Cooperating Events in Core Binding Factor Leukemia Development: A Dissertation

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COOPERATING EVENTS IN CORE BINDING FACTOR

LEUKEMIA DEVELOPMENT

A Dissertation presented

By

DMITRI MADERA

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COOPERATING EVENTS IN CORE BINDING FACTOR LEUKEMIA DEVELOPMENT

A Dissertation Presented By

Dmitri Madera

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Abstract

Leukemia is a hematopoietic cancer that is characterized by the abnormal differentiation and proliferation of hematopoietic cells. It is ranked 7th by death rate among cancer types in USA, even though it is not one of the top 10 cancers by incidence (USCS, 2010). This indicates an urgent need for more effective treatment strategies. In order to design the new ways of prevention and treatment of leukemia, it is important to understand the molecular mechanisms involved in development of the disease.

In this study, we investigated mechanisms involved in the development of acute myeloid leukemia (AML) that is associated with CBF fusion genes. The *RUNX1* and *CBFB* genes that encode subunits of a transcriptional regulator complex CBF, are mutated in a subset (20 – 25%) of AML cases. As a result of these mutations, fusion genes called *CBFB-MYH11* and *RUNX1-ETO* arise. The chimeric proteins encoded by the fusion genes provide block in proliferation for myeloid progenitors, but are not sufficient for AML development. Genetic studies have indicated that activation of cytokine receptor signaling is a major oncogenic pathway that cooperates in leukemia development. The main goal of my work was to determine a role of two factors that regulate cytokine signaling activity, the microRNA cluster *miR-17-92* and the thrombopoietin receptor MPL, in their potential cooperation with the CBF fusions in AML development.

We determined that the *miR-17-92* miRNA cluster cooperates with *Cbfb-MYH11* in AML development in a mouse model of human *CBFB-MYH11* AML. We found that

the miR-17-92 cluster downregulates Pten and activates the PI3K/Akt pathway in the leukemic blasts. We also demonstrated that *miR-17-92* provides an anti-apoptotic effect in the leukemic cells, but does not seem to affect proliferation. The anti-apoptotic effect was mainly due to activity of *miR-17* and *miR-20a*, but not *miR-19a* and *miR-19b*.

Our second study demonstrated that wild type Mpl cooperated with *RUNX1-ETO* fusion in development of AML in mice. Mpl induced PI3K/Akt, Ras/Raf/Erk and Jak2/Stat5 signaling pathways in the AML cells. We showed that PIK3/Akt pathway plays a role in AML development both *in vitro* and *in vivo* by increasing survival of leukemic cells. The levels of *MPL* transcript in the AML samples correlated with their response to thrombopoietin (THPO). Moreover, we demonstrated that MPL provides pro-proliferative effect for the leukemic cells, and that the effect can be abrogated with inhibitors of PI3K/AKT and MEK/ERK pathways.

Taken together, these data confirm important roles for the PI3K/AKT and RAS/RAF/MEK pathways in the pathogenesis of AML, identifies two novel genes that can serve as secondary mutations in CBF fusions-associated AML, and in general expands our knowledge of mechanisms of leukemogenesis.

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List of abbreviations

Abbreviation	Term
4E-BP1	4E-binding protein 1
AKT	v-Akt murine thymoma viral oncogene homolog
AMKL	Acute megakaryoblastic leukemia
AMP	Abnormal myeloid progenitor
APL	Acute promyelocytic leukemia
ARF	Alternative reading frame
BAD	BCL2-associated agonist of cell death
Bcl2	B-cell lymphoma 2
BCL2L11	BCL2-like 11
BM	Bone marrow
BMI	B lymphoma Mo-MLV insertion region
C/EBP	CCAAT-enhancer binding protein
CAMT	Congenital amegakaryocytic thrombocytopenia
CBF	Core binding factor
CCND	Cyclin D
CDK	Cyclin-dependent kinase
CEBPA	CCAAT/enhancer binding protein α
CIS3	Cytokine-inducible SH2 protein-1
c-KIT	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
CLL	Chronic lymphocytic leukemia
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
c-MPL	Myeloproliferative leukemia virus oncogene homolog
c-MYC	V-myc myelocytomatosis viral oncogene homolog
CR	Complete remission
CREB	cAMP response element-binding
CSF	Colony stimulating factor
CXCR4	C-X-C chemokine receptor type 4
d.p.c.	Days post conception
DN	Double negative
DNA	Deoxyribonucleic acid
DUSP2	Dual specificity protein phosphatase 2
e.g.	Exempli gratia
EBF	Early B-cell factor

EGR	Early growth response
EKLF	Erythroid Krüppel-like factor
EMSA	Electrophoretic mobility shift assay
ENU	N-ethyl-N-nitrosourea
EPO	Erythropoietin
EPOR	Erythropoietin receptor
ERK	Extracellular signal-regulated kinase
ES	Embryonic stem
ETO	Eight twenty one
ETP	Early T-lineage progenitor
ETS	V-ets avian erythroblastosis virus E26 oncogene homolog
FAB	French-American-British cooperative group
FLI	Friend leukemia integration
FLT	FMS-like tyrosine kinase
FOXO	Forkhead box
FOXP3	Forkhead Box P3
FPD	Familial platelet disorder
GAB	GRB2-associated binding protein
GATA	GATA binding factor
G-CSF	Granulocyte-colony stimulating factor
GEF	Guanine exchange factor
GEP	Gene expression profiling
GFI	Growth factor-independent
GFP	Green fluorescent protein
GM-CSF	Granulocytic-monocytic colony stimulating factor
GMP	Granulocytic-monocytic progenitor
GRB2	GTP-exchange complex growth factor receptor bound-2
GTP	Guanosine triphosphate
HABD	High affinity binding domain
HDAC	Histone deacetylase
HEK	Human embryo kidney
HIF	Hypoxia-induced factor
hMRP8	Human myeloid-restricted protein 8
HSC	Hematopoietic stem cell
i.e.	Id est
i. v.	Intravenous
ID	Inhibition domain
IGF2	Insulin-like growth factor 2
IKK	IκB kinase
IL	Interleukin
inv	Inversion
ITD	Internal tandem duplications

JAK	Janus kinase
JM	Juxtamembrane domain
kb	Kilobase
KO	Knock-out
LMPP	Lymphoid primed multipotent progenitor
LRF	Leukemia/lymphoma related factor
MAPK	MAP kinase
M-CSF	Monocytic-colony stimulating factor
MDS	Myelodysplastic syndrome
MEF2C	Myocyte enhancer factor 2C
MEK1	Mitogen-associated/extracellular regulated kinase 1
MEP	Megakaryocytic-erythroid progenitor
MID	pMSCV-IRES-humanCD4
MID-Mpl	pMSCV-IRES-humanCD4-Mpl
MID-PL2	MID-PlagL2
MIG	pMSCV-IRES-GFP
MIG-R1E	pMSCV-IRES-GFP-RUNX1-ETO
MiR	MicroRNA
MIR17HG	MiR-17-92 cluster host gene
MiRNA	MicroRNA
MLL	Mixed-lineage leukemia
MPL	Myeloproliferative leukemia virus oncogene
MPP	Multipotent progenitor
mRNA	Matrix RNA
mSin3A	Mammalian Sin3 protein A
mTOR	Mammalian target of rapamycin
mTORC	mTOR complex
MYH	Myosin heavy chain 11
MYT1	Myelin transcription factor 1
N-CoR	Nuclear receptor corepressor
NDE1	Nude nuclear distribution gene E homolog 1
NEB	New England Biolabs
NF1	Neurofibromin 1
NFI-A	Nuclear factor I type A
NFκB	Nuclear factor κB
NHR	Nervy homology region
NLS	Nuclear Localization Signal
NMTS	Nuclear matrix targeting signal
NOD-SCID	Nonobese diabetes-severe combined immunodeficiency
NOS	Not otherwise specified
NPM	Nucleophosmin
NRDB	Negative Regulatory region for DNA Binding

nt	Nucleotide
Nud	Nuclear distribution gene
NUDC	Nuclear distribution gene C
OS	Overall survival
PAX	Paired box
PB	Peripheral blood
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDK	3-phosphoinositide-dependent protein kinase
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PIG	Puromycin-IRES-GFP
PIM	Proviral integration site
PIP3	Phosphatidylinositol-3,4,5-triphosphate
pIpC	Polyinosinic-polycytidylic acid
PL2	PlagL2
PLAG	Pleomorphic adenoma gene
PLAGL	Pleomorphic adenoma gene-like
PLK2	Polo-like kinase 2
PTEN	Phosphatase and tensin homolog
PTPN6	Protein tyrosine phosphatase, non-receptor type 6
PU.1	Purine rich binding protein
qRT-PCR	Quantitative reverse transcriptase PCR
R1E	RUNX1-ETO
R1E9a	RUNX1-ETO9a
RAF	Rapidly Accelerated Fibrosarcoma
RAR	Retinoic acid receptor
RAS	Rat sarcoma
RHD	Runt homology domain
Rheb	GTPase Ras homolog enriched in brain
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal RNA
RTK	Receptor tyrosine kinase
RUNX	Runt-related transcription factor
S6K1	p70 S6 kinase 1
SCF	Stem cell factor
Ser	Serine
SH2	Scr homology 2
SMAD	MAD homolog
SMMHC	Smooth muscle myosin heavy chain

SMRT	Silencing mediator for retinoid and thyroid receptors
SOS	Son of sevenless
SOX2	SRY-box 2
STAT	Signal transducer and activator of transcription
SUV39H	Suppressor of variegation 3-9 homolog
t	Translocation
TAD	Transactivation domain
TCF3	Transcription factor 3
TCR	T-cell receptor
TF	Transcription factor
THPO	Thrombopoietin
Thr	Threonine
TKD	Tyrosine kinase domain
TLE	Transducing-like Enhancer of Split
TSC	Tuberous sclerosis protein
UTP3	U-three protein 3
UTR	Untranslated region
WBC	White blood cell
WHO	World Health Organization
WNT	Wingless-type MMTV integration site family
WT	Wild type
YAP	Yes-associated protein

Preface

This work included input from several people from Dr. Castilla''s laboratory and from our collaborators in other laboratories. Below I will specify my role in each project.

Chapter I "Introduction" was written by me and edited by Lucio Castilla.

All experiments described in Chapter II "Cooperation between *Cbfb-MYH11* and miRNA cluster *miR-17-92* in development of AML" were performed by me. I also analyzed data and wrote the manuscript. Lucio Castilla provided intellectual input and edited the manuscript.

In Chapter III "THPO/MPL signaling participates in the initiation and maintenance of *RUNX1-ETO* acute myeloid leukemia via PI3K signaling" the experiments that were performed by me are shown in figures 3.2 (with participation by Sean Landrette), 3.3, 3.5C, 3.7, 3.8D. I also participated in generation and analysis of *RUNX1-ETO/PlagL2* AML samples depicted in figure 3.6, although it was mainly done by Sean Landrette. I was also involved in data analysis and writing and editing of the manuscript. Lucio Castilla provided intellectual guidance and wrote the manuscript.

CHAPTER I

Introduction

Leukemia is a diverse family of blood diseases characterized by expansion of abnormal hematopoietic progenitors. It represents 4% of cancers and ranks as 7th most deadly cancer in the United States (American Cancer Society, www.cancer.org). These statistics underscore the importance of understanding the molecular mechanisms driving leukemia development and drug resistance in order to develop more specific and effective methods of treatment.

Regulation of hematopoietic differentiation

Hematopoiesis is the process of generation of blood cells by an organism. The mature hematopoietic cells, including macrophages, granulocytes, megakaryocytes and thrombocytes, erythrocytes, T- and B-cells, dendritic cells and natural killers, are generated from a rare cell population called hematopoietic stem cells (HSCs). In addition to providing multilineage differentiation, the HSCs have the capacity to generate more HSCs in a process called self-renewal.

Hematopoiesis is a tightly regulated process that balances self-renewal and differentiation, dormancy and proliferation of its progenitor cells. This regulation involves cellular and environmental cues, including the activity of transcription factors

(TFs), signaling cascades, and non-coding RNAs. Deregulation of hematopoiesis due to mutations in the regulating genes can result in blood disorders characterized by impaired differentiation and/or increased proliferation.

The characterization of hematopoietic differentiation has benefited from the development of antibodies, flow cytometry technology, and *in vitro* colony forming assays, which allowed researchers to specifically identify particular hematopoietic subpopulations of cells with defined differentiation capacity. The studies at Weissmann‟s laboratory led to the definition of a classic model of hematopoietic differentiation in mice (Figure 1.1A). They identified a population in the mouse bone marrow based on cell surface markers expression Thy1^{low}Sca1$^+$Lin$^-$ (where Lin$^-$ is defined as cells that are negative for lineage specific markers: CD4, CD8, B220, Mac1, Gr1) that was enriched for cells exhibiting properties of HSCs. The HSC population is mostly dormant (van der Wath et al., 2009; Wilson et al., 2008), and can reconstitute all hematopoietic lineages *in vitro* and *in vivo* (Spangrude et al., 1988). The same group later identified a multipotent progenitor (MPP) that derived from HSC and possessed limited self-renewal capacity but was able to provide multilineage differentiation (Morrison et al., 1997). The MPPs were suggested to differentiate into progenitors that were restricted to either myeloid or lymphoid lineages based on the identification of corresponding populations: a myeloid restricted IL-7Rα$^-$Lin$^-$c-Kit$^+$Sca1$^-$FcgR^{lo}CD34$^+$ progenitor called common myeloid progenitor (CMP (Akashi et al., 2000)), and a lymphoid restricted Lin$^-$IL-7R$^+$Thy1$^-$ Sca1^{low}c-Kit^{low} progenitor called common lymphoid progenitor (CLP (Kondo et al., 1997)). The CMPs were shown to differentiate into more restricted granulocytic-
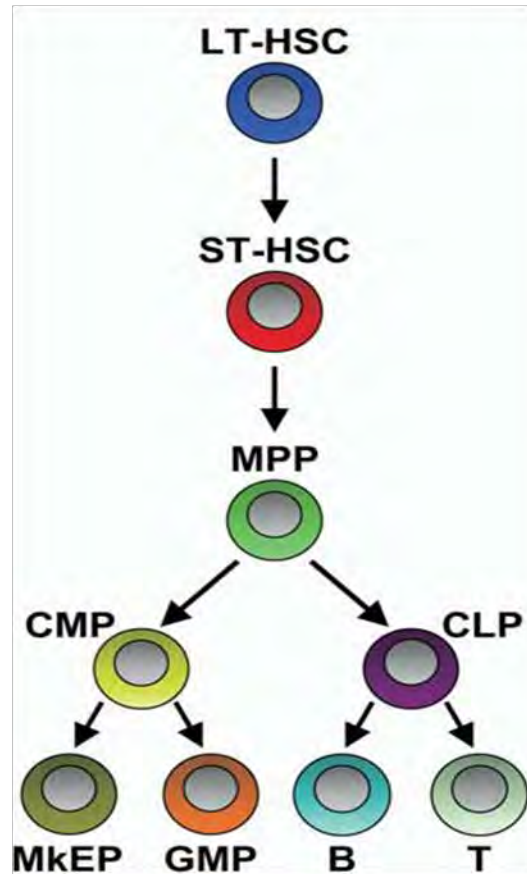
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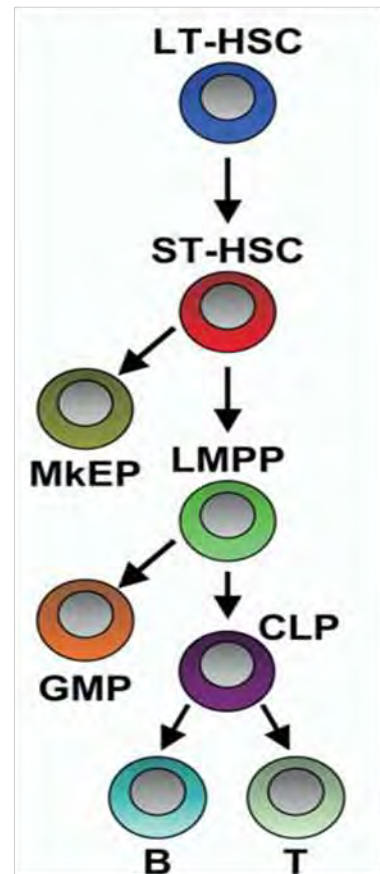
Figure 1.1. Models of hematopoietic differentiation.
A. Classic model. Long-term HSC (LT-HSC) capable of long term self-renewal and reconstitution of all hematopoietic lineages, gives rise to short-term HSC (LT-HSC) that is capable of limited self-renewal and reconstitution of all lineages. ST-HSC differentiates into multipotent progenitor (MPP) that is primed for terminal differentiation. MPP can differentiate into common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). CMP differentiates into megakaryocytic-erythroid progenitor (MkEP or MEP) and granulocytic-monocytic progenitor (GMP), eventually producing myeloid cells, erythrocytes and platelets/megakaryocytes. CLP differentiate into B- and T-cells through multiple stages of terminal differentiation. B. Alternative model. ST-HSC can differentiate into MkEP or lymphoid-primed multipotent progenitor (LMPP). In this model MkEP differentiates into erythroid and megakaryocytic lineages, whereas both myeloid and lymphoid lineages are produced from LMPP that gives rise to GMP and CLP. Adapted from Adolffson et al., 2005.

monocytic progenitors (GMPs) and megakaryocytic-erythroid progenitors (MEPs) (Akashi et al., 2000). In the thymus, an MPP-derived population, called early T-lineage progenitors (ETP) with $Sca2^+$ $Rh123^+$ $B220^-$ immunophenotype, is capable of T-cell differentiation (Wu et al., 1991b), but these cells do not seem to be restricted to T-cell differentiation in environments outside of the thymus (Wu et al., 1991a). Later, Kondo‚s group defined a lymphoid-primed MPP population, the $CCR9^+$ MPP, as the intermediate between MPP and ETP, with lymphoid (T, B and dendritic cell) and limited myeloid potential (Lai and Kondo, 2007).

This scheme of differentiation has recently been challenged by several studies that identified downstream of MPP two progenitor populations: $Lin^-c-kit^+Sca1^+Flt3^+$ lymphoid primed multipotent progenitor (LMPP) and megakaryocytic-erythroid progenitor (MEP). The LMPP then gives rise to both myeloid and lymphoid cells, but no megakaryocytic and erythroid cells (Adolfsson et al., 2005). This scheme suggests that differentiation of immune system cells is separated from megakaryocytic and erythroid differentiation (Figure 1.1B).

Many cell fate decisions are controlled by specific TFs that either cooperate or antagonize, thus resulting in a binary switch. An elegant example of a binary switch is the control of GMP (or LMPP) vs. MEP specification. The TFs Purine-rich binding protein (PU.1) and GATA-binding factor 1 (GATA-1) at the MPP stage exhibit antagonistic regulation, i.e. PU.1 inhibits GATA-1 targets, and GATA-1 inhibits PU.1 targets (Rekhtman et al., 1999; Zhang et al., 1999). This mutual regulation is based on physical binding of the proteins to each other at gene promoters and changing components of

protein complexes that regulate transcription (Rekhtman et al., 1999; Zhang et al., 2000). Additionally, both GATA-1 and PU.1 provide a positive feedback regulation of themselves (Okuno et al., 2005; Tsai et al., 1991). Therefore, when PU.1 and GATA-1 are co-expressed in a progenitor cell, minor changes in balance between PU.1 and GATA-1 will lead to switching to either activated PU.1 and repressed GATA-1 state leading to myeloid and/or lymphoid differentiation, or to activated GATA-1 and repressed PU.1 state leading to megakaryocytic and erythroid differentiation (Arinobu et al., 2007).

PU.1 is also involved in regulation of myeloid differentiation together with a regulator of myeloid differentiation CCAAT-enhancer binding protein (C/EBPα). PU.1 and C/EBPα cooperatively regulate the differentiation decision of GMPs to neutrophils and macrophages. The PU.1-null mice exhibit a mixed neutrophil/macrophage differentiation pattern, whereas addition of PU.1 results in upregulation of a macrophage-specific gene expression profile and a block in the expression of neutrophil-specific genes (Dahl et al., 2003). At the core of this regulation another binary switch was found that involved antagonizing TFs Early growth response 1 and 2 (Egr-1,2), and Growth factor independent 1 (Gfi-1). Expression of PU.1 activates Egr-1,2 leading to macrophage differentiation and inhibition of Gfi-1 targets, while low level of PU.1 activity allows Gfi-1 together with C/EBPα to activate neutrophil differentiation and inhibit targets of Egr-1,2 (Laslo et al., 2006).

The regulation of GMP differentiation to basophils and eosinophils depends on C/EBPα and GATA-binding factor 2 (GATA-2), and in this case, the order of activation

of the TFs determines an outcome (Iwasaki et al., 2006). When C/EBPα is exogenously expressed in CLPs (that do not express either of the TFs) followed by expression of GATA-2, the cells are reprogrammed to become eosinophils. When the order of expression is reversed (i.e. GATA-2 is expressed first, followed by expression of C/EBPα), the cells differentiate into basophils. This example illustrates that function of an individual TF depends on the expression of other factors in a particular cell context (Iwasaki et al., 2006).

The switch between myeloid and lymphoid lineages is complex and seems to require orchestrated functions of multiple TFs. Ectopic expression of C/EBPα in committed B cell progenitors induces cell type trans-differentiation, reprogramming them to macrophages (Xie et al., 2004). Additionally, PU.1 dosage seems to be a critical determinant of B-cell vs. macrophage development, as ectopic expression of low level of PU.1 in PU.1$^{-/-}$ mouse hematopoietic progenitors stimulates B-cell commitment, whereas 5-fold higher PU.1 expression level induces macrophage differentiation and blocks B-cell differentiation (DeKoter and Singh, 2000). Recent data suggest that the PU.1 block of lymphoid differentiation may be due to activity of a transcription factor upregulated by PU.1 called Myocyte enhancer factor 2C (Mef2c), but PU.1 role in regulation of lymphoid vs. myeloid fate is still under investigation (Stehling-Sun et al., 2009).

Several TFs are necessary for differentiation of B-cells, including Early B-cell factor 1 (EBF1), Ikaros, E2A (encoded by Transcription factor 3 (*Tcf3*) gene) and later B-cell determinants like Pax5. EBF1 is an essential early determinant of B-cell commitment, inhibiting expression of C/EBPα and PU.1, although it is not sufficient for

the commitment (Pongubala et al., 2008). Ectopic expression of Ebf1 can rescue B-cell differentiation in $Tcf3^{-/-}$ and $Ikaros^{-/-}$ background (Kikuchi et al., 2005; Seet et al., 2004), but in a context of wild type cells expression of EBF must be sustained by E2A (O'Riordan and Grosschedl, 1999), Ikaros (Reynaud et al., 2008) and in part by Pax5 (Nutt et al., 1999) proteins in order for normal B-cell commitment to occur. Recent work combining the expression profile of gain and loss of $Ebf1$ function, and genome wide chromatin immunoprecipitation determined that Ebf1 not only regulates Pax5 expression but it is also found in a third of promoter regions of Pax5 target genes, highlighting its participation in Pax5 regulatory role during B cell commitment (Treiber et al., 2010). This study also suggests that Ebf1 may act as a "pioneer in a hematopoietic chromatin context", remodeling chromatin before a cell undergoes further differentiation, therefore predisposing a particular gene expression profile that will play a role as the cell continues to differentiate into later stages.

Cell fate decision between B- and T-cell differentiation is mainly driven by the Notch-1 receptor in the thymus (Maillard et al., 2005). Regulation of Notch signaling in bone marrow (BM) depends on a TF called Leukemia/lymphoma related factor (LRF). Absence of LRF expression in BM results in increased Notch signaling and precocious appearance of T-cell progenitors (Maeda et al., 2007). GATA-binding factor 3 (GATA-3) that drives T-cell differentiation at multiple steps, also plays a role in emergence of early T-lineage progenitors (ETP) that upon Notch-1 signaling in the thymus can differentiate into mature T-cells (Hosoya et al., 2010). Activity of Notch-1 in early thymocyte development is tightly regulated by several transcription factors. Upon cleavage with

ADAM and γ-secretase, Intracellular Notch-1 (ICN) domain of Notch-1 is transported to the nucleus where it binds to Recombination signal binding protein for immunoglobulin κJ region (RBPJ, also known as CSL) and activates expression of multiple target genes by recruiting activators of transcription (CBP/p300, PCAF) (Yashiro-Ohtani et al., 2010). This regulation is counteracted by transcriptional repressor Ikaros that has the same DNA binding specificity as CSL (Kleinmann et al., 2008). Loss of Ikaros function results in increased Notch-1 signaling and development of T-cell leukemias (Beverly and Capobianco, 2003). Another example of Notch-1 regulation is E2A that activates Notch-1 expression and functions as a co-activator in a subset of Notch-1 target genes (Ikawa et al., 2006; Yashiro-Ohtani et al., 2009).

Interestingly, some proteins can be involved in regulation of several independent cell fate decisions. For example, in addition to the role of Gfi-1 in late myeloid development, it is involved in regulation of self-renewal vs. differentiation balance in HSCs, together with B lymphoma Mo-MLV insertion region 1 (Bmi-1) promoting self-renewal of HSCs, whereas C/EBPα and myelocytomatosis oncogene (c-Myc) promote HSC differentiation (Hock et al., 2004; Park et al., 2003; Wilson et al., 2004; Zhang et al., 2004).

The regulation of hematopoiesis by TFs is modulated by environmental cues, including cytokines, oxygen and calcium levels (Kaushansky, 2006). For example, the differentiation decision of MEPs into megakaryocytic vs. erythroid lineages is mediated by the antagonistic roles of Erythroid Krüppel-like factor (Eklf) and Friend leukemia integration 1 (Fli-1) TFs, both cooperating with GATA-1(Bouilloux et al., 2008; Frontelo

et al., 2007; Starck et al., 2003). Eklf promotes expression of erythroid-specific genes and inhibits activity of Fli-1, whereas Fli-1 promotes expression of megakaryocytic-specific genes and inhibits activity of Eklf. In megakaryocytic progenitors, GATA-1 and Fli-1 upregulate *Myeloproliferative leukemia virus oncogene homolog* (*c-Mpl*), a receptor for thrombopoietin that is a major cytokine promoting megakaryocytic maturation and platelet production (Deveaux et al., 1996; Jackers et al., 2004). Mature megakaryocytes and platelets can absorb thrombopoietin providing a feedback loop that supports steady state level of platelets in blood among other mechanisms (Yang et al., 1999). In erythroid cells GATA-1 induces expression of the erythropoietin receptor (EpoR), which in turn further activates GATA-1 (Zhao et al., 2006a; Zon et al., 1991). Erythropoietin (Epo) is a major regulator of erythrocyte production and stimulates proliferation and differentiation of erythroid progenitors. Synthesis of Epo is controlled in renal cells in an oxygen-dependent manner by regulation of stability of Hypoxia induced factor 1α (HIF1α) that upregulates *Epo* gene transcription (Ratcliffe et al., 1998). Therefore, EpoR and Epo provide sensitivity of erythropoiesis to oxygen levels in blood, allowing it to be responsive to environmental conditions. It is noteworthy that stem cell factor (SCF) and its receptor c-Kit play a synergistic role with Epo/EpoR in inducing differentiation and proliferation of erythroid progenitors (Munugalavadla and Kapur, 2005).

Other cytokines are involved in regulation of hematopoiesis, promoting differentiation of specific lineages. 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 and stimulate

proliferation and differentiation towards GMP, monocytes and granulocytes, correspondingly (Lieschke et al., 1994). Upon inflammation, release of inflammatory cytokines stimulates production of G-CSF by epithelial cells and monocytes, therefore increasing production and function of innate immunity cells when they are needed (Hareng and Hartung, 2002).

Two essential (but not exclusive) cytokines that regulate HSC are SCF and thrombopoietin. Null mutations in genes that encode SCF or its receptor c-KIT result in severe reduction of HSC numbers and their self-renewal ability (Bernstein, 1962; McCulloch et al., 1965). C-KIT receptor tyrosine kinase (RTK) type III is expressed in all hematopoietic progenitor cells at similar levels (Ashman et al., 1991; Cambareri et al., 1988; Papayannopoulou et al., 1991; Simmons et al., 1994) and, upon ligation with SCF activates signaling cascades that involve PI3K/AKT, RAS/RAF/MEK/ERK and JAK2/STAT3,5 pathways. Through these pathways (see below) c-KIT signaling regulates hematopoietic progenitor cell cycle (Leary et al., 1992), survival (Borge et al., 1997; Li and Johnson, 1994; Wineman et al., 1993), maintenance (Thorén et al., 2008) and adhesion to stromal cells in their niche (Kodama et al., 1994; Levesque et al., 1996; Levesque et al., 1995). Other hematopoietic functions of c-KIT involve myeloid (Sharma et al., 2007), erythroid (Munugalavadla and Kapur, 2005) and megakaryocytic differentiation (Avraham et al., 1992; Imai and Nakahata, 1994). To a lesser extent, lymphoid differentiation also depends on c-KIT function (Colucci and Di Santo, 2000; Sharma et al., 2007). *C-KIT* mutations leading to its constitutive receptor activation are common in leukemias (Zaker et al., 2010), and most AML cells express c-KIT on the cell

surface (Muroi et al., 1998; Wang et al., 1989).

Thromobpoietin (THPO) and its receptor Myeloproliferative leukemia virus oncogene (MPL) are also essential regulators of hematopoiesis and HSC function in particular, as absence of either of the genes results in diminished production of HSCs and their inability to compete with WT cells in lineage repopulation (Kimura et al., 1998a). Similar to c-KIT, MPL is also a cytokine receptor. MPL is a surface marker of a subpopulation within an HSC-enriched compartment, and the MPL$^+$ subpopulation exhibits higher multilineage repopulation potential than MPL$^-$ population, indicating that MPL is a surface marker of functional HSCs (Ninos et al., 2006; Solar et al., 1998). Detailed studies of MPL functions in HSCs suggest that it promotes self-renewal and maintenance, controls cell cycle entry, regulates HSC expansion after transplantation and interaction of HSC with their niche (Fox et al., 2002a; Qian et al., 2007; Yoshihara et al., 2007). MPL is also essential for megakaryocytic maturation (Alexander et al., 1996), erythroid and myeloid differentiation (Carver-Moore et al., 1996). An activating mutation W515L that disrupts an auto-inhibitory juxtamembrane domain of MPL (Staerk et al., 2006), is found in a fraction of primary myelofibrosis/idiopathic myelofibrosis and acute megakaryoblastic leukemias (AMKL) (Hussein et al., 2009a). Loss-of-function mutations in *THPO* and/or *MPL* genes result in development of a rare bone marrow failure syndrome called congenital amegakaryocytic thrombocytopenia (CAMT) (Ballmaier and Germeshausen, 2009).

Another receptor involved in regulation of hematopoiesis is FMS-like tyrosine kinase 3 (FLT3). It is expressed on early hematopoietic progenitor and stem cells and to a

lesser extent on B-cell committed progenitors (Brasel et al., 1995; Rosnet et al., 1996; Turner et al., 1996). Targeted disruption of the gene encoding this receptor, results in multiple hematopoietic abnormalities indicating a role for *Flt3* in lymphoid differentiation, as well as in myeloid development. Specifically, *Flt3*-null mice have reduced number of B-cell progenitors and exhibit deficiency in reconstitution of T-cell and myeloid lineages by HSCs after transplantation (Mackarehtschian et al., 1995). FLT3 promotes survival of early hematopoietic progenitors and, in cooperation with other factors, regulates proliferation (Rasko et al., 1995). *FLT3* activating mutations are commonly found in AMLs and myelodysplastic syndromes (Yokota et al., 1997), while high expression levels of FLT3 are found in a variety of hematopoietic cancers, including AML, B- and T-ALL and chronic myeloid leukemia (CML) (Drexler, 1996; Rosnet et al., 1996).

As we have discussed above, hematopoiesis is regulated by cytokines, their corresponding receptors and TFs. Another layer of hematopoietic regulation was recently discovered due to identification of miRNAs (see below) and their function in gene expression regulation. Hundreds of miRNAs have been described that are involved in regulation of hematopoiesis-related genes. While a role of miRNAs in HSCs is not yet determined, a crucial role of miRNAs in lymphoid differentiation was shown by lineage-specific ablation of Dicer that is a necessary effector of miRNA maturation (Koralov, 2008; Muljo et al., 2005). Several miRNAs are involved in differentiation of both B- and T-cell lineages, including *miR-150, miR-181, miR-17-92* and *miR-146* (Havelange and Garzon, 2010). Regulation of differentiation in other lineages is also affected by

miRNAs. For example, *miR-221* and *miR-222* downregulate *c-kit* expression, thereby inhibiting erythroid differentiation in an *in vitro* assay and hematopoietic stem cell activity in a NOD-SCID mouse transplantation assay (Felli et al., 2005). The *miR-17* miRNA family targets expression of *Runx1* TF (see below) and inhibits myeloid differentiation by failing to induce M-CSF receptor expression (Fontana et al., 2007). Recently an interesting feedback loop regulating both granulocytic and macrophage development was found involving downregulation of TF Nuclear factor I type A (NFI-A) by *miR-223* (in granulocyte progenitors) or *miR-424* (in macrophage progenitors). The miRNAs are in turn downregulated by NFI-A, but the downregulation is alleviated upon overexpression of C/EBPα or PU.1, correspondingly (Fazi et al., 2005; Rosa et al., 2007).

This brief introduction into regulation of normal hematopoiesis introduced major players involved in the regulation (except for CBF transcription complex, see below). As we will show later, many of these players are deregulated in leukemia development.

Core binding factor

The Core Binding Factor (CBF) is a master regulator of hematopoietic development, maintenance and differentiation, in embryonic and adult hematopoiesis. CBF is a heterodimeric TF that consists of subunits called α and β (Kamachi et al., 1990). In mammals, the α-subunit is encoded by three alternative genes with high level of homology (over 90% identity at the amino acid level) called *Runt-related transcription factors 1-3* (*RUNX1*, *RUNX2* and *RUNX3*). The nomenclature of the CBF factors has

been confusing due to its history until the year 2004 when researchers from these fields working with the CBF proteins proposed a unified nomenclature in which the mammalian homologs of the Drosophila gene *runt* would be officially called *RUNX1*, *RUNX2*, and *RUNX3*, while the cofactor would remain with the name given in the developmental field as *CBFB* (van Wijnen et al., 2004). Henceforth, we will use the *RUNX* and *CBFB* nomenclature.

