCGCTTGGA
Myc	Forward: ACAGCAGCTCGCCCAAATC Reverse: AGCAGCGAGTCCGAGGAA
Pim1	Forward: GATCATCAAGGGCCAAGTGT Reverse: GATGGTTCCGGATTTCTTCA
Flt3	Forward: CTTTCGGGAGGGTGATGAAC Reverse: CCGCCACCTGAATTGAGACT
β-catenin	Forward: AAACTCCTGCACCCACCATC Reverse: ACTATCTCCTCCATGCGCAC
Myb	Forward: CCATGAAAGCTCGGGCTTAG Reverse: CTCGACATTGGTGTCAGTTTGTG
Hoxa9	Forward: TGTCTCCTCTCCCCCAAACC Reverse: GAGATGAGGCCTGGGATTTAGA
Hoxa10	Forward: CCACAGGCCACTTCGTGTT Reverse: TCGTAGAGGCAGTAGGAGCTCTCT
RPL4	Forward: ATGATGAACACCGACCTTAGCA Reverse: CGGAGGGCTCTTTGGATTTC
RM41	5’ AAG GTA GAG TGA TGA AAG TTG TT 3’
RM42	5’ CAC CAT GTC CTC TGT CTA TTC 3’
RM43	5’ TAC ACT ATT GAA TCA CAG GGA CTT 3’
MuLV-LTR1	5’ GCT AGC TTG CCA AAC CTA CAG GTG G 3’

MuLV-LTR2	5’ CCA AAC CTA CAG GTG GGG TCT TTC 3’
Splink-NlaIII	5’ GTT GTT AGG ACT GCT TGG AGG GGA AAT CAA T 3’
Splink-MseI	5’ TAG TTG TTA GGA CTG CTT GGA GGG GA 3’
Splinkerette-LONG	5’ CCT CCA CTA CGA CTC ACT GAA GGG CA 3’
Splinkerette Primer 1	5’ GGG CAA CTA CGA CTC ACT GAA GGG C 3’
Splinkerette Primer 2	5’ GGG CAA GCA GTC CTA ACA AC 3’
β-catenin GSP1	5’ GAT TAC GCC AAG CTT CGG TTG TGA ACG TCC CGA GCA AGG ATG T 3’
β-catenin GSP2	5’ GAT TAC GCC AAG CTT CGT GGA ATA GCA CCC TGT TC 3’

FIGURES AND LEGENDS

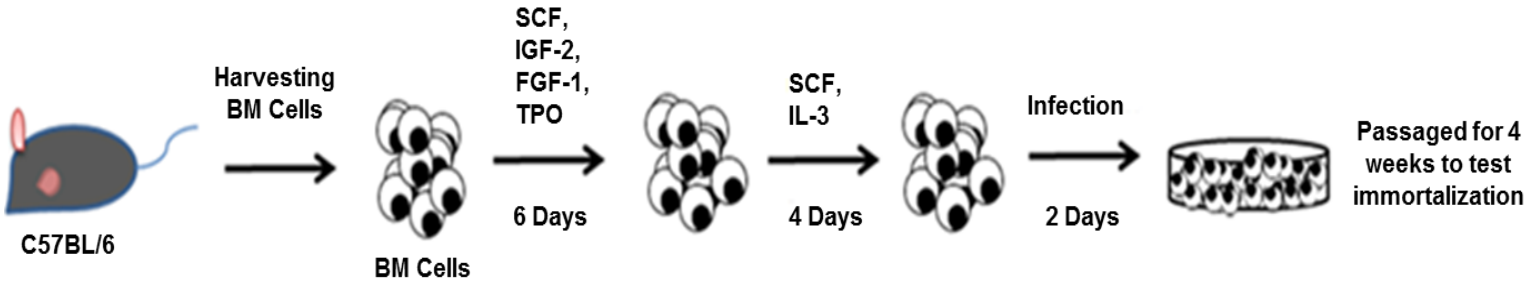


Figure 5: Schematic diagram of the immortalization procedure

Bone marrow cells were harvested from femur of C57BL/6 mice. These cells were cultured in serum free medium containing cocktail of four cytokines; SCF, TPO, FGF-1 and IGF-2 for a period of 6 days to expand HSCs. These cells were then induced to differentiate into myeloid progenitors by culturing the cells in SCF and IL-3 medium for a period of 4 days. This was done to enrich the harvested BM cells for primary myeloid progenitors. We then transduced these myeloid progenitors with *Hoxa9* expressing retrovirus using retronectin coated plates and passaged them for period of 4 weeks to assess immortalization of transduced myeloid progenitors.

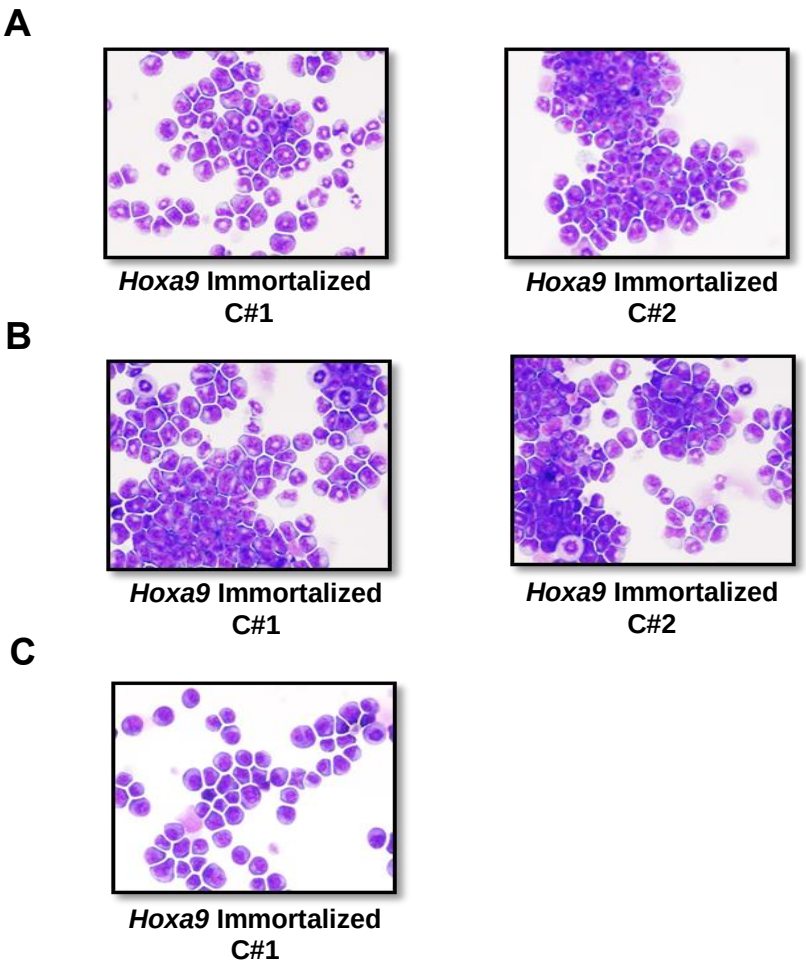


Figure 6: Ectopic expression of *Hoxa9* efficiently immortalizes primary myeloid progenitors in culture

(A) Cytospin preparation and Wright-Giemsa staining of *Hoxa9*-immortalized myeloid progenitors derived from myeloid progenitors generated by in vitro differentiation of HSCs. (B) Cytospin preparation and Wright-Giemsa staining of *Hoxa9*-immortalized myeloid progenitors derived from purified GMPs. (C) Cytospin preparation and Wright-Giemsa staining of Hoxa9-immortalized myeloid progenitors derived from 5-FU treated bone marrow progenitors. Original magnification 40X. Images were obtained using a Nikon Eclipse E800 microscope and a Qimaging Micropublisher 5.0 digital camera.

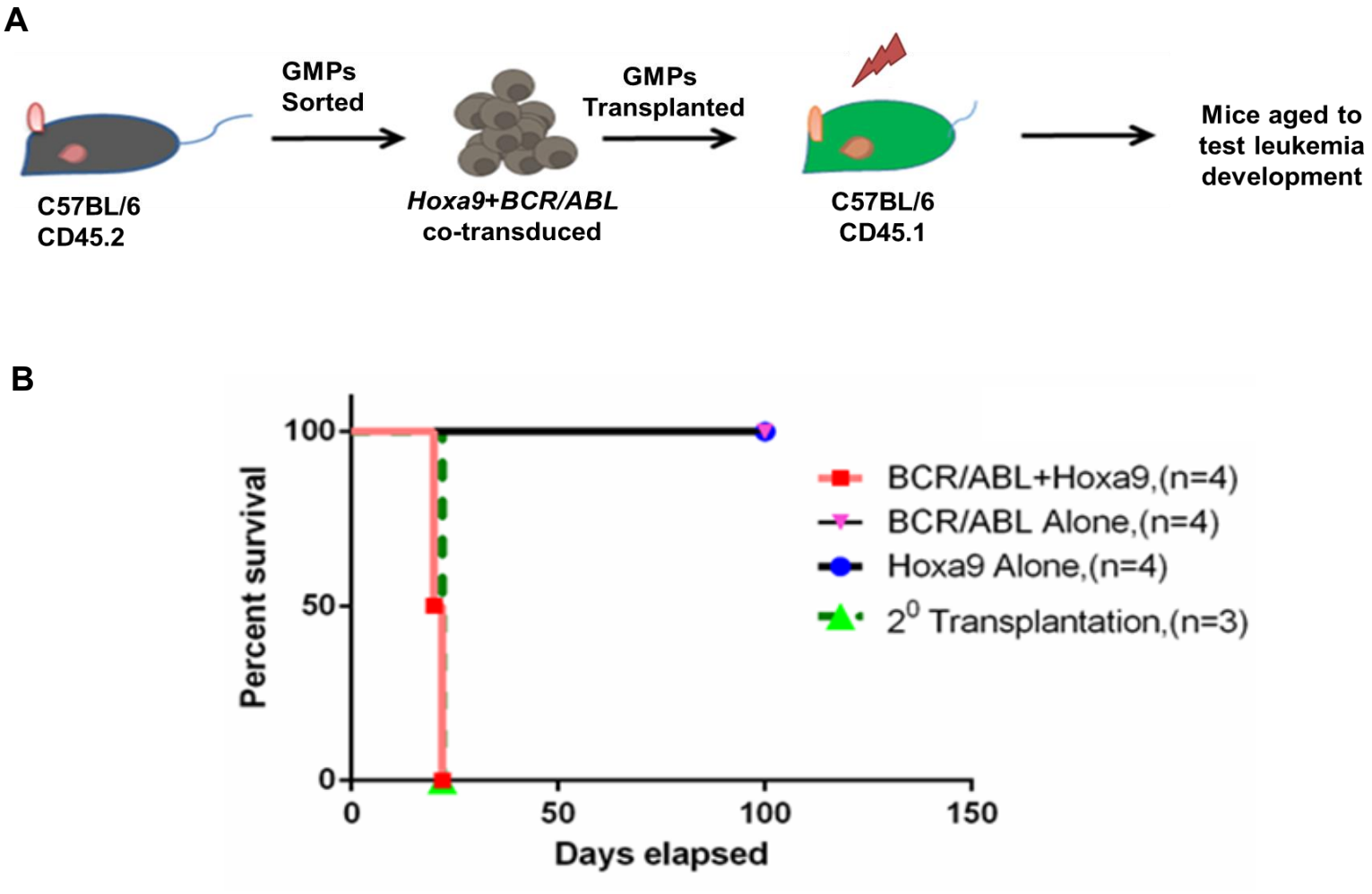
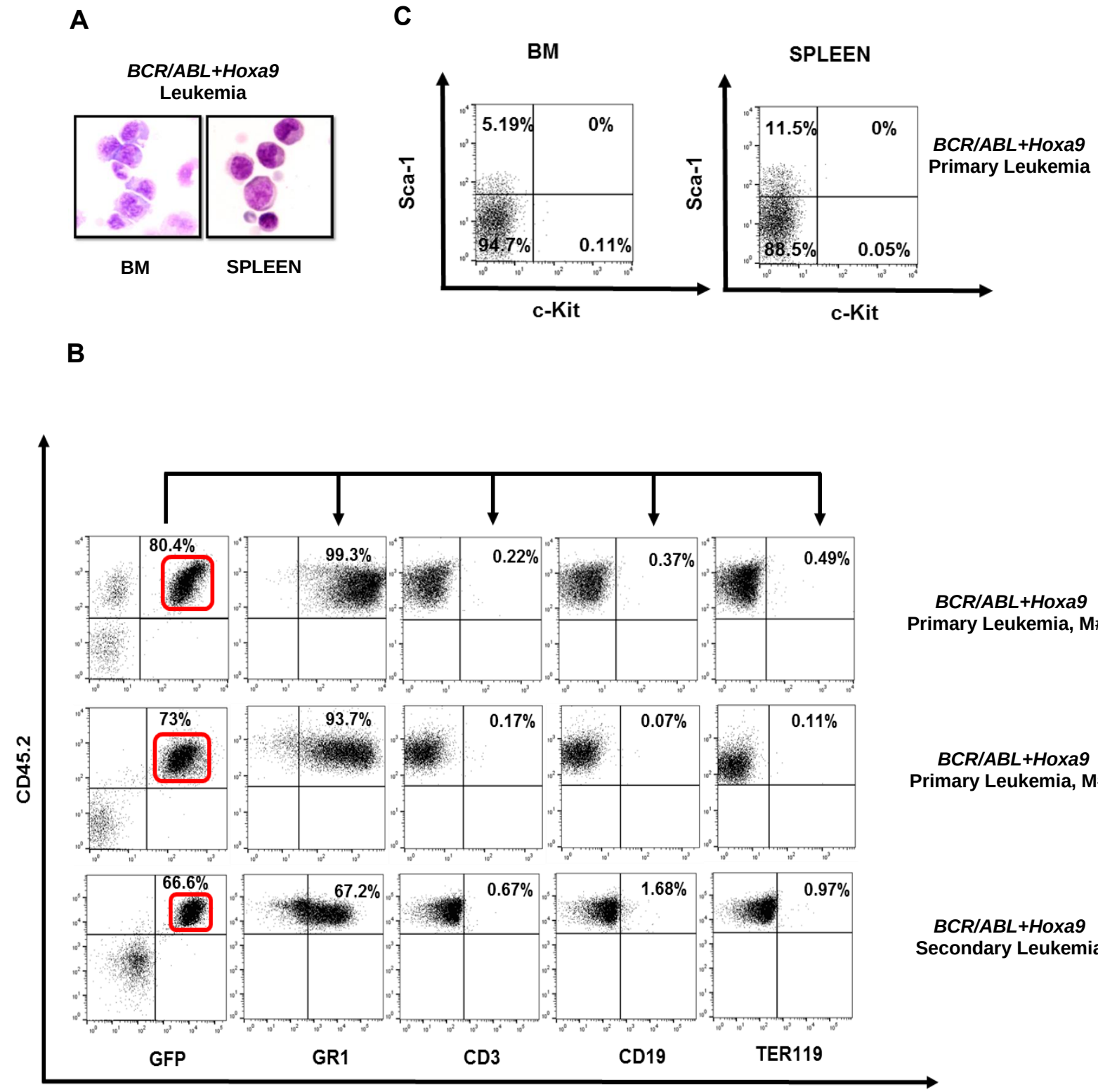


Figure 7: Mice transplanted with GMPs co-expressing *Hoxa9* and *BCR/ABL* show decreased survival rate

(A) Schematic diagram of bone marrow transduction transplantation assay. (B) Survival curves of irradiated C57BL6-Ly5.2 mice receiving GMPs co-transduced with pMSCV-*BCR/ABL*-IRES-GFP and pMSCV-*Hoxa9*-PGK-Neo virus for experimental condition and pMSCV-BCR/ABL-IRES-GFP alone and pMSCV-*Hoxa9*-PGK-Neo alone as control condition for primary leukemia, or 1 x 10^6 spleen cells from primary leukemic mice for serial transplantation for secondary leukemia.


