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Oncogene Function in Pre-Leukemia Stage of INV(16) Acute Myeloid Leukemia: A Dissertation

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ONCOGENE FUNCTION IN PRE-LEUKEMIA STAGE OF INV(16) ACUTE MYELOID LEUKEMIA

A Dissertation Presented

By

LITING XUE

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PROGRAM IN CANCER BIOLOGY

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DEDICATION

This work is dedicated to my great family:

my lovely daughter, Alicia Yin and my dear husband, Liusong Yin.

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Abstract

The CBFβ-SMMHC fusion protein is expressed in acute myeloid leukemia (AML) samples with the chromosome inversion inv(16)(p13;q22). This fusion protein binds the transcription factor RUNX with higher affinity than its physiological partner CBFβ and disrupts the core binding factor (CBF) activity in hematopoietic stem and progenitor cells. Studies in the Castilla laboratory have shown that CBFβ-SMMHC expression blocks differentiation of hematopoietic progenitors, creating a pre-leukemic progenitor that progresses to AML in cooperation with other mutations. However, the combined function of cumulative cooperating mutations in the pre-leukemic progenitor cells that enhance their expansion to induce leukemia is not known. The standard treatment for inv(16) AML is based on the use of non-selective cytotoxic chemotherapy, resulting in a good initial response, but with limited long-term survival. Therefore, there is a need for developing targeted therapies with improved efficacy in leukemic cells and minimal toxicity for normal cells.

Here, we used conditional *Nras*$^{+/LSL\text{-}G12D}$*; Cbfb*$^{+/56M}$*; Mx1Cre* knock-in mice to show that allelic expression of oncogenic N-Ras^{G12D} expanded the multi-potential progenitor (MPP) compartment by 8 fold. Allelic expression of Cbfβ-SMMHC increased the MPPs and short-term hematopoietic stem cells (ST-HSCs) by 2 to 4 fold both alone and in combination with N-Ras^{G12D} expression. In addition, allelic expression of oncogenic N-Ras^{G12D} and Cbfβ-SMMHC increases survival of pre-leukemic stem and progenitor cells. Differential analysis of bone marrow cells determined that *Cbfb*$^{+/MYH11}$ and *Nras*$^{+/G12D}$;

Cbfb$^{+/MYH11}$ cells included increased number of blasts, myeloblasts and promyelocytes and a reduction in immature granulocytes, suggesting that expression of N-Ras^{G12D} cannot bypass Cbfβ-SMMHC driven differentiation block.

N-Ras^{G12D} and Cbfβ-SMMHC synergized in leukemia, in which *Nras*$^{+/G12D}$; *Cbfb*$^{+/MYH11}$ mice have a shorter median latency than *Cbfb*$^{+/MYH11}$ mice. In addition, the synergy in leukemogenesis was cell autonomous. Notably, leukemic cells expressing N-Ras^{G12D} and Cbfβ-SMMHC showed higher (over 100 fold) leukemia-initiating cell activity *in vivo* than leukemic cells expressing Cbfβ-SMMHC (L-IC activity of 1/4,000 and 1/528,334, respectively).

Short term culture and biochemical assays revealed that pre-leukemic and leukemic cells expressing N-Ras^{G12D} and Cbfβ-SMMHC have reduced levels of pro-apoptotic protein Bim compared to control. The *Nras*$^{+/G12D}$; *Cbfb*^{MYH11} pre-leukemic and leukemic cells were sensitive to pharmacologic inhibition of MEK/ERK signaling pathway with increasing apoptosis and Bim protein levels but not sensitive to PI3K inhibitors. In addition, knock-down of *Bcl2l11* (*Bim*) expression in Cbfβ-SMMHC pre-leukemic progenitors decreased their apoptosis levels.

In collaboration with Dr. John Bushweller's and other research laboratories, we recently developed a CBFβ-SMMHC inhibitor named AI-10-49, which specifically binds to CBFβ-SMMHC, prevents its binding to RUNX proteins and restores CBF function. Biochemical analysis in human leukemic cells showed that AI-10-49 has significant specificity in reducing the viability of leukemic cells expressing CBFβ-SMMHC (IC_{50}= 0.83μM), and negligible toxicity in normal cells. Likewise, mouse *Nras*$^{+/G12D}$;

Cbfb$^{+/MYH11}$ leukemic cells were sensitive to AI-10-49 (IC_{50}= 0.93μM). By using the *Nras*$^{LSL\text{-}G12D}$*; Cbfb*^{56M} mouse model, we also show that AI-10-49 significantly prolongs the survival of mice bearing the leukemic cells. Preliminary mechanistic analysis of AI-10-49 activity has shown that AI-10-49 increased *BCL2L11* transcript levels in a dose and time dependent manner in murine and human leukemic cells, suggesting that the viability through BIM-mediated apoptosis may be targeted by both oncogenic signals.

My thesis study demonstrates that Cbfβ-SMMHC and N-Ras^{G12D} promote the survival of pre-leukemic myeloid progenitors primed for leukemia by activation of the MEK/ERK/Bim axis, and define *Nras*$^{LSL\text{-}G12D}$*; Cbfb*^{56M} mice as a valuable genetic model for the study of inv(16) AML targeted therapies. For instance, the novel CBFβ-SMMHC inhibitor AI-10-49 shows a significant efficacy in this mouse model. This small molecule will serve as a promising first generation drug for targeted therapy of inv(16) leukemia and also a very useful tool to understand mechanisms of leukemogenesis driving by CBFβ-SMMHC.

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Abbreviations

ACD	Assembly Competence Domain
AGM	Aorta-Gonad Mesonephros
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
AMP	Abnormal Myeloid Progenitor
APL	Acute Promyelocytic Leukemia
APT	Acyl Protein Thioesterase
BCL2L11	Bcl-2-like protein 11
BH	Bcl-2 Homology
BM	Bone Marrow
BOP	BH3-only protein
CB	Cord Blood
CBF	Core Binding Factor
CBL	Casitas B-cell Lymphoma
CFU	Colony Forming Unit
CKI	Cyclin-dependent kinase inhibitor
CLP	Common Lymphoid Progenitor
CM	CBFβ-SMMHC

CML	Chronic Myeloid Leukemia
CMML	Chronic Myelomonocytic Leukemia
CMP	Common Myeloid Progenitor
CR	Complete Remission
CREB	cAMP Response Element-Binding Protein
DMSO	Dimethyl Sulfoxide
E	Embryonic Day
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinase
ES	Embryonic Stem
ETP	Earliest Thymic Progenitor
ETS1	v-Ets Avian Erythroblastosis Virus E26 Oncogene Homolog 1
FAB	French-American-British
FACS	Fluorescence-Activated Cell Sorter
FL	FLT3 Ligand
FLT3	FMS-like Tyrosine Kinase 3
FOXP3	Forkhead Box P3
GAP	Guanine Activating Protein
G-CSF	Granulocyte-Colony Stimulating Factor

GEF	Guanine Nucleotide Exchange Factor
GFI1	Growth-Factor Independent 1
GM-CSF	Granulocytic-Monocytic Colony Stimulating Factor
GMP	Granulocyte-Macrophage Progenitor
GO	Gemtuzumab Ozogamicin
GRB2	Growth Factor Receptor-Bound protein 2
GSK3	Glycogen Synthase Kinase 3
H&E	Hematoxylin and Eosin
HABD	High-Affinity Binding Domain
DAC8	Histone Deacetylase 8
HiDAC	High Dose of Cytarabine
HIF-1α	Hypoxia-Inducible Factor-1α
HIPK2	Homeodomain interacting kinase 2
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic Stem Cell Transplantation
HSPC	Hematopoietic Stem and Progenitor Cell
HVR	Hypervariable Region
ICMT	Isoprenylcysteine-Carboxyl-Methyltransferase
ID	Inhibitory Domain
IFNγ	Interferon-γ

inv(16)	Chromosome 16 (p13; q22) Inversion
IRF8	Interferon-Fegulatory Factor 8
ITD	Internal Tandem Duplication
JM	Juxtamembrane
JMML	Juvenile Myelomonocytic Leukemia
LFS	Leukemia-Free Survival
L-IC	Leukemia-Initiating Cell
Lin	Lineage
LMP	pMSCV-LTRmiR30-PIG
LMPP	Lymphoid-primed Multipotent Progenitor
LSK^-	$Lin^-Sca1^-cKit^+$
LSK^+	$Lin^-Sca1^+cKit^+$
LT-HSC	Long-Term HSC
M-CSF	Monocytic-Colony Stimulating Factor
MDM2	Murine Double Minute 2
MDS	Myelodysplastic Syndrome
MEK	Mitogen-Activated Protein Kinase
MEP	Megakaryocyte-Erythrocyte Progenitor
MLP	Multilymphoid Progenitor

MPN	Myeloproliferative Neoplasm
MPP	Multipotent Progenitor
mTORC2	The Mammalian Target of Rapamycin Complex 2
NF1	Neurofibromin 1
NK	Natural Killer
NLS	Nuclear Localization Signal
NMTS	Nuclear-Matrix-Targeting Signal
NOS	Not Otherwise Specified
NS	Not Significant
NSG	*NOD-scid-IL2Rgc*$^{-/-}$
OS	Overall Survival
$p90^{Rsk-1}$	p90 Ribosomal Six Kinase-1
PB	Peripheral Blood
PCR	Polymerase Chain Reaction
PDK1	3-Phosphoinositide-Dependent Kinase-1
PEBP2α	Polyoma Enhancer Binding Protein 2α
PI3K	Phosphoinositide 3-Kinase
PIP_2	Phosphatidylinositol-4, 5-Bisphosphate

PIP_3	Phosphatidylinositol-3, -4, 5-Trisphosphate
PM	Plasma Membrane
poly(I:C)	Polyinosinic-Polycytidylic Acid
preBCR	preB-cell antigen receptor
PTEN	Phosphatase and Tensin Homologue
qRT-PCR	Quantitative Reverse Transcription-Polymerase Chain Reaction
RCE1	Ras-Converting Enzyme 1
RHD	Runt Homology Domain
RTK	Receptor Tyrosine Kinase
SCF	Stem Cell Factor
SFK	Src Family Kinase
SH2	Src Homology 2
SHC	Src Homology 2 Domain Containing Protein
SHP2	Src Homology-2 Domain –Containing Phosphatase 2
SMMHC	Smooth Muscle Myosin Heavy Chain
SOS	Son of Sevenless Homolog
SP	Spleen
STATs	Signal Transducers and Activators of Transcription
ST-HSC	Short-Term HSC

SUV39H1	Suppressor of Variegation 3-9 Homolog 1
TAD	Transactivation Domain
TE	Transactivation Element
TF	Transcription factor
TGFβ	Transforming Growth Factor β
TKB	Tyrosine Kinase Binding
TKD	Tyrosine Kinase Domain
TSC2	Tuberus Sclerosis Complex 2
UPD	Uniparental Disomy
WBC	White Blood Cell
WHO	World Health Organization
WT	Wild Type

Chapter I

Introduction

Acute Myeloid Leukemias (AMLs) comprise a variety of hematopoietic malignancies in the bone marrow, presenting impaired differentiation, deregulated proliferation and a survival advantage of myeloid progenitor cells. AML has an incidence of three to five cases per 100,000 population in the developed countries (Dores et al., 2012; Sant et al., 2010; Smith et al., 2011) with a poor clinical outcome (Estey and Dohner, 2006; Lowenberg et al., 1999). In the United States, there are approximately 19,000 new cases of AML diagnosed per year, with death rate close to 50% (American Cancer Society, www.cancer.org). Therefore, understanding the molecular basis of AML initiation and maintenance is critical for the development of improved treatment regimens of these aggressive hematopoietic malignancies.

I.A Hematopoietic differentiation

Hematopoiesis is the process that forms all the cellular components of the blood system in an organism. As one of the most highly regenerative tissues, approximately 10^{12} blood cells arise each day in adult human bone marrow (Doulatov et al., 2012). Bone marrow cells present a variety of morphologies, reflecting different lineage and stages of differentiation. The mature hematopoietic cells include megakaryocytes/platelets, erythrocytes, myeloid cells (monocytes/macrophages, dendritic cells and granulocytes) and lymphocytes (T cells, B cells and natural killer cells).

I.A.1 Road map of hematopoietic differentiation

The diverse composition of cell types in the blood is generated by the hematopoietic stem cells (HSCs), a rare cell population that resides in the bone marrow. The origin of HSCs has been the focus of developmental biologists. During mammalian embryo development, the hematopoietic system, together with vascular, cardiac, and skeletal muscle lineages, is derived from mesoderm (Murry and Keller, 2008). There are multiple waves of hematopoietic cells that emerge during embryo development. The initial wave in the mammalian yolk sac [embryonic day (E) 7.5-10.5] is termed "primitive" hematopoiesis, and produces nucleated primitive erythrocytes that facilitate tissue oxygenation during the rapid growth of embryo (Golub and Cumano, 2013; Orkin and Zon, 2008). The yolk sac also generates myeloid cells that migrate to the central nervous system and the skin to form the microglia and epidermal specific macrophages (Bertrand et al., 2005; Ginhoux et al., 2010; Palis et al., 1999; Schulz et al., 2012). This initial wave is rapidly replaced by the "definitive" hematopoiesis, which originates in the aorta-gonad-mesonephors (AGM) region and placenta (E8.5-13.5), generating HSCs from hemogenic endothelial cells (Gekas et al., 2005; Muller et al., 1994; Ottersbach and Dzierzak, 2005). Subsequent definitive hematopoiesis involves the colonization of HSCs to the fetal liver (E10.5 to after birth), thymus, spleen, and ultimately the bone marrow (Orkin and Zon, 2008).

The identification of bone marrow HSCs and subsequent progenitor cells has advanced significantly during decades of research. Studies in 1960s, using clonal *in vivo* repopulation assays, functionally suggested the existence of multipotent HSCs (Becker et

al., 1963; Till and Mc, 1961). With additional use of cell surface markers, flow cytometry analysis and *in vitro* colony forming assays, led by studies in the laboratory of Irving Weissman in the 1990s (Morrison et al., 1997; Morrison and Weissman, 1994), a detailed hematopoietic progenitor cell system has been revealed as a developmental hierarchy of progenitor cells, with the long-term HSCs (LT-HSCs) at the apex and terminally differentiated cells at the bottom (**Figure I.1**).

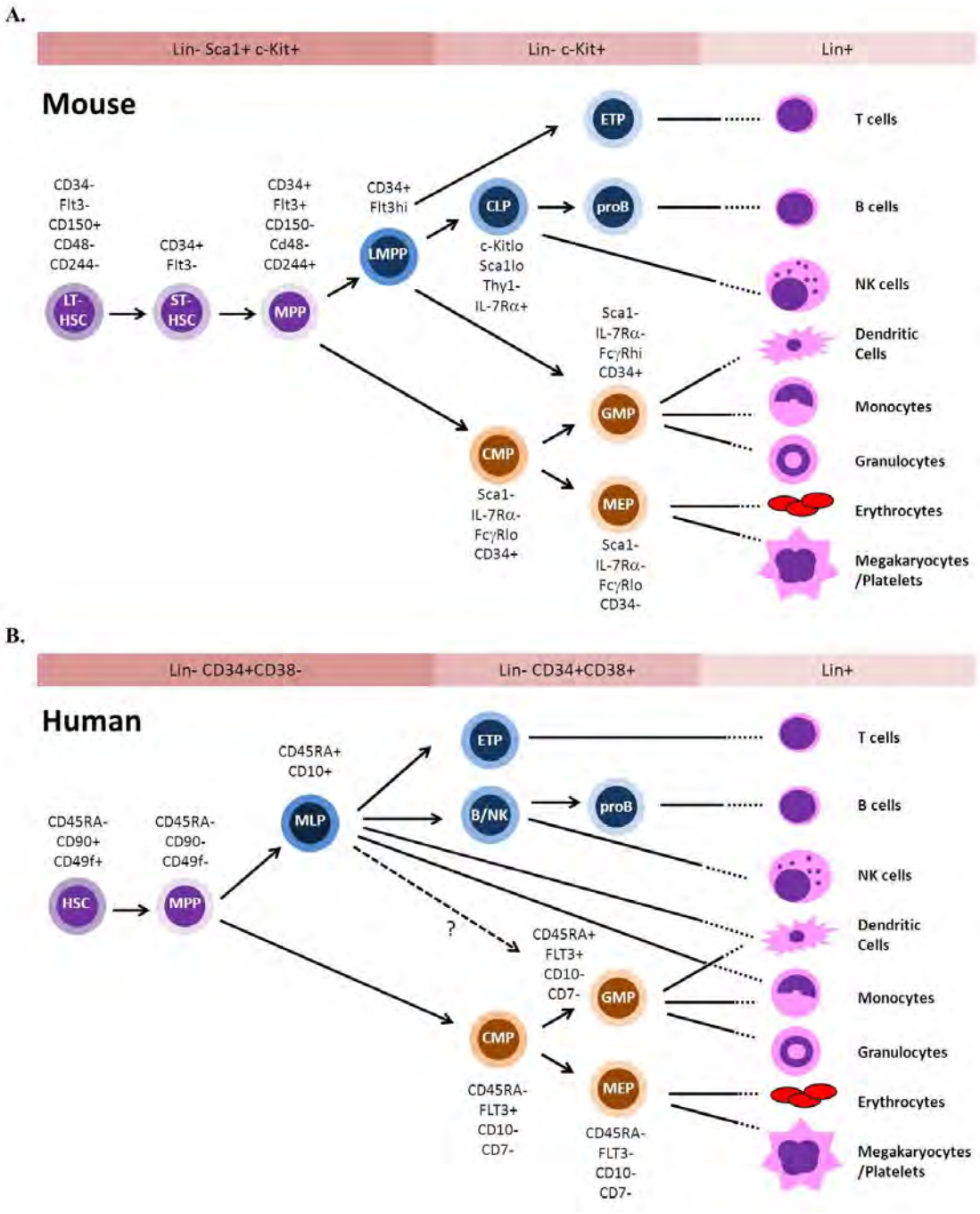

Figure I.1 Current models of hierarchies in the adult mouse and human hematopoiesis. The major classes of stem and progenitor cells described in the text are defined by cell surface phenotypes, which are listed next to each population and in the bars above each schematic plot. Terminally differentiated cells are shown on the right, and inferred lineage relationships are depicted with arrows. (A) Mouse; (B) Human. This figure was adapted from Doulatov et al., 2012.

In mouse studies, LT-HSCs have been historically defined as cells with two key functions: *multilineage repopulation* and *self-renewal*. Multilineage repopulation refers to the ability to produce long-term multi-lineage reconstitution in transplantation assays. Self-renewal is the process by which stem cells divide to make more stem cells, maintaining the undifferentiated state and perpetuating the stem cell pool throughout life (He et al., 2009). The LT-HSC activity is contained in cells presenting surface markers as Lineage$^-$(Lin$^-$, referring to negative for a cell surface marker cocktail of differentiated cells, such as markers for T cells, B cells, Monocytes, Granulocytes and Erythrocytes) Sca1$^+$c-Kit$^+$ (LSK$^+$) CD34$^-$Flt3$^-$(Adolfsson et al., 2001; Christensen and Weissman, 2001; Osawa et al., 1996). LT-HSCs have alternatively been defined by CD150$^+$CD48$^-$CD244$^-$ SLAM markers (Kiel et al., 2005). LT-HSCs can give rise to short-term HSCs (ST-HSCs) with cell surface markers as LSK$^+$CD34$^+$Flt3$^-$(Osawa et al., 1996; Yang et al., 2005), and then multipotent progenitors (MPPs) with cell surface markers as LSK$^+$CD34$^+$Flt3$^+$(Adolfsson et al., 2001). ST-HSCs and MPPs both give multi-lineage but transient reconstitution, and MPPs are responsible primarily for rapid lymphoid reconstitution (Yang et al., 2005).

The differentiation to the myeloid-erythroid compartment has also been defined in the mouse, by immunophenotyping. The MPPs give rise to the lymphoid-primed multipotent progenitors (LMPPs, LSK$^+$CD34$^+$Flt3^{hi}), with lymphoid bias and very low Erythro-Megakaryocytic potential (Adolfsson et al., 2005; Mansson et al., 2007). The LMPPs give rise to common lymphoid progenitors (CLPs, IL-7Rα^+Lin$^-$Sca-1^{lo}c-Kit^{lo}Thy-1$^-$) and granulocyte-macrophage progenitors (GMPs, IL-7Rα^-Lin$^-$Sca-1$^-$c-

$Kit^{+}Fc\gamma R^{hi}CD34^{+}$) (Akashi et al., 2000; Kondo et al., 1997). A variety of early lymphoid progenitors showing different states of lymphoid commitment have also been identified, suggesting the differentiation in the lymphoid progenitors is a gradual process (Welner et al., 2008). The CLPs can commit to differentiate into the B cell lineage, forming the pre-pro B, pro B, pre-B stages to give rise to mature B cells (Nutt and Kee, 2007). CLPs can also give rise to nature killer (NK) cells (Fathman et al., 2011; Rosmaraki et al., 2001). Though CLPs possess lineage B and T potential in transplantation assays (Kondo et al., 1997), more recent studies suggest that early thymic progenitors (ETPs, c-kit^{+}DN1, DN stands for "double negative") may derive from LMPPs in the circulation, differentiate through DN2, DN3, DN4, double positive stages and give rise to T cells in the thymus [reviewed in (Ye and Graf, 2007)]. For myeloid differentiation, the "classic" model (Reya et al., 2001) proposes that MPPs give rise to common myeloid progenitors (CMPs, IL-$7R\alpha^{-}Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}Fc\gamma R^{lo}CD34^{+}$), and then CMPs give rise to GMPs and megakaryocyte-erythrocyte progenitors (MEPs, IL-$7R\alpha^{-}Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}Fc\gamma R^{lo}CD34^{-}$) (Akashi et al., 2000). The GMPs differentiate to monocytes, granulocytes, and dendritic cells. The MEPs differentiate into erythrocytes and megakaryocytes and platelets. Particularly, the differentiation steps from GMPs to granulocytes include myeloblast, promyelocyte, myelocyte, metamyelocyte and neutrophils (Rosenbauer and Tenen, 2007), which have distinct cell morphologies (Yang et al., 2013).

Advances in the characterization of hematopoietic progenitors continue to redefine these models. A recent study using a single-cell transplantation system and marker mice have suggested a new myeloid bypass model in which the myeloid-restricted progenitors

with long-term repopulating activity, directly differentiated from HSCs without passing through an MPP stage (Yamamoto et al., 2013).

The cell surface markers of human HSCs and their progeny do not coincide with those in the mouse. The first marker identified in human HSCs is CD34, expressing on less than 5% of all blood cells (Civin et al., 1984). Over the past decades, using *in vitro* colony forming assays and xenograft experiments on immunodeficient mice, the combined markers as $CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$ for human HSCs have been identified (Baum et al., 1992; Bhatia et al., 1997; Conneally et al., 1997; Lansdorp et al., 1990; Murray et al., 1995). A more recent study has shown that human HSC activity was restricted in CD49f (integrin subunit α6) positive cells (Notta et al., 2011). In contrast, $CD90^{-}CD49f^{-}$ cells behave as MPPs by transplantation assay in NOD-*scid*-*IL2Rgc*$^{-/-}$ (NSG) mice. Human hematopoiesis does not follow a rigid model of myeloid-lymphoid segregation. A broader term, multilymphoid progenitor (MLP), has been given to describe any progenitor that gives rise to all lymphoid lineages, but also has myeloid potential (Doulatov et al., 2010). Similarly as in mice, CMPs, GMPs and MEPs have been isolated from human hematopoietic system (Doulatov et al., 2010; Manz et al., 2002). The cell surface markers for each progenitor compartment are reviewed in **Figure I.1**.

I.A.2 Regulation of hematopoietic differentiation

Hematopoiesis is a tightly regulated process that balances self-renewal and differentiation, dormancy and proliferation of its progenitor cells. This regulation

involves specific molecules in the microenvironment (Kiel and Morrison, 2008; Scadden, 2014), such as oxygen levels (Takubo et al., 2010), calcium levels (Adams et al., 2006), adhesion molecules (Sugiyama et al., 2006; Zou et al., 1998) and cytokines (Kondo et al., 2000; Metcalf, 1998; Rieger et al., 2009). It also involves signaling pathways (Karanu et al., 2000; Schreck et al., 2014; Varnum-Finney et al., 2000), epigenetic changes (Borghesi, 2014) and transcriptional factors (TFs) (Orkin and Zon, 2008; Rosenbauer and Tenen, 2007; Zhang et al., 2004). The deregulation of hematopoietic homeostasis can result from genetic mutations in genes encoding signaling molecules in cytokine response pathways and TFs, rendering hematopoietic progenitor cells with impaired differentiation, survival and proliferative capacity.

Role of cytokines

Cytokines in the microenvironment serve as extrinsic signals to direct hematopoietic proliferation and differentiation (Metcalf, 1998). These factors act via specific cell membrane receptors, such as the receptor tyrosine kinase (RTK) family, the cytokine receptor family and the transforming growth factor-β (TGFβ) receptor family.

Ligand binding induces intrinsic tyrosine kinase activity of RTKs and downstream intracellular signaling pathways leading to various biological effects (details about RTK signaling pathways and their deregulation in AMLs will be reviewed in Subchapter I.E). Stem Cell Factor (SCF) is an important cytokine for regulating HSC functions. Null-mutations in genes that encode SCF or its receptor c-KIT (a class III RTK) result in severe reduction of HSC numbers and self-renewal activity (Bernstein, 1962; McCulloch

et al., 1965). c-KIT signaling regulates hematopoietic progenitors' cell cycle (Leary et al., 1992), survival (Borge et al., 1997), maintenance (Thoren et al., 2008) and adhesion to stromal cells in their niche (Levesque et al., 1996; Levesque et al., 1995). FMS-like tyrosine kinase 3 (FLT3) ligand (FL) is another important cytokine for HSC and lymphoid differentiation. FLT3 also belongs to the class III RTK family. Flt3-null mice have reduced number of B-cell progenitors and exhibit deficiency in T-cell and myeloid lineage reconstitution in bone marrow transplantation (Mackarehtschian et al., 1995). FL promotes survival of early hematopoietic progenitor cells and, in combination with other factors, regulates proliferation of those cells (Rasko et al., 1995).

The cytokine receptor family and the TGFβ receptor family also play important roles in regulating hematopoietic differentiation. MPL is a type I cytokine receptor activated by the cytokine thrombopoietin. It regulates proliferation of HSCs and megakaryocytes (Kaushansky and Ranney, 2009). The number and function of HSCs are largely reduced in Mpl-null and Thpo-null mice (Alexander et al., 1996; Carver-Moore et al., 1996; Kimura et al., 1998). Nonsense mutations in the MPL and THPO genes cause congenital amegakaryocytic thrombocytopenia (Ballmaier and Germeshausen, 2009). In contrast, activating mutations in the MPL gene have been detected in a small fraction of myeloproliferative neoplasm and megakaryoblastic AML (Hussein et al., 2009; Pardanani et al., 2006). Interleukin-3 receptor is another important type I cytokine receptor in regulating hematopoiesis. Interleukin-3 is a product of $CD4^+$ T cells that acts on the most immature marrow progenitors (Ihle et al., 1983; Metcalf, 1989; Pierce, 1989). IL-3 is capable of inducing the growth and differentiation of multi-potential

hematopoietic stem cells, neutrophils, eosinophils, megakaryocytes, macrophages, lymphoid and erythroid cells (Reddy et al., 2000). Granulocytic-monocytic colony stimulating factor (GM-CSF), monocytic-colony stimulating factor (M-CSF) and granulocyte-colony stimulating factor (G-CSF) are involved in myeloid differentiation. The receptors of GM-CSF and G-CSF belong to the type I cytokine receptor family while the M-CSF receptor belong to the class III RTK family. These cytokines stimulate proliferation and differentiation of hematopoietic progenitors towards GMPs, monocytes and granulocytes, respectively (Gasson, 1991; Lieschke et al., 1994; Stanley et al., 1983). TGFβ has been shown to inhibit the growth and differentiation of quiescent early HSCs and stimulate the differentiation of late progenitors (Isufi et al., 2007). Disruption of TGFβ signaling has been observed in many myeloid and lymphoid neoplasms (Isufi et al., 2007).

Role of TFs

Triggered by multiple signals at different developmental stages, TFs activate lineage-specific programs in hematopoietic stem and progenitor cells dependent upon their expression levels and timing (Iwasaki and Akashi, 2007), and may direct cell fate decisions in collaboration or competition with other factors.

The regulation of lymphoid differentiation depends on the function of specific TFs. For example, Ikaros and PU.1 are required primarily for the formation of early lymphoid progenitors (DeKoter et al., 2002; Scott et al., 1994; Wang et al., 1996a). Other factors, such as E2A and EBF1, have direct roles in specifying the B cell gene expression while

PAX5 functions to promote B cell commitment by repressing lineage-inappropriate gene expression and reinforcing B cell specific gene expression (Nutt and Kee, 2007). In T cell development, Notch-Delta signaling behaves as a multilevel regulator. The success of T cell development depends on precise temporal and quantitative regulation of other factors, such as GATA3, TCF family factors, E2A, HEB, IKAROS, MYB, GFI1, RUNX family factors and PU.1, and on the modulating of Notch-Delta signals that buffer the cells against mechanisms promoting non-T outcomes (Rothenberg, 2007).

The regulation of myeloid differentiation programs depend on major TFs, such as PU.1, CEBP proteins (in particular, C/EBPα and C/EBPε), GFI1 and IRF8 (Rosenbauer and Tenen, 2007). The TF PU.1, encoded by the *SPI* gene, is critical in the formation of the earliest myeloid transcriptional network, generating CMPs from HSCs. Absence of PU.1 impairs HSC repopulation capacity and precludes differentiation into CMPs and CLPs (Dakic et al., 2005; Iwasaki et al., 2005; Scott et al., 1997). In addition, different levels of PU.1 expression are required for generation of different lineages. In the MPP stage, low levels of PU.1 promote B-cell development, whereas high levels favor macrophage development (DeKoter and Singh, 2000).

The TF C/EBPα , encoded by the *CEBPA* gene, plays an important role on the CMP to GMP transition. Genetic studies in mice have demonstrated that *Cebpa*-null HSCs are able to generate normal numbers of CMPs but they cannot produce GMPs and all following progenies to granulocytic lineage (Zhang et al., 1997a; Zhang et al., 2004). This phenotype correlates with the observation that C/EBPα regulates the expression of a number of myeloid-specific genes, similar as PU.1 (Tenen et al., 1997). Furthermore,

repression of E2F (a TF functioning in cell cycle control) activity by C/EBPα is critical for granulocytic differentiation (Porse et al., 2001).

The TFs PU.1 and its binding partner IRF8, determine granulocytic versus monocytic/macrophage fates from GMPs (Rosenbauer and Tenen, 2007). In the myeloid lineages, high levels of PU.1 support macrophage production, whereas low levels of PU.1 support granulocyte production (Dahl et al., 2003). Similarly, *Irf8*$^{-/-}$ mice strongly resembled human chronic myeloid leukemia (CML), showing a marked increase in the number of granulocytes and their precursors, as companied by fewer macrophages (Scheller et al., 1999). Accordingly, restoration of Irf8 expression in cell lines derived from *Irf8*$^{-/-}$ mice induced differentiation towards macrophages (Tamura et al., 2000).

Two additional TFs, GFI1 and C/EBPε are required in granulocyte specification. *Gfi1*-deficiency in mice impaired lymphoid differentiation and resulted in lack of neutrophilic granulocytes (Hock et al., 2003; Karsunky et al., 2002). However, the development of GMPs was not compromised and the block in neutrophilic differentiation was beyond the promyelocyte stage (Hock et al., 2003). The C/EBPε-deficient mice closely resembled this phenotype and had additional phenotype as abnormal granulopoiesis beyond the promyelocyte stage (Yamanaka et al., 1997).

These key TFs play dominant or negative roles, and in some cases antagonize each other to direct hematopoietic differentiation (Orkin, 2000). Disruptions of these critical TFs by chromosomal translocations or inversions are often seen in leukemia. Here, we will focus on the function of a critical TF family, core binding factors, which are frequently mutated in AMLs.

I.B Core binding factor

The core binding factor (CBF) is a heterodimeric transcription factor that regulates multiple cellular processes, including cell fate, differentiation, and survival. The CBF is composed of two core subunits: the RUNX protein, encoded by one of three genes (*RUNX1*, *RUNX2*, and *RUNX3*), binds to DNA in cis regulatory regions, and the non-DNA-binding partner, CBFβ, which stabilizes the complex and increases RUNX affinity to DNA (Blyth et al., 2005; Huang et al., 2001; Ogawa et al., 1993; Wang et al., 1993). In this section, I summarize the main concepts of CBF function, with focus on hematopoiesis.

I.B.1 CBF protein structure and general functions

The coincidental study of CBF function by scientists from fields of virology, leukemia and developmental biology resulted in a number of different names for the RUNX genes (Bae et al., 1994; Bae et al., 1995; Bae et al., 1993; Calabi et al., 1995; Kamachi et al., 1990; Levanon et al., 1994; Miyoshi et al., 1991; Wang and Speck, 1992; Zhang et al., 1997b). This confusion was resolved by the redefinition to "RUNX" names for the mammalian genes, and accepted by the HUGO Nomenclature Committee (van Wijnen et al., 2004). This nomenclature reflects an earlier discovery of *Drosophila Runt* as an essential gene in embryogenesis (Gergen and Butler, 1988), and the numbering as historically identified, which is summarized in **Table I.1**(van Wijnen et al., 2004).

Table I.1 Synonyms for human RUNX gene names and their chromosome location

RUNX	CBFα	AML	PEBP2α	Location
RUNX1	CBFA2	AML1	PEBP2αB	21q22
RUNX2	CBFA1	AML3	PEBP2αA	6p21
RUNX3	CBFA3	AML2	PEBP2αC	1p36

Adapted from van Wijnen et al., 2004

The most highly conserved feature of the RUNX proteins is the N-terminal 128-amnio-acid Runt Homology Domain (RHD), located between amino acids 50 and 177 in RUNX1 (Kagoshima et al., 1993; Levanon et al., 1994; Meyers et al., 1993) (**Figure I.2**). The three-dimensional structure for the RUNX1/CBFβ complex demonstrated the critical amino acids of the RHD participating in the interaction with CBFβ and DNA (Bravo et al., 2001; Huang et al., 1999; Warren et al., 2000). The CBFβ protein is a relatively small protein (21kD), with the RUNX binding domain as the only known functional domain (Huang et al., 1999; Wang et al., 1993). Mutation analysis has shown that the amino acids Gly-61 and Asn-104 of CBFβ are critical for binding to RUNX (Tang et al., 2000). Studies have also reported a number of co-factors that bind to the RHD, including the v-Ets avian erythroblastosis virus E26 oncogene homolog 1 (ETS1) (Giese et al., 1995) and suppressor of variegation 3-9 homolog 1 (SUV39H1) (Chakraborty et al., 2003). The nuclear localization signal (NLS), following the RHD, is responsible for predominant nuclear localization of RUNX proteins (Kanno et al., 1998). In addition to the RHD, RUNX proteins have three conserved functional domains/motifs: the transactivation/inhibitory domains (Gu et al., 2000; Ito, 1999; Kanno et al., 1998), nuclear-matrix-targeting signal (NMTS) (Zeng et al., 1997) and VWRPY motif at the C-terminus of the proteins (Aronson et al., 1997). The VWRPY motif is required for the interaction with the "transducin-like enhancer of split/groucho" family of transcription co-repressors (Aronson et al., 1997). The *RUNX* genes are expressed from two alternative promoters (P1 or distal, and P2 or proximal) and encode several isoforms by alternative splicing. For instance, *RUNX1* encodes a *RUNX1c* isoform (the longest isoform) using

P1, the predominant *RUNX1b* using P2, and the minor isoform *RUNX1a* using P2 with an early termination in exon 7 (Blyth et al., 2005; Miyoshi et al., 1995). RUNX1b and RUNX1c both possess the RHD at the N-terminal end as well as the C-terminal transcriptional regulatory domains, as considered to be broadly similar in function. In contrast, RUNX1a retains the RHD, but lacks the transcriptional regulatory domains, as considered a potential functional antagonist of RUNX1b and RUNX1c (Miyoshi et al., 1995). Since the isoform *RUNX1b* with the N-terminal pentapeptide MRIPV is the predominant isoform expressed in hematopoietic cells (Bae and Lee, 2006; Miyoshi et al., 1995), the amino acid numbering of this isoform will be utilized throughout this chapter.

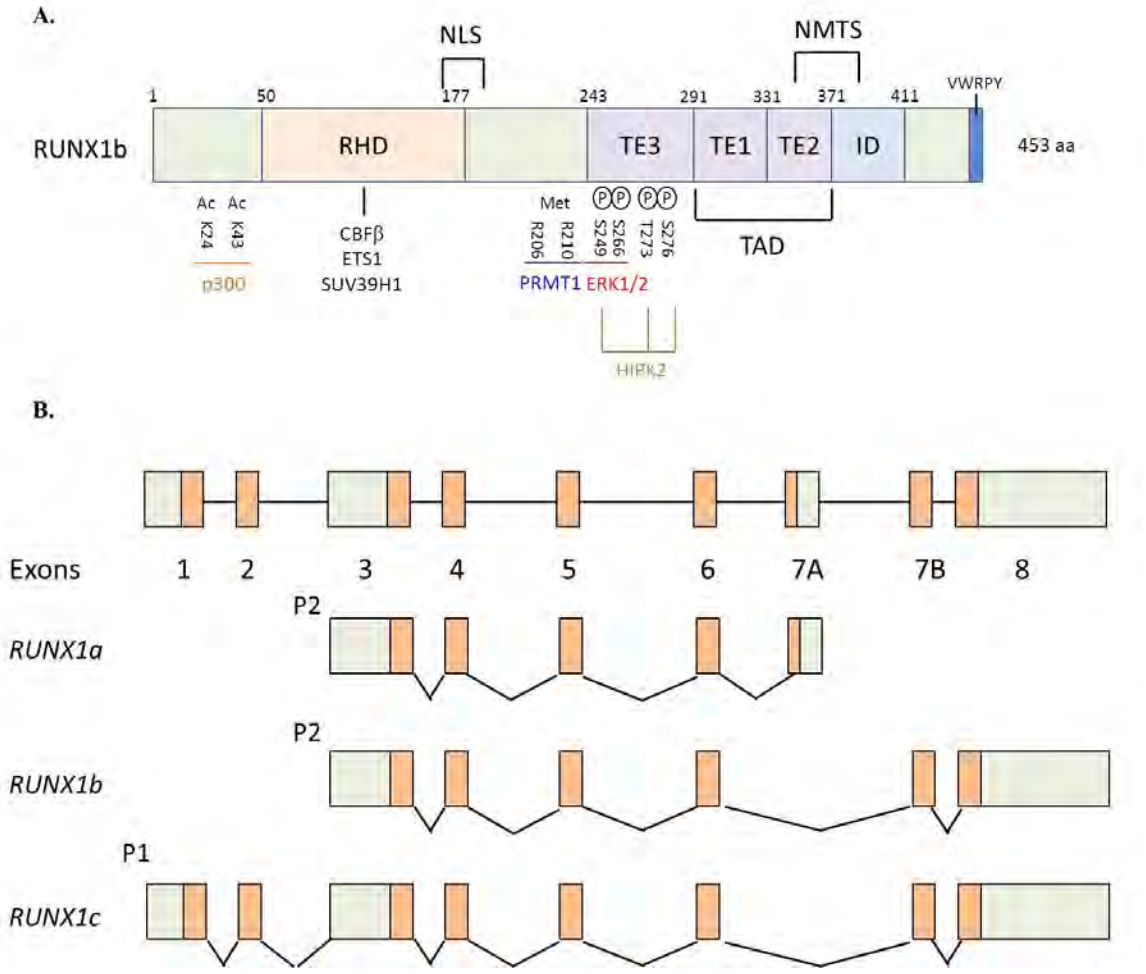

Figure I.2 Organization of human RUNX1 protein and alternative splicing transcripts. (A) the human RUNX1 has the Runt Homology Domain (RHD), three transactivation elements (TE1, TE2 and TE3) and an inhibitory domain (ID). The combination of TE1 and TE2 has also been called transactivation domain (TAD). RUNX1 contains a nuclear localization signal (NLS), a nuclear-matrix-targeting signal (NMTS) and a conserved C-terminal VWRPY motif. RHD is critical for binding to CBFβ, ETS1 and SUV39H1. Posttranslational modification sites of RUNX1 by p300, PRMT1, ERK1/2 and HIPK2 are also shown. Ac, acetylation; Met, methylation; P, phosphorylation. The labeling of amino acid number is based on the *RUNX1b* isoform (accession number NP_001001890), with 453 amino acids. (B) different isoforms of human *RUNX1* transcripts. *RUNX1a* and *RUNX1b* are transcribed from P2 promoter while *RUNX1c* is transcribed from P1 promoter. A schematic representation of the exons is shown at the top. Orange boxes represent the coding regions, and light green boxes represent the 5'- and 3'-untranslated regions.

CBFβ is predominantly present in the cytoplasm, whereas Runx has the nuclear localization signal (NLS) and translocates CBFβ into the nucleus (Adya et al., 1998). The RUNX proteins directly bind to the DNA consensus sequence TGYGGT (Y represents a pyrimidine) in promoters, enhancers and silencer regions (Kamachi et al., 1990; Melnikova et al., 1993; Meyers et al., 1993). CBFβ markedly increases RUNX DNA binding affinity (Ogawa et al., 1993; Wang et al., 1993), and protects it from proteasomal degradation (Huang et al., 2001).

Despite their structural similarity, the *RUNX* genes have different expression patterns and divergent biological roles in mammalian development. These roles are evident from phenotypes of *RUNX1-, RUNX2, and RUNX3*-deficient mice, presenting major defects in hematopoietic, osteoblastic and neuronal development respectively (Inoue et al., 2002; Komori et al., 1997; Levanon et al., 2002; Okuda et al., 1996; Otto et al., 1997; Wang et al., 1996b). In this section, I will summarize the role of Runx1 in hematopoiesis.

I.B.2 Runx1 function and regulation in hematopoiesis

The *Runx1*$^{-/-}$ embryos present normal morphogenesis and yolk sac–derived erythropoiesis at early stages of development, but lack fetal liver hematopoiesis and die at E11.5-12.5 due to necrosis and hemorrhaging in the developing central nervous system (Okuda et al., 1996; Wang et al., 1996b), indicating that the Runx1 is required for definitive hematopoiesis. In developing mouse embryo, Runx1 expression is found both in c-Kit^{+}CD34^{+} hematopoietic cells and hemogenic endothelium in sites from which

these hematopoietic cells are thought to emerge at E10.5 (North et al., 1999). Runx1 marks the HSC population, and deletion of Runx1 from the vascular endothelium prevents HSC emergence (Chen et al., 2009; North et al., 1999; North et al., 2002; Yokomizo et al., 2001). *Cbfb*$^{-/-}$ embryos lack of definitive hematopoiesis and die at E11.5-E13.5 due to hemorrhaging in the central nervous system, presenting very similar phenotypes as *Runx1*$^{-/-}$ embryo, which is a genetic evidence to support the heterodimerization of Cbfβ and Runx1 *in vivo* (Niki et al., 1997; Sasaki et al., 1996; Wang et al., 1996c).

At adult stage, RUNX1 and CBFβ are expressed in early human hematopoietic cells, with decreasing expression levels during differentiation (Bagger et al., 2012; Bagger et al., 2013). Accordingly, Runx1 is expressed in murine adult HSCs and differentiating myeloid and lymphoid cells, but has decreased expression during erythroid differentiation (North et al., 2004). Cbfβ is uniformly expressed in murine stem and progenitor cells, as well as mature myeloid and lymphoid cells (Kundu et al., 2002). Based on *Runx1* conditional knock-out mice, activated by *Mx1-Cre* system (Kuhn et al., 1995), studies have shown that Runx1 was not required for HSC maintenance but an expansion of HSC compartment (defined as $LSK^{+}CD34^{-}$) was observed (Growney et al., 2005; Ichikawa et al., 2004). A later study has shown that Runx1 negatively regulates quiescent HSCs in adult hematopoiesis, utilizing side population analysis and limiting dilution assays (Ichikawa et al., 2008). These data suggest that Runx1 may be a regulator of cell cycle in hematopoietic cells. Accordingly, exogenous RUNX1 stimulates G1 to S transition of murine myeloid progenitor 32D.3 cells (Strom et al., 2000). Activation of cyclin-

dependent kinase 4 (Lou et al., 2000) and cyclin D3 (Bernardin-Fried et al., 2004) transcription and repression of *Cdkn1a* promoter (Lutterbach et al., 2000) likely contribute to stimulation of cell cycle progression by Runx1. Furthermore, enforced expression of *RUNX1a*, a truncated isoform of RUNX1 with DNA-binding but no transactivation capacity, increased the competitive engraftment potential of murine long-term reconstituting stem cells, whereas the full-length isoform *RUNX1b* abrogated engraftment potential (Tsuzuki et al., 2007).

Runx1-deficient adult murine bone marrow also presented inhibition of megakaryocytic maturation, defective T- and B-lymphocyte development and a mild myeloproliferative phenotype (Growney et al., 2005; Ichikawa et al., 2004). These phenotypes are probably due to the regulation of lineage-specific genes by Runx1. During megakaryocytic differentiation, Runx1 activates expression of genes encoding the megakaryocytic integrin proteins αIIb and α2 (Elagib et al., 2003) and platelet myosin light chain (Jalagadugula et al., 2010). In immature myeloid cells, Runx1 activates expression of genes encoding myeloperoxidase (Nuchprayoon et al., 1994; Suzow and Friedman, 1993), neutrophil elastase (Nuchprayoon et al., 1994), the M-CSF receptor (Zhang et al., 1994) and C/EBPα (Guo et al., 2012). In the lymphocytic lineage, Runx1 activates T cell receptor subunits (Redondo et al., 1992) and silences the CD4 enhancer in T-lineage cells (Taniuchi et al., 2002). A recent study has also shown that Runx1 promotes survival and development of progenitors specified to the B-cell lineage and enables the developmental transition through the preB-stage triggered by the preB-cell antigen receptor (preBCR) (Niebuhr et al., 2013). Last but not least, Runx1 regulates

PU.1, a master hematopoietic differentiation regulator, both positively and negatively in a lineage dependent manner (Huang et al., 2008).

Activation or repression of the target transcription by RUNX1 is mediated by interaction with chromatin associated activators or repressors. These factors can also regulate RUNX1 activity by inducing specific post-translational modifications. For example, cytokines (IL-3 and thrombopoietin) can induce ERK1/2-mediated phosphorylation at Ser-249 and Ser-266 amino acids, in the transactivation element 3 of RUNX1 protein (Hamelin et al., 2006; Kanno et al., 1998; Tanaka et al., 1996). Such modification leads to RUNX1 dissociation from co-repressor SIN3A, activates its transcriptional activity and induces proteasome-mediated degradation of RUNX1 (Imai et al., 2004). Cyclin-dependent kinases can also phosphorylate RUNX1 and induce its anaphase-promoting complex mediated degradation (Biggs et al., 2006). RUNX1 can be methylated at amino acids Arg-206 and Arg-210 by the arginine methyltransferase PRMT1 (a transcriptional co-activator), which abrogates its association with SIN3A (Zhao et al., 2008). Another important transcriptional co-activator for RUNX1 is the lysine acetyltransferase p300. The interaction region of RUNX1 with p300 is between amino acid 178 and the C-terminus (Kitabayashi et al., 1998). The interaction of RUNX1 with CBFβ leads to phosphorylation of RUNX1 at Ser-249, Thr-273 and Ser-276 sites, which in turn induces the phosphorylation of p300 (Wee et al., 2008). Phosphorylation of RUNX1 and p300 is mediated by the homeodomain interacting kinase 2 (HIPK2) (Aikawa et al., 2006; Wee et al., 2008). Phosphorylation of p300 stimulates its acetyltransferase activity and specifically acetylates RUNX1 at Lys-24 and Lys-43 sites,

significantly augmenting DNA binding and transcription activity of RUNX1 (Aikawa et al., 2006; Yamaguchi et al., 2004). The modification sites of RUNX1 mentioned above are summarized in **FigureI.2**.

Taken together, RUNX1 and CBFβ are important TFs in definitive and adult hematopoiesis by regulating a variety of targets in a context of other cofactors. In the next subchapter I summarize the evidence implicating disruption of RUNX1/CBFβ complex in the promotion of hematopoietic malignancies.

I.C Acute myeloid leukemia

Leukemia is ranked as the 6th deadly cancer type in the US (Siegel et al., 2014), with approximately 52,000 cases diagnosed and 24,000 deaths every year (Cancer.org). From these, AML accounts for 19,000 diagnosed cases and 11,000 deaths. AML is predominantly a disease of adults, and patients over age 65 have especially poor outcomes (Estey and Dohner, 2006; Lowenberg et al., 1999). AML results from mutations that disrupt normal hematopoiesis with the clonal expansion of immature myeloid cells.

I.C.1 Classification of AML

Acute myeloid leukemia is characterized by an increase in the number of immature myeloid cells in the marrow. Patients with AML generally present with symptoms related to complications of pancytopenia (for example, anemia, neutropenia, and thrombocytopenia), including weakness and easy fatigability, infections, fever, and/or

hemorrhagic findings (Lowenberg et al., 1999; Meyers et al., 2005). Infiltration of leukemic cells into various tissues can produce a variety of other symptoms. Some patients also present metabolic and electrolyte abnormalities, many of which are due to a high turnover of the proliferating leukemic cells.

The classification of AML is complex due to the diversity of morphology, immunophenotype, genetics, and clinical features. The first proposal for the morphologic classification of acute leukemias was developed in 1976 by the French-American-British (FAB) cooperation group (Bennett et al., 1976)., and subsequently revised in the following years (Bennett et al., 1985a; Bennett et al., 1985b; Bennett et al., 1991b; Bloomfield and Brunning, 1985; Koike, 1984). This FAB classification divides AML into 9 distinct subtypes (M0-M7 including M4Eo, **Table I.2**) based on morphologic appearance of the blasts, their reactivity with histochemical stains and immunologic phenotypes. This distinction reflects the particular myeloid lineage involved and the degree of leukemic cell differentiation. Cytogenetic analysis of leukemic blasts identified nonrandom clonal chromosomal aberrations in a large percentage of AML patients (Look, 1997; Zeisig et al., 2012). Some of the lesions correlate with specific FAB subtypes, such as the chromosome 15/17 translocation [t(15;17)] is always presented in M3 subtype (Borrow et al., 1994; Tong et al., 1992), while the chromosome 16 (p13; q22) inversion [inv(16)] is always associated with the M4 subtype with abnormal eosinophils, named M4Eo subtype (Larson et al., 1986; Le Beau et al., 1983; Sole et al., 1992) (**Table I.2**). In addition, FAB subtypes are related to disease progression. For instance, the M0 subtypes had a significantly lower rate of complete remission (CR) and overall survival (OS) than

other subtypes in AML (Stasi et al., 1994). In contrast, M3 cases presented a significantly higher CR and OS rates (Bennett et al., 1991a).