Interaction between RUNX1 and CBFβ subunits is critical for CBF function (Wang et al., 1996b). The RUNX protein binds to the DNA consensus sequence YGYGGTY in promoters, enhancers and silencer regions (Wang and Speck, 1992). This binding is dependent on heterodimerization with CBFβ, as CBFβ increases RUNX affinity to DNA over five-fold (Golling et al., 1996; Wang et al., 1993), and protects it from proteasomal degradation (Huang et al., 2001).

All three RUNX proteins share similar conserved domains with defined functions (Figure 1.2). The highly conserved Runt-homology domain (RHD) between amino acids 50 and 177 is responsible for binding to DNA and CBFβ (Golling et al., 1996). The RHD also participates in RUNX binding a variety of cofactors. For example, the v-Ets avian erythroblastosis virus E26 oncogene homolog 1 (ETS1) TF binds to the RHD as RUNX1 recruits it to the *T-cell receptor α* (*TCRα*) enhancer in T cells (Giese et al., 1995). The RHD also binds to and is methylated by a methyltransferase Suppressor of variegation 3-9 homolog 1 (SUV39H1), which decreases RUNX1 affinity for DNA and, as a result, reduces its transactivation activity (Chakraborty et al., 2003). A similar competition is found in T-regulatory cells, as TF Forkhead Box P3 (FOXP3) binds to the RHD and
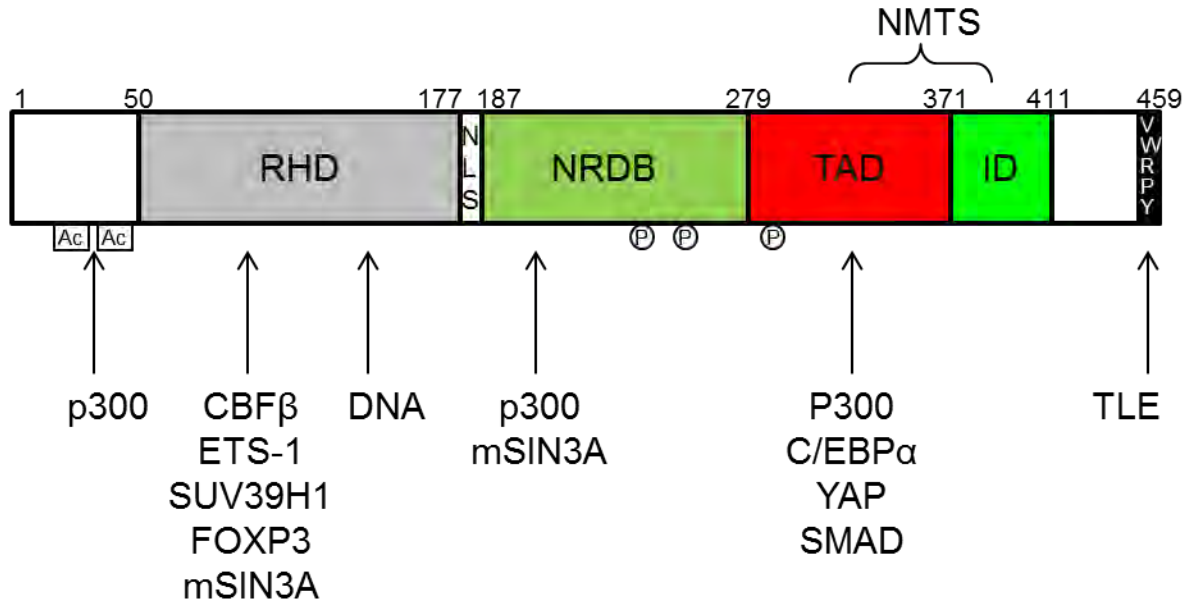
Figure 1.2. RUNX1 protein functional domains.
RUNX1 includes several defined functional domains from N- to C-terminus: Runt homology domain (RHD), nuclear localization signal (NLS), negative regulatory region for DNA binding (NRDB), trans-activation domain (TAD), inhibitory domain (ID), nuclear matrix targeting signal (NMTS), VWRPY motif. Approximate binding sites for protein interactions and the DNA binding site are shown by arrows with a list of interacting proteins. Phorsphorylaiton (P) and acetylation (Ac) sites are shown with circles and squares, respectively. Numbers above the domains indicate amino acid boundaries of the domains.

inhibits RUNX1 activation of IL-2 expression (Ono et al., 2007).

Another regulatory domain of RUNX proteins is the Negative Regulatory regionfor DNA Binding (NRDB) between amino acids 177 and 291, which affects the DNA binding affinity of RUNX proteins and is positively regulated upon acetylation by the acetyltransferase p300 (Yamaguchi et al., 2004). The Transactivation Domain (TAD) between amino acids 291 and 371 is critical for RUNX binding to a variety of co-factors that modulate its transactivation capacity (Kanno et al., 1998). For example, the TAD interacts with the aforementioned the p300 (Kitabayashi et al., 1998), C/EBPα (Petrovick et al., 1998), Yes-associated protein (YAP) (Yagi et al., 1999), and MAD homologs (SMADs) (Pimanda et al., 2007). Studies using RUNX1 deletion constructs have identified an Inhibition Domain (ID) between amino acids 371 and 411, which inhibits both CBFβ binding and affinity of RUNX1 to DNA, although the specific amino acids involved and the mechanism of action is poorly understood (Gu et al., 2000; Kanno et al., 1998).

RUNX proteins can both activate and silence target gene expression, depending on the cell context. The interactions listed above highlight some mechanisms of RUNX mediated transactivation. RUNX proteins can also repress the expression of target genes by interacting with mSin3A co-repressor via the RHD and NRDB (Imai et al., 2004; Satoh et al., 2008a) or with the Transducing-like Enhancer of Split 1-4 (TLE1-4) co-repressors on the RUNX C-terminal end amino acid VWRPY motif (Imai et al., 1998; Javed et al., 2000; Levanon et al., 1998).

The transport of CBF from the cytoplasm to the regulatory DNA regions is

regulated by two main RUNX domains: the Nuclear Localization Signal (NLS; amino acids 178-187) is essential for the translocation of CBF complex into the nucleus, and the Nuclear matrix targeting signal (NMTS; amino acids 346-387) acts on the transport of CBF into the RUNX1 nuclear foci (Harrington et al., 2002; Tang et al., 1999; Zeng et al., 1998; Zeng et al., 1997). This localization is functionally critical for activity of RUNX proteins in myeloid and lymphoid tissues (Telfer et al., 2004; Vradii et al., 2005). The nuclear localization of RUNX1 is also affected by a number of mechanisms, resulting in inhibition of RUNX function. For example, the three RUNX factors bind to Signal transducer and activator of transcription 5 (STAT5) with their RHD, which inhibits their nuclear transport (Ogawa et al., 2008). Interestingly, this interaction seems to have a mutual negative effect because it also inhibits STAT5 transactivation function on expression of its target, the *Cytokine-inducible SH2 protein-1* (*CIS3*).

Finally, RUNX activity is positively regulated by phosphorylation at multiple sites by several kinases. For example, the Extracellular signal-regulated kinase (ERK) potentiates RUNX-mediated transactivation by phosphorylating serines 249 and 266 (Tanaka et al., 1996), and Proviral integration site 1 (PIM-1) kinase provides a similar effect by phosphorylating serine 307 (Aho et al., 2006).

Contrary to the complexity in RUNX structure and protein organization, the organization and interactions of the 21 kD CBFB protein seems to be relatively simple. The CBFβ is encoded in three isoforms by alternative splicing, resulting in 187, 182, and 155 amino acid proteins, respectively (Ogawa et al., 1993). The CBFβ187 and CBFβ182 are thought to be functional, as these maintain an intact RUNX binding domain, its only

known domain. Deletion of this domain abrogates CBF function due to absence of CBFβ

binding to RUNX1 (Golling et al., 1996). In addition to RUNX binding, CBFβ was

shown to associate with Filamin A, which inhibits CBFβ association with RUNX1 and

prevents its nuclear transport due to retention in lipid rafts of the cell membrane (Yoshida

et al., 2005). Recently, the U-three protein 3 (UTP3), a member of the rRNA-processing

small subunit processome, was reported to bind to CBFβ and direct its localization to the

nucleus via an unknown mechanism (Park et al., 2009).

The CBF complex is essential for embryonic definitive hematopoiesis. Studies

using *Cbfb* and *Runx1* knock-out (KO) mice showed an absence of definitive

hematopoiesis and embryonic death at 12.5 days post conception (d.p.c.) due to severe

hemorrhages, (Okuda et al., 1996; Sasaki et al., 1996; Wang et al., 1996a). This critical

role of *Runx1* in establishment of definitive hematopoiesis was shown *in vitro* at the

hemangioblast stage (Lacaud et al., 2002). The fetal liver HSCs originate from the VE-

cadherin^{+} endothelium at day 10.5 d.p.c. (Zovein et al., 2008), and studies by Speck‟s

group demonstrated that Runx1 is required in endothelial cells on the ventral side of the

dorsal aorta for the formation of the hematopoietic clusters and emergence of the HSCs

(Chen et al., 2009). This critical role of Runx1 seems to be defined within two days (9.5

to 11.5 d.p.c.) of embryo development, and Runx1 may be dispensable thereafter.

Differentiation studies of 9.5 d.p.c. *Runx1*-null progenitors from the para-aortic

splanchnopleural region showed a rescue in differentiation in cells transduced with

Runx1 (Goyama et al., 2004) while this rescue failed in 11.5 d.p.c. *Runx1*-null progenitor

cells from the aorta-gonads-mesonephros (AGM) region (Mukouyama et al., 2000).

Similarly, definitive hematopoiesis is blocked when *Runx1* is deleted using the vascular endothelial cadherin *Cdh5* promoter driven-*Cre* transgene but not when using the *Vav1-Cre* transgene, expressed later in the fetal liver hematopoietic progenitors around day 11.5 d.p.c. (Chen et al., 2009). To date it is unclear whether Runx1 function ceases after fetal HSCs develop, or expression of other Runx proteins (Runx2 or Runx3) may compensate for Runx1 deficiency in regulation of its target genes.

In adult hematopoiesis, *Cbfb* or *Runx1* expression is not essential for maintenance of hematopoietic differentiation (Growney et al., 2005; Ichikawa et al., 2004). Instead, conditional deletion of *Runx1* and graded reduction of *Cbfb* have identified subtle defects in specific hematopoietic compartments, highlighting a lineage specific differential sensitivity for CBF function (Talebian et al., 2007).

In the HSC compartment, loss of the *Runx1* gene results in a moderate expansion of the HSC pool and a mild reduction of long-term HSC reconstitution (Ichikawa et al., 2004; Jacob et al., 2010; Sun and Downing, 2004). This expansion could be attributed to direct regulation of proliferation by Runx1, as *Runx1*-null HSCs exhibited higher levels of expression of *Bmi-1* and *B cell lymphoma 2* (*Bcl2*, a negative regulator of apoptosis) genes (Motoda et al., 2007). RUNX1 could also affect cell cycle progression of HSCs due to direct regulation of cell cycle regulators *p21* (Lutterbach et al., 2000) and *cyclins D* (*CCND*) (Bernardin-Fried et al., 2004), although in this case loss of *Runx1* would be expected to lead to decreased cycling of HSCs, as recently suggested (Ichikawa et al., 2008). Importantly, Runx1 also affects HSCs in their ability to interact with their niche by downregulating expression of a *C-X-C chemokine receptor type 4* (*CXCR4*) (Jacob et

al., 2010), therefore providing another potential mechanism of regulation of HSC quiescence, cell cycle and differentiation.

Several abnormalities were also found in lymphoid differentiation in the absence of normal CBF function. Reduced or absent function of either CBFB or RUNX1 genes resulted in impaired differentiation of T-cells with partial block at early double negative (DN) stages and decreased percentage of common lymphoid progenitors (CLP). Further studies identified *CD4* (Nishimura et al., 2004) and *GATA-3* (Komine et al., 2003) as some of the main targets of RUNX1 and RUNX3 in T-cell differentiation, implicating both RUNX proteins in regulation of T-cell development at two distinct stages (DN and CD8 single positive (Taniuchi et al., 2002)). B-cell development was also affected in conditional *Runx1* KO or *Cbfb* hypomorphic mice, but to a lesser extent, resulting in decreased percentage of B-cell progenitors, and inability to repopulate B220$^+$ cell compartment in competitive repopulation assays (Growney et al., 2005; Ichikawa et al., 2004; Talebian et al., 2007).

A significant difference between the *Cbfb* and *Runx1* genes was observed in monocyte/granulocyte differentiation, as it was almost undisturbed in *Runx1* conditional KO model, but reduction of *Cbfb* dosage resulted in 10-20-fold decrease in myeloid cells in fetal liver. The residual cells were enriched in immature monocytic progenitors, while mature bands and segmented neutrophils were not observed. This difference suggests that loss of *Runx1* activity may be compensated by other *Runx* family members in the myeloid development (Ichikawa et al., 2004; Talebian et al., 2007). It is also noteworthy that *Runx1* conditional KO mice exhibited a mild, but significant myeloproliferative

phenotype and myeloid extramedullary hematopoiesis (Growney et al., 2005).

Studies have also revealed critical roles for CBF in megakaryocytic maturation. Loss of *Runx1* or *Cbfb* genes resulted in lower number of platelets and, in case of *Cbfb*, appearance of abnormal small megakaryocytes (Ichikawa et al., 2004; Talebian et al., 2007). Data suggest that the effect of Runx1 on megakaryocytic maturation may be due to physical interaction and functional cooperation between Runx1 and GATA-1 (Elagib et al., 2003).

Taken together, CBF is an essential regulator of definitive hematopoiesis and is required for HSC emergence in the fetus and establishment of definitive hematopoiesis. It also plays multiple roles in differentiation of all hematopoietic lineages at different stages, with probably the exception of the erythroid lineage.

Acute myeloid leukemia

Acute myeloid leukemia is a diverse disease characterized by abnormal expansion and/or differentiation of hematopoietic progenitors. It arises as a result of genetic mutations that alter proliferation, survival and differentiation programs in the hematopoietic progenitor cells.

The classification of AML is complex due to the diversity of cytology, clinical prognosis, and genetic diversity. Two main classifications for AML have been defined over the years. The earliest was designed by a French-American-British cooperative group (FAB) in 1976, and was based on cytological and cytochemical criteria assessing

Table 1.1. French-American-British (FAB) classification of AML

FAB subtype	Common name	Frequency, % of AML cases	Associated translocations and rearrangements	Frequency, % of cases	Associated genes
M0	Acute myeloblastic leukemia with minimal differentiation	3	inv(3q26), 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	RUNX1-ETO, DEK-CAN
M3	Acute promyelocytic leukemia	5-10	(15;17) t(11;17) t(5;17)	98 1 1	PML-RARA PLZF-RARA NPM-RARA
M4	Acute myelomonocytic leukemia	20	inv(3q26), t(3;3) t(6;9) 11q23	3 1 20	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	

Adapted from Lowenberg et al., 1999

lineage of origin of leukemic cells and stage of differentiation block. The AMLs were divided into 8 groups from M0 to M7 with later groups presenting more differentiated cells than the earlier ones (Table 1.1).

Later findings confirmed the relevance of the FAB classification for disease prognosis. For example, the AML-M0 subtype had a significantly lower rate of complete remission (CR) and overall survival (OS) than other types (Stasi et al., 1994), while AML subtype M3 cases presented a significantly higher CR and OS rates (Bennett et al., 1991). Moreover, cytogenetic studies of genetic aberrations involved in AML (including karyotype changes or mutations in individual genes) showed some correlation between identified common mutations and FAB subtypes. For instance, an inversion of chromosome 16 [inv(16)] was found in all cases included in M4Eo subtype (acute myelomonocytic leukemia with abnormal eosinophils) (Larson et al., 1986; Solé et al., 1992), and genomic rearrangements involving a gene encoding *retinoic acid receptor α* (*RARα*) completely defined M3 subtype (acute promyelocytic leukemia) (Borrow et al., 1994; Tong et al., 1992), whereas the majority of cases within the M2 subtype carry the t(8;21)(q22;q22) chromosome translocation (Oshimura et al., 1982; Takeuchi et al., 1981). However, it was clear that in many cases FAB classification failed to distinguish between borderline cases, and prognostic values of AMLs classified according to other criteria (genomic rearrangements, secondary AMLs) were higher than those of the original M0 – M7 subtypes.

Later, identification of recurrent genetic abnormalities (chromosomal rearrangements and genetic mutations) resulted in definition of new, clinically relevant

AML subtypes that only partially correlated with the FAB subtypes. Additionally, clinical and biological differences between myelodysplastic syndromes (MDS)-related and MDS-unrelated AMLs was recognized, as well as between primary and therapy-related AMLs (Vardiman et al., 2002). These differences could not be adequately reflected in the FAB classification. In order to accommodate the body of new research, the World Health Organization (WHO) introduced a new leukemia classification that was revised several times, last time in 2008 (Jaffe, 2009; Vardiman, 2010; Vardiman et al., 2002; Vardiman et al., 2009). This classification is not limited to cytological or cytochemical characteristics of the cells, but was designed to include all currently available information, including morphology, cytochemistry, immunophenotype, genetics, and clinical features (Vardiman et al., 2009) in order to define clinically relevant diseases.

The latest WHO classification includes a number of entities that are defined by the common genomic rearrangements rather than other cellular characteristics. Two provisional entities were included that are defined based on mutations in *CCAAT/enhancer binding protein α* (*CEBPA)* and *nucleophosmin* (*NPM1)* genes. Although *FLT3* mutations did not define a separate category, screening was recommended due to their profound effect on prognosis. Separate entities include myeloid neoplasms with therapy-related changes, as they have significantly different CR, OS and response to treatment. Another interesting change was the introduction of a separate group for myeloid proliferations related to Down syndrome that are often associated with mutated *GATA-1*. As for not otherwise specified AMLs (AML, NOS),

Table 1.2. WHO classification of AML and related neoplasms

Acute myeloid leukemia with recurrent genetic abnormalities

    AML with t(8;21)(q22;q22); RUNX1-RUNX1T1

    AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11

    APL 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-EVI1

    AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1

    Provisional entity: AML with mutated NPM1

    Provisional entity: AML with mutated CEBPA

Acute myeloid leukemia with myelodysplasia-related changes

Therapy-related myeloid neoplasms

Acute myeloid leukemia, not otherwise specified

    AML with minimal differentiation

    AML without maturation

    AML with maturation

    Acute myelomonocytic leukemia

    Acute monoblastic/monocytic leukemia

    Acute erythroid leukemia

        Pure erythroid leukemia

        Erythroleukemia, erythroid/myeloid

    Acute megakaryoblastic leukemia

    Acute basophilic leukemia

    Acute panmyelosis with myelofibrosis

Myeloid sarcoma

Myeloid proliferations related to Down syndrome

    Transient abnormal myelopoiesis

    Myeloid leukemia associated with Down syndrome

Blastic plasmacytoid dendritic cell neoplasm

Adapted from Vardiman et al., 2009

they generally incorporate FAB classification based on stage of maturation of myeloid leukemia cells (Table 1.2).

It has been acknowledged that these classifications are useful for research and clinical purposes, but they are far from ideal because they may not in all cases include diseases that are clinically significant, mutually exclusive and diagnosable with currently available technologies (Vardiman, 2010). Clinical significance means that when a diagnosis is identified, it should dictate an optimal treatment that leads to the best prognosis possible for the disease. Therefore, response to treatment and accuracy of prognosis are very important in the significance of a classification.

Recently advances on the molecular characterization of AML provide valuable information on the expression signature of leukemias, which may further refine the current AML classifications. Examples are the expression profiling studies identifying either microRNA expression (Jongen-Lavrencic et al., 2008; Li et al., 2008), gene methylation (Bullinger et al., 2010; Figueroa et al., 2010) or gene expression profiling (GEP (Haferlach et al., 2005; Neben et al., 2004; Song et al., 2006; Valk et al., 2004c; Wilson et al., 2006)) in panels of AML. Some microarray studies focused on particular AML subtypes and refined them into additional categories (Bullinger et al., 2007; Silva et al., 2009). The classifications based on the microarray data correlate with WHO entities and identify new clusters that are novel. Importantly, in many cases the identified clusters of AML samples were shown to be clinically relevant due to specific prognostic characteristics (Radmacher et al., 2006; Wilson et al., 2006). The advantages of GEP classifications are that (1) they are quantitative and unbiased, and (2) they identify the

molecular characteristics of a disease, allowing putatively tailored approaches to treatment. The limitations of expression profiling classification include the high cost and requirement of special technology, precluding its routine use in hospital settings.

Treatment of AML

The treatment for AML is in most cases independent of the subtype, and has followed a similar treatment regime for several decades. The first standard treatment step in AML is called induction therapy with the goal of reducing the number of leukemic cells, and usually includes chemotherapy with cytosine arabinoside (also called Cytarabine or Ara-C) and anthracyclines. Cytarabine is a pyrimidine nucleoside analogue with an arabinose sugar moiety instead of a ribose. It was first shown to inhibit tumor cell growth in 1961 (Evans et al., 1961) and approved by the Food and Drug Administration in 1969 as a drug for treatment of AML (www.fda.gov). The main role of Cytarabine is to compete with the cytidine nucleotide to be incorporated into the new DNA strand during S-phase of cell cycle, stalling DNA replication and blocking its transition into mitosis.

The anthracyclines doxorubicin and daunorubicin were first isolated from the bacteria *Streptomyces sp.* and shown to act as antineoplastic drugs in the 1960"s and placed into clinical protocols with very effective outcome (Arcamone et al., 2000; Cassinelli and Orezzi, 1963; Dubost et al., 1963; Grein et al., 1963). It is thought that anthracyclins act as DNA intercalators, causing single stranded DNA breaks and apoptosis (Laurent and Jaffrézou, 2001). The use of these antibiotics has the limitations of (1) showing significant side effects when used in patients when administered

chronically or at high doses (chronic cardiomyopathy and congestive heart failure), and (2) spontaneous and acquired resistance. However, they are still used today in the treatment of a variety of cancers, including AML.

After the induction therapy, AML patients usually undergo a consolidation phase to further reduce the number of leukemic cells, and achieve a complete remission. The drug of choice is usually Cytarabine, and depending on the AML subtype complete remission can be achieved in approximately 64% of cases (Hamadani and Awan, 2010). The next step in standard AML treatment is called post-remission therapy and is aimed at complete eradication of the disease. Post-remission therapy has more options and is assessed based on a prognosis for a particular disease. The options include a low-dose maintenance therapy, intensive chemotherapy and hematopoietic stem cell transplantation (Rowe, 2009).

The most promising treatment approaches are molecularly targeted therapies, but their feasibility and efficiency vary between AML subtypes and for specific mutations found in samples (Hamadani and Awan, 2010). One prominent example is treatment of acute promyelocytic leukemia (APL) that in majority of cases carries *PML-RARA* fusion gene. As *RARA* encodes a receptor for retinoic acid, treatment of APL with all-trans retinoic acid or similar chemicals proved to be a very efficient treatment regimen, and it is now a standard treatment for APL (Hu et al., 2000; Jeddi et al., 2010; Liu et al., 2010b).

Other examples of targeted molecular treatment include treatment of chronic myeloid leukemia carrying *BCR-ABL* fusion gene with RTK inhibitor imatinib

(Buchdunger et al., 1996; Druker et al., 2001; le Coutre et al., 1999), AML treatment with c-KIT inhibitors (Faderl et al., 2009) and specific FLT3 inhibitors (Pratz and Levis, 2010). Activating mutations in FLT3 RTK (internal tandem duplications (ITD) or tyrosine kinase domain (TKD) mutations) are found in 30% of AMLs and commonly are associated with poor prognosis. They are not associated with any particular subtype of AML (Nakao et al., 1996; Schnittger et al., 2002). Inhibition of aberrant activation of FLT3 seems to be a good strategy, but currently the results are not clearly positive due to mechanisms of resistance that include effects of other mutations in the same AML, resistance mutations in FLT3 kinase domain and activation of compensatory signaling pathways (PI3K/AKT and MAPK) (Pratz and Levis, 2010).

A promising strategy for cancer treatment (including leukemia and specifically AML) is the use of rapamycin and rapamycin analogs (rapalogs) for inhibition of Mammalian target of rapamycin (mTOR), one of the main effectors of PI3K/AKT pathway (see below), alone or in combination with other inhibitors (Chapuis et al., 2010b; Martelli et al., 2007). Potential molecular feedback loops make this treatment less efficient than could be presumed. For example, mTORC1 complex that is a target of rapamycin inhibition, exists in a dynamic equilibrium with mTORC2 complex that is largely resistant to rapamycin (see below). mTORC2 activates PI3K/AKT pathway by phosphorylation of AKT, therefore treatment with rapamycin or rapalogs can result in a PI3K/AKT-activating feedback loop by shifting the equilibrium towards mTORC2. Another possible mechanism of rapamycin resistance is based on mTORC1-depedent indirect inhibition of PI3K subunit p110 (see below). Inhibition of mTORC1 activity with

rapamycin can, therefore, lead to activation of PI3K and its downstream pathway (Tamburini et al., 2008). Additional inhibitors targeting Tuberous sclerosis protein 1 and 2 (TSC1 and TSC2), mTOR itself (rather than as part of mTORC1 complex), and combinations of rapalogs with other PI3K/AKT pathway inhibitors should help in increasing efficiency of mTOR targeting (Chapuis et al., 2010a; Park et al., 2008).

CBF-related hematopoietic neoplasias

Approximately 20-25% of AML cases are associated with genomic alterations involving *CBFB* or *RUNX1* genes (Look, 1997). Specifically, 12% of cases of AML have chromosomal inversion inv(16)(p13q22), disrupting the *CBFB* gene and fusing it in-frame with the second half of the *Myosin heavy chain 11* (*MYH11*) gene that encodes the Smooth muscle myosin heavy chain (SMMHC) protein. The resulting gene is called *CBFB-MYH11*, and its encoded fusion protein is called CBFβ-SMMHC (Liu et al., 1993). In 12% of AML cases the translocation t(8;21)(q22;q22) results in the creation of the *RUNX1-ETO* fusion gene that fuses *RUNX1* in exon 5 with *Eight twenty one* (*ETO,* also known as *RUNX1T1*) gene starting from exon 2 (Rowley, 1973). In addition to these rearrangements, AML samples can also harbor point mutations in *RUNX1* that create a protein with dominant negative or hypomorphic function (Schnittger et al., 2011; Silva et al., 2009).

By FAB classification, 68% of cases associated with the *CBFB-MYH11* fusion gene fall into M4Eo group, while 20% are M4 and 12% are M2 (Marcucci et al., 2005; Schnittger et al., 2007a). There are rare cases of myelodysplastic syndrome and chronic

myeloid leukemia crisis also associated with *CBFB-MYH11* expression (Tirado et al., 2010), but these are temporary conditions that lead to development of AML. More than 90% of the M4Eo cases carry an abnormality that leads to *CBFB-MYH11* expression, whether it is inv(16), t(16;16) or del(16)(q22) (Hernandez et al., 2000; Pirc-Danoewinata et al., 2000). However, the fusion transcript *CBFB-MYH11* has been detected in all M4Eo cases tested, suggesting the presence of micro-rearrangements in some cases. Since the the presence of the *CBFB-MYH11* fusion in AML samples is unique at the molecular and clinical levels, the WHO classification defined it as a separate AML subtype (Vardiman, 2010). The *CBFB-MYH11*-expressing AML subtype is considered to have a favorable prognosis, with CR rate around 90% and OS rate around 50% (Appelbaum et al., 2006; Delaunay et al., 2003; Marcucci et al., 2005). The CR and OS rates were lower in older patients, patients with complex genotype and high white blood cell (WBC) counts (Appelbaum et al., 2006).

AML cases in M4Eo group are characterized by myeloblastic and/or monoblastic infiltration into BM, elevated monocytic counts in peripheral blood (PB), and the presence of atypical eosinophils in both BM and PB. The eosinophil population usually accounts for 5% or more of white blood cells and carries the inv(16), being therefore part of the leukemic population and not a secondary change in AML (Haferlach et al., 1996). Immunophenotype of the leukemic cells is somewhat heterogeneous, and while all AML cells express pan-myeloid CD13 marker, they are also partially positive for monocytic/myeloid markers CD11b, CD11c, CD14, CD33, marker of immature cells CD34 and a multilineage hematopoietic marker CD36. Surprisingly, some cells co-

expressed lymphoid CD2 marker together with the mentioned above myeloid markers, indicating either abnormal differentiation or a modified gene expression pattern. *In vitro* experiments indicated that the AML cells exhibit high spontaneous proliferation (Adriaansen et al., 1993). Taken together, the data from human patients suggest that *CBFB-MYH11* causes development of AML with partial abnormal differentiation into monocytic/myeloid lineage with small populations keeping expression of early hematopoietic stem/progenitor markers.

A majority (91%) of AML cases associated with the fusion gene *RUNX1-ETO* are in the FAB subtype M2, while only 6% are in the less differentiated subtype M1 and 3% in the more differentiated subtype M4 (Marcucci et al., 2005). The WHO classification defines a separate subtype based on the presence of t(8;21) translocation expressing *RUNX1-ETO*. The AML subtype is considered to have a favorable prognosis, but the OS rate is lower in the *RUNX1-ETO*-related AMLs than in *CBFB-MYH11*-related AMLs (Appelbaum et al., 2006; Marcucci et al., 2005), being around 45%. The risk factors were similar to those for *CBFB-MYH11* subtype: age, WBC count, complex genotype with additional genomic alterations. Race was an additional factor that was not found significant in *CBFB-MYH11* AML subtype, but was significant for OS of patients with *RUNX1-ETO* AMLs (Marcucci et al., 2005).

Morphologically, the *RUNX1-ETO*-carrying leukemic cells are immature basophilic myeloid cells that expressed myeloperoxidase and represented abnormal granulocytic development (Andrieu et al., 1996; Kita et al., 1994). The variable pattern of immunophenotypical markers shows that it is unreliable to determine a specific

immunophenotype of the AML cells, but CD19 and CD34 overexpression was shown to be a common feature of the AML populations with *RUNX1-ETO* expression (99% of the samples overexpressed the markers with specified thresholds). Conversely, CD7 and CD2 are rarely expressed. Although their expression varies, CD56 and CD54 were overrepresented, whereas CD45R0, CD33, CD36, CD11b and CD14 were expressed less than in control (Andrieu et al., 1996; Gustafson et al., 2009; Kita et al., 1994). Taken together, these data show the AML cells as a mixture of myeloid blasts and maturing myeloid cells.

Other hematopoietic disorders involving CBF genes include a subtype of AML with minimal differentiation that carries *RUNX1* mutations (Schnittger et al., 2011; Silva et al., 2009) and familial platelet disorder (FPD) – a hereditary disease that is associated with haploinsufficiency of *RUNX1* (Buijs et al., 2001; Kirito et al., 2008; Song et al., 1999). FPD patients frequently develop AMLs that often harbor additional *RUNX1* mutations including loss of heterozygocity (LOH) for *RUNX1* (Preudhomme et al., 2009).

Molecular functions of *CBFB-MYH11*

Due to a prominent role that the *CBFB-MYH11* fusion gene plays in human AML, it was thoroughly studied in cell lines and animal models. The main questions asked about this fusion were: what is its role in regulation of proliferation, apoptosis and differentiation in hematopoietic lineages; what are the specific molecular activities and pathways involved

in *CBFB-MYH11*-associated leukemogenesis; are there functional differences and interactions between CBFβ-SMMHC and wild type CBF complex components?

Studies performed in *Cbfb$^{+/MYH11}$* knock-in mice suggested that the fusion protein acted as a dominant negative factor over wild type CBF function. Indeed, heterozygous expression of the fusion gene under transcriptional control of the *Cbfb* promoter resulted in the absence of definitive hematopoiesis after embryonic day 10.5 post conceptus (d.p.c.), severe hemorrhages in the dorsal root ganglia, and death around d.p.c.12.5, recapitulating the phenotype of the homozygous *Cbfb$^{-/-}$* and *Runx1$^{-/-}$* KO mice (Castilla et al., 1996; Kundu et al., 2002; Okuda et al., 1998; Yergeau et al., 1997). Importantly, embryonic stem (ES) cells expressing the fusion protein failed to contribute to hematopoiesis in chimeric embryos despite high level of chimerism (over 70%), and they did not produce hematopoietic colonies *in vitro*, further confirming a block in definitive hematopoiesis caused by dominant negative function of Cbfβ-SMMHC. The dominant inhibition of myeloid differentiation by the fusion protein was confirmed *in vitro* in ES cells that failed to produce mature myeloid cells, instead producing immature myeloid blasts in the presence of Cbfbβ-SMMHC (Miller et al., 2001).

Besides recapitulation of *Cbfb* KO phenotype, *Cbfb$^{+/MYH11}$* knock-in embryos also presented a distinct phenotypic trait, as majority of primitive erythroid cells in the knock-in embyos also presented a delay in primitive (erythroid) hematopoiesis at 11.5 d.p.c. This delay was not observed in *Cbfb$^{-/-}$* embryos, suggesting that the fusion gene may have other activities besides dominant inhibition of CBF (Castilla et al., 1996).

The mechanism by which Cbfβ-SMMHC represses CBF function and induces

leukemogenesis is unclear. Two main models have been proposed: The sequestration model proposes that the fusion protein forms dimers and oligomers, and is localized in the cytoplasm, associated with the actin filaments. RUNX1 binds to the fusion protein at higher affinity than to CBFβ, thereby sequestering RUNX1 from the nucleus and the regulatory regions if the target genes (Adya et al., 1998; Kanno et al., 1998). This model is based on *in vitro* studies showing that CBFβ-SMMHC binds RUNX1 with higher affinity than wild type CBFβ due to the presence of a high affinity binding domain (HABD) found in CBFβ-SMMHC (Lukasik et al., 2002). This affinity imbalance is abrogated in the presence of DNA, probably because RUNX1 binding site responsible for the high-affinity interaction specifically with CBFβ-SMMHC is also involved in DNA binding (Lukasik et al., 2002). It was also shown that the fusion protein oligomerizes into large structures that supposedly sequester RUNX1 from its target promoters (Adya et al., 1998; Huang et al., 2004; Wijmenga et al., 1996). The ability of CBFβ-SMMHC to form oligomeric structures depends on an Assembly competence domain (ACD) (Zhang et al., 2006b) located in SMMHC (Figure 1.4A). Recently, the sequestration model has been challenged by the observation that a knock in mouse carrying a sequence of the fusion gene lacking the HABD region does not abrogate leukemia development but rather accelerates the disease (Kamikubo et al., 2010).