A
BCR/ABL+Hoxa9
Leukemia
BM
SPLEEN
C
BM
SPLEEN
Sca-1
c-Kit
5.19%
0%
94.7%
0.11%
11.5%
0%
88.5%
0.05%
BCR/ABL+Hoxa9
Primary Leukemia
B
CD45.2
80.4%
99.3%
0.22%
0.37%
0.49%
73%
93.7%
0.17%
0.07%
0.11%
66.6%
67.2%
0.67%
1.68%
0.97%
GFP
GR1
CD3
CD19
TER119
BCR/ABL+Hoxa9
Primary Leukemia, M#1
BCR/ABL+Hoxa9
Primary Leukemia, M#2
BCR/ABL+Hoxa9
Secondary Leukemia

(Figure 8)

Figure 8: *Hoxa9* cooperates with *BCR/ABL* to induce CML myeloid blast crisis *in vivo*

(A) Cytospin and Wright-Giemsa staining of Bone Marrow (BM) and Spleen cells from *BCR/ABL+Hoxa9* induced leukemic mice. Original magnification 40X. Images were obtained using a Nikon Eclipse E800 microscope and a Qimaging Micropublisher 5.0 digital camera. (B) FACS analysis of lineage specific markers on bone marrow cells of *BCR/ABL + Hoxa9* moribund mice and secondary transplanted *BCR/ABL + Hoxa9* moribund mice. Leukemic bone marrow cells were gated as CD45.2 and GFP positive cells (marked with red square). (C) FACS analysis of Sca-1 and c-Kit markers on GFP positive bone marrow cells of *BCR/ABL + Hoxa9* moribund mice.

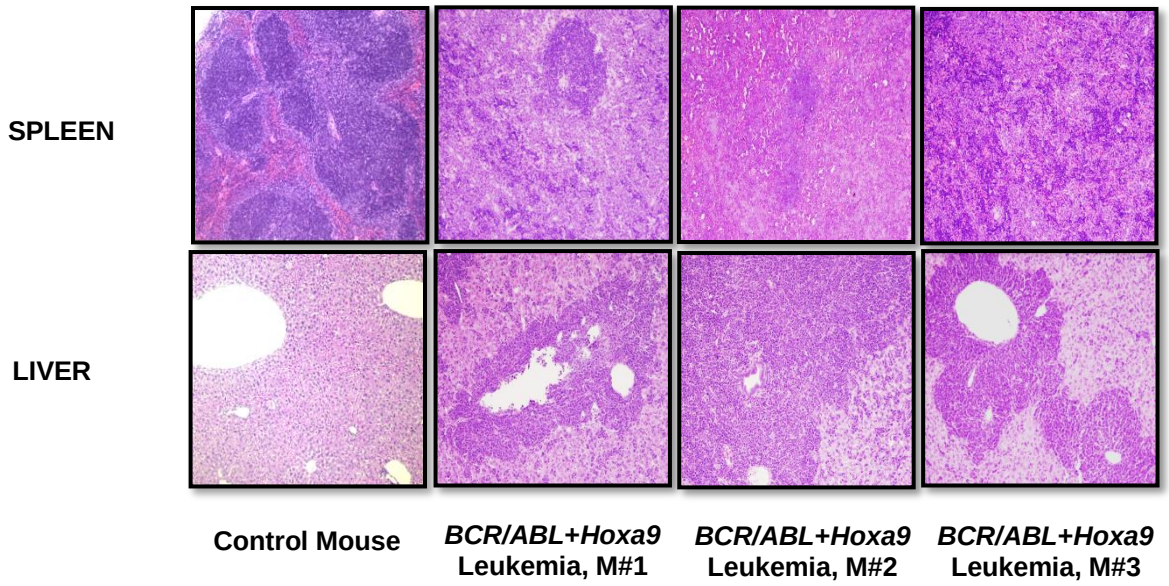


Figure 9: Pathological examination of leukemias induced by *BCR/ABL* and *Hoxa9*

H&E staining showing infiltration of myeloid blasts in spleen and liver of *BCR/ABL+Hoxa9* induced leukemic mice. Original magnification 10X. Images were obtained using a Nikon Eclipse E800 microscope and a Qimaging Micropublisher 5.0 digital camera.

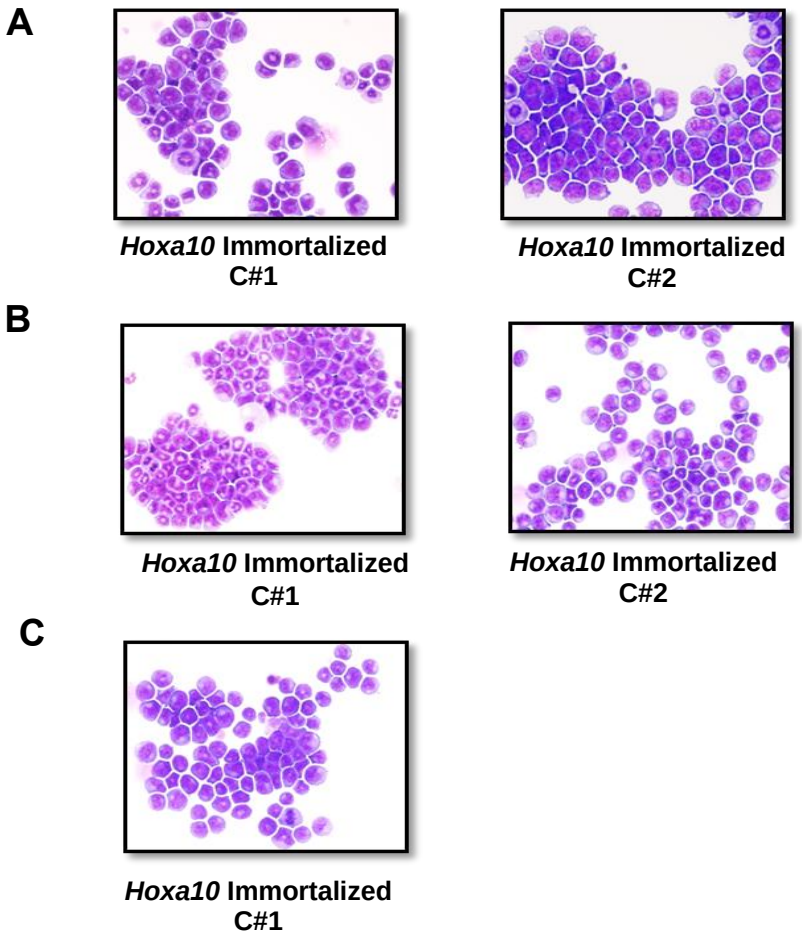


Figure 10: Ectopic expression of *Hoxa10* efficiently immortalizes primary myeloid progenitors in culture

(A) Cytospin preparation and Wright-Giemsa staining of *Hoxa10*-immortalized myeloid progenitors derived from myeloid progenitors generated by in vitro differentiation of HSCs. (B) Cytospin preparation and Wright-Giemsa staining of *Hoxa10*-immortalized myeloid progenitors derived from purified GMPs. (C) Cytospin preparation and Wright-Giemsa staining of *Hoxa10*-immortalized myeloid progenitors derived from 5-FU treated bone marrow progenitors. Original magnification 40X. Images were obtained using a Nikon Eclipse E800 microscope and a Qimaging Micropublisher 5.0 digital camera.

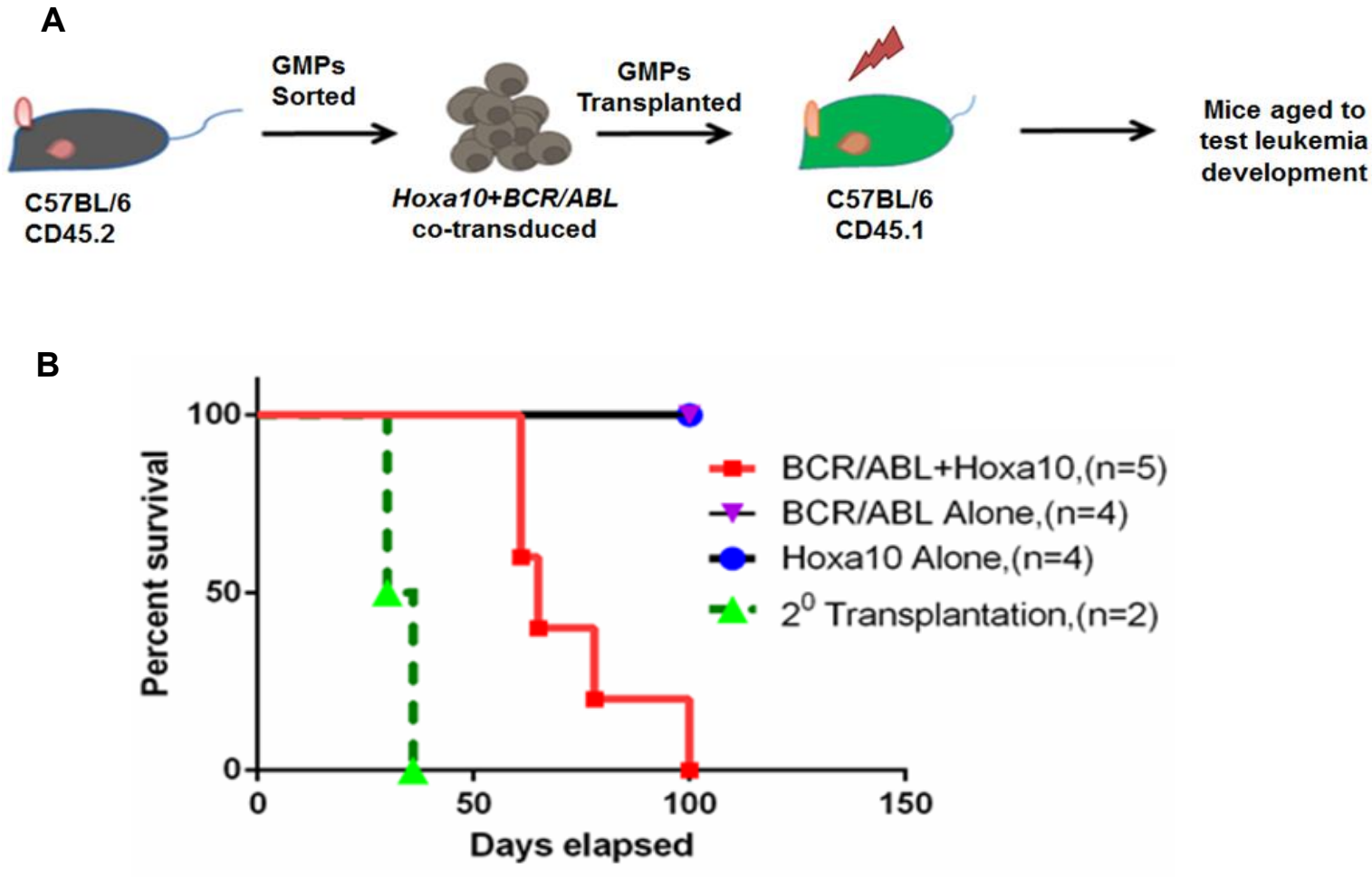
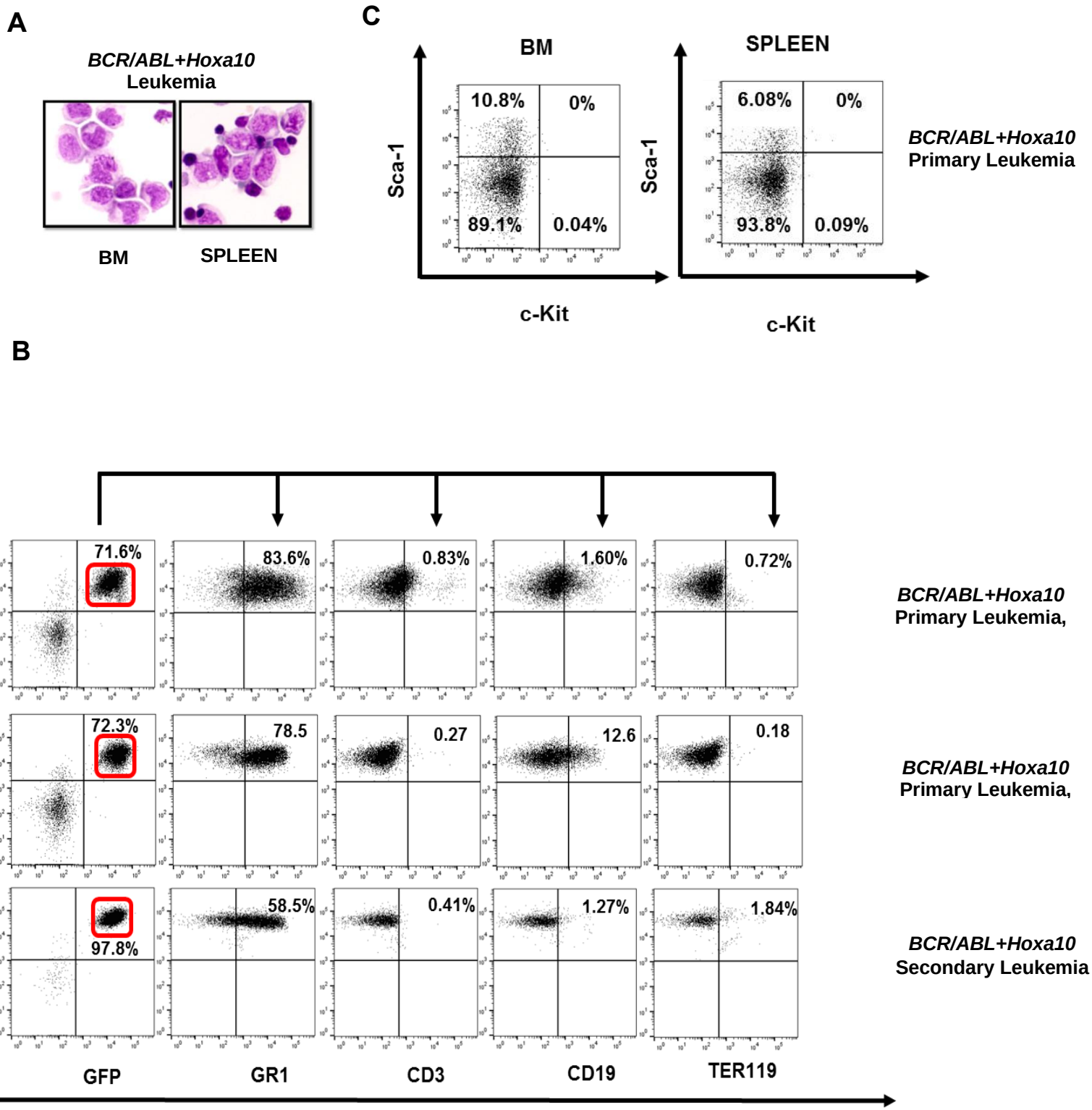


Figure 11: Mice transplanted with GMPs co-expressing *Hoxa10* and *BCR/ABL* show decreased survival rate

(A) Schematic diagram of bone marrow transduction transplantation assay. (B) Survival curves of irradiated C57BL6-Ly5.2 mice receiving GMPs co-transduced with pMSCV-*BCR/ABL*-IRES-GFP and pMSCV-*Hoxa10*-PGK-Puro virus for experimental condition and pMSCV-BCR/ABL-IRES-GFP alone and pMSCV-*Hoxa10*-PGK-Puro alone as control condition for primary leukemia, or 1 x 10^6 spleen cells from primary leukemic mice for serial transplantation for secondary leukemia.


A
BCR/ABL+Hoxa10
Leukemia
BM
SPLEEN
C
BM
SPLEEN
Sca-1
c-Kit
10.8%
0%
89.1%
0.04%
6.08%
0%
93.8%
0.09%
BCR/ABL+Hoxa10
Primary Leukemia
B
CD45.2
GFP
GR1
CD3
CD19
TER119
71.6%
83.6%
0.83%
1.60%
0.72%
72.3%
78.5
0.27
12.6
0.18
97.8%
58.5%
0.41%
1.27%
1.84%
BCR/ABL+Hoxa10
Primary Leukemia,
BCR/ABL+Hoxa10
Primary Leukemia,
BCR/ABL+Hoxa10
Secondary Leukemia

(Figure 12)

Figure 12: *Hoxa10* cooperates with *BCR/ABL* to induce CML myeloid blast crisis *in vivo*

(A) Cytospin and Wright-Giemsa staining of Bone Marrow (BM) and Spleen cells from *BCR/ABL+Hoxa10* induced leukemic mice. Original magnification 40X. Images were obtained using a Nikon Eclipse E800 microscope and a Qimaging Micropublisher 5.0 digital camera. (B) FACS analysis of lineage specific markers on bone marrow cells of *BCR/ABL + Hoxa10* moribund mice and secondary transplanted *BCR/ABL + Hoxa10* moribund mice. Leukemic bone marrow cells were gated as CD45.2 and GFP positive cells (marked with red square). (C) FACS analysis of Sca-1 and c-Kit markers on GFP positive bone marrow cells of *BCR/ABL + Hoxa10* moribund mice.