Table I.2 The FAB Classification of AML and associated genetic abnormalities

FAB Subtype	Common Name	% of Cases	Associated Chromosomal Abnormalities	% of Cases	Genes Involved
M0	Acute myeloblastic leukemia with minimal differentiation	3	inv(3q26) and t(3;3)	1	*EVI1*
M1	Acute myeloblastic leukemia without maturation	15-20			
M2	Acute myeloblastic leukemia with maturation	25-30	t(8;21) t(6;9)	40 1	*AML1-ETO* *DEK-CAN*
M3	Acute promyelocytic leukemia	5-10	t(15;17) t(11;17) t(5;17)	98 1 1	*PML-RARA* *PLZF-RARA* *NPM-RARA*
M4	Acute myelomonocytic leukemia	20	11q23 inv(3q26) and t(3;3) t(6;9)	20 3 1	*MLL* *EVI1* *DEK-CAN*
M4Eo	Acute myelomonocytic leukemia with abnormal eosinophils	5-10	inv(16), t(16;16)	80	*CBFB-MYH11*
M5	Acute monocytic leukemia	2-9	11q23 t(8;16)	20 2	*MLL* *MOZ-CBP*
M6	Erythroleukemia	3-5			
M7	Acute megakaryocytic leukemia	3-12	t(1;22)	5	*OTT-MAL*

Adapted from Lowenberg et al., 1999

However, it became clear that in many cases FAB classification failed to distinguish between borderline cases, and prognostic values of AMLs classified according to other criteria were higher than those of the original FAB subtypes. The identification of recurrent genetic abnormalities and new clinical finding lead to a second classification by World Health Organization (WHO) proposed in 2002, which was updated in subsequent years (Vardiman and Hyjek, 2011; Vardiman, 2010; Vardiman et al., 2002; Vardiman et al., 2009). The WHO classification includes recurrent chromosome inversions and translocations, as well as specific mutations as criteria to divide AMLs (**Table I.3**). It also pays attention to relationship between AMLs and precursor neoplasms.

Although the WHO classification is very useful for clinic and research purposes, recent progress on molecular characterization of AMLs has unraveled heterogeneity within these groups, and suggests that further refinement may be required. Examples include the expression profiling studies identifying microRNA expression (Jongen-Lavrencic et al., 2008; Li et al., 2008), epigenetic status (Bullinger et al., 2010; Figueroa et al., 2010) and gene expression profiling (Bullinger et al., 2004; Haferlach et al., 2010b; Valk et al., 2004b) in panels of AMLs. In addition, the next-generation sequencing technology also helps researchers to identify mutations in different subpopulations and understand the clonal evolution of leukemic cells (Ding et al., 2012; Shlush et al., 2014; Walter et al., 2012; Welch et al., 2012). These discoveries will, to a large extent, benefit the prognosis and treatment of AMLs.

Table I.3 The WHO Classification of AML and related precursor neoplasms

AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); *RUNX1-ETO*
AML with inv(16)(p13.1;q22) or t(16;16)(p13.1;p22); *CBFB-MYH11*
Acute promyelocytic leukaemia with t(15;17)(q22;q12); *PML-RARA*
AML with t(9;11)(p22;q23); *MLLT3-MLL*
AML with t(6:9)(p23;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3.3)(q21;q26.2); *RPN1-EVl1*
AML (megakaryoblastic) with t(1:22)(p13;q13); *RBM15-MKL1*
AML with mutated *NPM1*
AML with mutated *CEBPA*
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukaemia, NOS
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukaemia
Acute monoblastic and monocytic leukaemia
Acute erythroid leukaemia
Acute megakaryoblastic leukaemia
Acute basophilic leukaemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis
Myeloid leukaemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm

Note: NOS, not otherwise specified
Adapted from Vardiman et al., 2010

I.C.2 Treatment of AML

The prognostic factors of AML includes age, performance status (use to quantify cancer patients' general well-being and activities of daily life), antecedent hematologic disorders, cytogenetics and mutations (Basecke et al., 2006; Breems et al., 2008; Estey, 2001; Hamadani and Awan, 2010; Kantarjian et al., 2006; Olesen et al., 2005; Sekeres et al., 2004; Shah et al., 2013). The favorable factors include younger age, better performance status, and no antecedent hematologic disorder or prior therapy. According to cytogenetic abnormalities, patients have been classified into good [such as t(8;21), inv(16)/t(16;16), or t(15;17)], poor [such as those with -7, inv(3)/t(3;3), balanced translocations involving 11q23 other than t(9;11) or a complex karyotype] or intermediate cytogenetic risk groups (Hamadani and Awan, 2010). Favorable mutations include *NPM1* and *CEBPA* mutations (Falini et al., 2005; Preudhomme et al., 2002) and adverse mutations include *FLT3-ITD*, *MLL-PTD*, overexpression of *WT1*, *BAALC*, *ERG* and *EVI1* (Baldus et al., 2003; Bergmann et al., 1997; Dohner et al., 2002; Kottaridis et al., 2001; Marcucci et al., 2007).

The standard regimen for AML induction therapy is the so-called "7+3" regimen, which combines a seven-day continuous intravenous infusion of cytarabine with a short infusion or bolus of an anthracycline given on days one through three (Burnett, 2012). Cytarabine, initially developed in 1960s (Evans et al., 1961; Talley and Vaitkevicius, 1963), combines a cytosine base with an arabinose sugar. As a deoxycytidine analogue, it interferes with DNA synthesis during S-phase of cell cycle and blocks mitosis. Cytotoxicity of anthracyclines is thought to induce DNA damage and apoptosis (Laurent

and Jaffrezou, 2001). The commonly used anthracycline in this regimen include daunorubicin, doxorubicin, idarubicin or synthetic anthracycline analogues like mitoxantrone (Burnett, 2012; Roboz, 2012). CR may be achieved in approximately 60–80% of patients aged less than 60 years (Hann et al., 1997; Rees et al., 1996) and 40-60% of older patients (Rowe, 2009). Variations in this regimen have included the incorporation of high dose of cytarabine (HiDAC) (Bishop et al., 1996), double-dose induction (Castaigne et al., 2004), and the addition of other agents such as Cladribine, a purine analogue as a cytotoxic drug (Holowiecki et al., 2012; Holowiecki et al., 2004; Robak, 2003).

After the induction therapy, post-remission therapy aims to destroy leukemia cells that survived induction chemotherapy but are undetectable by conventional studies. There are three basic options for post-remission therapy (in order of increasing intensity): consolidation chemotherapy, autologous hematopoietic stem cell transplantation (HSCT), or allogeneic HSCT (Rowe, 2009). Still, treatment of older adults, representing the majority of patients with AML, remains quite unsatisfactory. Less than 10% are long-term survivors, and the cure rate of older patients has only minimally improved over the past three decades.

The most promising treatment approaches are to develop molecularly targeted therapies. The feasibility and efficacy may vary between AML subtypes and specific mutations found in samples (Hamadani and Awan, 2010). A good example is the usage of all-trans retinoic acid in treating acute promyelocytic leukemia (APL). APL was classified as AML-M3 in the older French-American-British (FAB) classification system

(**Table I.2**) and is currently classified as acute promyelocytic leukemia with t(15;17)(q24.1;q21.1); PML-RARA in the WHO classification system (**Table I.3**). All-trans retinoic acid promotes the terminal differentiation of malignant promyelocytes to mature neutrophils (Huang et al., 1988). It shows good efficacy and is now a standard regimen combined with chemotherapy in induction treatment (Hu et al., 2000; Jeddi et al., 2011; Liu et al., 2011).

I.C.3 CBF AML

CBF related hematopoietic malignancies account for about 10-16% of AML (Zeisig et al., 2012). Specifically, 5-8% of AML cases present with the translocation t(8;21)(q22;q22) (Zeisig et al., 2012). This chromosomal translocation creates the *RUNX1-ETO* fusion gene that fuses RUNX1, including the RHD, to the ETO gene (Rowley, 1973) (Miyoshi et al., 1991). Other chromosomal translocations associated with chromosome 21 implicate RUNX1 in fusion proteins as well, such as t(12;21) in acute T cell leukemias, creating the fusion protein TEL-RUNX1 (Look, 1997). In addition, RUNX1 point mutations have been identified in a variety of hematologic malignancies. Megakaryocytic maturation and platelet formation is regulated by RUNX1 (Bluteau et al., 2012; Elagib et al., 2003; Jalagadugula et al., 2010; Lordier et al., 2012). Cases with familial platelet disorder show point mutations (nonsense, missense and intragenic deletion mutations) of RUNX1, and present decreased platelet counts with high incidence of AML (Kirito et al., 2008; Song et al., 1999). RUNX1 point mutations, including nonsense, missense and frameshift mutations, also occur in 5-10% *de novo* AML and

MDS (Osato et al., 1999; Preudhomme et al., 2000) and are common in therapy-related MDS and AML (16-40%) (Christiansen et al., 2004; Harada et al., 2003). These mutations frequently cluster on RHD and C-terminal of RUNX1.

Another 5-8% of cases of AML have inv(16)(p13;q22) (Zeisig et al., 2012). This chromosomal inversion disrupts the *CBFB* gene (located on 16q22) and fuses it with the C-terminal half of the *MYH11* gene (located on 16p13) which encodes the smooth muscle myosin heavy chain (SMMHC) protein (Liu et al., 1993). The fusion gene *CBFB-MYH11* encodes a fusion protein called CBFβ-SMMHC. The karyotype inv(16)(p13;q22) or the related t(16;16)(p13;q22) was particularly associated with the M4Eo subtype in the FAB classification of AML (Larson et al., 1986; Le Beau et al., 1983; Sole et al., 1992). This AML subtype presents eosinophils with distinctly abnormal morphology, cytochemical staining and ultrastructure (Le Beau et al., 1983).

Overall, AMLs with t(8;21) and inv(16) have favorable prognosis. After induction therapy, frequently using anthracycline- and cytarabine, approximately 90% of the CBF AML patients achieve a CR (Lowenberg et al., 2011; Marcucci et al., 2005; Schlenk et al., 2004). However, the 5 year OS rate is around 60% to 70% in younger patients (Age<60), and 30% in elderly patients (Age >60) (Prebet et al., 2009). In addition, the 5 year cumulative incidence of relapse is 57% and 48% for inv(16) and t(8;21) AMLs, respectively (Marcucci et al., 2005). These statistics call for a better therapy even for those AMLs with favorable karyotypes. HiDAC has been used in treatment of CBF AMLs. However, HiDAC reduced relapse rates but not necessarily increased OS in some studies (Marcucci et al., 2005; Miyawaki et al., 2011) and the optimal dose of cytarabine

and number of chemotherapy courses is not commonly defined (Paschka et al., 2013). The immunoconjugate gemtuzumab ozogamicin (GO) combines a DNA-damaging toxin with an antibody directed against the CD33 antigen, which is expressed on leukemia cells from more than 90 % of patients with AML (Takeshita, 2013). A significant survival benefit was observed in CBF AML patients who did receive GO compared with those who did not receive GO (OS at 5 years 79% vs 51%) (Burnett et al., 2011). However, this drug was withdrawn from the market in 2010 due to safety concerns. Alternative anti-CD33 antibodies have been or are being explored in clinical trials (Paschka and Dohner, 2013). However, there has not been any molecular therapy specifically targeting CBFβ-SMMHC in clinics so far. These clinical data echo the important functions of CBF in hematopoiesis and raise the need for better therapies, which encourage researchers, like us to investigate the molecular mechanisms of CBFβ-SMMHC in hematopoiesis and leukemogenesis.

I.D Molecular Function of CBFβ-SMMHC

I.D.1 CBFβ-SMMHC structure

The CBFβ-SMMHC is a fusion protein with N-terminal amino acid (1-165) of CBFβ and various lengths of SMMHC C-terminal end, depending on the inversion breakpoints in the *MYH11* gene (Liu et al., 1995). All the fusion genes identified so far have the inversion breakpoints in the region encoding the coiled-coil domain of the myosin tail, which is responsible for dimerization and multimerization of the myosin

molecule (Shigesada et al., 2004). The multimerization process is known to depend on the assembly competence domain (ACD) located within exon 40 of SMMHC (Ikebe et al., 2001; Kummalue et al., 2002; Sohn et al., 1997). Wildtype CBFβ binds to RUNX1 via its heterodimerization interface (Warren et al., 2000), which is retained in CBFβ-SMMHC. Furthermore, the fusion between the coiled-coil domain of SMMHC and CBFβ creates a novel binding site for RUNX1, called the RUNX1 high-affinity binding domain (HABD) (Lukasik et al., 2002). As a result, CBFβ-SMMHC binds RUNX1 at two sites, which is associated with higher binding affinity. This high-affinity RUNX1 binding has been proposed to explain the dominant nature of CBFβ-SMMHC function over wild-type CBFβ (Lukasik et al., 2002). A following study confirmed the higher binding affinity of CBFβ-SMMHC to RUNX1 by one order of magnitude or more, and mapped a hyper-heterodimerization domain in residues 134-165 of CBFβ and exons 33-36 of SMMHC (**Figure I.3A**) (Huang et al., 2004). Also, a repression domain was mapped to exons 39-40 in SMMHC (Huang et al., 2004; Lutterbach et al., 1999). A bipartite model for the structure of CBFβ-SMMHC complexed with RUNX1 was proposed based on those studies (**Figure I.3B**) (Shigesada et al., 2004). Basically, the fusion protein binds to RUNX1 by binding sites in CBFβ and the hyper-heterodimerization domain. In the meantime, the complex of CBFβ-SMMHC and RUNX1 form dimer and ultimately multimer by the coiled-coil domain.

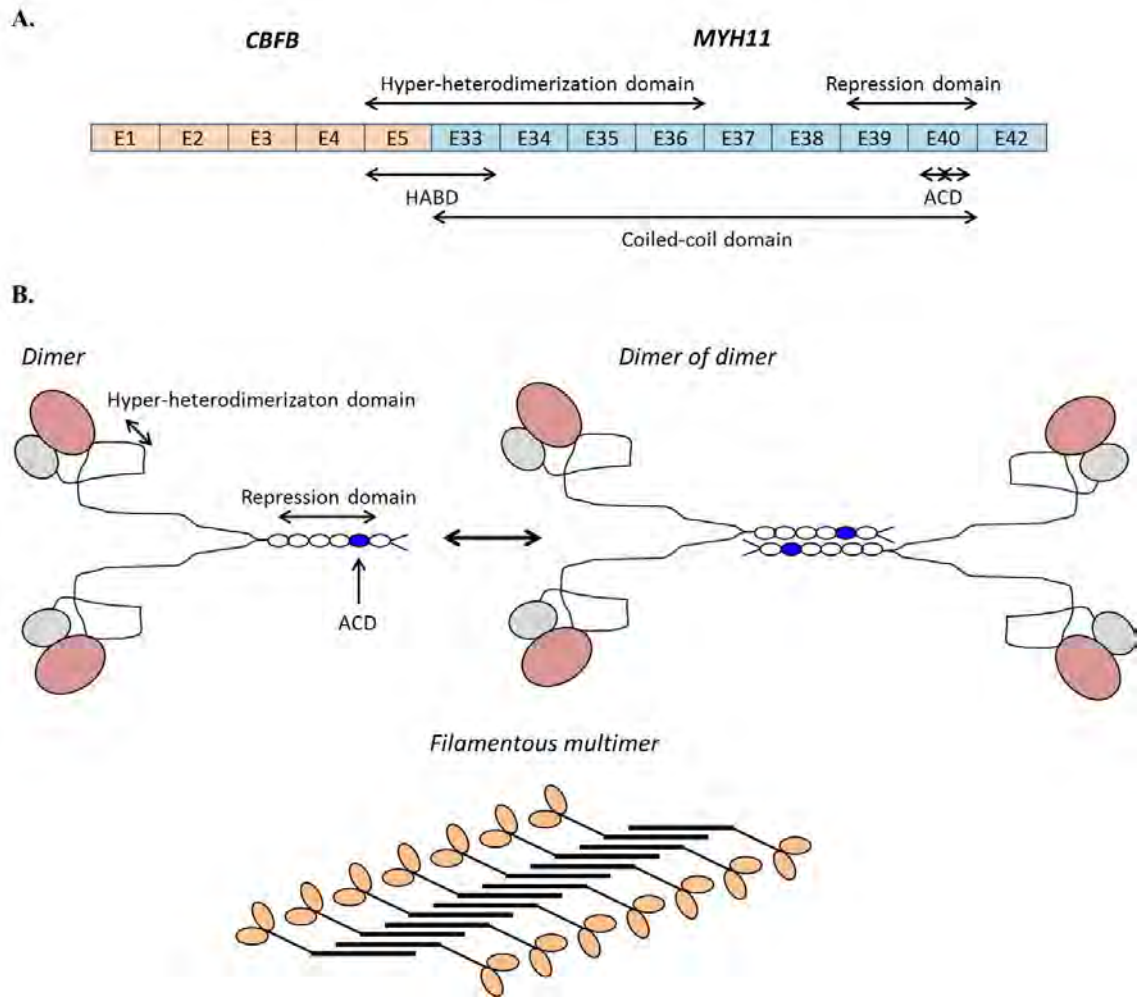

Figure I.3 CBFβ-SMMHC structure and bipartite model of multimer assembly. (A) Diagram of CBFβ-SMMHC structure: first 5 exons of *CBFB* (orange) fused with last 9 exons of *MYH11* (blue). Expansion of each domain was labeled as a double arrow line. HABD, High-Affinity Binding Domain; ACD, assembly competence domain. (B) A bipartite model for the structure of CBFβ-SMMHC complexed with RUNX1 with three stages. The red-colored and the gray-colored ovals represent RUNX1 and CBFβ, respectively. In the filamentous multimer, the dimeric unit is depicted in a simplified form. This figure was adapted from Shigesada et al., 2004.

I.D.2 Mechanisms of dominant inhibition

The strong evidence supporting the dominant inhibition of CBF functions by the fusion protein CBFβ-SMMHC is the embryonic lethality due to multiple hemorrhages (around E12.5) and lack of definitive hematopoiesis in the *Cbfb*$^{+/MYH11}$ embryos (Castilla et al., 1996), as this phenotype mimics the *Cbfb*$^{-/-}$ and *Runx1*$^{-/-}$ embryos (Niki et al., 1997; Okuda et al., 1996; Sasaki et al., 1996; Wang et al., 1996b; Wang et al., 1996c). Accordingly, definitive hematopoiesis of *Cbfb*$^{-/-}$ embryos is restored by ectopic expression of full-length *Cbfb* transgenes but not *Cbfb-MYH11* transgenes (Miller et al., 2001).

Exogenous expression of CBFβ-SMMHC inhibited the G1 to S cell cycle transition in myeloid and lymphoid cells (Cao et al., 1997). Viral expression of CBFβ-SMMHC also slowed proliferation of murine and human primary cells (D'Costa et al., 2005). These observations correlate to the promotion of cell cycle by exogenous expression of RUNX1 (Ichikawa et al., 2008; Strom et al., 2000).

There are two classic models to explain the dominant negative role of CBFβ-SMMHC on RUNX1 function:

The sequestration model was first proposed by Paul Liu in 1995 (Liu et al., 1995). This model states that CBFβ-SMMHC forms multimeric aggregates that tether RUNX1 from binding to CBFβ, and resulting in insufficient RUNX1/CBFβ available in regulatory regions of the genome. This model is supported by observations that CBFβ-SMMHC can cause altered subcellular localizations of Runx1 in a state closely colocalized with the fusion protein. This colocalization can happen in the nucleus as

multimerized complexes, which sometimes form large rod-like inclusion bodies (Wijmenga et al., 1996). It can also happen in the cytoplasm as deposits on cytoskeletal filaments or aggregates (Adya et al., 1998; Lu et al., 1995). The relative distributions of CBFβ-SMMHC/RUNX1 complexes between the nucleus and the cytoplasm were shown to depend on the level of expression of CBFβ-SMMHC and also the kind of cells studied (Adya et al., 1998; Cao et al., 1998; Kummalue et al., 2002). Particularly in inv(16) leukemic cells, approximately equal distributions between nucleus and cytoplasm were seen (Kanto et al., 2000; Liu et al., 1996).

The second model is the repression model, first named the "interference" model by Paul Liu, proposes that the RUNX1/CBFβ-SMMHC complex could alter the assembly of sequence-specific transcription factors on adjacent sites in the enhancers of certain target genes by either causing steric hindrance or participating in novel interactions with other proteins (Liu et al., 1995). A subsequent version of this model, called corepressors-recruiting model, was proposed in 1999 by Scott Hiebert (Durst et al., 2003; Lutterbach et al., 1999). This model is based on the findings that CBFβ-SMMHC formed ternary complexes with RUNX1 and repressor protein mSin3A, also associated with histone deacetylase 8 (HDAC8) and that the C-terminal 163 amino acids (exons 38-42) of the myosin tail in the fusion protein acted as a transcriptional repressor. In addition, it was also shown that multimerization of the fusion protein by the "assembly competence domain" (ACD) in SMMHC region is required for transcriptional repression and interaction with corepressors (Durst et al., 2003).

In summary, multiple studies may support each of these models, leaving to interpretation whether CBFβ-SMMHC may act by sequestration or repression of target gene expression. There is a unifying view that sequestration and repression may both be contributing mechanisms to CBFβ-SMMHC function. The relative importance of each depends on the cell type and experimental or physiological conditions. Further studies utilizing endogenous expression of CBFβ-SMMHC and RUNX factors are needed to provide compelling evidence in support of the relevant mechanism of action.

CBFβ-SMMHC can repress several RUNX related targets, such as *CDKN1A* (gene encodes p21) and *RUNX3* (Cheng et al., 2008; Lutterbach et al., 1999), but there are several studies suggesting that CBFβ-SMMHC may have *Cbfb/Runx1* -inhibition independent function. For example, the fusion protein can repress *CEBPA*, which is a critical TF for myeloid differentiation regulated by RUNX1/CBFβ. However, this repression may not result from a direct transcriptional regulation, but instead an indirect translational inhibition of *CEBPA* mRNA mediated by induction of calreticulin (Helbling et al., 2005). Interestingly, a recent study using Chip-sequencing technology disclosed that the fusion protein localized in RUNX1 occupied promoters and a set of genes was repressed upon CBFβ-SMMHC knock-down in ME-1 cells [an inv(16) patient derived cell line] (Mandoli et al., 2014). These data suggest that CBFβ-SMMHC may activate a distinct set of genes which may not necessarily be regulated by normal CBF. Also, during primitive hematopoiesis, *Cbfb-MYH11* delayed differentiation characterized by sustained expression of *Gata2*, *Il1rl1*, and *Csf2rb*, a phenotype not presented in *Cbfb* and *Runx1* knockout embryos (Castilla et al., 1996; Hyde et al., 2010).

I.D.3 Leukemogenesis

The expression of CBFβ-SMMHC in heterozygous *Cbfb*$^{+/MYH11}$ knock in embryos induced a block in definitive hematopoiesis and mid-gestation lethality (Castilla et al., 1996). In order to bypass this phenotype and study the role of CBFβ-SMMHC in leukemia initiation and maintenance in adult hematopoiesis, leukemia latency was studied in chimeric mice generated with *Cbfb*$^{+/MYH11}$ embryonic stem (ES) cells and treated with the mutagen N-ethyl-N-nitrosourea (Castilla et al., 1999). This study showed that the fusion protein CBFβ-SMMHC blocked adult myeloid and lymphoid differentiation and these mice developed AML only after gain of cooperating mutations by N-ethyl-Nnitrosourea mutagenesis. A following study using retroviral insertion mutagenesis in the chimeras also identified genes that synergized with CBFβ-SMMHC in the pathogenesis of AML (Castilla et al., 2004), strengthening the indication that CBFβ-SMMHC is necessary but not sufficient in leukemia development.

Using a conditional allele of *Cbfb*$^{56M/+}$ which can be restored to *Cbfb*$^{MYH11/+}$ with *Mx1-Cre* inducible system (Kuhn et al., 1995), our laboratory has previously found several hematopoietic abnormalities induced by the fusion protein in the adult stage (Kuo et al., 2008; Kuo et al., 2006). First, it expands the HSC compartments; second, it reduces HSC multilineage repopulation capacity under competitive conditions; third, it blocks megakaryocytic differentiation; forth, it induces distinct abnormal myeloid progenitors (AMPs) with reduced proliferation capacity able to develop AML after acquiring other mutations (Kuo et al., 2006). Furthermore, CBFβ-SMMHC impairs differentiation of common lymphoid progenitors, revealing an essential role for RUNX in early B-cell

development (Kuo et al., 2008). In addition, expression of Cbfβ reduces CBFβ-SMMHC induced AML as evidenced by the observation that loss of the wildtype allele of *Cbfb* accelerated the leukemogenesis in the *Cbfb*$^{-/MYH11}$ mice (Heilman et al., 2006).

A recent study indicated that the C-terminus of CBFβ-SMMHC is required to induce embryonic hematopoietic defects and leukemogenesis, utilizing a CBFβ-SMMHCΔC95 knock-in allele (Kamikubo et al., 2013). In contrast, the HABD seems not to be necessary for leukemogenesis, even though hematopoietic defects associated with Runx1-inhibition were partially rescued in knock-in mice expressing CBFβ-SMMHC with a HABD deletion (Kamikubo et al., 2010), which suggest that RUNX1 dominant inhibition may not be a critical step for leukemogenesis by CBFβ-SMMHC.

I.E Cooperating mutations with CBFβ-SMMHC

The driver oncogenic events in AMLs have been divided into two classes according to the two-hit hypothesis of leukemogenesis proposed by Gary Gilliland (Dash and Gilliland, 2001). Briefly, the class I mutations confer a proliferation or survival advantage to blast cells, while class II mutations block myeloid differentiation and give self-renewability. According to the molecular function of CBFβ-SMMHC, this fusion protein clearly behaves as a class II mutation and it requires other cooperating mutations (probably the Class I mutations) to drive leukemia.

The common secondary mutations found in inv(16) patients include activating mutations in *c-KIT* (10–45%) (Muller et al., 2008), *NRAS* (26–38%) (Bacher et al., 2006; Valk et al., 2004a), *KRAS* (7–17%) (Bowen et al., 2005; Valk et al., 2004a), *FLT3* (<10%)

(Muller et al., 2008) and inactivating mutations in *CBL* (3-5%) (Abbas et al., 2008; Reindl et al., 2009). Interestingly, these mutations all result in deregulation of a very important pathway in hematopoiesis, the RTK signaling pathways.

I.E.1 RTK signaling pathways

RTKs

Receptor tyrosine kinases (RTKs) are critical effectors of cell fate by initiating intracellular signaling events that elicit various cellular responses such as survival, proliferation, differentiation, and motility (Hubbard and Till, 2000). Fifty-eight RTKs are encoded within the human genome, which belong to 20 subfamilies as defined by genetic phylogeny (Lemmon and Schlessinger, 2010). Among that, class III RTK family, also named as PDGF receptor family is characterized by five extracellular immunoglobulin-like domains, a single transmembrane segment, a juxtamembrane (JM) domain and a split cytoplasmic tyrosine kinase domain (TKD) (Rosnet and Birnbaum, 1993). Some members of class III RTK family, including c-KIT and FLT3, play major roles in hematopoiesis and are frequently mutated in inv(16) AMLs (Muller et al., 2008). c-KIT is the receptor for SCF, which is a multipotent hematopoietic growth factor for early progenitor cells with strong synergistic activities with other growth factors (Martin et al., 1990; Williams et al., 1990). The FLT3 ligand (FL) is homologous to SCF and M-CSF (Hannum et al., 1994) and functions as a multipotent cytokine affecting a broad range of hematopoietic cell expansion (McKenna et al., 2000).

Binding of the ligand to RTKs induces dimerization of the receptors, followed by transphosphorylation of specific tyrosines which ultimately stabilizes the active conformation of the receptor (Masson and Ronnstrand, 2009). For c-KIT, autophosphorylation of the tyrosines in the JM domain occurs first (Mol et al., 2003). The JM domain is considered to sterically hold the activation loop in an inactive conformation in the absence of ligand binding (Mol et al., 2004). FLT3 was shown to be auto-inhibited by a similar mechanism (Griffith et al., 2004). Upon phosphorylation, the phosphate group interrupts the autoinhibited complex and the introduction of internal tandem duplications (ITDs) in FLT3 is thought to act in a similar manner, making a leaky autoinhibition (Schlessinger, 2003).

The RTK activities are regulated by Src family kinases (SFKs) and Casitas B-cell lymphoma (CBL). SFKs positively regulate the receptor via phosphorylation of a Src Homology 2 Domain Containing Protein (SHC) and negatively regulate the receptor via ligand-induced internalization and activation of E3 ubiquitin ligase CBL protein (Broudy et al., 1999; Corey and Anderson, 1999; Masson et al., 2006). Members of the CBL family including CBL, CBL-b and CBL-c, play a major role in the ligand-dependent ubiquitination of many RTKs, such as c-KIT and FLT3 (Reindl et al., 2009; Thien and Langdon, 2001; Zeng et al., 2005). They all possess a highly conserved N-terminal domain consisting of a tyrosine kinase binding (TKB) domain and a RING finger domain, both necessary for ubiquitin ligase activity. The TKB domain is required for CBL recruitment to tyrosine-phosphorylated proteins (Lill et al., 2000). The RING finger

domain associates with an E2 ubiquitin-conjugating enzyme (Yokouchi et al., 1999), leading to the transfer of ubiquitin molecules to lysine residues on the target protein.

The phosphotyrosine sites on the activated receptors can serve as docking sites that allow the binding of signaling molecules and triggers three major downstream pathways: MEK/ERK, PI3K and STAT pathways (**Figure I.4**).

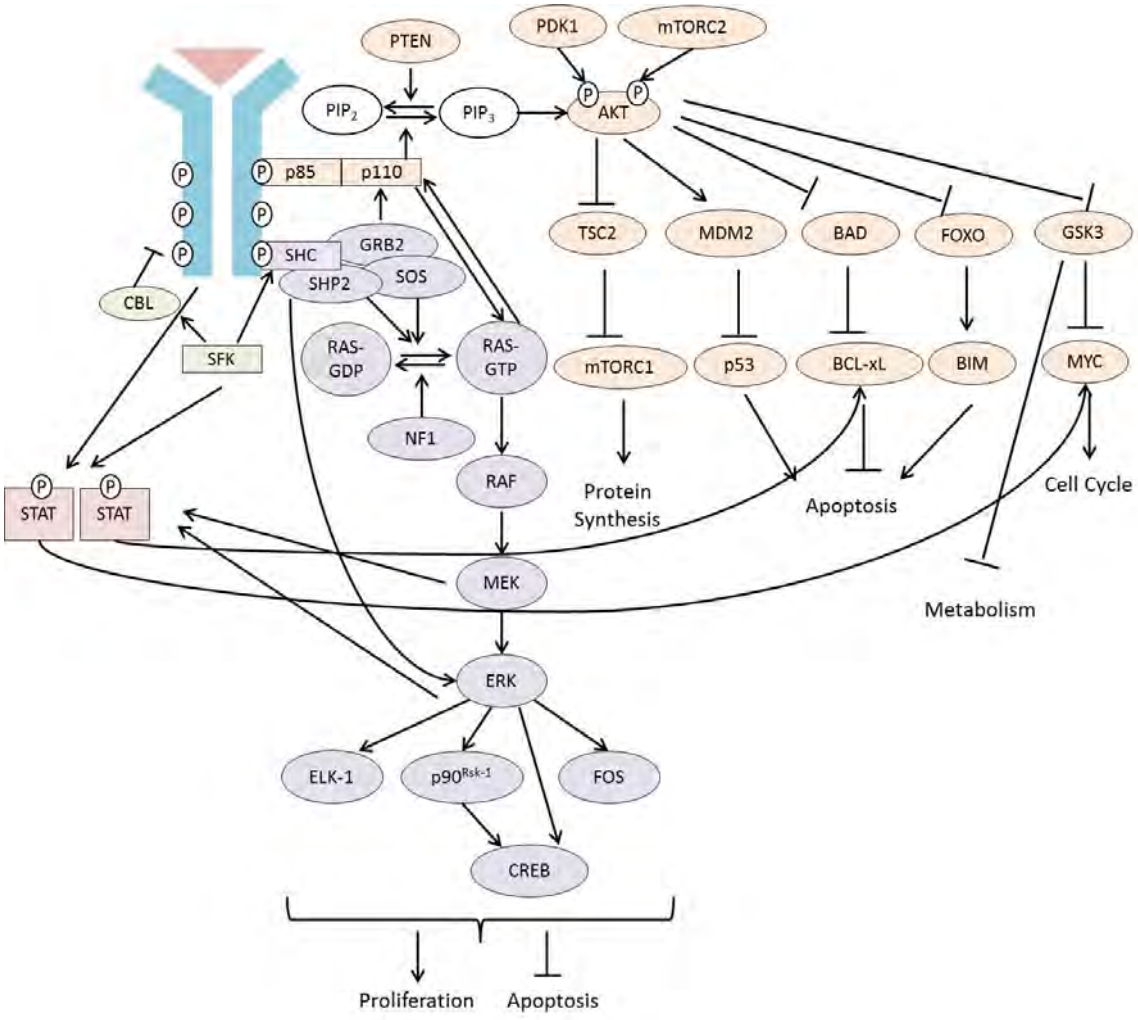

Figure I.4 RTK signaling pathways. The red reverted triangle represents the ligand. The cyan columns represent the RTK dimer. All the components in the MEK/ERK, PI3K and STAT pathways are in purple, orange and red, respectively. Other regulatory proteins are in green. Arrow stands for activation and flat arrow stands for inhibition. P, phosphorylation.

MEK/ERK pathway

The Ras/Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway has key roles in the transmission of proliferative and survival signals from membrane-bound receptors, such as c-KIT and FLT3 (Steelman et al., 2011).

The RAS protein belong to a family of small GTPases including H-RAS, N-RAS, K-RAS4A and K-RAS4B (if not specified, K-RAS in this dissertation is used to refer to the ubiquitously expressed K-RAS4B) that have identical guanine nucleotide and effector binding domains but diverge substantially within the hypervariable region (HVR) (Karnoub and Weinberg, 2008; Schubbert et al., 2007). The HVR of all RAS isoforms terminates with a CAAX motif, where the cysteine is prenylated by farnesyltransferase (Ward et al., 2012). After prenylation, the AAX is removed by Ras-converting enzyme 1 (RCE1), and the carboxyl group of isoprenylcysteine is methylated by isoprenylcysteine-carboxyl-methyltransferase (ICMT). These lipid modifications provide weak membrane binding affinity that is stabilized by a second signal motif. For K-RAS4B, this is provided by a polybasic lysine domain, which interacts strongly with membrane. In contrast, H-RAS, N-RAS, and K-RAS4A are palmitoylated at cysteine(s) adjacent to the CAAX motif. These posttranslational modifications are essential for correct trafficking and subcellular localization of each isoform (Mor and Philips, 2006). K-RAS4B localizes directly to the plasma membrane (PM) through an unknown mechanism. H-RAS, N-RAS, and K-RAS4A traffic from the endoplasmic reticulum (ER) though the Golgi to the PM in a dynamic cycle that is regulated by depalmitoylation and repalmitoylation.

Depalmitoylation is mediated by acyl protein thioesterase 1 (APT1) and APT2 (Mor and Philips, 2006).

The RAS proteins regulate cell fate by cycling between active GTP-bound and inactive GDP-bound conformations (RAS-GTP and RAS-GDP). Guanine nucleotide exchange factors (GEFs), such as son of sevenless homolog (SOS) protein, can activate RAS by releasing GDP from RAS. Guanine activating proteins (GAPs), such as Neurofibromin 1 (NF1), act as negative regulators of RAS by favoring the dephosphorylation of GTP.

After activation of RTK, the SHC adaptor protein associates with the C-terminus of the activated growth factor receptor after phosphorylation by SFKs (Steelman et al., 2011). SHC recruits the growth factor receptor-bound protein 2 (GRB2) and SOS, resulting in the loading of membrane-bound RAS with GTP (Downward, 2003). Src homology-2 domain –containing phosphatase 2 (SHP2) is also recruited to the receptors (Heiss et al., 2006), and dephosphorylates RAS-GAP binding sites, keeping RAS in an active state (Dance et al., 2008). RAS-GTP then recruits RAF to the membrane where it becomes activated, likely via a SFK (Franklin et al., 2006; Marais et al., 1995; Marais et al., 1997; Mason et al., 1999). RAF is responsible for phosphorylation of MEK1/2 and then MEK1/2 phosphorylates ERK1/2 (Derijard et al., 1995; Xu et al., 1995). Other means of ERK phosphorylation have been suggested to occur via its association with the tyrosine phosphatase SHP2 (Ronnstrand et al., 1999). Activated ERK1/2 phosphorylates and activates a group of substrates, including p90 ribosomal six kinase-1 ($p90^{Rsk-1}$) (Xing et al., 1996). $p90^{Rsk-1}$ can activate the cAMP response element-binding protein (CREB)

transcriptional factor, which can influence gene expression (Xing et al., 1996). Activated ERK can also translocate into the nucleus and phosphorylate additional TFs, such as ELK-1, CREB, FOS and others (Davis, 1995). These TFs bind promoters of many genes, including growth factor and cytokine genes that are important in promoting proliferation and preventing apoptosis in hematopoietic cells.

PI3K pathway

The phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases that phosphorylate the 3′-hydroxyl group of phosphoinositides. The members of class IA PI3K which are activated by RTKs, exist in a heterodimeric complex composed of a 110 kD (p110α, β or δ)-catalytic subunit and a 85 kD (p85α, β or p55γ)-regulatory subunit with two src homology 2 (SH2) domain which can bind to tyrosine-phosphorylated proteins (Klippel et al., 1994).

Stem cell factor (SCF) induces association of the SH2 domain of the p85α subunit with Y721 in c-Kit (Serve et al., 1995). Besides, GAB2 is also an important mediator of SCF-induced PI3-kinase activation (Sun et al., 2008; Yu et al., 2006). In the case of FLT3, the human receptor lacks a direct binding motif for the p85α, whereas the murine form contains this motif and has been verified to directly associate with p85α (Rottapel et al., 1994). Human FLT3 was found to phosphorylate docking proteins GAB1 and GAB2, which suggests that human FLT3 may activate the PI3K pathway indirectly through these intermediates (Zhang and Broxmeyer, 2000).

Upon activation, PI3K phosphorylate the membrane bound lipid phosphatidylinositol-4, 5-bisphosphate (PIP_2) to phosphatidylinositol-3, -4, 5-trisphosphate (PIP_3), which can be dephosphorylated by phosphatase and tensin homologue (PTEN) (Engelman et al., 2006). PIP_3 recruits serine/threonine kinase AKT to the membrane. AKT is the best known downstream target and a mediator of survival and proliferation (Datta et al., 1996). AKT is then phosphorylated on T308 by 3-phosphoinositide-dependent kinase-1 (PDK1) (Scheid et al., 2002). The second phosphorylation at S473 by the mammalian target of rapamycin complex 2 (mTORC2) locks AKT in an active conformation. Phosphorylation of both residues is required to activate AKT, which in turn activate/inactivate a variety of substrates by phosphorylation, such as FOXO proteins, Bad, glycogen synthase kinase 3 (GSK3), murine double minute 2 (MDM2) and tuberus sclerosis complex 2 (TSC2) (Engelman et al., 2006). These substrates further regulate other downstream targets, such as mTORC1, BCL-xL, BIM and MYC in protein synthesis, cell cycle, cell survival and metabolism.

A crosstalk between PI3K and RAS has been suggested in several studies. The PI3K regulatory subunit p85 inhibits p110 activation by RAS and this blockage is released by RTK stimulation (Jimenez et al., 2002). Thus, RTKs lacking a direct p85 binding site, such as human FLT3, are able to activate PI3K through GAB1, GAB2 or RAS. Conversely, PI3K has also been shown to activate RAS and MEK (Wennstrom and Downward, 1999).

STAT pathway

Normally, STATs are located in the cytoplasm. Upon phosphorylation directly by a RTK or SFK (Levy and Darnell, 2002), STATs dimerize and translocate into the nucleus. Activated STATs bind to specific DNA sequences to initiate transcription of target genes. For example, STAT3 activates the *c-MYC* gene in response to IL-6 (Coffer et al., 2000) and STAT5 induces expression of BCL-xL in response to erythropoietin (Socolovsky et al., 1999).

The crosstalk between signaling pathways, such as STAT and MEK/ERK, play an important role in hematopoiesis and leukemia. Phosphorylation of a conserved C-terminal serine residue (Ser-727) in STAT1 and STAT3 proteins by ERKs enhances the STAT transcriptional activity (Zhang et al., 1995), and STAT3 activity are also regulated by MEK 1 (Lim and Cao, 2001).

The ability of c-KIT to activate STAT seems to be cell type, time and context specific, since different studies give conflict results (Gotoh et al., 1996; Joneja et al., 1997; Linnekin et al., 1996; O'Farrell et al., 1996; Ryan et al., 1997). The findings with FLT3 are more consistent, as the FLT3-ITD receptor, but not the wild-type FLT3 is able to activate STAT5 (Grundler et al., 2005; Kiyoi et al., 2002; Rocnik et al., 2006).

I.E.2 Deregulation of RTK signaling in inv(16) AML

c-KIT mutations

In CBF leukemias, c-KIT mutations accumulate within exon 8 and exon 17 (Muller et al., 2008). Exon 8 encodes the fifth immunoglobulin-like unit in the extracellular

domain of c-KIT. Exon 8 mutations can be detected in about 10-20% of inv(16) AML patients (Boissel et al., 2006; Cairoli et al., 2006; Care et al., 2003) and usually result in single amino acid substitutions at codon D419, causing hyperactivation of the receptor in response to SCF (Kohl et al., 2005). Exon17 contains the activating loop and the mutations involving exon17 seem to occur exclusively at codon D816 in inv(16) AML. Retroviral transduction of Cbfβ-SMMHC knock-in bone marrow cells with c-KIT D816V and D816Y mutants induced leukemia in 60% and 80% recipient mice respectively but not in control mice, indicating cooperation between mutated *KIT* and *CBFB-MYH11* during leukemogenesis (Zhao et al., 2012).

The prognostic impact of c-KIT mutations in inv(16) AML patients remains controversial. Some studies find an association between c-KIT mutations and poor prognosis in t(8,21) AMLs but not inv(16) AMLs (Boissel et al., 2006; Cairoli et al., 2006). Other studies found that exon 8 mutations correlate with increased relapse rate but did not affect OS in inv(16) AMLs (Care et al., 2003) and exon 17 mutations of c-KIT increased relapse risk and adversely affect OS in inv(16) AMLs (Paschka et al., 2006).

FLT3 mutations

FMS-like tyrosine kinase 3 (FLT3) represents the single most common mutated gene in AML. It is affect by ITD mutations in the JM domain in 24% (Kottaridis et al., 2001) or by activating loop mutations in 7% of cases (Thiede et al., 2002). In contrast, inv(16) AMLs feature FLT3 mutations at a relative lower rate (<10%) (Muller et al., 2008).

The *Flt3*$^{+/ITD}$ and *Flt3*$^{ITD/ITD}$ mice develop a myeloproliferative disease with monocytic features (Lee et al., 2007). Analysis of this knock-in mouse model also revealed that Flt3-ITD conferred enhanced proliferation and survival properties to multipotent progenitors. Retroviral overexpression of FLT3-ITD and CBFβ-SMMHC in mice also indicated cooperation between those two oncogenes in leukemogenesis (Kim et al., 2008).

CBL mutations

The CBL adaptor protein is a negative regulator of RTK activity. *CBL* transcripts lacking exon 8 caused by splice donor or acceptor mutations have been identified in *de novo* inv(16) AML cases (Abbas et al., 2008; Reindl et al., 2009). The deleted transcripts encode protein lacking part of linker region and almost the entire RING finger domain, which is critical for the E3 ligase activity (Abbas et al., 2008; Swaminathan and Tsygankov, 2006). This CBL exon 8 mutant may transform hematopoietic cells by constitutively activating the FLT3 pathway (Reindl et al., 2009). .

I.F RAS mutations in myeloid neoplasms

Mutations in *NRAS* or *KRAS* occur nearly exclusively by one nucleotide substitutions in codons 12, 13 or 61, which abrogate intrinsic RAS GTPase activity, render resistance to GAPs (Schubbert et al., 2007) and thus constitutively activate RAS proteins and downstream effectors, such as RAF and MEK/ERK kinases (Beaupre and Kurzrock, 1999). Point mutations in *NRAS* and *KRAS* genes are found in approximately

10–15% and 5% of all AML patients, respectively. Codons most frequently mutated are *NRAS* codon 12 (43%), *NRAS* codon 13 (21%) and *KRAS* codon 12 (21%) (Bacher et al., 2006; Bowen et al., 2005). The RAS mutations identified in the AML patients were all heterozygous and do not influence outcome in patients younger than 60 years. In inv(16) AMLs, mutations in *NRAS* (26–38%) (Bacher et al., 2006; Valk et al., 2004a) are more commen than mutations in *KRAS* (7–17%) (Bowen et al., 2005; Valk et al., 2004a). The most frequent mutations in the *NRAS* gene of inv(16) AML patients are $NRAS^{G12D}$ and $NRAS^{Q61K}$ (Bacher et al., 2006).

Somatic *NRAS* and *KRAS* mutations also occur in other myeloid malignancies, including juvenile myelomonocytic leukemia (JMML), chronic myelomonocytic leukemia (CMML) and myelodysplastic syndrome (MDS) (Bos, 1989; Onida et al., 2002; Padua et al., 1988). Similar to inv(16) AMLs, *NRAS* is mutated 2 to 3 times more frequently than *KRAS* in other hematologic cancers (Bowen et al., 2005; Schubbert et al., 2007).

I.F.1 Mouse models of oncogenic Ras

The *Ras* oncogenes have been studied extensively in mice, both to elucidate the mechanisms underlying deregulated cell growth and to develop robust *in vivo* platforms for preclinical trials.

Allelic activation of oncogenic K-Ras in hematopoietic cells initiates a rapidly fatal myeloproliferative neoplasm (MPN) in $Kras^{LSL\text{-}G12D/+}$*/Mx1-Cre* mice with a medium latency of 84 days after oncogenic *Kras* induction (Braun et al., 2004). In a mouse bone

marrow transplantation model using the same knock-in mice, studies have shown that K-Ras^{G12D} induced hematopoietic malignancies in multiple lineages, including thymic T-cell lymphoblastic lymphoma, T-cell leukemia, and phenotypes closely resembling human JMML (Zhang et al., 2009). K-Ras^{G12D} expression induces hyperproliferation and aberrant signaling in primary hematopoietic stem/progenitor cells and spontaneous *Kras*$^{G12D/+}$ activation was observed even in the absence of pIpC treatment (Sabnis et al., 2009; Van Meter et al., 2007). Similarly, *KRAS*^{G12V}-transduced human cord blood (CB) CD34^{+} cells displayed a strong proliferative advantage over control cells (Fatrai et al., 2011). In addition, Allelic expression of K-Ras^{G12D} cooperates with loss of *Nf1* in acute myeloid leukemogenesis in mice (Cutts et al., 2009). A recent study has also shown that mice transplanted with K-Ras^{G12D} and RUNX1-ETO co-transduced bone marrow cells developed accelerated AMLs (Zhao et al., 2014).

In contrast to the aggressive MPN phenotype induced in *Kras*$^{LSL\text{-}G12D/+}$*/Mx1-Cre* mice (Braun et al., 2004), *Nras*$^{LSL\text{-}G12D/+}$*/Mx1-Cre* mice induces attenuated diseases with a medium latency of 363 days (Li et al., 2011). These diseases include MPN, a disorder mimicking human MDS, lymphoproliferation concomitant with myeloid disease, and histiocytic sarcoma. Recent studies have shown that *Nras*$^{G12D/+}$ HSCs undergo moderate hyperproliferation with increased self-renewal associated with hyperactivation of ERK1/2 (Wang et al., 2013). Effects of *Nras*$^{G12D/+}$ on HSCs are bimodal, increasing the frequency with which some HSCs divide and reducing the frequency with which others divide (Li et al., 2013). Bone marrow transplantation using the *Nras*$^{LSL\text{-}G12D/+}$ knock-in mice revealed a

similar prolonged latency, 96% myeloproliferative disease resembling human CMML (8% T-ALL) and aberrant GM-CSF signaling of the mutant cells (Wang et al., 2010a).

Different from *Nras*$^{G12D/+}$ mice, *Nras*$^{G12D/G12D}$ mice develop an acute MPN with a medium latency around 50 days, similarly to *Kras*$^{G12D/+}$ mice (Wang et al., 2011a). Recipient mice transplanted with 2.5x10^5 *Nras*$^{G12D/G12D}$ bone marrow cells all develop T-ALL (Wang et al., 2011a) and a higher dose (10^6) of *Nras*$^{G12D/G12D}$ bone marrow cell transplantation results in MPN, T-ALL and B-ALL (Wang et al., 2011b). Using a hemizygous *Nras*$^{G12D/-}$ mice, a recent study has shown that increased *Nras*^{G12D} gene dosage, but not inactivation of WT *Nras*, underlined the aggressive *in vivo* behavior of *Nras*$^{G12D/G12D}$ hematopoietic cells (Xu et al., 2013). Those different diseases developed in different experimental settings suggest distinct requirements of hematopoietic stem cell activity and N-Ras^{G12D} signaling in different cell types during leukemogenesis. In the retroviral and transplantation system, overexpression of oncogenic human N-RAS^{G12D} alone rapidly (medium latency around 50 days) and efficiently induces CMML- and AML-like disease in mice (Parikh et al., 2006). Retroviral overexpression of murine N-ras^{G12D} can cooperate with RUNX1-ETO9a in AML (Zuber et al., 2009).

I.F.2 Targeting oncogenic RAS

Targeting posttranslational modifications

In the 1990s, targeting posttranslational processing of RAS engendered intense interest. Drug discovery was first focused on farnesyltransferase inhibitors and they gave

disappointing results in clinical trials probably due to compensation by an alternative CAAX lipid modification enzyme, geranylgeranyl transferase (Downward, 2003; Karp et al., 2001). Unfortunately, combined inhibition of farnesyltransferase and geranylgeranyl proved too toxic (Downward, 2003).

Inactivating of a prenylated Ras C-terminal processing gene, *Rce1* in hematopoietic cells unexpectedly accelerated MPN in *Kras*$^{LSL\text{-}G12D/+}$*/Mx1-Cre* mice (Wahlstrom et al., 2007). In contrast, conditional inactivation of another C-terminal processing gene, *Icmt* attenuated MPN in these mice (Wahlstrom et al., 2008) and small molecules have been identified to block ICMT activity without *in vivo* test yet (Wang et al., 2010b).

The palmitoylation/depalmitoylation cycle is a potential therapeutic target for selectively inhibiting the growth of hematologic malignancies with *NRAS* mutations. Palmostain B is a novel inhibitor for depalmitoylation mediator APT that causes entropy-driven diffusion of H-Ras and N-Ras throughout the cell (Dekker et al., 2010). Exposing transduced hematopoietic cells from *Nras* and *Kras* mutant mice to palmostatin B specifically mislocalized N-Ras^{G12D} away from the plasma membrane and reduced CFU-GM and AML blast colony growth without affecting *kras* mutant cells (Xu et al., 2012). More potent APT inhibitors were reported, although systemic toxicities have not been fully described (Hedberg et al., 2011).