The competing repression model proposes that the CBFβ-SMMHC:RUNX1 heterodimer directly represses expression of target genes (Lutterbach et al., 1999). This model is based on the observation that the fusion protein can be found in the nucleus and associates with RUNX1 and a co-repressor mSin3A in transfection assays. A defined

Repression domain (RD) is required for the repression of gene expression (Lutterbach et al., 1999). Considering that RUNX1 is expressed at low levels, and that overexpression of CBFβ-SMMHC is toxic and may result in cellular mislocalization, the appropriate studies that can unequivocally establish the mechanism of action has proven challenging. Of note, the observation that the HABD loss accelerates AML development, raises a possibility that the leukemogenic role of CBFβ-SMMHC is not limited to a dominant negative inhibition of CBF function (via either sequestration or repression mechanisms). The fusion protein could be involved in gene regulation via RUNX-independent mechanisms. Chromatin immunoprecipitation microarrays for mouse or human promoter regions could determine whether CBFβ-SMMHC is associated with promoters that are not targets of RUNX1.

Expression of the fusion protein in *Cbfb-MYH11* chimeric mice showed to be present in HSCs but not in peripheral white blood cells, suggesting that the fusion protein induces a differentiation block in the bone marrow (Castilla et al., 1999). Lymphoid differentiation defects were detected in the CLP to pre-pro B cells transition (Kuo et al., 2008) and in the double negative T cell progenitors (Zhao et al., 2007). Expression of *CBFB-MYH11* from human myeloid-restricted *Myeloid-related protein 8* (*hMRP8*) promoter showed only mild impairment of myeloid differentiation suggesting that the block in myeloid differentiation occurs in earlier hematopoietic progenitors (Kogan et al., 1998).

Expression of *CBFB-MYH11* in chimeric mice did not result in leukemia development within the first year of life. When the mice were treated with N-ethyl-N-

nitrosourea (ENU), a potent mutagen, they developed a disease that recapitulated AML

with inv(16)(p13.1q22) (M4Eo by FAB classification). High WBC counts, infiltration of

the leukemic cells into spleen and other peripheral organs and well as presence of

abnormal granulocytes were evident in the mice (Castilla et al., 1999). Therefore, *CBFB-*

MYH11 is not sufficient for AML development that requires additional genetic mutations

to occur in the mouse model.

The understanding of *CBFB-MYH11* AML function benefited from the generation

of a conditional inducible knock-in mouse, which bypassed the embryonic lethality

associated with the fusion gene expression and allowed the expression of *CBFB-MYH11*

in the adult HSCs (Figure 1.3 (Kuo et al., 2006)). Examination of the hematopoietic

progenitor compartment in these mice revealed an impaired ability of HSCs for

multilineage repopulation, but did not affect HSC maintenance. Importantly, an abnormal

myeloid progenitor (AMP) population accumulates in the myeloid compartment of BM,

with MEP-like immunophenotype (lin$^-$c-kit$^+$Sca1$^-$CD34$^-$FcgRII/III$^-$) and CMP-like *in*

vitro differentiation potential. Limiting dilution transplantations showed that both HSCs

and AMPs could give rise to leukemia with similar efficiency and latency, indicating that

the AMPs as well as HSCs contain leukemia-initiating cells. Together, these studies

suggest that HSCs expressing CBFβ-SMMHC undergo partial differentiation to create a

myeloid leukemia precursor that induces leukemia in cooperation with additional

mutations. These findings are in concordance with the "two-hit hypothesis" posed by

Gary Gilliland (Deguchi and Gilliland, 2002; Kelly and Gilliland, 2002a). This

hypothesis postulates that at least two mutations are necessary for AML to develop: a
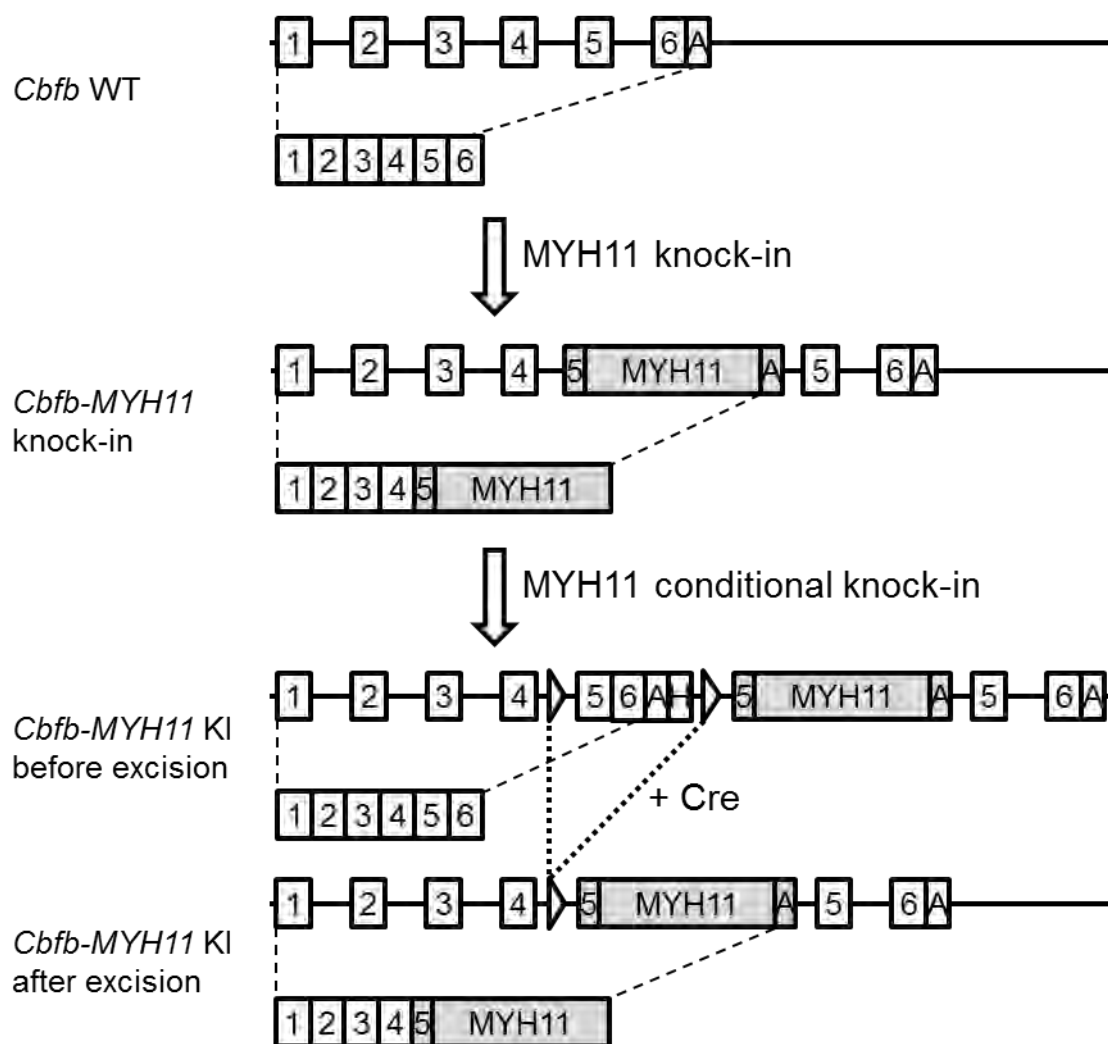
Figure 1.3. *Cbfb-MYH11* knock-in mouse models.
Schematic representations of wild type *Cbfb*, *Cbfb-MYH11* knock-in (KI) and conditional *Cbfb-MYH11* KI alleles. Exons are shown as closed boxes, with sequences from human AML shaded to gray. LoxP sites are indicated as triangles. Closed box labeled "A" is a transcription termination sequence. Thin dotted lines show splicing, and thick dotted lines show excision in DNA upon expression of Cre recombinase. For each part of the picture, top represents a scheme of DNA locus and bottom represents resulting ORF.
Wild type *Cbfb* has 6 exons that encode full-length Cbfβ protein. To obtain a straight knock-in for *Cbfb-MYH11* fusion gene, human *MYH11* sequence fused to exon 5 of *CBFB* was introduced between murine Cbfb exons 4 and 5. The straight knock-in was modified into conditional knock-in by insertion of wild type exons 5 and 6 between loxP sites upstream of sequence of human exon 5 fused to *MYH11*. Once Cre-mediated recombination in the conditional allele occurs, sequence between loxP sites is excised, leading to expression of Cbfβ-SMMHC fusion from first 4 endogenous exons and introduced sequence of exon 5 fused to human *MYH11* gene.

mutation that confers pro-survival and/or pro-proliferative advantage to the abnormal progenitor cell (class I mutation) and a mutation that blocks normal differentiation of a hematopoietic lineage (class II mutation), allowing proliferation and accumulation of leukemic cells. This hypothesis is useful for explaining various aspects of AML progression, although simplistic because some mutations can have functions from bothclasses and still required additional mutations for transformation, like the RUNX1-ETO fusion protein (see below). According to this hypothesis, *CBFB-MYH11* can be classified as a classic class II mutation because it does not provide self-renewal or proliferative advantage but induces a differentiation block. The search for the cooperating mutations both in human and mouse AMLs resulted in identification of a number of cooperating class I mutations, among which in human AMLs were *c-KIT*, *FLT3*, and *N-* and *K-RAS* mutations (see below). Using the conditional *Cbfb*$^{+/MYH11}$ knock-in mice our laboratory has identified the cooperating genes *Pleomorphic adenoma gene-like 2 (PlagL2)* (Landrette et al., 2005), *Runx2* (Kuo et al., 2009) and *Myeloproliferative leukemia virus oncogene* (*Mpl*) (Landrette et al., 2011), as well as other potential oncogenes (Castilla et al., 2004).

Despite the intensive research, the mechanism(s) of *CBFB-MYH11*-mediated transformation remains unclear. In addition to blocking CBF function, it is possible that inv(16) AML samples may have other genetic alterations that contribute to leukemic transformation. For example, the inv(16) rearrangement invariably breaks in half the gene *Nude nuclear distribution gene E homolog 1* (*NDE1*) transcribed from the inverse strand of the *MYH11* gene, resulting in decreased expression of *NDE1* transcript (Van der

Reijden et al., 2010). NDE1 is a scaffold protein known to control self-renewal and differentiation of cortical neuron progenitors (Pawlisz et al., 2008), suggesting that reduction in *NDE1* expression may contribute to inv(16) leukemogenesis. Of note, *Nde1* expression is not altered in the *Cbfb*$^{+/MYH11}$ knock in mice, and its participation in transformation has not yet been directly tested.

CBFβ-SMMHC may also have CBF-independent roles in leukemia development. Based on the observation that primitive hematopoiesis is delayed in *Cbfb*$^{+/MYH11}$, but not in *Cbfb*$^{-/-}$ embryos (Castilla et al., 1996), recent studies identify that Cbfβ-SMMHC, but not Cbfβ, upregulates expression of *Csf2b2* and *Gata2* during primitive hematopoiesis. These genes are also expressed in the inv(16) human AML-derived ME-1 cell line (Hyde et al., 2010). However, whether these genes play a role in leukemia development is unclear.

Molecular functions of *RUNX1-ETO*

The the t(8;21) fusion protein RUNX1-ETO also affects CBF function and causes AML in humans and in mouse models. It has been extensively studied in cell and mouse models in order to determine its effect on normal hematopoiesis and mechanisms of leukemic transformation. In some aspects this fusion proved to be similar to CBFβ-SMMHC, but there are important biological differences as well.

Similarly to *CBFB-MYH11*, a knock-in mouse model for *RUNX1-ETO* recapitulated the embryonic lethal phenotypes of *Cbfb*$^{-/-}$ and *Runx1*$^{-/-}$ mice, prompting a

conclusion that RUNX1-ETO functions as a dominant negative inhibitor of the CBF complex (Okuda et al., 1998; Yergeau et al., 1997). Unlike a complete failure to produce hematopoietic progenitors in *CBFB-MYH11* knock-in embryos, it was possible to obtain a few progenitor cells from fetal livers of embryos expressing *RUNX1-ETO* at 11.5 d.p.c. These progenitors did not undergo normal hematopoietic differentiation, but rather proliferated *in vitro* in a cytokine-dependent manner, giving rise to abnormal erythroid, megakaryocytic and myeloid cells. Serial replating assays revealed that while erythroid and megakaryocytic progenitors exhausted their self-renewal capacity, the myelo-monocytic progenitors displayed enhanced replating ability and readily immortalized into cytokine-dependent cell lines (Okuda et al., 1998). This increased *in vitro* self-renewal capacity of *RUNX1-ETO*-expressing myeloid progenitors was later confirmed in BM transplantations of progenitors transduced with a *RUNX1-ETO* retrovirus (de Guzman et al., 2002a).

Using conditional *Runx1-ETO* knock-in mouse model it became possible to bypass the embryonic lethality phenotype and determine the role of *Runx1-ETO* in adult hematopoiesis. Studies in these mice further confirmed that the fusion protein confers increased proliferation and self-renewal capabilities to myeloid progenitors *in vitro* without complete abrogation of myeloid differentiation (Higuchi et al., 2002; Rhoades et al., 2000).

The conditional *RUNX1-ETO* knock-in mice did not develop any hematopoietic abnormalities within the first 11 months of life despite high representation of *RUNX1-ETO*-expressing cells in BM and PB, although by the end of their first year 10% of mice

developed lymphoma (Higuchi et al., 2002). However, 47% of these mice developed

hematopoietic neoplasms 2-10 months after treatment with a chemical mutagen ethyl-

nitrosourea. Thirty three percent of affected mice developed T cell lymphomas, probably

unrelated to the fusion protein (Higuchi et al., 2002). The remaining mice developed

granulocytic sarcomas/AMLs similar to what is observed in patients that express

RUNX1-ETO fusion protein (Byrd et al., 1997; Tallman et al., 1993). Similar results

were obtained with a transgenic *RUNX1-ETO* mouse model where expression of the

fusion gene was driven by *hMRP8* promoter, restricting RUNX1-ETO expression to the

myeloid compartment (Yuan et al., 2001a). Taken together, these studies provide

evidence that *RUNX1-ETO* is an oncogene that predisposes mice to development of AML

in cooperation with other mutations. While in some studies *RUNX1-ETO* provides a

maturation deficiency in myeloid lineage (Higuchi et al., 2002; Rhoades et al., 2000) and

can therefore be considered a class II mutation according to the two-hit hypothesis, the

ability of RUNX1-ETO to simultaneously increase proliferation of immature myeloid

progenitors illustrates limitations of the two-hit hypothesis. Moreover, in a mouse model

expressing *RUNX1-ETO* from HSC-specific *Ly6A* promoter, the mice do not exhibit

differentiation block in myeloid lineage, but develop MPD characterized by abnormal

proliferation of mature myeloid cells (Fenske et al., 2004). While these data may suggest

that *RUNX1*-ETO is a class I mutation, human data in commonly found cooperating

mutations in t(8;21) AMLs (Boissel et al., 2006a; Care et al., 2003; Schessl et al., 2005b;

Speck et al., 1999; Valk et al., 2004a) indicate that RUNX1-ETO requires additional pro-

proliferative and pro-survival signaling for AML development. While *RUNX1-ETO* can

provide both class I and class II mutation functions, these functions are not sufficient for AML, and addition of a class I mutation results in AML development. Therefore, it can be functionally considered a class II mutation, but clearly illustrates limitations of the two-hit hypothesis.

The fusion protein contains N-terminal region of RUNX1 that includes the RHD and NLS, but does not include TA, ID, and repression domains (see Figures 1.2 and 1.4B) (Lenny et al., 1995). Therefore, while the fusion protein relies on CBFβ binding and using the CBF DNA-binding activity, its transregulation activity and association to cofactors is mediated by the ETO sequences (Lenny et al., 1995). ETO is a member of an E-box family of TFs, and it contains four domains called Nervy homology regions (NHR). The NHRs recruit several repressors of transcription, including mSin3A, Histone deacetylases 1-3 (HDAC1-3), Nuclear receptor corepressor (N-CoR), Silencing mediator for retinoid and thyroid receptors (SMRT) mainly through cooperative function of NHR2 and NHR4 (Amann et al., 2001; Gelmetti et al., 1998; Hildebrand et al., 2001; Wang et al., 1998), as well as GFI-1, which can also exhibit transcriptional repression activity (McGhee et al., 2003). Importantly, the NHR2 domain is required for ETO homooligomerization and for interaction with other ETO family proteins. Mutations of NHR2 that abrogate oligomerization dramatically reduce effects of RUNX1-ETO on proliferation and differentiation of murine BM cells, indicating that oligomerization plays an important role in RUNX1-ETO function, probably by providing a interacting surface for several RUNX1-ETO-binding corepressors (Liu et al., 2006; Zhang et al., 2001).

The RUNX1-ETO protein acts, at least in part, as a repressor of CBF targets.

A.
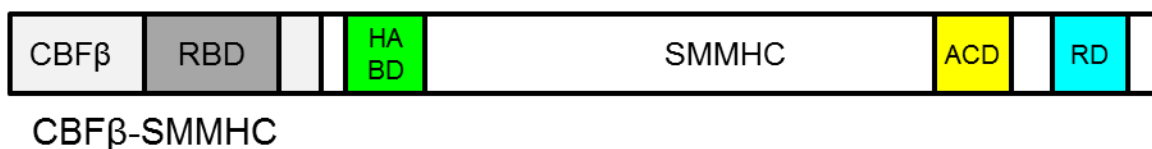
B.
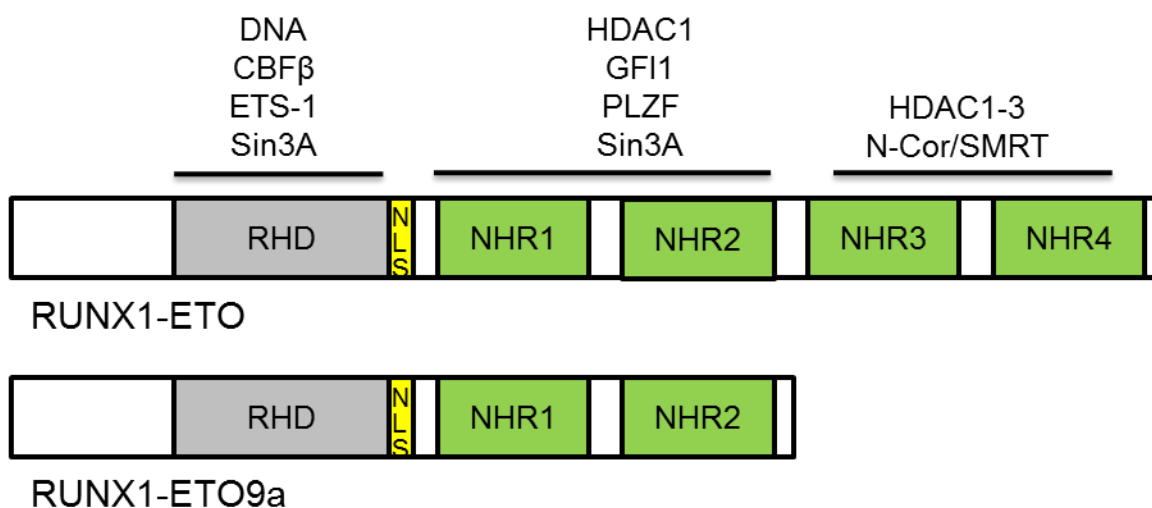
Figure 1.4. Functional domains of CBFβ-SMMHC, RUNX1-ETO and RUNX1-ETO9a proteins. A. Schematic representation of CBFβ-SMMHC domains: Runx binding domain (RBD), Hyperaffinity binding domain (HABD), Repression domain (RD), and Assembly competence domain (ACD). CBFβ part of the fusion protein is filled with light gray, SMMHC part is white. B. Full length RUNX1-ETO includes several defined functional domains from N- to C-terminus: Runt homology domain (RHD) and nuclear localization signal (NLS) from RUNX1 part of the fusion, and nervy homology regions 1-4 (NHR1-4) domains from ETO part of the fusion. RUNX1-ETO9a splicing isoform of RUNX1-ETO lacks NHR3 and NHR4 domains. Approximate binding sites for protein interactions and the DNA binding site are shown by horizontal lines above the protein diagrams with a list of interacting proteins. Adapted from Peterson et al., 2007.

Examples of CBF targets repressed by the fusion protein include the *Alternative reading frame p14* (*p14^{ARF}*) (Linggi et al., 2002), *T-cell receptor β* (*TCRβ*) (Meyers et al., 1995), and *GM-CSF* (Frank et al., 1995). The activity of RUNX1-ETO also seems to regulate gene expression by a CBF-independent mechanism. Studies have shown that RUNX1-ETO upregulates expression of *BCL2* (Klampfer et al., 1996), and *c-Jun* (Elsasser et al., 2003; Frank et al., 1999). The CBF-independent gene regulation hypothesis is further supported by the observation that RUNX1 and RUNX1-ETO localize to different regions of the nucleus (Bakshi et al., 2008). Considering that ETO is part of the fusion protein, it is possible that this cofactor may guide it to other transcriptionally active loci.

Acute myeloid leukemia samples carrying the t(8;21) express two *RUNX1-ETO* isoforms by alternative splicing (Yan et al., 2006b). In addition to the full length isoform into exon 11, some transcripts show splicing into an alternative exon 9a, terminating the transcript prematurely. The RUNX1-ETO9a protein lacks the C-terminal NHR3 and NHR4 domains, associated with binding to HDAC1/2/3 corepressors and the NCor/SMRT complex (Figure 1.4B). The loss of these domains creates a protein with strong leukemogenic function, since RUNX1-ETO9a induces leukemia in BM-transplantation assays, and does not inhibit proliferation *in vitro* (Yan et al., 2004). Indeed, significant differences were found between the isoforms on the regulation of *Cyclin-dependent kinase 4* (*CDK4*), *cyclin D3* (*CCND3*) and *p27^{kip1}* (*CDKN1B*). The leukemogenic ability of RUNX1-ETO9a is dependent on the DNA binding capacity from the RHD and the oligomerization function of the NHR2 domain (Yan et al., 2009). In addition, a single point mutation that disrupts the zinc finger structure of the NHR4

domain is sufficient to strongly enhance leukemogenic activity of RUNX1-ETO protein, making it similar to that of RUNX1-ETO9a (Ahn et al., 2008). Therefore, considering the relevance of the RUNX1-ETO9a function to AML development, and that patient AML blasts carrying t(8;21) express both isoforms at different proportions, it is possible that RUNX1-ETO9a may be the main isoform contributing to AML development *in vivo* at least in a fraction of t(8;21) AMLs. Of note, RUNX1-ETO9a provides a more potent myeloid differentiation block in a mouse model (Yan et al., 2006b). This suggests that ambiguous data obtained for RUNX1-ETO in its effect on differentiation and proliferation (see above) may not accurately represent the RUNX1-ETO(9a) function in actual human AMLs. Stronger effect of RUNX1-ETO9a on myeloid differentiation may also explain why class I and not class II mutations most commonly are found in t(8;21) AML.

Tyrosine Kinase Receptor Signaling

The C-KIT and FLT3 are receptor tyrosine kinase receptors, which together with Platelet-derived growth factor (PDGF) and Colony stimulating factor-1 (CSF-1) receptors are members of the structurally related type III RTKs. These RTKs contain five extracellular immunoglobulin-like domains, the transmembrane and juxtamembrane domains (JM), and a cytoplasmic tyrosine kinase domain (TKD). Upon binding to their ligands, c-KIT and FLT3 homodimerize (Blume-Jensen et al., 1991; Verstraete et al., 2011) and undergo autophosphorylation at several tyrosine residues in JM and TKD (Heiss et al., 2006;

Razumovskaya et al., 2009; Reith et al., 1991; Rottapel et al., 1991; Serve et al., 1994).

The autophosphorylation step is critical for determination of the specific downstream

signaling for each RTK because the phosphorylated tyrosines provide a substrate for

phospho-tyrosine specific Src homology 2 (SH2) domains found in proteins that bind to

the RTKs and recruit additional components of signaling pathways (Figure 1.5)

(Anderson et al., 1990; Kanner et al., 1991). One of the SH2-containing proteins that bind

to both c-KIT and murine Flt3 is p85 regulatory subunit of Phosphatidylinositol 3-kinase

(PI3K) (Lev et al., 1992; Rottapel et al., 1994). Importantly, human FLT3 does not

directly bind p85, but instead recruits it via the SH2 adaptor proteins GTP-exchange

complex growth factor receptor bound-2 (GRB-2), GRB2-associated binding proteins 1

and 2 (GAB-1/2) and Protein tyrosine phosphatase, non-receptor type 6 (PTPN6, also

known as SHP1) (Zhang and Broxmeyer, 1999; Zhang and Broxmeyer, 2000). The

recruitment of p85 to the RTK results in its phosphorylation and release of its inhibitory

function over PI3K catalytic subunit p110 (Cuevas et al., 2001). Activation of PI3K

results in accumulation of phosphatidylinositol-3,4,5-triphosphate (PIP3) in cell

membrane and subsequent recruitment and activation of Pleckstrin homology (PH)

domain-containing proteins, including 3-phosphoinositide-dependent protein kinase 1

(PDK1). Accumulation of PIP3 is negatively regulated by a tumor suppressor

Phosphatase and tensin homolog (PTEN) (Li et al., 1997) that is a phosphatase that

dephosphorylates PIP3 (Maehama and Dixon, 1998) and is a major negative regulator of

PI3K pathway (Stambolic et al., 1998). The best known PI3K downstream target is v-Akt

murine thymoma viral oncogene homolog (AKT) that is phosphorylated by PDK1 at
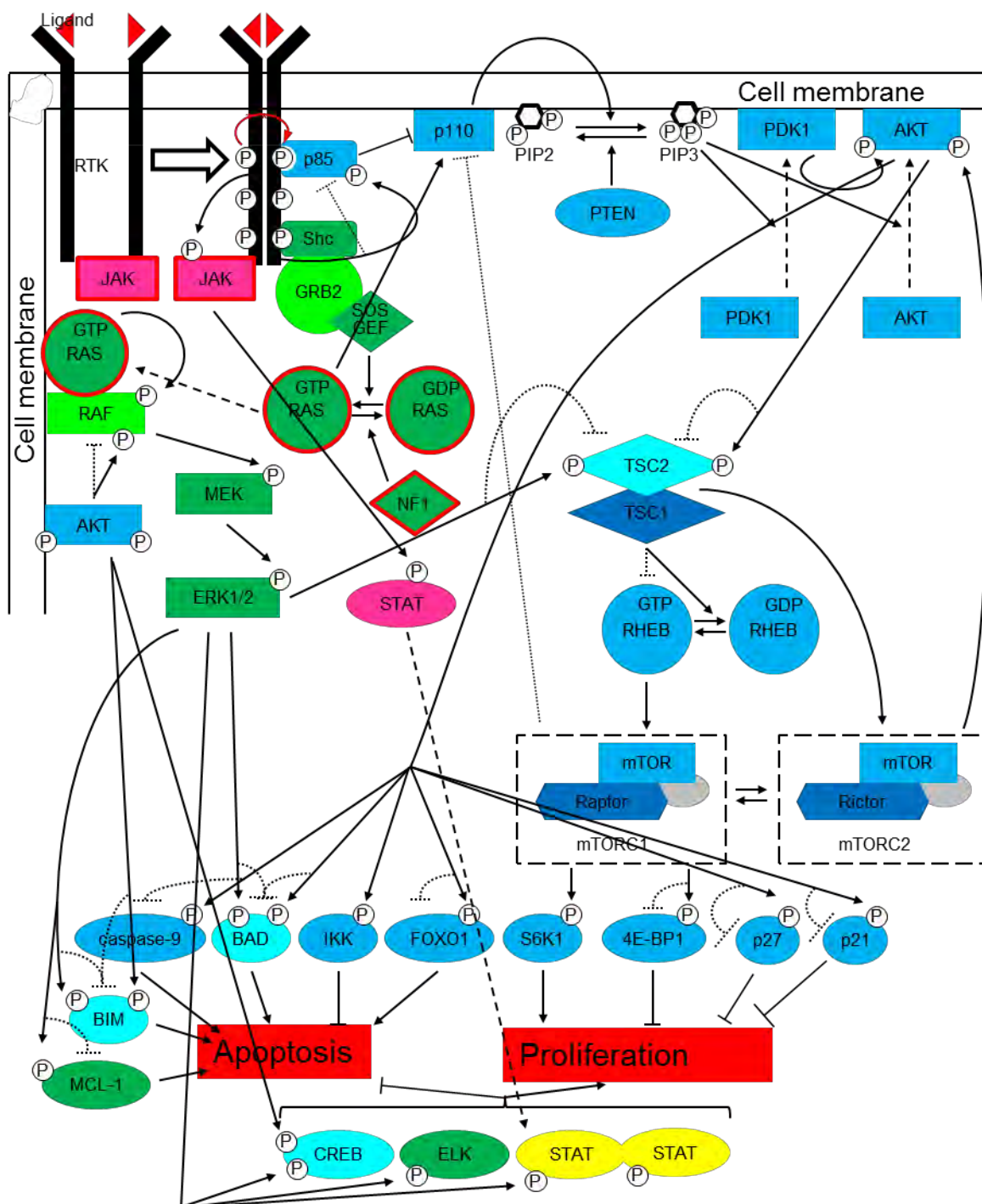
Figure 1.5. Pathways commonly activated by RTKs type III.
For description of the pathways see text. Proteins involved in PI3K/AKT pathway are depicted in blue, RAS pathway in green, and JAK/STAT pathway in crimson. Common targets and players are depicted in mixed colors. Phosphates are shown as Ⓟ, activation and/or phosphorylation are shown as solid arrows. If phosphorylation leads to inhibition, a dotted flat arrow is shown next to an arrow indicating phosphorylation. Intracellular re-localization is shown as punctuated arrows. Proteins encoded by genes commonly mutated in AML are emphasized by a red edge. * indicates activating RTK mutatins in AML.

Thr308 (Carnero, 2010). AKT kinase phosphorylates a variety of substrates involved in control of cell proliferation and apoptosis. One of the AKT substrates is Tuberous sclerosis complex 2 (TSC2) that together with TSC1 negatively regulates a small GTPase Ras homolog enriched in brain (Rheb). Phosphorylation of TSC2 by AKT inhibits its activity and allows Rheb to accumulate in an active GTP-bound state, which in turn activates mammalian Target of Rapamycin (mTOR) and stimulates its assembly into a protein complex called mTORC1. mTORC1 exists in dynamic equilibrium with another mTOR complex mTORC2 (whose assembly is stimulated by TSC1/2) that provides an important feedback loop by stimulating AKT activity by phosphorylating AKT at Ser473. mTORC1 indirectly negatively regulates AKT by influencing PI3K activity (Huang and Manning, 2009). Importantly, mTORC1, one of the main effectors of AKT, phosphorylates two substrates: p70 S6 kinase 1 (S6K1) and 4E-binding protein 1 (4E-BP1), stimulating protein synthesis and proliferation (Shah et al., 2000). In addition, PI3K/AKT signaling prevents apoptosis due to direct phosphorylation and inhibition of pro-apoptotic TFs of the Forkhead box (FOXO) family (Rena et al., 1999), BCL2-associated agonist of cell death (BAD) (Peso et al., 1997), caspase-9 (Cardone et al., 1998). Nuclear factor κB (NFκB) signaling pathway is also activated by phosphorylation of IκB kinase (IKK) with AKT (Nidai Ozes et al., 1999; Romashkova and Makarov, 1999). While NFκB pathway can provide both pro- and anti-apoptotic signaling, there is evidence for an anti-apoptotic effect of the pathway in several types of hematopoietic cancers, including a fraction of CBF-associated AML (Lück et al., 2010).

The PI3K/AKT signaling also increases cell proliferation as a result of inhibitory

phosphorylation of cyclin-dependent kinase inhibitors p27 and p21, and indirect

activation of Cyclin-dependent kinase 1 (CDK1) via inhibition of the protein kinases

WEE1 and Myelin transcription factor 1 (MYT1) (Martelli et al., 2006). In general,

PI3K/AKT signaling pathway provides major functions necessary for uncontrolled

proliferation and survival of a transformed cell. Not surprisingly, activating mutations in

its components (and loss-of-function mutations of PTEN) are commonly found as

oncogenic mutations in solid tumors (Samuels and Ericson, 2006). The reason for low

incidence of such mutations in AML is unknown, although a large fraction of AMLs

exhibit constitutive activation of PI3K/AKT pathway (Tamburini et al., 2007). It was also

shown that knocking-out *PTEN* (Yilmaz et al., 2006; Yu et al., 2010a; Zhang et al.,

2006a) or constitutive activation of AKT (Kharas et al., 2010) leads to leukemogenesis in

mice.

Upon RTK activation, Shc protein is recruited to the TKD domains of c-KIT and

FLT3 via its SH2 domain. This in turn results in recruitment of adaptor protein GRB-2,

guanine exchange factor (GEF) Son of sevenless (SOS), and Rat sarcoma (RAS),

resulting in RAS activation by its binding to guanosine triphosphate (GTP) (Dosil et al.,

1993; Duronio et al., 1992; Thommes et al., 1999; Zhang and Broxmeyer, 2000). The

activation of RAS is negatively regulated by GTPase activating protein Neurofibromin 1

(NF1) that stimulates RAS GTPase activity (Xu et al., 1990). Active GTP-bound RAS

recruits Rapidly Accelerated Fibrosarcoma (RAF) proteins to the cell membrane where

they become activated and besides other activities phosphorylate Mitogen-

associated/extracellular regulated kinase 1 (MEK1), which in turn activates by

phosphorylation Extracellular regulated kinases 1 and 2 (ERK1/2). ERK1/2 serine

kinases phosphorylate variety of substrates resulting in anti-apoptotic signaling by

directly inhibiting activity of BIM, BAD and MCL-1. One of the main functions of ERK

is phosphorylation and activation of several transcriptional regulators in the nucleus,

including cAMP response element-binding (CREB) TF, ELK and STAT. These

regulators alter the expression of multiple target genes promoting cell proliferation and

anti-apoptotic signaling (Steelman et al., 2008). Importantly, ERK also phosphorylates

TSC2 at a site distinct from the AKT phosphorylation site, providing a synergistic effect

between PI3K/AKT and RAS signaling pathways on TSC2 and mTOR regulation

(Winter et al., 2011). Other mechanisms of cross-talk between the pathways include

interaction between RAS and p110 resulting in induction of PI3K activity (Kodaki et al.,

1994; Rodriguez-Viciana et al., 1994), inhibition of RAF by AKT via direct

phosphorylation (Rommel et al., 1999; Zimmermann and Moelling, 1999), indirect

activation of RAF-1 by AKT (Majewski et al., 1999), and a number of common

phosphorylation targets, including CREB and BCL2-like 11 (BCL2L11, also known as

BIM) (Steelman et al., 2008). The activation of PI3K by RAS proteins is of particular

importance, as it is critical for oncogenic activity of RAS (Gupta et al., 2007), indicating

PI3K/AKT as an important RAS downstream effector pathway.