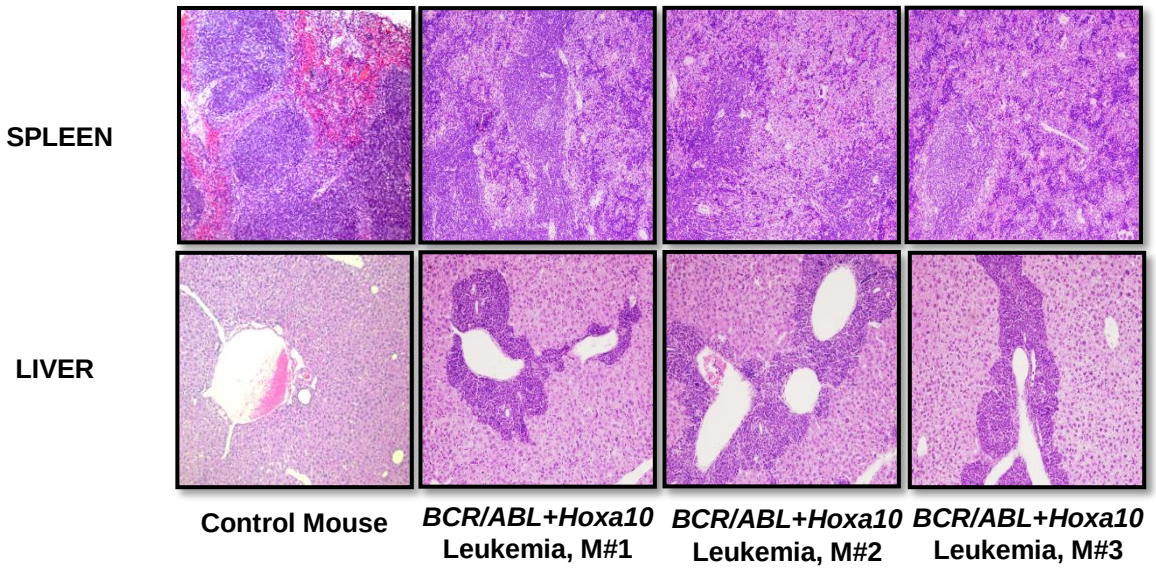
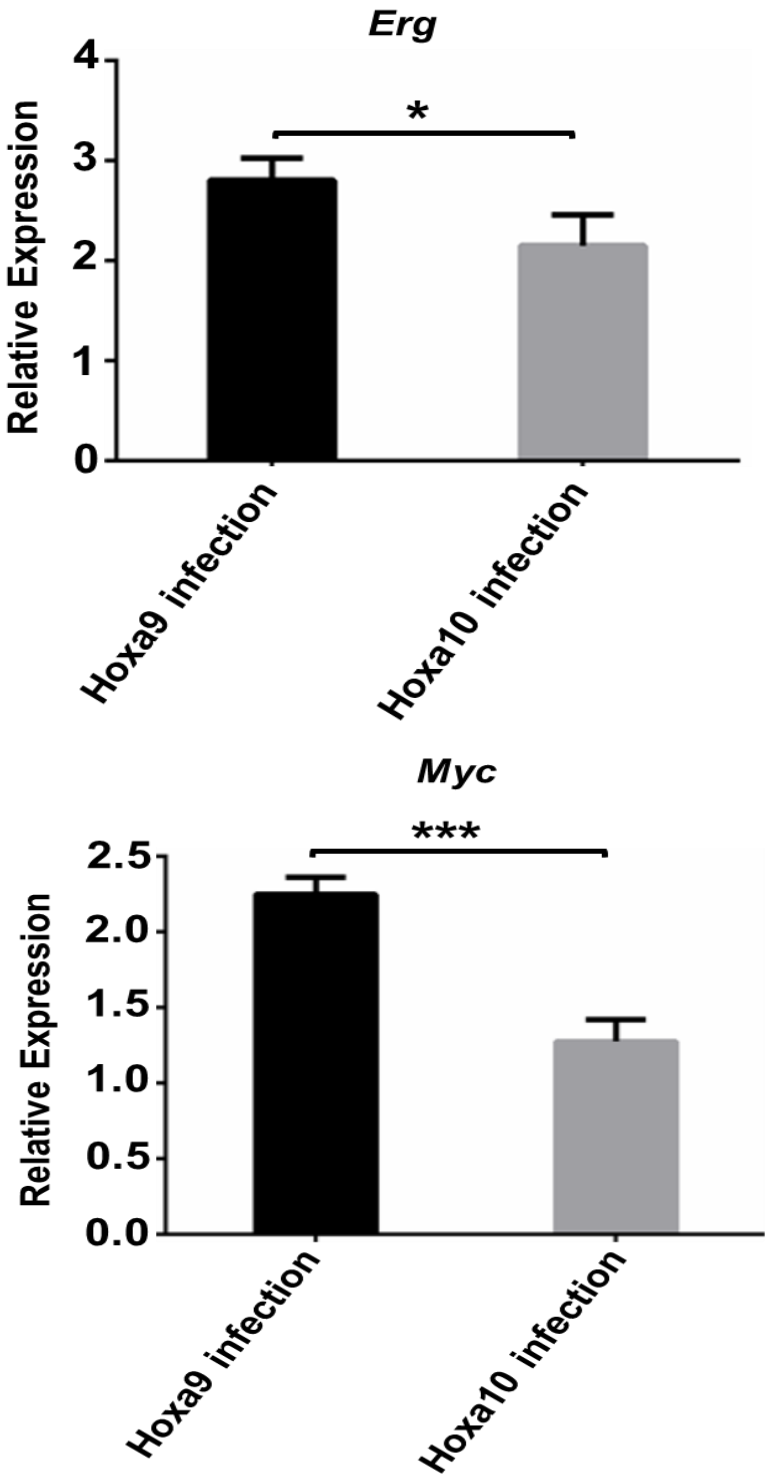


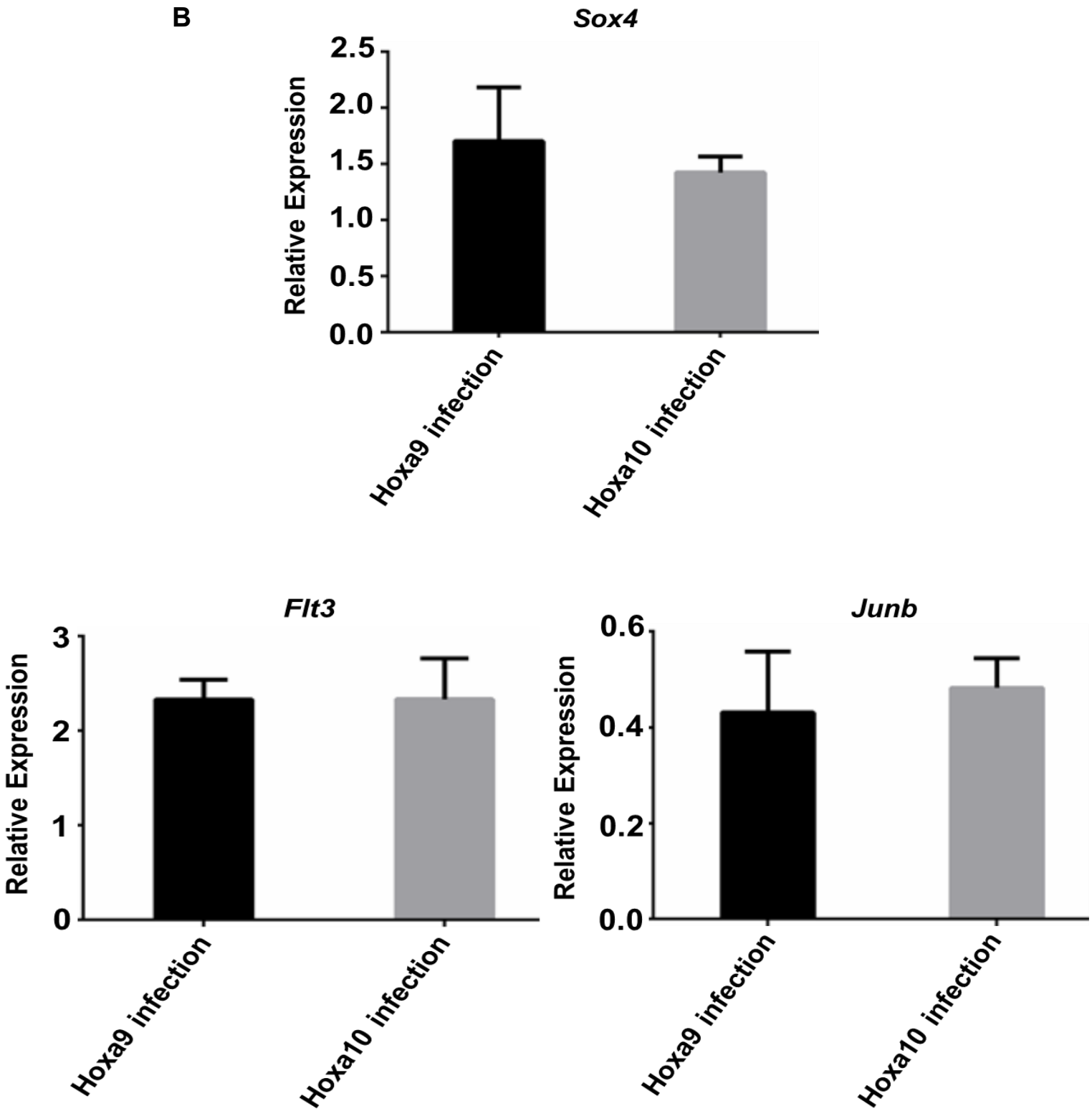
Figure 13: Pathological examination of leukemias induced by *BCR/ABL* and *Hoxa10*

H&E staining showing infiltration of myeloid blasts in spleen and liver of *BCR/ABL+Hoxa10* induced leukemic mice. Original magnification 10X. Images were obtained using a Nikon Eclipse E800 microscope and a Qimaging Micropublisher 5.0 digital camera.

A

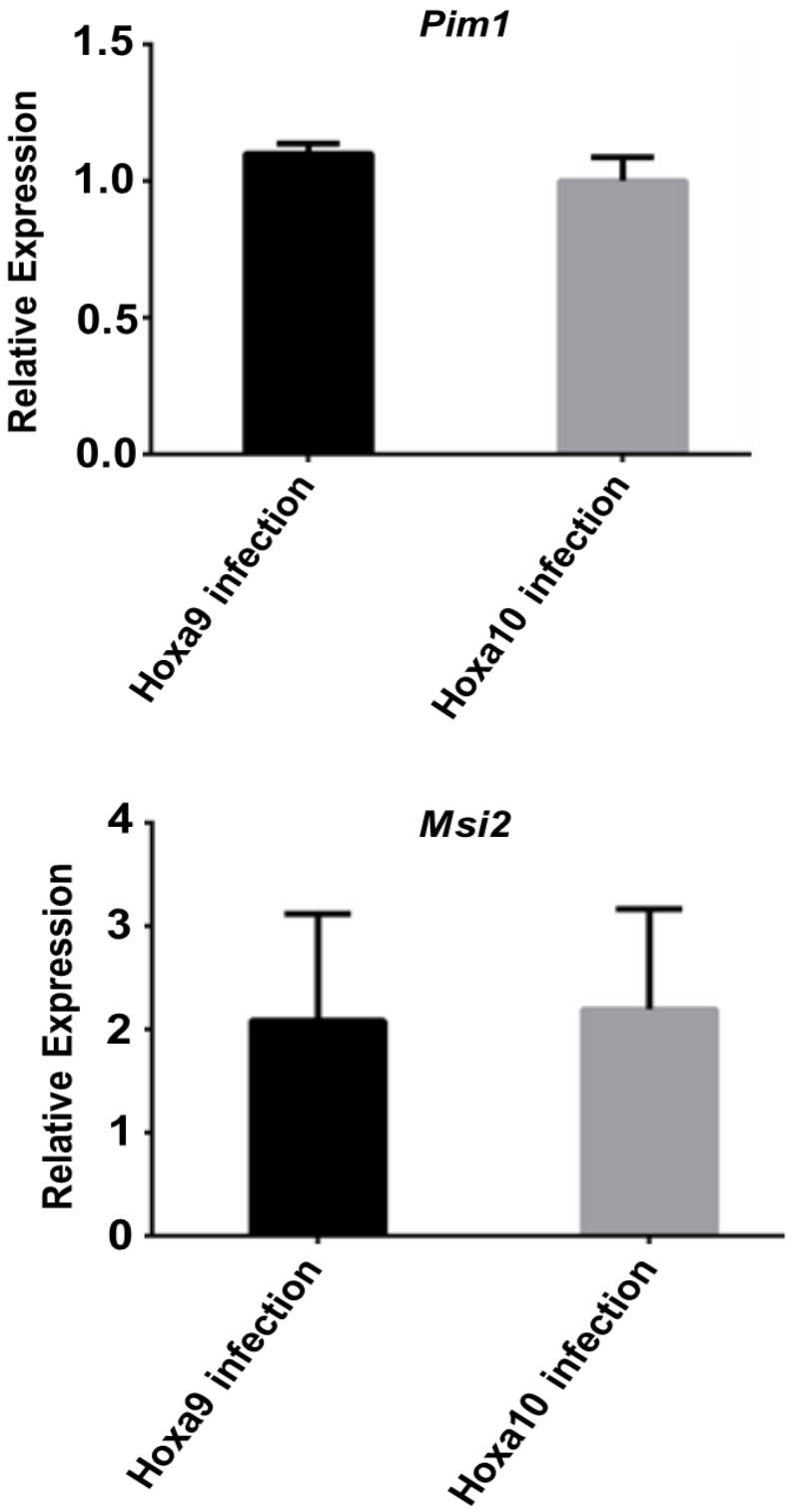

Erg
Relative Expression
4
3
2
1
0
*
Hoxa9 infection
Hoxa10 infection
Myc
***
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
Hoxa9 infection
Hoxa10 infection

(Figure 14)



(Figure 14 continued)

B



(Figure 14 continued)

Figure 14: Expression level of *Hoxa9* and *Hoxa10* targets

(A) *Erg and Myc* mRNA levels in primary hematopoietic progenitor cells 48 hrs. after *Hoxa9* and *Hoxa10* infection. Real-time RT-PCR analysis was done from total RNA isolated from these cells. (B) *Sox4, Flt3, Junb, Pim1 and Msi2* mRNA levels in primary hematopoietic progenitor cells 48 hrs. after *Hoxa9* and *Hoxa10* infection. Real-time RT-PCR analysis was done from total RNA isolated from these cells. Relative expression levels were calculated by normalizing to *RPL4* mRNA levels in the same samples. The mean and SD of each relative expression level is shown.