Blocking upstream growth-promoting signals that converge to activate GEFs is another potential therapeutic strategy. In the case of oncogenic Ras, though the oncoproteins are thought be constitutively active, primary hematopoietic cells from *Kras*$^{LSL\text{-}G12D/+}$*/Mx1-Cre* mice show little basal activation of MEK/ERK or PI3K signaling

and respond robustly to growth factor stimulation (Braun et al., 2004; Van Meter et al., 2007). A compound DCA1 was screened to inhibit Ras activation by interfering with SOS-mediated guanine nucleotide exchange, and probably also has activity against other exchange factors (Maurer et al., 2012). Although the long-term therapeutic potential of this approach is uncertain, treatment with drugs that block upstream proteins might be considered in hematologic cancers with RAS mutations that respond to growth factor stimulation.

Targeting Ras effectors

Intensive efforts have been focused on targeting RAF/MEK/ERK and PI3K/AKT/mTOR cascades. CI-1040 and PD0325901 are allosteric MEK inhibitors. Whereas CI-1040 was ineffective in *Nf1*$^{flox/flox}$*/Mx1-Cre* mice with MPN (Lauchle et al., 2009), PD0325901 induced dramatic hematologic improvement and greatly prolonged survival in both *Kras*$^{LSL\text{-}G12D/+}$*/Mx1-Cre* and *Nf1*$^{flox/flox}$*/Mx1-Cre* models of MPN (Chang et al., 2013; Lyubynska et al., 2011). MEK inhibitors were also tested in insertional mutagenesis models of AML initiated by Nf1 inactivation (Lauchle et al., 2009). In contrast to no beneficial therapeutic index in MPN, CI-1040 induced dramatic disease regression and markedly extended survival in recipients transplanted with primary Nf1-deficient AMLs. This suggests that cooperating mutations acquired during progression to AML rendered these aggressive cancer cells more dependent on Raf/MEK/ERK signaling. However, all of these mice that initially responded to MEK inhibition ultimately relapsed despite continued treatment. Due to minimal activity in RAS-mutant tumors and

unexpected ocular toxicity (Solit et al., 2006), clinical development of CI-1040 and PD0325901 was suspended. Newer MEK inhibitors, such as AZD6244 showed only modest and transient antitumor acitivity in advanced AMLs (Ward et al., 2012). Another novel potent MEK inhibitor GDC-0973 was developed with a biochemical IC_{50} estimate of 4.2 nmol/L against MEK1 and has antitumor efficacy in *BRAF* and *KRAS* mutant human xenograft tumor models (Hoeflich et al., 2012).

Two well-known PI3K inhibitors, LY294002 and wortmannin, prevent ATP to bind to and activate PI3K. Preclinical experiments indicate that both drugs induce apoptosis in leukemic cells (Polak and Buitenhuis, 2012). However, it has also been demonstrated that both inhibitors are detrimental for normal cells (Buitenhuis et al., 2008; Gunther et al., 1989). A novel PI3K p110δ inhibitor, IC87114, has been shown to reduce proliferation and survival of both AML blasts and acute promyelocytic leukemia cells without affecting the proliferation of normal hematopoietic progenitors (Billottet et al., 2009; Sujobert et al., 2005). Another potent PI3K inhibitor GDC-0941 has biochemical IC_{50} values of 3 to 75 nmol/L for the 4 class I isoforms of PI3K (Folkes et al., 2008). GDC-0941 strongly inhibited growth of breast tumor cells *in vitro* and *in vivo*, especially those with activated PI3K pathway signaling (O'Brien et al., 2010) and presented improved efficacy in combination with MEK inhibitor GDC-0973 in xenograft models (Hoeflich et al., 2012).

Downstream effectors such as AKT and mTOR also serve as therapeutic targets in the PI3K pathway. AKT phosphorylation inhibitor perifosine induces apoptosis in multidrug-resistant human T-ALL cells and primary AML cells, but does not affect

normal cells (Chiarini et al., 2008; Papa et al., 2008). The efficacy of perifosine in treatment of leukemia is currently examined in several Phase II clinical trials (NCT00391560 and NCT00873457) (Polak and Buitenhuis, 2012). As in case of mTOR inhibitors, although both rapamycin and its analog CCI-779 exhibit strong anti–tumor capacities *in vitro* (Meyer et al., 2011; Recher et al., 2005; Teachey et al., 2008), only a partial response was observed in clinical trials with rapamycin or its analog AP23573 in hematologic malignancies (Recher et al., 2005; Rizzieri et al., 2008).

Due to the modest response of single-specificity inhibitors in patients, the efficacy of combination therapy targeting different intermediates of the PI3K signaling module is under investigation. Combined inhibition of mTOR and p110δ with RAD001 and IC87114, respectively synergistically reduces proliferation of AML blasts (Tamburini et al., 2008). A Dual-specificity inhibitor like BEZ235 has also been identified, inhibiting the activity of both PI3K and mTOR (Maira et al., 2008). This compound significantly reduced proliferation and survival in both primary AML cells without affecting the clonogenic capacity of normal hematopoietic progenitors (Chapuis et al., 2010). GDC-0980 is another dual inhibitor targeting both PI3K and mTOR. Low doses of GDC-0980 potently inhibited tumor growth in xenograft models including those with activated PI3K and loss of PTEN (Wallin et al., 2011).

I.G General objectives of this Thesis

The focus of this Thesis is on how allelic expression of *Cbfb-MYH11* and $Nras^{G12D/+}$ in mouse hematopoietic cells altered the pre-leukemic compartments and progress to

leukemia by developing a triple mutant *Cbfb*$^{56M/+}$/*Nras*$^{LSL\text{-}G12D/+}$/*Mx1-Cre* mice. By using this valuable preclinical tool, we also want to identify downstream targets of those two oncogenes and evaluate small molecule inhibitors targeting Cbfβ-SMMHC and N-Ras^{G12D} signaling effectors.

Preface of Chapter II

The work presented in chapter II "N-Ras^{G12D} oncoprotein inhibits apoptosis of pre-leukemic cells expressing Cbfβ-SMMHC via activation of MEK/ERK axis" is aimed at providing insights into the molecular mechanisms of inv(16) AML with the *NRAS*^{G12D} mutation, using genetic and pharmacologic approaches. Most of the data described in this chapter has been published in:

> Xue, L., Pulikkan, J.A., Valk, P.J., and Castilla, L.H. (2014). NrasG12D oncoprotein inhibits apoptosis of preleukemic cells expressing Cbfβ-SMMHC via activation of MEK/ERK axis. Blood 124, 426-436 (Xue et al., 2014).

All the experiments described in this chapter were performed by me. I also analyzed the data and wrote the manuscript. Dr. Lucio Castilla provided intellectual input and edited the manuscript. Dr. John Pulikkan participated in the editing of the manuscript.

Chapter II

N-Ras^{G12D} oncoprotein inhibits apoptosis of pre-leukemic cells expressing Cbfβ-SMMHC via activation of MEK/ERK axis

II.A Abstract

Acute myeloid leukemia (AML) results from the activity of driver mutations that deregulate proliferation and survival of hematopoietic stem cells (HSCs). The fusion protein CBFβ-SMMHC impairs differentiation in hematopoietic stem and progenitor cells, and induces AML in cooperation with other mutations. However, the cumulative function of cooperating mutations in pre-leukemic expansion is not known. Here, we used *Nras$^{LSL-G12D}$; Cbfb^{56M}* knock-in mice to show that allelic expression of oncogenic N-Ras^{G12D} and Cbfβ-SMMHC increases survival of pre-leukemic short-term HSCs and myeloid progenitor cells and maintains the differentiation block induced by the fusion protein. N-Ras^{G12D} and Cbfβ-SMMHC synergize to induce leukemia in mice in a cell autonomous manner with a shorter median latency and increased leukemia-initiating cell activity than that of mice expressing Cbfβ-SMMHC. Furthermore, *Nras$^{LSL-G12D}$; Cbfb^{56M}* leukemic cells were sensitive to pharmacologic inhibition of MEK/ERK signaling pathway, increasing Bim protein levels and apoptosis. This study demonstrates that Cbfβ-SMMHC and N-Ras^{G12D} promote the survival of pre-leukemic myeloid progenitors primed for leukemia by activation of the MEK/ERK/Bim axis, and define *Nras$^{LSL-G12D}$;*

Cbfb^{56M} mice as a valuable genetic model for the study of inv(16) AML targeted therapies.

II.B Introduction

Acute myeloid leukemia (AML) results from the accumulation of mutations that deregulate self-renewal, proliferation, and differentiation in hematopoietic stem and progenitor cells (HSPCs) (Reilly, 2005). The understanding of the mechanism dictated by "driver" mutations during leukemia development is essential for the identification of targets for therapeutic intervention, inducing cell death of AML cells with minimal detriment of normal cells.

The fusion protein CBFβ-SMMHC is expressed in up to 5-8% of AML, and results from a chromosome 16 (p13; q22) inversion [inv(16)], which fuses the first five exons of *CBFB* with the last exons of *MYH11* to create the fusion gene *CBFB-MYH11* (Le Beau et al., 1983; Liu et al., 1993; Zeisig et al., 2012). The standard treatment for patients with inv(16) AML includes chemotherapy with cytotoxic agents, such as cytarabine and anthracyclines. This treatment has a favorable initial response, but the 5 year overall survival remains at approximately 50% to 60% (Pulsoni et al., 2008; Ravandi et al., 2007), and reduced to 20% in older patients (Cancer et al., 2006; Marcucci et al., 2005).

Studies in mice and inv(16) AML patient samples have shown that CBFβ-SMMHC is a "driver" mutation in AML. Expression of the fusion protein in *Cbfb*$^{+/MYH11}$ knock-in mouse embryos blocked definitive hematopoiesis (Castilla et al., 1996), and is necessary for leukemic development in cooperation with other mutations (Castilla et al., 1999;

Castilla et al., 2004). Using conditional *Cbfb*$^{+/56M}$ knock-in mice, we have shown that Cbfβ-SMMHC induces expansion of hematopoietic stem cells (HSCs), establishes a myeloid pre-leukemic progenitor population in the bone marrow, and predisposes to leukemia (Kuo et al., 2006). The leukemic cells of practically all patients diagnosed with subtype M4Eo AML have the inv(16), and this inversion is invariably present in relapse samples. Furthermore, the expression profile of inv(16) AML samples defines a unique signature, suggesting that CBFβ-SMMHC re-defines the molecular activity of targeted cells (Valk et al., 2004b).

The inv(16) AML blasts have "secondary mutations" that cooperate with CBFβ-SMMHC in leukemia. Frequently, these oncogenic mutations target components of cytokine signaling, including small GTPases (N-RAS and K-RAS), receptors (c-KIT and FLT3), and adaptor molecules (CBL) (Haferlach et al., 2010a; Reilly, 2005). For example, the predominant *NRAS* mutations are present in up to 45% of inv(16) AML (Muller et al., 2008; Paschka et al., 2013), with prevalence of G12D and Q61K missense mutations (Bacher et al., 2006; Renneville et al., 2008). N-RAS^{G12D} can promote proliferation and survival in cancer via activation of MEK/ERK and PI3K/AKT pathways (Braun and Shannon, 2008), although its function appears to be cell type specific. Recent genetic studies have shown that N-Ras^{G12D} induces a spectrum of hematologic malignancies in mice, including myeloid- and lympho-proliferation after a long latency, but not AML (Li et al., 2011; Wang et al., 2013; Wang et al., 2010a; Xu et al., 2013). However, the role of N-Ras^{G12D} in pre-leukemic progenitor cells primed by other oncoproteins remains unclear.

In this chapter, we have taken a genetic approach to define the role of N-Ras^{G12D} in inv(16) pre-leukemic HSPCs and leukemia development. Using *Cbfb^{56M} and Nras$^{LSL-G12D}$* conditional knock-in alleles, we show that N-Ras^{G12D} cooperates with Cbfβ-SMMHC in survival of pre-leukemic myeloid progenitors and in leukemia development. We use transplantation assays to evaluate the median leukemia latency and leukemia-initiating cell (L-IC) activity of AML blasts carrying *Cbfb^{56M} or Cbfb^{56M} and Nras$^{LSL-G12D}$* alleles to show that N-Ras^{G12D} contributes to produce aggressive leukemia. The pharmacologic inhibition of N-Ras^{G12D} activated pathways suggests that leukemic cell survival depends on MEK/ERK activity, and that PI3K/AKT signaling may not play a significant role. Furthermore, we show that N-Ras^{G12D} modulates Bcl-2 pro-apoptotic protein Bim expression in the pre-leukemic progenitor and leukemic cells, suggesting that survival advantage could be, at least partly, mediated by Bim inhibition.

II.C Materials and Methods

Mouse strains and treatment

Transgenic mice carrying the *Mx1-Cre, Cbfb^{56M}, Nras$^{LSL-G12D}$* and *Bim knock-out* alleles have been previously described (Bouillet et al., 1999; Haigis et al., 2008; Kuhn et al., 1995; Kuo et al., 2006). *Mx1-Cre, Nras$^{+/LSL-G12D}$* and *Bim$^{+/-}$* mice were in C57BL/6 background. *Cbfb$^{+/56M}$* mice, originally in 129SvEv background, were backcrossed 6 times into C57BL/6 background for this study. All mice were treated in accordance with federal and state government guidelines, and the University of Massachusetts Medical School Institutional Animal Care and Use Committee.

Genotyping of mice was performed as previously described (Haigis et al., 2008; Kuo et al., 2006). The primers for genotyping include:

For *Nras*$^{+}$ allele (500bp PCR product) and *Nras*$^{LSL\text{-}G12D}$ allele (345bp PCR product)

Nras F2 (5'-AGACGCGGAGACTTGGCGAGC-3')

Nras SD5' (5'-AGCTAGCCACCATGGCTTGAGTAAGTCTGCA-3')

Nras R1 (5'-GCTGGATCGTCAAGGCGCTTTTCC-3')

For *Cbfb*$^{+}$ allele (250bp PCR product)

WT Fwd (5'-CATCCCTGGGAATGGAGGTA-3')

WT Rev (5'-GAGTGGACCATTGTGGCAAG -3')

For *Cbfb*^{56M} allele (263bp PCR product)

Hyg Fwd (5'-GTTCAGCGAGAGCCTGACCT-3')

Hyg Rev (5'-CGGTGTCGTCCATCACAGTT-3')

For *Mx1-Cre* allele (450bp PCR product)

CreA.F (5'-CCGGGCTGCCACGACCAA-3')

CreA.R (5'-GGCGCGGCAACACCATTTTT-3')

For *Bim*$^{+}$ allele (400bp PCR product) and *Bim*$^{-}$ allele (540bp PCR product)

223 (5'-CATTCTCGTAAGTCCGAGTCT-3')

224 (5'-GTGCTAACTGAAACCAGATTAG-3')

225 (5'-CTCAGTCCATTCATCAACAG-3')

Transient Cre activation was induced in mice carrying the *Mx1Cre* transgene with three intraperitoneal injections of 250μg polyinosinic-polycytidylic acid [poly(I:C), Amersham Biosciences, Piscataway, NJ] every other day at 6 to 8 weeks of age. Mice

were initially monitored daily and early signs of leukemia were detected by the presence of c-Kit$^+$/Lineage$^-$ cells in peripheral blood by flow cytometry. Mice with signs of leukemia were under observation twice daily, and moribund leukemic mice were euthanized when presenting limited motility, pale paws, and dehydration.

Flow cytometry Analysis

Pre-leukemic progenitors: bone marrow cells were harvested from femurs and tibiae of each mouse 20 days after Cre activation [treatment with poly(I:C) as described] and stained for surface markers: Lineage (Lin) cocktail [Gr1, Mac1, Ter119, B220, CD3, CD19 and IgM; IL-7Rα are also added to the cocktail to exclude common lymphoid progenitors (CLPs)], c-Kit, Sca1, CD34, Flt3, and FcγRII/III (all from BD Biosciences, San Diego, CA). Immunophenotypic characterization of hematopoietic compartments included: stem and progenitor compartment (LSK$^+$): Lin$^-$Sca1$^+$c-Kit$^+$, LT-HSCs: LSK$^+$Flt3$^-$CD34$^-$ (Adolfsson et al., 2001), ST-HSC: LSK$^+$Flt3$^-$CD34$^+$, MPPs: LSK$^+$Flt3$^+$CD34$^+$ (Yang et al., 2005), myeloid progenitor cells (LSK$^-$): Lin$^-$Sca1$^-$c-Kit$^+$ (Akashi et al., 2000), CMP: LSK$^-$CD34$^+$FcγRII/III$^-$, GMP: LSK$^-$CD34$^+$FcγRII/III$^+$, and MEP: LSK$^-$CD34$^-$FcgRII/III$^-$.

Apoptosis analysis: bone marrow cells were harvested and stained with indicated cell surface markers, and stained with Annexin V and 7-AAD (BD Biosciences, San Diego, CA) according to manufacturer's instructions. Early apoptotic cells were determined as Annexin V$^+$/7AAD$^-$ by flow cytometry.

Cell cycle analysis: bone marrow cells were harvested and stained with cell surface markers, fixed and permeabilized (BD Cytofix / Cytoperm™ Fixation / Permeabilization

Solution Kit; BD Biosciences, San Diego, CA). Cells were stained with Ki67 (BD Biosciences, San Diego, CA) and Hoechst (Immunochemistry Technologies, Bloomington, MN) for 1 hour on ice, and cell cycle analysis was determined by flow cytometry. Fluorescence-activated cell sorter (FACS) data were acquired with LSRII (BD Biosciences, San Diego, CA) using FACSDiva software and analyzed with FlowJo software (TreeStar, Ashland, OR).

Morphology analysis of bone marrow cells

The bone marrow cells from control, Nras, CM, and Nras/CM mice were harvested from femurs and tibiae, 20 days after Cre activation. Cells were suspended in PBS, cytospun onto glass slides (500 rpm, 1 min), and stained using Wright Giemsa (Fisher Scientific Company, Kalamazoo, MI). The representation of progenitor cells was quantified using differential counting following the cell morphology description previously reported (Fredrickson, 2000; Yang et al., 2013).

Pathology

Peripheral blood, bone marrow and spleen cells were harvested from moribund leukemic mice, lysed in RBC lysis buffer (5 PRIME, Gaithersburg, MD) and analyzed by flow cytometry, using Lineage and c-Kit cell surface markers. White blood cell counts were measured using a Hemavet (Drew Scientific Group, Dallas, TX). Peripheral blood smears were stained using Wright Giemsa (Fisher Scientific Company, Kalamazoo, MI). The preparation of bone marrow, spleen and liver sections and Hematoxylin and Eosin (H&E) staining were performed by Histoserv (Germantown, MD). The images were

captured using an Axioskop 40 microscope, Axio CamMRC camera (ZEISS, Oberkochen, Germany) and the MRGrab software (ZEISS, Oberkochen, Germany).

Limiting dilution assay

Spleen cells were harvested from moribund CM or Nras/CM leukemic mice and 160, 800, $4X10^3$, $2X10^4$, $1X10^5$, $5X10^5$ or $2X10^6$ cells as indicated in the figures were transplanted by intravenous injection into sub-lethally irradiated (650 rads) recipient mice (8 to 10 week old CD45.1+ B6.SJL-ptprc<a>model#004007 females, Taconic Farms, NY). Recipient mice were monitored for leukemia development up to 27 weeks or 36 weeks as indicated.

Colony PCR

Bone marrow cells from 3 independent Nras/CM primary leukemic mice were harvested and plated onto MethoCult Medium (M3434, StemCell Technologies, BC, Canada) in a concentration of $5X10^4$ cells per 35mm culture dish according to manufacturer's instructions. Individual colonies (20 colonies per sample) were picked 7 days after incubation, washed in PBS and lysed in 50μl KAPA express extract buffer to extract genomic DNA (KAPA Biosystems, Wilmington, MA).

Five microliter of the extracted DNA solution was load for polymerase chain reaction (PCR). Each reaction consists of a denaturation at 95^oC for 3 minutes, followed by 35 cycles of amplification (95^oC 15 seconds, 60^oC 15seconds, 72^oC 30 seconds), and a final extension step at 72^oC 10 minutes. Primer sequences for each allele are listed below:

For $Nras^+$ and $Nras^{G12D}$ alleles

Nras F2 (5'-AGACGCGGAGACTTGGCGAGC-3')

Nras R1 (5'-GCTGGATCGTCAAGGCGCTTTTCC-3')

For $Nras^{LSL\text{-}G12D}$ allele

Nras SD5' (5'-AGCTAGCCACCATGGCTTGAGTAAGTCTGCA-3')

Nras R1 (5'-GCTGGATCGTCAAGGCGCTTTTCC-3')

For $Cbfb^{+}$, $Cbfb^{MYH11}$ and $Cbfb^{56M}$ alleles

mCi4.1F (5'-ATTGCATCCCTGGGAATGG-3')

mCi4.1R (5'-AGGCTCAACAGTATCAAGAGTCG-3')

hEx5/6n R (5'-TTGTCTTCTTGCCTCCATTTCC-3')

Sequencing for mutations in *Nras* and *Kras*

Nine CM leukemic cells were harvest from spleens. The RNA extraction and cDNA synthesis (500ng of RNA was used to make cDNA using Oligo-dT primers) were performed as previously described (Pulikkan et al., 2012). *Nras* and *Kras* cDNA were amplified from the 5μl total cDNA by Vent Polymerase (New England Biolabs, Ipswich, MA). PCR conditions are the same as above and primer sequences included:

For *Nras* cDNA

Nras Fs (5'-TCTGCGGAGTTTGAGGTTTT-3')

Nras Rs (5'-CTGTCCTTGTTGGCAAGTCA-3')

For *Kras* cDNA

Kras Fs (5'-CTGAGACGGCAGGGGAAG-3')

Kras Rs (5'-CCAGGACCATAGGCACATCT-3')

The PCR products were purified using Gel Purification Kit (Qiagen, Valencia, CA) and sequenced by Eton Bioscience (Charlestown, MA). The sequencing primers included:

For mutations in *Nras* cDNA

Nras seq new (5'-GACTGAGTACAAACTGGTGGTGG-3')

Nras seq rev (5'-CTTAATTTGCTCCCTGTAGAGGTT-3')

For mutations in *Kras* cDNA

Kras seq (5'-AGGCCTGCTGAAAATGACTG-3')

Kras seq rev (5'-CCCTCCCCAGTTCTCATGTA-3')

The sequencing results were viewed and analyzed by Chromas software (Technelysium, Australia).

Bone marrow transplantation

Five hundred thousand or two million bone marrow cells from Nras, CM, or Nras/CM (all $CD45.2^+$) mice were mixed with $2x10^5$ $CD45.1^+$ wild type (B6.SJL-ptprc<a>model#004007) bone marrow cells, and transplanted by intravenous injection into sub-lethally irradiated recipient mice (8 to 10 week old $CD45.1^+$ B6.SJL-ptprc<a>model#004007 females, Taconic Farms, NY). Engraftment of donor $CD45.2^+$ cells was analyzed two weeks post-transplantation in peripheral blood by flow cytometry. The recipient mice were then treated with 3 doses of poly(I:C), and monitored for early signs of leukemia up to 37 or 47 weeks as indicated.

In vitro assays of pre-leukemic and leukemic cells

Inhibitor response: the bone marrow cells from three Nras/CM mice 20 days after Cre activation were harvested, pooled, enriched for Lin- progenitors using "Negative Selection Mouse Hematopoietic Progenitor Enrichment Kit", and grown in cytokine-free StemSpan Medium (both from StemCell Technologies, BC, Canada) in the presence of

DMSO, MEK (PD325901; LC Laboratories, Woburn, MA) or PI3K inhibitor (GDC-0980; Selleckchem, Houston, TX) for 5 hours, and Bim levels were revealed by qRT-PCR and immunoblot analysis. Apoptosis analysis was performed in cells with the same treatment for 24 hours. The leukemic cells from three Nras/CM mice were independently cultured in serum-free medium and treated with PI3K and MEK inhibitors as described above.

Cell viability assay: wild type bone marrow or spleen cells from CM and Nras/CM leukemic mice were enriched for Lin^- progenitors, and treated in cytokine-free StemSpan Medium with DMSO, MEK or PI3K inhibitor for 48 hours in 96 well white plates (Corning Incorporated, Corning, NY), in triplicates or quadruplicates for each sample. The cell viability was tested using CellTiter-Glo reagent (Promega, Madison, WI) according to manufacturer's instructions.

Reverse transcription and quantitative PCR (qRT-PCR) analyses

The RNA extraction, cDNA synthesis (random primers were used), and qPCR were performed as previously described (Pulikkan et al., 2012). Primer sequences included:

For *Actb* cDNA

b-actin F1 (5'-CGAGGCCCAGAGCAAGAGAG-3')

b-actin R1 (5'-CGGTTGGCCTTAGGGTTCAG-3')

For *Bcl2l11* cDNA

mBim F (5'-GCTGGTGGGACCTGTTTCTA-3')

mBim R (5'-TTCAGTGAGCCATCTTGACG-3')

Immunoblot analyses

Cells were washed with PBS and resuspended in modified RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.25% Sodium Deoxycholate) with protease inhibitor cocktail III (Calbiochem, Darmstadt, Germany) and phosphatase inhibitor cocktail II and III (Sigma-Aldrich, St. Louis, MO). Antibodies included anti-Bim (cat#2819), anti-Bcl2 (cat#2870), anti-Bcl-xl (cat#2762), anti-Mcl-1 (cat#5453), anti-Phospho-Akt (Ser473) (cat#4058), anti-Akt (cat#9272), anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (cat#9101), anti-p44/42 MAPK (Erk1/2) (cat#9102), anti-Phospho-Stat5 (Tyr694) (cat#9359), and anti-Stat5 (cat#9358, all from Cell Signaling Technology, Danvers, MA).

Bim knock-down in bone marrow cells

Retroviral production: shRNA for *Scrambled* (5'-TACTATCTCTAAGACTTTCT-3' for antisense sequence) or mouse *Bcl2l11* (5'-ATATATTTAAGTACAAAGGCCT-3' for antisense sequence) were cloned into retroviral vector pMSCV-LTRmiR30-PIG (LMP) following manufacturer directions (LMP, Thermo Scientific, Billerica, MA). Stable GP+E-86 viral-packaging cells (Markowitz et al., 1988) were transfected with LMP constructs using JetPRIME reagent (Polyplus Transfection, New York, NY), and selected with 4μg/ml puromycin (InvivoGen San Diego, CA). Retroviral supernatants were concentrated and titered in 3T3 cells by GFP expression. Knock-down efficiency was tested in puromycin selected 3T3 cells by quantitative RT-PCR and immunoblot analyses.

Bone marrow cell infection: bone marrow progenitor cells were harvested from CM mice pretreated with poly(I:C) and 5-fluorouracil, spin-infected twice with retroviral supernatants in 7.5mM Hepes Buffer Solution as well as 5μg/ml Polybrene (American

Bioanalytical, Natick, MA) and cultured for 24 hours. The cells were transferred to serum free media for 24 hours to induce Bim expression and were analyzed for apoptosis by flow cytometry or sorted for $GFP^{+}7\text{-}AAD^{-}$ cells using a BD FACSAria cell sorter (BD Biosciences, San Diego, CA) to assay knock-down efficiency.

Statistics

Medium latencies of Kaplan-Meier survival curves were calculated using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). The P values of the Kaplan-Meier survival curves were calculated by Log-rank (Mantel-Cox) test using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). Paired P values as indicated in the experiments on inhibitor response of leukemic cells were calculated by two-tailed paired Student t-test using Excel (Microsoft Cooperation, Redmond, WA). All other P values were calculated by two-tailed heteroscedastic Student t-test using Excel (Microsoft Cooperation, Redmond, WA). The leukemia initiating cell activity (L-IC) was estimated by limiting dilution assay and calculated based on Poisson distribution using L-Calc software (StemCell Technologies, BC, Canada). The nonlinear regression curve fit for viability assay was generated using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA).

II. D Results

1. Cbfβ-SMMHC and N-Ras^{G12D} expand pre-leukemic stem and progenitor cells in mice

The allelic (*Cbfb*^{56M}) expression of CBFβ-SMMHC in HSCs induces pre-leukemic progenitor cells with impaired differentiation capacity, and progresses to leukemia after the accumulation of additional mutations (Kuo et al., 2006). *Nras*^{G12D} is a frequent mutation found in human inv(16) AML, and has been shown to promote the expansion of HSCs and myeloid progenitors (Li et al., 2011; Wang et al., 2013). To understand the role of N-Ras^{G12D} in leukemia progression, we analyzed the frequency of pre-leukemic stem and progenitor cells in bone marrow of *Mx1Cre* (Control), *Nras*$^{+/LSL\text{-}G12D}$*/Mx1Cre* (Nras), *Cbfb*$^{+/56M}$*/Mx1Cre* (CM), and *Nras*$^{+/LSL\text{-}G12D}$*/Cbfb*$^{+/56M}$*/Mx1Cre* (Nras/CM) mice, by flow cytometry 20 days after Cre activation. The bone marrow cellularity was increased in Nras mice, primarily increased in the late (Lin^{+}) progenitor cells (**Figure II.1A-C**; $p<0.05$). Conversely, CM and Nras/CM mice showed a reduction in bone marrow cellularity, with a significant increase in the immature (Lin^{-}) and decrease in the mature (Lin^{+}) bone marrow cells (**Figure II.1A-C**; $p<0.01$), suggesting a block in myeloid differentiation. The number of early progenitor [Lin^{-}Sca1^{+}c-Kit^{+}, (LSK^{+})] cells in Nras, CM, and Nras/CM increased to 5, 3 and 2 fold respectively (**Figure II.1D,** representative FACS plots are shown in **Figure A.1** in Appendix A). Activation of N-Ras^{G12D} increased the multi-potential progenitors (MPPs) by 8 fold, with minimal change of the long-term and short term HSCs (LT-HSCs and ST-HSCs; **Figure II.1E**). Activation of Cbfβ-SMMHC increased the MPPs and ST-HSCs by 2 to 4 fold, and reduced the LT-HSCs by 2 to 3 fold in CM and Nras /CM mice (**Figure II.1E**; $p<0.05$). The percentage of ST-HSCs and MPPs increased similar in CM and Nras/CM, suggesting that Cbfβ-SMMHC directed the expansion of ST-HSC and early progenitors in bone marrow (**Figure II.2A-**

B). The total number of myeloid progenitor cells (LSK^-) increased 2 fold in the three groups compared to Controls (**Figure II.1F**, $p<0.05$). The $N\text{-}Ras^{G12D}$ expression expanded all myeloid compartments proportionally (**Figure II.1G** and **Figure II.2C-D**) as previously shown (Li et al., 2011). The expression of Cbfβ-SMMHC increased primarily the CMP compartment in the bone marrow of CM and Nras/CM mice (**Figure II.1G** and **Figure II.2C-D**) and hindered $N\text{-}Ras^{G12D}$ mediated expansion of GMPs in Nras/CM mice.

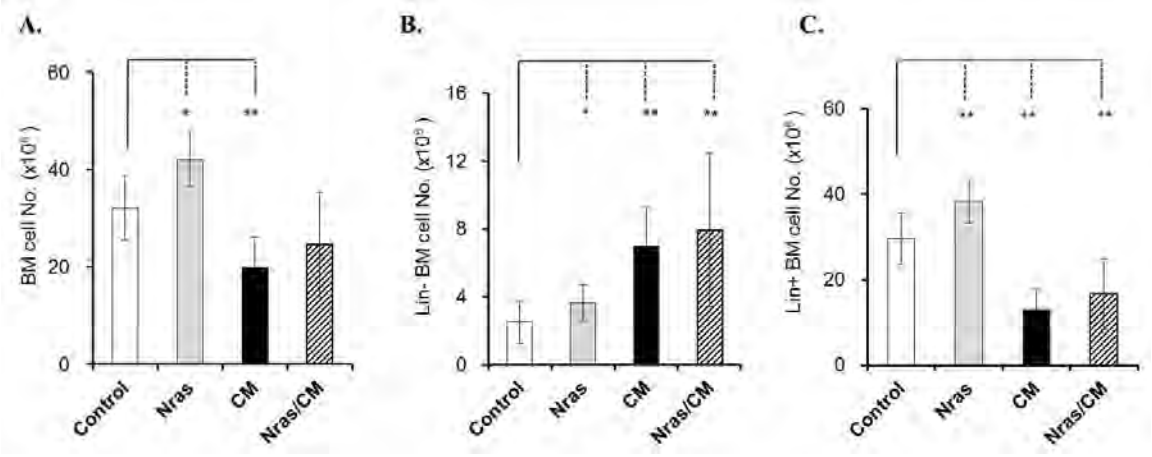

Figure II.1 Cbfβ-SMMHC and N-Ras^{G12D} expand pre-leukemic stem and progenitor cells. Quantification of bone marrow cells from *Mx1Cre* (Control, white), *Nras$^{+/LSL\text{-}G12D}$/Mx1Cre* (Nras, grey), *Cbfb$^{+/56M}$/Mx1Cre* (CM, black), and *Nras$^{+/LSL\text{-}G12D}$/Cbfb$^{+/56M}$/Mx1Cre* (Nras/CM, diagonal) mice by flow cytometry, 20 days after Cre activation. (A-C) n=8-14 mice per group: (A) bone marrow cell numbers (per mouse) in each group; (B) lineage negative (Lin$^-$) bone marrow cell numbers in each group; (C) lineage positive (Lin$^+$) bone marrow cell numbers in each group. BM, bone marrow. *, P<0.05; **, P<0.01.

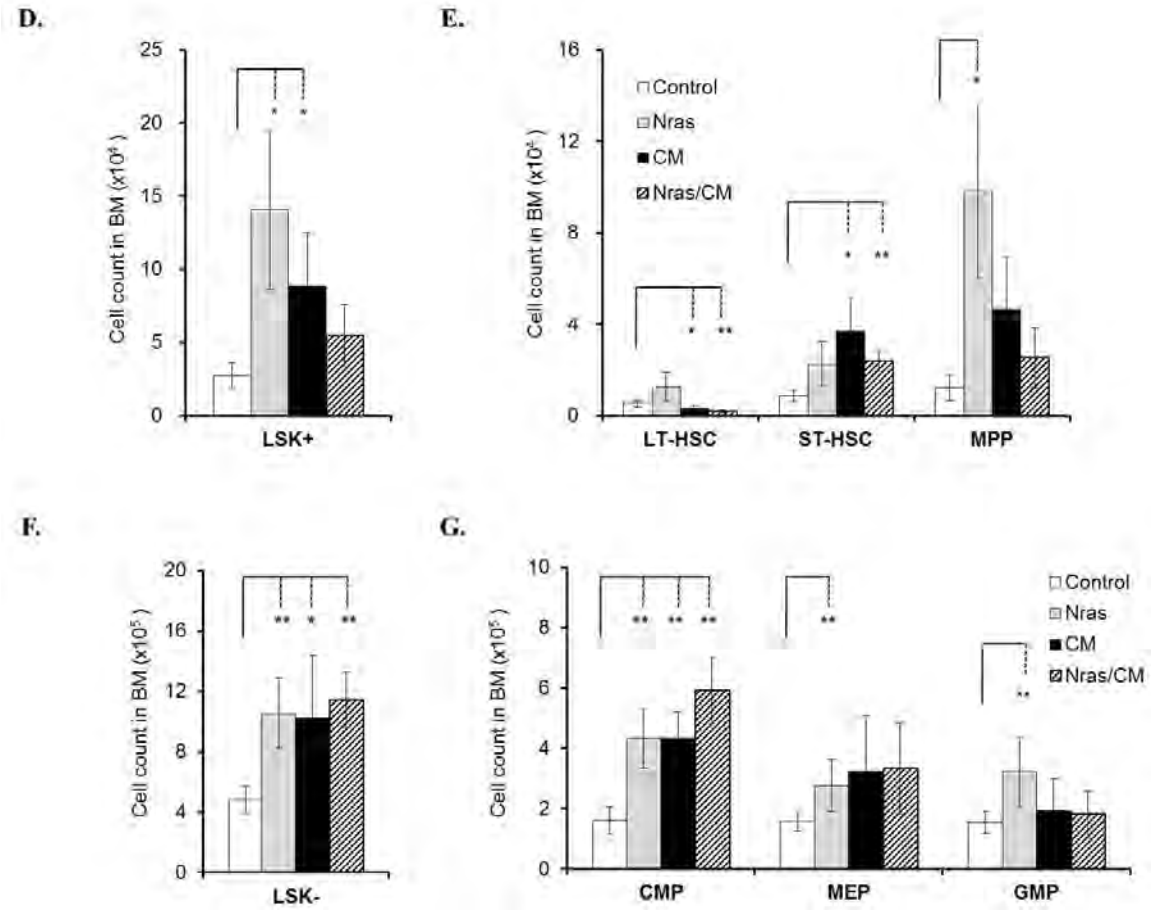

Figure II.1 (Cont.) Cbfβ-SMMHC and N-Ras^{G12D} expand pre-leukemic stem and progenitor cells. (D-E) n=4-8 mice per group: (D) LSK^{+} cell numbers in bone marrow; (E) long-term (LT-HSCs, LSK^{+}CD34^{-}FLT3^{-}), short-term (ST-HSCs, LSK^{+}CD34^{+}FLT3^{-}) hematopoietic stem, and multipotential progenitor (MPPs, LSK^{+}CD34^{+}FLT3^{+}) cell number in each group. (F-G) n=5-12 mice per group: (F) LSK^{-} cell numbers; (G) common myeloid progenitor (CMP, LSK^{-}CD34^{+}FcγRII/III^{-}), megakaryocyte-erythroid progenitor (MEP, LSK^{-}CD34^{-}FcγRII/III^{-}), and granulocyte-monocyte progenitor (GMP, LSK^{-}CD34^{+}FcγRII/III^{+}) cell numbers in each group. BM, bone marrow. *, P<0.05; **, P<0.01.

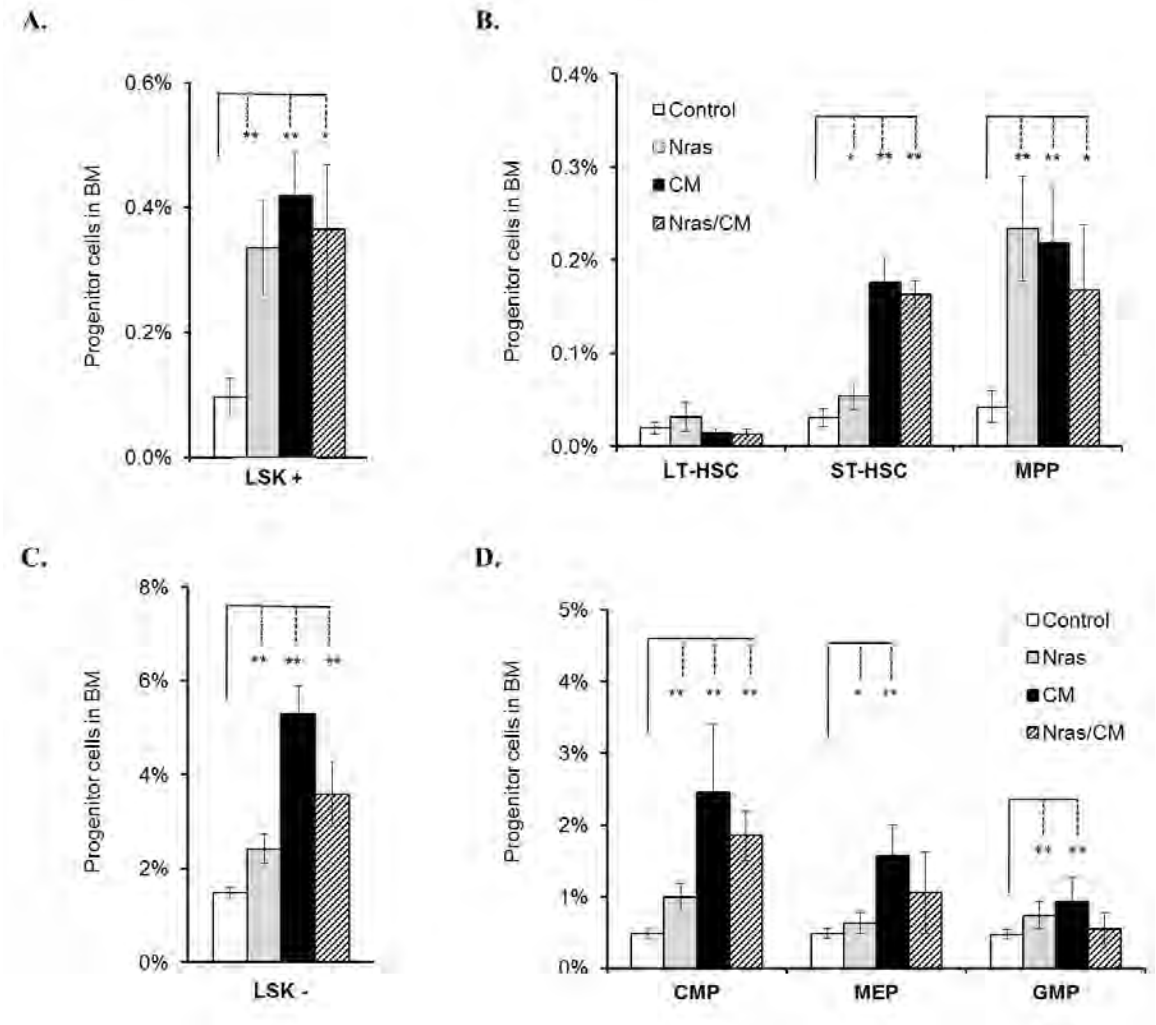

Figure II.2 Cbfβ-SMMHC and N-Ras^{G12D} expand pre-leukemic stem and progenitor cells in mice. (A-B) n=4-8 mice per group: (A) percentage of LSK^{+} cells in bone marrow; (B) percentage of LT-HSC, ST-HSC and MPP in bone marrow. (C-D) n=5-12 mice per group: (C) percentage of LSK^{-} compartment; (D) percentage of CMP, MEP and GMP compartments in each group. BM, bone marrow. *, P<0.05; **, P<0.01.

The morphology (differential counts) analysis of bone marrow cells from each genetic group revealed that CM and Nras/CM samples had an increase in blast/myeloblasts and promyelocytes and a reduction in immature neutrophils and eosinphils (**Figure II.3**).

These results show that N-Ras^{G12D} and Cbfβ-SMMHC expression increase short-term HSCs, MMPs and common myeloid progenitor cells in the early pre-leukemia stage *in vivo*, and suggest that this expansion is mainly driven by Cbfβ-SMMHC activity in Nras/CM mice.

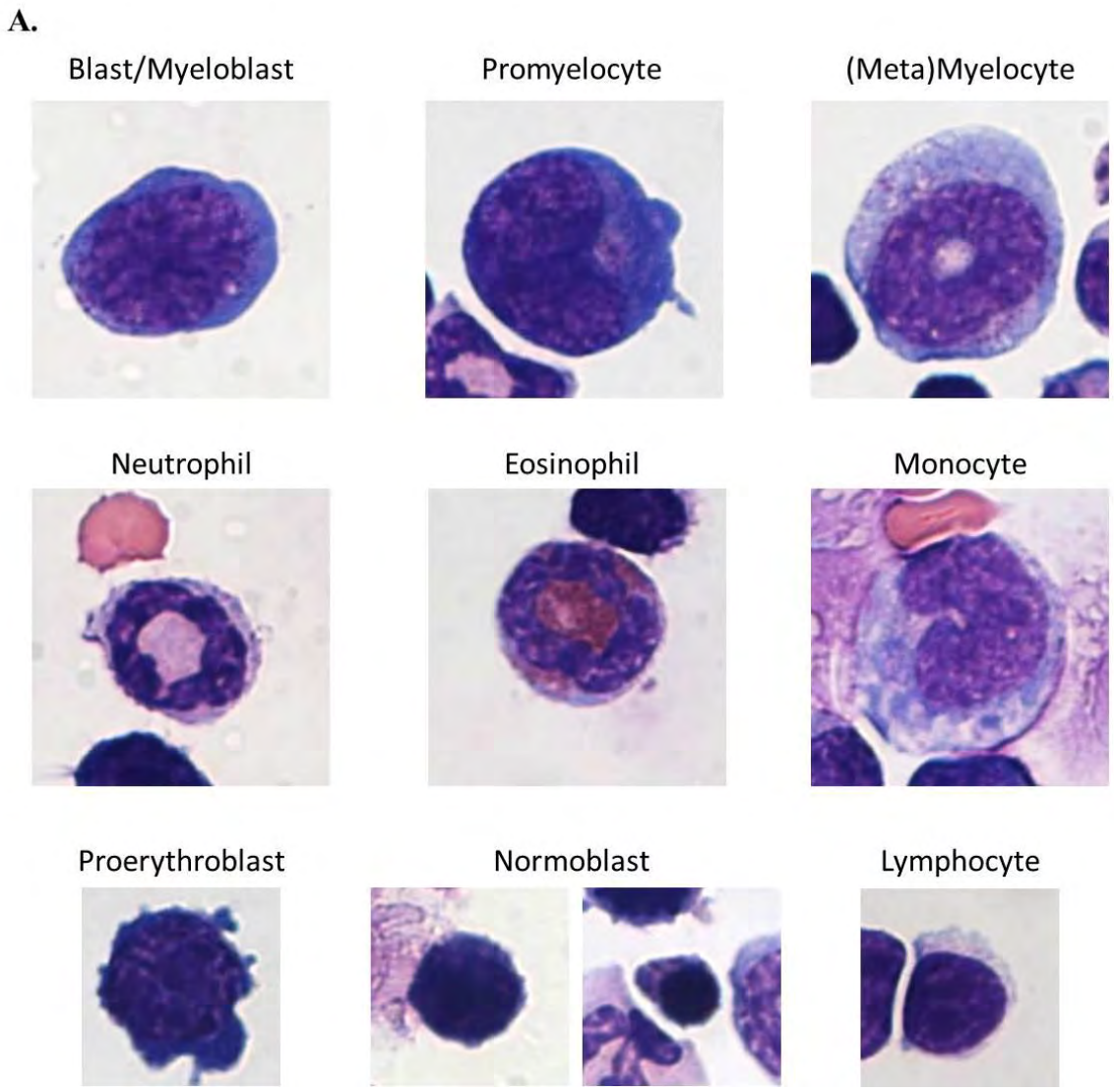

Figure II.3 Expression of Cbfβ-SMMHC increases blasts, myeloblasts as well as promyelocytes and reduces neutrophils and eosinophils. Bone marrow cells from Control, Nras, CM and Nras/CM mice were harvest, cytospun and stained 20 days after Cre activation. (A) representative pictures of different bone marrow cell morphologies.

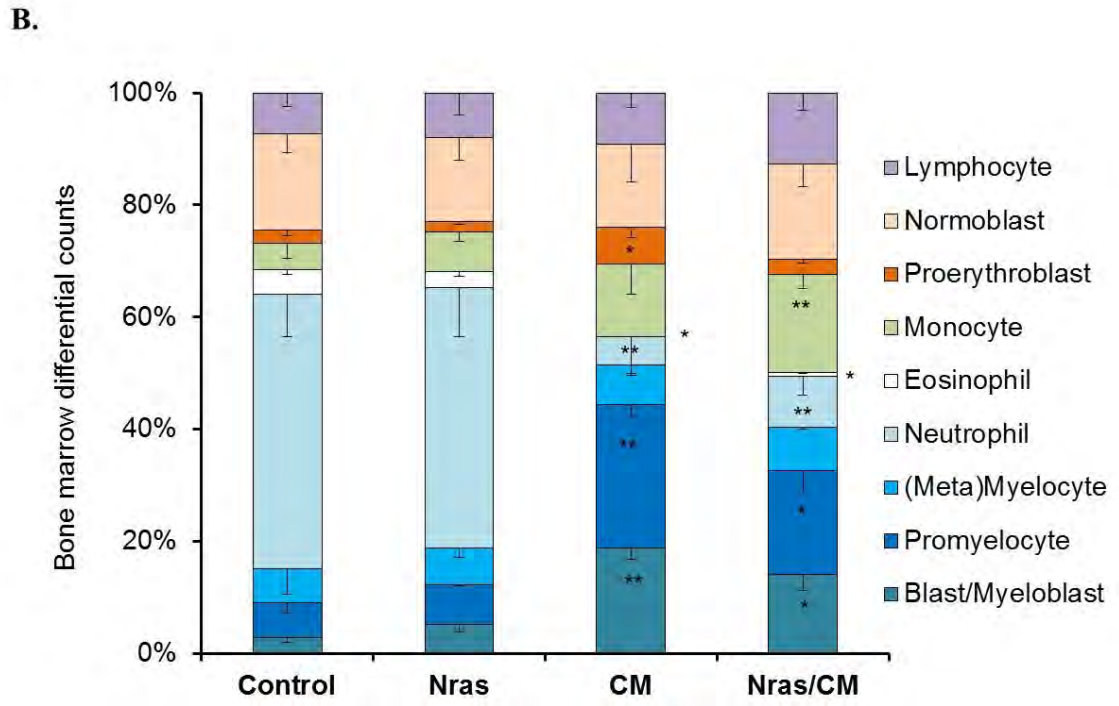

Figure II.3 (Cont.) Expression of Cbfβ-SMMHC increases blasts, myeloblasts as well as promyelocytes and reduces neutrophils and eosinophils. (B) bone marrow cell differential counts in each group (n=3 mice per group). *, P<0.05; **, P<0.01.

2. Cbfβ-SMMHC and N-Ras^{G12D} provide survival advantage to pre-leukemic progenitor cells

To better understand the role of N-Ras^{G12D} and Cbfβ-SMMHC on pre-leukemic progenitor cells, we inquired their functions on proliferation and apoptosis of bone marrow progenitors *in vivo*. Early apoptosis (AnnexinV$^+$/7AAD$^-$) was analyzed in bone marrow cells from mice by flow cytometry 20 days after Cre activation. Apoptosis was significantly reduced by N-Ras^{G12D} or Cbfβ-SMMHC in both LSK$^+$ and LSK$^-$ compartments (**Figure II.4**; $p<0.05$), with an additive effect in cells expressing N-Ras^{G12D} and Cbfβ-SMMHC (**Figure II.4**; $p<0.01$). A similar result was obtained based on a different gating strategy (**Figure A.2** in Appendix A). The analysis of cell cycle (Hoechst and Ki67 staining) of stem and early progenitor cells (LSK$^+$) revealed a reduction in the G_0 fraction in CM and Nras/CM mice and an increase in the S/G2/M fraction increased in Nras/CM mice (**Figure II.5**). However, proliferation of myeloid progenitor cells (LSK$^-$) was reduced (increased G_0/G_1 vs $S/G_2/M$) in mice expressing Cbfβ-SMMHC (CM and Nras/CM groups; **Figure II.5**).

These results suggest that N-Ras^{G12D} and Cbfβ-SMMHC increase the survival of pre-leukemic stem and progenitor cells. In addition, Cbfβ-SMMHC increases proliferation of stem and early progenitor cells but reduces proliferation of myeloid progenitor cells.