Wild type C-KIT RTK also activates Janus kinase 2 (JAK2) that is constantly

associated with the receptor and is phosphorylated upon SCF binding (Linnekin et al.,

1996; Weiler et al., 1996). Unlike c-KIT, wild type FLT3 can activate JAK/STAT

pathway only upon overexpression of FLT3 or if it is constitutively activated by

mutations (Spiekermann et al., 2003). In this case, though, the mechanism of JAK/STAT pathway activation occurs through direct, JAK-independent (Choudhary et al., 2007), but GRB2-dependent (Masson et al., 2009) activation of STAT5. JAK/STAT pathway is also involved in oncogenesis, as JAK2 mutations are commonly found in CML and MDS (Reuther, 2008). JAK is an intracellular kinase that is activated by different receptors and in turn phosphorylates STAT proteins. The phosphorylated STAT TFs dimerize, are transported into nucleus and become active as regulators of transcription. Among different genes regulated by STAT is anti-apoptotic BCL family member BCL-xL. STAT5 also promotes cell survival by inducing PIM1 expression (Steelman et al., 2008). Additionally, it is shown that activation of STAT5 by mutant FLT3 increases proliferation and alters differentiation of hematopoietic stem cells (Moore et al., 2007). Activation of STAT5 may be enhanced by ERK phosphorylation, indicating a possible cross-talk between JAK/STAT and MAPK pathways (David et al., 1995; Winston and Hunter, 1995).

The most commonly mutated genes found in human AMLs, as well as many of the genes constantly upregulated in AMLs, are involved in the described pathways. C-KIT RTK, which is almost ubiquitously expressed on AML cells, results in direct activation of PI3K/AKT pathway and activates STAT3 (Ning et al., 2001a; Ning et al., 2001b). RAS GTPases are also directly activated by c-KIT receptor, leading to upregulated RAF/MEK/ERK cascade (Scholl et al., 2008a). FLT3-ITD and other activating mutations of FLT3 activate AKT phosphorylation, RAS/RAF/MEK/ERK and STAT5 in AML cells (Brandts et al., 2005; Hayakawa et al., 2000; Kim et al., 2005;

Mizuki et al., 2000; Spiekermann et al., 2003). JAK2, as described above, is a member of JAK/STAT oncogenic pathway. Commonly mutated in AML RAS proteins are downstream targets of type III RTKs (including c-KIT and FLT3) and activators of RAF/MEK/ERF pathway. Finally, a major player in PI3K/AKT pathway AKT is overexpressed in a large human AML (Tamburini et al., 2007). Many of these players of leukemic transformation are targets for specific inhibitors therapy (see above), although cross-talks between the pathways and autoregulatory feedback loops make a task of cancer prevention by signaling inhibition challenging (Breitenbuecher et al., 2009; Chu and Small, 2009; Huang et al., 2003; Huang and Houghton, 2001).

Receptor tyrosine kinase pathways are frequently deregulated in CBF fusions-associated leukemias

Mutations in components of the RTK signaling pathway are the most common mutations found in CBF leukemias (Haferlach et al., 2010). Approximately 90% of CBF AML samples present cooperating mutations in *NRAS, KRAS, KIT,* and *FLT3*. The *KIT* and *FLT3* genes play crucial roles in proliferation and survival of hematopoietic progenitors (Ikeda et al., 1991; Pollard et al., 2010; Wang et al., 1989; Yokota et al., 1997). The oncogenic mutations that activate the FLT3 receptor are found in 5% to 7% of CBF AML cases, including the ITD and TKD mutations (Boissel et al., 2006b; Care et al., 2003; Schnittger et al., 2002; Shih et al., 2008), and have an unfavorable prognosis (Döhner et al., 2010; Mrózek et al., 2007). The activating mutations in *KIT* (the most common are

D816V and N822K) are found in approximately 25% of CBF AML and are rare in other

AMLs (Boissel et al., 2006b; Care et al., 2003; Goemans et al., 2005; Paschka, 2008;

Shih et al., 2008). It was also proven by using mouse models that both *FLT3-ITD* (Kim et

al., 2008; Schessl et al., 2005a) and mutant *KIT* (Wang et al., 2011b) cooperate with CBF

fusions in leukemia development in mice.

The CBF AMLs can also present oncogenic mutations in *NRAS* and *KRAS*, but

rarely in *HRAS* (Neubauer et al., 2008). *Rat sarcoma* (*RAS*) genes encode membrane-

anchored GTPases that are involved in multiple signaling pathways and were shown to

have oncogenic activity (McCubrey et al., 2007). In inv(16) AML cases around 30%

harbored constitutively active mutated *RAS* genes with the most commonly mutated

NRAS. The incidence of *RAS* mutations in t(8;21) AML cases is around 10% (Boissel et

al., 2006b; Care et al., 2003; Goemans et al., 2005; Shih et al., 2008). It is noteworthy

that *KIT*, *FLT3* and *RAS* mutations in inv(16) and t(8;21) human AML cases were

mutually exclusive, thus supporting the idea that they act as class I mutations in the "two-

hit" model for leukemia development (Care et al., 2003; Shih et al., 2008).

Approximately 8 to 10% of t(8;21) AML cases show oncogenic mutations in the

tyrosine kinase *Janus kinase 2* (*JAK2*) gene, frequently at residue V617F (Dohner et al.,

2006; Schnittger et al., 2007b), but this occurs less frequently in inv(16) AML.

Interestingly, these mutations in JAK2 are often found in CML and MDS, but are rare in

non-CBF AMLs (Illmer et al., 2007). Although JAK2 is a non-receptor tyrosine kinase,

the signaling pathways activated by the constitutively active JAK2V617F are similar to

those activated by RTKs (Reuther, 2008). Finally, heterozygous and homozygous loss of

tumor suppressor Neurofibromin 1 (*NF1*) expression have been identified in 10% of CBF AMLs. NF1 catalyzes GTPase activity of RAS proteins, thereby reducing RAS signaling, so identification of the NF1 loss-of-function mutations in CBF AMLs further strengthens the relevance of RTK signaling in leukemia development (Haferlach et al., 2010).

Taken together, these studies highlight the critical involvement of constant RTK activation in AML development. They also underscore functional connection between commonly found class I mutations in AML and provide mechanisms by which the mutations confer leukemic cells proliferative and survival advantages.

Prognostic significance of RTK pathways mutations in CBF AML

Frequent deregulation of RTK signaling in CBF AML may present an opportunity for efficient treatment strategies of AML cases by targeting the RTK pathways. The decision for use of particular inhibitors, however, should take into account both potential benefits for a patient and risks due to toxicity. Below I will briefly review the prognostic values of RTK pathways mutations in CBF AMLs and current or potential treatments based on RTK targeting.

While *KIT* activating mutations are frequently found in CBF AML (see above), the effect of the mutations on the disease prognosis is not clear. Some studies of adult AML determined there is reduced survival in t(8;21)-associated AML cases carrying *KIT* mutations, but not in inv(16)-associated AML (Boissel et al., 2006b; Cairoli et al., 2006). Other studies showed increased rate of relapse, but no changes in survival of CBF AML positive for KIT mutations (Care et al., 2003). Finally, a study by Pashka e al. showed

effects of KIT mutations on survival and relapse of both inv(16)- and t(8;21)-associated AMLs (Paschka et al., 2006). Disease prognosis for childhood CBF AMLs was not shown to be affected by *KIT* mutations (Goemans et al., 2005; Pollard et al., 2010; Shih et al., 2008). Due to a relatively good prognosis and an unclear effect of *KIT* mutations on CBF AML, the treatment of the CBF AML cases is generally not changed when presence of the mutations in KIT is detected. Occasional trials that used RTK inhibitor imatinib on the CBF AMLs carrying mutations in KIT indicate mixed results and suggest that efficiency of imatinib may depend on the particular mutation of KIT (Cairoli et al., 2005; Nanri et al., 2005). Further studies are necessary to determine whether inhibition of KIT activity is beneficial for the treatment of CBF AML. More potent inhibitors that affect a wider range of *KIT* mutations than imatinib may prove to be effective in the future (Schittenhelm et al., 2006).

Activating *FLT3* mutations are less commonly found in CBF AML than in other subtypes of AML (see above). However, presence of the mutations in AML in general significantly worsens the prognosis (Schnittger et al., 2002), and screening for *FLT3* mutations was recommended for all AML subtypes in the latest WHO classification of hematopoietic diseases (Vardiman et al., 2009). The adverse effect of *FLT3* mutations on survival and relapse rate was also detected in CBF AML (Boissel et al., 2006b; Goemans et al., 2005). Therefore, efficient inhibition of mutated FLT3 signaling should theoretically benefit the patients that carry mutation in this RTK. To our knowledge, no clinical trials using FLT3 inhibitors focusing on CBF AML were conducted probably due to relatively low incidence of FLT3 mutations in CBF AML. Trials conducted in AML in

general determined that use of FLT3 inhibitor sorafenib can be beneficial in some cases, but many AML cases were resistant to it indicating presence of widespread resistance mechanisms (Parmar et al., 2011; Pratz and Levis, 2010). More efficient FLT3 inhibitors are required to make the treatment more beneficial to the patients. In the meantime, due to adverse effect of *FLT3* mutations on survival of CBF AML patients, it may be necessary to evaluate effectiveness of bone marrow transplantation in these patients. The bone marrow transplantation is usually not used for CBF AML patients, but it is used for other subtypes of AMLs with more poor prognosis (Rowe, 2009).

Presence of the activating mutations of *RAS* genes in CBF AML is not considered a reason for altering standard therapy. Although these mutations are frequently found in CBF AML, they do not have prognostic significance (Boissel et al., 2006b; Goemans et al., 2005). Moreover, AMLs carrying *RAS* mutations have more favorable prognosis when treated with high-dose cytarabine during post-remission phase (Neubauer et al., 2008), therefore it is unlikely that introduction of *RAS* inhibitors in AML treatment will lead to a better prognosis. Rather, CBF AMLs carrying *RAS* mutations are some of the least aggressive secondary mutations in AML and may benefit from novel treatment strategies targeting the CBFβ-SMMHC or RUNX1-ETO fusion proteins functions. These strategies have not been developed yet, but potentially can be very effective in treatment of CBF AML.

Novel and potentially cooperating genes in CBF-related AML

The identification and characterization of factors that cooperate with CBF fusion proteins in AML is an important task that can be beneficial in several aspects: (1) they can identify genes mutated or deregulated in human leukemia cells, and (2) they can represent valid targets of therapeutic inhibitors for the reversal of the pathways activated by mutations. A previous study in the Castilla laboratory used a retorviral insertional mutagenesis approach in *Cbfb-MYH11* knock-in mice to identify candidate cooperating genes in leukemia (Castilla et al., 2004). Some of the common retroviral insertion sites (CIS) identified were located in the promoter regions of the related transcription factors *PLAG1* and *PLAGL2* and the micro-RNA cluster *miR-17-92*. The *PLAG* genes were later shown to synergize with *Cbfb-MYH11* in leukemia development in mice (Landrette et al., 2005). Since these genes are the focus of this thesis, I will summarize their function in the following sections.

PLAG Transcription Factors are oncogenes that cooperate with CBFB-MYH11 in AML development

The Pleomorphic adenoma of salivary gland (PLAG) is a small family of transcription factors, including the PLAG1, PLAGL1 and PLAGL2. The PLAG1 protein has an NLS signal at the N-terminus and 7 C2H2 type zinc finger domains, three of which bind to the DNA consensus sequence $GRGGC(N)_{6-8}RGGK$ (Hensen et al., 2002; Kas et al., 1998), and the only known PLAG1 target is *Insulin-like growth factor 2* (*IGF2*) at its promoter 3

(Voz et al., 2000). The upregulation of *PLAG1* expression is the most frequent mutation in pleomorphic adenomas of the salivary gland, and results from a translocation-mediated promoter swapping involving the 8q12 locus (Kas et al., 1998). Its oncogenic function was later extended to other tumor types, including chronic lymphocytic leukemia (CLL) (Pallasch et al., 2009; Patz et al., 2010), salivary gland tumors (Matsuyama et al., 2011), lipoblastomas (Astrom et al., 2000), hepatoblastomas (Zatkova et al., 2004), and breast cancer (Declercq et al., 2008). In NIH3T3 cells the PLAG genes were shown to provide pro-proliferative and oncogenic activity upon overexpression (Hensen et al., 2002). Further, a 250 kb region on chromosome 20q11 was found amplified in MDS/AML cases. This region includes *PLAGL2*, and the amplifications resulted in upregulation of its transcript levels (MacKinnon et al., 2010). In addition, PLAG1 and PLAGL2 have been involved in the regulation of Wingless-type MMTV integration site family (WNT) signaling pathway in neuronal stem cells, gliomas, and salivary gland tumors (Zhao et al., 2006b; Zheng et al., 2010).

After the identification of retroviral insertions upstream of *Plag1* and *Plagl2* genes in *Cbfb-MYH11*-associated leukemias in mice (Castilla et al., 2004), our laboratory demonstrated that upregulation of both genes act as efficient cooperating oncogenes in leukemia development (Landrette et al., 2005). Furthermore, we have recently shown that PlagL2 induces expression of *Mpl* and activates the Mpl signaling pathway and thereby cooperates with *Cbfb-MYH11* in AML development (Landrette et al., 2011).

Mutations in the Thrombopoietin/MPL signaling pathway are associated with hematopoietic malignancies

The Myeloproliferative leukemia virus oncogene MPL (also known as CD110) is the cognate receptor for the cytokine thrombopoietin (THPO), and both are essential for megakaryocyte function and HSC maintenance (Kaushansky and Ranney, 2009a). The MPL receptor belongs to the type I tyrosine kinase receptor family, characterized by the conserved WSWXWS motif in the extracellular region, and the lack of intrinsic kinase activity in the intracellular region (Kaushansky, 2009a). Upon interaction with Thpo ligand, homodimers of MPL receptor are formed to activate a signaling cascade. Cytoplasmic domains of the MPL units change conformation and become closer to each other, allowing cross-phosphorylation of specific tyrosines of MPL cytoplasmic domains by JAK2 kinase (Drachman et al., 1995; Drachman et al., 1999). Upon activation, phosphorylated tyrosine residues of MPL serve as docking sites for SHP2 and GAB2 SH2 motif-containing proteins that in turn bind p85 subunit of PI3K, leading to PI3K/AKT pathway induction (Miyakawa et al., 2001). Similarly, MPL binds GRB2 and SOS1 that serve to activate MAP kinase (MAPK) signaling (RAS/RAF/MEK/ERK) (Sasaki et al., 1995). As mentioned above, MPL and THPO are involved in regulation of HSCs and megakaryocytes (Kaushansky and Ranney, 2009b), supporting HSC maintenance (Kaushansky, 2009b) and self-renewal capacity (Fox et al., 2002b; Qian et al., 2007) and megakaryocytic maturation (Broudy et al., 1995; de Sauvage et al., 1994; Kaushansky et al., 1994). THPO also induces proliferation in several hematopoietic cell types (Ku et al., 1996; Pang et al., 2009; Rasko et al., 1997; Tang et al., 2008).

Although THPO is the best studied and accepted MPL ligand, studies in human and mouse cells have reported the activation of MPL signaling via the mammalian homolog of the *nuclear distribution gene* (*nud*) from *Aspergilus nidulans* (*Nuclear distribution gene C*, *NUDC*). The *NUDC* is expressed in early myeloid and erythroid progenitors, and in acute leukemia samples (Miller et al., 1999). As a cytokine, NudC promotes megakaryocyte maturation and platelet formation (Wei et al., 2006) by activation of MPL signaling (Pang et al., 2009; Tang et al., 2008; Zhang et al., 2007), providing an explanation for the residual platelet levels in *Tpo*-null mice (Pan et al., 2005). Although the role of hNUDC in normal and neoplastic hematopoiesis remains unclear, involvement of hNUDC in AML is an interesting possibility that may complement the results obtained in this study showing cooperation of wild type Mpl with RUNX1-ETO in AML development. If the observed hNUDC upregulation in human leukemia (Miller et al., 1999) is functional in upregulating MPL signaling (Tang et al., 2008), this could be one of the ways for increasing MPL signaling and, therefore, cooperation of wild type MPL with other oncogenes in leukemia.

Loss of function mutations in *MPL* or *THPO* genes are associated with congenital amegakaryocytic thrombocytopenia, severe thrombocytopenia and aplastic anemia (Ballmaier and Germeshausen, 2009). Somatic activating mutations in *MPL* (most commonly W515L/K) cause constitutive JAK2 activation and are associated with myeloproliferative neoplasms, including myelofibrosis with myeloid metaplasia and essential thrombocythemia (Pardanani et al., 2006; Pikman et al., 2006). Mutations in *MPL* have only been detected in a quarter of acute megakaryoblastic leukemia cases

(Hussein et al., 2009b).

MicroRNA cluster miR-17-92 is an oncogene that affects cell proliferation and survival

The microRNAs (miRNAs, miRs) are small non-coding RNA molecules (21-25 nucleotides) that regulate a variety of cellular processes, including apoptosis, metabolism, cell cycle and differentiation (Ambros, 2004; Bartel, 2004; Bushati and Cohen, 2007). The miRNAs can be located in intergenic regions of the genome or within gene structures. Intergenic miRNAs are thought to be regulated by specific promoters and transcribed using RNA polymerases II or III (Borchert et al., 2006; Ozsolak et al., 2008). The miRNAs located in the gene introns are preferentially co-transcribed with the host gene (Rodriguez et al., 2004).

Most miRNAs are present as individual miRNAs, but in some cases they are grouped in clusters and transcribed from a single primary miRNA molecule (pri-miRNA). In either case, the pri-miRNAs are then processed by the ribonuclease Drosha into a precursor miRNA (pre-miRNA) of about 70 nt and a stem-loop structure (Figure 1.6) (Lee, 2003). Processing of different pre-miRNAs from one cluster can be independently regulated, involving specific factors and leading to different expression patterns of mature miRNAs from the same cluster (Guil and Caceres, 2007; Wang et al., 2011a). The precursors are then transported into cytoplasm where they are further processed into mature miRNAs by the Ribonuclease (RNase) III enzyme Dicer. The mature miRNAs are incorporated into the RNA-induced silencing complex (RISC) to
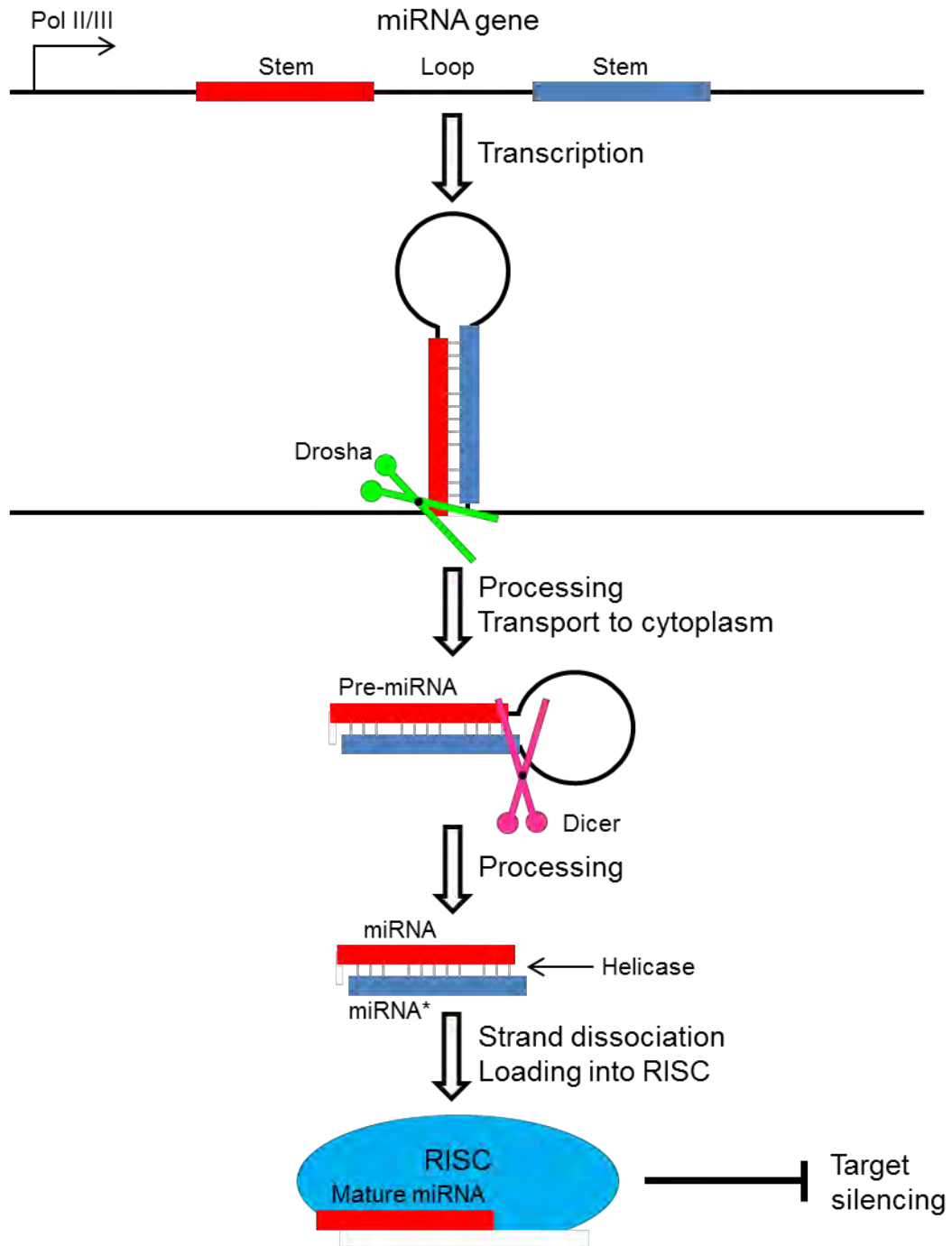
Figure 1.6. Schematic representation of miRNA processing.
See text for details. The miRNA is transcribed with RNA Pol II or Pol III, cleaved by RNAse III Drosha, transported into cytoplasm and cleaved by RNAse III Dicer into miRNA:miRNA* complex. The complex dissociates with helicase, and the mature miRNA is loaded in the RISC complex. DNA is shown as a black line, miRNA sequence is shown as red box, miRNA* sequence is shown as blue box. Small gray lines between the boxes indicate imperfect pairing.

destabilize mRNA targets of the miRNAs (Olena and Patton, 2010). The targeting is

based in part on homology to a seed sequence within the miRNA (6-8 nt) that imperfectly

base-pairs to a miRNA binding site(s) usually located at the 3"-UTR (untranslated region)

of an mRNA target. To date, more than 500 miRNAs were described in human genome,

36% of them localized in clusters. They are estimated to regulate up to a third of all

protein coding genes (Griffiths-Jones et al., 2008).

The miRNAs have been shown to play a role in tumorigenesis, acting as tumor

suppressors or "oncomiRs" (i.e., promoting cancer development). The earliest example of

tumor suppressor miRNAs are the closely located *miR-15* and *miR-16* in chromosome

13q14, commonly deleted in CLL (Calin et al., 2002; Calin et al., 2005), and also found

to be also deleted in other cancers, such as high-stage prostate cancer (Bonci et al., 2008;

Dong et al., 2001). They have been shown to induce apoptosis via negative regulation of

an anti-apoptotic protein Bcl2 (Cimmino et al., 2005). Other examples of miRNA tumor

suppressors are *miR-451* in gliomas (Nan et al., 2010), and *miR-96* in pancreatic cancers

(Yu et al., 2010c). The oncomiR role, mainly when upregulated by transcription factors

or chromosome amplifications, include *miR-21* in breast cancer (Si et al., 2007), *miR-155*

in B-cell lymphoma (Eis et al., 2005), and *miR-17-92* cluster in leukemias (He et al.,

2005; Xiao et al., 2008).

The miRNA cluster *miR-17-92* is transcribed from the intronic sequence of the

non-coding *MIR17 host gene* (*MIR17HG*), and encodes 6 miRNAs (*miR-17-5p*, *miR-18a*,

miR-19a, *miR-20a*, *miR-19b-1* and *miR-92a*). Two paralogous clusters present in the

mammalian genome, are the *miR-106a-363* cluster in chromosome Xq26.2 and *miR-*
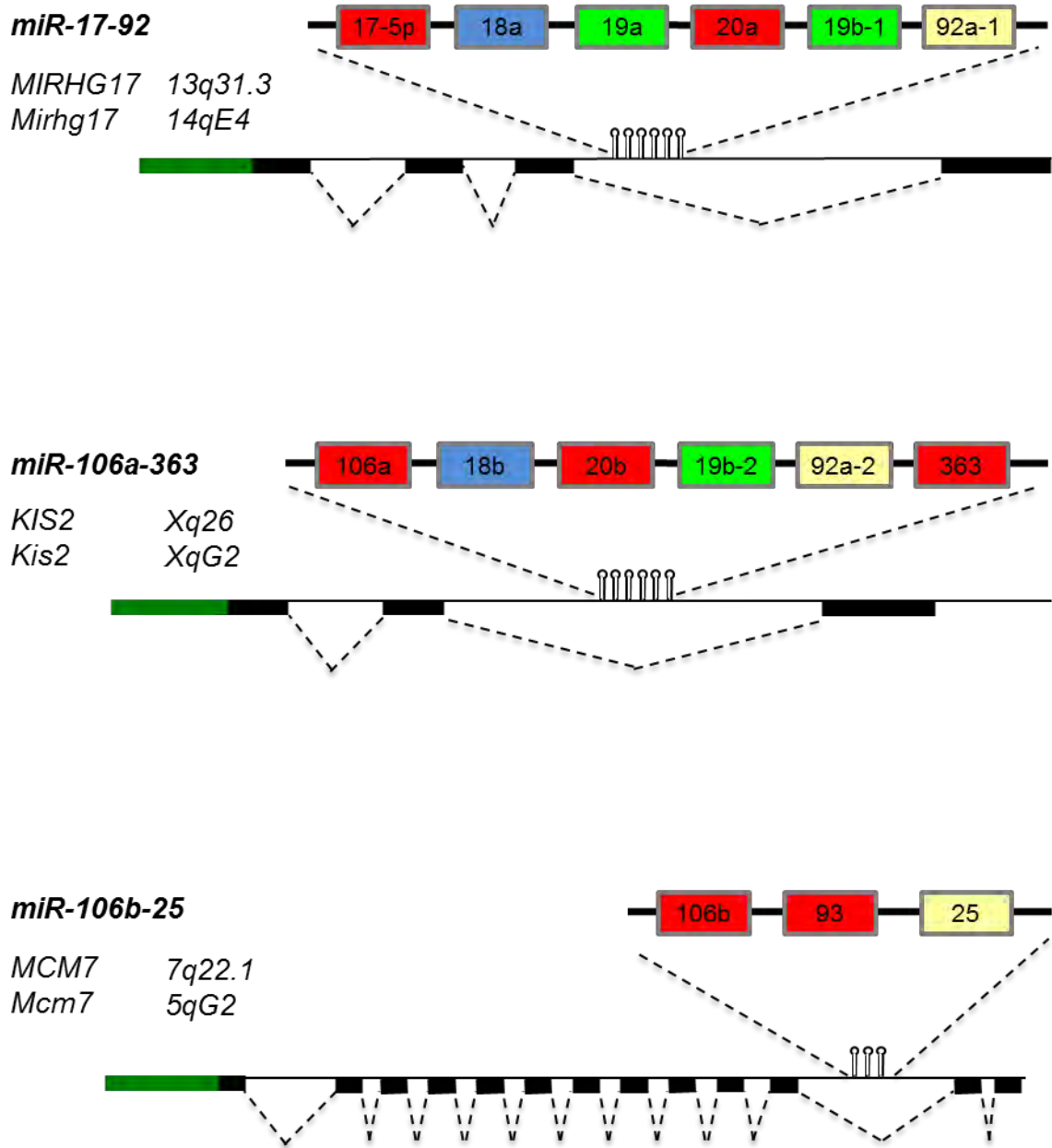
Figure 1.7. Schematic representation of the paralogous microRNA clusters.
The *miR-17-92* (top), *miR-106a-363* (middle) and *miR-106b-25* (bottom). The chromosome location and host gene name (human and mouse) are shown below the miRNA name. CpG island at the promoter regions (green box), exons (black box), introns (thin black lines) and microRNA clusters (stem loops) are shown. MicroRNA families are color coded: *miR-17* (red), *miR-18* (blue), *miR-19* (green) and *miR-92* (yellow).
Modified from Mendell, 2008, and UCSC Genome Browser (NCBI37/mm9).

106b-25 in chromosome 7q22.1, with specific tissue expression patterns (Figure 1.7 (Mendell, 2008)). They are thought to have emerged through a complex series of duplications and losses of individual members (Tanzer and Stadler, 2004).

The miRNAs from this cluster can be divided into four functional families according to their target seed sequences: *miR-17* family (*miR-17-5p, miR-20a*), *miR-19* (*miR-19a, miR-19b-1*), *miR-18a* and *miR-92*. Since these families also include the miRs of the paralog clusters, the expression of either miR of a family is expected to regulate the same group of targets (Mendell, 2008). The *miR-17-92* cluster plays important roles in development of various tissues by regulating differentiation, proliferation and/or apoptosis. For example, it inhibits differentiation and promotes proliferation of lung epithelial cells (Lu et al., 2007), promotes cell cycle during neuronal differentiation *in vitro* (Trompeter et al., 2011), and enhances differentiation in an *in vitro* model of adipocyte differentiation (Wang, 2008).

In hematopoietic tissues, *miR-17* family of miRNAs was found to be downregulated during monocytic differentiation. Forced overexpression of *miR-17* miRNAs inhibited monocytic differentiation due to downregulation of RUNX1 and consequently M-CSFR expression (Fontana et al., 2007). Clues to the role of the *miR-17-92* cluster role in B-cell development were found in a study of Dicer ablation in B cell lineage (Koralov, 2008). The study showed upregulation of *miR-17-92* targets in B-cells and a subsequent block of B-cell differentiation at the pro-B to pre-B cell transition. The block could be rescued by ablation of *Bim* or upregulation of *Bcl2*, suggesting an anti-apoptotic role of *miR-17-92* in B-cell development. Both lung and B-cell defects were

evident in a *miR-17-92*-null study. These KO mice exhibited lung hypoplasia and B-cell

defects similar to the ones observed in B-cell specific Dicer KO mice. Other tissues

appeared normal, suggesting possible compensation of *miR-17-92* function by *miR-17-92*

paralogs, as double KO of *miR-17-92* and its paralog *miR-106b-25* had a much more

severe phenotype resulting in midgestation lethality (Ventura et al., 2008).

The *miR-17-92* cluster is located on chromosome 13q31 that is often amplified in

several types of human cancers, including diffuse large B-cell lymphoma (Lenz et al.,

2008), primary cutaneous B-cell lymphoma (Inomata et al., 2009), follicular lymphoma,

mantle cell lymphoma (Ota et al., 2004). While invariably correlating with amplification

of the 13q31, *miR-17-92* cluster is also upregulated in small cell lung cancer (Hayashita

et al., 2005), pancreatic cancer and some other cancers (Chow et al., 2010; Guo et al.,

2009; Novotny et al., 2007; Takakura et al., 2008). The presumption that *miR-17-92* may

be an oncogene was further supported by the fact that *miR-17-92* was a frequent target of

RIS in RIM screenings for erythroleukemia and T cell leukemia (Cui et al., 2007; Wang

et al., 2006). In addition, it was shown that *miR-17-92* overexpression increases

penetrance and shortens latency in an Eμ-*Myc* mouse model of B-cell lymphoma (He et

al., 2005). Enhanced leukemia development upon increased *miR-17-92* expression was

also shown in a mouse model of Mixed-lineage leukemia (MLL) fusions-associated

leukemia (Mi et al., 2010; Wong et al., 2010). Finally, lymphocyte-specific conditional

overexpression of the *miR-17-92* cluster in mice induced a lympoproliferative disease by

providing lymphocytes with pro-proliferative and anti-apoptotic signaling. The

mechanism of this signaling was shown to involve downregulation of *Pten* and *Bim* by

the *miR-17-92* cluster, although other unidentified effectors were likely involved (Xiao et al., 2008).

MiR-17-92* is predicted to regulate hundreds of genes based on seed sequence homology, although it is now accepted that this regulation is highly cell type specific. Known targets that were shown to be downregulated by the cluster include PTEN, a negative regulator of PI3K/AKT pathway, the cell cycle regulators E2f1, Rb2, and p21, the pro-apoptotic protein Bim, the transcription factor Runx1, and members of TGFβ signaling pathway Tgfβ type II receptor, Smad2 and Smad4 (Table 1.3) (Cloonan et al., 2008; Ernst et al., 2010; Hong et al., 2010; Mestdagh et al., 2010; Sun et al., 2010; Trompeter et al., 2011). All aforementioned genes have been implicated in cancer regulation by providing anti-proliferative and/or pro-apoptotic signaling. Besides direct regulation of target genes, *miR-17-92* is also involved in regulatory feedback loops. For example, c-Myc and E2f proteins upregulate *miR-17-92* expression by binding to the *miR-17-92* cluster promoter and activating its transcription. In turn, *miR-17-92* downregulates E2F1 protein expression. (Novotny et al., 2007; O'Donnell et al., 2005; Sylvestre et al., 2007). Another example is a feedback loop between *miR-17/20a* and STAT3: *miR-17/20a* downregulate STAT3 expression, whereas STAT3 activates transcription of *miR-17-92* (Dai et al., 2011; Zhang et al., 2011).