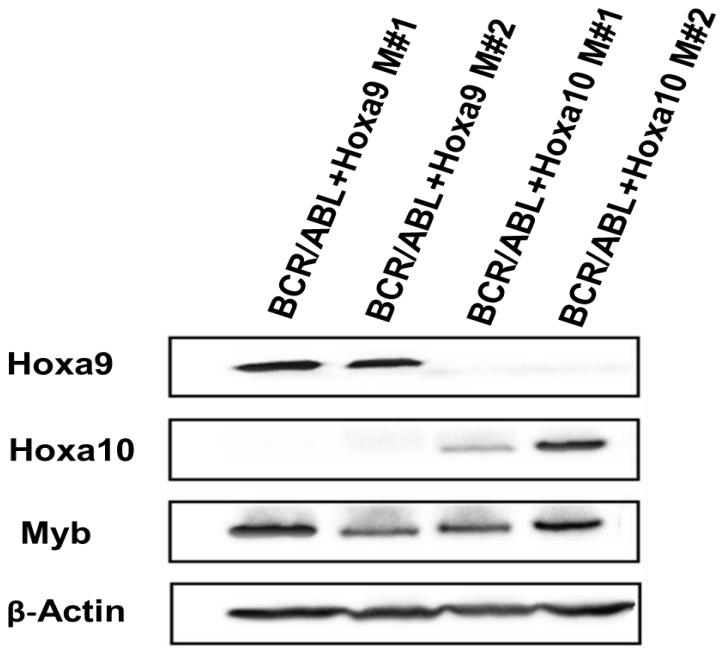


Figure 15: *Myb* is a target in *Hoxa9* and *Hoxa10* induced leukemias

Western blot analysis of *Hoxa9*, *Hoxa10*, *Myb* and *Actin* protein in *BCR/ABL* + *Hoxa9* and *BCR/ABL* + *Hoxa10* leukemic samples. Leukemic samples isolated from spleen of moribund mice.

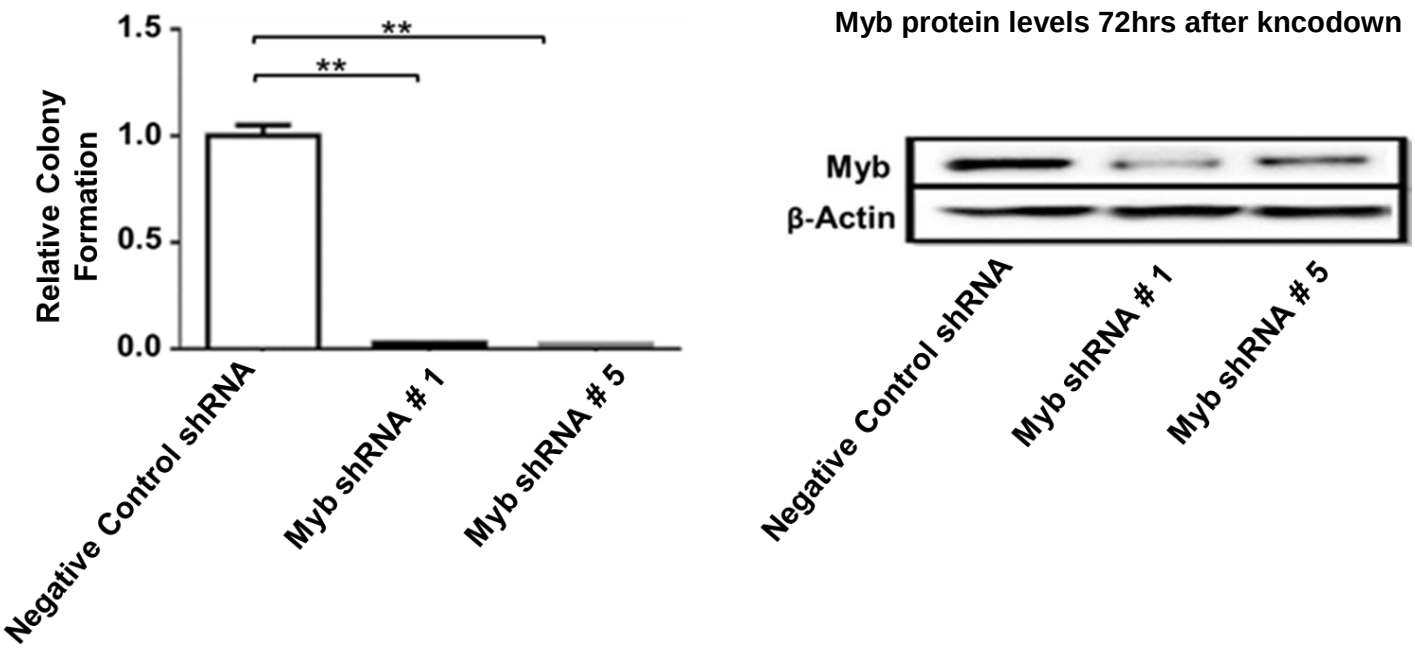


Figure 16: *Myb* is critical for the colony-forming potential of *Hoxa9* immortalized myeloid progenitors

Mean and SD of colony formation potential of *Hoxa9* immortalized primary hematopoietic progenitor cells in the presence of SCF, IL-3 and Blaticidin (14ug/ml) at 48 hours after infection with *Myb* specific shRNA (Myb shRNA#1 and Myb shRNA#5) and control shRNA (Negative Control shRNA). Western blot analysis of *Myb* and actin protein in the same infected cells at 72 hours after shRNA infection. Relative *Myb* protein levels after normalization to actin levels in the same sample are indicated.

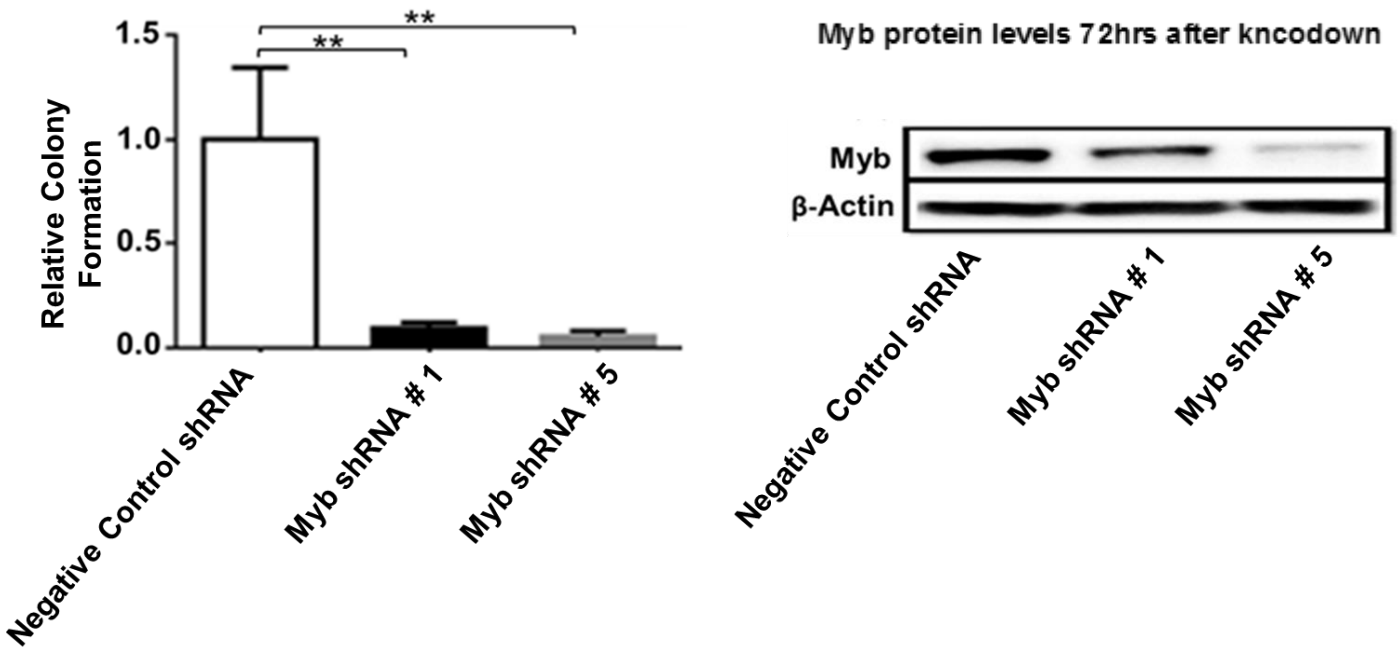


Figure 17: *Myb* is critical for the colony-forming potential of *Hoxa10* immortalized myeloid progenitors

Mean and SD of colony formation potential of *Hoxa10* immortalized primary hematopoietic progenitor cells in the presence of SCF, IL-3 and Blaticidin (14ug/ml) at 48 hours after infection with *Myb* specific shRNA (Myb shRNA#1 and Myb shRNA#5) and control shRNA (Negative Control shRNA). Western blot analysis of *Myb* and actin protein in the same infected cells at 72 hours after shRNA infection. Relative *Myb* protein levels after normalization to actin levels in the same sample are indicated.

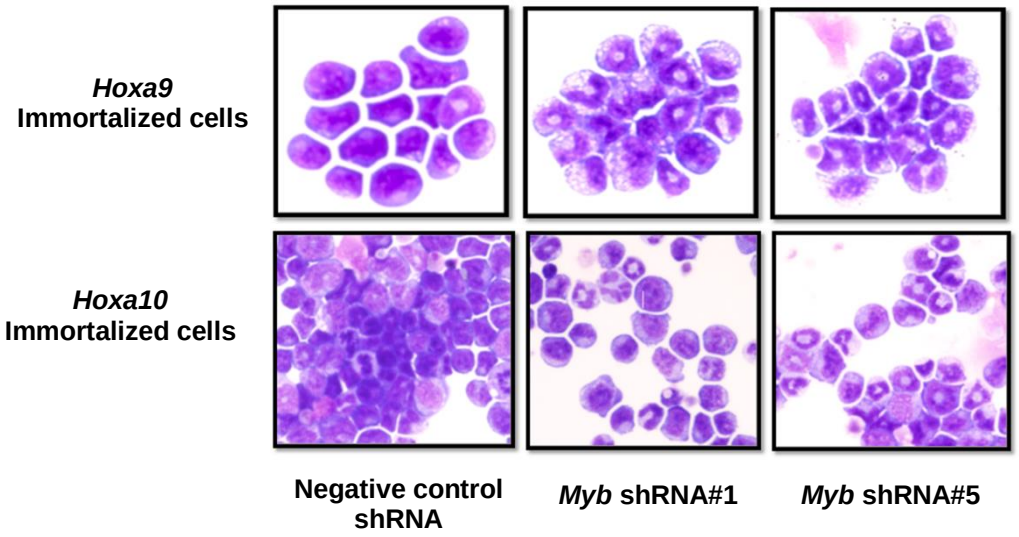
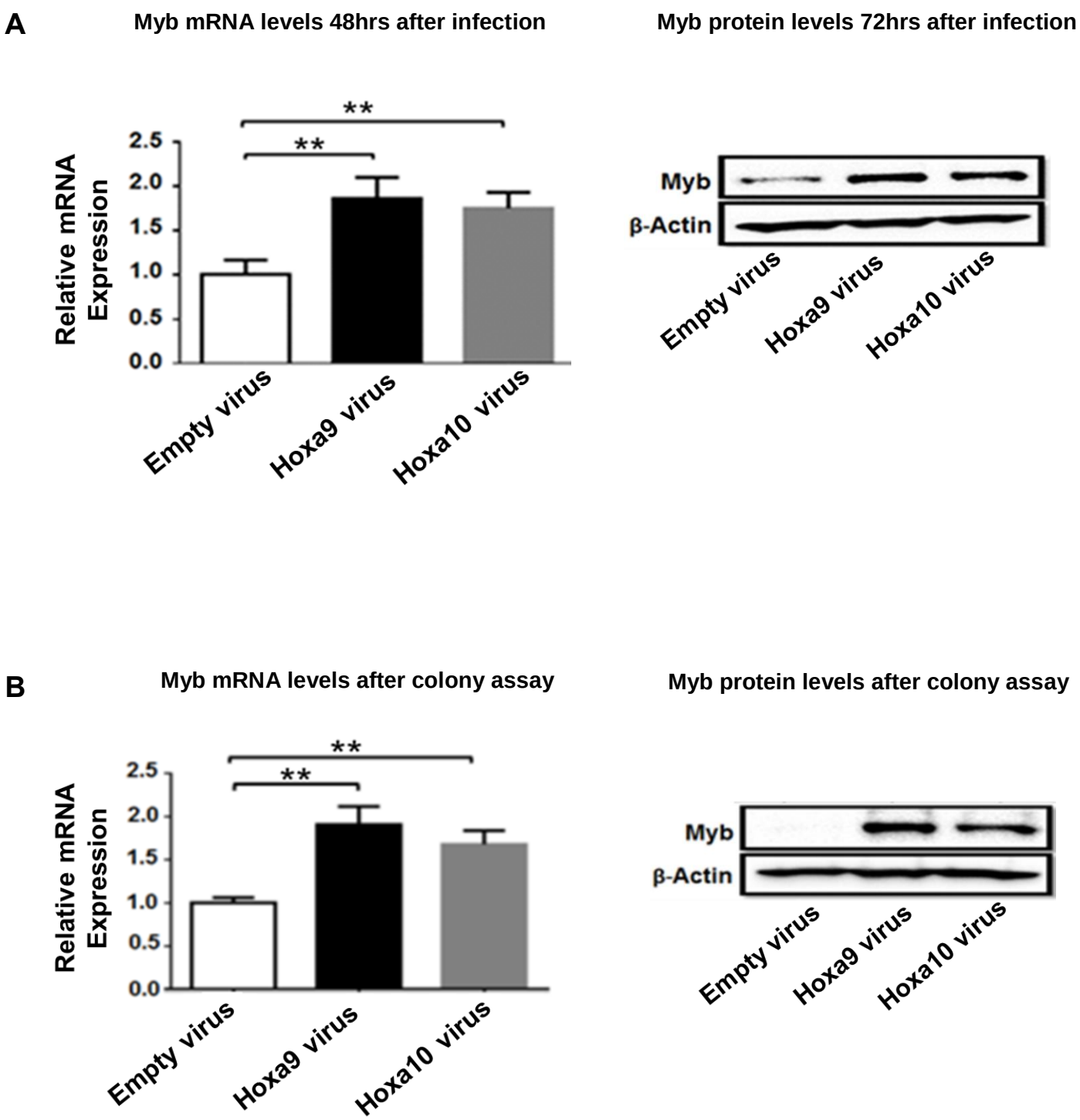


Figure 18: *Myb* knockdown induces differentiation of *Hoxa9* and *Hoxa10* immortalized myeloid progenitors

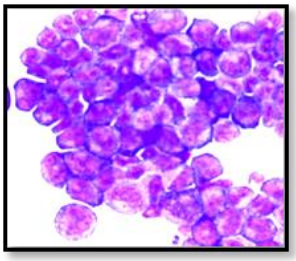
Cytospin and Wright-Giemsa staining of *Hoxa9* and *Hoxa10* immortalized cells after *Myb* knockdown. Original magnification 40X. Images were obtained using a Nikon Eclipse E800 microscope and a Qimaging Micropublisher 5.0 digital camera.


A
Myb mRNA levels 48hrs after infection
Myb protein levels 72hrs after infection
Relative mRNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
**
**
Empty virus
Hoxa9 virus
Hoxa10 virus
Myb
β-Actin
Empty virus
Hoxa9 virus
Hoxa10 virus
B
Myb mRNA levels after colony assay
Myb protein levels after colony assay
Relative mRNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
**
**
Empty virus
Hoxa9 virus
Hoxa10 virus
Myb
β-Actin
Empty virus
Hoxa9 virus
Hoxa10 virus

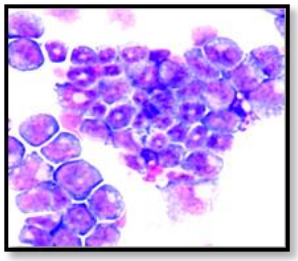
(Figure 19)

Figure 19: Increase in *Myb* mRNA levels in primary hematopoietic progenitor cells after *Hoxa9* and *Hoxa10* infection

(A) Real-time RT-PCR analysis of total RNA isolated from puromycin selected primary hematopoietic progenitor cells 48 hours after infection with *Hoxa9, Hoxa10* and empty retrovirus. (B) Real-time RT-PCR analysis of total RNA isolated from cells after colony assay. Relative expression levels were calculated by normalizing to *RPL4* mRNA levels in the same samples. The mean and SD of each relative expression level is shown. Western blot analysis of *Myb* and actin protein in the same infected cells at 72 hours after infection or from colony assay cells. Relative *Myb* protein levels after normalization to actin levels in the same sample are indicated.