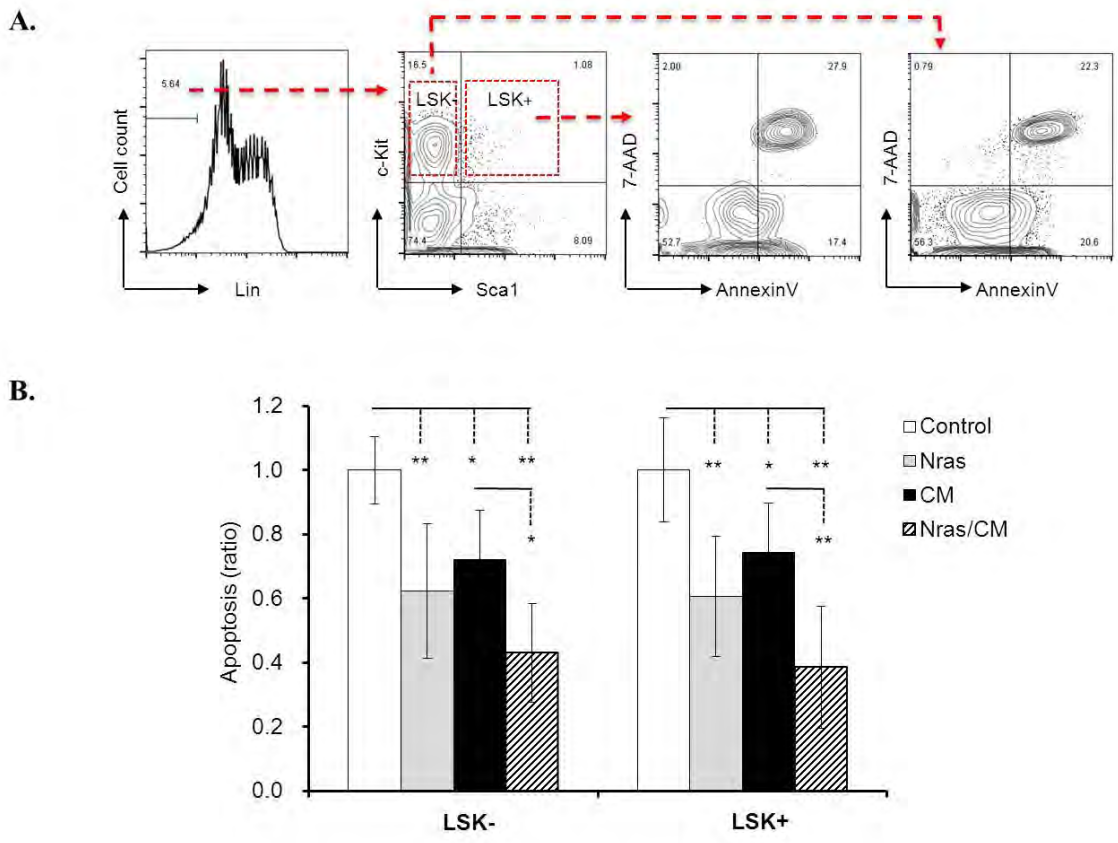

Figure II.4 Apoptosis analyses of bone marrow pre-leukemic cells expressing Cbfβ-SMMHC and N-Ras^{G12D}. Bone marrow cells from mice treated as in Figure II.1 were stained with 7-AAD and Annexin V, and analyzed by flow cytometry. (A) cells were first gated by FSC-A/SSC-A, then gated for single cells by FSC-A/FSC-H, and the following gating of each compartment is shown; (B) quantification of the ratio of apoptosis levels for LSK$^-$ (left) and LSK$^+$ (right) cells of each group normalized to control group (n=5-11, *, P<0.05; **, P<0.01).

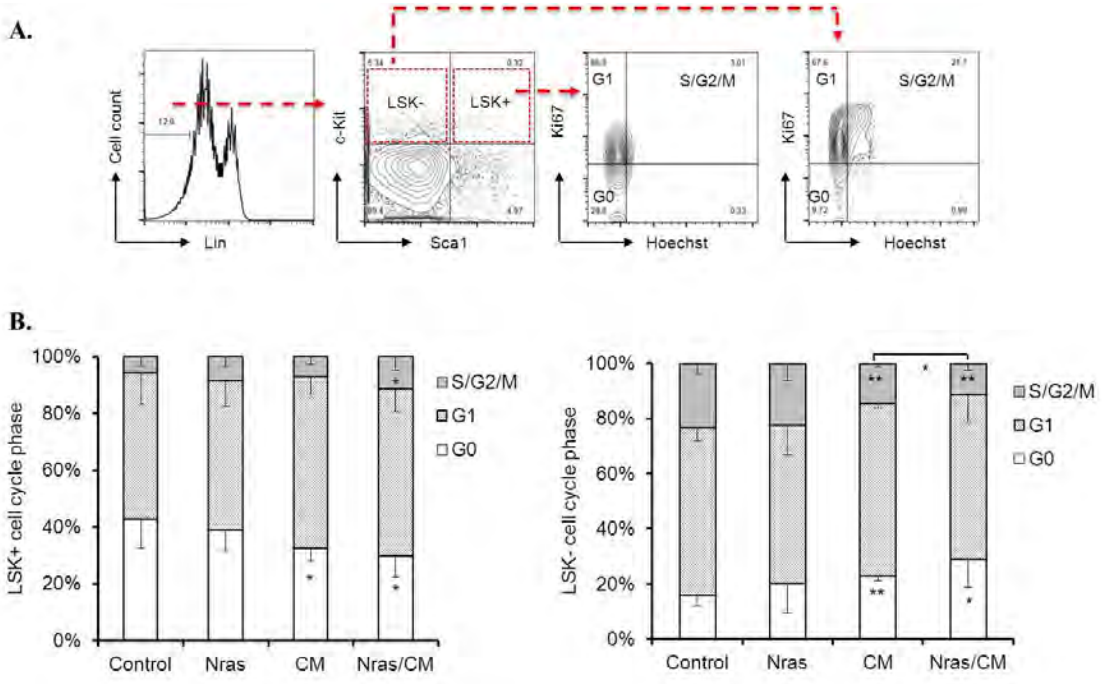

Figure II.5 Cell Cycle analyses of bone marrow pre-leukemic cells expressing Cbfβ-SMMHC and N-Ras^{G12D}. Bone marrow cells from mice treated as in Figure II.1 were stained with Ki67 and Hoechst and analyzed by flow cytometry for cell cycle phase. (A) cells were first gated by FSC-A/SSC-A, then gated for single cells by FSC-A/FSC-H, and the following gating of each compartment is shown; (B) quantification (percentage) of cells in G_0 (white), G_1 (dotted), or $S/G_2/M$ (grey) phase for LSK^+ (left) and LSK^- (right) groups (n=4-8, *, $P<0.05$; **, $P<0.01$).

3. N-Ras^{G12D} cooperates with Cbfβ-SMMHC to induce acute myeloid leukemia

To determine whether N-Ras^{G12D} synergizes with Cbfβ-SMMHC in leukemia initiation, leukemia latency was analyzed in CM, Nras and Nras/CM mice after Cre activation. Nras mice remained healthy up to 35 weeks (experimental endpoint), and CM mice developed leukemia with a median latency of 21.43 weeks and 89% penetrance (**Figure II.6A**, dashed and solid lines, respectively; **Table II.1**). Nras/CM mice developed leukemia with a reduced median latency (13.72 weeks, P= $8x10^{-4}$) and full penetrance (**Figure II.6A**, dotted line; **Table II.1**). The median leukemia latency of secondary transplantation assays ($5x10^5$ leukemic cells) from Nras/CM mice was consistently shorter than that of CM mice even with some heterogeneity in each group (Nras/CM: 3 weeks, 100% penetrance, CM: 19 weeks, 62.5% penetrance, P<0.0001; **Figure II.6B-D** and **Table II.1**). The leukemia initiating cell activity (L-IC) was estimated using limiting dilution assays in two representative leukemic cells from CM and Nras/CM mice, respectively. Concordant to the median latency data, the L-IC activities in CM leukemias were 1/528,334 (range: 1/1,268,433 to 1/220,064; 95% confidence) and 1/68,380 (range: 1/152,523 to 1/30,657; 95% confidence) for two independent clones (B92 and A287), while L-IC activities in Nras/CM leukemias were 1/1,283 (range: 1/2,927 to 1/562; 95% confidence) and higher than 1/160 leukemic cells for two independent clones (B81 and B286) (**Figure II.6E-H**), which are over 400 fold higher than CM leukemias.

The mice that appear health at the end of experiments (36 weeks) transplanted with either A287 or B81 primary leukemic clone were analyzed for appearance of c-kit$^+$ cells

in peripheral blood by flow cytometry, existence of cells that harboring *Cbfb*^{MYH11} alleles in white blood cells in peripheral blood and bone marrow by PCR of genomic DNA and spleen weight (**Figure A.3** and **Figure A.4** in Appendix A). There is no sign of leukemia development revealed in all the mice (around or less than 1% c-kit^{+} cells in peripheral blood, no *Cbfb*^{MYH11} allele revealed by PCR in peripheral blood or bone marrow cells, spleen weight around 0.1g) except that one mouse transplanted with 160 Nras/CM B81 leukemic cells. That mouse appeared normal and presented elevated percentage of c-kit^{+} cells (10.2%, **Figure A.3C** in Appendix A) and enlarged spleen (0.3418g, **Figure A.3D** in Appendix A) in peripheral blood with no detection of recombined Cbfβ-SMMHC allele in genomic DNA from peripheral blood, bone marrow and spleen cells (**Figure A.4B** in Appendix A). That mouse was still counted as leukemia free survival (**Figure II.6H**).

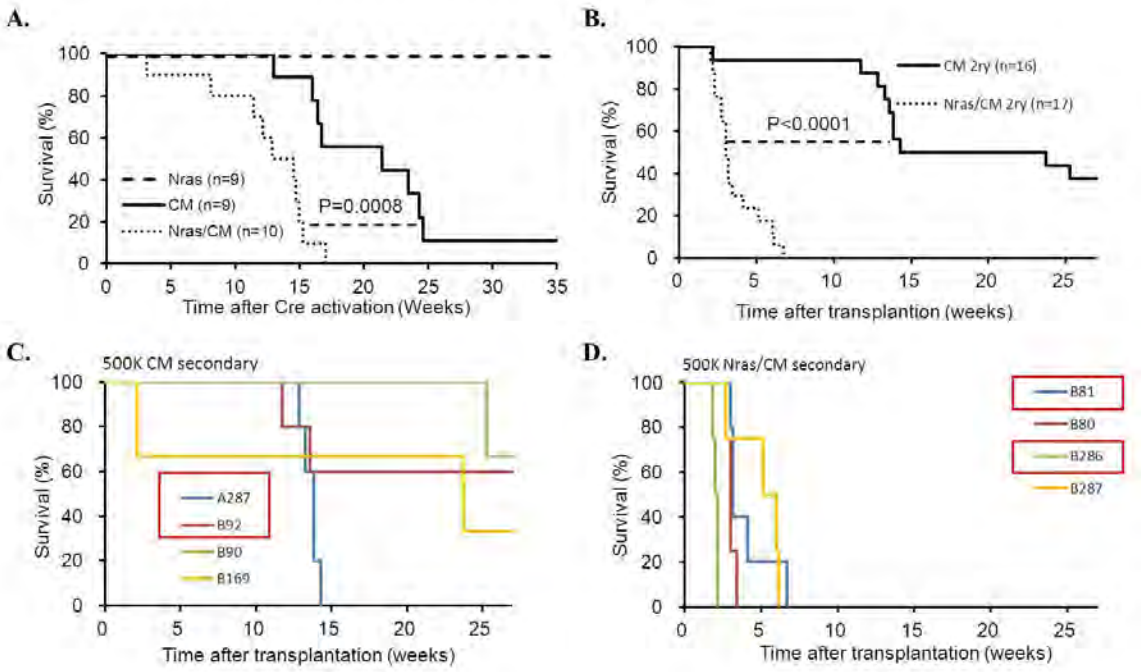

Figure II.6 N-Ras^{G12D} induces an accelerated leukemia with higher L-IC activity in cooperation with Cbfβ-SMMHC. (A) Kaplan-Meier curves of leukemia-free survival in Nras (dash), CM (solid) and Nras/CM (dotted) mice after Cre activation (time 0); (B) Kaplan-Meier survival curves of recipients transplanted with leukemic cells from CM (solid) and Nras/CM (dotted) mice; (C-D) Kaplan Meyer survival curves of recipients transplanted with leukemic cells from $5x10^5$ (500K) CM (C) or Nras/CM (D) mice, plotted as individual clones; red blocks represent the clones used for limiting dilution assays.

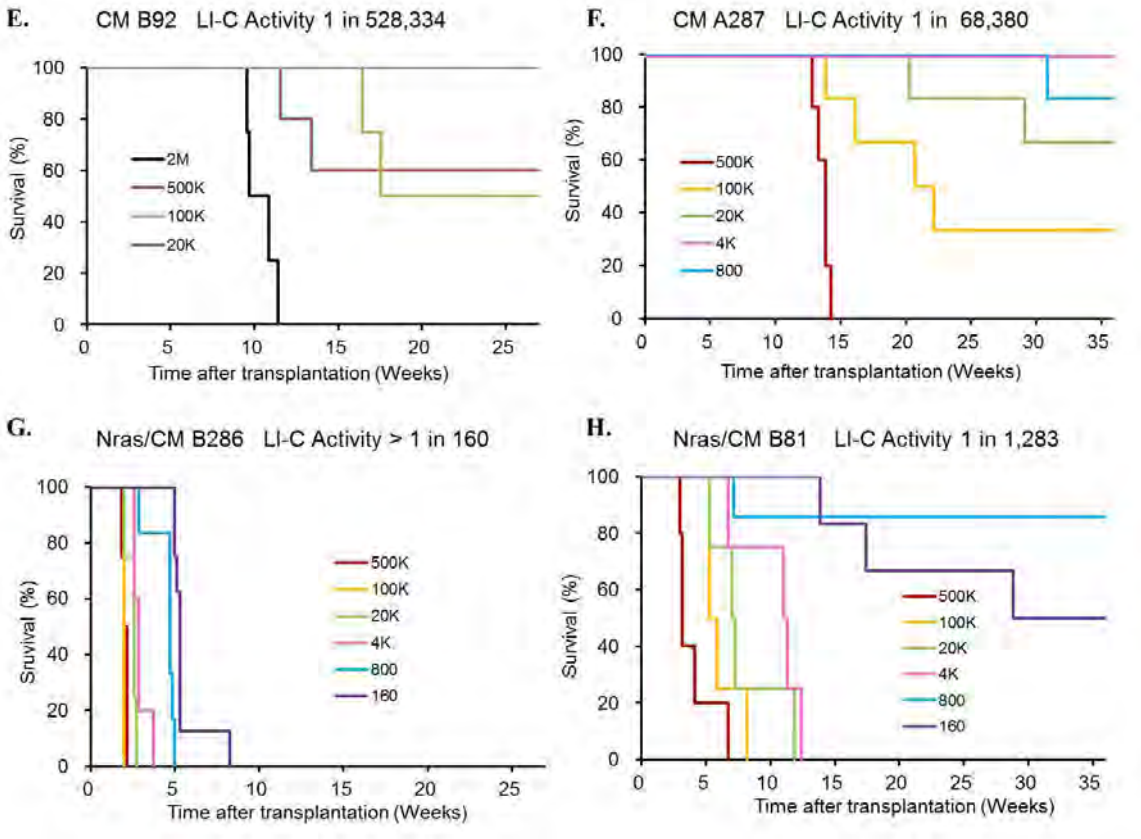

Figure II.6 (Cont.) N-Ras^{G12D} induces an accelerated leukemia with higher L-IC activity in cooperation with Cbfβ-SMMHC. (E-F) Kaplan Meyer survival curves for limiting dilution assays, using four concentrations, of two CM leukemia samples; (G-H) Kaplan Meyer survival curves for limiting dilution assays, using four concentrations, of two Nras/CM leukemia samples. Note: For mice transplanted with 800 B81 leukemic cells, one mouse was found dead one day after bleeding at the end of experiment, presenting no sign of leukemia (Figure A.3C-D and Figure A.4B in Appendix A) and counted as leukemia free survival; for mice transplanted with 160 B81 leukemic cells, one mouse presented enlarged spleen (0.3418g) and elevated percentage of c-kit^{+} cells (10.2%) in peripheral blood with no detection of recombined Cbfβ-SMMHC allele in genomic DNA from peripheral blood, bone marrow and spleen cells at the end of experiment (Figure A.3C-D and Figure A.4B in Appendix A) and counted as leukemia free survival.

Table II.1 Leukemia burden in mice carrying the *Cbfb*^{56M}; *NRas*$^{LSL\text{-}G12D}$ knock-in alleles

Groups	Median latency (w)	Spleen weight (g)	WBC (range $10^3/\mu L$)
WT	Na	0.08 ± 0.01	5.76 - 9.56
CM	21.43	1.59 ± 1.21	17.00 - 558.00
Nras/CM	13.72	2.94 ± 1.37	22.88 - 638.60
CM 2^{ry}	19	0.31 ± 0.18	6.36 - 32.60
Nras/CM 2^{ry}	3	0.28 ± 0.11	35.38 - 454.20
Nras/CM Trans	28.15	0.34 ± 0.06	40.08 - 306.00

Note: wild type (WT); *Cbfb*$^{+/56M}$*/Mx1Cre* (CM); *Nras*$^{+/LSL\text{-}G12D}$*/Cbfb*$^{+/56M}$*/Mx1Cre* (Nras/CM); weeks (w); grams (g); White blood cell count (WBC); secondary transplantation (2^{ry}); Recipient mice engrafted with bone marrow cells carrying *Nras*$^{+/LSL\text{-}G12D}$*/Cbfb*$^{+/56M}$*/Mx1Cre* (Nras/CM Trans).

The pathology of leukemia was similar between genetic groups (CM and Nras/CM) and between primary and secondary leukemias. Analysis of peripheral blood cellular composition from leukemic mice revealed a consistent increase in white blood cell (WBC) count and increased presence of leukocytes with immature morphology (**Figure II.7A** and **Table II.1**). Immunophenotypic analysis of peripheral blood revealed the predominant presence of immature (c-Kit^{+}, Lin^{-}) cells (**Figure II.7B-C**). The spleen of primary and secondary leukemic mice was enlarged (**Table II.1**), with evident disruption in splenic architecture (**Figure II.7D**). Histological staining also revealed that leukemic cells infiltrated into bone marrow and liver. Quantification of leukemic cell burden was confirmed in peripheral blood, spleen and bone marrow from Nras/CM, CM and Nras/CM secondary leukemic mice (**Figure II.7C**).

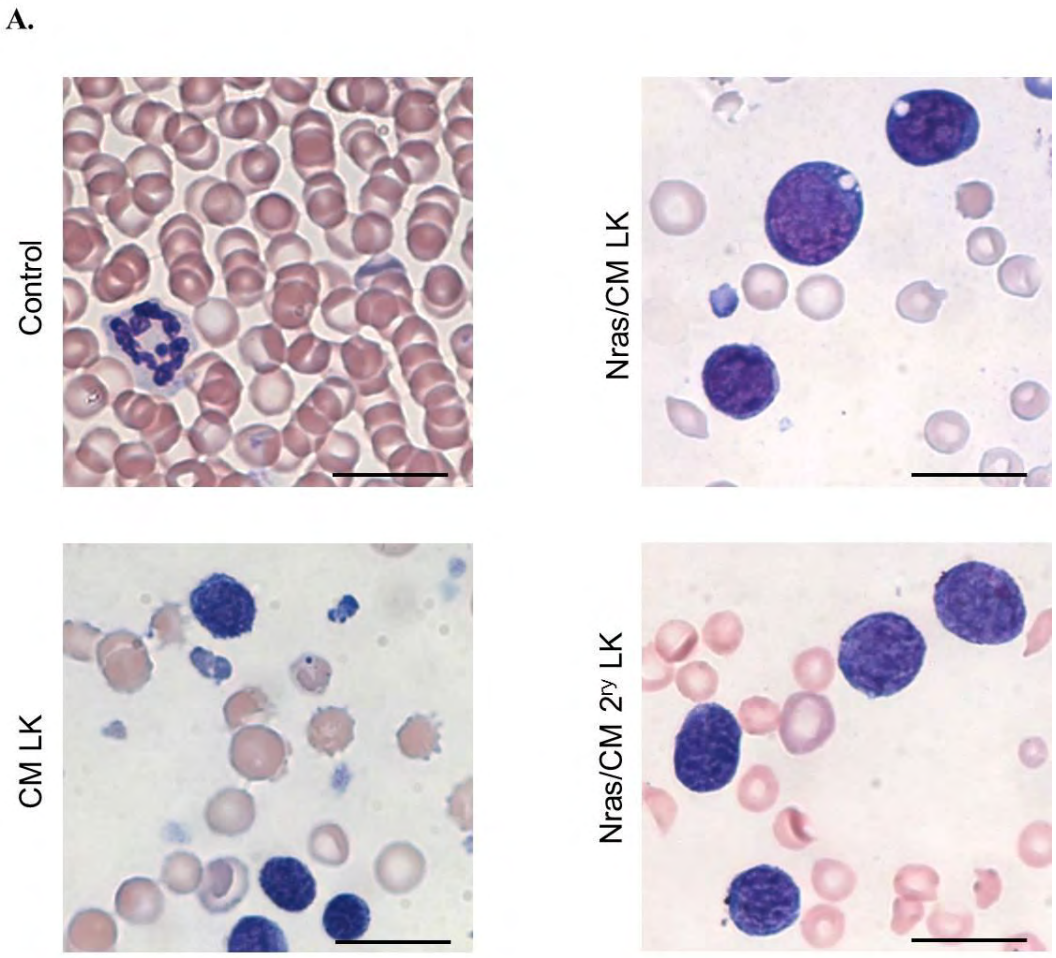

Figure II.7 Characterization of leukemia in mice expressing Cbfβ-SMMHC and N-Ras^{G12D}. (A) representative Wright Giemsa staining of peripheral blood smears from wild type (control), moribund Nras/CM, CM, and Nras/CM secondary leukemic mice. Scale bars, 5μm.

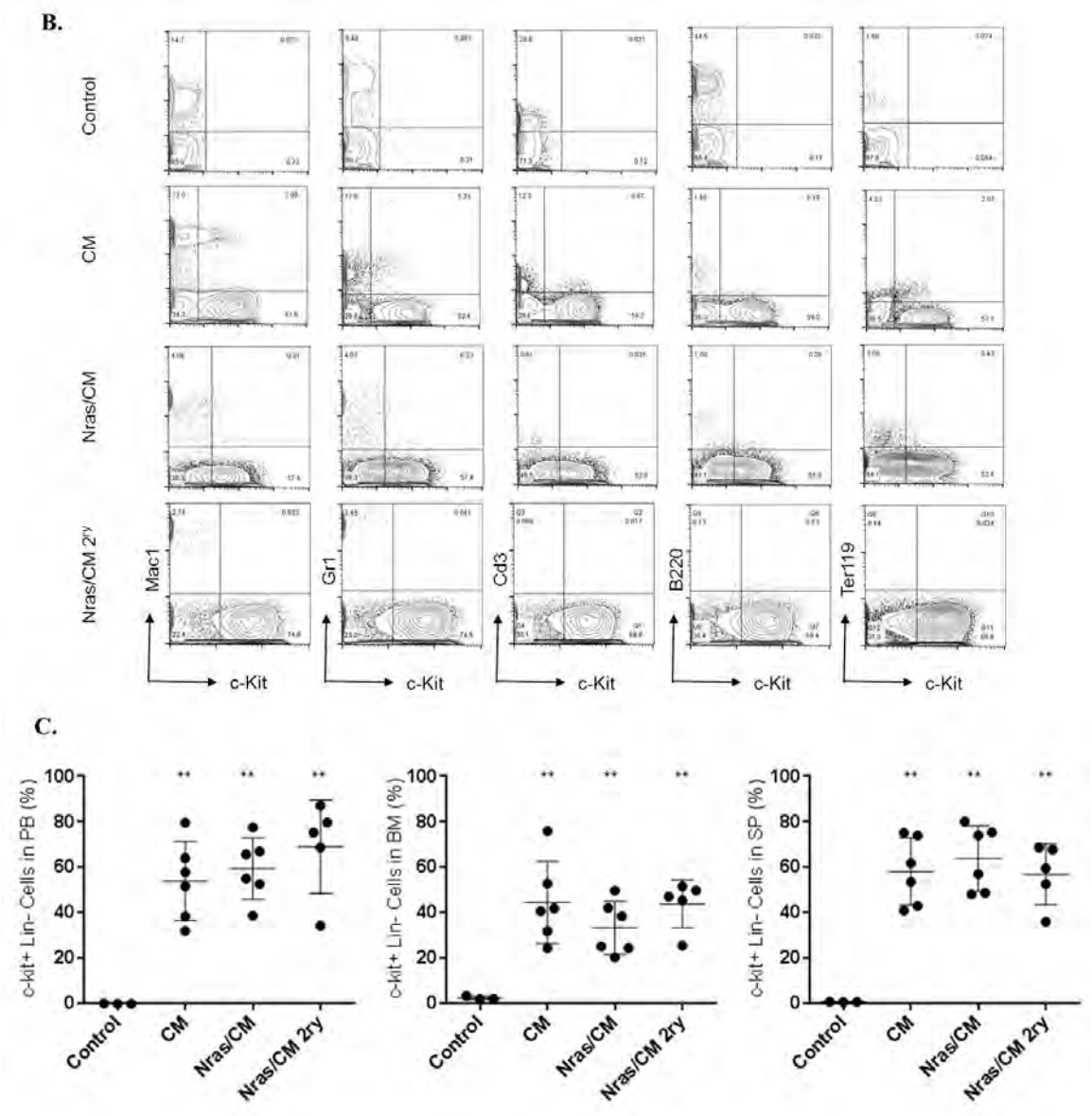

Figure II.7 (Cont.) Characterization of leukemia in mice expressing Cbfβ-SMMHC and N-Ras^{G12D}. (B) representative FACS analysis of peripheral blood cells from wild type Control, and moribund CM, Nras/CM or Nras/CM secondary leukemic mice, showing progenitor cell marker c-Kit (x-axis) and lineage markers (y-axis); (C) quantification of FACS analysis in Figure II.7B and similar FACS analysis of peripheral blood (PB, left), bone marrow (BM, center) and spleen (SP, right) cells from wild type control (WT), moribund CM, Nras/CM, and Nras/CM secondary leukemic mice (**, P<0.01).

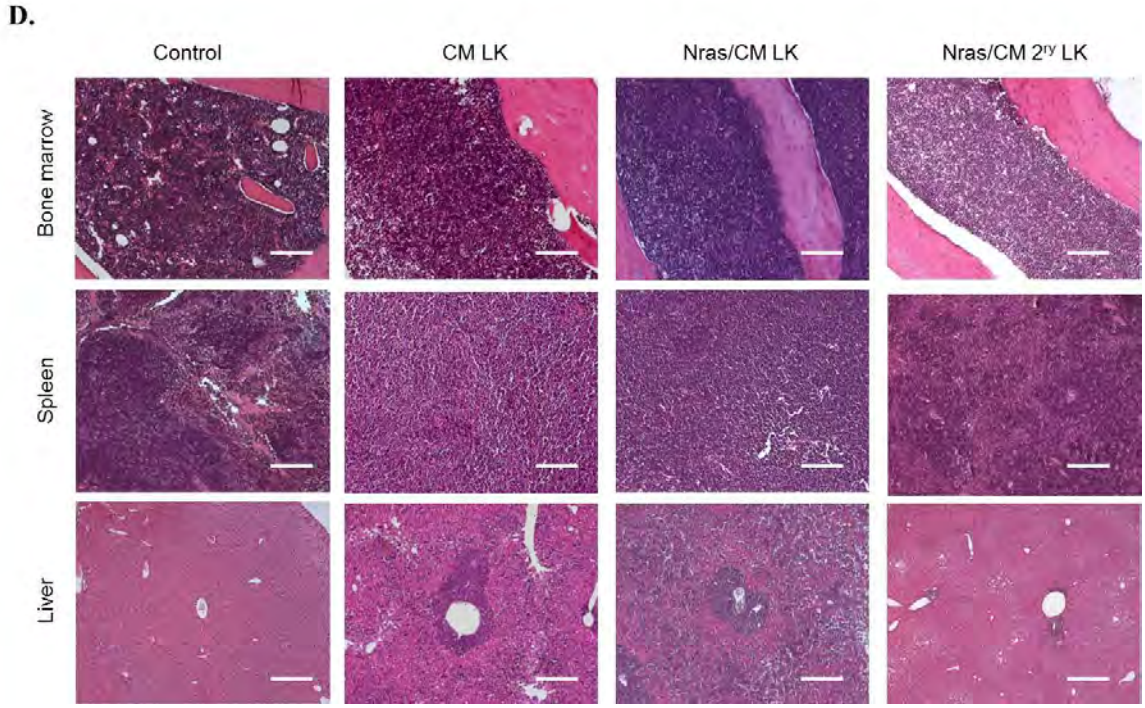

Figure II.7 (Cont.) Characterization of leukemia in mice expressing Cbfβ-SMMHC and N-Ras^{G12D}. (D) representative H&E histology sections of bone marrow, spleen and liver from wild type control, and moribund CM, Nras/CM mice, and Nras/CM secondary leukemic mice (LK, leukemia). Scale bar in bone marrow and spleen: 50μm; in liver: 100μm.

Juvenile myelomonocytic leukemia can present homozygous mutant *NRAS* expression by uniparental disomy (UPD) (Dunbar et al., 2008). The phenomenon was replicated in the N-Ras^{G12D} chronic myelomonocytic leukemia (CMML) and AML mouse models (Wang et al., 2010a; Xu et al., 2013). Genomic PCR analysis of colony forming units (CFUs) from 3 independent Nras/CM leukemia samples confirmed the presence the mutant *Nras*^{G12D} and wild type alleles as well as *Cbfb*^{MYH11} alleles in all cases (*Nras*$^{+/G12D}$/*Cbfb*$^{+/MYH11}$ genotype; **Figure II.8**), suggesting that UPD is not a frequent event in our AML model. In addition, we sequenced 9 CM leukemic samples and we did not find any spontaneous mutations in codon 12, 13 or 61 of *Nras* or *Kras* transcripts (**Figure II.9** and **Figure A.5** in Appendix A). The dilution experiment showed a sensitivity of detecting as low as 11% to 20% *Nras* heterozygous mutations by this sequencing method (**Figure II.9**).

Together, these data suggest that allelic *Nras*^{G12D} expression synergizes with Cbfβ-SMMHC to induce myeloid leukemia in mice.

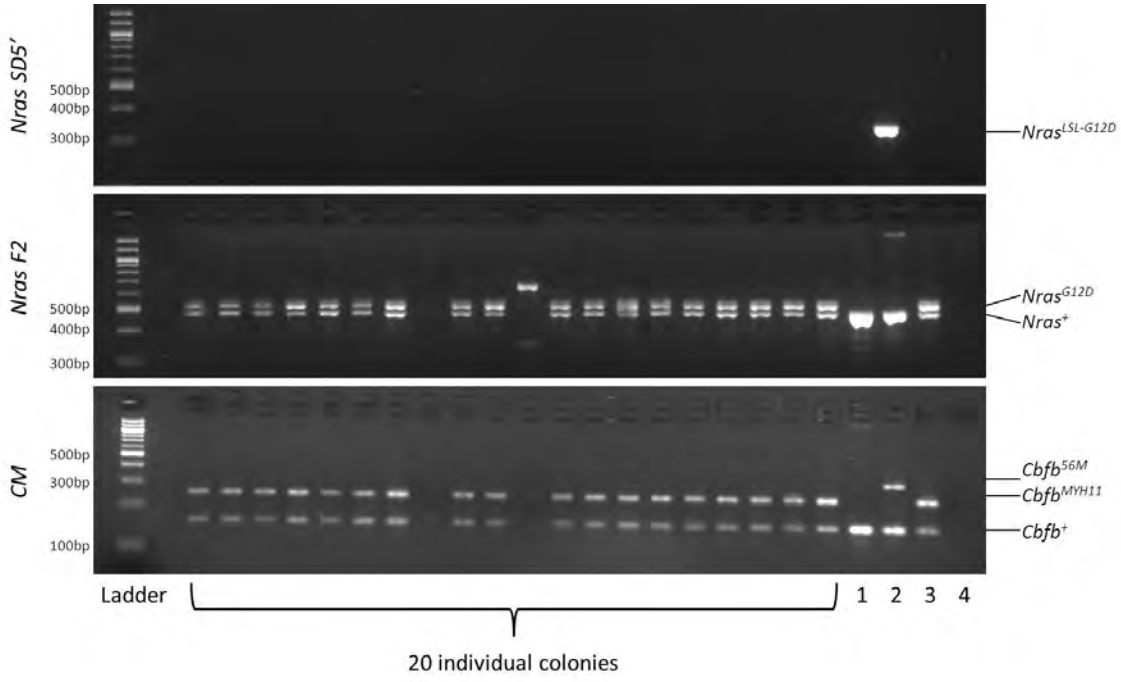

Figure II.8 Colony PCR confirms presence of both *Nras*$^{+}$ and *Nras*^{G12D} alleles. Individual colonies formed from three Nras/CM leukemic samples were picked. Genomic DNA were extracted and amplified for specific alleles. Result from a representative sample is shown. Lanes 1-4 are PCR products from control genomic DNA samples: Lane 1, *Mx1-Cre*; Lane 2, *Nras*$^{+/LSL\text{-}G12D}$*/Cbfb*$^{+/56M}$; Lane 3, bulk Nras/CM Leukemic cells; Lane 4, water. All of colonies present *Nras*$^{+/G12D}$*/Cbfb*$^{+/MYH11}$ genotype except for two. Those two colonies do not give conclusive PCR products probably due to limited cell number in the colonies.

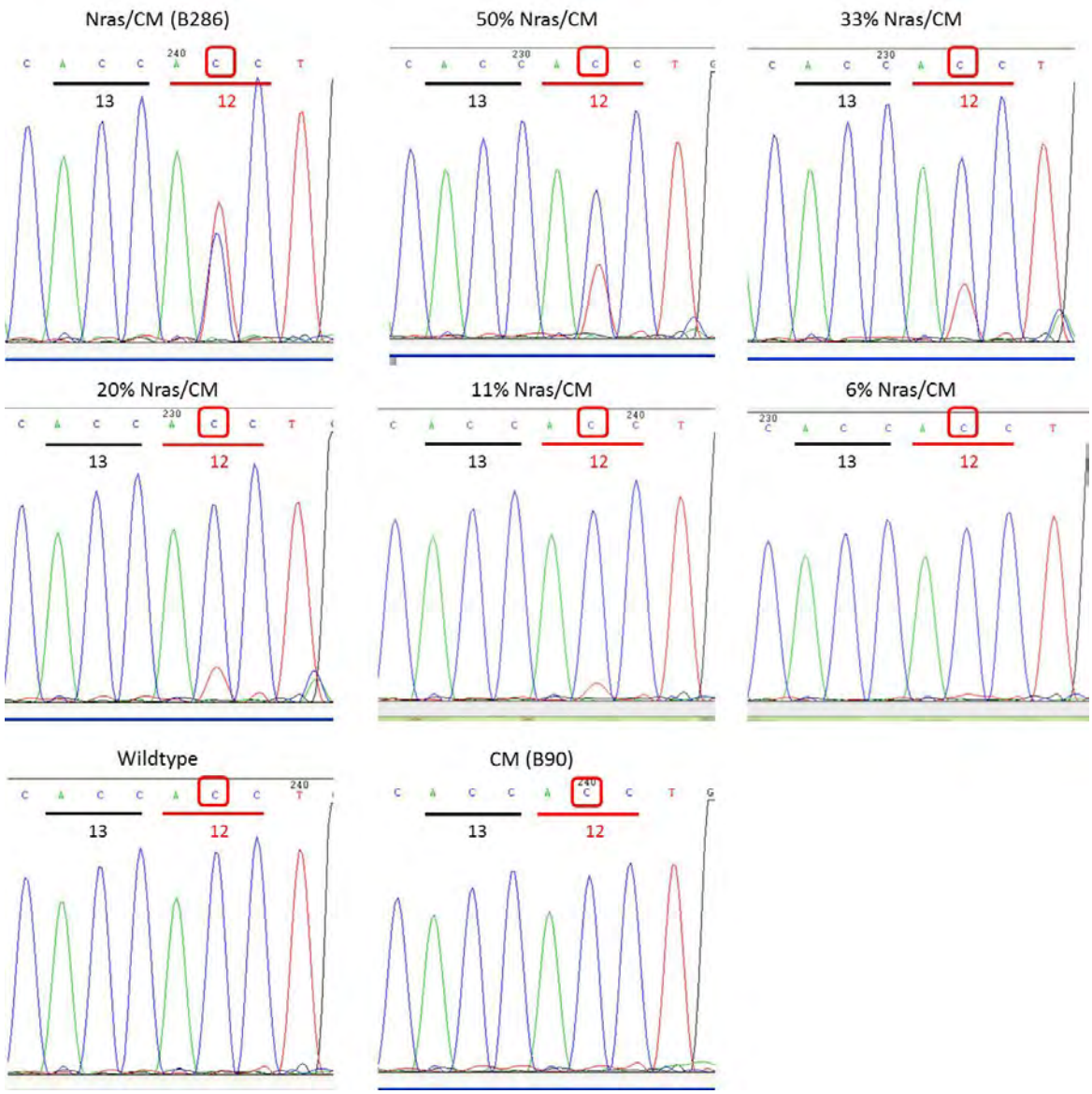

Figure II.9 No mutations in codon 12, 13, 61 of *Nras* or *Kras* genes were identified in CM leukemic cells. *Nras* and *Kras* cDNA of 9 CM leukemic cells were sequenced for mutations in codon 12, 13 and 61. *Nras* cDNA from Nras/CM leukemic cells (clone B286) serves as a positive control for $Nras^{G12D}$ mutation sequencing with half C (blue) and half T (red) in the ACC codon while *Nras* cDNA from wildtype lineage negative bone marrow cells serves as a negative control. Serial dilution was performed by diluting *Nras* cDNA from Nras/CM leukemic cells with *Nras* cDNA from wildtype lineage negative bone marrow cells (1:1, 1:2, 1:4, 1:8 and 1:16 ratio corresponding to 50%, 33%, 20% 11% and 6% of Nras/CM cDNA). Representative sequencing results from *Nras* codon 12 and 13 of CM leukemic cells (clone B90) are also shown; the complete results of CM leukemic cell sequencing are shown in Figure A.5 in Appendix A.

4. N-Ras^{G12D} and Cbfβ-SMMHC drive cell autonomous AML in mice

We inquired whether expression of N-Ras^{G12D} and Cbfβ-SMMHC is sufficient to induce leukemia using a donor-cell leukemia assay (**Figure II.10A**). Briefly, 5x10^5 untreated CD45.2$^+$ bone marrow cells from CM, Nras or Nras/CM mice were transplanted with 2 x10^5 CD45.1$^+$ wild type bone marrow cells into sub-lethal irradiated CD45.1$^+$ recipient mice. The engraftment efficiency of CD45.2$^+$ donor cells was evaluated two weeks after transplantation by flow cytometry analysis of peripheral blood cells [donor (CD45.2) cell range: 42-60%, 17-37%, and 38-54% for Nras, CM and Nras/CM respectively]. Cre-mediated activation of oncoprotein expression was induced by poly(I:C) treatment, and the leukemia latency was monitored thereafter. Under this experimental design, Nras/CM recipient mice developed leukemia with a median latency of 28.15 weeks and 75% penetrance (**Figure II.10B**, dotted line; **Table II.1**). Conversely, Nras and CM recipient mice remained healthy for 37 weeks (experimental endpoint; **Figure II.10B**, dashed and solid lines). To test whether the incomplete penetrance in the Nras/CM group was due to the limited number of bone marrow cells transplanted, we increased the number of donor cells to 2x10^6 and observed a median latency of 24.72 weeks and full penetrance of disease in Nras/CM group (**Figure II.10C**, dotted line). In this context, 40% and 50% disease penetrance was observed in Nras and CM groups 47 weeks after Cre activation, respectively (**Figure II.10C**, dashed and solid lines). The Nras/CM group presented accelerated leukemia development compared to both Nras and CM groups, with P value as 0.0031 and 0.0015, respectively (**Figure II.10C**).

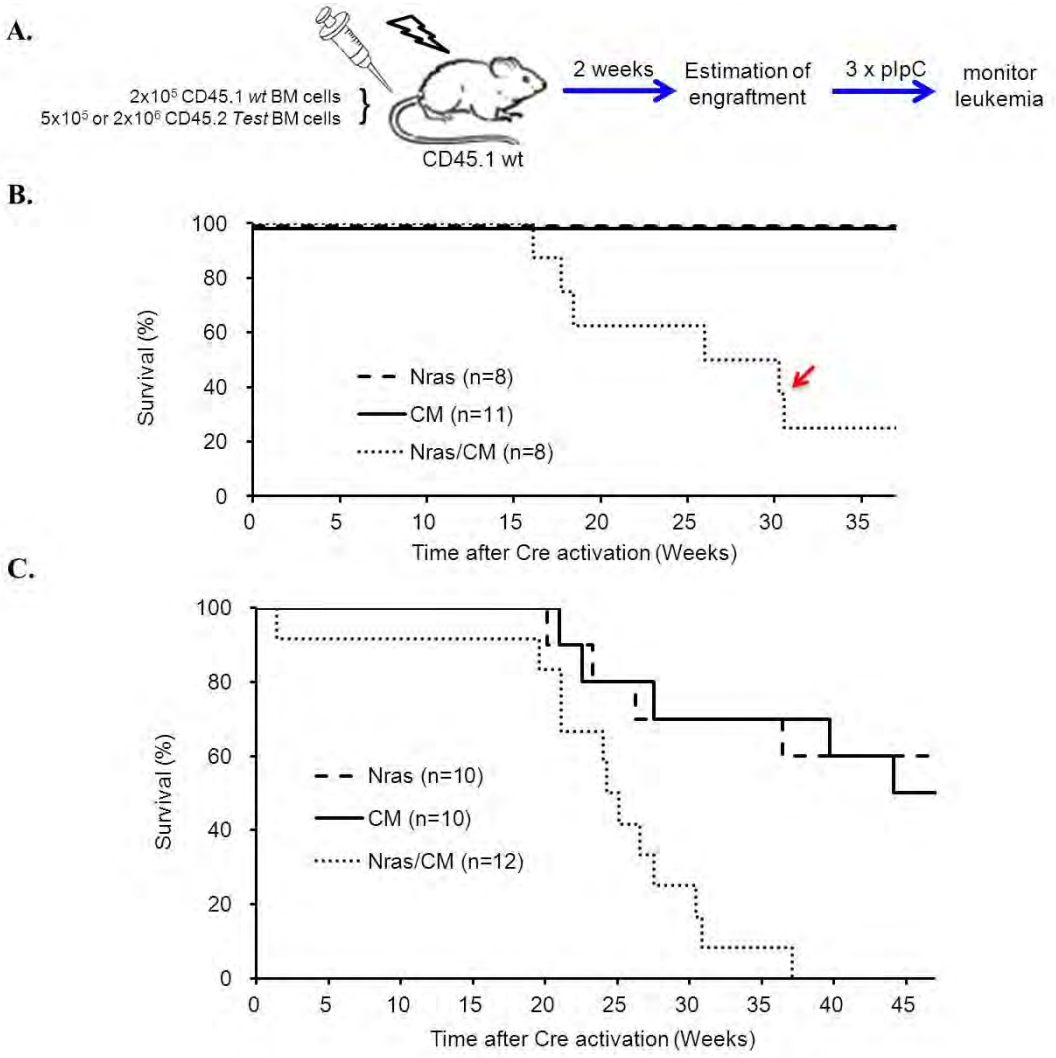

Figure II.10 N-Ras^{G12D} and Cbfβ-SMMHC drive cell autonomous AML in mice. (A) experimental design of leukemia analysis in mice transplanted with CD45.2^{+} test [*Nras*$^{+/LSL\text{-}G12D}$*/Mx1Cre (Nras),* *Cbfb*$^{+/56M}$*/Mx1Cre* (CM), or *Nras*$^{+/LSL\text{-}G12D}$*/Cbfb*$^{+/56M}$*/Mx1Cre* (Nras/CM)] bone marrow cells. The lightning bolt stands for sub-lethal irradiation; (B) Kaplan-Meier survival curves of recipient mice transplanted with 5x10^{5} Nras (dash), CM (solid) or Nras/CM (dotted) bone marrow cells and Cre-activated after 2 weeks. Red arrow depicts the disease onset of a mouse with mix leukemic and myelo-proliferative cells; (C) Kaplan-Meier survival curves of recipient mice transplanted with 2x10^{6} Nras (dash), CM (solid) or Nras/CM (dotted) bone marrow cells and Cre-activated after 2 weeks; P value (Nras vs Nras/CM) = 0.0031, P value (CM vs Nras/CM) = 0.0015. Note: Cells from the peripheral blood of mice that appeared health in the group transplanted with either 2x10^{6} Nras or CM bone marrow cells still showed presence of mutated alleles during the thesis preparation (data not shown). These mice are monitored for the development of disease beyond 47 weeks as shown in the thesis.

Immunophenotypic analysis of the peripheral blood cells from mice transplanted with $5x10^5$ Nras/CM bone marrow cells revealed a myeloid leukemia phenotype similar to that described for the Nras/CM mice (5/6 mice), including increased number of leukocytes with immature morphology (**Figure II.11A** and **Table II.1**), and increased number of Lin^-kit^+ cells (as illustrated in **Figure II.11B**, and quantified in **Figure II.11C**). These mice showed splenomegaly (**Table II.1**) and infiltration of leukemic cells in bone marrow, spleen and liver (**Figure II.11C-D**). One mouse (**Figure II.10B**, red arrow) presented a mixed AML and myelo-proliferative phenotype, with intermediate levels of Lin^-kit^+ cells and high levels of $Gr1^+Mac1^+$ cells in peripheral blood, bone marrow and spleen (**Figure II.11A** right, **Figure II.11B** bottom row and **Figure II.11C** open-square).

Together, these studies show that N-Ras^{G12D} accelerates Cbfβ-SMMHC mediated leukemia in the presence of competitor wild type bone marrow cells.

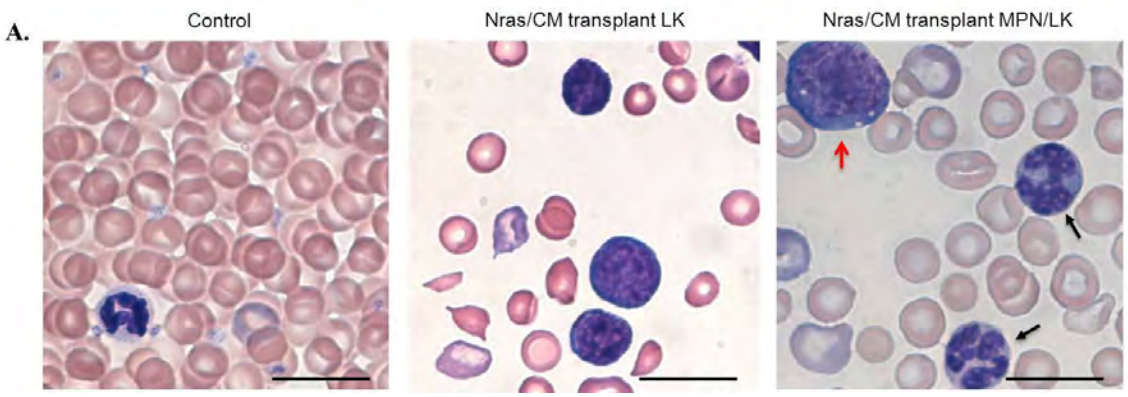

Figure II.11 Characterization of donor-cell leukemia expressing Cbfβ-SMMHC and N-Ras^{G12D}. (A) representative Wright Giemsa staining of peripheral blood smears from control (left) and moribund Nras/CM recipient mice with leukemia (LK, center) or with mixed myelo-proliferative and leukemia (MPN/LK, right); leukemic cells (red open arrow) and granulocytes (black arrow) are shown. Scale bars, 5μm.

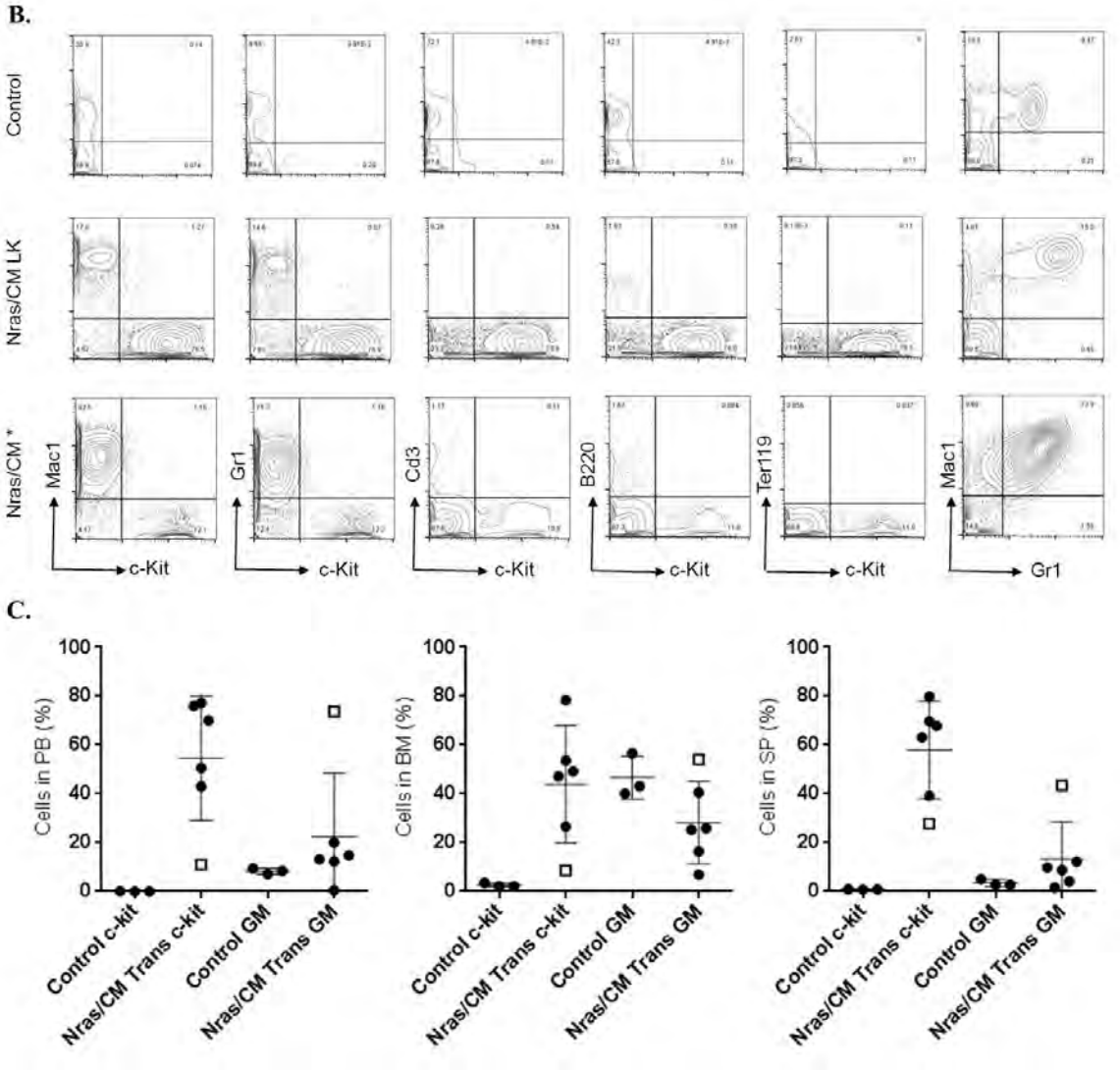

Figure II.11 (Cont.) Characterization of donor-cell leukemia expressing Cbfβ-SMMHC and N-Ras^{G12D}. (B) FACS analysis of peripheral blood cells from control (top), representative leukemic moribund Nras/CM recipient mice (5/6) showing progenitor cell marker c-kit (x-axis) and lineage markers (y-axis), and the Nras/CM* (bottom) mouse with a mix of leukemia and myelo-proliferative phenotypes; (C) quantification of percentage of wild type (Control) and Nras/CM leukemic cells (Nras/CM Trans) in peripheral blood (PB), bone marrow (BM) and spleen (SP) cells. Open-square represents the mouse that developed mixed AML and myelo-proliferative phenotypes.