As mentioned above, the *miR-17-92* cluster can affect both cell cycle and apoptosis in cell type specific manner. In HEK293 (human embryo kidney) cells overexpression of *miR-17-5p* alone accelerates transition from G1 to S phases of cell cycle resulting in increased proliferation (Cloonan et al., 2008). Acceleration of cell cycle

Table 1.3. Confirmed target genes of miRNAs expressed from *miR-17-92* cluster

Gene	miRNA family	Reference
AIB1	miR-17	(Hossain et al., 2006)
BCL2L11	miR-17	(Cloonan et al., 2008)
CDKN1A	miR-17	(Hong et al., 2010)
E2F1	miR-17	(O'Donnell et al., 2005)
E2F2	miR-17	(Sylvestre et al., 2007)
E2F3	miR-17	(Sylvestre et al., 2007)
FN1	miR-17	(Shan et al., 2009)
FNDC3A	miR-17	(Shan et al., 2009)
GAB1	miR-17	(Cloonan et al., 2008)
IRF	miR-17	(Cloonan et al., 2008)
JNK2	miR-17	(Cloonan et al., 2008)
NR4A3	miR-17	(Cloonan et al., 2008)
PCAF	miR-17	(Cloonan et al., 2008)
PKD1	miR-17	(Cloonan et al., 2008)
PKD2	miR-17	(Sun et al., 2010)
PPARA	miR-17	(Cloonan et al., 2008)
PTEN	miR-17	(Xiao et al., 2008)
RB	miR-17	(Volinia et al., 2006)
RBL1	miR-17	(Cloonan et al., 2008)
RBL2	miR-17	(Lu et al., 2007)
RUNX1	miR-17	(Fontana et al., 2007)
STAT3	miR-17	(Zhang et al., 2011)
TGFBR2	miR-17	(Volinia et al., 2006)
TSG101	miR-17	(Cloonan et al., 2008)
CTGF	miR-18	(Dews et al., 2006)
SMAD2	miR-18	(Mestdagh et al., 2010)
SMAD4	miR-18	(Mestdagh et al., 2010)
BIM	miR-19	(Mavrakis et al., 2010)
BNIP3	miR-19	(Mavrakis et al., 2010)
PPP2R5E	miR-19	(Mavrakis et al., 2010)
PRKAA1	miR-19	(Mavrakis et al., 2010)
PTEN	miR-19	(Olive et al., 2009)
TNF	miR-19	(Liu et al., 2011)
TSP1	miR-19	(Dews et al., 2006)
BIM	miR-92	(Xiao et al., 2008)

by *miR-17-5p* was also shown on human primary pancreatic cancer cells (Yu et al., 2010b). Yet in lung cancer cells the *miR-17* and *miR-20a* miRNAs provide anti-apoptotic effect (Matsubara et al., 2007). An interesting illustration of cell type specificity on an example of B-cell lymphoma cell lines was shown by Inomata et al. They showed that *miR-17-92* downregulated BIM in Burkitt''s lymphoma derived cell line Raji, but not in mantle cell lymphoma derived Jeko1 cells, where Bim is homozygously deleted. Instead, *miR-17-92* downregulated p21 in Jeko1 cell, resulting in cell cycle progression (Inomata et al., 2009). Taken together, the data suggest that *miR-17-92* cluster could potentially serve as class I mutation in cancer development by providing either or both pro-proliferative and anti-apoptotic signals.

General research objectives of this Thesis

In this study I focused on the identification of novel cooperating mutations that cooperate with CBF-related fusion proteins in development of AML, and investigated their potential mechanisms of cooperation. First I hypothesize that upregulation of the *miR-17-92* cluster is a novel cooperating mutation in CBF-related AMLs. I provide functional evidence that the *miR-17* family is responsible for the oncomiR activity within the cluster. Second, I hypothesize that the oncogenic role of PlagL2 is, at least in part, the upregulation of the Mpl receptor. Furthermore, based on similarity of signaling pathways involved in MPL signaling and signaling of known cooperating receptor kinases KIT and FLT3, I proposed that *MPL* overexpression can synergize with *RUNX1-ETO* to induce

AML. Therefore, this thesis research identifies *miR-17-92* and *Mpl* genes as cooperating genetic factors in CBF leukemia.

CHAPTER II

The *miR-17* family miRNAs cooperate with CBFβ-SMMHC in leukemia development by activating antiapoptotic responses

Introduction

The microRNAs (miRs) are small non-coding RNA molecules (21-23 nucleotides) that regulate protein levels by reducing translation and stability of target mRNAs (Djuranovic et al., 2011; Lee and Ambros, 2001). They have been shown to regulate cellular processes, such as apoptosis, metabolism, cell cycle and differentiation (Ambros, 2004; Bartel, 2004; Bushati and Cohen, 2007), and their deregulation can participate in tumorigenesis functioning as oncogenic miRNAs (oncomiRs) or tumor suppressors (Cimmino et al., 2005; Eis et al., 2005; Esquela-Kerscher and Slack, 2006; Kent and Mendell, 2006; Nan et al., 2010; Si et al., 2007).

The miR cluster *miR-17-92* is an intronic miR transcribed by the *miR-17-92 cluster host gene* (*MIR17HG*) as a single primary transcript, and encodes 6 mature miRs: *miR-17-5p*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19b-1* and *miR-92a*. This miR cluster (also called *oncomiR-1*) is implicated as an oncogene in different tumors, including B-cell lymphoma (Inomata et al., 2009; Lenz et al., 2008), small cell lung cancer (Hayashita et

al., 2005), pancreatic cancer and some other cancers (Chow et al., 2010; Guo et al., 2009; Novotny et al., 2007; Takakura et al., 2008). The presumption that *miR-17-92* may be an oncomiR was further supported by studies of murine hematopoietic cancer models suggesting that *miR-17-92* cooperate with variety of oncogenes in development of erythroid, lymphoid and myeloid malignancies (Cui et al., 2007; He et al., 2005; Mi et al., 2010; Wang et al., 2006; Wong et al., 2010). This cluster has 4 functional families, based on their seed sequences that determine DNA target specificity: The *miR-17* (*miRs-17-5p* and *-20a*), *miR-18* (*miR-18a*), *miR-19* (*miR-19a* and *-19b-1*), and *miR-92* (Mendell, 2008).

Individual members of this cluster have been shown to regulate a variety of targets, including Pten, E2f1, Rb2, p21, Bim, Runx1 (Cloonan et al., 2008; Ernst et al., 2010; Hong et al., 2010; Mestdagh et al., 2010; Sun et al., 2010; Trompeter et al., 2011). In normal hematopoiesis, *miR-17* members regulated differentiation of monocytes and B cells (Fontana et al., 2007; Lu et al., 2007; Ventura et al., 2008). The *miR-17* family promoted proliferation in human primary pancreatic cancer cells and inhibited apoptosis in lung cancer cells (Cloonan et al., 2008; Matsubara et al., 2007; Yu et al., 2010b).

The activation of the cluster *miR-17-92* has been shown to induce lymphoid neoplasia in cooperation with *MYC*, using mouse models and human samples (He et al., 2005; Mu et al., 2009), and later reported that *miR-19b*, but not *miR-17* family acted as an oncomiR by hindering Pten levels (Olive et al., 2009).

Mutations in components of the heterodimeric transcription factor CBF *RUNX1* and *CBFB* are frequently present in acute myeloid leukemia (AML) samples (Paschka,

2008). One of these alterations is the inversion in chromosome 16 inv16(p13;q22), generating the fusion oncogene *CBFB-MYH11* (Liu et al., 1993). The encoded chimeric protein CBFβ-SMMHC blocks myeloid differentiation and induces leukemia in cooperation with other mutations (Castilla et al., 1999; Castilla et al., 2004). Considering that the fusion protein does not provide self-renewal or proliferative advantage, these functions acquired in the leukemic cells may be provided by the cooperating mutations (Kuo et al., 2006).

In order to identify candidate mutations that cooperate with CBFβ-SMMHC in leukemia development we screened for candidate oncogenes using a retroviral insertional mutagenesis (RIM) approach (Castilla et al., 2004). In the present study we identify and characterize two retroviral insertions upstream of the miR cluster *miR-17-92* in mice and human AML samples. We validated the upregulation of 4 miRs in the cluster, including *miRs-17-5p*, *-20a*, *19a*, and *-92a*. Expression analysis of a panel of inv(16) human AML samples shows expression of these miRs in a fraction of samples. We use a conditional *CBFB-MYH11* knock in mouse to show that the miR cluster induces myeloid leukemia. Furthermore, we show that expression of *miR-17-92* upregulates PI3K/Akt pathway, and that *miRs-17-5p*, *-20a*, and *-19a* can regulate Pten and increase Bad phosphorylation levels in the leukemic blasts. Finally, we show that *miR-17* and *miR-20a*, but not *miR-19a* and *-19b*, provide anti-apoptotic capacity to the leukemic samples. These studies provide evidence that miRNAs of the *miR-17* family are the oncomiRs in AML.

Materials and methods

Linker-mediated PCR. A collection of previously obtained by RIM *Cbfb-MYH11* AML

samples (Castilla et al., 2004) was used for identification of retroviral insertion sites with

linker-mediated genomic PCR as described elsewhere (Schmidt et al., 2001). Briefly, 1μg

of tumor genomic DNA was digested with NlaIII (cat. #R0125, NEB, Ipsich, MA) or

MseI (cat. #R0525, NEB, Ipswich, MA). After heat inactivation of the restriction

enzymes, the DNA was ligated with T4 DNA ligase (cat. #M0202, New England Biolabs

(NEB), Ipswich, MA), and ligated with double stranded NlaII or MseI linkers. The

linkers were obtained by annealing the following sense and antisense DNA oligos: NlaIII

linker+ 5′-GTA ATA CGA CTC ACT ATA GGG CTC CGC TTA AGG GAC CAT G-3′

and NlaIII linker- 5′-Phos-GTC CCT TAA GCG GAG-C3spacer-3′; MseI linker+ 5′-

GTA ATA CGA CTC ACT ATA GGG CTC CGC TTA AGG GAC-3′ and MseI linker-

5′-Phos-TAG TCC CTT AAG CGG AG-C3spacer-3′. Ligation reaction was digested

with EcoRV (cat. #R0195, NEB, Ipswich, MA) and used for PCR amplification with the

following primers: MuLV LTR3 5′-GCT AGC TTG CCA AAC CTA CAG GTG G-3″

and Linker long primer 5′-GTA ATA CGA CTC ACT ATA GGG CTC CG-3′. The PCR

product was diluted and used for secondary PCR amplification with the following

primers: MuLV LTR1 5′-CCA AAC CTA CAG GTG GGG TCT TTC-3′ and Linker

nested primer 5′-AAG GCT CCG CTT AAG GGA C-3′. PCR conditions are 94°C for 2

min, 94°C for 15 sec, 60°C for 30 sec, 72°C for 90 sec (25 cycles) 72°C for 5 min for

both primary and secondary PCR. The polymerase used was Platinum Taq polymerase (SKU #10966, Invitrogen, Carlsbad, CA).

The secondary PCR product was separated on 3% agarose gel, individual bands were isolated with QIAquick gel extraction kit (cat. #28706, QIAgen, Valencia, CA) and used for cloning into pGEM-T vector system (cat. #A3600, Promega, Madison, WI). The *E. coli* clones that contained the plasmid were selected with blue/white screening, plasmid DNA was isolated with QIAprep Miniprep kit (cat. #27106, QIAgen, Valencia, CA) and sent for sequencing (Macrogen, Rochville, MD) with universal pUC/M13 primers.

Genomic PCR. The following primers were used to identify presence and direction of retroviral insertions in the *miR-17-92* genomic locus: genome-specific primers C (*miR-17*flnk.Fwd) 5"-CTT GTG GTC CTG GCT CTC TC-3", D (*miR-17*flnk.Rev) 5"-GGT GGG GTG AAG ACA GAG AA-3", and retrovirus-specific primers A (LTR5) 5"-TCG TGG TCT CGC TGT TCC TTG G-3", B (LTR4) 5"-GCG TTA CTT AAG CTA GCT TGC-3" (Figure 2.1A). For identification of the presence of the retroviral insertion in the *miR-106a-363* genomic locus the following primers were used: E 5"- AGT GCT GGC ACT GCA GTA GA-3", F 5"- TGG ACA GCC GTC TAT CAG TG-3", G 5"-GTC TGA TGC CAC CTT CTG GT-3". PCR was performed using the following prigram: 94°C for 5 min, 94°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec (40 cycles) 72°C for 5 min. The polymerase used was Platinum Taq polymerase (SKU #10966, Invitrogen, Carlsbad, CA).

MiRNA quantification. Total RNA was isolated with Trizol reagent (cat. #15596-018, Invitrogen, Carlsbad, CA) from cryopreserved AML cells isolated from BM or spleen.

MiRNAs *miR-17-5p*, *miR-20a*, *miR-19a*, *miR-19b-1*, *miR-18a* and *miR-92a* were quantified using TaqMan miRNA assays (part #4427975, Applied Biosystems, Carlsbad, CA) according to the manufacturer''s protocol. MiRNA microarrays were performed and analyzed by LC Sciences (Houston, TX).

Vectors and cloning. *MiR-17-92* genomic sequence was amplified from murine genomic DNA using the following primers: 5''-CTG GTC AAT GTG AGG CTT TC-3'' and 5''-CCG TTT TAC ACA CCA ACG AA-3''. Vent DNA polymerase (cat. #M0254, NEB, Ipswich, MA) was used with the following PCR program: 94°C for 5 min, 94°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec (30 cycles), 72°C for 5 min. The PCR fragment was purified using QIAquick PCR purification kit (cat. #28106, QIAgen, Valencia, CA) and cloned into pGEM-T vector (cat. #A3600, Promega, Madison, WI). After isolation of plasmid DNA from positive clones, the insert was cut out of the vector with EcoRI restriction enzyme (cat. #R0101, NEB, Ipswich, MA) and cloned into pMSCV-PIG vector (generously provided by Scot Lowe, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) by the same site. Obtained clones were screened for forward orientation and sequenced.

Retroviral construct that expressed shRNA targeting human and mouse Pten was purchased from Open Biosystems (oligo ID #V2SHS_231477, Huntsville, AL). The vector backbone for the construct is pSMP.

Retroviral production. Phoenix packaging cells (generously provided by Gary Nolan, Stanford University, CA) were co-transfected with 2 µg miR-17-92-pMSCV-PIG or shPten-pSMP constructs and 2 µg ψ-Eco packaging plasmid with Effectene reagent (cat.

#301425, QIAgen, Valencia, CA) according to manufacturer's protocol. Retrovirus supernatants were collected at 48, 56, and 72 hrs and titered in NIH3T3 cells by FACS analysis of GFP expression or by serial dilutions and selection with puromycin at 4 mkg/ml.

Bone marrow transduction and transplantation (tBMT). *Cbfb*$^{+/56M}$/Cre or *Cbfb*$^{+/56M}$ conditional knock-in mice were treated with polyinosinic-polycytidylic acid (pIpC, cat. #tlrl-pic, Invivogen, San Diego, CA). BM progenitor cells from both groups of mice were spin-infected twice with retrovirus supernatants, and $5x10^5$ to $1x10^6$ BM cells transplanted by intravenous injection (i. v.) into 6- to 8-week of age sublethally (650 rads) irradiated 129SvEv wild-type recipient mice. Mice were under daily observation for early signs of leukemia, and peripheral blood was taken periodically to monitor for the presence of immature cells. The early signs included limited motility, pale paws, and dehydration. FACS analysis of peripheral blood was performed by using antibodies to cell-surface markers Sca1, Gr1, CD11b, B220, CD3, Ter119, CD71, and c-kit (BD Biosciences, San Diego, CA). Staining of peripheral blood smears and spleen sections were ordered from Histoserv (Germantown, MD).

For secondary transplantations of *miR-17-92/Cbfb-MYH11* AML, leukemic cells were harvested from the BM or spleen of affected mice in PBS, and 200,000 to 500,000 cells were transplanted iv. into sublethally irradiated 6- to 8-week-old 129SvEv recipients.

Akt induction. For induction of Akt phosphorylation via IL-3 stimulation, cryopreserved *miR-17-92/Cbfb-MYH11* or spontaneous *Cbfb-MYH11* AML cells were thawed and

immediately serum starved in RPMI media with 1% BSA for 60 min at 37°C (Invitrogen, Carlsbad, CA). Cells were then incubated with RPMI media with 0.1% BSA and 0, 10, or 20 ng/ml IL-3 (cat. #213-13, Peprotech, Rockyhill, NJ) for 10 min. Cells were washed with PBS and resuspended in RIPA buffer with protease-inhibitor-cocktail III (Calbiochem, Darmstadt, Germany) and phosphatase inhibitor cocktails 2 and 3 (cat. # P5726, P0044, Sigma, St. Louis, MO). Antibodies included anti-phospho-Akt S473 (cat. #4058 Cell Signaling Technology, Danvers, MA) and anti-Akt (cat. #9272).

Inhibition of miRNA function with antagomirs. Cryopreserved *miR-17-92/Cbfb-MYH11* AML cells were thawed and plated into Accell medium (cat. #B-005000-500, Thermo Scientific, Lafayette, CO) supplemented with 1% FBS (cat. #SH30088.03, HyClone, Logan, UT) and 6 ng/ml IL-3 (cat. # 213-13, Peprotech, Rockyhill, NJ), at 1,000,000 cells/ml in 12- or 24-well plates. 24 hrs later the cells were treated with antagomirs at final concentration of 1 μM. 72 hrs after soaking with antagomirs, the cells were analyzed for apoptosis, cell cycle distribution or protein expression.

The antagomirs are 2"-OMe modified RNA oligos covalently linked to cholesterol moiety at 3"-end. They were ordered from Dharmacon (custom RNA systhesis, Thermo Scientific, Lafayette, CO) and have sequences complementary to mature miRNA sequence of targeted miRNA.

For immunoblot analysis, cells were washed with PBS and resuspended in RIPA buffer with protease-inhibitor-cocktail III (Calbiochem, Darmstadt, Germany) and phosphatase inhibitor cocktails 2 and 3 (cat. # P5726, P0044, Sigma, St. Louis, MO). Antibodies included anti-Pten (cat#9559, Cell Signaling Technology, Danvers, MA),

anti-phospho-Bad S136 (cat. #9295), anti-Bad (cat. #9292), anti-β-actin (cat. #5125).

Immunoblot analysis of miRNA targets. Primary *Cbfb-MYH11* spontaneous or *miR-17-92*/*Cbfb-MYH11* cryopreserved AML cells were thawed, washed with PBS and enriched for c-kit$^+$ cells with EasySep mouse CD117 positive selection cocktail kit (cat. #18757, StemCell Technologies, Vancouver, Canada). The enriched cells were resuspended in RIPA buffer with protease-inhibitor-cocktail III (Calbiochem, Darmstadt, Germany) and phosphatase inhibitor cocktails 2 and 3 (cat. # P5726, P0044, Sigma, St. Louis, MO). Antibodies included anti-Pten (cat#9559, Cell Signaling Technology, Danvers, MA), anti-Mcl-1 (cat. #sc-819, Santa Cruz Biotechnology, Santa Cruz, CA), anti-p21 (cat. #ab7960, Abcam, Cambridge, MA), anti-Rb2 (cat. #610261, BD Transduction laboratories, Franklin Lakes, NJ), anti-Runx1 (cat. #PC285, Calbiochem) and anti-β-actin (cat#5125, Cell Signaling Technology, Danvers, MA).

Apoptosis and cell cycle analyses. *Cbfb$^{+/MYH11}$*/*MiR-17-92* AML cells treated with antagomirs for 72 hrs (see above) were analyzed for apoptosis by flow cytometry as Annexin V$^+$/7AAD$^-$ (BD Pharmingen, San Jose, CA) according to manufacturer's instructions. Alternatively, apoptosis was analyzed at the same time point by flow cytometry with Caspase Detection Kit (cat. #QIA92-1KIT, Calbiochem, Merck Biosciences, Darmstadt, Germany). For cell cycle analysis, the cells were fixed in 70% etnanol, 24 hrs later stained with propidium iodide and analyzed by flow cytometry.

Results

Retroviral insertions upstream of miR-17-92 cluster upregulate expression of miRNA-17-5, -20a, and -19a

A retroviral insertional mutagenesis (RIM) screen in mice expressing *Cbfb-MYH11* identified candidate cooperating genes in leukemia development (Castilla et al., 2004). Two retroviral insertions (called R1 and R2) were located within 1.2 kb upstream of *miR-17-92* cluster. Another insertion was identified upstream of *miR-17-92* paralog *miR-106a-363* (Figure 2.1A, Table 2.1). We pursued further analysis with tumor samples with the R1 and R2 insertions. The presence of the insertions at *miR-17-92* locus was confirmed by genomic PCR using virus-specific and genome-specific primers, and their forward orientation was identified by using alternative viral primers A and B (Figure 2.1B). Furthermore, Southern blot analysis of genomic DNA from spleens of affected mice using a probe from the viral sequence identified an insertion in the R1 but not the R2 samples (Figure 2.1C). These results show that both leukemia samples include retroviral insertions upstream of the *miR-17-92* cluster, and that in one case the majority of the sample included a leukemia cell population with the R1 insertion, while the other included a minor cell population with the R2 insertion.

The expression of mature miRNAs in samples R1 and R2 was determined using a murine miRNA microarray plate assay. The expression of *miRs-17-5p, -17-3p, -20a, -19b* and *-92a* were upregulated in R1 ($p < 0.01$) in comparison to a control AML sample

**Table 2.1. Retroviral insertion sites identified upstream of *miR-17-92*
and *miR-106a-363* miRNA clusters**

Insertion	Clone	Location	Sequence
R1	pc26	846 bp upstream of *miR-17*-5p	<u>TTTCA</u>AGTAATTTTGAGCAAATGTGCTTACC CTTTCTCTGTCTTCACCCCACCCCCAACCCT AGTCATACACGTGGACCTAGCAGCACCCGA AGCATTGCCCAAG
R2	A26	425 bp upstream of *miR-17*-5p	<u>TTTCA</u>ACAATAATCATTAATCTTGTCGAGTA TCTGACAATGTGGAGGACAGAAGAAAGGG ATTGCTGCCTGGTCAATGTGAGGCTTTCCTC TAAAGGTAGTACCA
R3	A21	1472 bp upstream of *miR-106a*	<u>TTTCA</u>CCACCAACCCGAGAATGGAGGGAGA GGCTTAAGCTTGGCGCGTTTTGGGGGCTTT GAATGGAGAACTCCGTTTCCCATAATGCTT TGTTTCTTTCTGTTT

Insertion sites correspond to a genomic position following the LTR sequence TTTCA.

A.

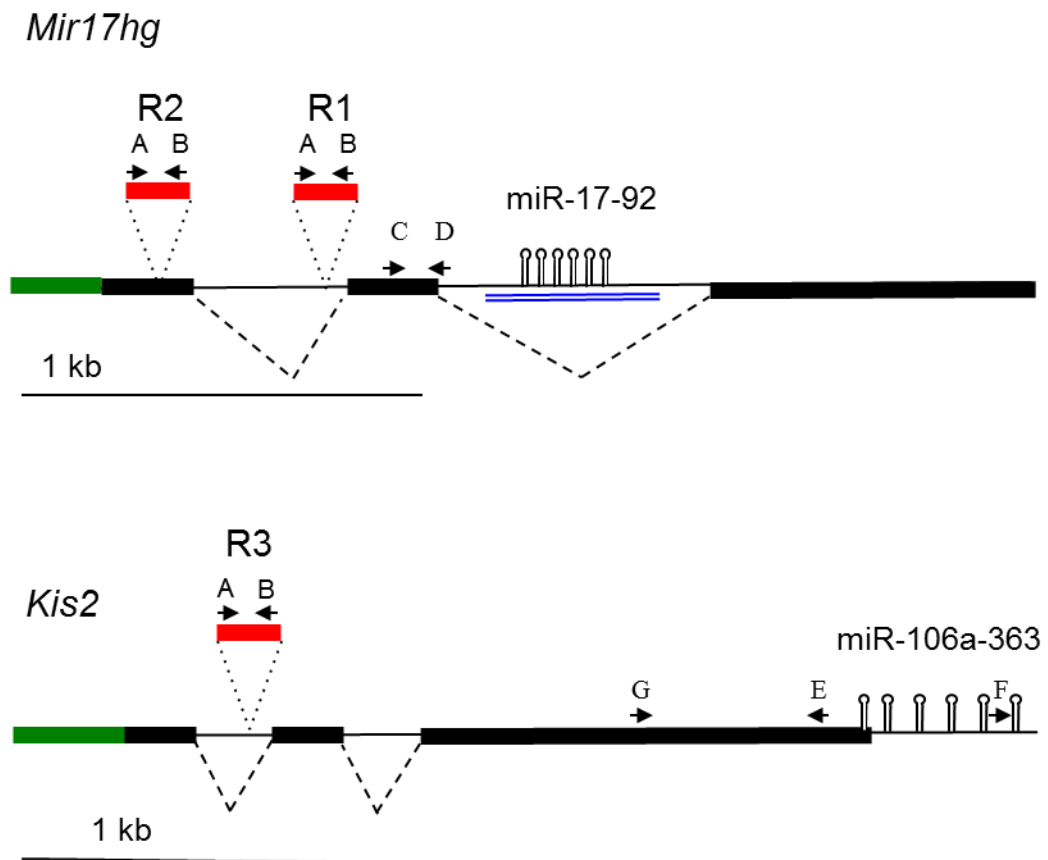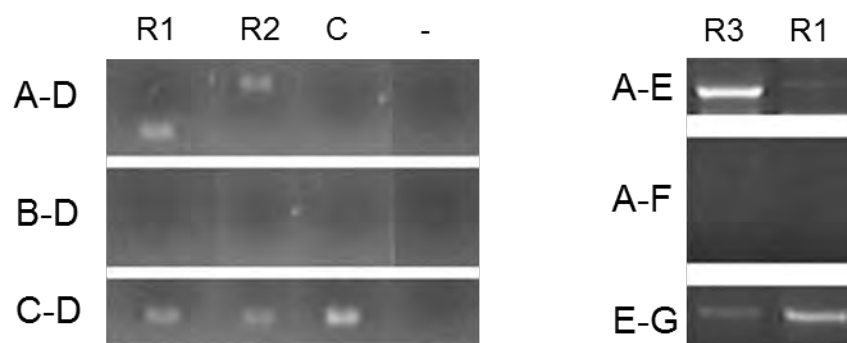**Figure 2.1. Identification and Expression analysis of retroviral insertions near the *miR-17-92* cluster family.** A. Schematic representation of retroviral insertions (red line) at *Mir17hg* locus in samples R1 and R2 (top) and at *Kis2* locus in sample R3 (bottom). Primers A-G (arrows), CpG island (green line), exons (black line), splicing (dashed line), miRNAs (stem loops). Southern blot probe (blue double line).

B.

C.
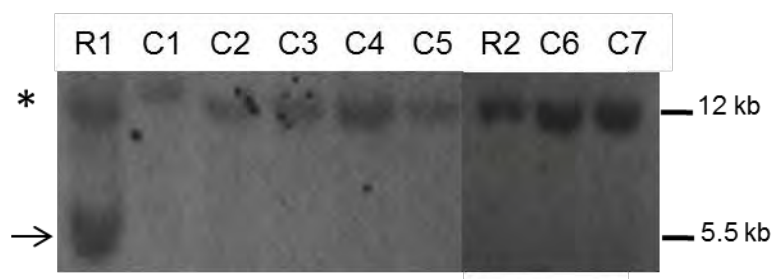
Figure 2.1 (cont.) Identification and Expression analysis of retroviral insertions near the *miR-17-92* cluster family. B. Genomic PCR with DNA of AML samples R1, R2, R3, control AML sample (C) from RIM with unrelated retroviral insertions, and negative control (NC) with no DNA. Primer pairs used for each PCR are shown to the left of each row, and correspond to arrows in Figure 2.1A. C. Southern blot analysis of AML samples using probe for the *miR-17-92* locus. Shifted band corresponding to retroviral insertion in R1 (arrow), genomic band (asterisks); unrelated AML samples (C1-7); R2 samples with insertion identified by PCR (R2).

D.
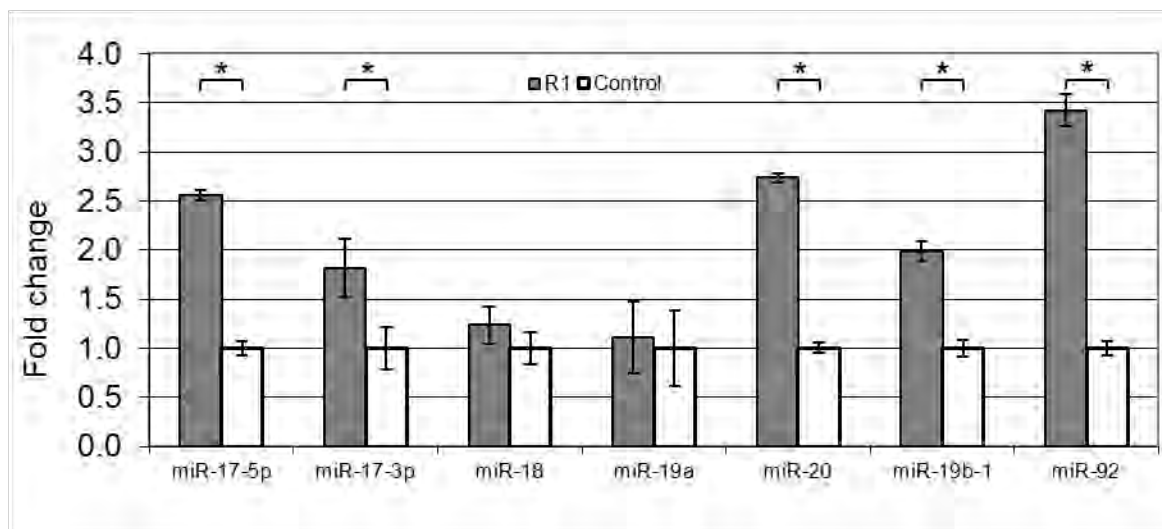
E.
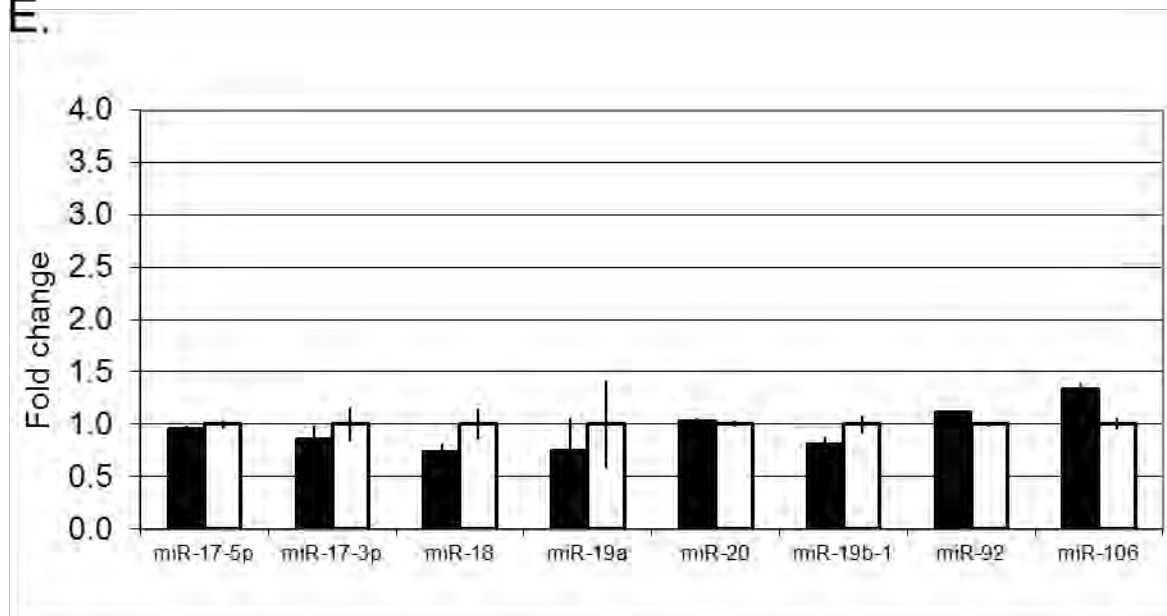
Figure 2.1 (cont.). Expression of *miR-17-92* cluster in R1 and R2 leukemia samples. Microarray expression of miRs from the *miR17-92* cluster of control sample (control: leukemia sample with no retroviral insertion near miR17-92 cluster) compared to R1 (D, grey) or R2 (E, black) samples. The expression in the control (white) sample is normalized to 1. Error bars indicate SD. The Student's t-test was used to determine the statistical differences between various experimental and control groups. p < 0.01 (*).

F.
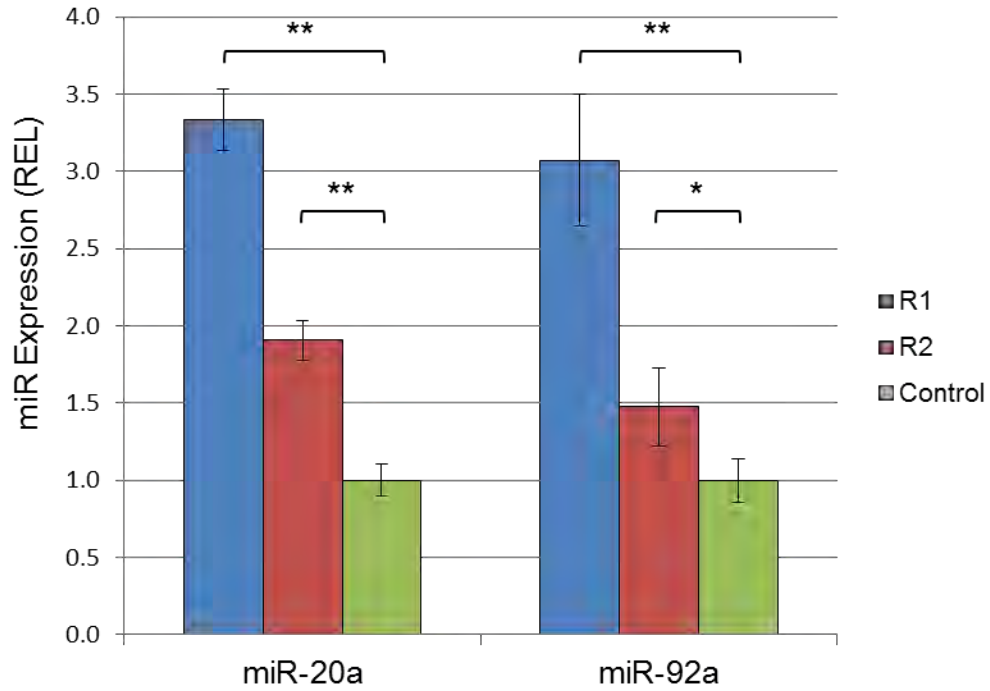
Figure 2.1 (cont.). Expression of *miR-17-92* cluster in R1 and R2 leukemia samples. F. Relative Expression Levels (normalized to control) of *miR-20a* and *miR-92a* in R1, R2, and control AML samples. Assays were tested in quadruplicate. Error bars indicate SD. The Student's t-test was used to determine the statistical differences between various experimental and control groups. $p = 0.016$ (*), $p < 0.0001$ (**).

with no retroviral insertion in the locus, and in R2 there were no significant changes

detected (Figure 2.1D, E). The expression changes were confirmed using a miRNA qPCR

for *miR-20a* and *miR-92a* (p < 0.0001). In addition, we also identified low, but significant
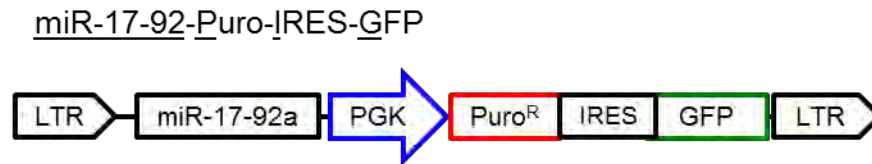
upregulation of *miR-20a* (p < 0.0001) and *miR-92a* (p = 0.016) in AML sample R2

(Figure 2.1F). Taken together, these data show the retroviral insertions R1 and R2 within

1.2 kb upstream of the *miR-17-92* cluster in a forward direction, inducing upregulation of

its miRNAs.