***Myb* Immortalized C#1**



***Myb* Immortalized C#2**

Figure 20: Ectopic expression of *Myb* efficiently immortalizes primary myeloid progenitors in culture

Cytospin preparation and Wright-Giemsa staining of primary myeloid cells infected with *Myb* Short Isoform retrovirus at 5 weeks after infection. Original magnification X40. Images were obtained using a Nikon Eclipse E800 microscope and a Qimaging Micropublisher 5.0 digital camera.

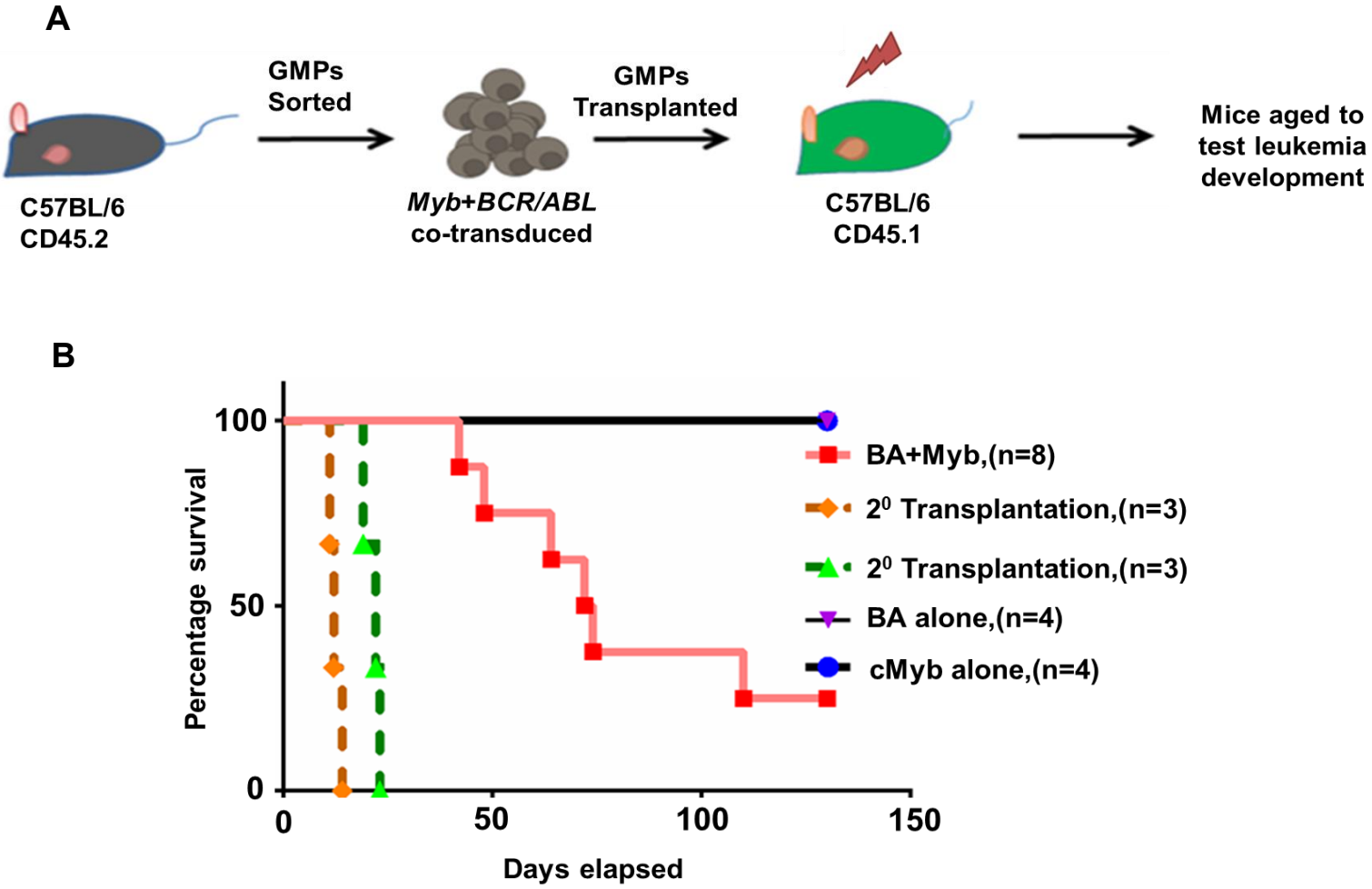
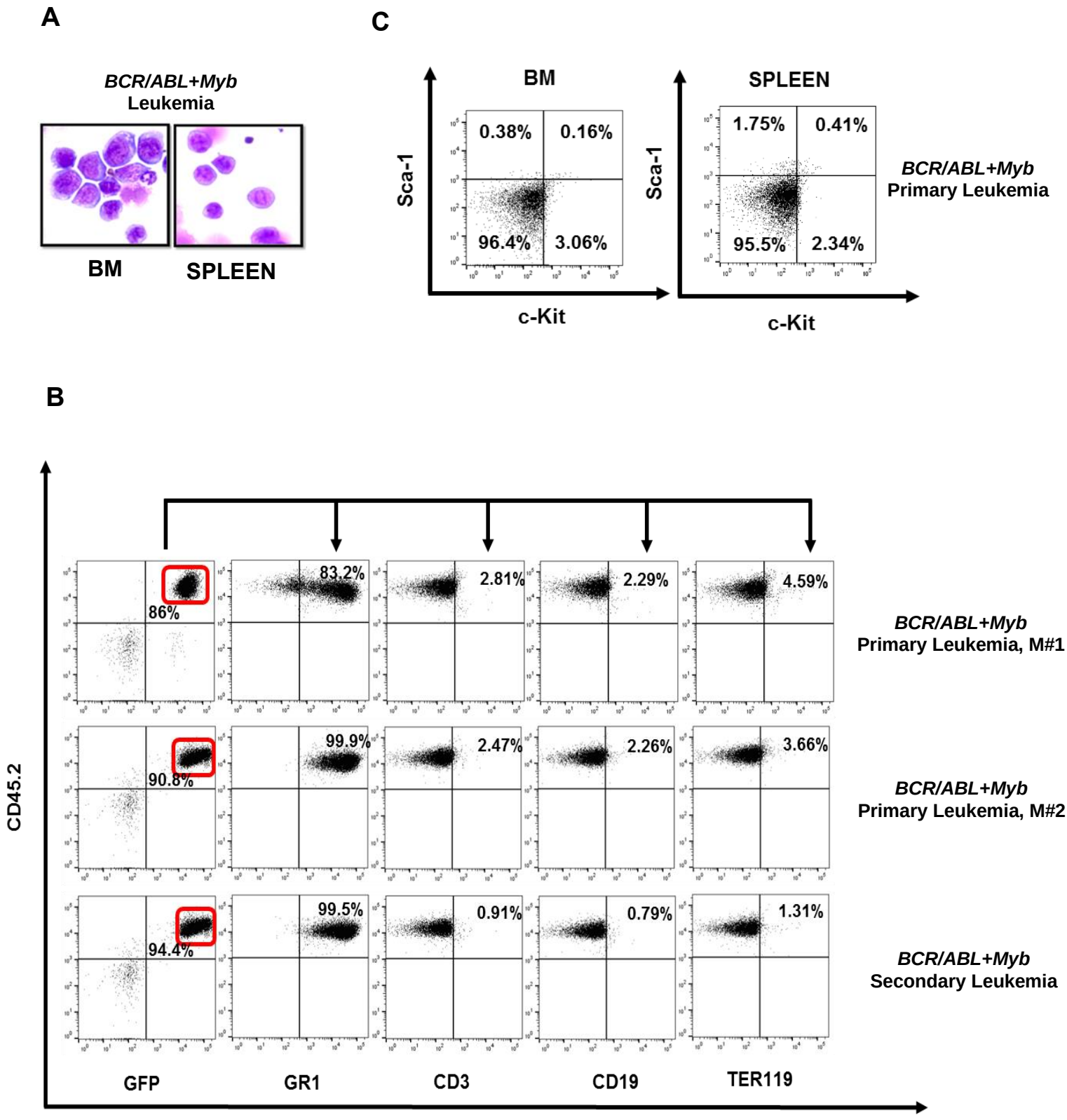


Figure 21: Mice transplanted with GMPs co-expressing *Myb* and *BCR/ABL* show decreased survival rate

(A) Schematic diagram of bone marrow transduction transplantation assay. (B) Survival curves of irradiated C57BL6-Ly5.2 mice receiving GMPs co-transduced with MigR1-BCR/ABL-IRES-GFP and MSCV-MybSI-IRES-GFP for experimental condition and MigR1-BCR/ABL-IRES-GFP alone and MSCV-MybSI-IRES-GFP alone as control condition for primary leukemia, or 1 x 10^6 spleen cells from primary leukemic mice for serial transplantation for secondary leukemia.


A
BCR/ABL+Myb
Leukemia
BM
SPLEEN
C
BM
SPLEEN
Sca-1
c-Kit
0.38%
0.16%
96.4%
3.06%
1.75%
0.41%
95.5%
2.34%
BCR/ABL+Myb
Primary Leukemia
B
CD45.2
GFP
GR1
CD3
CD19
TER119
86%
83.2%
2.81%
2.29%
4.59%
90.8%
99.9%
2.47%
2.26%
3.66%
94.4%
99.5%
0.91%
0.79%
1.31%
BCR/ABL+Myb
Primary Leukemia, M#1
BCR/ABL+Myb
Primary Leukemia, M#2
BCR/ABL+Myb
Secondary Leukemia

(Figure 22)

Figure 22: *Myb* cooperates with *BCR/ABL* to induce CML myeloid blast crisis *in vivo*

(A) Cytospin and Wright-Giemsa staining of Bone Marrow (BM) and Spleen cells from *BCR/ABL+Myb* induced leukemic mice. Original magnification 40X. Images were obtained using a Nikon Eclipse E800 microscope and a Qimaging Micropublisher 5.0 digital camera. (B) FACS analysis of lineage specific markers on bone marrow cells of *BCR/ABL + Myb* moribund mice and secondary transplanted *BCR/ABL + Hoxa10* moribund mice. Leukemic bone marrow cells were gated as CD45.2 and GFP positive cells (marked with red square). (C) FACS analysis of Sca-1 and c-Kit markers on GFP positive bone marrow cells of *BCR/ABL + Myb* moribund mice.

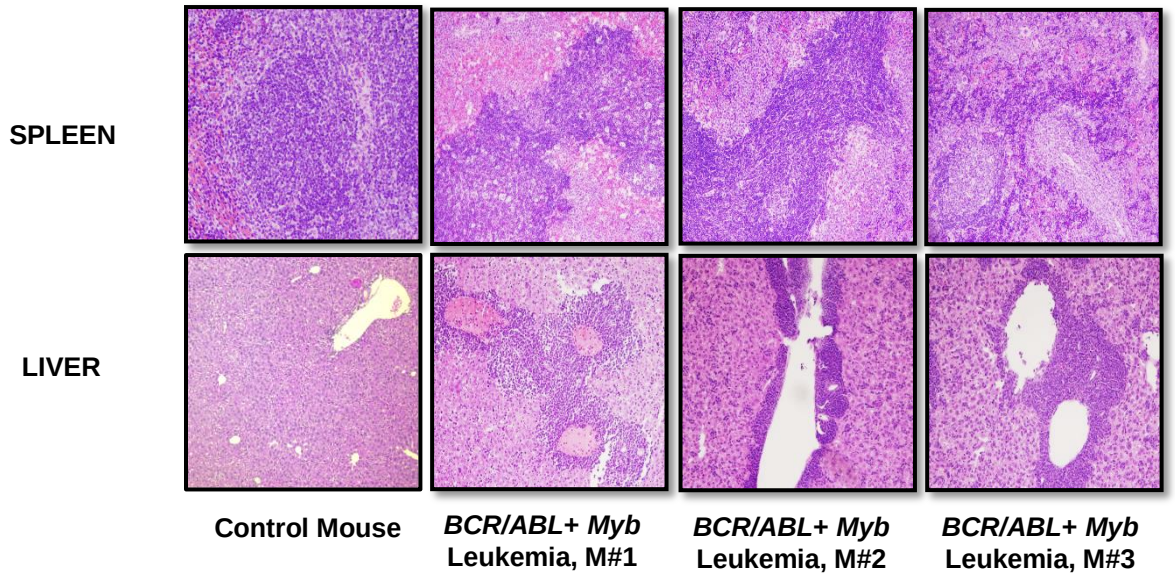


Figure 23: Pathological examination of leukemias induced by *BCR/ABL* and *Myb*

H&E staining showing infiltration of myeloid blasts in spleen and liver of *BCR/ABL+Myb* induced leukemic mice. Original magnification 10X. Images were obtained using a Nikon Eclipse E800 microscope and a Qimaging Micropublisher 5.0 digital camera.

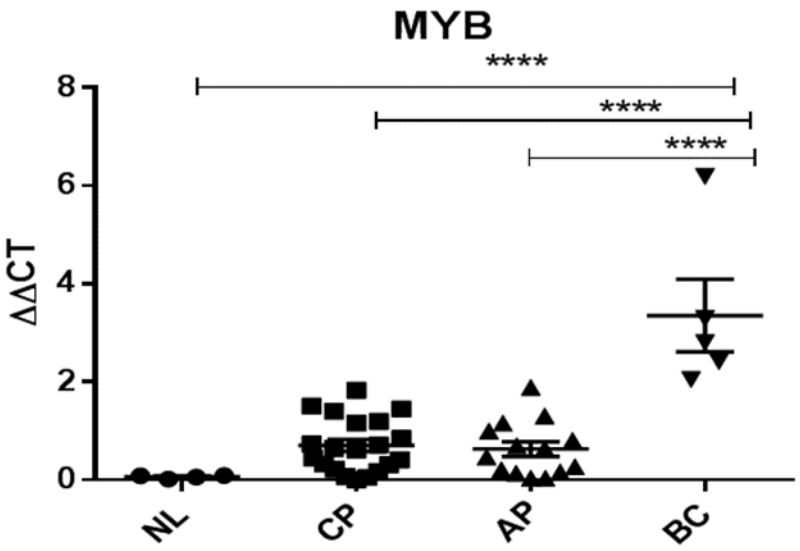
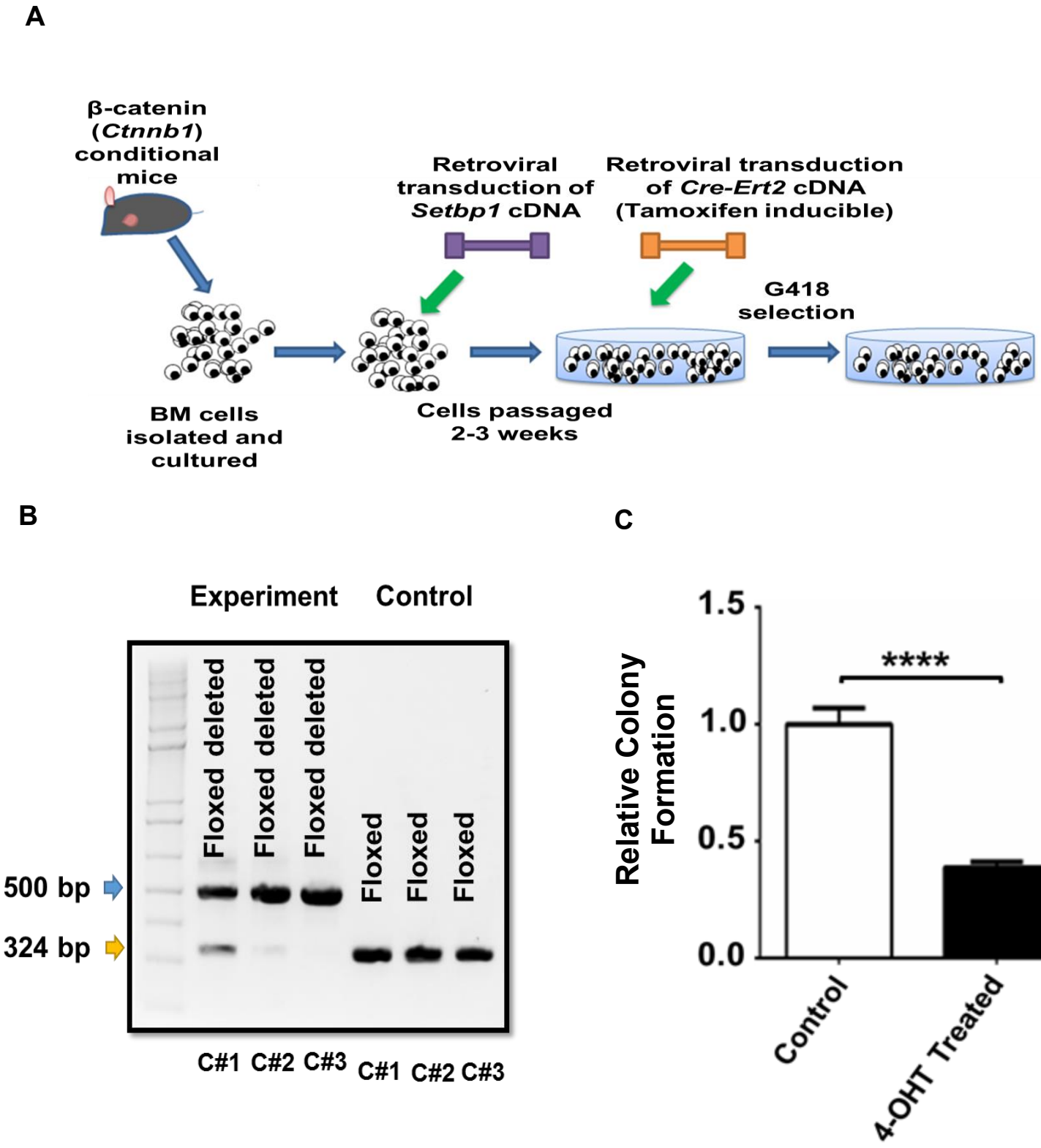