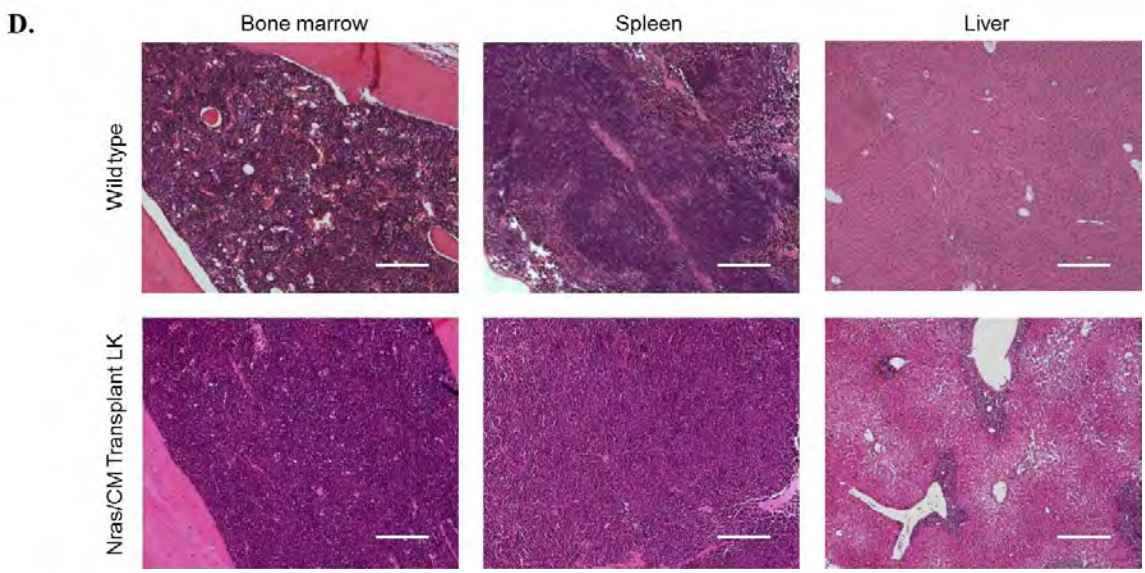

Figure II.11 (Cont.) Characterization of donor-cell leukemia expressing Cbfβ-SMMHC and N-Ras^{G12D}. (D) representative H&E histology sections of bone marrow, spleen and liver from control and moribund Nras/CM recipient mice (Nras/CM transplantation LK). Scale bars of bone marrow and spleen sections: 50μm; for liver sections: 100μm.

5. Endogenous N-ras^{G12D} and Cbfβ-SMMHC co-regulate Bim protein levels

Our results showed a reduction in apoptosis on the pre-leukemic progenitor cells expressing N-Ras^{G12D} and Cbfβ-SMMHC (**Figure II.4**). To understand the mechanism underlying this survival advantage, we tested the expression of apoptosis-associated proteins from the Bcl-2 family, including Bim, Bcl2, Bcl-xl and Mcl-1, in pre-leukemic Lin$^-$ bone marrow cells (**Figure II.12A-B**). The expression levels of Bcl2, Bcl-xL, and Mcl-1 were inconsistent in Nras/CM pre-leukemic cells, and similar in leukemic cells (**Figure II.12B** and **F**). However, Bim-EL protein levels were reduced 50% in Nras and CM and 80% in Nras/CM Lin$^-$ pre-leukemic cells when compared to controls (**Figure II.12A**). Interestingly, levels of Bim transcript, *Bcl2l11*, were reduced 30% by the Cbfβ-SMMHC and 50% by both oncoproteins in pre-leukemic Lin- bone marrow cells, but were unchanged in Lin- cells expressing N-Ras^{G12D} (**Figure II.12C**). Similarly, Nras/CM leukemic cells showed lower apoptosis and reduced Bim-EL levels when compared to CM leukemic cells (**Figure II.12D-E**).

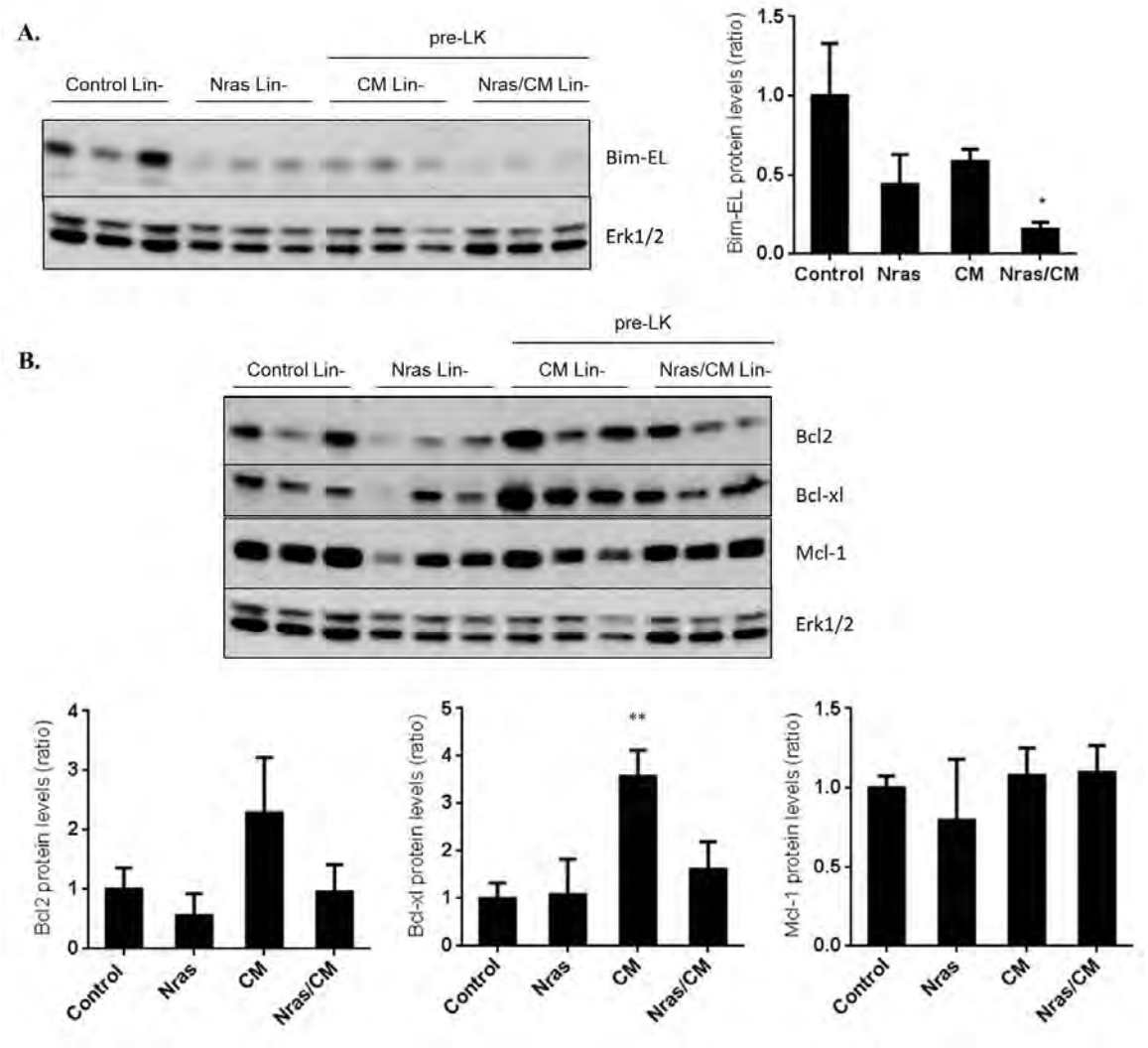

Figure II.12 N-Ras^{G12D} and Cbfβ-SMMHC reduce Bim-EL levels in pre-leukemic and leukemic cells. (A) immunoblot analysis of Bim-EL expression in Lin$^-$ bone marrow cells from Control and Nras, and pre-leukemic cells from CM and Nras/CM mice (three independent samples per group); quantification relative to total Erk level (right panel), *, $P<0.05$. (B) immunoblot analysis of Bcl2, Bcl-xl, and Mcl-1 expression in Lin$^-$ progenitor cells from control, Nras, CM, and Nras/CM mice (top, three independent samples per group); quantification relative to total Erk level (bottom), **, $P<0.01$.

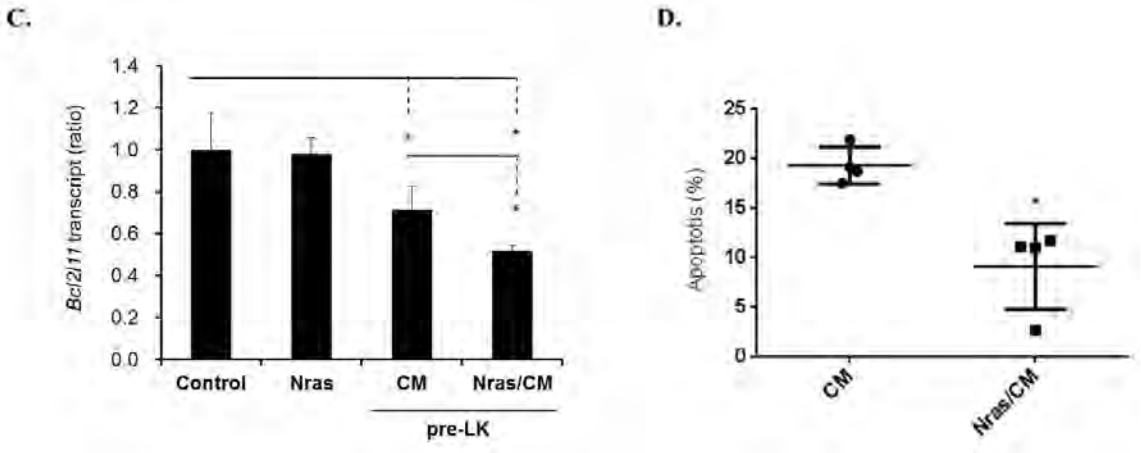

Figure II.12 (Cont.) N-Ras^{G12D} and CBFβ-SMMHC reduce Bim-EL levels in pre-leukemic and leukemic cells. (C) *Bcl2l11* transcript levels in pre-leukemic (Lin$^-$) bone marrow cells from Control, Nras, CM, and Nras/CM mice, relative to *Actb* by qRT-PCR (n=4, *, P<0.05). (D) apoptosis analysis (Annexin V$^+$/7-AAD$^-$) of c-Kit$^+$ gated CM or Nras/CM leukemic cells (n=4, *, P<0.05).

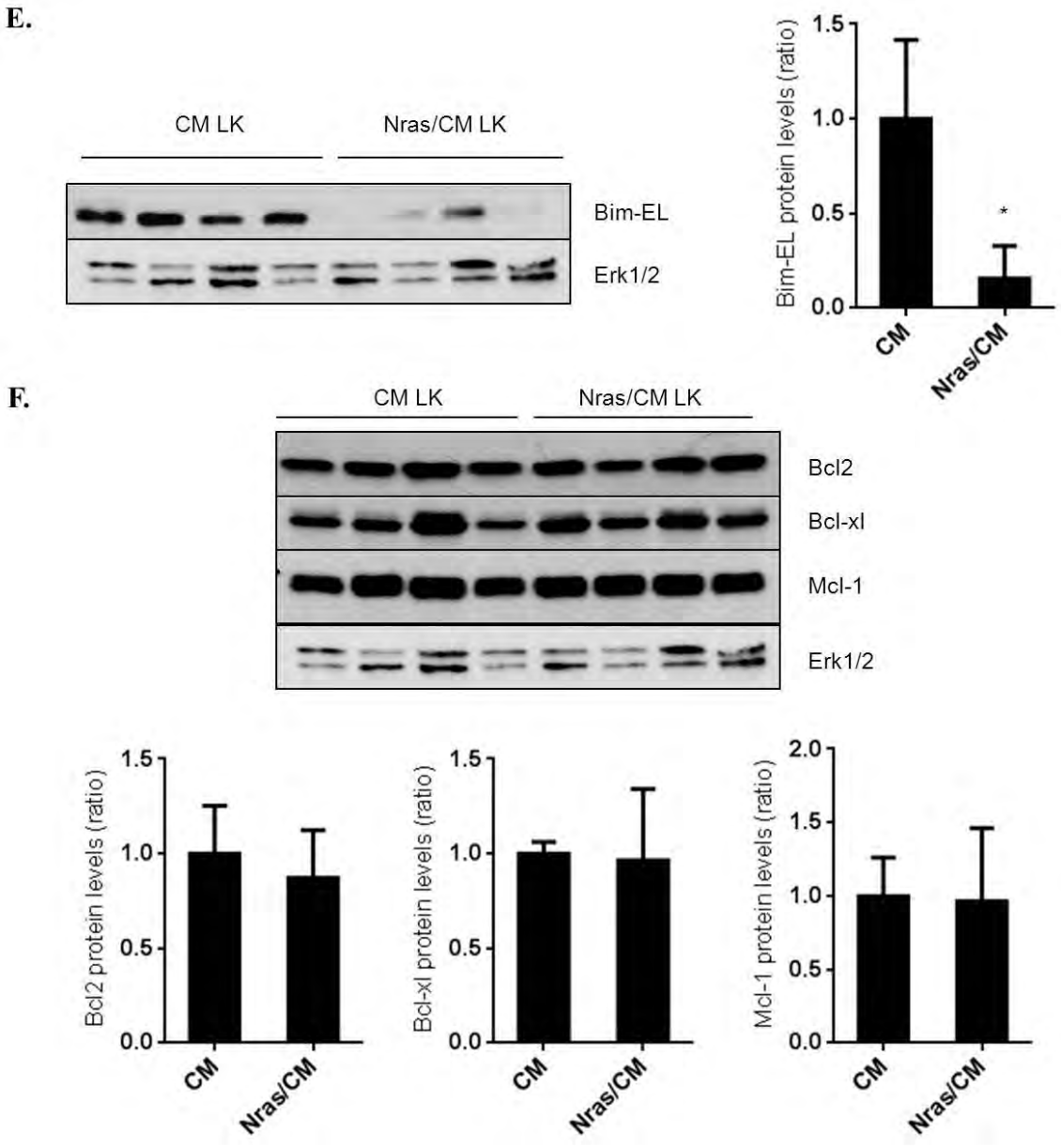

Figure II.12 (Cont.) N-Ras^{G12D} and CBFβ-SMMHC reduce Bim-EL levels in pre-leukemic and leukemic cells. (E) immunoblot analysis of Bim-EL expression in CM or Nras/CM leukemic cells used in (D), and quantification relative to total Erk levels (right panel), *, $P<0.05$. (F) immunoblot analysis of Bcl2, Bcl-xl, and Mcl-1 expression in CM or Nras/CM leukemic cells used in (D; top); quantification of protein levels normalized to Erk levels (bottom).

6. N-Ras^{G12D} induces survival of Nras/CM leukemic cells through activation of MEK/ERK

Previous studies have shown that Erk1/2 can induce proteasomal degradation of the Bim-EL isoform (Luciano et al., 2003). Since N-Ras^{G12D} reduced Bim-EL protein but not transcript levels, we investigated if N-Ras^{G12D} regulates Bim-EL levels post-translationally in Nras/CM leukemic cells via the PI3K or MEK/ERK pathways. Biochemical analysis of Erk, Akt and Stat5 phosphorylation revealed that CM leukemic cells had a heterogeneous activation of these proteins. The first two clones showed phosphorylation of all three proteins; the third clone showed a strong phosphorylation of Stat5 but not the other two; the fourth clone show only phosphorylation of Erk. Nras/CM leukemic cells showed a consistent activation of ERK and low activation of AKT but not STAT5 phosphorylation (**Figure II.13A**). Five-hour treatment of Nras/CM leukemic cells from 3 mice with the MEK inhibitor PD325901, a potent inhibitor of myelo-proliferative neoplasm in *Kras^{G12D}* and *Nf1$^{-/-}$* mice (Chang et al., 2013; Lauchle et al., 2009; Lyubynska et al., 2011), increased *Bcl2l11* transcript levels in a dose dependent manner, and increased Bim-EL protein levels (**Figures II.13B-C**). However, treatment with the PI3K inhibitor GDC-0980 (Wallin et al., 2011) did not significantly affect Bim transcript or protein levels. In addition, apoptosis of Nras/CM leukemic cells was significantly increased by treatment with the MEK inhibitor (paired t-test $P<0.05$), but not consistently increased by treatment with the PI3K inhibitor at 24 hours (**Figure II.13D**).

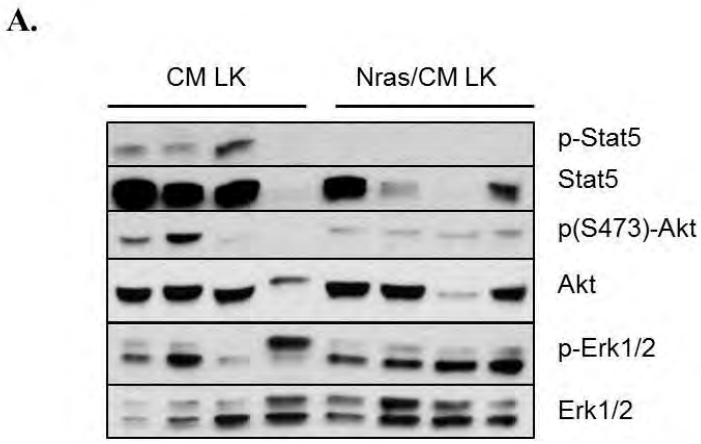

Figure II.13 Inhibition of MEK/ERK pathway increases Bim-EL levels and induces apoptosis. (A) immunoblot analysis of Stat5, Akt and Erk phosphorylation in four CM and Nras/CM leukemic samples.

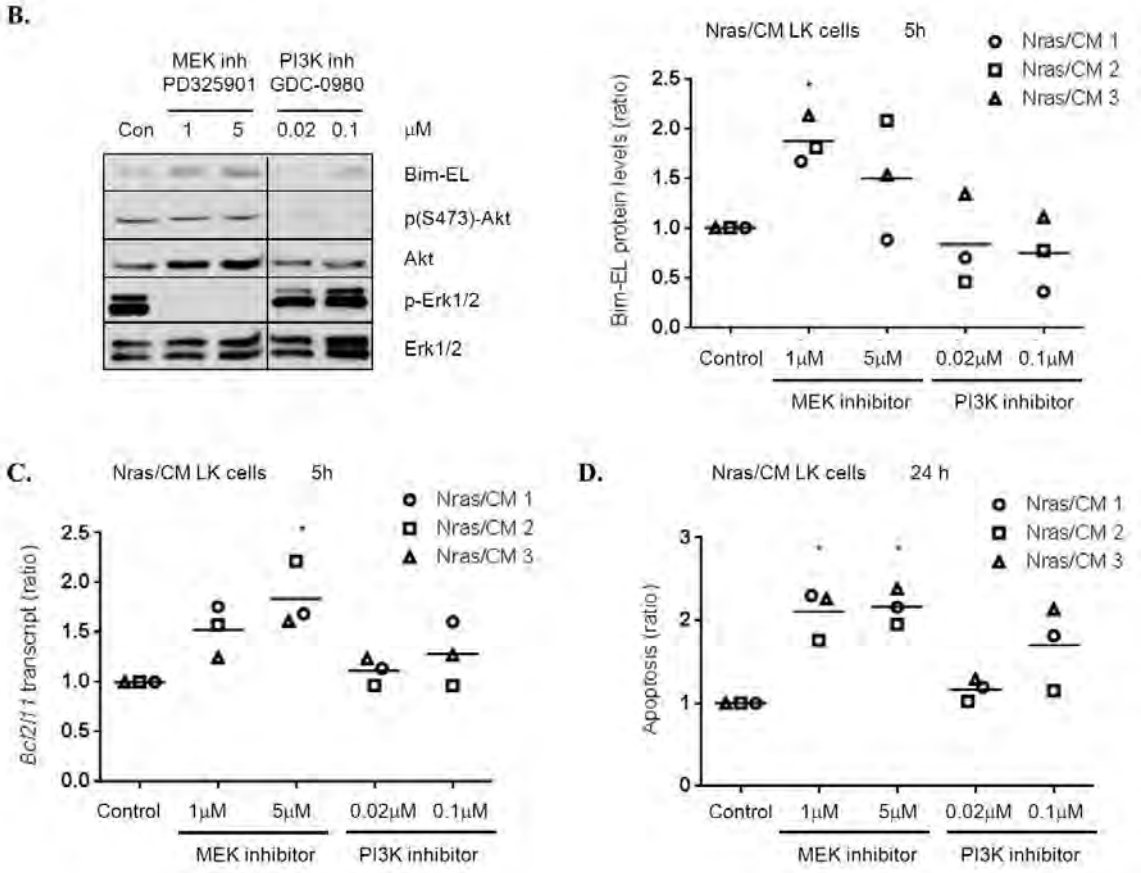

Figure II.13 (Cont.) Inhibition of MEK/ERK pathway increases Bim-EL levels and induces apoptosis. (B) immunoblot analysis of Bim-EL, phospho-Akt, Akt, phospho-Erk1/2 and Erk1/2 levels in Nras/CM leukemic cells treated for 5 hours with DMSO (Con.), MEK inhibitor PD325901 or PI3K inhibitor GDC-0980 (left); quantification of Bim-EL protein levels normalized to Erk1/2 is shown for 3 independent Nras/CM clones (right). A vertical line has been inserted to indicate a repositioned gel lane. (C) *Bcl2l11* transcript levels after the same 5 hour treatment were analyzed by qRT-PCR and values normalized to *Actb;* this experiment was performed in 3 independent Nras/CM clones, each by triplicates (average value of each clone relative to control was shown in the figure). (D) quantification of apoptosis (Annexin V^+/7AAD$^-$) levels in c-Kit^+ gated Nras/CM leukemic cells treated for 24 hours; this experiment was performed in 3 independent Nras/CM clones, each by triplicates (average value of each clone relative to control was shown in the figure). *, paired t-test $P<0.05$.

We inquired whether inhibition of MEK/ERK or PI3K is important for leukemic cell growth. Treatment of Nras/CM leukemic cells with 0.032μM PD325901 reduced significantly their viability when compared to CM leukemic and wild type Lin- bone marrow cells (35.4±3.7%, 74.9±11.5% and 75.1±6.9% viability, respectively; **Figure II.14A**). The dose response treatment of Nras/CM leukemic cells with GDC-980 was similar to that of CM leukemic and wild type Lin- bone marrow cells (**Figure II.14B**).

The sensitivity of pre-leukemic progenitors to MEK/ERK and PI3K inhibitors was tested in vitro using Lin- bone marrow cells from Nras/CM mice 20 days after Cre-activation. Five-hour treatment with MEK inhibitor increased 50% Bim-EL protein and 30% *Bcl2l11* transcript levels, and increased apoptosis 30% at 24 hours (**Figure II.15**). Similar treatment with the PI3K inhibitor significantly increased *Bcl2l11* transcript levels to 3 fold but the protein levels were not increased accordingly, and the apoptosis was increased 30% (**Figure II.15**).

Taken together, these results show that the viability of Nras/CM leukemic cells depend on MEK/ERK signaling and not on PI3K signaling in vitro, and correlate with the expression of the apoptotic protein Bim-EL.

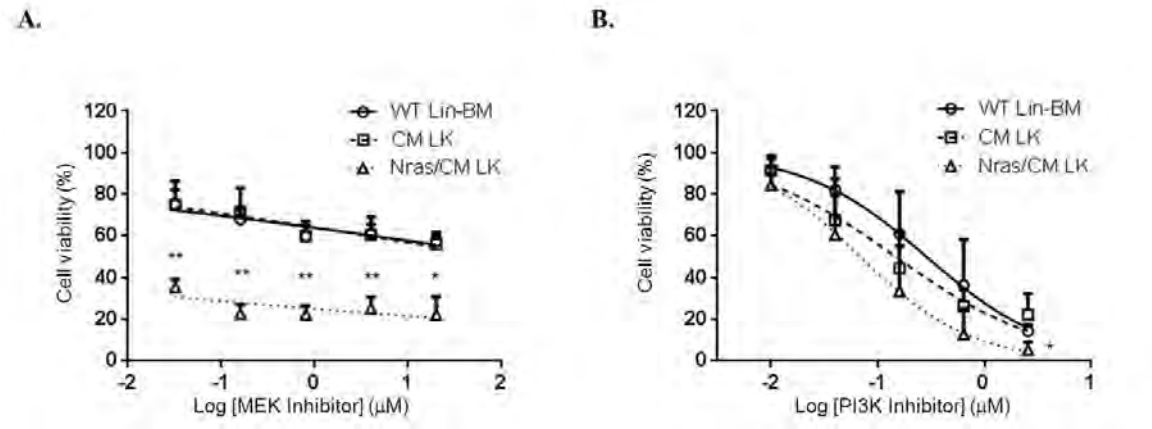

Figure II.14 Inhibition of MEK/ERK pathway specifically reduced cell viability of Nras/CM leukemic cells. Dose response curve of Lin$^-$ wild type bone marrow (WT Lin-BM, open circle), CM leukemic cells (CM LK, open square), or Nras/CM leukemic cells (Nras/CM LK, open triangle) to (A) MEK inhibitor (PD325901), or (B) PI3K inhibitor (GDC-0980), for 48 hours; cell viability were measured by CellTiter-Glo assays. Nonlinear regression curve fit was generated by inhibitor (log) vs. normalized response-variable slope analysis; the mean of 3 independent clones from each group is shown. *, $P<0.05$; **, $P<0.01$.

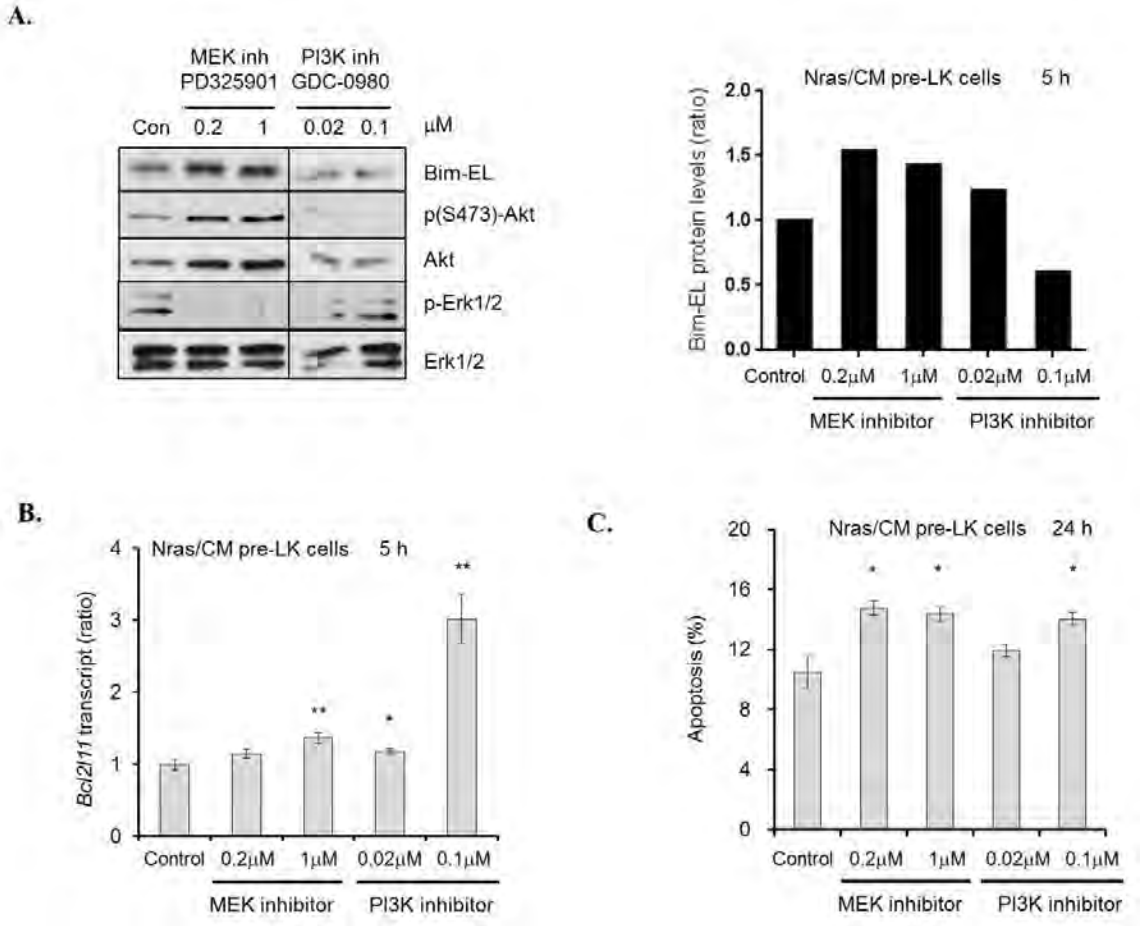

Figure II.15 Response of Nras/CM pre-leukemic cells to MEK/ERK or PI3K inhibitors. (A) immunoblot analysis of Bim-EL, phospho-Akt, Akt, phospho-Erk1/2 and Erk1/2 levels (left), and quantification of relative Bim-EL protein levels normalized to Erk1/2 from pre-leukemic Nras/CM samples treated with MEK inhibitor PD325901 and PI3K inhibitor GDC-0980 for 5 hours (right). A vertical line has been inserted to indicate a repositioned gel lane. (B) *Bcl2l11* transcript levels after 5h treatment with MEK inhibitor PD325901 and PI3K inhibitor GDC-0980, by qRT-PCR; values were normalized to *Actb* in the same treatment and are expressed relative to untreated control; assays were performed by triplicate of the pooled sample. (C) apoptosis analysis (Annexin V^+/7AAD$^-$) in c-Kit^+ gated Nras/CM Lin^- bone marrow cells treated for 24 hours; assays were performed by triplicate of the pooled sample. *, $P<0.05$; **, $P<0.01$.

7. Bim knock down reduces apoptosis of CM bone marrow progenitor cells

To investigate if Bim is an important mediator of survival downstream of N-Ras^{G12D} in Cbfβ-SMMHC-associated leukemia, we tested apoptosis in pre-leukemic bone marrow cells expressing Cbfβ-SMMHC and reduced Bim levels (**Figure II.16A**). The Bim shRNA vectors reduced 70% of Bim transcript and protein levels in NIH3T3 cells (**Figure II.16B-C**), and reduced 60% of transcript levels in sorted GFP^{+}, 7-AAD^{-} CM bone marrow cells (protein levels from cells infected with Bim shRNA was below detection threshold, **Figure II.16B** and **D**). Accordingly, CM bone marrow pre-leukemic cells transduced with Bim shRNA showed 45% reduction in apoptosis when compared to cells transduced with Scrambled shRNA (**Figure II.16E**). These data suggest that Bim is an important mediator of apoptosis in pre-leukemic cells expressing Cbfβ-SMMHC.

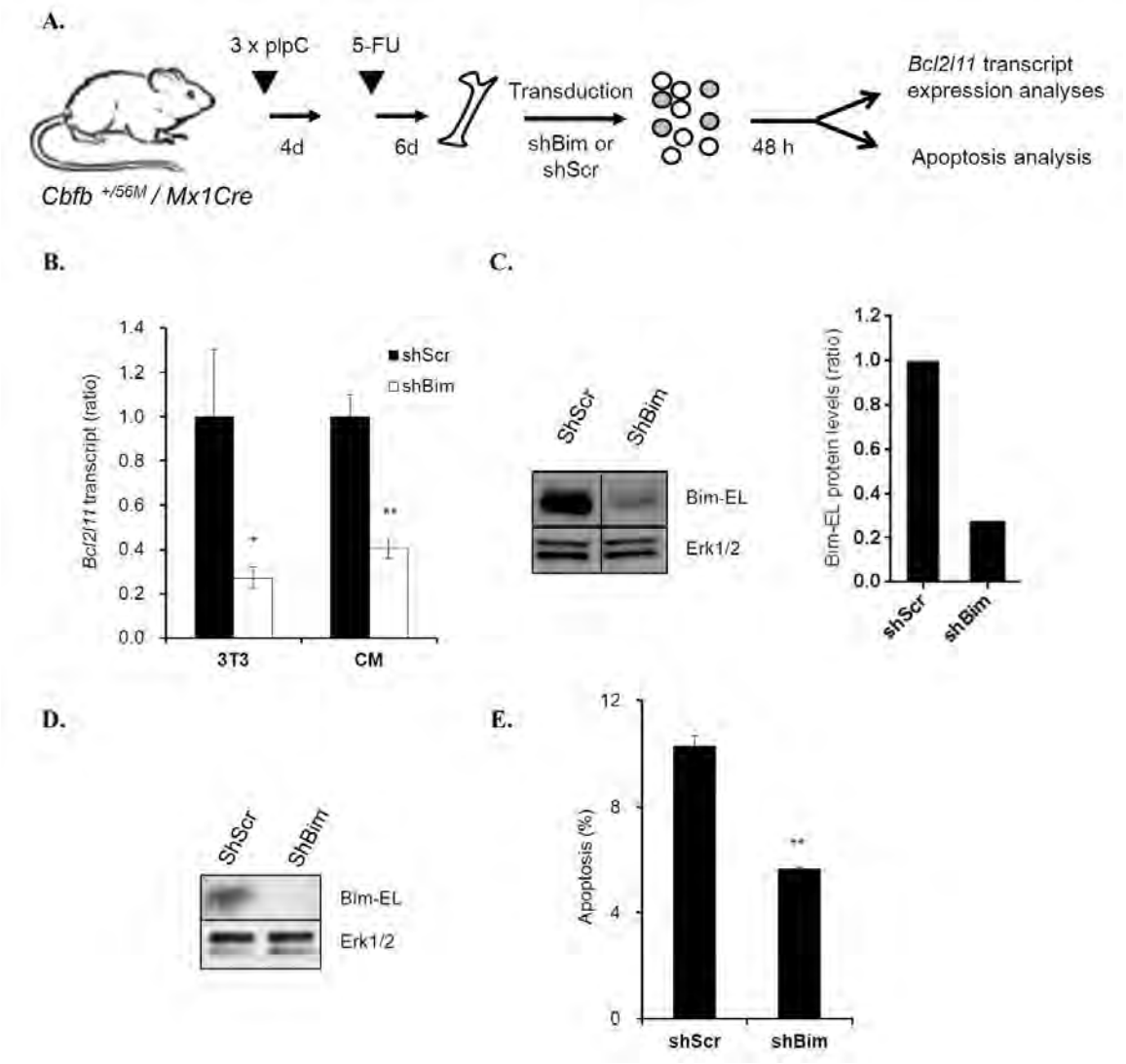

Figure II.16 Reduction of Bim-EL levels inhibits apoptosis in CM pre-leukemic cells. (A) Experimental design of Bim knock-down strategy. (B) relative *Bcl2l11* transcript levels (qRT-PCR) in puromycin-selected NIH-3T3 (3T3) or sorted $GFP^{+}7\text{-}AAD^{-}$ CM pre-leukemic cells infected with either Scrambled shRNA (shScr) or Bim shRNA (shBim); *, $P<0.05$; **, $P<0.01$. (C) immunoblot analysis of Bim-EL levels in puromycin selected NIH-3T3 cells (left panel); quantification of Bim-EL protein levels normalized to Erk1/2 (right panel). A vertical line has been inserted to indicate a repositioned gel lane. (D) immunoblot analysis of Bim-EL levels in sorted $GFP^{+}7\text{-}AAD^{-}$ CM pre-leukemic cells. (E) quantification of apoptosis (Annexin $V^{+}/7\text{-}AAD^{-}$) levels in c-Kit^{+} GFP^{+} CM pre-leukemic cells infected with either Scrambled (shScr) or Bim (shBim) shRNA; **, $P<0.01$.

8. Heterozygous knock-out of *Bcl2l11* does not cooperate with Cbfβ-SMMHC in leukemogenesis

To test whether reduced Bim levels could serve as a cooperating event in Cbfβ-SMMHC mediated leukemogenesis, we monitored leukemia development in *Bim*$^{+/-}$*/Mx1-Cre* (Bim-h) and *Bim*$^{+/-}$*/Cbfb*$^{+/56M}$*/Mx1-Cre* (Bim-h/CM) mice after Cre activation and compared to leukemia development in *Cbfb*$^{+/56M}$*/Mx1-Cre* (CM) and *Nras*$^{+/LSL\text{-}G12D}$*/Cbfb*$^{+/56M}$*/Mx1-Cre* (Nras/CM) mice which we have shown in **Figure II.6A**. Bim-h mice did not develop leukemia up to 35 weeks (experimental endpoint, **Figure II.17**, orange dashed line). Bim-h/CM mice developed leukemia with a medium latency of 28.14 weeks and 71% penetrance (**Figure II.17**, orange solid line) which was not significantly different from CM mice (median latency of 21.43 weeks and 89% penetrance, **Figure II.17**, black solid line). These results suggest that inactivation of one allele of *Bcl2l11* gene does not accelerate Cbfβ-SMMHC mediated leukemogenesis.

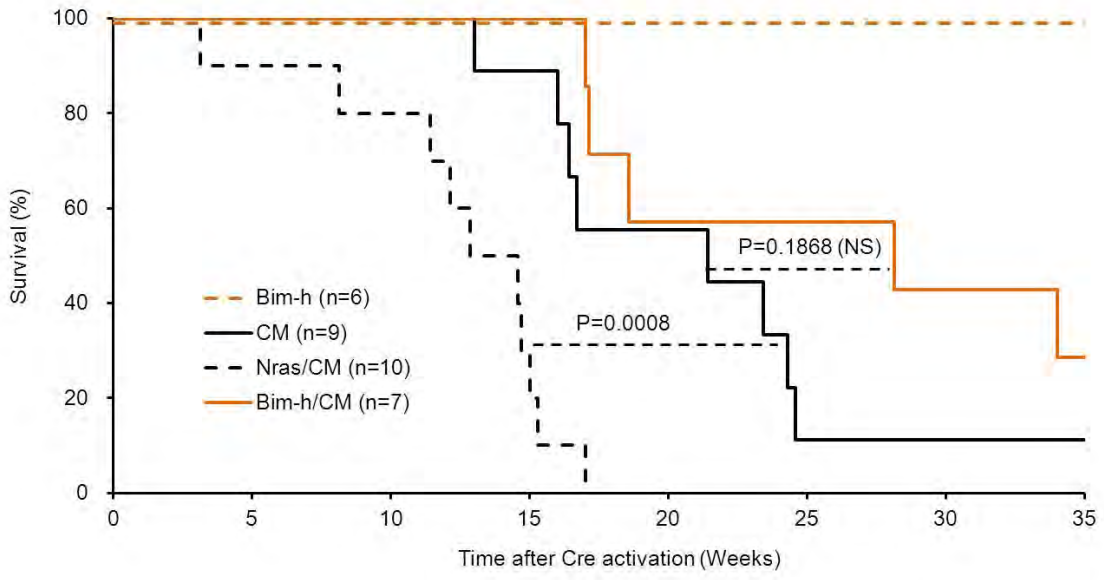

Figure II.17 Loss of one allele of *Bcl2l11* gene does not accelerate Cbfβ-SMMHC mediated leukemogenesis. Kaplan-Meier curves of leukemia-free survival in *Bim*$^{+/-}$*/Mx1-Cre* (Bim-h, orange dashed), *Cbfb*$^{+/56M}$*/Mx1-Cre* (CM, black solid), *Nras*$^{+/LSL\text{-}G12D}$*/Cbfb*$^{+/56M}$*/Mx1-Cre* (Nras/CM, black dashed) and *Bim*$^{+/-}$*/Cbfb*$^{+/56M}$*/Mx1-Cre* (Bim-h/CM, orange solid) mice after Cre activation (time 0). NS, not significant.

II.E Discussion

Acute myeloid leukemia results from the stepwise accumulation of mutations that drive transformation of hematopoietic stem and progenitor cells. The pre-leukemic compartment is thought to include a group of clonal progenitor cells with common founding mutation(s) and a combination of different subsequent mutations that deregulate their proliferation, survival, and differentiation programs (Jan et al., 2012; Walter et al., 2012; Welch et al., 2012). Recent studies demonstrated that pre-leukemic progenitors found in human AML survive chemotherapy and are a reservoir for relapse AML (Corces-Zimmerman et al., 2014; Shlush et al., 2014). Therefore, the elucidation of the cumulative functional contribution of each mutation towards leukemia is essential for the understanding of disease progression and the design of effective therapies targeting driver mutations. The inv(16) chromosomal rearrangement is a founding mutation that creates a pre-leukemic myeloid population in the bone marrow (Kuo et al., 2006). However, inv(16) is necessary but not sufficient for leukemogenesis (Castilla et al., 1999; Castilla et al., 2004; Kuo et al., 2006). In this study, we show that *Nras*^{G12D}, a frequent mutation found in inv(16) AMLs, may regulate the survival of the pre-leukemic cells though Mek/Erk/Bim pathway, and synergizes with Cbfβ-SMMHC in leukemia development.

Recent studies have shown that *Nras*^{G12D} conditional knock-in mice develop an indolent myelo-proliferative phenotype, and eventually develop a diverse spectrum of hematologic cancers with long disease latency (Li et al., 2011). Similarly, mice transplanted with *Nras*^{G12D} bone marrow develop CMML-like phenotype only after a prolonged latency (Wang et al., 2010a). Here, we show that co-expression of N-Ras^{G12D}

and Cbfβ-SMMHC modified the cellular composition of the bone marrow significantly, with a reduction in its cellularity due to a block in myelo-lymphoid differentiation (lineage positive cells). However, the pre-leukemic early progenitors (ST-HSCs and MPPs) and myeloid progenitors (CMPs) were increased. These results showed that CBFβ-SMMHC is dominant over N-Ras^{G12D} because N-Ras^{G12D} was unable to overcome the differentiation block imposed by Cbfβ-SMMHC to induce myelo-proliferation, and are consistent with data demonstrating that *NRAS* mutations are acquired as a secondary event in human inv(16) AML (Paschka et al., 2013). The Nras/CM pre-leukemic progenitors remained in the bone marrow and transformed to leukemia with a latency reduced from 21.43 weeks, as seen in the CM group, to 13.72 weeks, indicating that allelic expression of N-Ras^{G12D} cooperated with Cbfβ-SMMHC in leukemia. In addition, the synergy was evident when expression of these oncoproteins was activated after engraftment into recipient mice, demonstrating that the synergy is cell autonomous.

We have found that N-Ras^{G12D} and Cbfβ-SMMHC contribute on the reduction of apoptosis in the pre-leukemic bone marrow progenitors. This survival signal may direct the expansion of pre-leukemic cells, and may participate in leukemia transformation. N-RAS^{G12D} was shown to provide survival advantage for human umbilical cord blood cells expressing AML1-ETO (Chou et al., 2011), suggesting that N-RAS^{G12D} –mediated pro-survival activity could be a general mechanism in leukemia. In addition, considering that Cbfβ-SMMHC has been shown to impair Runx1 function (Castilla et al., 1996) and that *Runx1*-loss reduced HSC apoptosis (Cai et al., 2011), it is possible that the pro-survival activity of Cbfβ-SMMHC could be mediated by Runx1 inhibition.

The analysis of protein levels of the BCL-2 family members revealed that Bim-EL protein levels correlated with apoptosis in pre-leukemic and leukemic cells. In pre-leukemic cells, N-Ras^{G12D} expression decreased Bim-EL protein levels but not transcript levels, indicating that N-Ras^{G12D} may affect Bim-EL levels by proteasome degradation though MEK/ERK pathway as previous reported (Hubner et al., 2008; Luciano et al., 2003). Interestingly, N-Ras^{G12D} synergized with Cbfβ-SMMHC to decrease Bim-EL protein and transcript levels, which correlated with the increase in Bim-EL protein and transcript levels by the MEK inhibitor. On the other hand, PI3K inhibitor increased Bim-EL transcript but not protein levels, probably because Mek/Erk pathway still actively decreased *Bcl2l11* transcripts and degraded Bim-EL protein. Consistent with these results, knock-down of Bim-EL levels decreased apoptosis in pre-leukemic progenitor cells expressing Cbfβ-SMMHC. The pharmacologic experiments also revealed that expression of Bim-EL was regulated by Mek/Erk pathway in the Nras/CM leukemic cells. These results suggest that Bim-EL may be a critical effector of apoptosis in the transformation of pre-leukemic progenitor cells. However, heterozygous knock-out of *Bim* was not sufficient in accelerating leukemia mediated by Cbfβ-SMMHC, suggesting existence of other downstream targets of N-Ras^{G12D} which may participate in leukemogenesis, though a quantification of Bim expression levels in *Bim*$^{-/+}$ bone marrow cells compared to wildtype cells is needed.

Consistent with the regulation of Bim-EL levels, pharmacologic inhibition of N-Ras^{G12D} activity showed that the oncogenic N-Ras activity seems to be modulated during leukemia progression. Our results show that leukemic cell viability depends primarily on

the activity of the Mek/Erk pathway, suggesting that MEK inhibitors may efficiently reduce leukemia in the treatment of inv(16) / *NRAS*^{G12D} AML.

In conclusion, allelic co-expression of Cbfβ-SMMHC and N-Ras^{G12D} promote the survival of pre-leukemic progenitors by activation of the MEK/ERK pathway and inhibition of Bim-EL protein levels. The transgenic mice characterized in this study are an attractive genetic tool for mechanistic studies and for the development of efficacious AML targeted therapies.

II.F. Acknowledgements

This work was supported by grants from the National Cancer Institute (R01 CA140398) and the Alex's Lemonade Stand Foundation for Childhood Cancer to L.H.C.. L.H.C. was the recipient of a Scholar Award from the Leukemia & Lymphoma Society (grant 1334-08) and J.A.P. of a Scholar Award from the American Society of Hematology.

Preface of Chapter III

The work presented in chapter III "Pharmacologic inhibition of CBFβ-SMMHC activity abrogates inv(16) leukemia" is aimed at providing additional insights in molecular mechanisms of inv(16) AML using pharmaceutical approaches. The studies included in this chapter are unpublished. Part of these studies have been included in a manuscript, currently under review for publication, and describe the development of a CBFβ-SMMHC specific inhibitor, AI-10-49, as part of a collaborative effort by four laboratories.

Chapter III only includes the experiments performed by me, with the exception of the *in vivo* treatment experiments described in Figure III.5, which were performed by me and Dr. John Pulikkan.

Chapter III

Pharmacologic inhibition of CBFβ-SMMHC activity abrogates inv(16) leukemia

III.A. Abstract

Inv(16) generates a fusion protein called CBFβ-SMMHC in acute myeloid leukemia (AML). This oncogene disrupts transcriptional factor core binding factor (CBF) function and drives leukemia by cooperation with other secondary genes. Here, we report a small molecule called AI-10-49 which selectively binds to CBFβ-SMMHC and thus restores normal CBF function. This CBFβ-SMMHC inhibitor AI-10-49 specifically reduced viability of mouse and human leukemic cells expressing CBFβ-SMMHC with minimal toxicity to normal cells and induces expression of a pro-apoptotic gene *BCL2L11*. This process is not mediated by increasing RUNX3 expression. In addition, AI-10-49 significantly prolongs the survival of mice bearing Cbfβ-SMMHC/N-Ras^{G12D} leukemic cells. This study is a proof-of-principle that transcriptional factors can be targeted in leukemia therapy. This small molecule AI-10-49 will serve as a promising drug for treating inv(16) leukemia and also a very useful tool to understand mechanisms of leukemogenesis driving by CBFβ-SMMHC.

III.B. Introduction

AML is the most common form of adult leukemia (Kumar, 2011). Long-term survival for AML remains poor and varies with the mutational composition. The transcription factor fusion protein CBFβ-SMMHC, is expressed in AML with the chromosome inversion inv(16)(p13;q22) (Liu et al., 1993)). It cooperates with activating mutations in components of cytokine signaling pathways in leukemia transformation, such as *c-KIT*, *NRAS*, *KRAS* and *FLT3* (Castilla et al., 1999; Castilla et al., 2004; Muller et al., 2008; Ravandi et al., 2007).

CBFβ is a component of the heterodimeric transcription factor CBF, where it binds to RUNX proteins and enhances their affinity for DNA (Adya et al., 2000). The CBF complex plays a key role in regulating hematopoiesis (de Bruijn and Speck, 2004). CBFβ-SMMHC outcompetes CBFβ for binding to RUNX1 (Lukasik et al., 2002), deregulates RUNX1 transcription factor activity in hematopoiesis (Liu et al., 1995; Shigesada et al., 2004), and induces AML (Castilla et al., 1999; Kuo et al., 2006).

Current inv(16) AML treatment using non-selective cytotoxic chemotherapy results in a good initial response, but limited long-term survival. Studies in mice and patient samples support the concept that inv(16) is a driver mutation that generates pre-leukemic progenitor cells that, upon acquisition of additional cooperating mutations, progress to leukemia (Castilla et al., 1999; Castilla et al., 2004; Kottaridis et al., 2002; Kuo et al., 2006; Nakano et al., 1999; Shih et al., 2008). Furthermore, they suggest that inhibition of CBFβ-SMMHC activity is essential to eliminate leukemic cells effectively.

In Chapter II, we established a leukemic mouse model expressing both Cbfβ-SMMHC and N-Ras^{G12D} mutations. We have shown that pre-leukemic cells expressing Cbfβ-SMMHC had reduced apoptosis and pro-apoptotic protein Bim levels than wildtype bone marrow progenitors. However, the mechanisms of the survival benefits provided by Cbfβ-SMMHC need further investigation.

In collaboration with Dr. John Bushweller's and other research laboratories, we developed a small molecule called AI-10-49 which specifically disrupts binding of CBFβ-SMMHC to RUNX proteins and restores normal CBF function (Illendula et al., manuscript submitted). In this chapter, we show that AI-10-49 specifically reduced viability of mouse and human leukemic cells expressing CBFβ-SMMHC. It also restores *BCL2L11* and *RUNX3* transcript levels. However, repression of RUNX3 does not affect the efficacy of AI-10-49. In addition, treatment of AI-10-49 significantly prolongs the survival of mice harboring leukemic cells expressing both Cbfβ-SMMHC and N-Ras^{G12D}. This small molecule will serve as a very promising first-generation drug targeting CBFβ-SMMHC and a useful tool for understanding mechanisms of inv(16) leukemogenesis.