MiR-17-92 cooperates with Cbfb-MYH11 in development of AML in vivo

Based on identification of retroviral insertions at the *miR-17-92* locus, and corresponding

upregulation of miR17-92 miRNAs, we hypothesized that expression of *miR-17-92*

cluster could cooperate with *Cbfb-MYH11* in development of AML. We utilized a

conditional *Cbfb$^{+/MYH11}$* knock in mouse that activated *Cbfb-MYH11* expression after Cre-

mediated recombination of the *Cbfb^{MYH11}* allele in hematopoietic progenitors (Kuo et al.,

2006). The protein Cre was temporarily induced from the *Mx1-Cre* transgene (Kuhn,

1995). The expression of Cre is regulated by Infα/β, induced by treatment with the

double stranded ribonucleic polyinosinic-polycytidylic acid (pIpC). We performed bone

marrow transplantation assays using donor *Cbfb$^{+/MYH11}$; Mx1Cre* BM cells transduced

with pMSCV-PIG retrovirus expressing the *miR-17-92* cluster under the LTR regulation

and Puromycin resistance gene and GFP regulated by the PGK promoter (Figure 2.2A).

Control groups included floxed *Cbfb$^{+/MYH11}$* BM transduced with pMSCV-PIG or

A.

miR-17-92-Puro-IRES-GFP

B.

| Harvest BM from Cbfb+/MYH11, Mx1-Cre Cbfb+/MYH11 mice | Transduction with PIG or miR-17-92 PIG | Transplantation into irradiated recipients |
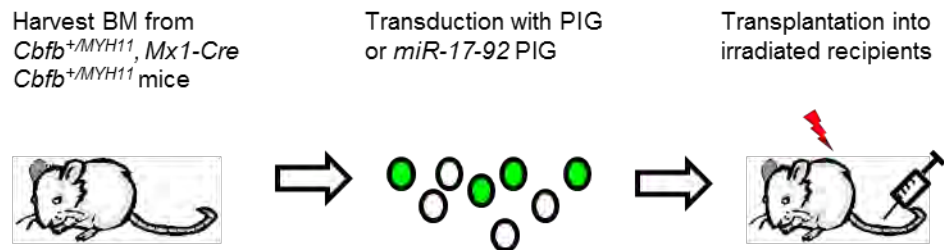
Figure 2.2 Expression of *miR-17-92* cluster miRNAs cooperates with Cbfβ-SMMHC in AML development. Schematic representations of (A) *miR-17-92*-expressing PIG retroviral vector. Letters used in abbreviation are underlined. The two LTR elements (arrowheads), Puromycin-resistance gene (puro[R] in red) and GFP (green) gene are expressed from PGK promoter (blue), and separated by an IRES; (B) experimental design of BM transplantation experiment of BM *Cbfb+/MYH11, Mx1-Cre* (expressing CBFβ-SMMHC) and *Cbfb+/MYH11* (control). Transduced cells (green); Recipients were transplanted with BM cells after sublethal irradiation (red lightning) by i.v. tail injection.

C.
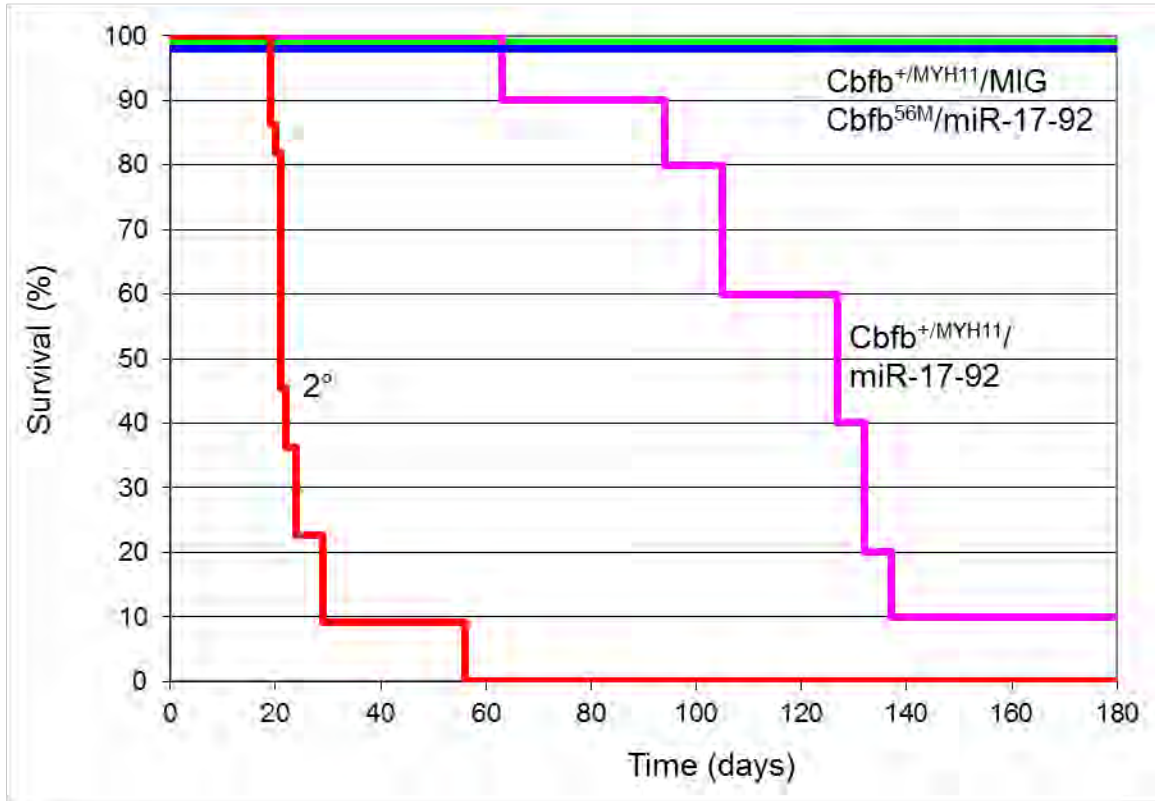
Figure 2.2. (cont.) Expression of *miR-17-92* cluster miRNAs cooperates with Cbfβ-SMMHC in development of AML. C. Kaplan-Meier survival curve of mice transplanted with *Cbfb*[+/*MYH11*]/*miR-17-92* (pink line, n=10), *Cbfb*[*MYH11*]/PIG (green, n=8), or *Cbfb*[*56M*]/*miR-17-92* (blue, n=8) BM cells. Each mouse was transplanted with 5×10^5 BM cells transduced (10% to 20% efficiency). Survival curve for secondary leukemia cells (5×10^5 cells per mouse) is shown (red, n=22). X-axis indicates time in days, and Y-axis shows percentage of live.

pMSCV-PIG-miR17-92 viruses. The three groups of cells were transplanted with i. v. injections into sublethally irradiated wild type recipient mice (Figure 2.2B).

Ninety percent of mice (9/10) transplanted with $Cbfb^{+/MYH11}$/miR-17-92 BM cells succumbed to leukemia 64 to 134 days after transplantation (Figure 2.2 C, pink line). None of the control groups (WT BM transduced with miR-17-92, and $Cbfb^{+/MYH11}$ BM transduced with vector alone developed a disease for over 180 days (experimental endpoint). Secondary transplantation of $5x10^5$ leukemic cells reproduced leukemia with a median latency of 3 weeks, and all recipients developed leukemia within 7 weeks (Figure 2.2C, red line). The pathology of leukemia was characterized by elevated WBC count in PB (Figure 2.3B; $p < 0.0001$), and the presence of immature myeloid blasts (Figure 2.3A). The PB was enriched with Lin⁻c-kit⁺ cells ($p < 0.0001$), other lineages were decreased except for $Gr1^+$ and $Ter119^+$ populations that did not change significantly (Figure 2.3C). The GFP⁺ cells (carrying miR-17-92-expressing retorvirus) were Lin⁻c-kit⁺ with a small subpopulation of c-kit⁺Sca1⁺ cells (Figure 2.3C), reminiscent of early hematopoietic progenitors that include the HSCs. In addition, leukemic mice presented marked splenomegaly due to infiltration of the leukemic blasts into peripheral organs (Figure 2.3D). Taken together, the cooperation between $CBFB$-$MYH11$ and miR-17-92 expression results in a myeloid leukemia with a pathology that is driven by the expression of $CBFB$-$MYH11$, as it has previously been reported (Kuo et al., 2006; Kuo et al., 2009; Landrette et al., 2005).

A.
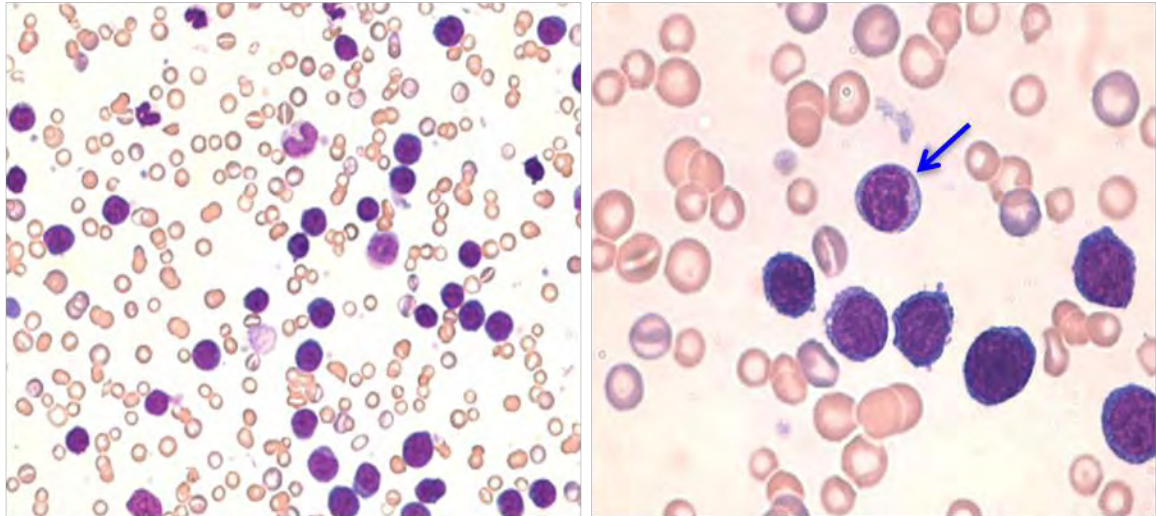
B.
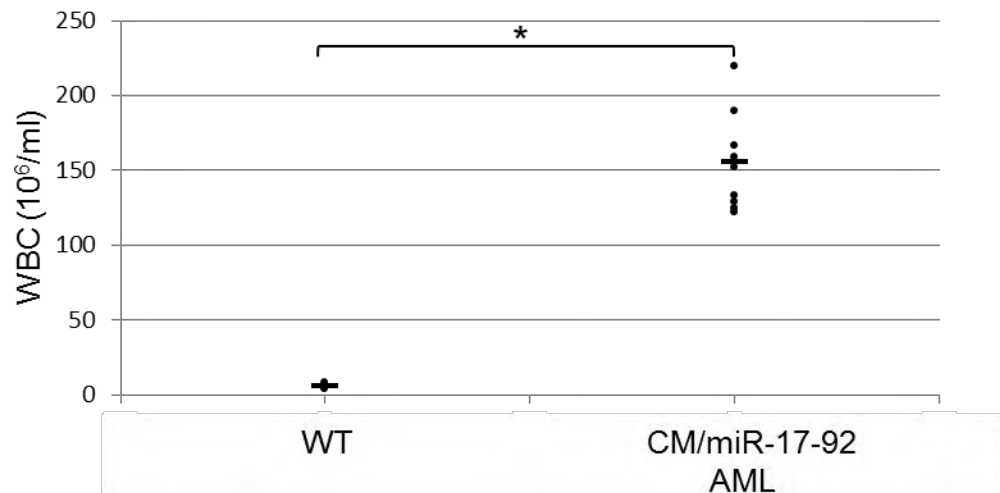
Figure 2.3. Phenotypic characterization of *Cbfb*[+MYH11]/*miR-17-92* AML. A. Wright-Giemsa staining of PB smears of leukemic mouse, using 10x (left) and 100x (right) magnification. Representative immature blast (Arrow). B. Quantification of WBC counts in PB of WT (n=5) and *Cbfb*[+/MYH11]/*miR-17-92* AML mice (n=9). Dots represent individual WBC counts, lines represent average WBC count in each group. The Student's t-test was used to determine the statistical differences between various experimental and control groups. p < 0.0001 (*).

C.
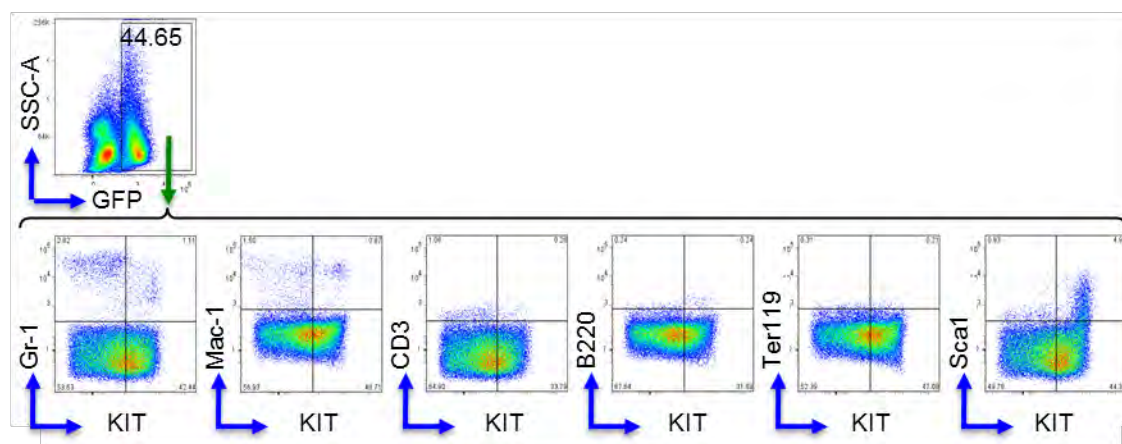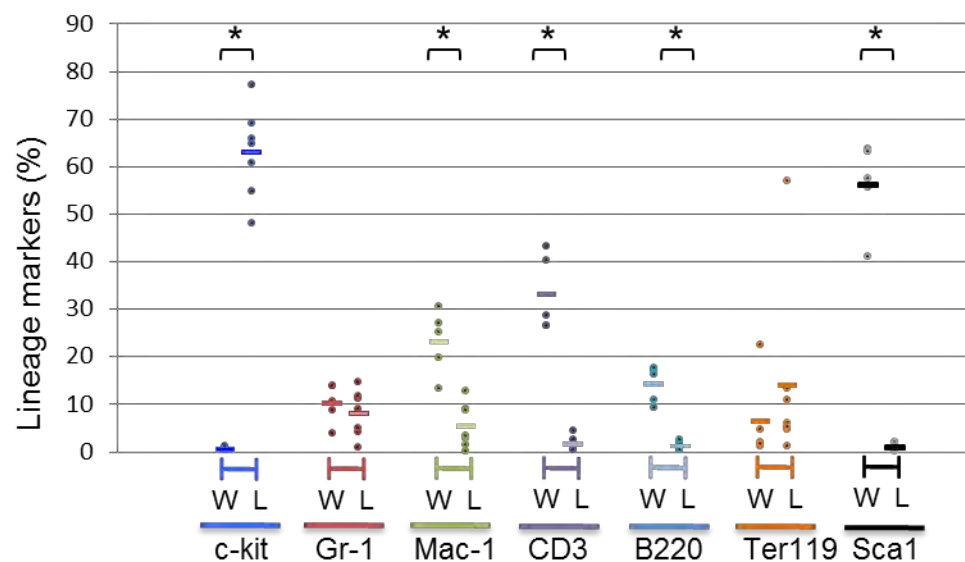
Figure 2.3. (cont.) Phenotypic characterization of *Cbfb$^{+/MYH11}$/miR-17-92* AML. C. Top: Percentage of cells expressing lineage-specific markers in WT (left, n=5) and *Cbfb$^{+/MYH11}$/miR-17-92* AML (right, n=7) PB. Dots represent individual percentages in each mouse, lines represent average percentages in each group. The Student's t-test was used to determine the statistical differences between AML and WT groups. p < 0.001 (*). Bottom: Representative FACS analysis from *Cbfb$^{+/MYH11}$/miR-17-92* AML mice. GFP$^+$ PB cells from total PB were analyzed for lineage (y-axis) and c-kit (x-axis).

D.
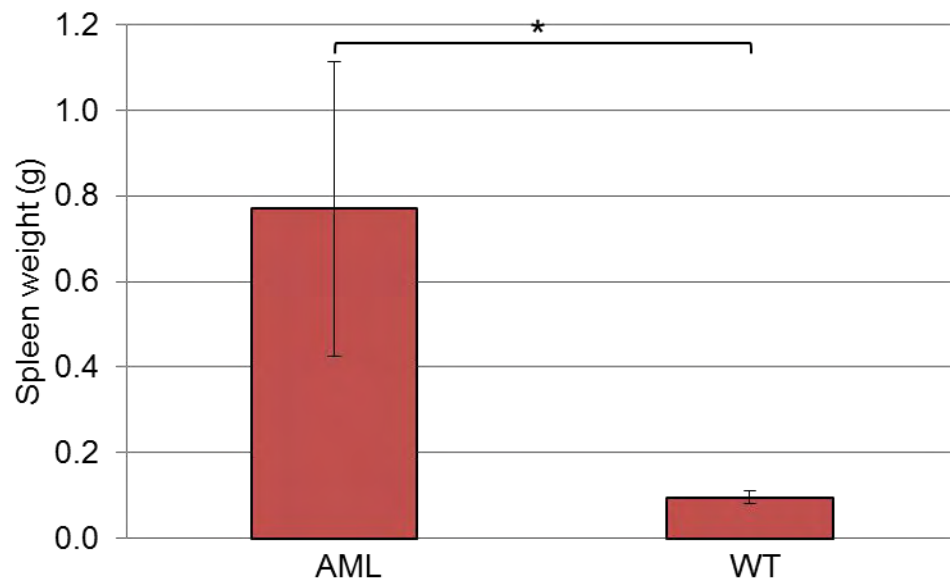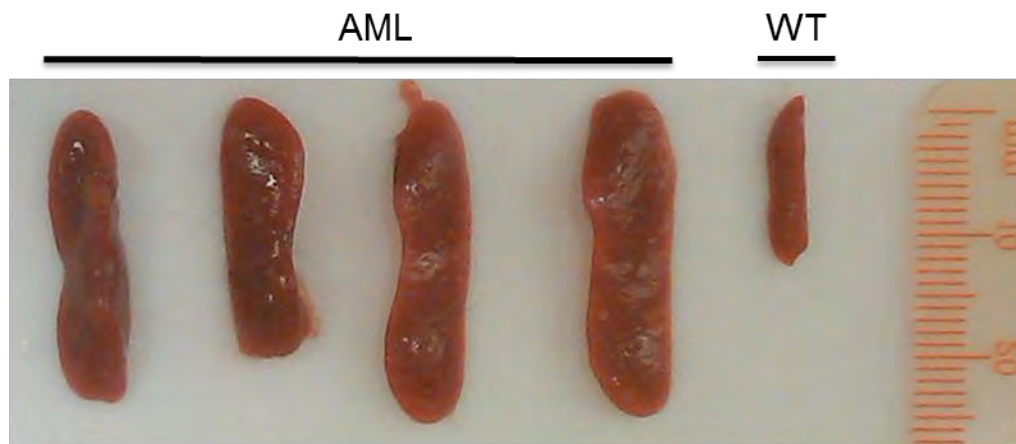
Figure 2.3. (cont.) Phenotypic characterization of *Cbfb*+/*MYH11*/*miR-17-92* AML. D. Top: Photographs of spleens isolated from *Cbfb*+/*MYH11*/*miR-17-92* leukemic (AML) and healthy (WT) mice. Bottom: Quantification of spleen weight from *Cbfb*+/*MYH11*/*miR-17-92* leukemic (AML; n=10) and healthy (WT; n=5). Error bars indicate SD. The Student's t-test was used to determine the statistical differences between AML and WT groups. $p < 0.01$ (*).

Expression of miR-17-92 associated microRNAs in Cbfb-MYH11 leukemia cells

The expression levels of different miRs from the *miR-17-92* cluster vary, suggesting differential posttranscriptional processing. The expression of *miR-17*, *-20a*, and *-19a/b* seems to follow a similar pattern, while that of *miR-18a* and *-92a* is different (Hayashita et al., 2005; Lu et al., 2007). In collaboration with Dr. Mojca Jongen-Lavrencic, we analyzed the expression levels of miRs from the three paralog clusters in human AML samples (Jongen-Lavrencic et al., 2008). Analysis of 15 inv(16) AML samples showed expression of miRs from the *miR-17* (7/15), and *miR-18* (6/15) families (Figure 2.4A). The analysis in $Cbfb^{+/MYH11}$/*miR-17-92* AML cells in comparison to WT sorted myeloid progenitors and to unrelated $Cbfb^{+/MYH11}$ AMLs using miRNA qPCR showed that *miRs-17-5*, *-20a*, *-19a* and *-19b* were upregulated in all $Cbfb^{+/MYH11}$/*miR-17-92* samples (p < 0.04), while *miR-18a* and *miR-92a* were not significantly changed (Figure 2.4B). This expression pattern provides correlative evidence suggesting that these miRs are expressed in a fraction of human inv(16) AMLs and that *miR-17* and/or *-19* family miRs may direct the leukemic signal in these samples.

Inhibition of miR-17 and miR-20a in $Cbfb^{+/MYH11}$/miR-17-92 AML cells results in increased level of apoptosis

Based on the miRNA expression data, we focused our studies on the role of miR families -17 and -19 in leukemia development. We tested the survival of leukemic cells expressing

A.

Inv(16) AML cells

hsa-miR-106a
hsa-miR-106b
hsa-miR-17-5
hsa-miR-18a
hsa-miR-19a
hsa-miR-19b
hsa-miR-20a
hsa-miR-20b
hsa-miR-25
hsa-miR-92
hsa-miR-93

-4.4   0   +2.3
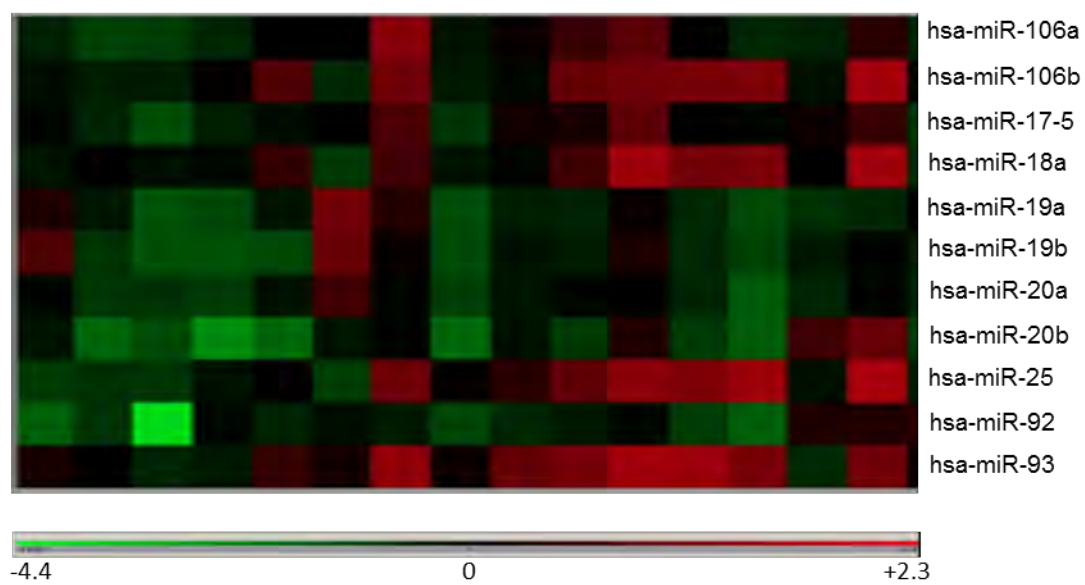
Figure 2.4. Expression of miRNAs in *Cbfb*[+/MYH11]/*miR-17-92* AMLs. A. Expression profile of microRNAs associated with *miR-17-92* in 15 human AML samples with inv(16). Expression range -4.40 to +2.3 fold change, normalized to geometric mean of expression.
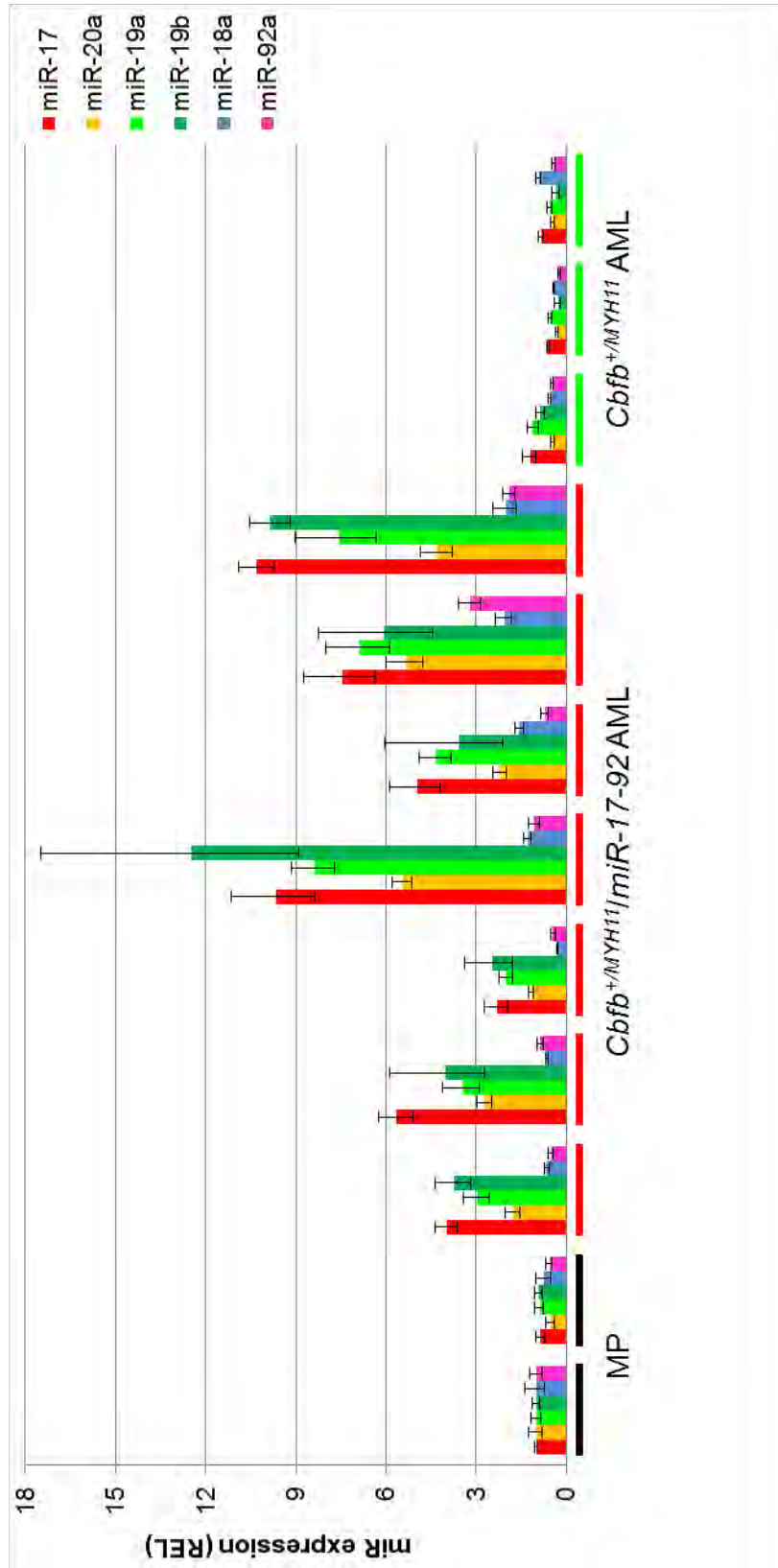
Figure 2.4 (cont.) Expression of miRNAs in *Cbfb*^{+/MYH11}/*miR-17-92* AML. B. Expression of miRNAs of *miR-17-92* cluster determined by miRNA qPCR. Myeloid progenitors (MP, black, n=2), *Cbfb*^{+/MYH11}/*miR-17-92* positive AML samples (AML, red, n=7), *Cbfb*^{+/MYH11}/*miR-17-92* negative AML samples (AML, green; n=3). Each assay was performed in quadruplicate, error bars indicate SD.

Cbfb-MYH11 and *miR-17-92* after downregulation of *miR-17-5p*, *miR-20a*, *miR-19a* and *miR-19b*. Antagomir-mediated inhibition of *miR-17* or *-20a* significantly increased the number of apoptotic cells (p<0.01) as measured by percentage of Annexin V$^+$/7AAD$^-$ cells compared to scramble oligo or day zero values (Figure 2.5A). Treatment with combined anti-*miR-17* and *-20a* further increased cell death (p<0.001). Conversely, cells treated with anti-*miRs-19a* or *-19b* did not show altered cell death rate. Apoptosis was also estimated in a similar experiment measuring the levels of caspase cleavage by means of the percentage of PE-FMK-VAD$^+$ label (Figure 2.5B). Cells treated with antagomirs for single or combined *miR-17* and *-20a* significantly increased caspase activity, while treatment with anti-*miR-19a/b* had no significant effect. The anti-apoptotic effect of *miR-17* and *miR-20a* was specific to *Cbfb*$^{+/MYH11}$/*miR-17-92* AMLs, as unrelated *Cbfb-MYH11* AML cells did not exhibit changes in apoptosis upon treatment with any of the antagomirs (Figure 2.5C). The cell cycle phases distribution was not altered by treatment of *Cbfb*$^{+/MYH11}$/*miR-17-92* AML cells with antagomirs (Figure 2.5D). These data indicate that *miR-17* family miRs and not *miR-19* family miRs provide an anti-apoptotic signal in *Cbfb*$^{+/MYH11}$/*miR-17-92* leukemic cells, sugesting a possible mechanism of *miR-17-92* cooperation with *Cbfb-MYH11*.

MiR-17-92 cluster downregulates Pten expression in the Cbfb$^{+/MYH11}$/miR-17-92 leukemic blasts

The miRs of families *miR-17* and *-19* regulate protein levels of factors associated with proliferation, differentiation and survival. Considering that such regulation is cell type

A.
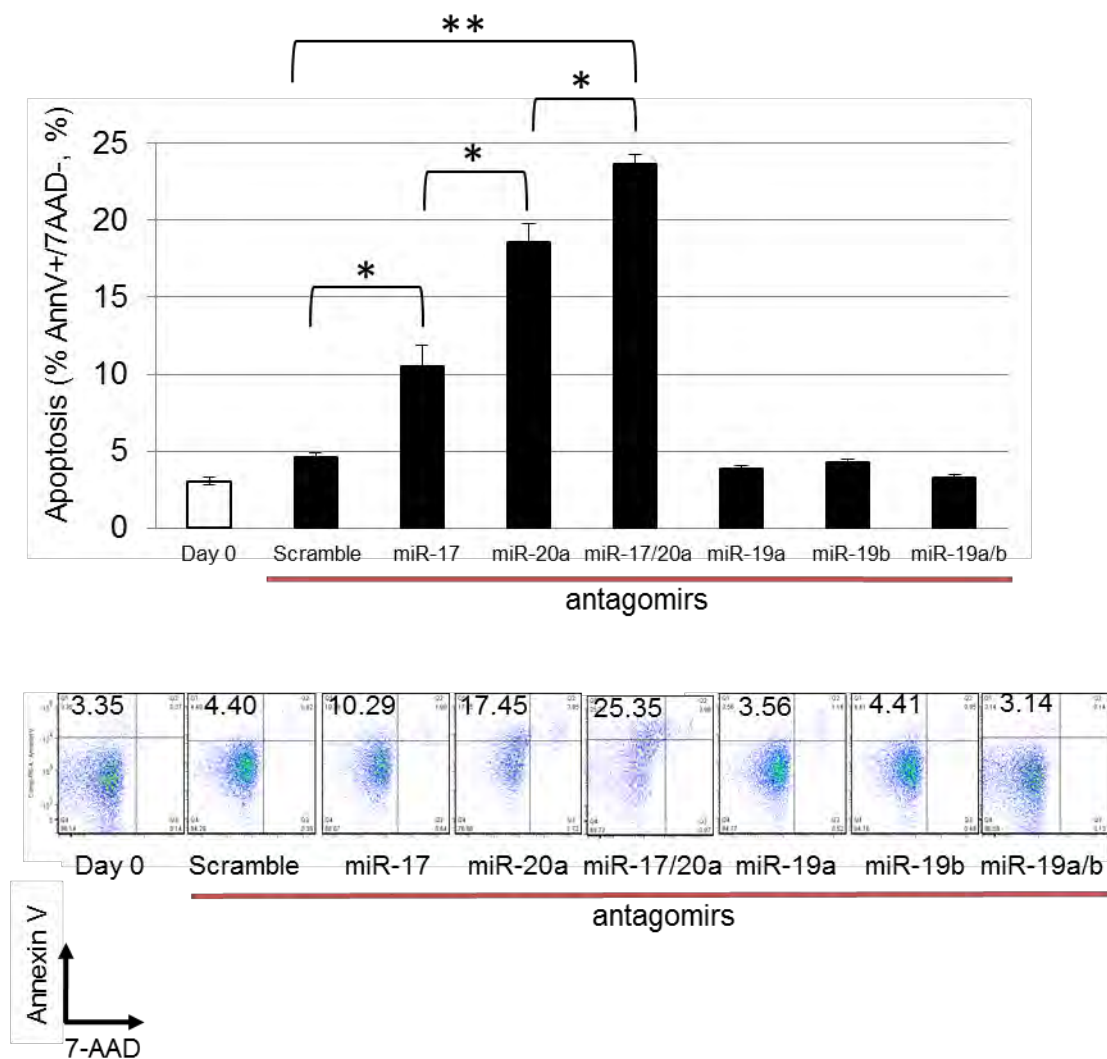
Figure 2.5. *miR-17/20a* provide anti-apoptotic response in *Cbfb*[+/MYH11]/*miR-17-92* AML cells. A. Apoptosis assay of *Cbfb*[+/MYH11]/*miR-17-92* AML cells. Top: Percentage of Annexin V[+]/7-AAD^{-} cells before transfection (white bar, Day 0), and transfected with scramble oligo or miR-specific antagomirs. Experiments were performed in triplicate, error bars indicate SD; $p < 0.01$ (*), $p < 0.001$ (**). Bottom: Representative FACS plots of apoptosis assay showing fraction of apoptotic cells.