Figure 24: Increased *Myb* expression is detected in CML Blast Crisis Patients

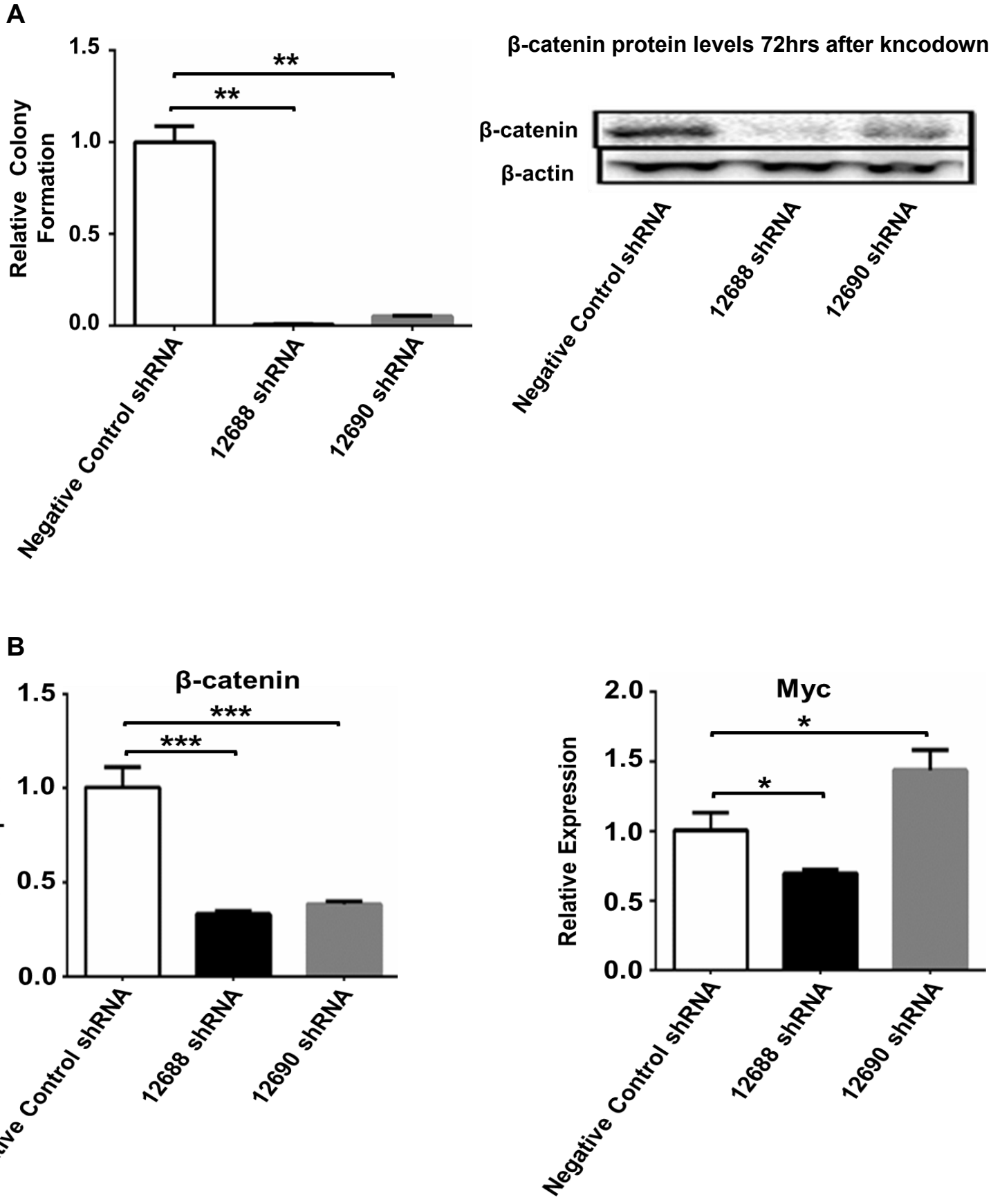
Real-time RT-PCR analysis of *Myb* mRNA levels in total RNA isolated from whole BM of healthy volunteers (NL) and CML chronic phase (CP) and CML accelerated phase (AP) and blast crisis phase (BC) patients.


A
β-catenin (Ctnnb1) conditional mice
Retroviral transduction of Setbp1 cDNA
Retroviral transduction of Cre-Ert2 cDNA (Tamoxifen inducible)
G418 selection
BM cells isolated and cultured
Cells passaged 2-3 weeks
B
Experiment
Control
Floxed deleted
Floxed deleted
Floxed deleted
Floxed
Floxed
Floxed
500 bp
324 bp
C#1 C#2 C#3 C#1 C#2 C#3
C
Relative Colony Formation
1.5
1.0
0.5
0.0
****
Control
4-OHT Treated

(Figure 25)

Figure 25: β-catenin deletion induces decrease in self-renewal capabilities of *Setbp1* immortalized myeloid progenitors

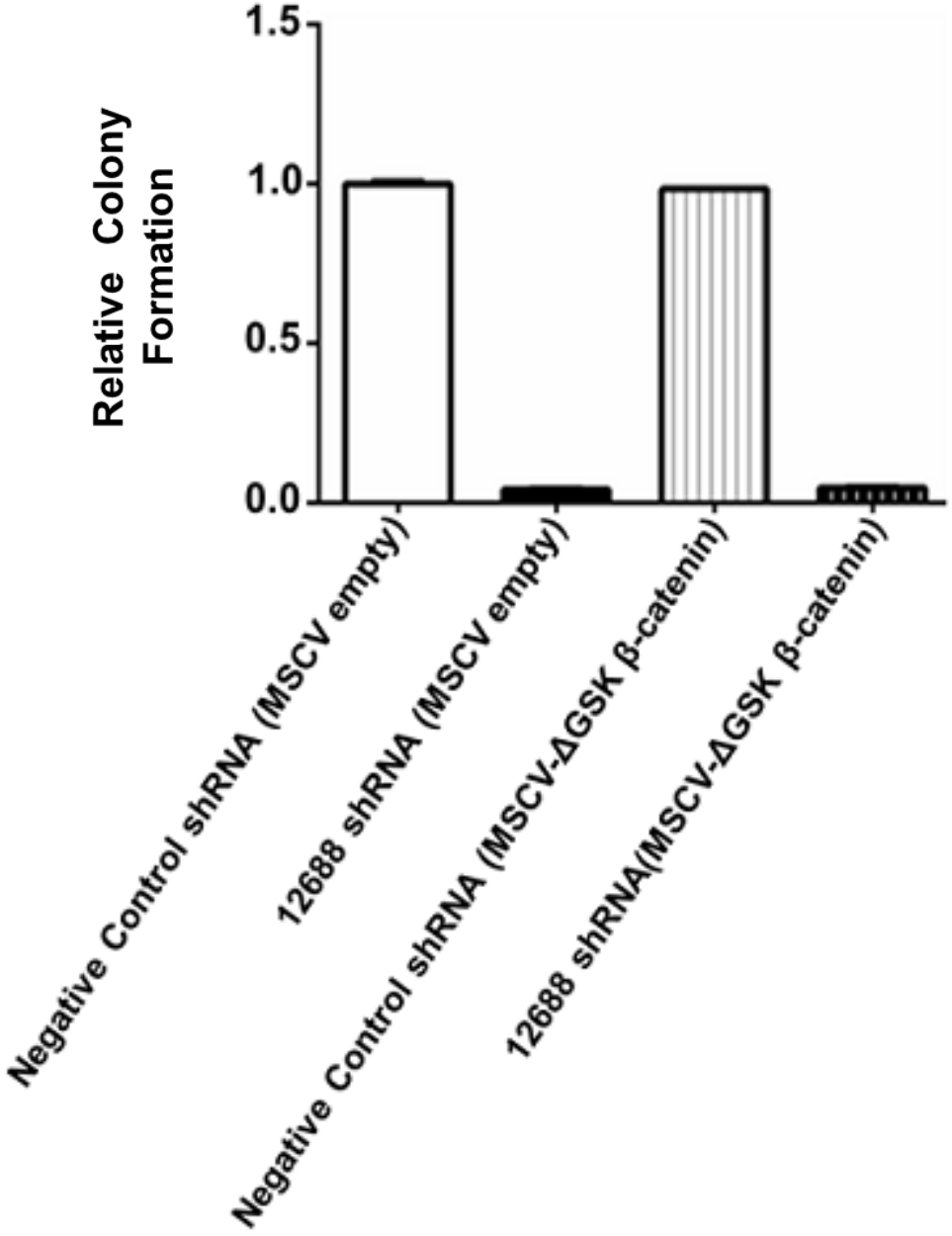
(A) Schematic diagram for the generation of 4-OHT inducible β-catenin conditional *Setbp1* immortalized cell line (2176-cre). (B) Genotyping analysis gel run for 2176-cre cells. Experiment cells treated with 4-OHT, control cells treated with ethanol. For β-catenin deletion the combination of these 3 primers RM 41/42/43 generates a 500bp PCR product (blue arrow) and for the undeleted allele a 324bp (yellow arrow) PCR product is generated. (C) β-catenin deletion inhibits the proliferation of 2176-cre cells. Mean and SD of colony formation potential of 2176-cre cells in the presence of SCF, IL-3 and G418 at 48 hours after 4-OHT (1mM) or ethanol (as control) treatment.


A
Relative Colony Formation
1.5
1.0
0.5
0.0
**
**
Negative Control shRNA
12688 shRNA
12690 shRNA
β-catenin protein levels 72hrs after kncodown
β-catenin
β-actin
Negative Control shRNA
12688 shRNA
12690 shRNA
B
β-catenin
Relative Expression
1.5
1.0
0.5
0.0
***
***
Negative Control shRNA
12688 shRNA
12690 shRNA
Myc
Relative Expression
2.0
1.5
1.0
0.5
0.0
*
*
Negative Control shRNA
12688 shRNA
12690 shRNA

(Figure 26)

Figure 26: β-catenin knockdown inhibits the proliferation of *Setbp1* immortalized cells

(A) Mean and SD of colony formation potential of *Setbp1* immortalized cells (23923 cells) in the presence of SCF, IL-3 and Puromycin at 48 hours after infection with β-catenin specific shRNA; 12688 (β-catenin shRNA#1) and 12690 (β-catenin shRNA#2) and control shRNA (Negative Control shRNA). Western blot analysis of β-catenin and Actin protein in the same infected cells at 72 hours after infection. Relative β-catenin protein levels after normalization to Actin levels in the same sample are indicated. (B) β-catenin and *Myc* mRNA levels in *Setbp1* immortalized cells (23923 cell line) after β-catenin knockdown. Real-time RT-PCR analysis of total RNA isolated from puromycin selected 23923 cells 48 hours after infection with 12688 shRNA, 12690 shRNA and control shRNA. Relative expression levels were calculated by normalizing to *RPL4* mRNA levels in the same samples. The mean and SD of each relative expression level is shown.


Relative Colony Formation
1.5
1.0
0.5
0.0
Negative Control shRNA (MSCV empty)
12688 shRNA (MSCV empty)
Negative Control shRNA (MSCV-ΔGSK β-catenin)
12688 shRNA(MSCV-ΔGSK β-catenin)

(Figure 27)

Figure 27: Ectopic expression of full length β-catenin is unable to rescue decrease in colony forming capability in *Setbp1* immortalized cells

Mean and SD of colony formation potential of *Setbp1* immortalized cells (23923 cells) in the presence of SCF, IL-3 and Puromycin at 48 hours after infection with 12688 shRNA/negative control shRNA and rescue with constitutively active full length β-catenin (MSCV-ΔGSK β-catenin) or empty MSCV virus.

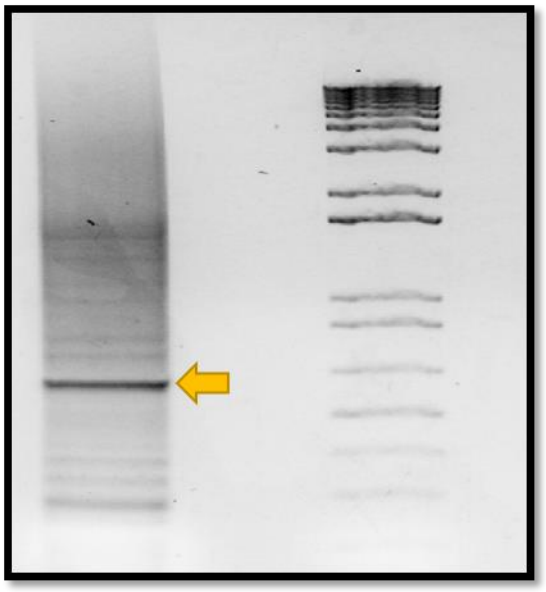
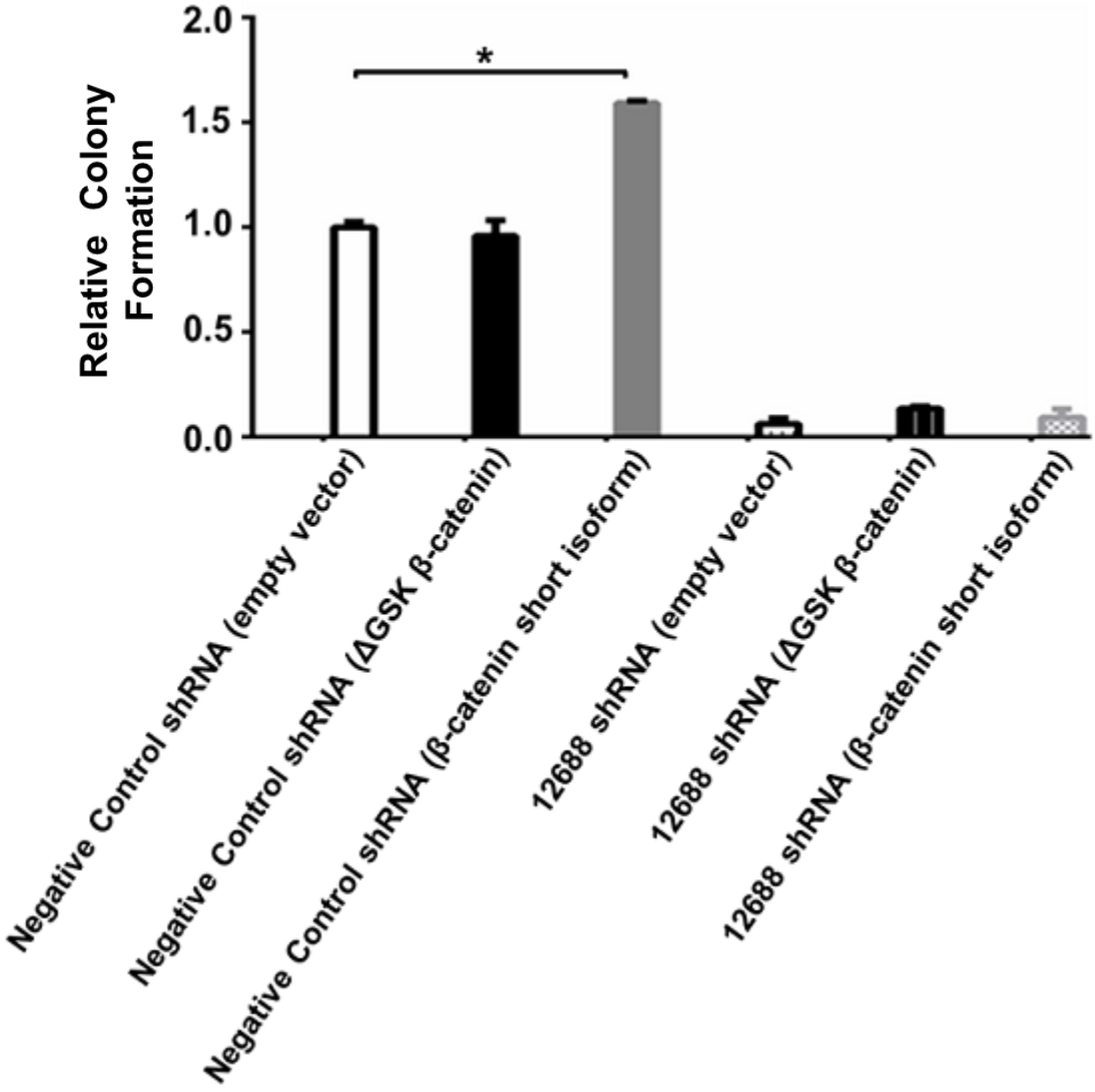


Figure 28: Isolation of potential smaller isoform of β-catenin

5’ RACE nested PCR for isolating potential smaller isoform of β-catenin. PCR product resolved on 1.2% agarose gel. Yellow arrow shows the major 5’RACE fragment indicating presence of potential smaller isoform of β-catenin.