III.C. Materials and Methods

Cell viability assay

For mouse cells: bone marrow cells from wild type mice or spleen cells from *Nras*$^{+/LSL\text{-}G12D}$*/Cbfb*$^{+/56M}$*/Mx1Cre* (Nras/CM) leukemic mice were enriched for Lin$^-$ progenitors, and treated in cytokine-free StemSpan Medium with DMSO or AI-10-49 (Cbfβ-SMMHC inhibitor) for 48 hours in triplicates.

For the human cells: mobilized human bone marrow enriched for progenitor cells were treated in RPMI medium + 20%FBS with DMSO or AI-10-49 for 48 hours in 96 well plates in quadruplicates. Similarly, ME-1 cells [a human inv(16) leukemic cell line (Yanagisawa et al., 1991)] were treated in RPMI medium + 20% FBS + 15mM HEPES + Penicillin/Streptomycin with DMSO or AI-10-49 for 24 hours in quadruplicates.

Cells were all plated in 96 well white plates (Corning Incorporated, Corning, NY). The cell viability was tested using CellTiter-Glo reagent (Promega, Madison, WI) according to manufacturer's instructions.

***RUNX3* knock-down**

Lentiviruses: Lentiviral supernatants (pLKO.1 vector) expressing shRNA for *eGFP* or human *RUNX3* were purchased from UMASSMED stock (RNAi Core Facility, http://www.umassmed.edu/shrna/) of Open Biosystems (GE Healthcare, Lafayette, CO). The clone number and sense sequences of RUNX3 shRNA are as below:

TRCN0000013668 (668) 5'-CCCAGCACTTTGTAGTCTCAT-3'

TRCN0000013670 (670) 5'-CCAAGGCACCTCGGAACTGAA-3'

TRCN0000013671 (671) 5'-CGACCGCTCACCTACCCGCAT-3'

RUNX3 knock-down in HeLa cells: HeLa cells were infected with the lentiviral supernatants in 4μg/ml Polybrene (American Bioanalytical, Natick, MA). Three days after infection, HeLa cells were selected in 4μg/ml puromycin for three days until all the uninfected cells (negative control) were dead. The puromycin resistant HeLa cells were lysed for RNA and protein extraction and assayed for RUNX3 knock-down by subsequent quantitative RT-PCR and immunoblot analysis.

RUNX3 knock-down in ME-1 cells: ME-1 cells were infected twice with lentiviral supernatants in 40μg/ml Polybrene (American Bioanalytical, Natick, MA). Two days after infection, ME-1 cells were selected in 5μg/ml puromycin for three days until all the uninfected cells (negative control) were dead. The puromycin resistant ME-1 cells were cultured in the presence of DMSO, or 1μM AI-10-49 for either 6 hours to extract RNA and perform quantitative RT-PCR or 24 hours to perform CellTiter AQueous One Solution Cell Proliferation Assay (MTS, Promega, Madison, WI). For the MTS assay, 20μl reagent was added to 100μl cell culture in a 96 well plate. The plate was incubated at 37^{o}C for three hours and read for 490 nm absorbance.

Reverse transcription and quantitative PCR (qRT-PCR) analyses

The RNA extraction, cDNA synthesis (random primers were used), and qPCR were performed as previously described (Pulikkan et al., 2012). Primer sequences included:

Mouse

For *Actb* cDNA

b-actin F1 (5'-CGAGGCCCAGAGCAAGAGAG-3')

b-actin R1 (5'-CGGTTGGCCTTAGGGTTCAG-3')

For *Bcl2l11* cDNA

mBim F (5'-GCTGGTGGGACCTGTTTCTA-3')

mBim R (5'-TTCAGTGAGCCATCTTGACG-3')

Human

For *ACTB* cDNA

hBactin F (5'-AGAAAATCTGGCACCACACC-3')

hBactin R (5'-AGAGGCGTACAGGGATAGCA-3')

For *BCL2L11* cDNA

hBim F (5'-TGGTCTGCAGTTTGTTGGAG-3')

hBim R (5'-CAGACATTTGGGGGAACAAG-3')

For *RUNX3* cDNA

a. Used in HeLa cells

hRx3 F2 (5'-CGCTGTTATGCGTATTCCC-3')

hRx3 R (5'-CAATGCCACCACCTTGAAG-3')

b. Used in ME-1 cells

Rx3 Cheng F (5'-CAGAAGCTGGAGGACCAGAC-3')

Rx3 Cheng R (5'-GTCGGAGAATGGGTTCAGTT-3')

For this particular reaction above, samples were amplified for 40 cycles of 30 seconds at 95^{o}C and 30 seconds at 62^{o}C according to a reference (Cheng et al., 2008). All other reactions were performed as default settings of the StepOne Plus Sequence Detection System (Applied Biosystems, Grand Island, NY).

Immunoblot analyses

Cells were washed with PBS and resuspended in modified RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.25% Sodium Deoxycholate) with protease inhibitor cocktail III (Calbiochem, Darmstadt, Germany) and phosphatase inhibitor cocktail II and III (Sigma-Aldrich, St. Louis, MO). Antibodies included anti-RUNX3 (cat#9647) and anti-p44/42 MAPK (ERK1/2) (cat#9102, all from Cell Signaling Technology, Danvers, MA).

AI-10-49 *in vivo* treatment

One hundred thousand or two thousand spleen cells from Nras/CM secondary leukemic mice were transplanted by intravenous injection into sub-lethally irradiated (550 rads) recipient mice (8 to 10 week old CD45.1^{+} B6.SJL-ptprc<a>model#004007 females, Taconic Farms, NY). The recipient mice were treated with either 50μl DMSO or AI-10-49 (207.5mg/kg) by intraperitoneal injections everyday starting from Day 5 to Day 14 after transplantations. Then mice were monitored twice everyday for early signs of leukemia.

Statistics

Medium latencies of Kaplan-Meier survival curves were calculated using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). The P values of the Kaplan-Meier survival curves were calculated by Log-rank (Mantel-Cox) test using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). All other P values were calculated by two-tailed heteroscedastic Student t-test using Excel (Microsoft Cooperation, Redmond, WA). The nonlinear regression curve fit for viability assay was generated using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA).

III.D. Results

1. A small molecule AI-10-49 was developed to specifically inhibit binding of CBFβ-SMMHC to RUNX

In collaboration with Dr. John Bushweller's and other research laboratories, we developed a small molecule called AI-10-49 which can specifically inhibit binding of

CBFβ-SMMHC to RUNX (Illendula et al., manuscript submitted). AI-10-49 is a bivalent version of a lead compound AI-10-47 (**Figure III.1A**). AI-10-47 binds to CBFβ and disrupts its binding to RUNX. CBFβ-SMMHC was proposed to assemble from dimer to multimer (Shigesada et al., 2004). We have shown in the submitted manuscript that the bivalent AI-10-49 selectively bond to CBFβ-SMMHC, disrupted the binding of the fusion protein to RUNX, and thus restored the normal binding of CBFβ to RUNX as well as transcription of downstream targets (**Figure III.1B**).

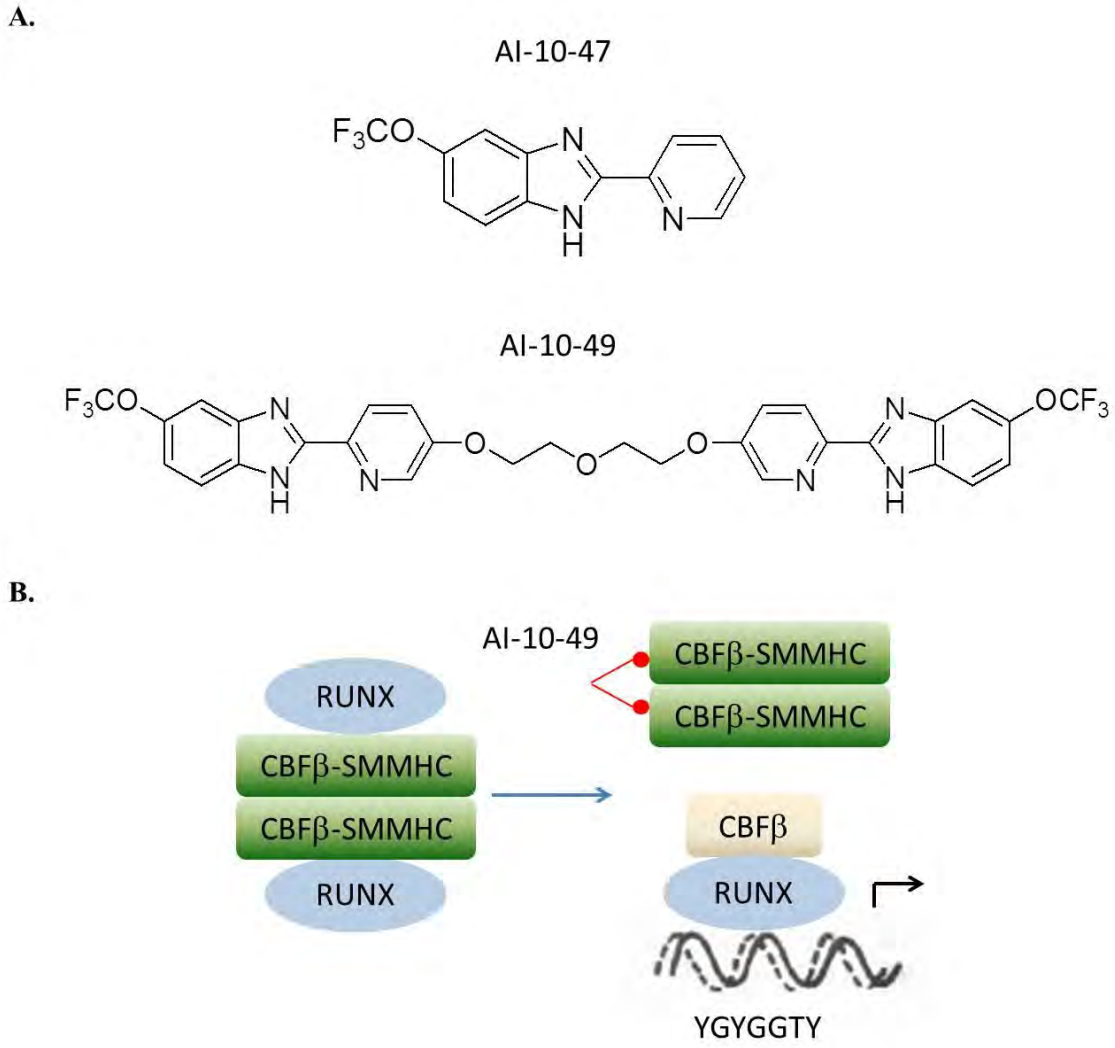

Figure III.1 A small bivalent molecule AI-10-49 can specifically inhibit CBFβ-SMMHC binding to RUNX. (A) structure of monovalent AI-10-47 and bivalent AI-10-49; (B) mechanism of AI-10-49 function based on the dimerization/multimerization of CBFβ-SMMHC.

2. AI-10-49 specifically affects viability of mouse and human leukemic cells expressing CBFβ-SMMHC and increases *BCL2L11* transcript levels

To assay if the CBFβ-SMMHC inhibitor AI-10-49 can specifically affect viability of inv(16) leukemic cells, Lin^- wild type bone marrow or *Nras*$^{+/LSL\text{-}G12D}$/*Cbfb*$^{+/56M}$/*Mx1Cre* (Nras/CM) leukemic cells were treated with different concentration of AI-10-49 for 48 hours. The IC_{50} for AI-10-49 was reduced 4.5 fold in Nras/CM leukemic cells compared to wild type bone marrow cells (0.93 μM versus 4.20 μM; **Figure III.2A**). In Chapter II, we have shown that Cbfβ-SMMHC pre-leukemic progenitors had reduced *Bcl2l11* transcript levels than wildtype bone marrow progenitors. To ask whether CBFβ-SMMHC regulates *Bcl2l11* transcription, Lin^- Nras/CM leukemic cells were treated with DMSO, 1μM or 4μM AI-10-49 for 24 hours. We found that *Bcl2l11* transcript levels increased upon CBFβ-SMMHC inhibition dose dependently (**Figure III.2B**). Accordingly, viability of human inv(16) patient derived ME-1 cells (Yanagisawa et al., 1991) were also selectively reduced by AI-10-49 with a 24-hour IC_{50} of 0.83μM, compared to 48-hour IC_{50} of 42.18μM in normal human bone marrow progenitors (**Figure III.3A-B**). Similar to mouse cells, *BCL2L11* transcript levels increased upon CBFβ-SMMHC inhibition in ME-1 cells treated with AI-10-49 (**Figure III.3C**). These results show that inhibition of CBFβ-SMMHC/RUNX binding specifically affects leukemic cell viability and increases pro-apoptotic gene *BCL2L11* expression in murine and human cells.

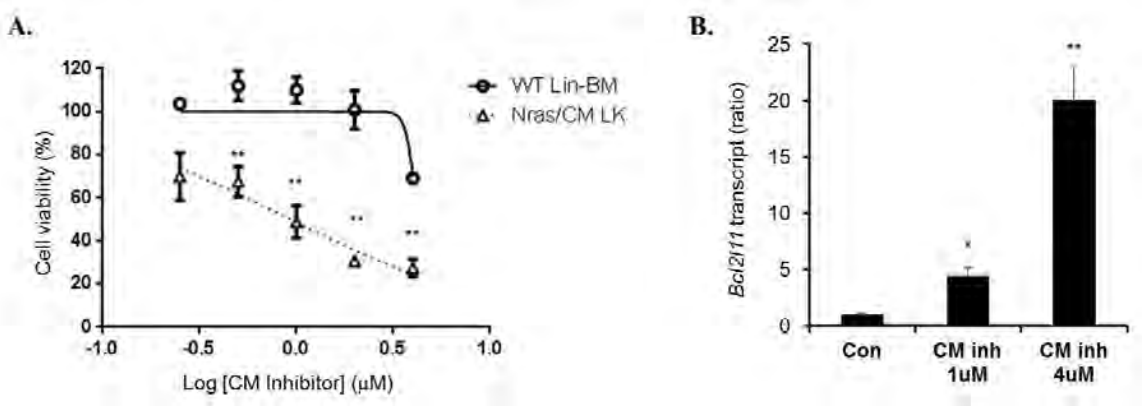

Figure III.2 AI-10-49 specifically reduces viability of Nras/CM leukemic cells and increases *Bcl2l11* transcript levels. (A) Lin$^-$ wild type bone marrow cells (WT Lin- BM) or Nras/CM leukemic (Nras/CM LK) cells were treated with different concentration of CBFβ-SMMHC (CM) inhibitor AI-10-49 for 48 hours and assayed for cell viability. The IC_{50} for WT and LK cells are 4.20μM and 0.93μM, respectively. (B) Lin$^-$ Nras/CM leukemic cells was treated with DMSO, 1μM or 4μM of CM inhibitor (CM inh) AI-10-49 for 24 hours and *Bcl2l11* transcripts were measured by qRT-PCR and values were normalized to *Actb* transcripts. *, P<0.05; **, P<0.01.

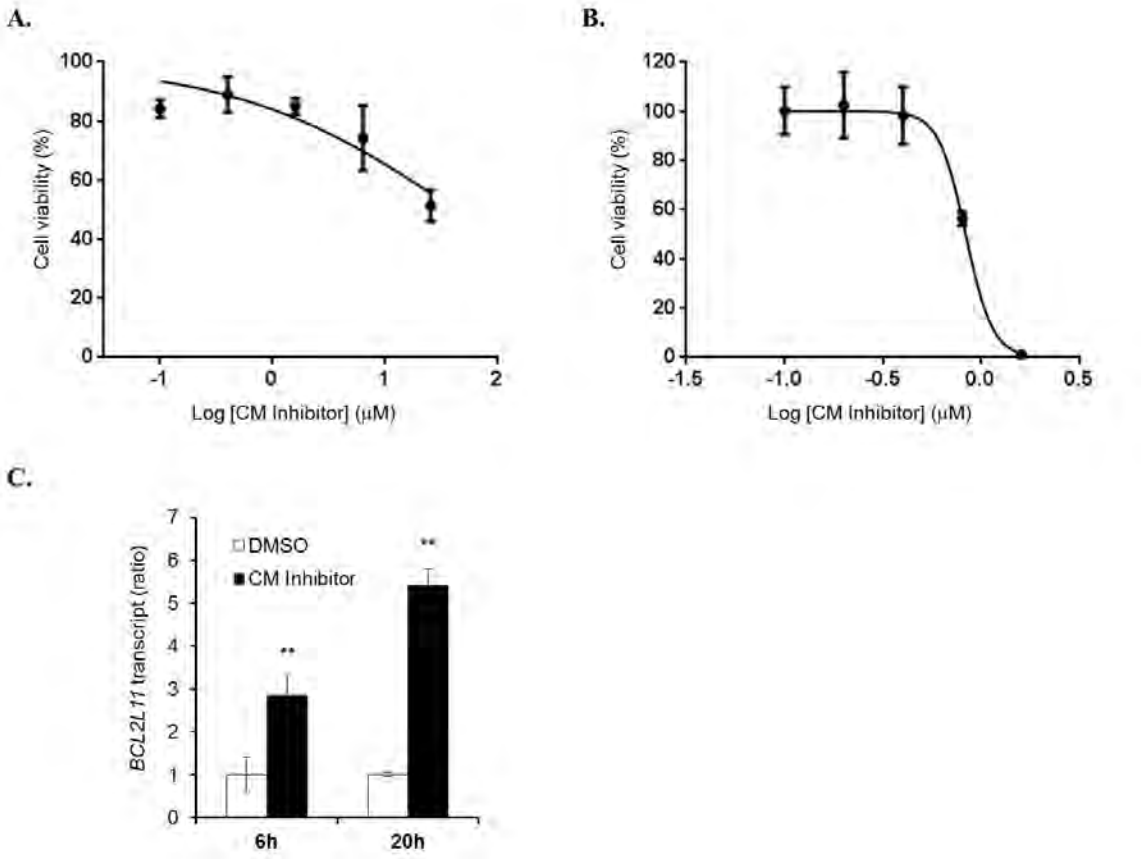

Figure III.3 AI-10-49 specifically reduces viability of ME-1 cells and increases *BCL2L11* transcript levels. (A) human mobilized bone marrow cells were treated with CM inhibitor AI-10-49 for 48 hours and assayed for cell viability, presenting an IC_{50} of 42.18μM. (B) ME-1 cells were treated with AI-10-49 for 24 hours and assayed for cell viability, presenting an IC_{50} of 0.83μM. (C) ME-1 cells were treated with 1μM AI-10-49 for 6 or 20 hours and *BCL2L11* transcripts were measured by qRT-PCR and values were normalized to *ACTB* transcripts; **, P<0.01.

3. The survival signals provided by CBFβ-SMMHC may not be solely mediated by *RUNX3* repression

RUNX3 has been shown to be transcriptionally repressed by CBFβ-SMMHC in AML cells (Cheng et al., 2008). Accordingly, we have shown that upon AI-10-49 treatment, RUNX1 occupancy on *RUNX3* promoter and *RUNX3* transcription was restored in ME-1 cells (Illendula et al., manuscript submitted). Furthermore, RUNX3 transcriptionally upregulates pro-apoptotic gene *BCL2L11* to induce apoptosis in gastric cells (Yamamura et al., 2006; Yano et al., 2006). Since CBFβ-SMMHC inhibition by AI-10-49 increased *Bcl2l11* transcript levels and affected viability of leukemic cells (**Figure III.2** and **Figure III.3**), we want to study whether RUNX3 plays an important role in AI-10-49 mediated eliminating of leukemic cells. Three different shRNA lentiviruses targeting *RUNX3* were tested in HeLa cells. All of them showed a significant knock-down of RUNX3 transcript and protein levels compared to shRNA targeting eGFP (**Figure III.4A-B**). Two shRNA lentiviruses targeting *RUNX3* were selected to infect ME-1 cells and significantly reduced RUNX3 transcripts to basal levels even upon AI-10-49 treatment (**Figure III.4C**). However, AI-10-49 can still efficiently affect ME-1 cell viability with reduced *RUNX3* transcript levels (**Figure III.4D**). These data suggested that RUNX3 may not be a critical mediator in reducing viability of inv(16) leukemic cells upon CBFβ-SMMHC inhibition.

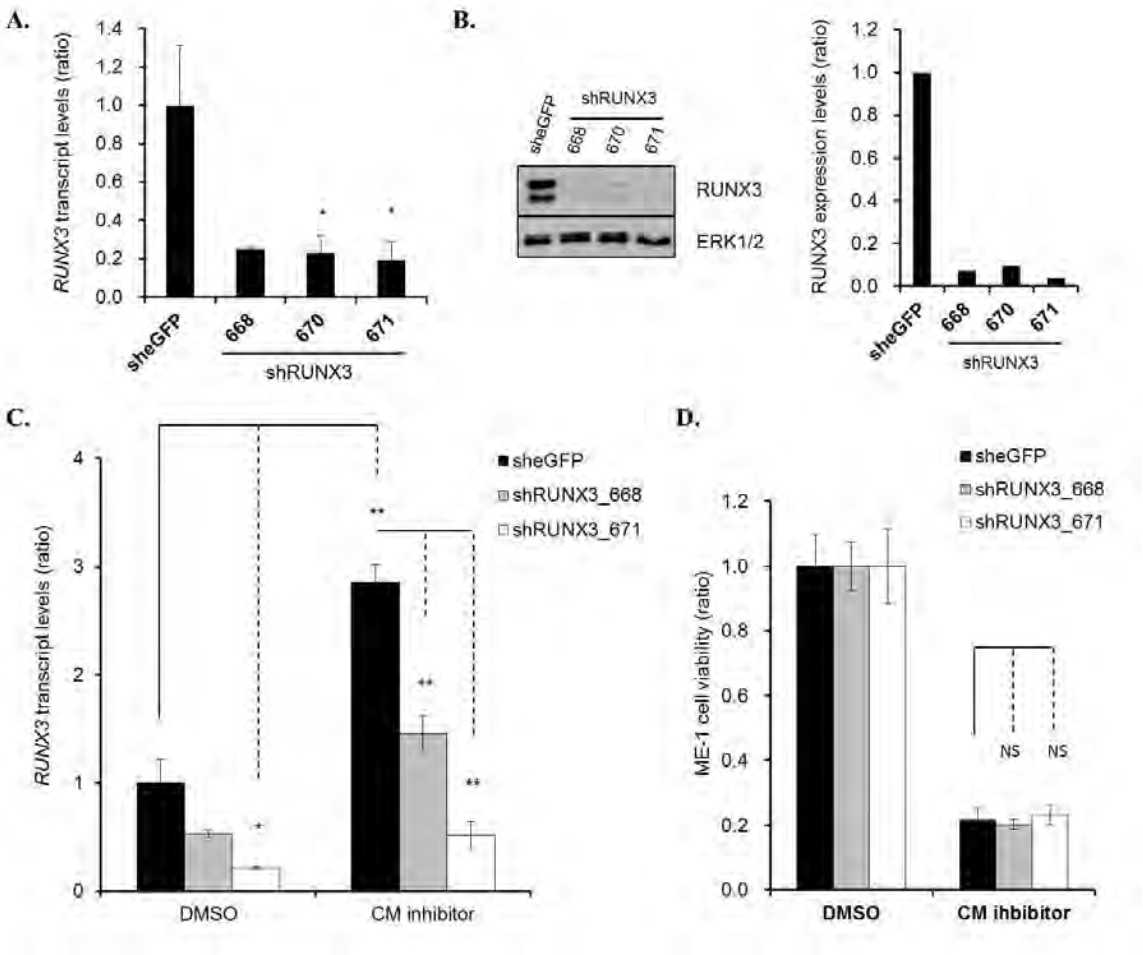

Figure III.4 Efficacy of AI-10-49 is not affected upon *RUNX3* knock-down in ME-1 cells. (A) HeLa cells were infected with lentiviruses expressing shRNA for *eGFP* or *RUNX3*, selected for puromycin resistance and assayed for *RUNX3* transcript levels by qRT-PCR with values normalized to *ACTB* transcripts. (B) immunoblot analysis of RUNX3 and ERK1/2 levels in puromycin resistant HeLa cells (left panel); quantification of RUNX3 protein levels normalized to ERK1/2 (right panel). (C) ME-1 cells were infected with lentiviruses expressing shRNA for *eGFP* or *RUNX3*, selected for puromycin resistance and treated with 1μM CM inhibitor AI-10-49 for 6 hours. *RUNX3* transcript levels were assayed by qRT-PCR and values normalized to *ACTB* transcripts. (D) Puromycin resistant ME-1 cells were treated with AI-10-49 for 24 hours and cell viabilities were measured by the MTS assay. *, P<0.05; **, P<0.01; NS, not significant.

4. AI-10-49 significantly prolonged survival of Nras/CM leukemic mice

To test whether inhibition of Cbfβ-SMMHC can benefit leukemia treatment, mice were transplanted with either 100,000 or 2,000 of Nras/CM leukemic cells and treated with 10 doses of AI-10-49 (207.5mg/kg). In both cases, AI-10-49 significantly prolonged survival of the mice bearing Nras/CM leukemic cells ($P < 0.0001$; **Figure III.5**). Medium latency was prolonged from 21 to 30 days with AI-10-49 treatment when 100,000 leukemic cells were transplanted (**Figure III.5A**). Similarly, medium latency was prolonged from 34 to 61 days with AI-10-49 treatment when 2000 leukemic cells were transplanted (**Figure III.5B**). These results demonstrated that AI-10-49 has a significant survival benefit to Nras/CM leukemic mice.

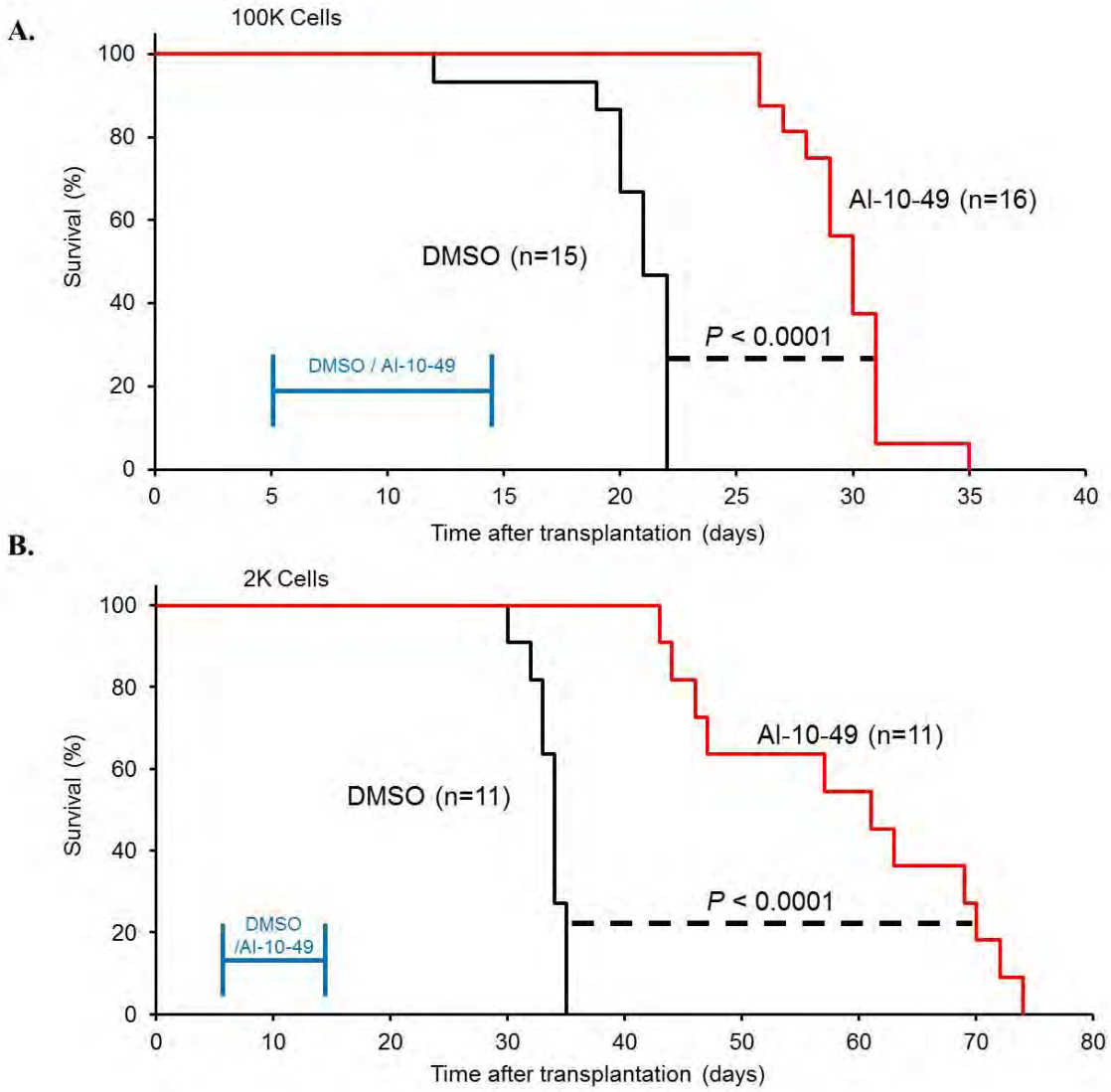

Figure III.5 AI-10-49 prolongs survival of Nras/CM leukemic mice. Mice were transplanted with either 100,000 (A) or 2000 (B) Nras/CM leukemic cells, and treated with either DMSO (black) or 207.5mg/kg AI-10-49 (red) everyday from Day 5 to Day 14 after transplantation. Kaplan-Meier survival curves show significant prolonged medium latencies due to AI-10-49 treatment in both cases.

III.E. Discussion

Dysregulated gene expression is a hallmark of cancer and is particularly important for the maintenance of cancer stem cell properties, such as self-renewal. As such, the targeting of proteins that drive transcriptional dysregulation, so called "transcription therapy", represents an avenue for drug development with immense potential. A number of fusion proteins involving transcription factors have been identified as drivers of disease in leukemia (Look, 1997; Zeisig et al., 2012), which provide excellent targets for therapeutic intervention.

Our results provide a clear proof-of-principle for this approach, as AI-10-49 specifically inhibits CBFβ-SMMHC/RUNX binding and shows efficacy against CBFβ-SMMHC driven leukemia *in vitro* and *in vivo* with no apparent side-effects to normal cells. Specificity of action is a key component of the development of a targeted drug. Imatinib, for example, inhibits ABL kinase and its efficacy in CML is clearly a result of effective inhibition of the BCR-ABL fusion protein that drives CML. However, even this agent inhibits both the BCR-ABL fusion protein as well as wildtype ABL. The "holy grail", as it were, of targeted therapy with fusion or mutated protein drivers of cancer is to achieve inhibition of the fusion or mutated protein with little to no effect on the wildtype protein, thereby minimizing any toxicity or cancer promotion associated with inhibition of the wildtype protein. This study demonstrates that AI-10-49 represents a clear example of such selectivity for inv(16) leukemia, inhibiting CBFβ-SMMHC activity while having a minimal effect on CBFβ function. In addition, AI-10-49 clearly possesses the key properties of a high quality chemical probe recently outlined by Frye (Frye, 2010),

namely a clear molecular profile of activity, mechanism of action, identity of active species, and proven utility.

In the Chapter II, we established that CBFβ-SMMHC and N-Ras^{G12D} cooperates in providing survival advantage and reduced expression of a pro-apoptotic gene *Bcl2l11* in pre-leukemic cells. Here we found that inhibition of CBFβ-SMMHC by AI-10-49 in mouse or human leukemic cells expressing CBFβ-SMMHC restored the *Bcl2l11* transcript levels. These suggested that CBFβ-SMMHC directly or indirectly reduces *Bcl2l11* transcript levels. Studies have shown that *BCL2L11* transcript levels are induced by RUNX3 and FOXO3 in gastric cells (Yamamura et al., 2006; Yano et al., 2006), and CBFβ-SMMHC represses *RUNX3* levels in AML cells (Cheng et al., 2008). However, based on our results, though AI-10-49 treatment did restore *RUNX3* transcript levels in ME-1 cells, repression of *RUNX3* expression did not affect the efficacy of AI-10-49. Therefore, the survival signals provided by CBFβ-SMMHC are not only mediated by repression of *RUNX3*. It will be interesting to investigate whether *BCL2L11* transcript levels are essential in the survival of the inv(16) leukemic cells and also search for other potential mediators downstream of CBFβ-SMMHC.

In summary, AI-10-49 is a direct, potent, and specific first generation CBFβ-SMMHC inhibitor that induces cell death in inv(16) leukemic cells and establishes a proof-of-principle that transcription factor fusion oncoproteins can be directly targeted for leukemia treatment. It will also serve as a very useful tool to further understand the functions of CBFβ-SMMHC in pre-leukemic and leukemic cells.

III.F. Acknowledgements

This work was supported by grants from the National Cancer Institute (R01 CA140398) to J.H.B., L.H.C., and R.A.R., (R01 CA096983) to L.H.C., and a SCOR grant from the Leukemia and Lymphoma Society to J.H.B. (SCOR 7006). L.H.C. is the recipient of a Scholar Award from the Leukemia & Lymphoma Society (grant 1334-08) and J.A.P. of a Scholar Award from the American Society of Hematology. M.L.G. is funded by the US National Institutes of Health (NIH) through the NIH Director's New Innovator Award Program, 1 DP2 OD007399-01.

Chapter IV

Discussion

The work presented in this thesis was directed at understanding the molecular mechanisms of CBFβ-SMMHC in inv(16) AML. In Chapter II, I described the alterations in pre-leukemic progenitor cells expressing both CBFβ-SMMHC and N-Ras^{G12D} oncproteins, using a mouse model, and investigated into downstream targets. In Chapter III, I characterized a small molecule targeting CBFβ-SMMHC and tested its efficacy in the mouse model developed in Chapter II. In this chapter, I provide additional discussion, overall conclusions and future directions for the results presented.

IV.A Pre-leukemic alteration by CBFβ-SMMHC and N-RAS^{G12D}

Acute myeloid leukemia (AML) is an aggressive hematopoietic malignancy with a 5-year overall survival (OS) between 30 and 40% (Estey and Dohner, 2006; Lowenberg et al., 1999). AMLs predominantly occur in adults and patients over age 65 have especially poor outcome (Estey and Dohner, 2006; Lowenberg et al., 1999). Even in patients with favorable cytogenetics as inv(16), the 5 year probabilities of OS and leukemia-free survival for the elderly (Age >60) patients are only 31% and 27% (Prebet et al., 2009). This relative low rate of OS is mainly due to the relapsed, treatment-refractory disease. The 5 year cumulative incidence of relapse is 57% for inv(16) AMLs (Marcucci et al., 2005).

This situation raises important questions of how AMLs develop and progress as well as what is the cellular basis of relapse. The existence of a pre-leukemia stage in human AML has been suggested in two recent studies, utilizing next generation sequencing technologies in AML patient genomes and xenograft in immunodeficient (*NOD-scid-IL2Rgc*$^{-/-}$, NSG) mice (Jan et al., 2012; Shlush et al., 2014). The first study revealed that a small proportion of residual hematopoietic stem cells (HSCs) in AML patient samples presented some of mutations also identified in the leukemic cells (Jan et al., 2012). Detailed single-cell analysis disclosed a clonal evolution of the pre-leukemic HSCs to full-blown leukemia and a potential mechanism contributing to relapse. The second study showed that these mutated pre-leukemic HSCs had higher multilineage repopulation advantage over non-mutated HSCs in xenografts. They survived in chemotherapy, accumulated multiple oncogenic mutations, and served as a cellular reservoir for developing *de novo* and relapse AML (Shlush et al., 2014). Therefore, investigation on the pre-leukemia stage altered by different driver and secondary mutations are essential for understanding the mechanisms of leukemogenesis and providing insights for treatment of *de novo* and relapse leukemias.

The pre-leukemia stage for AML in humans is asymptomatic, and the patient is first diagnosed with *de novo* AML after the full-blown leukemia has occurred. In addition, expansion of pre-leukemic cells is limited because they are usually outcompeted by their malignant descendants (Greaves, 2009). Therefore, mouse models harboring recurrent mutations observed in human AMLs are valuable genetic tools to study the dynamics of the pre-leukemia stage. The chromosome 16 inversion [inv(16)(p13;q22)] is thought to be

a founding mutation in the HSCs, creating a pre-leukemic population of cells, which persist asymptomatic in the bone marrow for years. The first insight on the role of the fusion protein in pre-leukemic hematopoiesis was reported by our laboratory (Kuo 2006). Considering that inv(16) AML patients frequently present mutations in receptor tyrosine kinase (RTK) signaling pathways, such as *NRAS*, *KRAS* and *FLT3* (Muller et al., 2008), we studied the role of $NRAS^{G12D}$ mutation in the pre-leukemic progenitor cells, using a mouse model with allelic expression of Cbfβ-SMMHC and $N\text{-}Ras^{G12D}$. Our results showed that both Cbfβ-SMMHC and $N\text{-}Ras^{G12D}$ expanded the stem and early progenitor compartments (LSK^+) as well as the myeloid progenitor compartments (LSK^-), consistent with previous and recent studies on mice harboring individual oncogenes (Kuo et al., 2006; Li et al., 2011; Wang et al., 2013). Importantly, the co-expression of these mutated genes is not sufficient to induce an aggressive leukemia. The introduction of the $Nras^{G12D}$ into $Cbfb^{+/MYH11}$ mice did not induce a proliferative expansion of maturing myeloid cells, as observed in $Nras^{+/G12D}$ mice, probably due to the dominant role of Cbfβ-SMMHC. It will also be interesting to analyze extramedullary hematopoiesis in $Nras^{+/LSL\text{-}G12D}/Cbfb^{+/56M}/Mx1Cre$ mice since $Nras^{+/LSL\text{-}G12D}/Mx1Cre$ present an increased stem and progenitor cells in spleen (Wang et al., 2013).

The question of what drives the expansion of pre-leukemic cells led us to perform a detailed analysis of this compartment.

Though $N\text{-}Ras^{G12D}$ has recently been reported to increase proliferation of LT-HSCs ($LSK^+CD48^-CD150^+$) but not of whole bone marrow (Li et al., 2013; Wang et al., 2013), we did not observe any alteration by $N\text{-}Ras^{G12D}$ in cell cycle of stem and early progenitor

cells (LSK^+) or myeloid progenitor cells (LSK^-). Another study reported that $K\text{-}Ras^{G12D}$ oncoprotein significantly increased cell cycle entry of HSCs ($Flt3^-LSK^+$) (Sabnis et al., 2009). In consequence, a small population of cells expressing $K\text{-}Ras^{G12D}$ dominated both primitive and differentiated hematopoietic compartments in *Kras*$^{+/LSL\text{-}G12D}$*/Mx1Cre* mice in the absence of polyI:C treatment. This phenotype correlated with the an aggressive myeloproliferative neoplasm (MPN, medium latency as 84 days after polyI:C) observed in *Kras*$^{+/LSL\text{-}G12D}$*/Mx1Cre* mice (Braun et al., 2004), in contrast to the indolent hematologic disorders (medium latency as 342 days after polyI:C) observed in *Nras*$^{+/LSL\text{-}G12D}$*/Mx1Cre* mice (Li et al., 2011). The cytokine sensitivity and downstream effector activation were also different between *Kras*$^{+/LSL\text{-}G12D}$*/Mx1Cre* and *Nras*$^{+/LSL\text{-}G12D}$*/Mx1Cre* myeloid progenitor cells (Li et al., 2011). Our data suggest that the primary role of Nras in the pre-leukemic cells is to provide survival capacity, while Kras may maintain a primary proliferative phenotype. The difference may be explained in that $K\text{-}Ras^{G12D}$ is expressed at higher levels and has more GTP binding forms than $N\text{-}Ras^{G12D}$ in bone marrow cells (Li et al., 2011). In this regard, it remains unclear how $K\text{-}Ras^{G12D}$ alters the pre-leukemic cells in the presence of Cbfβ-SMMHC, compared to what we found in the *Nras*$^{+/LSL\text{-}G12D}$*/Cbfb*$^{+/56M}$*/Mx1Cre* mice.

The allelic expression of Cbfβ-SMMHC showed a bimodal behavior since we have observed that it increased the proliferation of stem and early progenitor cells but reduced the proliferation of myeloid progenitor cells. The reduced $S/G_2/M$ phase in the myeloid progenitors correlated with previous studies, which have shown that ectopic expression of Cbfβ-SMMHC inhibited G1 to S transition in murine myeloid or lymphoid cell lines and

slowed cell cycle of primary murine and human myeloid progenitors (Cao et al., 1998; Cao et al., 1997; D'Costa et al., 2005).

In this context, $NRas^{G12D}$ reduced apoptosis of pre-leukemic stem and progenitor cells expressing Cbfβ-SMMHC. Our results are consistent with studies revealing a critical role of oncogenic *NRAS* mutations in providing survival advantages to pre-leukemic and leukemic cells (Chou et al., 2011; Kim et al., 2009). Furthermore, HSCs from *Runx1*-null mice also presented reduced apoptotic cells (Cai et al., 2011). These data suggest that the survival advantage mediated by Cbfβ-SMMHC is probably due to inhibition of Runx functions. We will discuss in details on regulation of pro-apoptotic protein Bim by these two oncogenes in the subchapter IV.C. Other potential targets could be discovered by expression profiling analysis of $N\text{-}Ras^{G12D}$ / $Cbfb^{+/MYH11}$ pre-leukemic cells compared to, $Nras^{G12D}$, and $Cbfb^{MYH11}$ cells. It would provide additional valuable information to compare Cbfβ-SMMHC pre-leukemic progenitor cells treated with and without AI-10-49, the small molecule disrupting Cbfβ-SMMHC/Runx binding.

We have previously shown that Cbfβ-SMMHC induced an abnormal myeloid progenitor population (Kuo et al., 2006). This pre-leukemic population was maintained in bone marrow of $Nras^{G12D}/Cbfb^{MYH11}$ mice. Significant reduction of lineage positive cells and increase of lineage negative cells in pre-leukemic $Cbfb^{MYH11}$, and $Nras^{G12D}/Cbfb^{MYH11}$ cells led us to conduct a detailed morphology analysis of the whole bone marrow cells in addition to the immunophenotypic analysis focusing on the small percentage of stem and progenitor cells. This analysis revealed a similar accumulation of blasts/myeloblasts and promyelocytes and a reduction of neutrophils in pre-leukemic $Cbfb^{MYH11}$ and

Nras^{G12D}/*Cbfb*^{MYH11} cells. These data suggest that the block of differentiation ocurrs at the myeloblast / promyelocyte stage and the activity of N-Ras^{G12D} cannot by pass the differentiation block set by Cbfβ-SMMHC. Analysis of expression levels of master myeloid differentiation regulators, such as *Cebpa*, *Pu.1* and *Gfi1* in Cbfβ-SMMHC pre-leukemic cells and the usage of RNA interference or retroviral overexpression coupled with differentiation assays to test the roles of those potential targets will help us to better understand the mechanisms of the differentiation block.

In summary, we have shown that different oncogenes deregulate specific properties of the hematopoietic stem and progenitor cells before full blown transformation occurs. In addition, we have developed a valuable mouse model for studying functional and genetic changes that drive leukemia development. Studies on the molecular alteration of the pre-leukemic cells by oncogenic mutations will help in understanding the functions of those critical genes in normal hematopoiesis, the progression of leukemia from pre-leukemia stage, possible mechanisms of relapse and development of targeted therapies to those pre-leukemic clones.

IV.B Cooperation between CBFβ-SMMHC and N-RAS^{G12D} in leukemogenesis

As a driver mutation in inv(16) AML, CBFβ-SMMHC dictates the leukemic phenotype in our *Nras*^{G12D}/*Cbfb*^{MYH11} mouse model. The acceleration and higher leukemia initiating-cell (L-IC) activity provided by N-Ras^{G12D} probably result from the enhanced survival capacity observed in the pre-leukemic cells. Accordingly, studies using

serial transplantation assays have noted an advantage of N-Ras^{G12D} in serial multi-lineage repopulation activities (Li et al., 2013).

The related concept of whether these two mutations are sufficient to induce leukemia was addressed in our experiments. The median latency of leukemia in Nras$^{+/G12D}$/*Cbfb*$^{+/MYH11}$ mice was significantly shorter than in *Cbfb*$^{+/MYH11}$ mice. However the extended time needed for leukemia development suggests that additional mutations may be required for leukemia development. To address this question, future studies may utilize a retroviral mutagenesis approach on transplantation assays of *Nras*^{G12D}/*Cbfb*^{MYH11} pre-leukemic cells and assess whether the emerging leukemia is poly- or olig-oclonal .

Does the activation of Cbfβ-SMMHC and N-Ras^{G12D} need to occur in hematopoietic progenitor cells before the stage of Cbfβ-SMMHC-mediated differentiation block? Our finding that one out of six Nras$^{+/G12D}$/*Cbfb*$^{+/MYH11}$ mice developed a mixed phenotype that included MPN and AML cells. Since the MPN population did express Cbfβ-SMMHC (data not shown), it is possible that the fusion protein-mediated differentiation block may not be effective in myeloid cells after the myeloblast/promyeloblast stage, and Nras^{G12D} may direct enhanced myeloproliferative phenotype.

The presence of homozygous oncogenic NRAS alleles has been reported in juvenile myelomonocytic leukemia patients, resulting from uniparental disomy (Dunbar et al., 2008). This phenomenon was recaptured in the N-Ras^{G12D} chronic myelomonocytic leukemia and AML mouse models (Wang et al., 2010a; Xu et al., 2013). However, we found that the three AML clones analyzed maintained one wild type allele. Our results

correlate with studies of human AML cases that all the patients were found heterozygous for mutated *NRAS* or *KRAS* genes (Bacher et al., 2006; Bowen et al., 2005).

Mutations in *NRAS* or *KRAS* are frequently found in inv(16) AMLs, with 26–38% and 7-17%, respectively (Bacher et al., 2006; Bowen et al., 2005; Valk et al., 2004a). However, no spontaneous mutations in codon 12, 13 or 61 of *Nras* or *Kras* transcripts were revealed in sequencing of 9 murine Cbfβ-SMMHC leukemic samples in our study. As we are aware, approximately only half of the cells are c-kit^{+} in each sample and we were not performing a deep sequencing analysis in those samples. Therefore it is possible that any small subclone but not the predominant one which contained the *Ras* mutations could be overlooked in this type of analysis due to sensitivity issues. Also, we could not exclude the existence of other types of *Ras* mutations or mutations in other factors related to the receptor tyrosine kinase signaling pathways.

IV.C Bim is a potential downstream target of CBFβ-SMMHC and N-RAS^{G12D}

The CBFβ-SMMHC directed a reduction in apoptosis of pre-leukemic progenitor cells. Analysis of apoptosis related proteins revealed that the pro-apoptotic Bcl-2-like protein 11 (Bcl2l11 or Bim) protein levels correlated with the reduced apoptosis in the pre-leukemic and leukemic cells expressing both CBFβ-SMMHC and N-Ras^{G12D}.

The BIM protein is a BCL-2 homology 3 (BH3) - only protein (BOP), a pro-apoptotic member of the BCL-2 protein family (Ewings et al., 2007b). Alternative splicings of *BCL2L11 (BIM)* mRNA give rise to the short, long and extra long protein variants (BIM-S, BIM-L and BIM-EL) (Bouillet et al., 2001). The BCL-2 family proteins

are evolutionarily conserved regulators of the cell intrinsic apoptosis pathway, which act to regulate the integrity of the outer mitochondrial membrane (Cory and Adams, 2002; Strasser, 2005).

Upon stress induced signaling, BOPs, such as BIM, bind to the pro−survival BCL-2 proteins, such as BCL-xL and MCL-1, neutralizing them and thereby relieving their inhibition of BAX and BAK (Chen et al., 2005; Ewings et al., 2007a; Puthalakath and Strasser, 2002; Willis et al., 2005). BAX and BAK are also BCL-2 family members. They are critical downstream effectors of apoptosis since cells lacking both proteins are resistant to a wide variety of apoptotic insults (Wei et al., 2001; Willis and Adams, 2005). In viable cells, BAX and BAK are restrained by their physical interaction with the pro-survival BCL-2 proteins (Ewings et al., 2007b). After activation mediated by BOPs, BAX and BAK promote the release of cytochrome c through the outer mitochondrial membrane, leading to activation of caspase 9 (initiator caspase) and caspase 3 (executioner caspases), and eventually cell death (McCubrey et al., 2008; McIlwain et al., 2013).

The levels of BIM are regulated at both transcriptional and post-translational levels. Transcription of the *BCL2L11* gene is normally repressed by serum, growth factors and cytokines while increased expression of *BCL2L11* is required for optimal cell death following cytokine withdrawal (Bouillet et al., 1999; Ewings et al., 2007a; Whitfield et al., 2001). This increase is promoted by the FOXO3 transcription factor (Gilley et al., 2003; Yamamura et al., 2006). FOXO3 is inhibited by both ERK and AKT pathways. AKT phosphorylates and sequesters FOXO3 in the cytosol by binding to 14-3-3 proteins

to prevent it from activating BIM transcription (Fu and Tindall, 2008). ERK1⁄2-dependent phosphorylation of FOXO3 targets it for proteasome-dependent degradation (Yang et al., 2008). BIM-EL is the most abundant isoform and undergoes the most dynamic changes in expression upon withdrawal of survival factors (Ewings et al., 2007a). Many studies have shown that BIM-EL is phosphorylated at multiple sites in response to activation of the ERK1⁄2 pathway, and this has the effect of promoting its ubiquitination and proteasome dependent degradation (Hubner et al., 2008; Ley et al., 2003; Ley et al., 2005; Luciano et al., 2003; Marani et al., 2004). Accordingly, our results showed that in leukemic cells expressing Cbfβ-SMMHC and N-Ras^{G12D}, Bim transcript and protein levels increased upon MEK inhibitor treatment and the leukemic cells are more sensitive to MEK inhibitor than wild type cells or leukemic cells only expressing Cbfβ-SMMHC. In the contrast, PI3K inhibitor did not significantly affect Bim levels and there is no difference in the sensitivity to the inhibitor between groups. The observation in the pre-leukemic cells is similar except the sensitivity to MEK inhibitor is lower and PI3K inhibitor did induce an increase in *Bcl2l11* transcript levels but not necessarily protein levels. These results suggest a rewiring of pathway dependence during leukemic progression.

As we should be aware, even though reduced Bim levels were observed in pre-leukemic and leukemic cells expressing N-Ras^{G12D} and were increased upon MEK inhibitor treatment, whether this regulation is dependent on the activity of N-Ras^{G12D} needs further investigation. The usage of RNA interference selectively targeting N-Ras^{G12D} but not wildtype N-Ras in pre-leukemic or leukemic cells expressing N-Ras^{G12D}

will provide useful information about whether the activation of MEK/ERK pathway, the reduction in Bim levels and the survival advantage are mediated by N-Ras^{G12D} activity.