B.
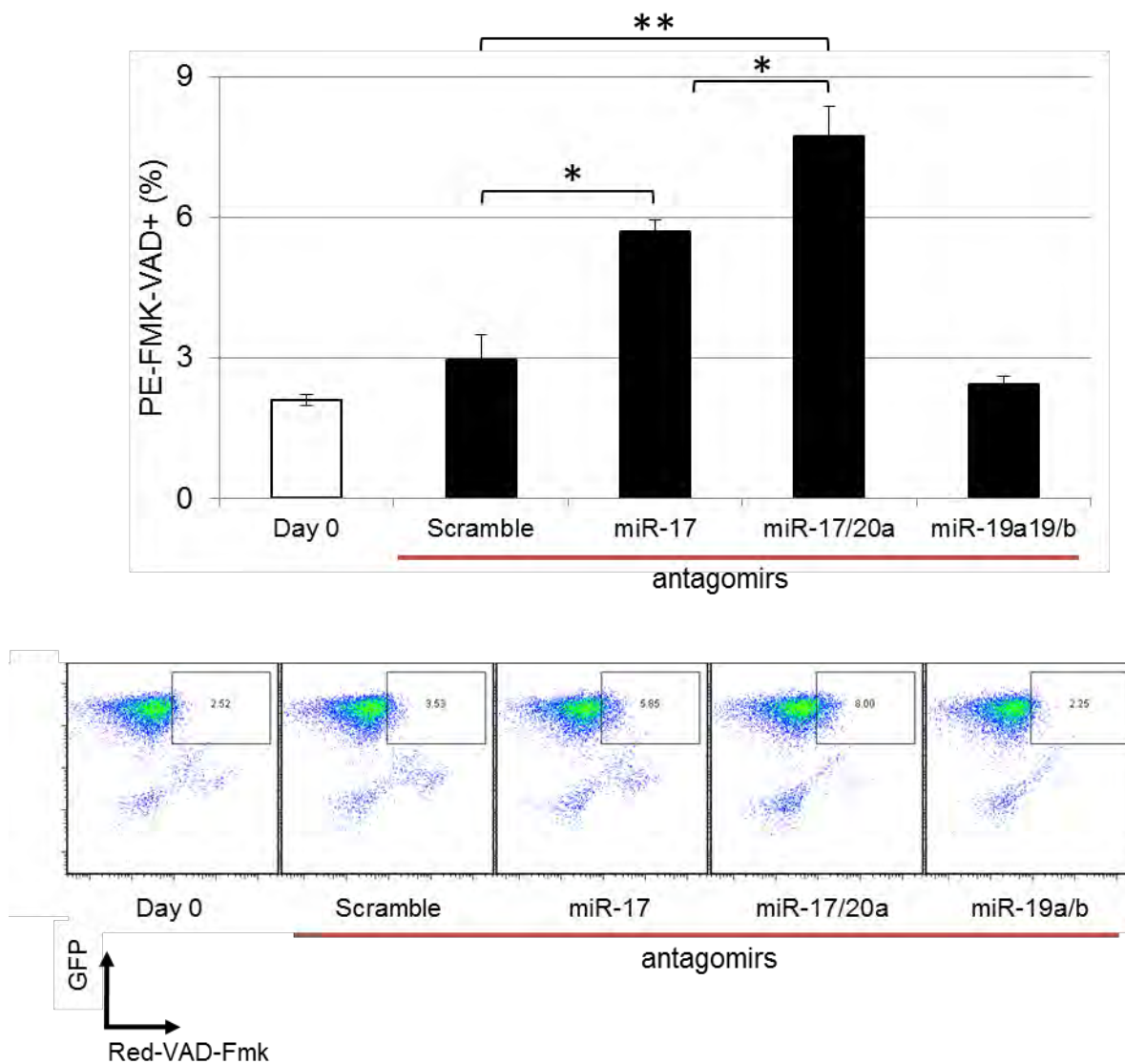
Figure 2.5. (cont.) *miR-17/20a* **provide anti-apoptotic response in** *Cbfb^{+/MYH11}/miR-17-92* **AML cells.**
B. Top: Percentage of apoptotic (Red-VAD-Fmk⁺) cells from *Cbfb^{+/MYH11}/miR-17-92* AML untreated (day 0, white bar), and treated (black bars) with scramble or miR-specific oligos. The experiment was performed in triplicate, error bars indicate SD. Statistical significance shown in asterisks (p < 0.01 (*), p < 0.001(**)). Bottom: Representative FACS plots that were quantified in the bar graph are shown.

C.
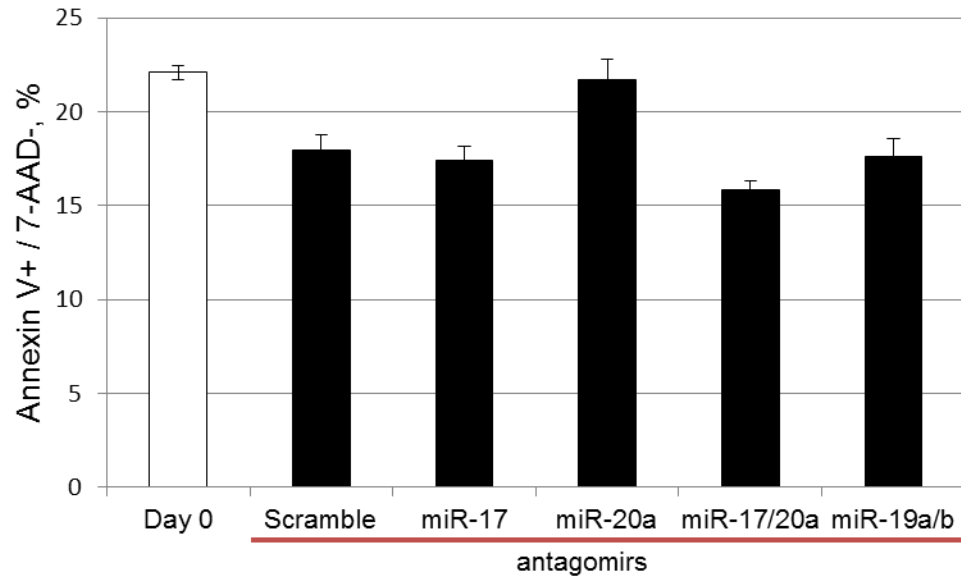
Figure 2.5. (cont.) *miR-17/20a* **provide anti-apoptotic response in** *Cbfb$^{+/MYH11}$/miR-17-92* **AML cells.**
C. Apoptosis assay of unrelated *Cbfb$^{+/MYH11}$* AML cells. Percentage of Annexin V$^+$/7-AAD$^-$ cells before transfection (white bar, Day 0), and transfected with scramble oligo or miR-specific antagomirs. Experiments were performed in triplicate, error bars indicate SD.

D.
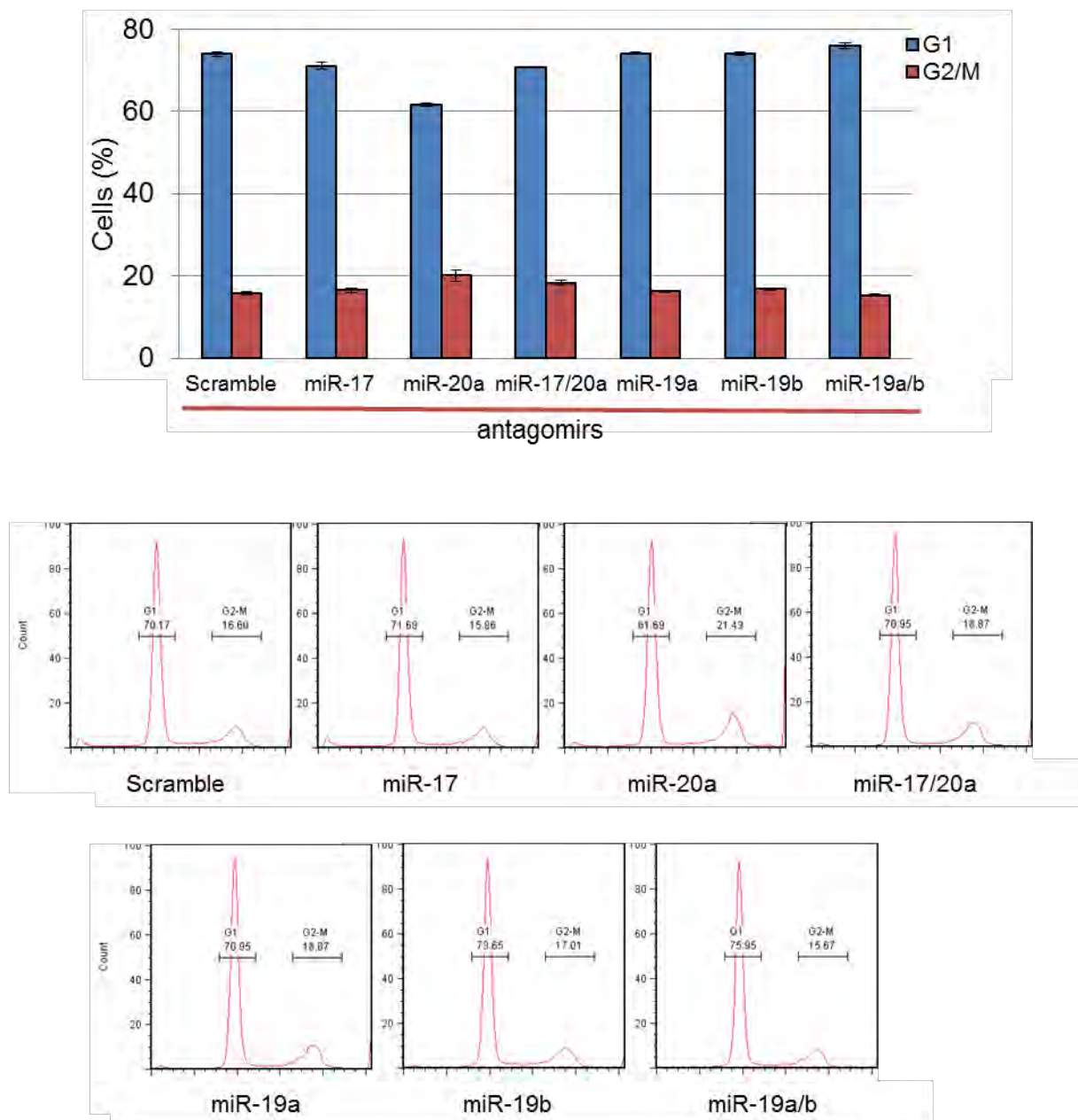
Figure 2.5. (cont.) *miR-17/20a* **provide anti-apoptotic response in** *Cbfb*^{+/MYH11}/*miR-17-92* **AML cells.**
D. Top: percentage of transfected *Cbfb*^{+/MYH11}/*miR-17-92* AML cells in G1 (blue) or G2/M (red) phases of
a cell cycle as determined by DNA content with PI staining. Non-targeting antagomir (Scramble) as a
control. Experiments were performed in triplicate, error bars indicate SD. Bottom: Representative PI
staining analysis with gates for G1 and G2/M phases.

specific, we tested the expression of *miR-17* targets in a panel of *Cbfb-MYH11* AML cells

with or without overexpression of the *miR-17-92* cluster using Western blot analysis. In

order to minimize contamination from non-leukemic cells, leukemic cells were enriched

from spleen for c-kit$^+$ using magnetic beads to obtain highly GFP$^+$c-kit$^+$ cells with more

than 90% purity (Figure 2.6A). The expression of Rb2 and Runx1 in *Cbfb$^{+/MYH11}$* AML

cells (n=3) and *Cbfb$^{+/MYH11}$/miR17-92* AML (n=7) cells showed an irregular expression

pattern in both groups (Figure 2.6 B). Similarly, levels of p21 protein and the anti-

apoptotic Mcl-1L isoform were unchanged, while expression of the pro-apoptotic short

Mcl-1S isoform was also inconsistently changed (Figure 2.6.C). However, we found

consistent and significant decrease (p=0.02) in Pten protein levels only in AML samples

expressing the *miR-17-92* cluster, when compared to AML cells not expressing the

cluster and to hematopoietic progenitor and myeloid progenitor controls (quantification

of the Western blot in Figure 2.6D). These data suggest that *miR-17-92* overexpression in

the AML cells results in down-regulation of Pten with possible activation of PI3K/Akt

pathway.

miR-17-92 activates PI3K/Akt signaling via Pten downregulation

The 3''-UTR of *Pten* contains two target sites for *miR-17* family and one target site for

miR-19 family, and it has been shown to be a target of these miRs (Olive et al., 2009;

Takakura et al., 2008; Xiao et al., 2008). Since Pten levels are reduced in

Cbfb$^{+/MYH11}$/miR-17-92 leukemias, we reasoned that *miR-17/20a* and *-19a/b* may affect

Akt phosphorylation. Leukemic cells from *Cbfb$^{+/MYH11}$* and *Cbfb$^{+/MYH11}$/miR-17-92*

A.
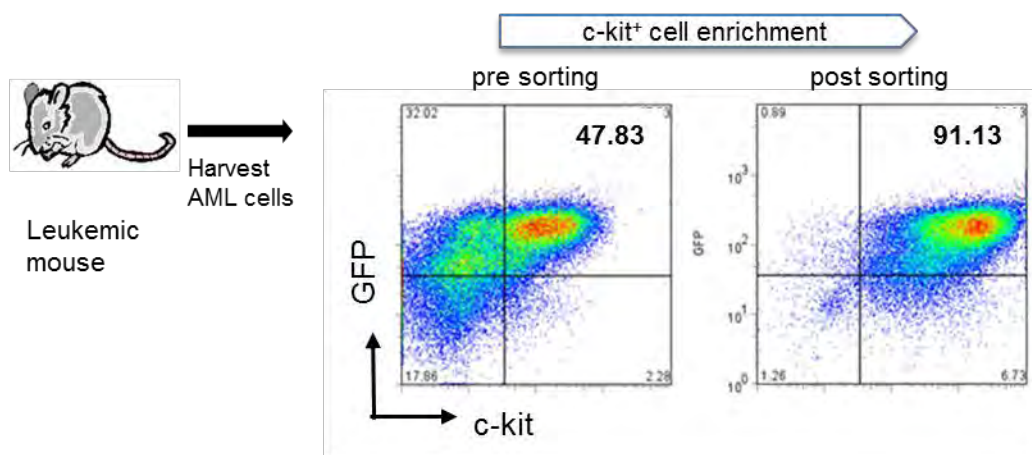
B.
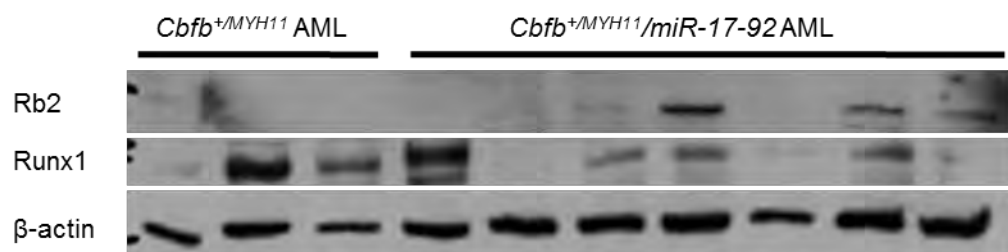
Figure 2.6. *MiR-17-92* inhibits PTEN levels in AML cells. A. AML cell-enrichment for protein analysis from BM and spleen cells from leukemic mice, using magnetic beads for c-kit selection. B. Western blot analysis of Rb2, Runx1 and β-actin protein levels from *Cbfb*[+/MYH11] AML cells (n=3) and *Cbfb*[+MYH11]/*miR-17-92* AML cells (n=7).

C.
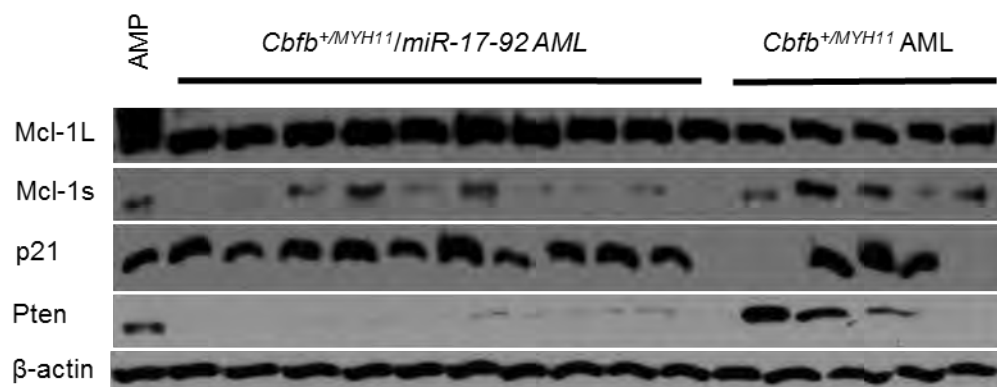
D.
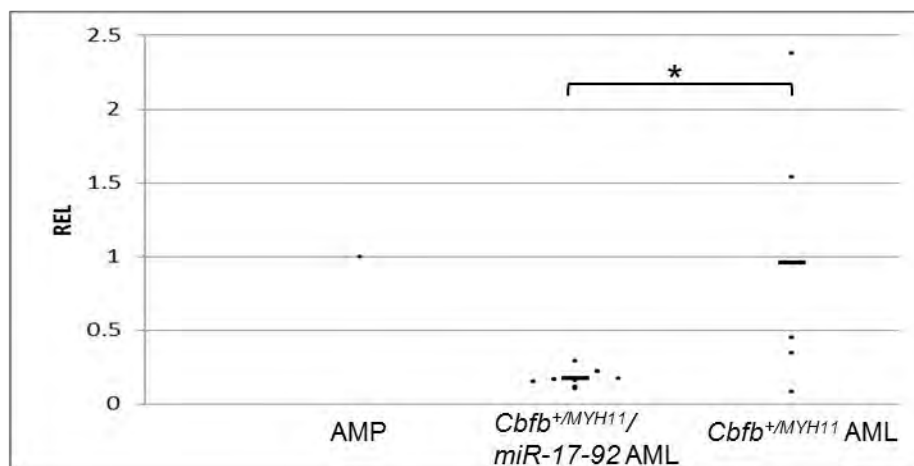
Figure 2.6. (cont.) *MiR-17-92* **inhibits PTEN levels in AML cells.** C. Western blot analysis of Mcl-1L and Mcl1-s, p21, Pten, and β-actin (control) in lysates from myeloid progenitor expressing Cbfβ-SMMHC (AMP), and leukemic cells expressing *Cbfb+/MYH11/miR-17-92* (n = 10), and C*bfb+/MYH11* (n = 5). D. Dot plot quantification of Pten protein level in AML samples from "C". Lines indicate average protein level per group. Expression is normalized to control AMP. The Student's t-test was used to determine the statistical differences between various experimental and control groups. p = 0.02 (*).

groups were stimulated with IL3, and levels of Akt and Akt phosphorylation at S473 residue was tested. We found that while Akt levels were not significantly altered, S473-phospho Akt was increased only in $Cbfb^{+/MYH11}$/miR-17-92 AML cells upon stimulation with IL-3 in a dose-dependent manner (Figure 2.7A).

An Akt-mediated anti-apoptotic signaling occurs in part through inhibiting activity of the BCL2-related Bad protein via phosphorylation at its S136 residue. We sought to determine whether inhibition miR-17 or miR-19 families in $Cbfb^{+/MYH11}$/miR-17-92 cells increase S136 phospho-Bad level. We found that leukemic cells treated with miR-$17/20a$ or $-19a/b$ antagomirs presented a significant decrease in S136-pBad levels, indicating that both miR-$17/20a$ and miR-$19a/b$ activity results in phosphorylation of Bad in the AML cells (Figure. 2.7B). Taken together, the data suggest that both miR-17 and miR-19 family of miRNAs from the miR-17-92 cluster induce PI3K/Akt signaling in the AML cells.

Discussion

The work presented in this chapter demonstrates that upregulation of the miR-17-92 cluster cooperates with the leukemia fusion protein CBFβ-SMMHC as an oncomiR in mouse AML. We determined that the miR-17-$5p$ and miR-$20a$ provide an anti-apoptotic advantage, a function that seems to be independent of Bad phosphorylation.

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A.
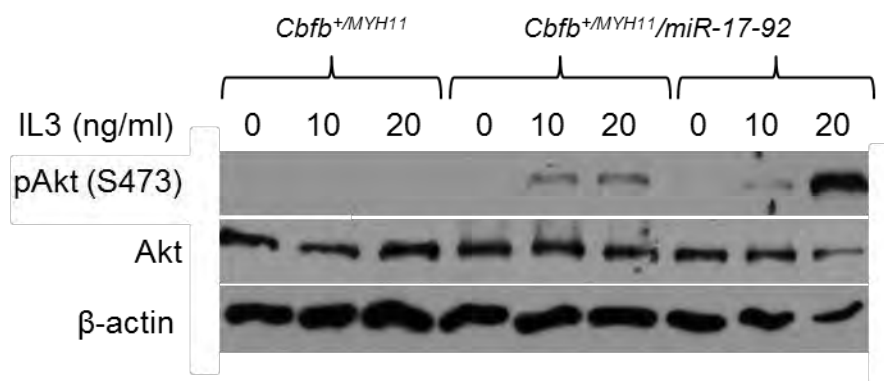
B.
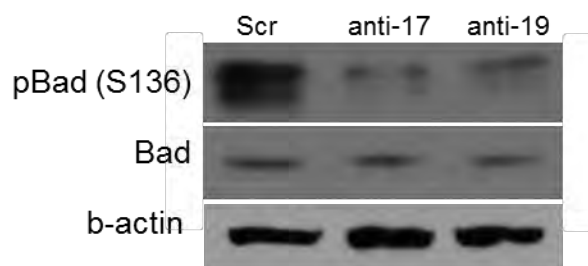
Figure 2.7. *MiR-17-92* cluster increases cytokine sensitivity to PI3K/Akt signaling in AML. A. Levels of Akt and pAkt (Ser473) in *Cbfb+/MYH11* AML and two *Cbfb+/MYH11/miR-17-92* AML samples after IL3 stimulation. B. Bad and pBad (Ser136) levels after treatment with scramble (Scr), anti-*miR-17/20a* (anti-17) and anti-*miR-19a/b* (anti-19) antagomirs.

MiR-17-92 cooperates with Cbfb-MYH11 in AML development

Identification of the retroviral insertions upstream of *miR-17-92* cluster suggests that this cluster can act as an oncomiR. Overexpression of the cluster has been shown to promote B-cell lymphomas in a mouse model overexpressing the oncogene *Myc* (He et al., 2005). The cluster was also identified in a RIM screening for T-cell ALL and was shown to be overexpressed in a large fraction of human T-cell lymphomas (Landais et al., 2007; Landais et al., 2005). MicroRNA expression profiling in human AML showed that these miRs are consistently upregulated in MLL-fusions associated leukemia cases bearing chromosomal 11q23 translocations, but not CBF AML cases (Jongen-Lavrencic et al., 2008; Li et al., 2008; Mi et al., 2010). These data suggest that MLL-fusion proteins may directly regulate the expression of the cluster, and that *miR-17-92* upregulation in CBF AMLs may emerge as a cooperating event. Supporting this idea, over 40% of human inv(16) AML cases expressed some of the *miR-17-92* miRs, mainly *miR-17-5p*, *miR-18a*, and *miR-20a*. While a panel of *Cbfb$^{+/MYH11}$* mouse leukemias did not overexpress these miRs, the R1 sample from the RIM screening and the *Cbfb$^{+/MYH11}$*/*miR-17-92* leukemias overexpressed *miR-17-5p*, *-18a*, *-20a*, and *-19a/b*. In addition, the identification of a third retroviral insertion at the *Kis2* locus upstream of *miR-106a-363* cluster suggests that upregulation of miRs from this *miR-17-92* paralog could also function as an oncomiR in AML.

We did not find any insertions near another *miR-17-92* paralog *miR-106b-25*. This cluster is overexpressed in human prostate cancers and is involved in targeting of the tumor suppressor PTEN in the prostate cancer cells (Poliseno et al., 2010). *miR-106b-25*

cluster expresses *miR-106b, miR-93* (*miR-17* family) and *miR-25* (*miR-92* family), but lacks *miR-19* family miRNAs. The reason why *miR-17-92* was found most frequently out of all three paralogs might be explained by small number of samples screened, although knock-out studies for all three miRNA clusters indicate that some functions of *miR-17-92* cannot be compensated by other paralogs. Importantly, both *miR-17-92* and *miR-106b-25* had similar expression pattern, therefore the observed lack of compensation has to be explained by functional differences of the clusters and not just tissue specificity. Therefore, it is possible that miRNAs from the same miRNA families still have distinct functions, and *miR-17-92* provides more efficient cooperation than *miR-106a-363* or *miR-106b-25*, if they provide any. Another explanation is that retroviral integration may not be completely random, and some loci are more frequently integrated than others. Finally, absence of insertions that would upregulate *miR-106b-25* expression could potentially indicate that *miR-19* function is required for the cooperation. The potential oncogenic functions of the *miR-106a-363* and *miR-106b-25* alone or in cooperation with Cbfβ-SMMHC can be tested by utilizing murine BM transduction and transplantation into recipient mice as described above for *miR-17-92*.

Using a conditional *Cbfb$^{+/MYH11}$* knock-in a mouse model of inv(16) AML, we proved that *miR-17-92* cooperates with *Cbfb-MYH11* in development of AML *in vivo*. The leukemia phenotype was similar to the one described in previous *Cbfb-MYH11*-associated studies (Kuo et al., 2006; Landrette et al., 2005), supporting the model that the fusion protein regulates the disease progression and differentiation block and the *miR-17-92* provides survival and/or proliferative advantage in the myeloid compartment (Fontana

et al., 2007). In contrast, in a mouse *Eμ-myc* model of B-cell lymphoma, *miR-17-92* changed phenotype of leukemic cells by causing earlier differentiation block than *c-Myc* itself (He et al., 2005). Spontaneous *Eμ-myc*-associated B-cell lymphomas have various B-cell phenotypes from pre-B to mature B-cells indicating that the *Myc* oncogene prevents normal B-cell differentiation at later stages than *miR-17-92* that blocks pro-B to pre-B transition (Ventura et al., 2008). Therefore, effects of *miR-17-92* on differentiation are dependent on cellular context.

Importantly, in mice *miR-17-92* overexpression by itself was not sufficient for AML development, thus it needs a pre-leukemic environment in order to efficiently support oncogenic transformation. Unlike ability of *miR-17-92* alone to cause lymphoproliferative phenotype when overexpressed in lymphoid progenitors (Xiao et al., 2008), we did not observe any hematopoietic disease, including myelo- or lymphoproliverative disorders, upon overexpression of *miR-17-92*. This discrepancy can be explained by the experimental setup: in the Xiao et al. study all or majority of lymphoid progenitors expressed *miR-17-92*, and the expression the miRNAs was strongly upregulated. Even in these conditions the mice started to develop lymphoproliferative diseases close to 20 weeks after induction of miRNA expression. In our studies only 15-20% of BM cells expressed *Cbfb-MYH11* at the time of transduction (Kuo et al., 2006), and the transduction efficiency with *miR-17-92* construct was 15% (data not shown). Moreover, upregulation of *miR-17-92* was less efficient than in the Xiao et al. study. Therefore, the latency of the disease could be longer than a 6-months period that the mice were monitored.

Pten is miR-17-92 target in AML cells

PTEN phosphatase regulates PIP3 to PIP2 transition in a cell membrane and inhibits activity of PDK/AKT signaling. It acts as a tumor suppressor that is mutated in multiple types of cancers, and it is a target of *miR-17-92* in B- and T-cell leukemias (Mavrakis et al., 2010; Olive et al., 2009). *PTEN* mutations are not common in AML, yet the PI3K/AKT pathway is frequently affected downstream of major mutations that are found in AML, including RAS and RTK mutations (Martelli et al., 2006).

We show that Pten was the only *miR-17-92* target protein tested that was consistently downregulated in the $Cbfb^{+/MYH11}$/*miR-17-92* AML in comparison to $Cbfb^{+/MYH11}$ AML, whereas other targets, including *Runx1*, *Cdkn1a* and *Rbl2* were not consistently changed. This is possibly due to a cell type specific regulation of the miR targets. For example, RUNX1 is regulated by *miR-17/20a* in the cell fate decision of granulocytic-myeloid progenitors and human monocytic cell lines that undergo granulocytic differentiation (Fontana et al., 2007), a differentiation step posterior to the myelomonocytic block directed by the CBFβ-SMMHC. Another possibility is that *Cbfb-MYH11* may need residual RUNX function for its oncogenic activity (as discussed in Chapter I), therefore downregulation of RUNX1 with *miR-17-92* could be selected against in a fraction of AML samples. Thus, *miR-17-92* targets that are found in cancers may not only reflect ability of the miRNAs to downregulate their targets in a given tissue, but also be a result of selection for those targets that promote or at least do not impede the tumorigenesis.

We analyzed effectors downstream of Pten in the PI3K/Akt pathway. The

phosphorylation of Akt was increased in response to IL-3 cytokine in $Cbfb^{+/MYH11}$/miR-17-92 AML cells but not $Cbfb^{+/MYH11}$ AML cells. Furthermore, inhibition of either *miR-17/20a*, or *miR-19a/b* decreased inactive (phosphorylated) form of the proapoptotic protein Bad. These results are in line with the model that miRs from the *miR-17-92* cluster target Pten levels, triggering signaling through Akt and Bad. This corresponds to studies in lymphoid leukemias identifying Pten as a key target in *miR-17-92*-mediated leukemia (Matsubara et al., 2007; Olive et al., 2009; Takakura et al., 2008; Yu et al., 2010b).

MiR-17-92 cluster provides anti-apoptotic signal in $Cbfb^{+/MYH11}$/miR-17-92 AML

The functional assays on survival of $Cbfb^{+/MYH11}$/miR-17-92 AML cells after treatment with *miR-17* or *miR-19* inhibitors by measuring caspase cleavage and accumulation of Annexin V-positive cells suggest that at least part of the anti-apoptotic response is specific to *miR-17* family and independent of *miR-19*. The apparent contradiction with the Pten/Bad results seems to point at a second mechanism of survival in the leukemic cells. The possible mechanism underlying these results is still unclear. Anti-apoptotic function of *miR-17-92* was shown in different tissues to be due to E2F1 that is involved in a feedback loop with *miR-17/20a*, and is not regulated by *miR-19a/b* (Phillips and Vousden, 2001; Sylvestre et al., 2007). Another possible mechanism can be BIM downregulation that was shown to be an effector in *miR-17-92* anti-apoptotic signaling (Dai et al., 2011; Molitoris et al., 2011). However, it is not evident that Bim plays a role

in these particular AMLs. Bim was not consistently downregulated in the AML samples, and it can also be theoretically regulated by both *miR-17/20a* and *miR-19a/b*. Examples of putative *miR-17* specific targets include the MAP2K3 (MEKK2) kinase, which signals through JNK and NFκB pathways, and the hematopoiesis-specific Dual specificity protein phosphatase (DUSP2), which negatively regulates ERK1 and ERK2 (Ward et al., 1994).

An open question is how relevant the observed role of *miR-17-92* in Cbfb$^{+/MYH11}$ AML is in the context of human AML. The antagomir technology provides an excellent opportunity to test a response of human AML samples to inhibition of the miRNAs from the *miR-17-92* cluster *in vitro*. If a pro-apoptotic and/or anti-proliferative response that correlates with the miRNA expression was determined upon treatment of human AML samples with the miRNA inhibitors, this would suggest relevance of the observed data to the pathology of human AML.

Role of individual miRNAs

We have shown that not all miRNAs from the *miR-17-92* cluster are overexpressed in the AML samples. The original R1 sample identified from RIM as carrying a retroviral insertion next to the miRNA cluster, had most miRNAs from the cluster overexpressed, but levels of *miR-18a* and *miR-19a* were the same as in a control AML sample. In the murine *Cbfb$^{+/MYH11}$/miR-17-92* AMLs obtained as a result of cooperation between the fusion protein and *miR-17-92*, *miR-18a* and *miR-92a* were not consistently upregulated (although they were upregulated in a subset of the samples). Reports indicate that

individual miRNAs from a cluster can be regulated independently, as it was shown for *miR-18a* (Guil and Caceres, 2007). Importantly, absence of overexpression of *miR-18a* and *miR-92a* from the full length *miR-17-92* cluster does not prevent the cooperation between *Cbfb-MYH11* and *miR-17-92*, suggesting that the cooperation occurs mostly due to the function of members of *miR-17* and/or *miR-19* miRNA families. In a study of cooperation between *miR-17-92* and *Eμ-myc* in B-cell lymphoma development, the authors used *miR-17-19b* truncated version of the cluster that was as efficient in cooperation as the full length cluster (He et al., 2005), suggesting that *miR-92a* is not playing a role in the cooperation, which goes in line with our results in AML cells.