Relative Colony Formation
2.0
1.5
1.0
0.5
0.0
*
Negative Control shRNA (empty vector)
Negative Control shRNA (ΔGSK β-catenin)
Negative Control shRNA (β-catenin short isoform)
12688 shRNA (empty vector)
12688 shRNA (ΔGSK β-catenin)
12688 shRNA (β-catenin short isoform)

(Figure 29)

Figure 29: Ectopic expression of shorter isoform of β-catenin is unable to rescue decrease in colony forming capability in *Setbp1* immortalized cells

Mean and SD of colony formation potential of *Setbp1* immortalized cells (BM70 cells) in the presence of SCF, IL-3 and Puromycin at 48 hours after infection with 12688 shRNA/negative control shRNA and rescue with constitutively active full length β-catenin (MSCV-ΔGSK β-catenin) or β-catenin short isoform (MSCV- β-catenin short isoform) or empty MSCV virus.

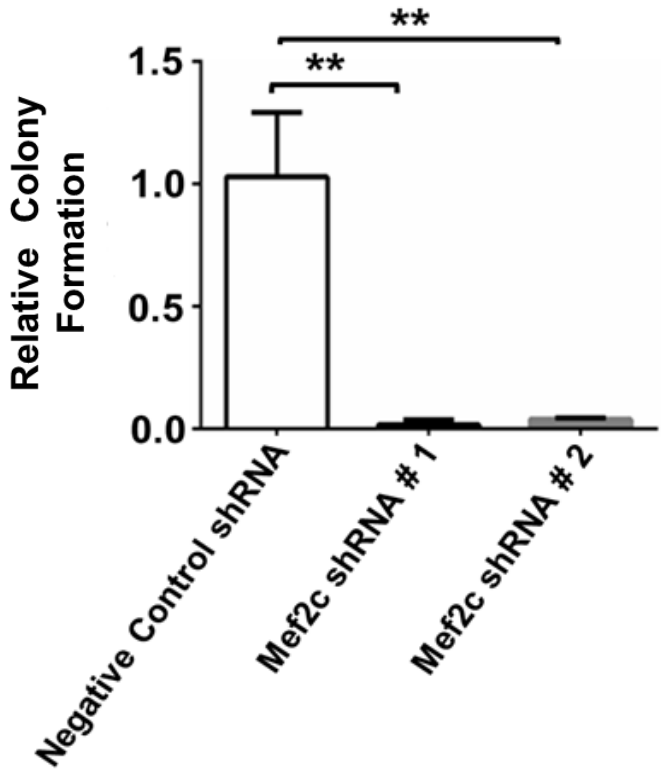


Figure 30: *Mef2c* knockdown inhibits the proliferation of *Setbp1* immortalized bone marrow cells

Mean and SD of colony formation potential of *Setbp1* immortalized (BM70) cells in the presence of SCF, IL-3 and puromycin at 48 hours after infection with *Mef2c* specific shRNA (Mef2c shRNA#1 and Mef2c shRNA#2) and control shRNA (Negative Control shRNA).

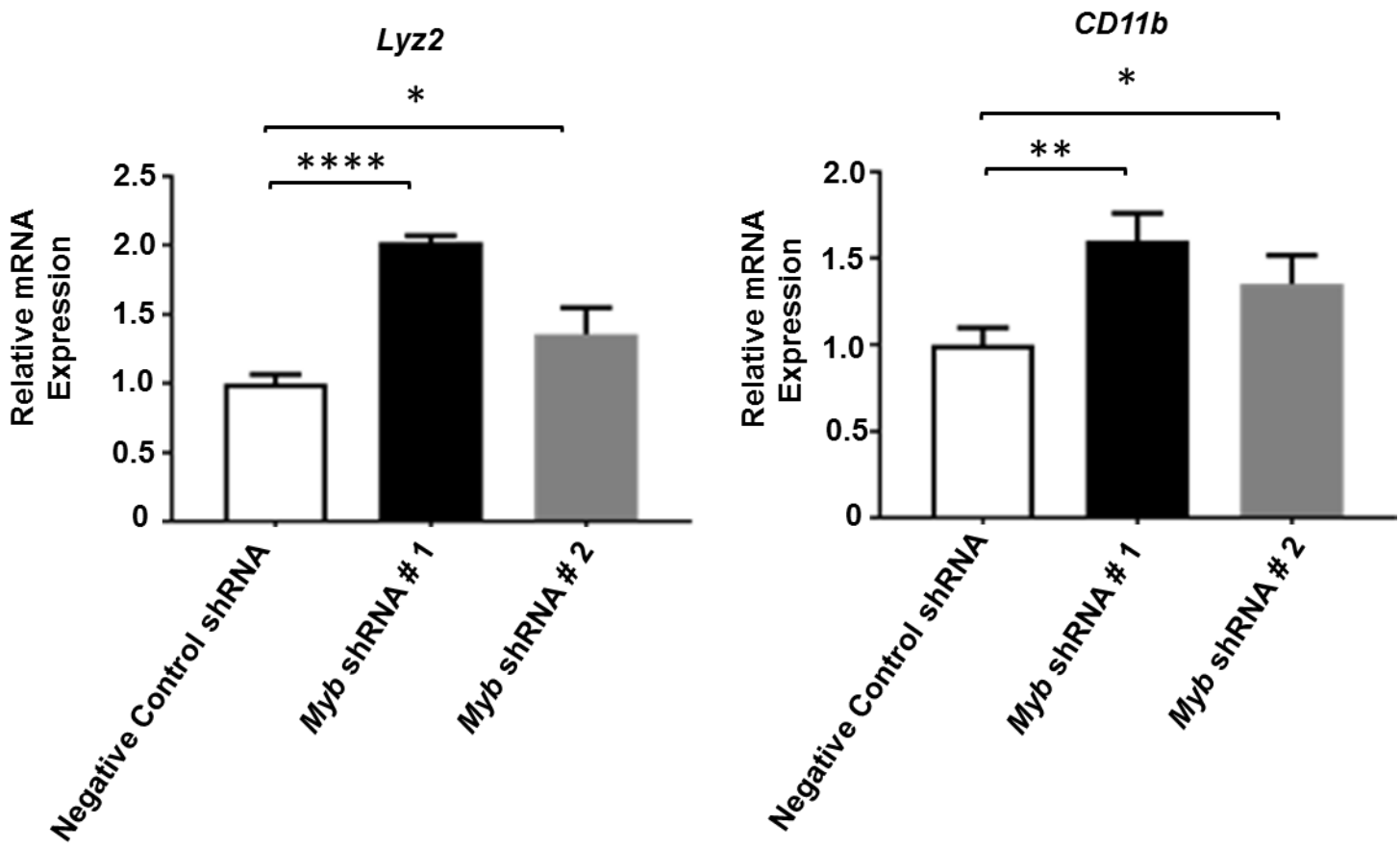


Figure 31: *Myb* knockdown induces differentiation of *Hoxa9* immortalized myeloid progenitors

Lyz2 and *CD11b* mRNA levels in *Hoxa9* immortalized primary hematopoietic progenitor cells after *Myb* knockdown. Real-time RT-PCR analysis of total RNA isolated from Blaticidin (14ug/ml) selected *Hoxa9* immortalized myeloid progenitor cells at 72 hours after infection with *Myb* specific shRNAs (Myb shRNA#1 and Myb shRNA#5) and control shRNA (Negative Control shRNA). Relative expression levels were calculated by normalizing to *RPL4* mRNA levels in the same samples.

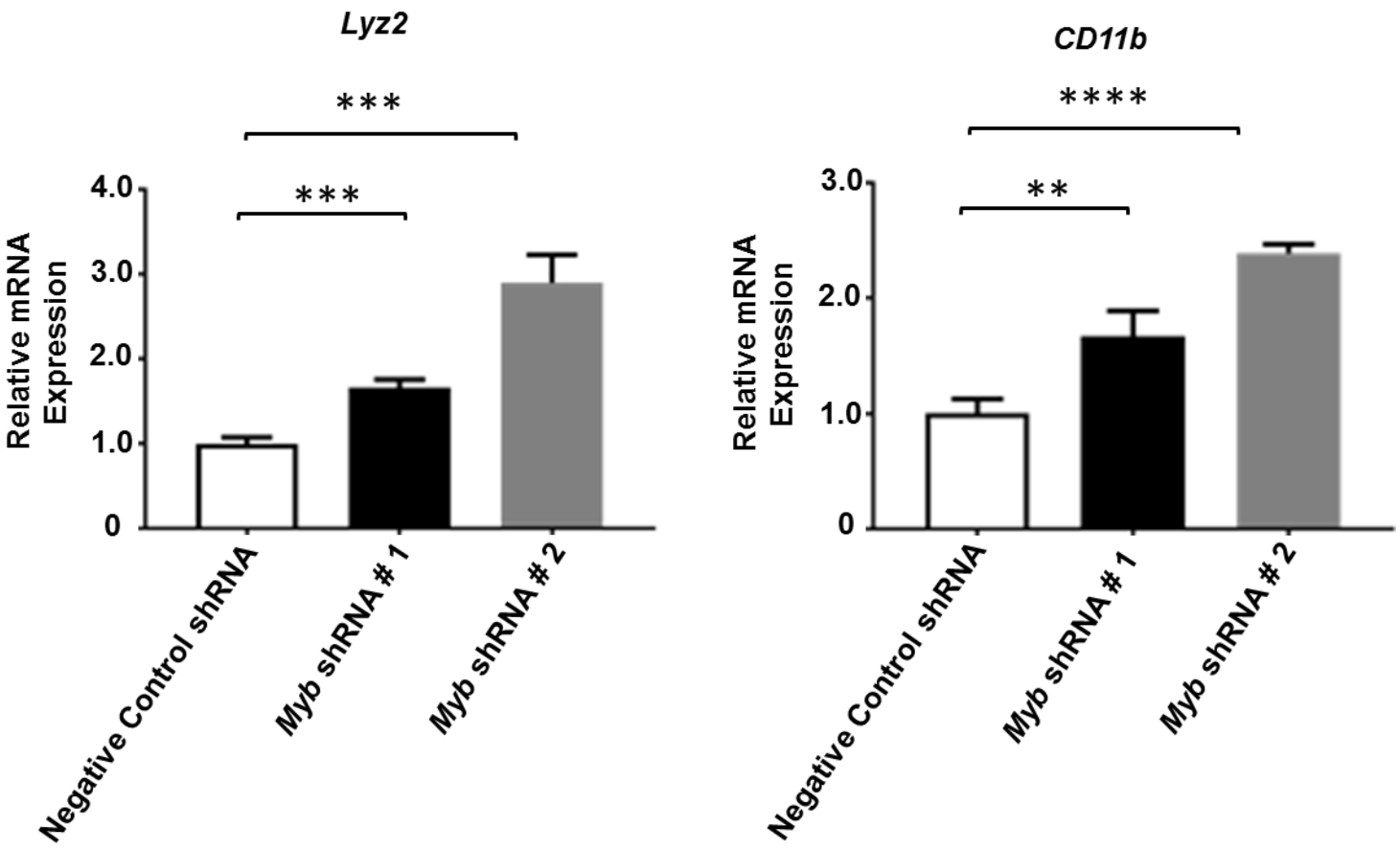
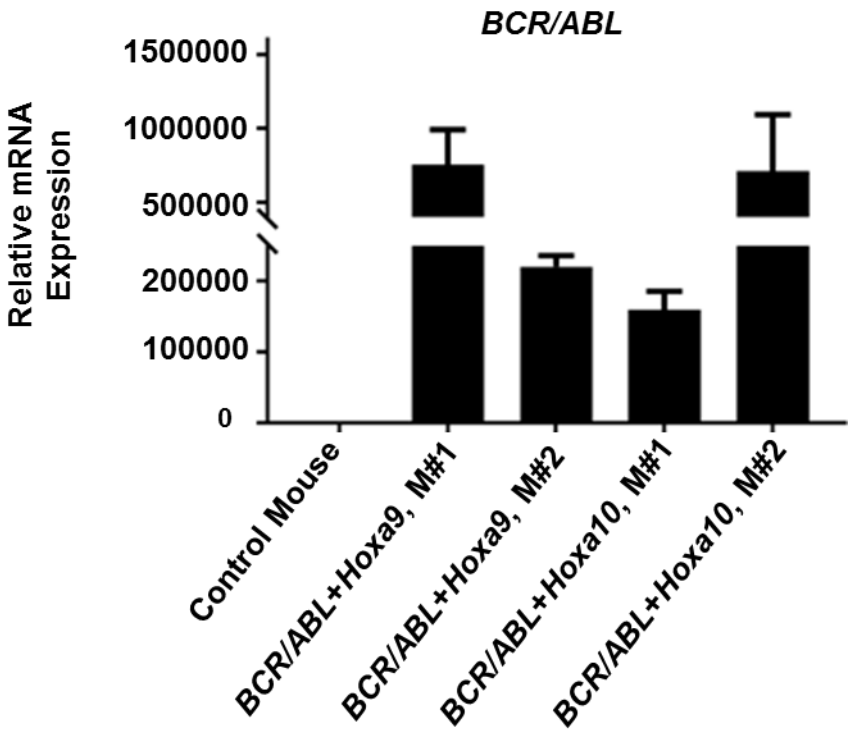
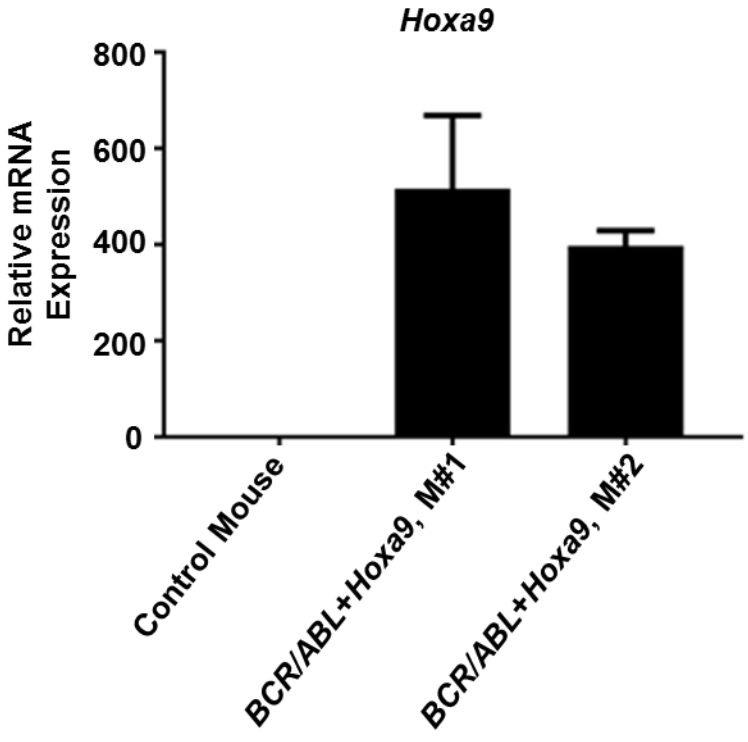


Figure 32: *Myb* knockdown induces differentiation of *Hoxa10* immortalized myeloid progenitors

Lyz2 and *CD11b* mRNA levels in *Hoxa10* immortalized primary hematopoietic progenitor cells after *Myb* knockdown. Real-time RT-PCR analysis of total RNA isolated from Blaticidin (14ug/ml) selected *Hoxa10* immortalized myeloid progenitor cells at 72 hours after infection with *Myb* specific shRNAs (Myb shRNA#1 and Myb shRNA#5) and control shRNA (Negative Control shRNA). Relative expression levels were calculated by normalizing to *RPL4* mRNA levels in the same samples.