The RUNX3 and RUNX1 proteins are also potential regulators of *BCL2L11* transcripts. RUNX3 inhibits AKT transcription and cooperates with FOXO3 to induce *BCL2L11* transcripts in gastric epithelial cells (Lin et al., 2012; Yamamura et al., 2006; Yano et al., 2006). *RUNX3* is transcriptionally repressed by CBFβ-SMMHC (Cheng et al., 2008) and can be induced by treating ME-1 with CBFβ-SMMHC/RUNX inhibitor AI-10-49 (Illendula et al., manuscript submitted). Therefore, inhibition of the RUNX3/BIM axis could be a potential survival mechanism provided by CBFβ-SMMHC. However, we found that though AI-10-49 induced expression of *BCL2L11* and specifically reduced the viability of murine and human leukemic cells expressing Cbfβ-SMMHC, decrease of *RUNX3* expression by RNA interference did not affect the efficacy of AI-10-49 in ME-1 cells. RUNX1 has also been shown to cooperate with FOXO3 in mediating transforming growth factor β (TGFβ)-induced *BCL2L11* transcription in hepatic cells (Wildey and Howe, 2009). The RUNX1/FOXO3/BIM axis could be another potential mechanism to be investigated. We have shown that knock-down of *Bcl2l11* in pre-leukemic cells expressing Cbfβ-SMMHC reduced apoptosis. Testing whether those cells or pre-leukemic Cbfβ-SMMHC cells in a *Bcl2l11*$^{-/-}$ background are resistant to AI-10-49 treatment will provide more information on the how critical Bim is for pre-leukemic cell survival downstream of Cbfβ-SMMHC.

We have shown that loss of one copy of wildtype *Bcl2l11* allele did not cooperate with Cbfβ-SMMHC in leukemogenesis. The first possibility is that the Bim levels in the

Bim$^{+/-}$ background are not low enough to cooperate with Cbfβ-SMMHC in leukemia development. To test that, a comparison of Bim levels between *Bim*$^{-/+}$ and *Nras*$^{+/G12D}$ hematopoietic progenitor cells will provide very useful information. If the Bim levels are higher in *Bim*$^{+/-}$ cells, it will be necessary to analyze leukemogenesis in *Bim*$^{-/-}$*Cbfb*$^{+/MYH11}$ mice, keeping in mind the possible autoimmune kidney diseases induced by loss of Bim (Bouillet et al., 1999). Regarding that, monitoring leukemogenesis in mice transplanted with *Cbfb*$^{+/MYH11}$ cells harboring shRNAs targeting Bim is an alternative option. The second possibility is that there are other potential targets downstream of N-Ras^{G12D} involved or not involved in apoptosis that are critical in cooperation with Cbfβ-SMMHC in leukemia development. I am aware that we have not performed an exhaustive analysis of all cell death associated proteins/pathways that may be involved in N-Ras^{G12D} and Cbfβ-SMMHC-mediated pro-survival of pre-leukemic and leukemic cells. We have not explored other possible mechanisms besides survival signals in leukemia initiation and maintenance, either. Therefore, future efforts may be required to further explore other important factors driving the anti-apoptotic response and leukemogenesis.

IV.D Target the MEK/ERK pathway in AMLs with *NRAS* mutations

Oncogenic RAS mutations are dominant mutations that are found in many cancers, and represent compelling targets for the development of small molecule inhibitors. However, constitutive activation of RAS results from an inactivation of its intrinsic enzyme activity and restoration of this activity is a daunting proposition that has not been achieved to date (Braun and Shannon, 2008). Therefore, inhibition of downstream

components of RAS signaling pathway, such as MEK, becomes a recent trend in targeted therapies for cancer patients with *RAS* mutations. However, studies of knockout mice raised concerns about potential toxicity of inhibition the MEK/ERK pathway. *Erk1*$^{-/-}$ mice presented a twofold reduction in the number of mature thymocytes and T-cell proliferation was reduced in response to activating factors (Pages et al., 1999). *Mek1*$^{-/-}$ mice underwent embryonic lethality, resulting from impaired placental development (Giroux et al., 1999).

Our results demonstrate that MEK inhibitor PD0325901 specifically affected viability of leukemic cells expressing both Cbfβ-SMMHC and N-Ras^{G12D} with minimal toxicity to wild type bone marrow progenitor cells and leukemic cells expressing only Cbfβ-SMMHC. In contrast, PI3K inhibitor GDC-0980 reduced the viability of all the three biological groups. This observation represents an example of signaling network remodeling by cancer cells. The dependence of Cbfβ-SMMHC/N-Ras^{G12D} leukemic cells on MEK/ERK pathway but not on PI3K/AKT pathway points out a clinical implication that targeting MEK/ERK pathway using MEK inhibitors may be an efficient complimentary therapy to inv(16) AMLs with *NRAS* mutations.

A variety of MEK inhibitors have been developed and tested in preclinical models and clinical trials in patients with solid tumors and hematopoietic malignancies presenting activated RAS/RAF/MEK pathway. PD098059 was the first MEK inhibitor to be disclosed (Dudley et al., 1995). It has been mostly used in an *in vitro* to understand the role of MEK/ERK pathway in carcinogenesis (Dudley et al., 1995; Murphy et al., 2001; Xing and Imagawa, 1999). Two other potent MEK inhibitors, U0126 (Favata et al., 1998)

and Ro 09-2210 (Williams et al., 1998), were subsequently identified in cell-based assays. These inhibitors were highly selective in inhibiting activation of MEK (Davies et al., 2000; Williams et al., 1998). However, none of these compounds was moved to clinical evaluation because of their pharmaceutical limitations (Fremin and Meloche, 2010).

PD184352 (subsequently named CI-1040) was the first MEK inhibitor reported to inhibit tumor growth *in vivo* (Sebolt-Leopold et al., 1999). CI-1040 is a potent (IC_{50} of 17 nM against purified MEK1) and highly selective inhibitor of MEK1/2 (Sebolt-Leopold et al., 1999). Though CI-1040 was ineffective in preclinical MPN model with inactivation of Nf1, it induced markedly extended survival in insertional mutagenesis models of AMLs initiated by Nf1 inactivation (Lauchle et al., 2009). However, insufficient antitumor activity, poor solubility and low bioavailability of CI-1040 precluded further clinical development of this compound from Phase II study (Rinehart et al., 2004).

PD325901 was developed as a significantly more potent MEK inhibitor (IC_{50} of 1nM against purified MEK1/2) with longer *in vivo* duration than CI-1040 (Barrett et al., 2008; Lauchle et al., 2009; Sebolt-Leopold and Herrera, 2004; Solit et al., 2006). PD0325901 have been shown to prolong survival of $Kras^{G12D}$ or $Nf^{-/-}$ MPN mice (Chang et al., 2013; Lyubynska et al., 2011). However, PD0325901 has minimal activity in *RAS*-mutant tumors, and was associated with more severe toxicity than CI-1040, including blurred vision as well as acute neurotoxicity (Haura et al., 2010; Solit et al., 2006; Wang et al., 2007). The clinical development of PD0325901 has been suspended. Prompted by the successful use of this inhibitor in MPN mouse models, we have tested the efficacy of PD0325901 in mice transplanted with $N\text{-}Ras^{G12D}$/Cbfβ-SMMHC leukemic cells. In line

with the toxicity found in clinical trials and contrary to the MPN studies in mice, we have also observed toxicity and no efficacy in our studies (data not shown). These results suggest a possibility that targeting hyperactive Ras signaling could be differentially effective in MPN, where *RAS* and *NF1* mutations seem to initiate the diseases, versus AML, where RAS and FLT3 mutations are thought to serve as cooperating events.

AZD6244 is another highly selective MEK1/2 inhibitor with an IC_{50} of 14nM against purified MEK1/2 (Yeh et al., 2007). AZD6244 has an potent antitumor activity against a panel of mouse xenograft models of colorectal, pancreatic, liver, skin and lung cancer (Davies et al., 2007; Haass et al., 2008; Huynh et al., 2007; Yeh et al., 2007). Some phase II studies of AZD6244 in subjects with solid tumors showed clinical improvements companied by a high rate of adverse events (Carvajal et al., 2014; Janne et al., 2013; Patel et al., 2013). However, a recent phase II study of AZD6244 in advanced AML showed only modest and transient antitumor activity [NCT00588809, (Ward et al., 2012)].

GDC-0973 is a potent, selective, orally active MEK inhibitor with a biochemical IC_{50} of 4.2nM against MEK1 (Hoeflich et al., 2012; Rice et al., 2012). GDC-0973 has antitumor efficacy in *BRAF* and *KRAS* mutant human xenograft tumor models (Hoeflich et al., 2012). A phase I dose-escalating of study of GDC-0973 was initiated in subjects with solid tumors and preliminary results indicated the drug was well tolerated [NCT00467779, (Fremin and Meloche, 2010)]. However, though GDC-0973 was as potent as PD0325901 in affecting viability of Cbfβ-SMMHC/N-Ras^{G12D} leukemic cells *in*

vitro, we did not observe a significant survival benefit by GDC-0973 *in vivo* (data not shown).

GSK1120212 is an orally available, selective MEK inhibitor [nanomolar activity against purified MEK 1 and MEK 2 kinases, (Yamaguchi et al., 2011)] with antitumor activity in mouse xenograft models (Gilmartin et al., 2011). A recent study has revealed a high efficacy of GSK1120212 in combination with imatinib mesylate in a murine model of BCR-ABL–independent imatinib mesylate-resistant chronic myeloid leukemia with sustained MEK/ERK signaling (Ma et al., 2014). A Phase III study of GSK1120212 has shown that it improved rates of progression-free and overall survival among patients who had metastatic melanoma with a BRAF V600E or V600K mutation, compared to chemotherapy [NCT01245062, (Flaherty et al., 2012)]. Another recent Phase II clinical trial demonstrated utility of GSK1120212 in relapsed/refractory myeloid neoplasm [NCT00920140, (Bachegowda et al., 2013)]. This MEK inhibitor could be a good candidate to be tested in our preclinical $Cbfb^{MYH11}/Nras^{G12D}$ leukemic mouse model.

MEK162 is a novel small molecule inhibitor of the kinases MEK1/2. A recent Phase II study disclosed that MEK162 was the first targeted therapy to show activity in patients with *NRAS*-mutated melanoma [NCT01320085, (Ascierto et al., 2013)]. There are several Phase I/II clinical trials just starting to recruit patients for applying MEK162 in advanced AMLs (NCT02089230 and NCT01885195). Taken together, it will provide valuable information for future clinical trials utilizing MEK inhibitors as a cooperating drug in AMLs to test efficacy of those inhibitors in our preclinical inv(16) AML mouse model harboring $Nras^{G12D}$ mutation.

Even though KRAS and NRAS only differ in the hypervariable region (HVR) (Karnoub and Weinberg, 2008), constitutively activated mutant K-Ras induced a more aggressive MPN than constitutively activated mutant N-Ras (Braun et al., 2004; Li et al., 2011). A recent report has revealed the dependency of K-Ras^{G12D} mediated MPN on activities of PI3K p110α isoform in mice (Gritsman et al., 2014), suggesting that cells harboring mutant K-RAS may have different dependency on downstream signaling pathways compared to cells harboring mutant N-RAS. Another interesting result from this study is that THP1 cells [a human AML cell line harboring a *NRAS*^{G12D} mutation (Morgan et al., 2001)] were sensitive to low dose of MEK inhibitor MEK162 but not PI3K inhibitor BYL719, but those two inhibitors synergized in affecting proliferation of THP1 cells (Gritsman et al., 2014). Therefore, it will be interesting to test in our mouse model if a similar synergy between MEK inhibitor and PI3K inhibitor can be achieved, even though we did not observe a significant dependency on PI3K signaling for survival of Cbfβ-SMMHC/N-Ras^{G12D} leukemic cells.

IV.E The novel small molecule AI-10-49 targeting CBFβ-SMMHC

Acute myeloid leukemia is a polyclonal disease that different clones share a founding mutation(s) and differ in the presence of secondary mutations (Jan and Majeti, 2013). As such, AML follows a clonal evolution, as recently revealed by studies performing whole-genome sequencing of patient samples (Ding et al., 2012; Welch et al., 2012). Studies have shown that chemotherapy failed to eradicate the founding clone and the pre-leukemic cells harboring the driver mutations (Ding et al., 2012; Shlush et al.,

2014). Accordingly, current chemotherapy treatment induce a high complete remission (CR) rate in the initial treatment but a low 5 year overall survival (OS) rate and high incidence of relapse even in the favorable karyotype inv(16) AML patients (Lowenberg et al., 2011; Marcucci et al., 2005; Pulsoni et al., 2008; Ravandi et al., 2007; Schlenk et al., 2004).

The inv(16) is a driver mutation invariably present in *de novo* and relapse samples (Castilla et al., 1999; Kuo et al., 2006; Laczika et al., 1998; Shih et al., 2008). It induces AML upon acquisition of other cooperating mutations (Castilla et al., 1999; Castilla et al., 2004; Kuo et al., 2006; Kuo et al., 2009; Paschka et al., 2013). The development of inhibitors that target CBFβ-SMMHC activity without affecting the normal CBF function would represent an ideal therapy in treating inv(16) AMLs and might reduce the relapse risk. In collaboration with four research laboratories we have recently developed the small molecule AI-10-49, a first generation CBFβ-SMMHC/RUNX inhibitor with great efficacy in reducing viability of human and murine inv(16) AML cells. This inhibitor shows significant efficacy *in vivo*, using our N-Ras^{G12D}/CBFβ-SMMHC mouse model with minimal toxicity to normal hematopoietic cells. However, we should point out that even though the medium latency was significantly delayed in mice treated with AI-10-49 compared with mice treated with DMSO, mice treated with AI-10-49 eventually died of leukemia. It would be unlikely for those mice to develop drug resistance in just 10 days of treatment. The possible explanation is that the 10-day treatment with AI-10-49 only reduced the pool of leukemic-initiating cells but not eradicated them. AI-10-49 has poor water solubility and precipitation of the inhibitor was observed in the peritoneal of the

treated mice where the injection was performed (data not shown). Therefore, the future direction for modifying AI-10-49 will be to improve the solubility of it without affecting the efficacy.

This work serves as a proof-of-principle that the transcriptional factors, which are often disrupted in AML, can become therapeutic targets. In the next subchapter, I will discuss some future directions on this exciting inhibitor in both basic research and therapeutic aspect.

IV.F Future perspectives

IV.F.1 Studying downstream targets in Cbfβ-SMMHC and N-Ras^{G12D} leukemias

My thesis has identified the pro-apoptotic protein *Bim* as a potential downstream target of both Cbfβ-SMMHC and N-Ras^{G12D}. Conceivably, other potential targets could also participate in the anti-apoptotic phenotype observed.

We have found that the expression level of Bcl2 significantly increased in *Cbfb*$^{+/MYH11}$ pre-leukemic cells but not in *Nras*$^{+/G12D}$/*Cbfb*$^{+/MYH11}$ pre-leukemic cells compared to wildtype bone marrow progenitor cell. Notably, AML1-ETO activates transcription of the BCL-2 gene in U937 cells (Klampfer et al., 1996). These data suggest that Bcl-2 may be an important target downstream of Cbfβ-SMMHC in providing survival advantage to pre-leukemic and leukemic cells. To test this hypothesis, we could treat the bone marrow cells harboring Cbfβ-SMMHC with AI-10-49 or shRNA specifically targeting the fusion protein and then assay the levels of Bcl-2. In addition,

transplantation of *Cbfb*$^{+/MYH11}$ or *Nras*$^{+/G12D}$/*Cbfb*$^{+/MYH11}$ leukemic cells with reduced Bcl-2 levels into recipient mice will evaluate whether up-regulation of Bcl-2 is required for maintenance of leukemic cells. Other Bcl-2 family proteins could also serve as potential downstream targets of Nras^{G12D} in providing survival benefits, such as Bad, Puma, and Noxa, which are regulated by either MEK/ERK or PI3K pathways (McCubrey et al., 2008). In addition, a RNA sequencing analysis comparing pre-leukemic and leukemic cells harboring either Cbfβ-SMMHC and/or Nras^{G12D} mutations to normal hematopoietic progenitor cells will systemically unravel potential downstream targets downstream not only involved in pro-survival but also involved in other potential mechanisms related to leukemogenesis.

IV.F.2 Dissecting differential roles of Cbfβ-SMMHC between pre-leukemic and leukemic cells

The concept of oncogene addiction postulates that despite the diverse array of genetic lesions typical of cancer, some tumors rely on a single dominant oncogene for growth and survival, so that inhibition of this specific oncogene is sufficient to halt the neoplastic phenotype. This concept was introduced by Bernard Weinstein in 1990s (Weinstein, 2000; Weinstein, 2002; Weinstein et al., 1997) and has been demonstrated *in vivo* for several oncogenes. For example, mouse models using an inducible *MYC* oncogene have shown that MYC-driven skin papillomas, lymphomas, and osteosarcomas can all be reversed upon inactivation of MYC (Felsher and Bishop, 1999; Jain et al., 2002;

Pelengaris et al., 1999). Other experimental examples of oncogene addiction include *H-RAS*, *K-RAS*, *ABL*, *HER2* and *EGFR* [reviewed in (Torti and Trusolino, 2011)].

We have found that Cbfβ-SMMHC is necessary but not sufficient to induce acute myeloid leukemia (Castilla et al., 1999; Castilla et al., 2004; Kuo et al., 2006). Experiments presented in my thesis, performed by a collaborative effort, have shown that inhibition of binding between CBFβ-SMMHC and RUNX1 specifically reduced the viability of murine and human inv(16) leukemic cells with minimal toxicity in normal hematopoietic cells. In contrast, while retroviral overexpression of Cbfβ-SMMHC in wild type murine hematopoietic progenitor cells blocked differentiation, overexpression of Cbfβ-SMMHC with two point mutations (G61A and N104A) which abrogate binding to Runx proteins (Lukasik et al., 2002; Tang et al., 2000) did not block differentiation of the progenitor cells (unpublished data, Castilla laboratory). These observations lead to a hypothesis that inhibition of CBFβ-SMMHC in pre-leukemic cells restores the normal differentiation of hematopoietic progenitor cells while inhibition of CBFβ-SMMHC in inv(16) leukemic cells induces cell death because the leukemic cells are "addicted" to the presence of the fusion protein for survival.

To test this hypothesis, differentiation and apoptosis of wild type and pre-leukemic cells expressing Cbfβ-SMMHC could be tested after treatment with the specific inhibitor. To study the mechanism in the differential responses to inhibition of the fusion protein, a RNA sequencing analysis in pre-leukemic and leukemic cells treated with either DMSO or CBFβ-SMMHC inhibitor AI-10-49 could be valuable to identify potential targets. CBFβ-SMMHC has been shown to suppress the granulocytic differentiation factor

C/EBPα in inv(16) AML (Helbling et al., 2005). Thus, the transcriptional factor C/EBPα could be a potential target in differentiation block induced by Cbfβ-SMMHC. In addition, based on my thesis, Bcl-2 family proteins such as Bim and Bcl-2 could be potential targets in survival advantage provided by Cbfβ-SMMHC. We can verify candidate targets revealed by the analysis and investigate into the functions of those candidates by means of retroviral overexpression and/or RNA interference.

IV.F.3 Combination therapies and drug resistance

We have established the efficacy of CBFβ-SMMHC inhibitor AI-10-49 in murine and human inv(16) leukemic cells and *Cbfb*^{MYH11}/*Nras*^{G12D} mouse models. The next step is to combine AI-10-49 with conventional and/or other targeted therapies in our preclinical leukemic mouse models.

The standard regimen for AML induction therapy involves cytotoxic reagents, cytarabine and anthracycline. It will be informative to test the combination of AI-10-49 with either cytarabine or doxorubicin (one type of anthracycline) *in vitro* and *in vivo*. A recent report showed that the sequential application of targeted and cytotoxic anticancer drugs were critical for enhanced cell death (Lee et al., 2012). Therefore, the order of application between AI-10-49 and conventional drugs should be taken into account. Furthermore, combination of AI-10-49 with other targeted therapies is another future direction. We did not observe efficacy of PD0325901 and GDC-0973 in the conditions we tested *in vivo*. We should modify the dose and duration of these two inhibitors and also assess other MEK inhibitors which have been using in the clinical trials, such as

GSK1120212 and MEK162. Finally, drug resistance is a major concern during therapy. In the case of AI-10-49, it will be important to develop drug resistance studies in order to define the potential mutations that can drive drug resistance. Based on the suggested binding targets within the CBFβ-SMMHC protein, mutations blocking the binding of the compound to the protein or compensatory mutations elsewhere in the protein that compensate the allosteric modifications in the runt homology domain binding domain of CBFβ region induced by AI-10-49 (Illendula et al., manuscript submitted) should be explored.

IV.G Closing remarks

My thesis establishes a very useful mouse model with allelic expression of two important oncogenes recurrently found in human inv(16) AMLs cases: *Cbfb*^{MYH11} and *Nras*^{G12D} and demonstrates that Nras^{G12D} and Cbfβ-SMMHC provide survival advantage to murine pre-leukemic progenitors. The activity of Nras^{G12D} cannot bypass the myeloid differentiation block by Cbfβ-SMMHC. These two oncogenes cooperate in leukemogenesis and repress the pro-apoptotic gene *Bcl2l11*. The leukemic cells depend on MEK/ERK pathway to survival.

In collaboration with other research laboratories, we develop and test a novel bivalent compound AI-10-49 which selectively disrupts CBFβ-SMMHC/RUNX binding and restores normal CBF function. My thesis indicates the specificity of AI-10-49 in affecting viability of leukemic cells without affecting normal hematopoietic cells. This compound also significantly prolongs the survival of the Nras^{G12D}/Cbfβ-SMMHC

leukemic mice. AI-10-49 will be a very promising first-generation drug in inhibiting transcriptional factors and also benefits the research on mechanisms of inv(16) AMLs.

Appendix A

This appendix lists all the supplementary figures for Chapter II.

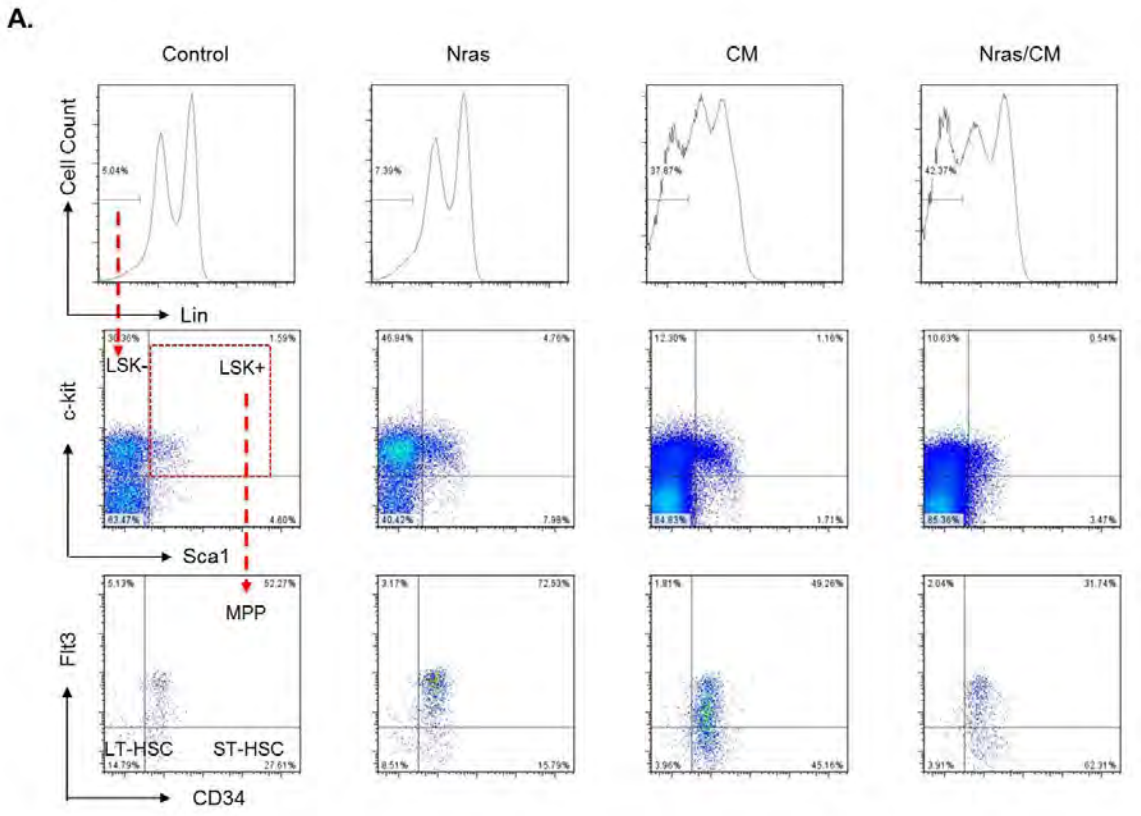

Figure A.1 Cbfβ-SMMHC and N-Ras^{G12D} expand pre-leukemic stem and progenitor cells. Representative FACS plots of Figure II.1 and Figure II.2. (A) cells were first gated by FSC-A/SSC-A, then gated for single cells by FSC-A/FSC-H, then gated for 7-AAD^{-} population and the following gating of LSK^{+} compartment from Control, Nras, CM and Nras/CM mice is shown.

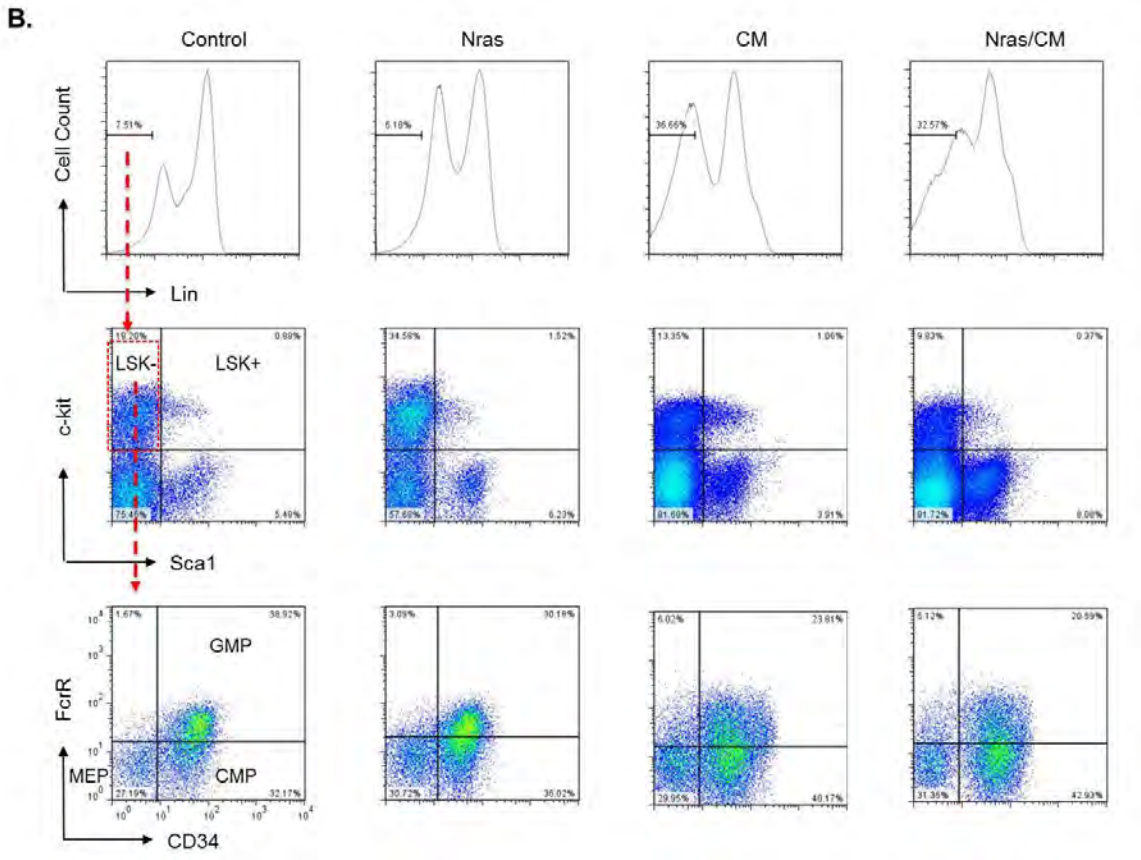

Figure A.1 (Cont.) Cbfβ-SMMHC and N-Ras^{G12D} expand pre-leukemic stem and progenitor cells. (B) cells were first gated by FSC-A/SSC-A, then gated for single cells by FSC-A/FSC-H, then gated for 7-AAD^{-} population and the following gating of LSK^{-} compartment from Control, Nras, CM and Nras/CM mice is shown.

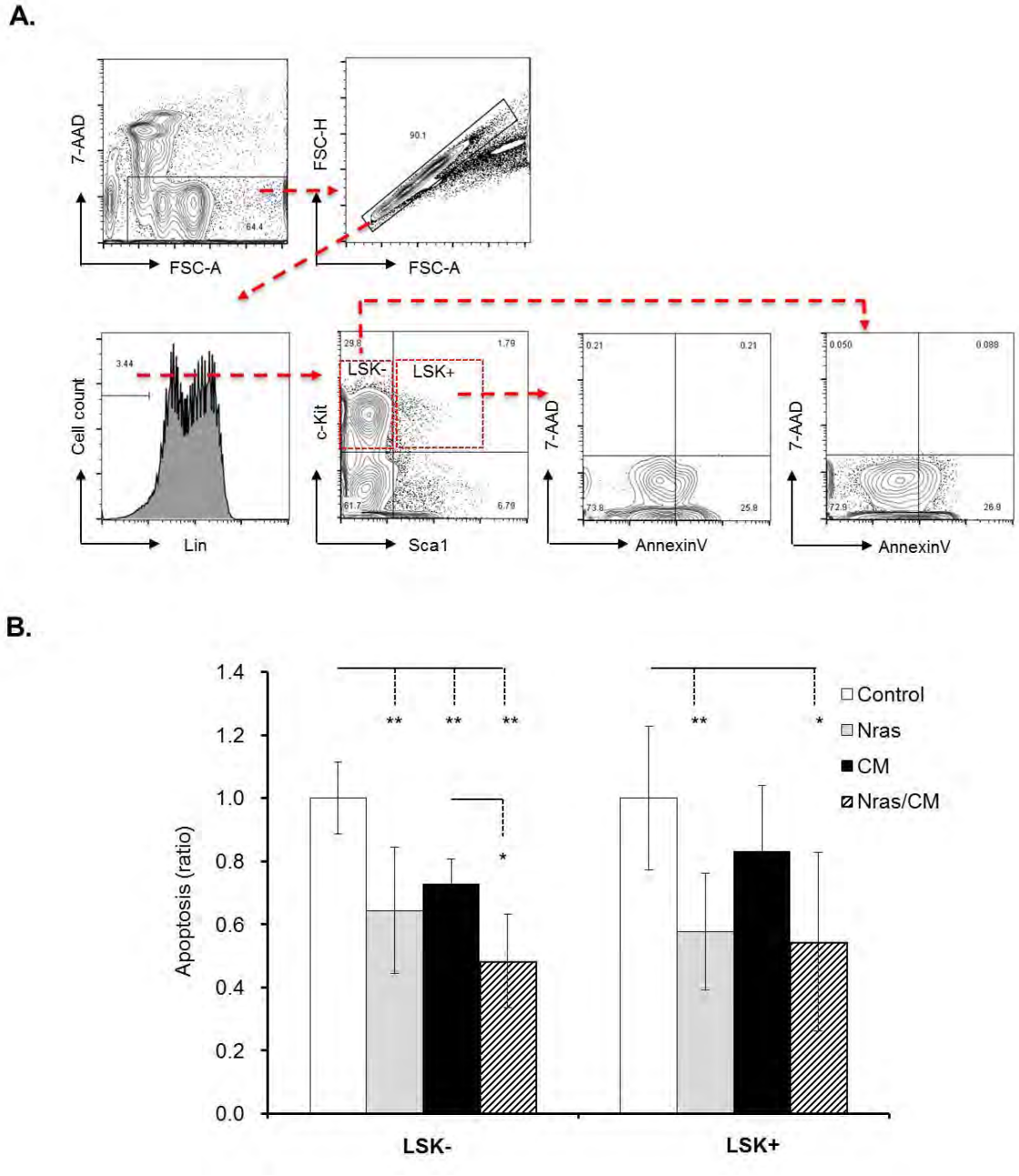

Figure A.2 Apoptosis analyses of bone marrow pre-leukemic cells expressing Cbfβ-SMMHC and N-Ras^{G12D}. Data from Figure II.4 were reanalyzed by a different gating strategy. (A) the gating of each compartment is shown; (B) quantification of the ratio of apoptosis levels for LSK$^-$ (left) and LSK$^+$ (right) cells of each group normalized to control group (n=4-9, *, P<0.05; **, P<0.01). Note: since it is a reanalysis of the previous data and one of the data file was corrupted, the dataset shown here is incomplete compared to the dataset in Figure II.4.

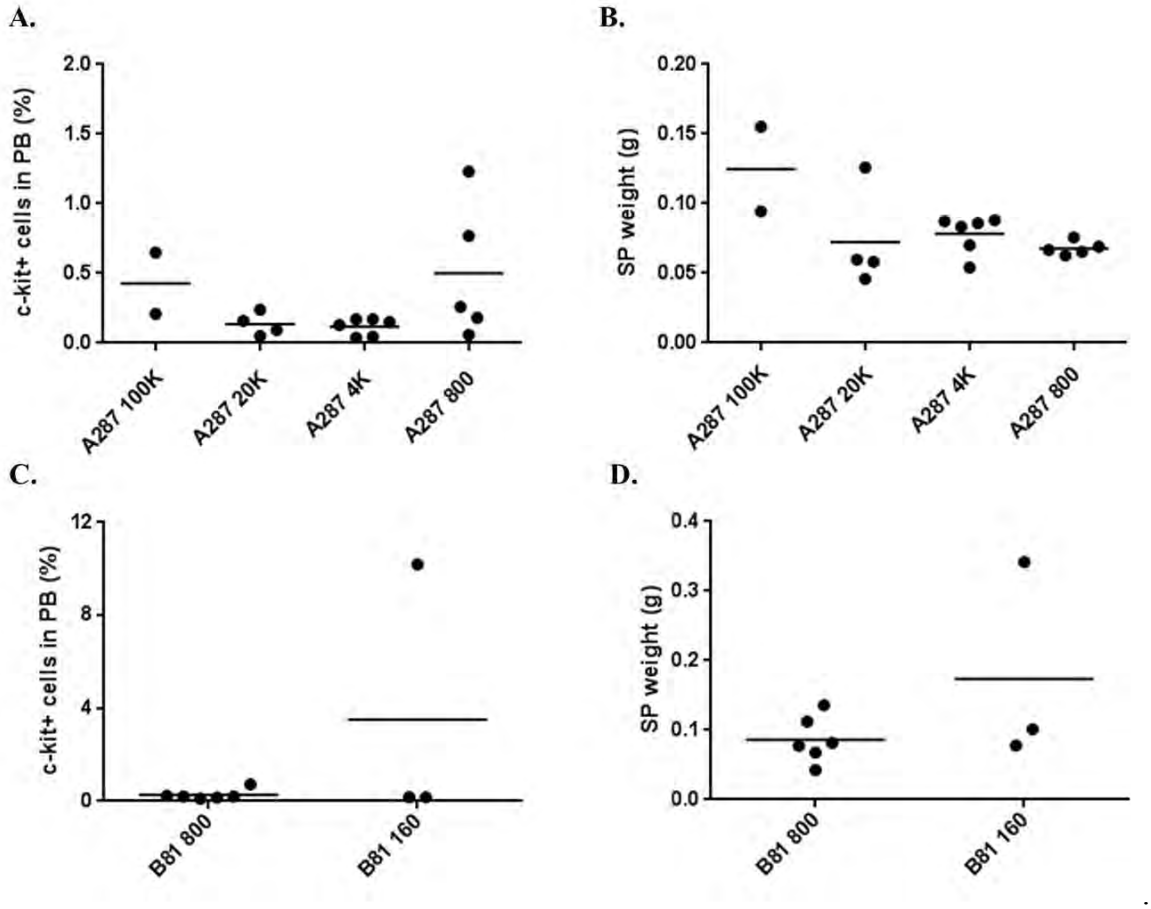

Figure A.3 Percentage of c-kit positive cells in peripheral blood and spleen weight of mice transplanted with either CM or Nras/CM leukemic clones. At the end of limiting dilution experiments (36 weeks) shown in Figure II.6F and H, mice that shows no appearance of diseases were analyzed for c-kit positive cells in peripheral blood (PB) by flow cytometry (A, C) or spleen (SP) weight (B, D). (A-B) CM A287 leukemic clone (C-D) Nras/CM B81 leukemic clone.

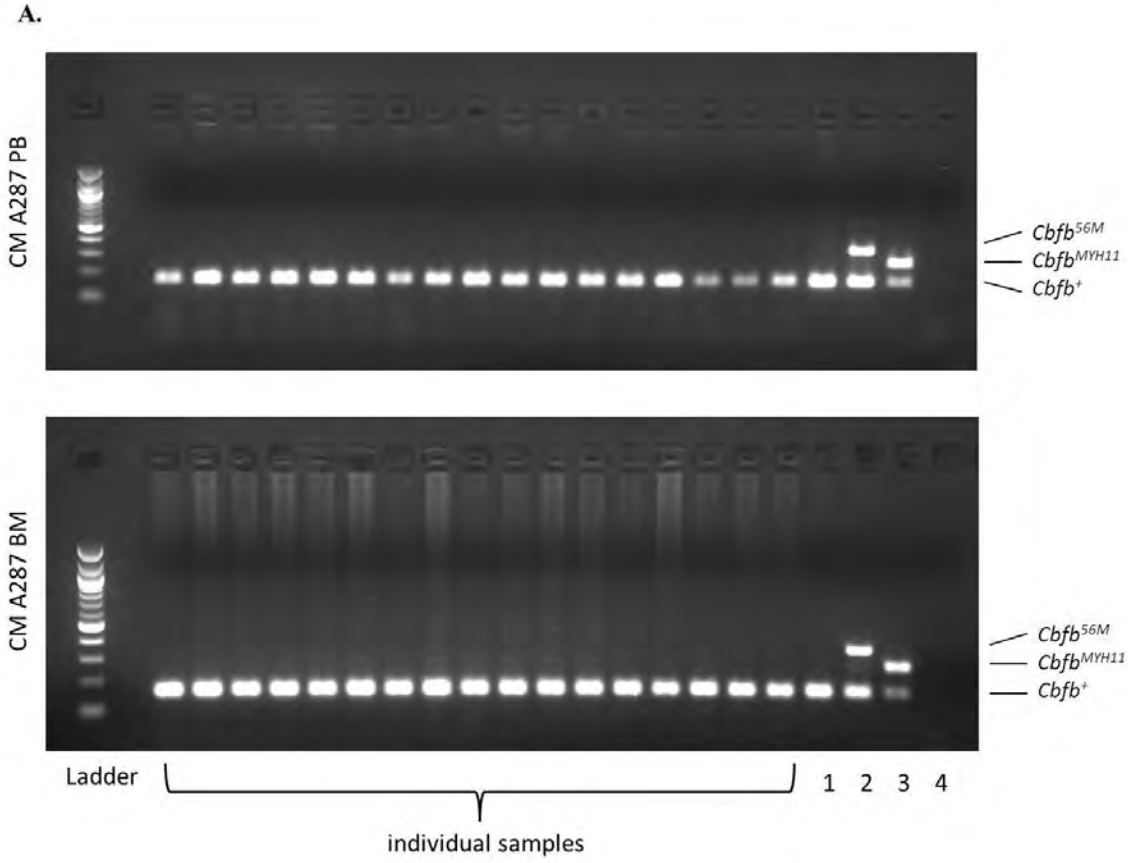

Figure A.4 Genomic PCR of peripheral blood and bone marrow cells from mice transplanted with either CM or Nras/CM leukemic clones. At the end of limiting dilution experiments (36 weeks) shown in Figure II.6F and II6.H, mice that shows no appearance of diseases were analyzed for recombination of *Cbfb*^{MYH11} alleles in white blood cells from peripheral blood (PB) and bone marrow (BM) by genomic PCR. (A) CM A287 leukemic clones. Lanes 1-4 are PCR products from control genomic DNA samples: Lane 1, *Mx1-Cre*; Lane 2, *Nras*$^{+/LSL-G12D}$*/Cbfb*$^{+/56M}$; Lane 3, bulk Nras/CM Leukemic cells; Lane 4, water.

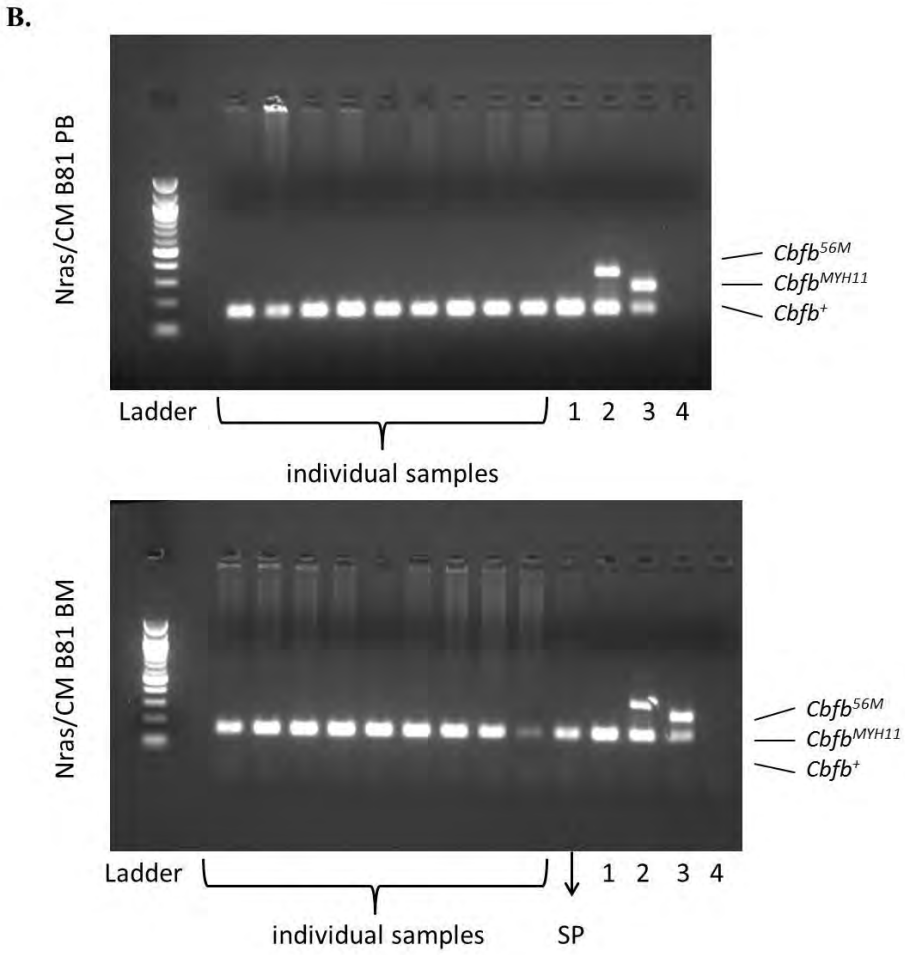

Figure A.4 (Cont.) Genomic PCR of peripheral blood and bone marrow cells from mice transplanted with either CM or Nras/CM leukemic clones. (B) Nras/CM A287 leukemic clones. Lanes 1-4 are PCR products from control genomic DNA samples: Lane 1, *Mx1-Cre*; Lane 2, $Nras^{+/LSL\text{-}G12D}/Cbfb^{+/56M}$; Lane 3, bulk Nras/CM Leukemic cells; Lane 4, water. SP represent the PCR reaction of spleen cells from the mice that transplanted with 160 Nras/CM B81 leukemic cells and present splenomegaly (spleen weight is 0.3418g as shown in Figure A.2D).

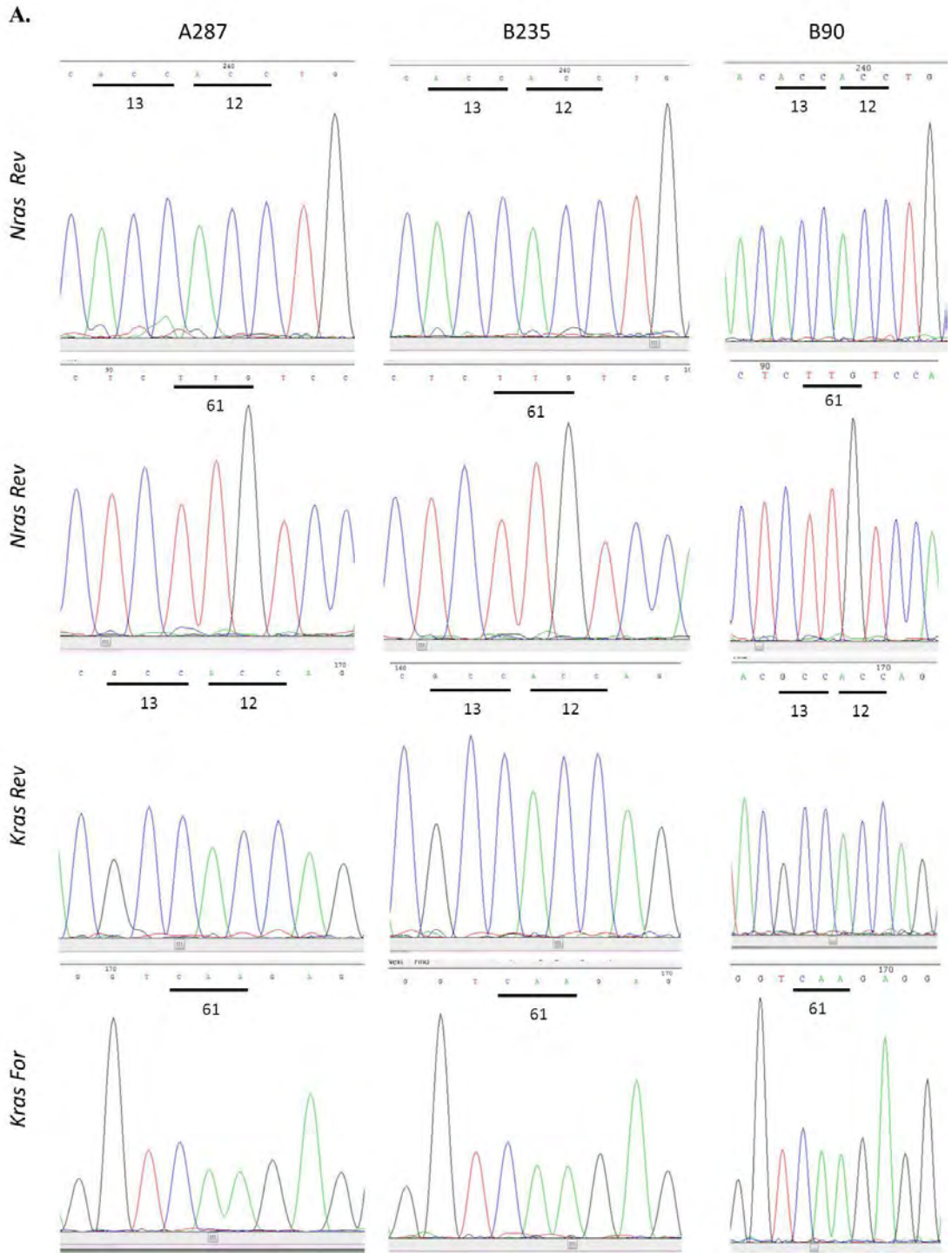

Figure A.5 No mutations in codon 12, 13, 61 of Nras or Kras genes were identified in CM leukemic cells. Complete sequencing results of Figure II.9 for 9 CM leukemic cells. (A) Clone A287, B235 and B90.

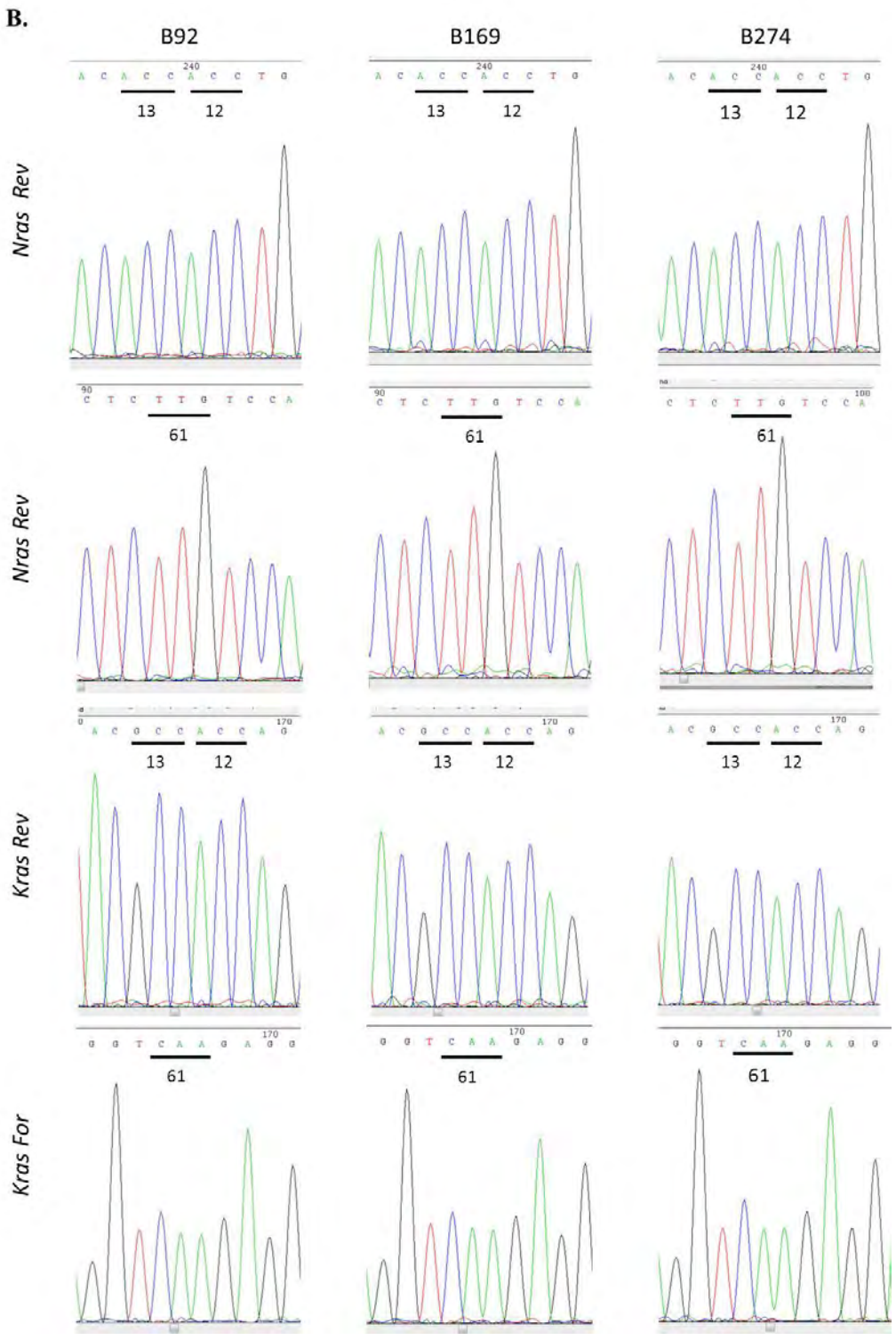

Figure A.5 (Cont.) No mutations in codon 12, 13, 61 of Nras or Kras genes were identified in CM leukemic cells. (B) Clone B92, B169 and B274.