BCR/ABL
Relative mRNA Expression
1500000
1000000
500000
200000
100000
0
Control Mouse
BCR/ABL+Hoxa9, M#1
BCR/ABL+Hoxa9, M#2
BCR/ABL+Hoxa10, M#1
BCR/ABL+Hoxa10, M#2


Hoxa9
Relative mRNA Expression
800
600
400
200
0
Control Mouse
BCR/ABL+Hoxa9, M#1
BCR/ABL+Hoxa9, M#2

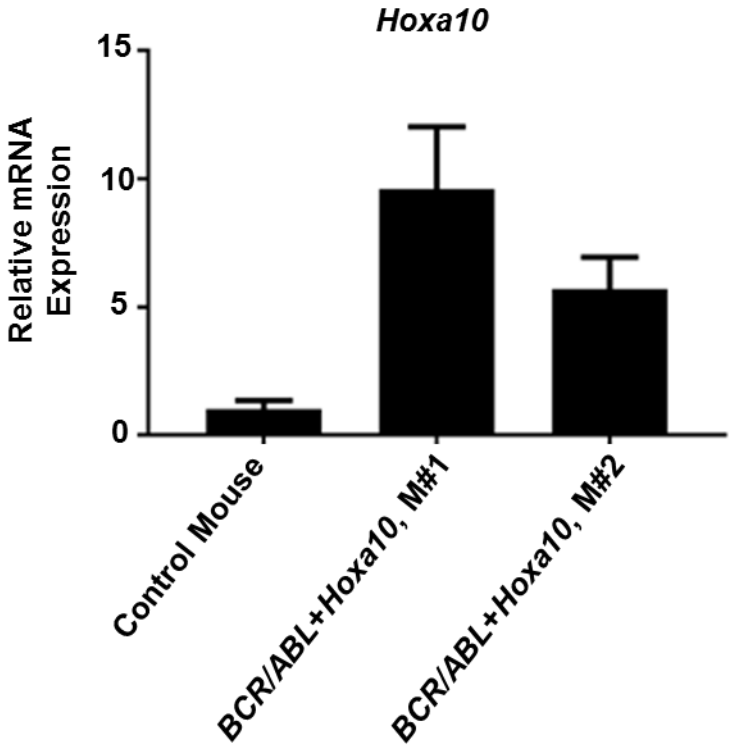

Hoxa10
Relative mRNA Expression
15
10
5
0
Control Mouse
BCR/ABL+Hoxa10, M#1
BCR/ABL+Hoxa10, M#2

Figure 33: *BCR/ABL, Hoxa9* and *Hoxa10* expression in *BCR/ABL + Hoxa9* and *BCR/ABL + Hoxa10* leukemic mice

BCR/ABL, *Hoxa9* and *Hoxa10* mRNA levels in *BCR/ABL + Hoxa9* and *BCR/ABL + Hoxa10* leukemic mice. Real-time RT-PCR analysis of total RNA isolated from moribund mice bone marrow tissue. Relative expression levels were calculated by normalizing to *RPL4* mRNA levels in the same samples. For *BCR/ABL,* relative mRNA expression was calculated by assigning *BCR/ABL* CT value of control mice as 40. For *Hoxa9* and *Hoxa10*, relative mRNA expression was calculated by using CT values of control mice as determined by the sample.

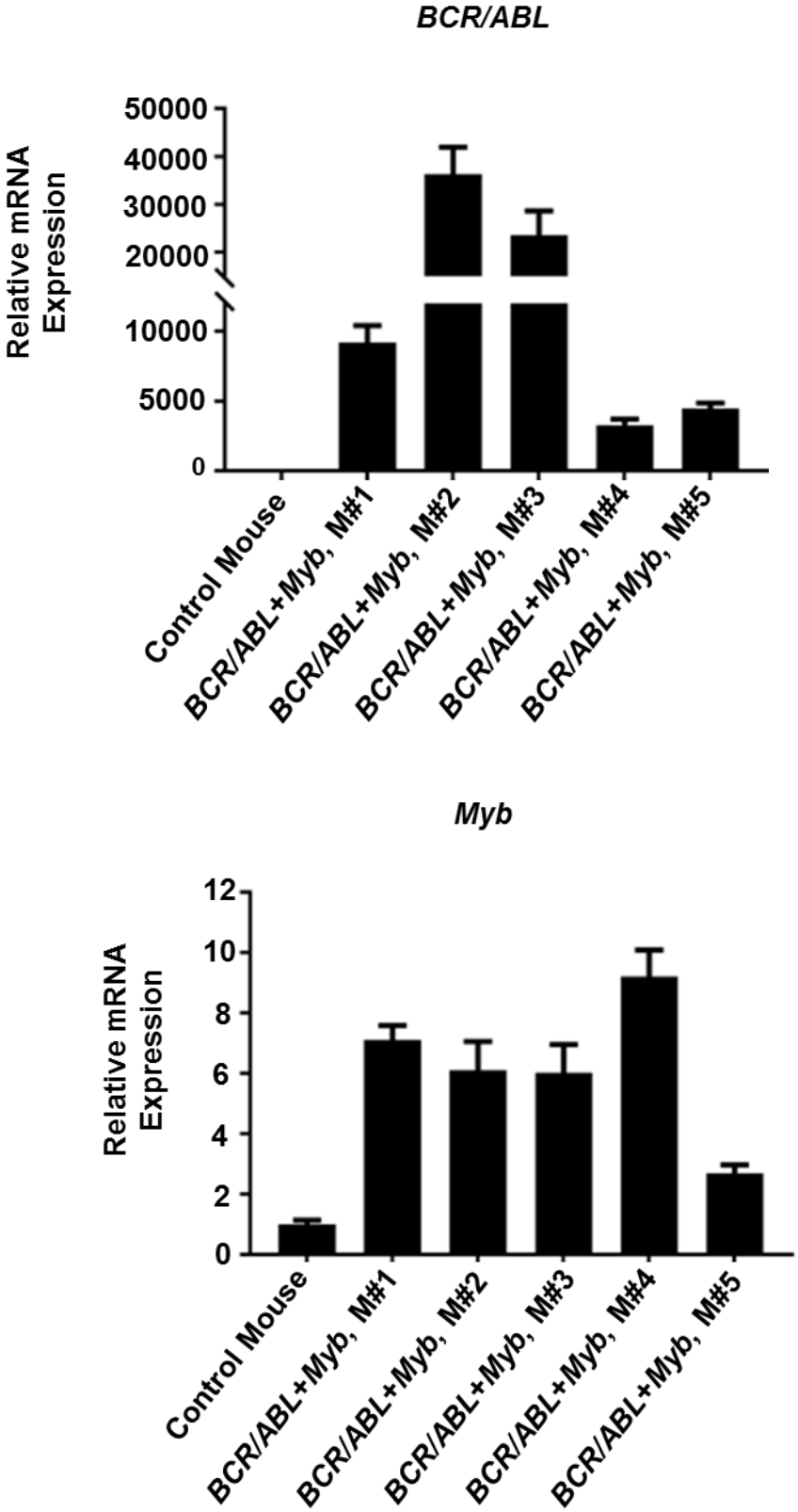

BCR/ABL
Relative mRNA Expression
50000
40000
30000
20000
10000
5000
0
Control Mouse
BCR/ABL+Myb, M#1
BCR/ABL+Myb, M#2
BCR/ABL+Myb, M#3
BCR/ABL+Myb, M#4
BCR/ABL+Myb, M#5
Myb
Relative mRNA Expression
12
10
8
6
4
2
0
Control Mouse
BCR/ABL+Myb, M#1
BCR/ABL+Myb, M#2
BCR/ABL+Myb, M#3
BCR/ABL+Myb, M#4
BCR/ABL+Myb, M#5

Figure 34: *BCR/ABL* and *Myb* expression in *BCR/ABL+ Myb* leukemic mice

BCR/ABL, and *Myb* mRNA levels in *BCR/ABL + Myb* leukemic mice. Real-time RT-PCR analysis of total RNA isolated from moribund mice bone marrow tissue. Relative expression levels were calculated by normalizing to *RPL4* mRNA levels in the same samples. For *BCR/ABL*, relative mRNA expression was calculated by assigning *BCR/ABL* CT value of control mice as 40. For *Myb*, relative mRNA expression was calculated by using CT value of control mice as determined by the sample.

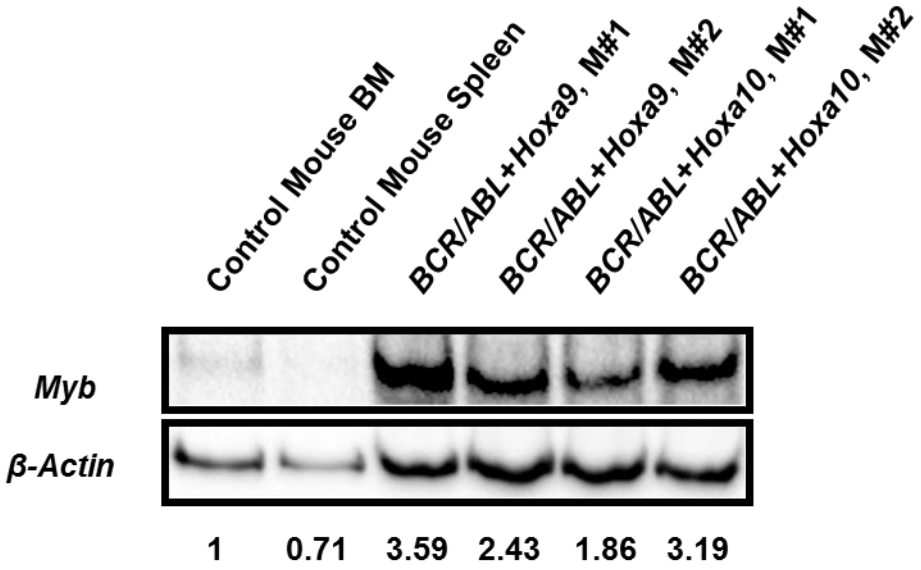


Figure 35: *Myb* expression in *BCR/ABL + Hoxa9* and *BCR/ABL + Hoxa10* leukemic mice

Western blotting analysis of Myb and β-Actin protein levels in protein lysates prepared from the spleens of *BCR/ABL + Hoxa9* and *BCR/ABL + Hoxa10* leukemic mice. Relative Myb protein levels after normalization to β-Actin levels in the same sample are indicated.

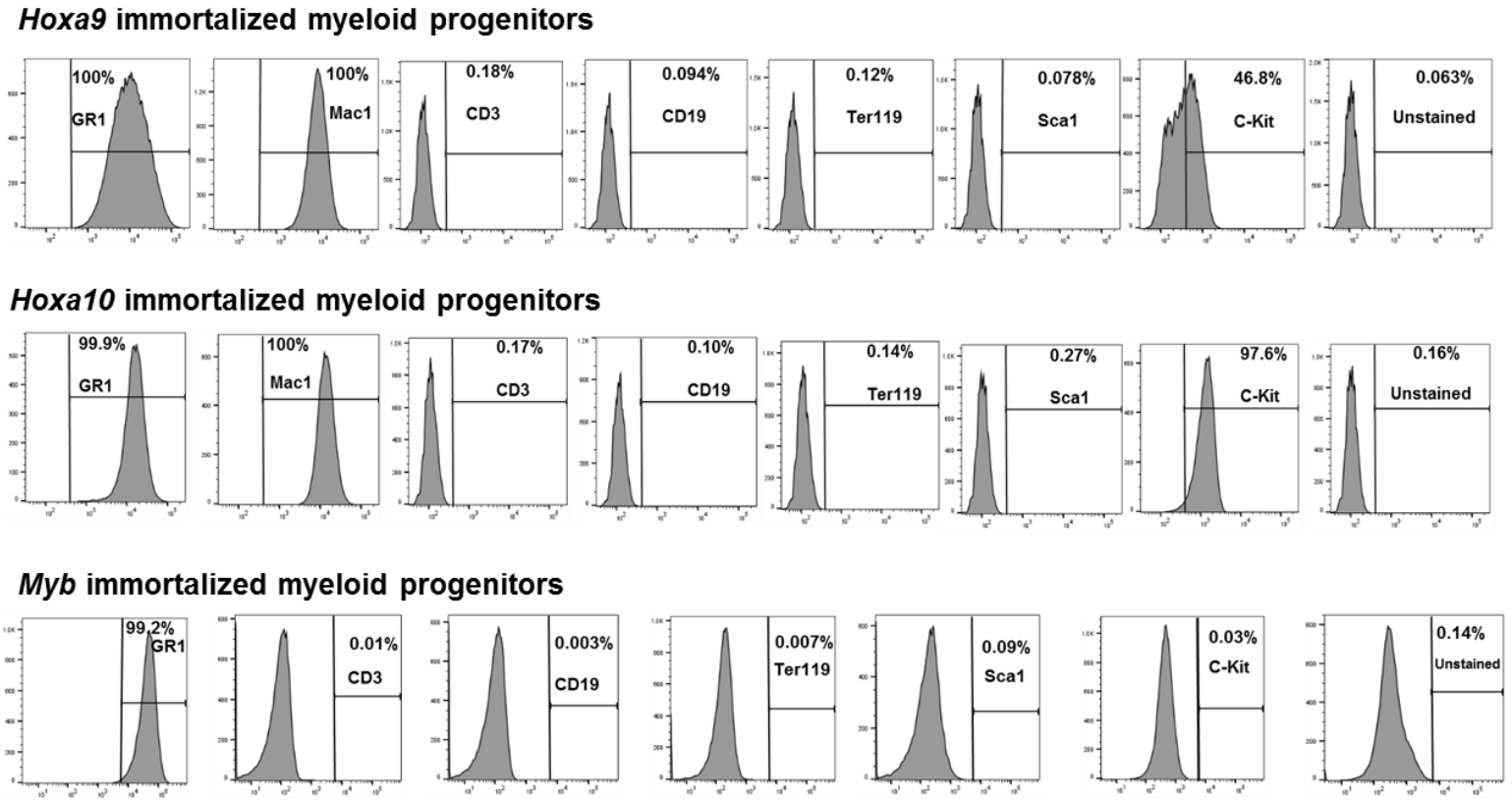


Figure 36: Lineage determination of immortalized cells

Flow cytometric analysis for lineage determination of *Hoxa9*, *Hoxa10* and *Myb*-immortalized myeloid progenitors using antibodies specific for myeloid lineage markers (Mac-1 and Gr-1), lymphoid lineage markers (CD-3, CD-19) and erythroid marker (Ter-119).

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