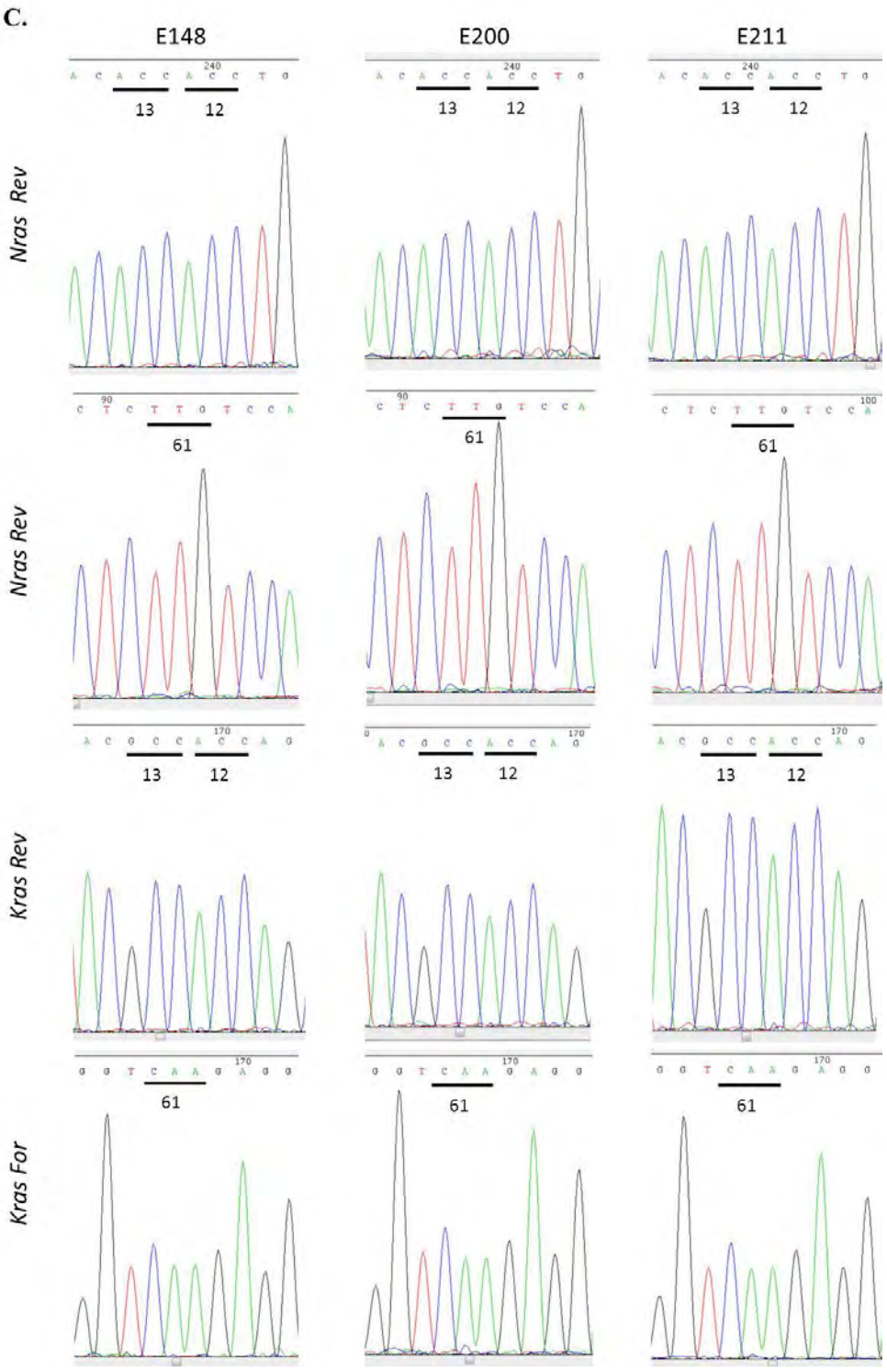

Figure A.5 (Cont.) No mutations in codon 12, 13, 61 of Nras or Kras genes were identified in CM leukemic cells. (C) Clone E148, E200 and E211.

Appendix B

This appendix lists all the plasmid constructs that I made during my stay in the Castilla laboratory.

B.1 Construct information

Table B.1 List of the construct information

Construct	Size	Insert Name	Insert Species	Insert Size	Insert Origin
pMSCV2.2-CBL WT	9.4 kb	*CBL* wildtype cDNA	Human	2800 bp	pCR2.1-CBL WT
pMSCV2.2-CBL Major	9.3 kb	*CBL* Major mutant cDNA	Human	2668 bp	pCR2.1-CBL Major
pMSCV2.2-CBL Minor	9.4 kb	*CBL* Minor mutant cDNA	Human	2840 bp	pCR2.1-CBL Minor
pMSCV2.2-Bim-EL	7.2 kb	*Bcl2l11* extra-long isoform cDNA	Mouse	618 bp	pCMV-Tag2B Flag Bim-EL
pMSCV2.2-mSt18	10.9 kb	*St18* full length cDNA	Mouse	4340 bp	pCR-BluntII-TOPO mSt18
LMP-shScrambled	8.0 kb	*Scrambled* shRNA	/	100 bp	97mer oligonucleotide
LMP-shId2-1	8.0 kb	*Id2* shRNA-1	Mouse	104 bp	97mer oligonucleotide
LMP-shId2-2	8.0 kb	*Id2* shRNA-2	Mouse	104 bp	97mer oligonucleotide
LMP-shId2-3	8.0 kb	*Id2* shRNA-3	Mouse	104 bp	97mer oligonucleotide
LMP-shId2-4	8.0 kb	*Id2* shRNA-4	Mouse	104 bp	97mer oligonucleotide
LMP-shId2-5	8.0 kb	*Id2* shRNA-5	Mouse	104 bp	97mer oligonucleotide
LMP-shCM	8.0 kb	*CBFB-MYH11* shRNA	Human (Junction)	104 bp	97mer oligonucleotide
LMP-shBim-1	8.0 kb	*Bcl2l11* shRNA-1	Mouse	104 bp	97mer oligonucleotide
LMP-shBim-3	8.0 kb	*Bcl2l11* shRNA-3	Mouse	104 bp	97mer oligonucleotide
LMP-shBim-4	8.0 kb	*Bcl2l11* shRNA-4	Mouse	104 bp	97mer oligonucleotide

Note: All the plasmids listed have Ampicillin antibiotic resistance markers.

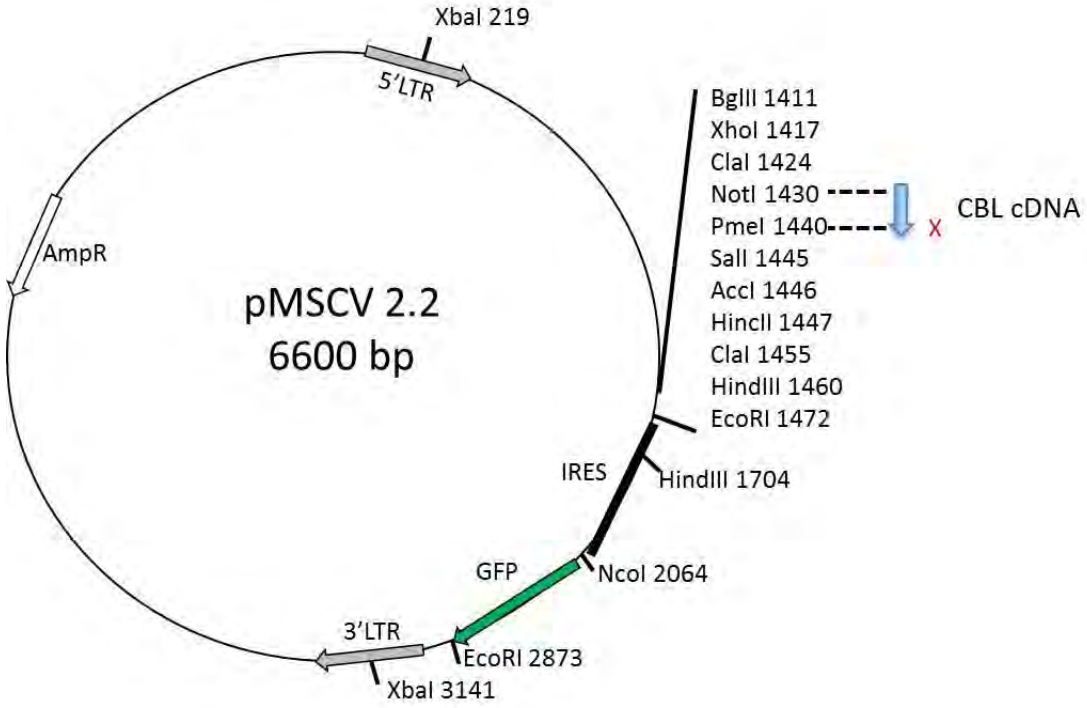

Figure B.1 Construction of pMSCV2.2-CBL. All the *CBL* wild type or mutant forms (*CBL* WT, Major and Minor) were cloned from original pCR2.1-CBL constructs by digesting with NotI and SpeI restriction enzymes. SpeI site of the inserts were blunted and the inserts were ligated into the pMSCV2.2 plasmids between NotI and PmeI sites. The red crossing sign means the PmeI site was destroyed by this cloning method.

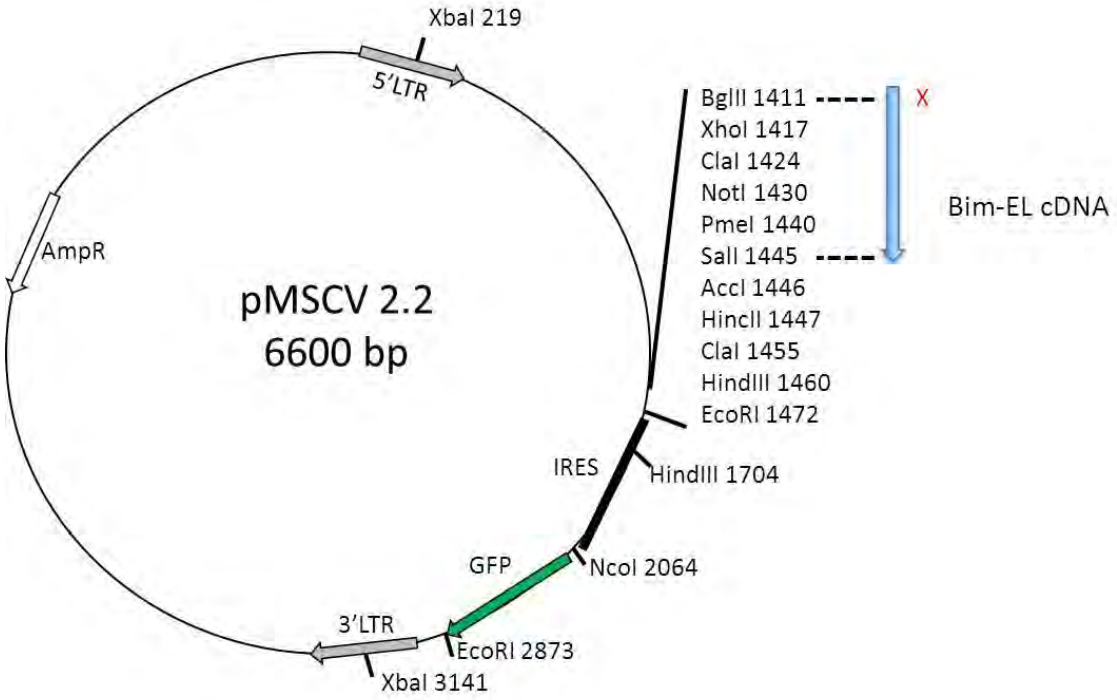

Figure B.2 Construction of pMSCV2.2-Bim-EL. The *Bcl2l11* extra-long isoform cDNA was cloned from original pCMV-Tag2B Flag Bim-EL construct by digesting with BamHI and SalI restriction enzymes. The insert was ligated into the pMSCV2.2 plasmid between BglII and SalI sites. The red crossing sign means the BglII site was destroyed by this cloning method.

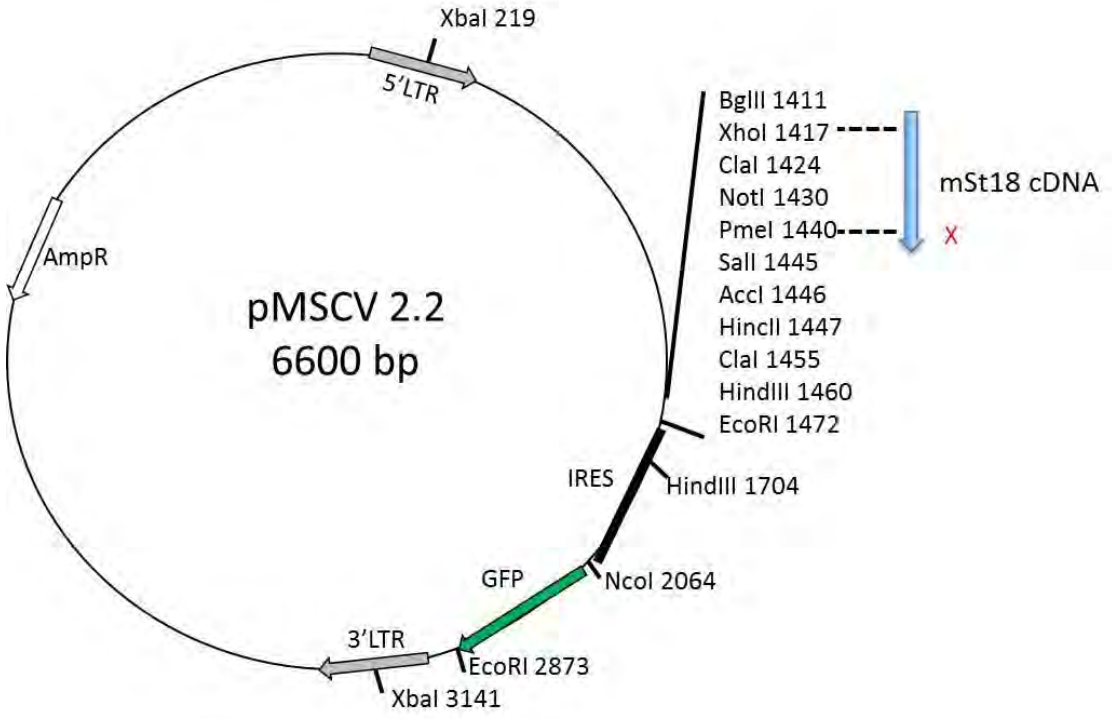

Figure B.3 Construction of pMSCV2.2-mSt18. Mouse St18 full length cDNA were cloned from the original pCR-BluntII-TOPO mSt18 construct by digesting with XhoI and BamHI restriction enzymes. BamHI site of the insert was blunted and the insert was ligated into the pMSCV2.2 plasmid between XhoI and PmeI sites. The red crossing sign means the PmeI site was destroyed by this cloning method.

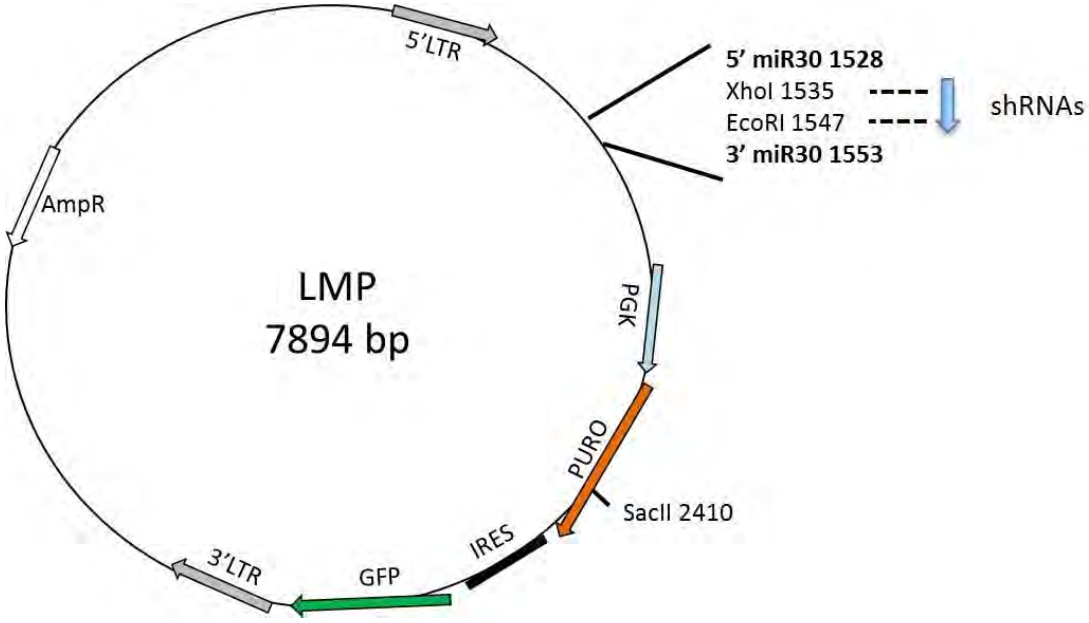

Figure B.4 Construction of LMP-shRNA. All the LMP-shRNAs were constructed according to LMP technical manual (Thermo Scientific, Billerica, MA). Basically, 97mer (or 90mer as in the Scrambled shRNA case) oligonucleotides containing unique shRNA sequences were amplified using primers containing XhoI and EcoRI restriction sites. The amplification products and LMP plasmid were digested by XhoI and EcoRI restriction enzymes and ligated to create LMP-shRNAs constructs.

B.2 Sequence of Inserts

B.2.1 cDNA inserts:

Note: all the sequences of the cDNAs below are color-coded. Blue, restriction enzyme sites of the inserts; Red, start and stop codons of open reading frame.

a. *CBL* wild type cDNA

GC_GGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTCAGGCCATGGCC
GGCAACGTGAAGAAGAGCTCTGGGGCCGGGGGCGGCAGCGGCTCCGGGGGC
TCGGGTTCGGGTGGCCTGATTGGGCTCATGAAGGACGCCTTCCAGCCGCACC
ACCACCACCACCACCACCTCAGCCCCCACCCGCCGGGGACGGTGGACAAGAA
GATGGTGGAGAAGTGCTGGAAGCTCATGGACAAGGTGGTGCGGTTGTGTCAG
AACCCAAAGCTGGCGCTAAAGAATAGCCCACCTTATATCTTAGACCTGCTAC
CAGATACCTACCAGCATCTCCGTACTATCTTGTCAAGATATGAGGGGAAGAT
GGAGACACTTGGAGAAAATGAGTATTTTAGGGTGTTTATGGAGAATTTGATG
AAGAAAACTAAGCAAACCATAAGCCTCTTCAAGGAGGGAAAAGAAAGAATG
TATGAGGAGAATTCTCAGCCTAGGCGAAACCTAACCAAACTGTCCCTCATCTT
CAGCCACATGCTGGCAGAACTAAAAGGAATCTTTCCAAGTGGACTCTTTCAG
GGAGACACATTTCGGATTACTAAAGCAGATGCTGCGGAATTTTGGAGAAAAG
CTTTTGGGGAAAAGACAATAGTCCCTTGGAAGAGCTTTCGACAGGCTCTACA
TGAAGTGCATCCCATCAGTTCTGGGCTGGAGGCCATGGCTCTGAAATCCACT
ATTGATCTGACCTGCAATGATTATATTTCGGTTTTTGAATTTGACATCTTTACC
CGACTCTTTCAGCCCTGGTCCTCTTTGCTCAGGAATTGGAACAGCCTTGCTGT
AACTCATCCTGGCTACATGGCTTTTTTGACGTATGACGAAGTGAAAGCTCGGC
TCCAGAAATTCATTCACAAACCTGGCAGTTATATCTTCCGGCTGAGCTGTACT
CGTCTGGGTCAGTGGGCTATTGGGTATGTTACTGCTGATGGGAACATTCTCCA
GACAATCCCTCACAATAAACCTCTCTTCCAAGCACTGATTGATGGCTTCAGGG
AAGGCTTCTATTTGTTTCCTGATGGACGAAATCAGAATCCTGATCTGACTGGC
TTATGTGAACCAACTCCCCAAGACCATATCAAAGTGACCCAGGAACAATATG
AATTATACTGTGAGATGGGCTCCACATTCCAACTATGTAAAATATGTGCTGAA
AATGATAAGGATGTAAAGATTGAGCCCTGTGGACACCTCATGTGCACATCCT
GTCTTACATCCTGGCAGGAATCAGAAGGTCAGGGCTGTCCTTTCTGCCGATGT
GAAATTAAAGGTACTGAACCCATCGTGGTAGATCCGTTTGATCCTAGAGGGA
GTGGCAGCCTGTTGAGGCAAGGAGCAGAGGGAGCTCCCTCCCCAAATTATGA
TGATGATGATGATGAACGAGCTGATGATACTCTCTTCATGATGAAGGAATTG
GCTGGTGCCAAGGTGGAACGGCCGCCTTCTCCATTCTCCATGGCCCCACAAG
CTTCCCTTCCCCCGGTGCCACCACGACTTGACCTTCTGCCGCAGCGAGTATGT
GTTCCCTCAAGTGCTTCTGCTCTTGGAACTGCTTCTAAGGCTGCTTCTGGCTCC
CTTCATAAAGACAAACCATTGCCAGTACCTCCCACACTTCGAGATCTTCCACC

ACCACCGCCTCCAGACCGGCCATATTCTGTTGGAGCAGAATCCCGACCTCAA
AGACGCCCCTTGCCTTGTACACCAGGCGACTGTCCCTCCAGAGACAAACTGC
CCCCTGTCCCCTCTAGCCGCCTTGGAGACTCATGGCTGCCCCGGCCAATCCCC
AAAGTACCAGTATCTGCCCCAAGTTCCAGTGATCCCTGGACAGGAAGAGAAT
TAACCAACCGGCACTCACTTCCATTTTCATTGCCCTCACAAATGGAGCCCAGA
CCAGATGTGCCTAGGCTCGGAAGCACGTTCAGTCTGGATACCTCCATGAGTA
TGAATAGCAGCCCATTAGTAGGTCCAGAGTGTGACCACCCCAAAATCAAACC
TTCCTCATCTGCCAATGCCATTTATTCTCTGGCTGCCAGACCTCTTCCTGTGCC
AAAACTGCCACCTGGGGAGCAATGTGAGGGTGAAGAGGACACAGAGTACAT
GACTCCCTCTTCCAGGCCTCTACGGCCTTTGGATACATCCCAGAGTTCACGAG
CATGTGATTGCGACCAGCAGATTGATAGCTGTACGTATGAAGCAATGTATAA
TATTCAGTCCCAGGCGCCATCTATCACCGAGAGCAGCACCTTTGGTGAAGGG
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ATGATGGGTATGATGTCCCAAAGCCACCTGTGCCGGCCGTGCTGGCCCGCCG
AACTCTCTCAGATATCTCTAATGCCAGCTCCTCCTTTGGCTGGTTGTCTCTGGA
TGGTGATCCTACAACAAATGTCACTGAAGGTTCCCAAGTTCCCGAGAGGCCT
CCAAAACCATTCCCGCGGAGAATCAACTCTGAACGGAAAGCTGGCAGCTGTC
AGCAAGGTAGTGGTCCTGCCGCCTCTGCTGCCACCGCCTCACCTCAGCTCTCC
AGTGAGATCGAGAACCTCATGAGTCAGGGGTACTCCTACCAGGACATCCAGA
AAGCTTTGGTCATTGCCCAGAACAACATCGAGATGGCCAAAAACATCCTCCG
GGAATTTGTTTCCATTTCTTCTCCTGCCCATGTAGCTACCTAGCACACCATCTC
CAAGCCGAATTCCAGCACACTGGCGGCCGTTACTAG_AAAC

b. *CBL* Major mutant cDNA (missing exon8)

GC_GGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTCAGGCCATGGCC
GGCAACGTGAAGAAGAGCTCTGGGGCCGGGGGCGGCAGCGGCTCCGGGGGC
TCGGGTTCGGGTGGCCTGATTGGGCTCATGAAGGACGCCTTCCAGCCGCACC
ACCACCACCACCACCACCTCAGCCCCCACCCGCCGGGGACGGTGGACAAGAA
GATGGTGGAGAAGTGCTGGAAGCTCATGGACAAGGTGGTGCGGTTGTGTCAG
AACCCAAAGCTGGCGCTAAAGAATAGCCCACCTTATATCTTAGACCTGCTAC
CAGATACCTACCAGCATCTCCGTACTATCTTGTCAAGATATGAGGGGAAGAT
GGAGACACTTGGAGAAAATGAGTATTTTAGGGTGTTTATGGAGAATTTGATG
AAGAAAACTAAGCAAACCATAAGCCTCTTCAAGGAGGGAAAAGAAAGAATG
TATGAGGAGAATTCTCAGCCTAGGCGAAACCTAACCAAACTGTCCCTCATCTT
CAGCCACATGCTGGCAGAACTAAAAGGAATCTTTCCAAGTGGACTCTTTCAG
GGAGACACATTTCGGATTACTAAAGCAGATGCTGCGGAATTTTGGAGAAAAG
CTTTTGGGGAAAAGACAATAGTCCCTTGGAAGAGCTTTCGACAGGCTCTACA
TGAAGTGCATCCCATCAGTTCTGGGCTGGAGGCCATGGCTCTGAAATCCACT
ATTGATCTGACCTGCAATGATTATATTTCGGTTTTTGAATTTGACATCTTTACC
CGACTCTTTCAGCCCTGGTCCTCTTTGCTCAGGAATTGGAACAGCCTTGCTGT
AACTCATCCTGGCTACATGGCTTTTTTGACGTATGACGAAGTGAAAGCTCGGC
TCCAGAAATTCATTCACAAACCTGGCAGTTATATCTTCCGGCTGAGCTGTACT
CGTCTGGGTCAGTGGGCTATTGGGTATGTTACTGCTGATGGGAACATTCTCCA

GACAATCCCTCACAATAAACCTCTCTTCCAAGCACTGATTGATGGCTTCAGGG
AAGGCTTCTATTTGTTTCCTGATGGACGAAATCAGAATCCTGATCTGACTGGC
TTATGTGAACCAACTCCCCAAGACCATATCAAAGTGACCCAGGAATCAGAAG
GTCAGGGCTGTCCTTTCTGCCGATGTGAAATTAAAGGTACTGAACCCATCGTG
GTAGATCCGTTTGATCCTAGAGGGAGTGGCAGCCTGTTGAGGCAAGGAGCAG
AGGGAGCTCCCTCCCCAAATTATGATGATGATGATGATGAACGAGCTGATGA
TACTCTCTTCATGATGAAGGAATTGGCTGGTGCCAAGGTGGAACGGCCGCCT
TCTCCATTCTCCATGGCCCCACAAGCTTCCCTTCCCCCGGTGCCACCACGACT
TGACCTTCTGCCGCAGCGAGTATGTGTTCCCTCAAGTGCTTCTGCTCTTGGAA
CTGCTTCTAAGGCTGCTTCTGGCTCCCTTCATAAAGACAAACCATTGCCAGTA
CCTCCCACACTTCGAGATCTTCCACCACCACCGCCTCCAGACCGGCCATATTC
TGTTGGAGCAGAATCCCGACCTCAAAGACGCCCCTTGCCTTGTACACCAGGC
GACTGTCCCTCCAGAGACAAACTGCCCCCTGTCCCCTCTAGCCGCCTTGGAGA
CTCATGGCTGCCCCGGCCAATCCCCAAAGTACCAGTATCTGCCCCAAGTTCCA
GTGATCCCTGGACAGGAAGAGAATTAACCAACCGGCACTCACTTCCATTTTC
ATTGCCCTCACAAATGGAGCCCAGACCAGATGTGCCTAGGCTCGGAAGCACG
TTCAGTCTGGATACCTCCATGAGTATGAATAGCAGCCCATTAGTAGGTCCAG
AGTGTGACCACCCCAAAATCAAACCTTCCTCATCTGCCAATGCCATTTATTCT
CTGGCTGCCAGACCTCTTCCTGTGCCAAAACTGCCACCTGGGGAGCAATGTG
AGGGTGAAGAGGACACAGAGTACATGACTCCCTCTTCCAGGCCTCTACGGCC
TTTGGATACATCCCAGAGTTCACGAGCATGTGATTGCGACCAGCAGATTGAT
AGCTGTACGTATGAAGCAATGTATAATATTCAGTCCCAGGCGCCATCTATCAC
CGAGAGCAGCACCTTTGGTGAAGGGAATTTGGCCGCAGCCCATGCCAACACT
GGTCCCGAGGAGTCAGAAAATGAGGATGATGGGTATGATGTCCCAAAGCCAC
CTGTGCCGGCCGTGCTGGCCCGCCGAACTCTCTCAGATATCTCTAATGCCAGC
TCCTCCTTTGGCTGGTTGTCTCTGGATGGTGATCCTACAACAAATGTCACTGA
AGGTTCCCAAGTTCCCGAGAGGCCTCCAAAACCATTCCCGCGGAGAATCAAC
TCTGAACGGAAAGCTGGCAGCTGTCAGCAAGGTAGTGGTCCTGCCGCCTCTG
CTGCCACCGCCTCACCTCAGCTCTCCAGTGAGATCGAGAACCTCATGAGTCA
GGGGTACTCCTACCAGGACATCCAGAAAGCTTTGGTCATTGCCCAGAACAAC
ATCGAGATGGCCAAAAACATCCTCCGGGAATTTGTTTCCATTTCTTCTCCTGC
CCATGTAGCTACCTAGCACACCATCTCCAAGCCGAATTCCAGCACACTGGCG
GCCGTTACTAG_AAAC

c. *CBL* Minor mutant cDNA (with exon 8 + 40 bp from intron 8-9)

GC_GGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTCAGGCCATGGCC
GGCAACGTGAAGAAGAGCTCTGGGGCCGGGGGCGGCAGCGGCTCCGGGGGC
TCGGGTTCGGGTGGCCTGATTGGGCTCATGAAGGACGCCTTCCAGCCGCACC
ACCACCACCACCACCACCTCAGCCCCCACCCGCCGGGGACGGTGGACAAGAA
GATGGTGGAGAAGTGCTGGAAGCTCATGGACAAGGTGGTGCGGTTGTGTCAG
AACCCAAAGCTGGCGCTAAAGAATAGCCCACCTTATATCTTAGACCTGCTAC
CAGATACCTACCAGCATCTCCGTACTATCTTGTCAAGATATGAGGGGAAGAT
GGAGACACTTGGAGAAAATGAGTATTTTAGGGTGTTTATGGAGAATTTGATG

AAGAAAACTAAGCAAACCATAAGCCTCTTCAAGGAGGGAAAAGAAAGAATG
TATGAGGAGAATTCTCAGCCTAGGCGAAACCTAACCAAACTGTCCCTCATCTT
CAGCCACATGCTGGCAGAACTAAAAGGAATCTTTCCAAGTGGACTCTTTCAG
GGAGACACATTTCGGATTACTAAAGCAGATGCTGCGGAATTTTGGAGAAAAG
CTTTTGGGGAAAAGACAATAGTCCCTTGGAAGAGCTTTCGACAGGCTCTACA
TGAAGTGCATCCCATCAGTTCTGGGCTGGAGGCCATGGCTCTGAAATCCACT
ATTGATCTGACCTGCAATGATTATATTTCGGTTTTTGAATTTGACATCTTTACC
CGACTCTTTCAGCCCTGGTCCTCTTTGCTCAGGAATTGGAACAGCCTTGCTGT
AACTCATCCTGGCTACATGGCTTTTTTGACGTATGACGAAGTGAAAGCTCGGC
TCCAGAAATTCATTCACAAACCTGGCAGTTATATCTTCCGGCTGAGCTGTACT
CGTCTGGGTCAGTGGGCTATTGGGTATGTTACTGCTGATGGGAACATTCTCCA
GACAATCCCTCACAATAAACCTCTCTTCCAAGCACTGATTGATGGCTTCAGGG
AAGGCTTCTATTTGTTTCCTGATGGACGAAATCAGAATCCTGATCTGACTGGC
TTATGTGAACCAACTCCCCAAGACCATATCAAAGTGACCCAGGAACAATATG
AATTATACTGTGAGATGGGCTCCACATTCCAACTATGTAAAATATGTGCTGAA
AATGATAAGGATGTAAAGATTGAGCCCTGTGGACACCTCATGTGCACATCCT
GTCTTACATCCTGGCAGCAGCGACTTTTTTCAGCTATGTAATAACCTTGGAAA
ATTCGAATCAGAAGGTCAGGGCTGTCCTTTCTGCCGATGTGAAATTAAAGGT
ACTGAACCCATCGTGGTAGATCCGTTTGATCCTAGAGGGAGTGGCAGCCTGT
TGAGGCAAGGAGCAGAGGGAGCTCCCTCCCCAAATTATGATGATGATGATGA
TGAACGAGCTGATGATACTCTCTTCATGATGAAGGAATTGGCTGGTGCCAAG
GTGGAACGGCCGCCTTCTCCATTCTCCATGGCCCCACAAGCTTCCCTTCCCCC
GGTGCCACCACGACTTGACCTTCTGCCGCAGCGAGTATGTGTTCCCTCAAGTG
CTTCTGCTCTTGGAACTGCTTCTAAGGCTGCTTCTGGCTCCCTTCATAAAGAC
AAACCATTGCCAGTACCTCCCACACTTCGAGATCTTCCACCACCACCGCCTCC
AGACCGGCCATATTCTGTTGGAGCAGAATCCCGACCTCAAAGACGCCCCTTG
CCTTGTACACCAGGCGACTGTCCCTCCAGAGACAAACTGCCCCCTGTCCCCTC
TAGCCGCCTTGGAGACTCATGGCTGCCCCGGCCAATCCCCAAAGTACCAGTA
TCTGCCCCAAGTTCCAGTGATCCCTGGACAGGAAGAGAATTAACCAACCGGC
ACTCACTTCCATTTTCATTGCCCTCACAAATGGAGCCCAGACCAGATGTGCCT
AGGCTCGGAAGCACGTTCAGTCTGGATACCTCCATGAGTATGAATAGCAGCC
CATTAGTAGGTCCAGAGTGTGACCACCCCAAAATCAAACCTTCCTCATCTGCC
AATGCCATTTATTCTCTGGCTGCCAGACCTCTTCCTGTGCCAAAACTGCCACC
TGGGGAGCAATGTGAGGGTGAAGAGGACACAGAGTACATGACTCCCTCTTCC
AGGCCTCTACGGCCTTTGGATACATCCCAGAGTTCACGAGCATGTGATTGCG
ACCAGCAGATTGATAGCTGTACGTATGAAGCAATGTATAATATTCAGTCCCA
GGCGCCATCTATCACCGAGAGCAGCACCTTTGGTGAAGGGAATTTGGCCGCA
GCCCATGCCAACACTGGTCCCGAGGAGTCAGAAAATGAGGATGATGGGTATG
ATGTCCCAAAGCCACCTGTGCCGGCCGTGCTGGCCCGCCGAACTCTCTCAGA
TATCTCTAATGCCAGCTCCTCCTTTGGCTGGTTGTCTCTGGATGGTGATCCTAC
AACAAATGTCACTGAAGGTTCCCAAGTTCCCGAGAGGCCTCCAAAACCATTC
CCGCGGAGAATCAACTCTGAACGGAAAGCTGGCAGCTGTCAGCAAGGTAGTG
GTCCTGCCGCCTCTGCTGCCACCGCCTCACCTCAGCTCTCCAGTGAGATCGAG
AACCTCATGAGTCAGGGGTACTCCTACCAGGACATCCAGAAAGCTTTGGTCA

TTGCCCAGAACAACATCGAGATGGCCAAAAACATCCTCCGGGAATTTGTTTC
CATTTCTTCTCCTGCCCATGTAGCTACCTAGCACACCATCTCCAAGCCGAATT
CCAGCACACTGGCGGCCGTTACTAG_AAAC

d. *Bcl2l11* extra-long isoform cDNA

A_GATCCATGGCCAAGCAACCTTCTGATGTAAGTTCTGAGTGTGACAGAGAA
GGTGGACAATTGCAGCCTGCTGAGAGGCCTCCCCAGCTCAGGCCTGGGGCCC
CTACCTCCCTACAGACAGAACCGCAAGGTAATCCCGACGGCGAAGGGGACC
GCTGCCCCCACGGCAGCCCTCAGGGCCCGCTGGCCCCACCGGCCAGCCCTGG
CCCTTTTGCTACCAGATCCCCACTTTTCATCTTTGTGAGAAGATCTTCTCTGCT
GTCCCGGTCCTCCAGTGGGTATTTCTCTTTTGACACAGACAGGAGCCCGGCAC
CCATGAGTTGTGACAAGTCAACACAAACCCCAAGTCCTCCTTGCCAGGCCTT
CAACCACTATCTCAGTGCAATGGCTTCCATAAGACAGTCTCAAGAGGAACCT
GAAGATCTGCGCCCGGAGATACGGATTGCACAGGAGCTACGGCGGATCGGA
GACGAGTTCAACGAAACTTACACAAGGAGGGTGTTTGCAAATGATTACCGCG
AGGCTGAAGACCACCCTCAAATGGTTATCTTACAACTGTTACGCTTTATCTTC
CGTCTGGTATGGAGAAGGCATTGAGAATTCGATATCAAGCTTATCGATACCG_
TCGAC

Note: This sequence containing 229-819 (coding sequence) of mouse *Bcl2l11* extra-long isoform cDNA with silence mutations.

e. *St18* full length cDNA

C_TCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGCCCTTTACACGC
AGGTGCTTATCTCCGAGCAGTCAAAGGCACTGGATGTCATATCATGGAGACT
ACCTTTCAAGGCAACAGCTTTAGTGTCTCCTGTCAGCACACCAGAGAGAAGA
GAAATAGTTTTGGAAACAAACACAAAAAACTCCAGCTCCAGATGAACCAGG
GTAGTGTGTGGTGGCAGGAACAGCTGACTCATTAGTAGAGAGCAGTGAAAGC
AATGACATTTCTTCTGTTCAAGTTCAGTAGCTGCTAGAGAAGATGGATGCCGA
GGTTGAAGATAAAACGTTGCATACTCTTTCAAAAGGAACTGAAGTTCCAATG
GATTCTCTGATCCCAGAGCTCAGGGTTCCATATGATTGCTCTATGGCGAAGAA
GAGGAGAGCTGAAGAGCAGGCATCGGGAGTTCCAATTAACAAGAGGAAATC
TCTGCTAATGAAACCCCGGCACTACAGCCCAGACATGGGCTGCAAGGAAAGC
CCAGACAACAGGAACGAGGATGATGGCCTCCTAGAAACAAACGACCATGCC
ACTGCAGATGAAATCATGGTAAAATCCATGGATGAAACTCTTCATTTACCTGC
ACAAGACAGCTCACTCCAGAAGAAAGACCAATACACATGTTATCCAGAGCTC
ATGGTCAAATCTCTGGTGCACTTAGGCAAATTTGAGGAAAGTGAGTCTGTGC
AGACCACATGTGAGAACTTAAATGGCAGCAGTATCCAGTCTTTAAAAGCAGA
GAGTGATGAAGCACATGAAGGTTCTATGGTTCATTCCGACAATGGAAGAGAC
AAAGTCCACCATTCCCAGCCACCTTTCTGCTCTTCTGGTGACAGCGAAAGTGA
CTCTGACAGTGCAGAGAATGGGTGGGGCAATGGCTCCAACTCATCAGAAGAC
ACTGACACTCACAAAGGCCCCAAACACAAGCTGACATACAATAGAAAGGAC

CTGTTGGAAGTTCCTGAGATAAAAGCTGAAGATGACAAGTTTATCCCTTGTG
AGAACAGGTGTGATTCTGACACAGATGGGAGAGACCCACAGAACTCTCATAT
GGAACCCTTGGTTGTGAAAGCCCAGCCTTCCTTCCCAGAGGTTGAAGAGGGT
GAGAGCCTGGCCACAGTAACAGAAGAGCCTGCTGAGGTGGAGAAAGCAAAG
GGGAACCTGAGTTTGCTGGAACAGGCTATTGCTCTACAGGCTGAACGAGGTT
CTGTCTTCCATCACACTTATAAGGAGCTGGACCGATTTTTCCTGGACCACCTG
GCAAGGGAACGGAGGCAACCTAGAGTTACTGATGCAAATGGAAGACAAATC
TTTACTAATAAACATTCACCAAGACCTGAAAGGAGGGAGGCCAAGTGCCCCA
TTCCTGGCTGTGATGGCACGGGGCACGTAACAGGGCTCTACCCTCACCACCG
CAGCCTTTCTGGGTGCCCCCACAAAGTGCGAGTTCCTCTGGAAATTCTCGCCA
TGCATGAGAACGTGCTCAAGTGTCCCACGCCAGGATGCACAGGAAGGGGAC
ATGTGAACAGCAATCGCAACACTCACAGAAGTCTTTCTGGTTGTCCGATTGCT
GCAGCTGAAAAGCTGGCAATGACCCAGGACAAAAGTCAACTTGATTCTTCTC
AGACGGGGCAGTGTCCTGAGCAGGCACACAGGGTGAATTTGGTGAAGCAAA
TTGAATTTAATTTCCGCTCACATGCCATCACTTCTCCAAGAGCCTCTGCATCA
AAAGAACAAGAGAAGTTTGGGAAAGTACCGTTTGATTATGCCAGTTTTGATG
CACAAGTATTTGGGAAACGTCCTCTCCTACAAACAGGGCAAGGACAAAAAGC
ACCACCATTTCCTGAATCAAAGCATTTTTCAAATCCAGTGAAATTTCCTAATG
GACTGCCTAGTGCTGGCGCCCACACACAGAGCACAGTCCGTGCCAGCTCTTA
TGGCCATGGTCAGTACAGTGAAGACACCCACATAGCAGCAGCTGCTGCCATC
CTGAACCTTTCCACCCGCTGCAGGGAAGCCACAGACATCCTCTCCAACAAAC
CACAAAGCCTGCGTGCCAAGGGAGCTGAGATAGAAGTAGATGAAAACGGCA
CATTGGACTTAAGCATGAAAAAAAATCGAATCCACGACAAGTCTATACCCCC
AACTTCCTCACCTACTACAATTACAACCCCATCCTCATCCCCATTCAACGCAA
GCAGCCTTCTGGTCAATGCTGCCTTCTATCAGGCCCTCTCTGATCAAGAAGGC
TGGAATGTGCCCATCAACTATAGCAAATCCCATGGGAAGACAGAGGAGGAG
AAAGAGAAAGATCCTGTGAACTTCCTAGAAAATTTAGAGGAAAAAAAGTTTG
CTGGAGAGGCCTCTATCCCAAGCCCAAAGCCCAAGCTGCATACAAGAGATCT
CAAGAAAGAATTAATCACCTGTCCAACACCAGGATGTGATGGAAGCGGCCAT
GTCACAGGCAACTATGCATCTCACCGCAGTGTCTCTGGATGTCCTCTAGCAGA
TAAGACTCTGAAGTCTCTCATGGCTGCCAACTCTCAAGAGCTTAAGTGTCCAA
CCCCAGGTTGTGATGGTTCTGGTCATGTGACTGGAAACTATGCCTCCCACCGA
AGCTTGTCTGGTTGCCCTCGTGCAAGGAAAGGTGGCATCAAAATGACCCCAA
CAAAAGAAGAAAAAGAAGACTCTGAACTTAGATGCCCTGTAATAGGGTGTG
ACGGCCAAGGCCACATATCAGGTAAATACACATCACACCGCACAGCTTCTGG
CTGTCCTCTGGCTGCCAAAAGACAGAAAGAGAATCCCCTCAATGGGGCACCT
CTCTCCTGGAAACTGAACAAGCAAGAGCTTCCTCACTGTCCTCTGCCAGGAT
GCAATGGTCTGGGTCATGTAAACAACGTTTTTGTCACCCACAGAAGCTTATCT
GGATGCCCTCTTAATGCACAAGCTATCAAAAAAGTCAAGGTCTCTGAGGAAC
TAATGACTATCAAGCTCAAAGCAACTGGGGGTATTGATGGTGATGAAGAAAT
TAGGCATCTGGATGAAGAAATCAAGGAACTGAATGAATCCAACCTTAAAATT
GAAGCAGATATGATGAAACTTCAGACCCAGATAACATCTATGGAGAGCAACT
TGAAGACCATAGAGGAGGAGAACAAGCTTATAGAACAGAGCAATGAGAGTC
TGCTGAAGGAGCTGGCAGGGCTTAGCCAGGCTCTCATCTCCAGCCTTGCTGA

CATCCAACTTCCACAGATGGGGCCTATCAATGAGCAGAATTTTGAAGCATAT
GTAAATACTCTCACAGACATGTACAGCAATCTGGAACAGGACTATTCCCCAG
AATGCAAAGCTCTACTGGAAAGCATCAAGCAGGCGGTGAAGGGCATCCATGT
GTAGAACGACAGTATGCAGGAAACAGAGGTCACTACGGCCATGAACTCCCA
CAAATCTGCAGGGGCCTAATACCCCTGGAGCCCAGAGGCTGTCCCTGTGTTT
ATAATTGCAACATTGCACTAATTTTTCCCCAGCTGACATAAAAAGGAAAGAA
AAACTATGATAAACTCTTTGGATTAAAAGCAATGCAGTCAATTATTAAACTC
GTTTATTTTCATATCTTTTTCTTTTCTTTCTTCATTGCACTCTTTATTTTGTAAA
GTATATGTAAAAATAAATGTGACATTTTATATTTTATTTATTACTAATCAAAG
AGTTTTTTATCTTTTAACTGCATTTTGAAGTCTACTATATTTTTACAGGTGTGT
TTATTAATTTATCTTCTAATAGGATTTAAATAGAAATGCTGTTCTCAAATCAC
ATATCTTTCTGGATTTAAATGAGAAAAACAAAACAAAAGAAATGTATGAAGG
AAATCCTGATGTCAGGACTACACTGTGGTCAAGCTTGGTTTCTACTGTGCACT
TCCATTTCTCCTTTACTGGTTTTGTTTTTATAAATGGCTTTTGGTAATGTGAGC
TGTGAAGGATTATGAAGATAAATGGCGCCCACTAGTGGGAAATCCACACTCA
CAAAAGCACAGGATGTTAGCAAACAGCGCACTCAGAACTTGATAGCAATAAT
TAAATAAAATAGTCAGCATAGTATTCCACATGGCTGCAGAGATTTAATTAAT
ATGAATTATTTATTTAGACTGCTTAAAGCATACATTTTAAGTGAATCAGATTT
GTCTTCTTGTTTTTCAAGTCATATTGGCTAGTTGGCAACCCTTTCCCAAAATGA
GAAATAAGCTTTGATGGGACCAGCTTGGCTAAGGGCGAATTCCAGCACACTG
GCGGCCGTTACTAGTGGATC_AAAC

B.2.2 shRNA inserts

Note: all the sequences of the shRNAs below are color-coded. Black, mir30 context; Red, sense sequence of the shRNA; Green, mir30 loop; Blue, anti-sense sequence of shRNA.

a. *Scrambled* shRNA (93mer, used in Chapter II)

TGCTGTTGACAGTGAGCGCGAAAGTCTTAGAGATAGTATAGTGAAGCCACAG
ATGTATACTATCTCTAAGACTTTCTTGCCTACTGCCTCGGA

b. *Id2* shRNA-1 (97mer)

TGCTGTTGACAGTGAGCGCGCAGCACGTCATCGATTACATTAGTGAAGCCAC
AGATGTAATGTAATCGATGACGTGCTGCATGCCTACTGCCTCGGA

c. *Id2* shRNA-2 (97mer)

TGCTGTTGACAGTGAGCGCGCAAAGTACTCTGTGGCTAAATAGTGAAGCCAC
AGATGTATTTAGCCACAGAGTACTTTGCTTGCCTACTGCCTCGGA

d. *Id2* shRNA-3 (97mer)

TGCTGTTGACAGTGAGCGCCGACTGCTACTCCAAGCTCAATAGTGAAGCCAC
AGATGTATTGAGCTTGGAGTAGCAGTCGTTGCCTACTGCCTCGGA

e. *Id2* shRNA-4 (97mer)

TGCTGTTGACAGTGAGCGCCTGAGCTTATGTCGAATGATATAGTGAAGCCAC
AGATGTATATCATTCGACATAAGCTCAGATGCCTACTGCCTCGGA

f. *Id2* shRNA-5 (97mer)

TGCTGTTGACAGTGAGCGCATGTCGAATGATAGCAAAGTATAGTGAAGCCAC
AGATGTATACTTTGCTATCATTCGACATATGCCTACTGCCTCGGA

g. *CBFB-MYH11* shRNA (97mer)

TGCTGTTGACAGTGAGCGCCGGGAGGAAATGGAGGTCCATTAGTGAAGCCAC
AGATGTAATGGACCTCCATTTCCTCCCGATGCCTACTGCCTCGGA

h. *Bcl2l11* shRNA-1 (97mer)

TGCTGTTGACAGTGAGCGCGATGTAAGTTCTGAGTGTGACTAGTGAAGCCAC
AGATGTAGTCACACTCAGAACTTACATCATGCCTACTGCCTCGGA

i. *Bcl2l11* shRNA-3 (97mer)

TGCTGTTGACAGTGAGCGACCTCAAATGGTTATCTTACAATAGTGAAGCCAC
AGATGTATTGTAAGATAACCATTTGAGGGTGCCTACTGCCTCGGA

j. *Bcl2l11* shRNA-4 (97mer, used in Chapter II)

TGCTGTTGACAGTGAGCGCGGCCTTTGTACTTAAATATATTAGTGAAGCCACA
GATGTAATATATTTAAGTACAAAGGCCTTGCCTACTGCCTCGGA

Appendix C

This appendix lists all the inhibitors that I used during my stay in the Castilla laboratory.

Table C.1 List of inhibitors

Inhibitor	Target	*in vivo*		Comments
		Dose	Delivery	
PD098059	MEK	/	/	High efficacy, i*n vitro* only
PD325901	MEK	5 mg/kg	O.G.	Toxic *in vivo*
GDC-0973	MEK	10 mg/kg	O.G.	Minimal efficacy *in vivo*
LY294002	PI3K	/	/	High efficacy, i*n vitro* only
GDC-0941	PI3K	100 mg/kg	O.G.	No efficacy *in vivo*
GDC-0980	PI3K/mTOR	/	/	High efficacy *in vitro*
BEZ235	PI3K/mTOR	45 mg/kg	O.G.	Toxic *in vivo*
Rapamycin	mTOR	0.8mg/kg	IP	No efficacy *in vivo*

Notes: O.G., oral gavage; IP, intraperitoneal injection.

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