We have shown the functional difference between *miR-17/20a* and *miR-19a/b* in apoptosis regulation in the $Cbfb^{+/MYH11}/miR$-*17-92* AML cells. In lymphoid leukemias with *miR-17-92* role of *miR-19* miRNA family was shown to be pivotal for leukemia development and cooperation with other oncogenes, e.g. c-Myc in a model of B-cell lymphoma (Olive et al., 2009). Yet, in lung cancers *miR-17* and *miR-20a* provided the strongest anti-apoptotic effect leading to "oncomiR addiction", when cancer cells constantly need high level of *miR-17/20a* expression in order to keep apoptosis at a low level (Matsubara et al., 2007). Our data suggest that despite both *miR-17* and *miR-19* families regulating Bad phosphorylation in the AML cells, *miR-17* family provides additional signaling essential for apoptosis regulation in $Cbfb^{+/MYH11}/miR$-*17-92* AMLs. Yet, we cannot exclude a potential role of *miR-19a/b* in the process that could provide changes in gene expression enhancing *miR-17/20a* effects, or create a correct gene expression context that allows *miR-17/20a* to determine the anti-apoptotic effect.

Individual expression of each miRNA alone or in pools in the context of the *Cbfb*$^{+/MYH11}$ conditional knock-in mouse model could clarify this question.

In summary, the studies presented in this chapter provide evidence for a role of *miR-17-92* microRNA cluster in acute myeloid leukemia development in cooperation with the fusion oncoprotein CBFβ-SMMHC. Furthermore, we provide evidence for the survival role of microRNAs of the *miR-17* family in the leukemic cells.

CHAPTER III

THPO/MPL signaling participates in the initiation and maintenance of *RUNX1-ETO* acute myeloid leukemia via PI3K signaling

Foreword

The first part of the work described in this chapter has been published (Landrette et al., 2011), in which I am a co-first author. My contribution included (1) the investigation of activity of different PlagL2 binding sites in *Mpl* promoter using electrophoretic mobility shift assay (EMSA) (Figure 3.5C), (2) participation in the BMT assays of cells expressing $Cbfb^{+/MYH11}/PlagL2$, (3) participation on the BMT assays involving donor cells expressing $Cbfb^{+/MYH11}/Mpl$, and (4) writing and editing parts of the discussion and writing of the manuscript.

The second part of the work described in this chapter is part of a manuscript that is currently under revision for publication. This work includes a collaboration with Peter Valk, and I am the first author.

Introduction

Acute myeloid leukemia (AML) results from the alteration of genes associated with proliferation, differentiation, and survival. These functions can be altered by mutations in genes encoding for transcription factors and cytokine receptors that are essential for normal hematopoietic function. The leukemia fusion protein RUNX1-ETO is created by the chromosome translocation t(8;21), and is found in 12% of AML samples (Look, 1997). The fusion protein increases survival of hematopoietic progenitors *in vitro*, and inhibits differentiation of hematopoietic progenitors (Mulloy et al., 2002; Okuda et al., 1998). Expression of RUNX1-ETO is not sufficient for leukemic transformation in animal models (Yuan et al., 2001b), but it induces AML in cooperation with oncogenic mutations in proteins associated with cytokine signaling. Examples are the oncogenic mutations in genes encoding the receptor tyrosine kinases c-KIT and FLT3, and their downstream GTPases NRAS and KRAS (Boissel et al., 2006a; Care et al., 2003; Schessl et al., 2005b; Speck et al., 1999; Valk et al., 2004a).

The myelo-proliferative leukemia virus oncogene MPL (c-MPL or CD110) is a homodimeric receptor activated by the cytokine THPO, and regulates proliferation in HSCs and megakaryocytes (Kaushansky and Ranney, 2009b). The MPL receptor is expressed in 70% of cells within the HSC compartment and is markedly reduced upon HSC differentiation (Kaushansky et al., 1996; Solar et al., 1998). The THPO/MPL signaling regulates the expansion and maintenance of HSCs and early progenitors via activation of JAK/STAT, ERK/MEK and PI3K/AKT pathways (Kaushansky, 2009b).

The number and function of HSCs is markedly reduced in *Mpl*-null and *Thpo*-null mice (Alexander et al., 1996; Carver-Moore et al., 1996; Gurney and de Sauvage, 1996; Kimura et al., 1998b). Furthermore, the self-renewal capacity of long-term HSCs is reduced 10- to 20-fold in *Thpo*-null recipients, an effect that can be rescued with the addition of recombinant-THPO (Fox et al., 2002b).

Mutations identified in human disease also highlight the importance of MPL signaling in homeostasis of the hematopoetic compartment. Nonsense mutations in the *MPL* and *THPO* genes cause congenital amegakaryocytic thrombocytopenia, with severe thrombocytopenia and aplastic anemia (Ballmaier and Germeshausen, 2009). Somatic activating mutations in *MPL* cause constitutive JAK2 activation and are associated with myeloproliferative neoplasms, including myelofibrosis with myeloid metaplasia and essential thrombocythemia (Pardanani et al., 2006; Pikman et al., 2006). In AML, mutations in *MPL* gene have been detected in a quarter of AMKL (Hussein et al., 2009b). However, the functional role of increased levels of wild type MPL in the initiation and maintenance of AML is unknown.

In this study, we used human AML cells and mouse transplantation models to study the role of MPL in leukemia development. We find that a large fraction of t(8;21) AML samples expresses *MPL* transcript, and proliferates in response to THPO. We use transplantation assays in mice to show that expression of *RUNX1-ETO* and *Mpl* synergize to induce leukemia. We find that *Mpl* levels are not regulated by RUNX1-ETO in mouse hematopoietic progenitors and leukemic cells, and show negative correlation with RTK mutations in human AML cells. Biochemical analysis of the mouse leukemic cells

showed that Mpl expression sensitizes leukemic blasts to JAK2, ERK, STAT and AKT phosphorylation. Furthermore, the survival signal promoted by THPO can be abrogated with a PI3K inhibitor, and leukemia expansion in mice can be delayed by mTOR-inhibitor rapamycin. These studies show that *MPL* expression provides an oncogenic signal that cooperates with *RUNX1-ETO* in leukemia development and maintenance.

Materials and methods

Reverse transcriptase and quantitative RT-PCR analyses. RNA from mouse BM and AML cells was extracted with Trizol (Invitrogen, Carlsbad, CA). First-strand cDNA was generated by using 2 µg RNA, 1 U Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), and 0.5 µM oligo-dT or random hexamer primers in a 20-µL reaction. SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used for qPCR according to the manufacturer's instructions. *Mpl* primers were Mplx1 (5‟-ACTTTGATCCAGCGGGTGCT-3‟) and Mplx2 (5‟-CAGGAAGTCACTGATTTCAG-3‟). The β-actin primers were ActbF1 (5‟-CGAGGCCCAGAGCAAGAGAG-3‟) and ActbR1 (5‟-CGGTTGGCCTTAGGGTTCAG-3‟). QPCR was performed in a StepOne Plus Sequence Detection System (Applied Biosystems Foster City, CA). Samples were normalized to β-actin transcript levels, and relative values were determined using standard curve or comparative C_T methods.

DNA sequence analysis. The search for PLAG consensus site GRGGC(6–8)RGGK was analyzed using the UCSC Genome Browser on Mouse July 2007 (NCBI37/mm9)

Assembly (http://genome.ucsc.edu). Promoter regions were defined as 1000 bp upstream and 200 bp downstream of transcription start site, with this site as position 1000. Evolutionary conservation of PLAG sites among six mammal species (human, orangutan, mouse, rat, dog and horse) was estimated. The sequence conservation analysis at the Mpl proximal promoter was performed using ClustalW-Alignment Analysis from MacVector9.5.2 (MacVector Inc., Cary, NC, USA).

Luciferase assays. A 300 bp fragment of the Mpl proximal promoter was amplified with primers MPLFLPromkpnF and MPLFLpromBglR using PFU (Stratagene, La Jolla, CA, USA), and the amplicon was digested with KpnI and BglII and cloned into pGL3-Basic (Promega, Madison, WI, USA). Mutants were constructed by overlapping PCR with primers Mpls1F and Mpls1R for „site-1", Mplps2F and Mplps2R for „site-2", and Mplps3F and Mplps3R for „site-3" (for primer sequences, see (Landrette et al., 2011)). The mutant primers were used with primers MPLFLpromkpnF and MPLFLpromBglR to amplify overlapping fragments that were annealed, digested with KpnI and BglII and cloned into the PGL3-basic vector. Constructs were transfected into NIH3T3 cells along with MIhCD4 or MIhCD4-PLAGL2 and PRL-TK with Effectene (Qiagen). Cells were analyzed for luciferase levels with the Dual-Luciferase Reporter Assay System (Promega).

Electrophoretic mobility shift assay. The electrophoretic mobility shift assay was performed as previously described (Javed et al., 2004). Briefly, 3T3 cells transfected with MIG-PlagL2 were used for isolation of nuclear proteins and added to protein–DNA binding reactions with double-stranded DNA oligos representing PlagL2-binding sites in

the Mpl promoter. The sequence for DNA oligos P1ss and P1as, P2ss and P2as, P3ss and P3as, mutP1ss and mutP1as, mutP2ss and mutP2as, and mutP3ss and mutP3as are shown in (Landrette et al., 2011). The P1ss, P2ss and P3ss oligos were radioactively labeled with 32P using OptiKinase (USB, Cleveland, OH, USA), and the excess 32P-γATP was removed by purifying the labeled oligos using G-25 Mini Quick Spin Oligo columns (Roche, Basel, Switzerland). The oligos were annealed with equal amounts of antisense oligos (P1as, P2as, P3as respectively) to obtain a double stranded labeled oligo with a PlagL2-binding site. In all, 100 pg of the oligo was used for each binding reaction. Cold competitors and mutant competitors were obtained the same way, but without labeling the oligos. When using cold competitors (wild type or mutant), a 1:200 molar ratio of labeled and cold oligo was used. Constant DNA amount was maintained by adding scrambled oligos Sss and Sas. The protein–DNA binding reactions were loaded onto a 4% polyacrylamide gel, ran until unlabeled 32P-gATP went out into buffer, dried and exposed for 24 h.

Analysis of human AML samples. <u>Expression Analysis</u>: Bone marrow leukemia blasts from 162 patients with AML at diagnosis (classified according to the French-American-British nomenclature) and 6 healthy volunteers were obtained after informed consent. Approval was obtained from the Erasmus Medical Ethical Review Committee for these studies. Blasts from patients with AML and mononucleated fractions from normal BM specimens were isolated by Ficoll-Hypaque (Nygaard, Oslo, Norway) centrifugation and then cryopreserved. After thawing, cells were washed with Hanks Balanced Salt Solution (HBSS) and further processed for RNA isolation. AML samples treated according to this

procedure usually contain more than 90% blasts after thawing. Total RNA was extracted with guanidium thiocyanate followed by centrifugation in cesium chloride solution. RNA (1 µg) was transcribed into cDNA by using Superscript (Life Technologies, Merelbeke, Belgium) and random hexamers in a 40-µL reaction, under standard conditions. The qPCR amplification was performed in an ABI PRISM 7900 HT Sequence Detector, using 12.5 µL SYBR Green PCR Master Mix (Applied Biosystems, Nieuwerkerk a/d IJsel, The Netherlands), 2 µL (1/20th aliquot) cDNA, 2.5 pmol primer mix (Life Technologies), and 10 µL water. The PCR conditions included 2 min at 50°C and 10 min at 95°C followed by 45 cycles of amplification (each with a 15 sec denaturation step at 95°C and a 1 min annealing/extension step at 60°C). The *MPL* primers were hMPLx1 (5"- CCAGCCAGGGGAACTTC-3") and hMPLx2 (5"-GCTTTGGTCCATCTTGCC-3").

MPL expression was determined using the assay-on-demand Hs00180489-m1 (Applied Biosystems, Foster City, CA). To determine *MPL* relative expression levels, the average C_T (threshold cycle) values from duplicate readings were normalized for endogenous reference ($\Delta Ct = Ct$ target - Ct PBGD) and compared with a calibrator using the "delta-delta Ct method" ($\Delta\Delta Ct = \Delta Ct$ Sample - ΔCt Calibrator). As calibrator we used the average Ct value of *MPL* in 6 CD34+ samples from healthy volunteers. The relative expression was calculated ($2-\Delta\Delta Ct$). Pearson correlation analysis was performed to assess the linear relationship between *MPL* expression and induction of growth by THPO. Log transformed data were used for this analysis in order for the three variables to be approximately normal.

Mutation Analysis: The analysis for mutations in *KIT*, *FLT3*, *NRAS* and *KRAS*

genes in AML samples was performed as previously described (Care et al., 2003; Valk et al., 2004a). <u>Microstimulation assay with cytokine THPO</u>: Ficoll-isolated mononuclear cells of the primary AMLs were suspended in IMDM/1% BSA and plated on tissue culture dishes for 1 hour at 37ºC. Non-adherent cells were collected and $2x10^4$ cells were stimulated with 100ng/ml THPO for 3 days. 3H-thymidine incorporation was measured after an 8 hrs incubation period.

Retroviral production. The MIG-Mpl construct was generously provided by Harvey Lodish (Massachusetts Institutes of Technologies, Cambridge MA). *Mpl* was subcloned into the pMSCV-IRES-hCD4 (MID) vector via the BglII and XhoI sites. The RUNX1-ETO cDNA (provided by Peter Westervelt, Washington University, Saint Louis MO) was cloned into the XhoI site of MIG. *PLAGL2* was cloned into MID via the BglII and XhoI sites. Phoenix packaging cells (provided by Gary Nolan, Stanford University, Stanford CA) were co-transfected with 2 μg retroviral constructs, 2 μg ψ-Eco packaging plasmid with Effectene reagent (Qiagen, Valencia, CA) according to manufacturer's protocol. Retrovirus supernatants were collected at 48, 56, and 72 hrs and titered in NIH3T3 cells by FACS analysis of GFP or hCD4 expression.

Bone marrow transduction and transplantation (tBMT). Wild type BM progenitor cells were spin-infected twice with retrovirus supernatants, and $5x10^5$ to $1x10^6$ BM cells transplanted by intravenous injection (iv.) into 4- to 6-week of age sublethally (650rads) or lethally (split dose of 500 rads) irradiated 129SvEv wild-type recipient mice. Mice were under daily observation for early signs of leukemia, and peripheral blood was taken periodically to monitor for the presence of immature cells. The early signs included

limited motility, pale paws, and dehydration. FACS analysis of peripheral blood was performed by using antibodies to cell-surface markers Gr1, CD11b, B220, CD3, Ter119, CD71, and c-kit (BD Biosciences, San Diego, CA). For flow cytometry analysis of Mpl receptor expression, we used a polyclonal rabbit anti-Mpl extracellular domain antibody (provided by Harvey Lodish, Whitehead Institute for Biomedical Research, Cambridge MA) and a PE-anti rabbit secondary antibody (BD Biosciences, San Diego CA). Staining of peripheral blood smears and spleen sections were ordered from Histoserv (Germantown, MD).

For secondary transplantations of *RUNX1-ETO/MPL* AML, leukemic cells were harvested from the BM or spleen of affected mice in PBS and sorted for hCD4$^+$ and GFP$^+$hCD4$^+$ populations by FACS. 80,000 sorted cells were transplanted i. v. into sublethally irradiated 4- to 6-week-old 129SvEv recipients.

The rapamycin experiments consisted of transplantation of 1×10^5 *RUNX1-ETO/PL2* AML cells into sublethally irradiated recipients. Recipient mice were daily treated intra-peritoneally with 200 μL of 80 μg/mL rapamycin (Calbiochem, San Diego, CA) as previously described (Yilmaz et al., 2006). Moribund animals were analyzed as described above.

Immunoblot analysis. Cryopreserved AML cells were thawed and immediately serum starved in RPMI media with 1% BSA for 60 min at 37°C (Invitrogen, Carlsbad, CA). Cells were then incubated with RPMI media with 0.1% BSA and 0, 5, 25, or 50 ng/ml THPO (Peprotech, Rockyhill, NJ) for 10 min. Cells were washed with PBS and resuspended in RIPA buffer with protease-inhibitor-cocktail III (Calbiochem, Darmstadt,

Germany). Antibodies included anti-Jak2 (cat#sc-294; Santa Cruz Biotechnology Inc., Santa Cruz, CA), and antiphospho-Jak2-Tyr221 (cat#3774), anti-phospho-Stat5-Tyr694 (cat#9351), anti-Stat5 (cat#9310), anti-phospho-Akt-Ser273 (cat#4058), anti-Akt (cat#9272), anti-phospho-Erk1/2 -Thr202/Tyr204 (cat#9101), and anti-Erk1/2 (cat#9102; all from Cell Signaling Technology, Danvers MA).

Apoptosis analysis of mouse AML cells in presence of Thpo. Leukemic cells carrying RUNX1-ETO and MPL, as well as RUNX1-ETO and MIG were cultured in RPMI medium with 20% FBS, 3 ng/ml IL3, 10 ng/ml SCF and 10 ng/ml IL6 (Peprotech, Rockyhill, NJ) for 24 hrs. Cells (10^6/ml) were then resuspended in serum-free medium (STEMSPAN SFEM, Stem Cell Technologies) and treated with THPO (20 ng/ml) for 48 hours. Cell proliferation was measured using Trypan blue exclusion method. Apoptotic cells were determined by flow cytometry as Annexin V^+ / 7AAD$^-$ at 48 hours (BD Pharmingen, San Jose, CA) according to manufacturer's instructions. The following inhibitors were used: MEK (PD98059) at 25 uM and PI3K (LY294002; both from Gibco, Carlsbad, CA) at 20 uM. For experiments with inhibitor and THPO, cells were first treated with inhibitor for 1 hour followed by THPO induction.

Results

Expression of MPL is increased in t(8;21)-positive human AML samples and promotes proliferation

A previous unsupervised clustering of gene expression in 285 human AML samples defined 16 distinct clusters displaying similar gene expression signatures (Valk et al., 2004b). In order to study the significance of the THPO receptor MPL in AML, we queried the *MPL* expression in this dataset using the 3 independent *MPL* probe sets (Figure 3.1A). We found that *MPL* transcript is expressed in a fraction of AML samples across all clusters, with a marked overrepresentation particularly in cluster #13. This cluster aggregates all samples carrying the chromosome translocation t(8;21), which expresses the leukemia oncogene *RUNX1-ETO*. To test if human AML blasts respond to THPO, the proliferation of 86 AML cells, including 7 t(8;21) samples, was measured in the presence of THPO as single cytokine. After 3 day cultures, cell proliferation was estimated by 3H-Thymidine incorporation and THPO specificity adjusted by normalizing to the values in the absence of cytokine, thus excluding immortalized samples.

Approximately 20% of samples (17/86) induced proliferation over 3-fold when compared to no-cytokine controls (Figure 3.1B), including 85% (6/7) of t(8;21) samples (Figure 3.1B, black bars). The expression of *MPL* transcript was determined by quantitative RT-PCR in the 86 AML samples (Figure 3.1C). The MPL expression showed significant correlation with THPO response (R = 0.572, P < 0.0001), and *MPL*

A.
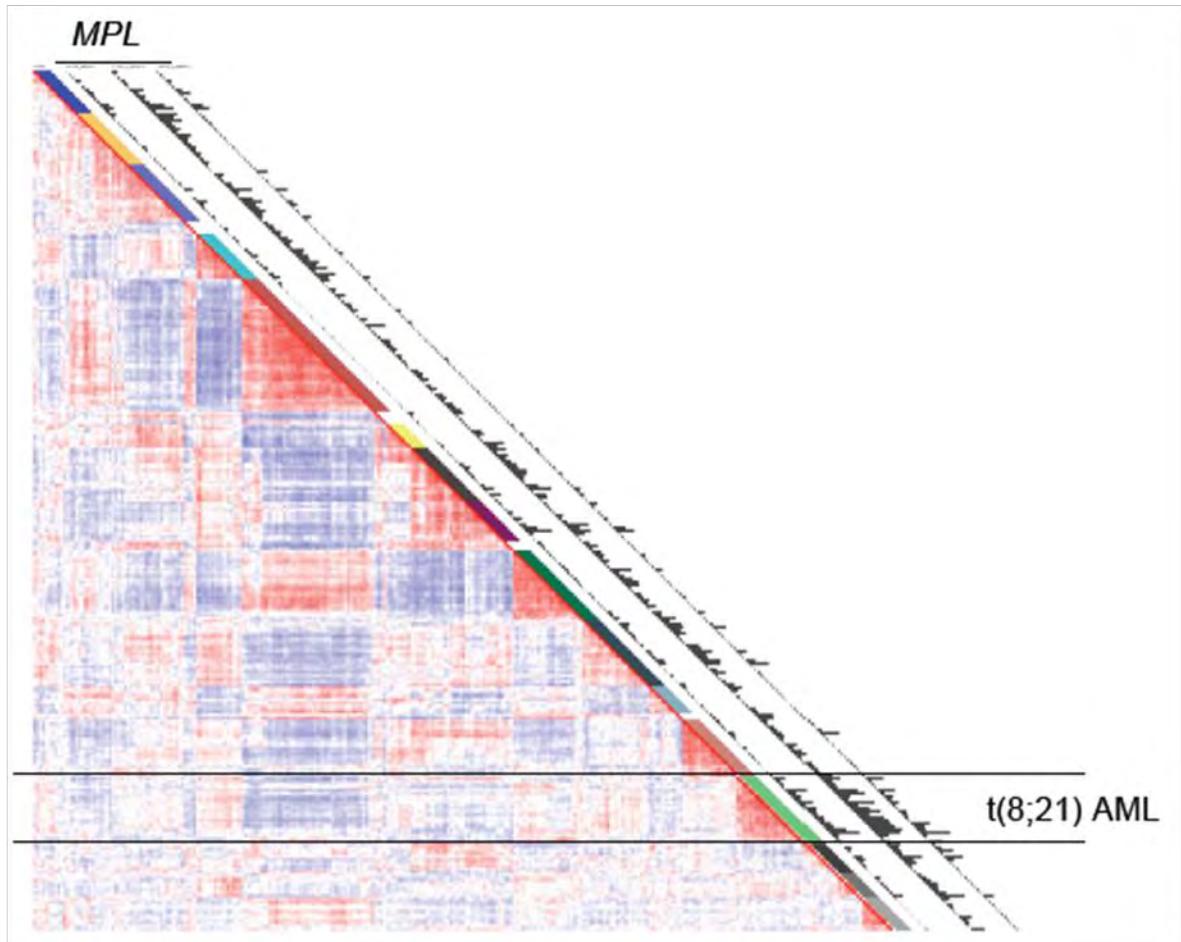
Figure 3.1. t(8;21)-positive acute myeloid leukemia cells express MPL and respond to THPO induction. A. Expression correlation view (2856 probe sets) of 285 AML patients, adapted from (Valk et al., 2004b). The 16 clusters identified on the basis of the Correlation View are depicted by different colors alongside the Correlation View (1 to 16). Cluster #13, aggregating all AML cells with t(8;21), is indicated by horizontal lines. The expression of *MPL* (detected by 3 independent probe sets 207550_at, 211903_s_at, 216825_s_at) is plotted in the latter 3 columns in which the bars are proportional to the *MPL* expression level.
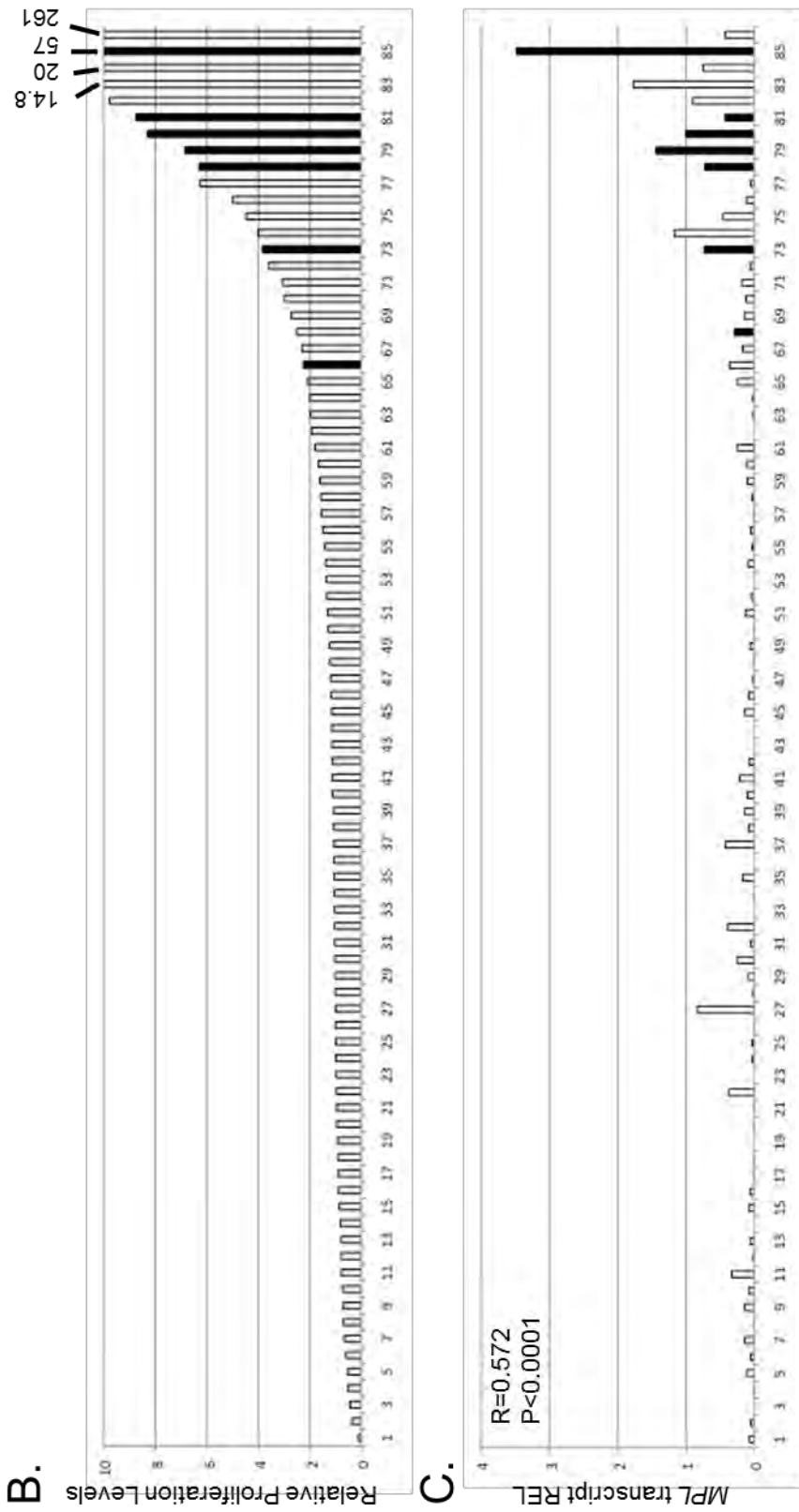
Figure 3.1 (cont.). t(8;21)-positive acute myeloid leukemia cells express MPL and respond to THPO induction. B. Relative proliferation levels of 86 human AML cells in response to THPO as singly cytokine. Samples were incubated with THPO or PBS for 3 days, and proliferation was tested by 3H-thymidine incorporation. The fold increase proliferation with THPO were normalized to that of values with no cytokine, and ordered in increasing response. Black bars indicate t(8;21) positive samples. C. *MPL* transcript levels determined by quantitative RT-PCR in human AML samples that were subjected to proliferation assays. The *MPL* levels are the average of duplicate values and relative to the average value of 6 healthy CD34+ bone marrow samples (REL). Correlation between MPL expression and THPO response was performed by R statistics (P<0.001).

D.
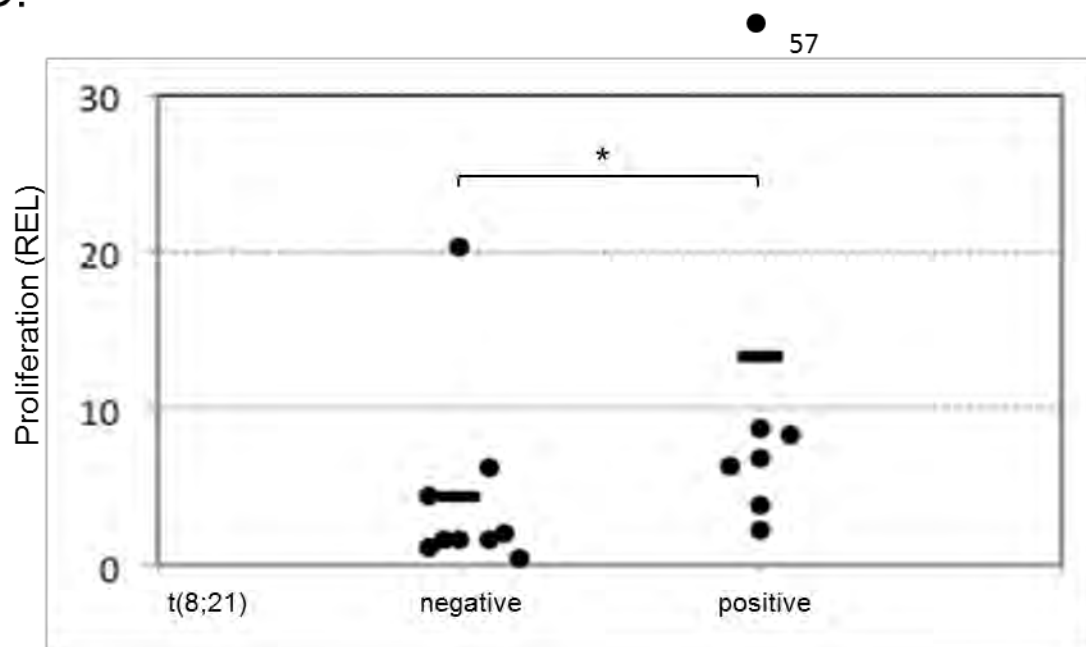
E.
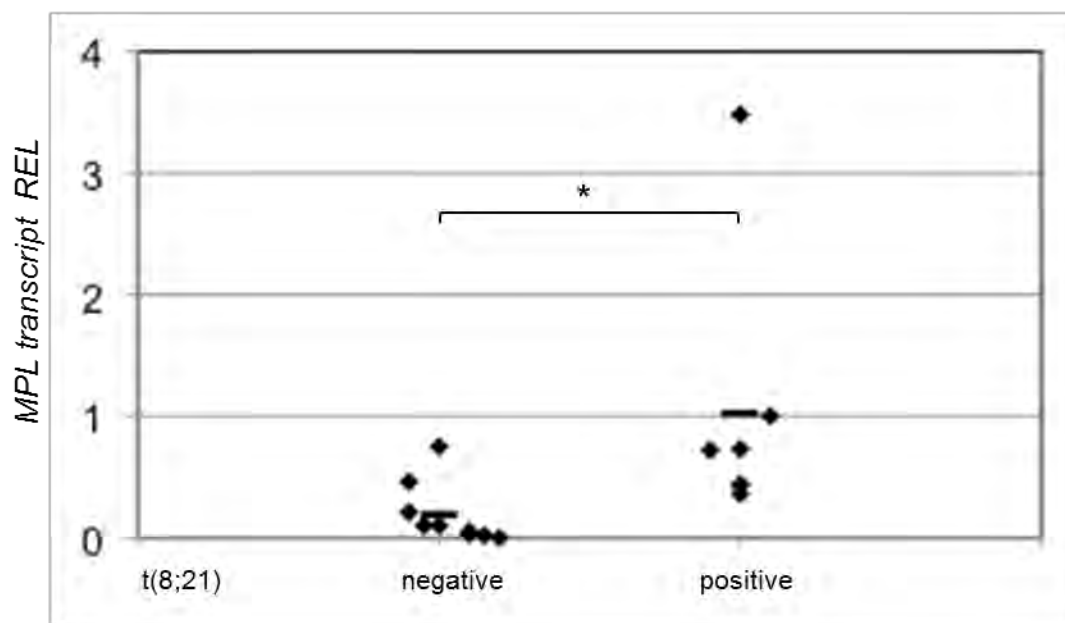
Figure 3.1 (cont.). t(8;21)-positive acute myeloid leukemia cells express MPL and respond to THPO induction. D. Relative proliferation levels, and E. Relative MPL levels of AML-M2 samples lacking (negative) or carrying (positive) the translocation t(8;21). The average value of individual samples (dot) and group mean (line) are shown. The Student's t-test was used to determine the statistical differences between various experimental and control groups. $p < 0.05$ (*).

levels of the 7 t(8;21) samples were significantly higher than that of the t(8;21) negative group. The panel of AML samples included 16 subtype M2 samples, composed by 7 t(8;21) and 9 non-t(8;21) samples. Analysis of THPO response and *MPL* expression in the M2 samples also shows a marked correlation of active THPO/MPL signaling in t(8;21) positive (85%; 6/7) versus t(8;21) negative (33%; 3/9) samples (Figure 3.1D, E).

Mutation analysis of 83 of the 86 AML samples for oncogenic mutations in *FLT3* (-ITD and -TKD), *NRAS*, *KRAS*, and *KIT* genes indicated that 5 out of the 7 t(8;21) THPO-responsive samples were wild type for these genes, suggesting that *MPL*-upregulation was the only known proliferative signal in these AML samples (Tables 3.1 and 3.2). Together, these results show that human AML cells expand in presence of THPO/MPL signaling, and that this effect is preferentially present in *RUNX1-ETO* expressing samples.

Mpl cooperates with RUNX1-ETO in AML development in mice

To determine if *Mpl* expression synergizes with *RUNX1-ETO* in leukemia development, 5-FU treated bone marrow cells were co-transduced with pMSCV-IRES-GFP-RUNX1-ETO (MIG-R1E) and either pMSCV-IRES-humanCD4-Mpl (MID-Mpl) or pMSCV-IRES-humanCD4 (MID) retroviruses. Since t(8;21) patients express two isoforms of RUNX1-ETO fusion protein: the full length (R1E) and a truncated isoform (RUNX1-ETO9a; R1E9a) missing the C-terminal exons and inducing more aggressive leukemia (Yan et al., 2006a), we tested leukemia onset with both isoforms. Transduced cells were sorted for GFP and transplanted into irradiated isogenic recipients (Figure 3.2A). Mice

Table 3.1.

Distribution of *FLT3, NRAS, KRAS,* and *KIT* mutations in AML based on their karyotype

Karyotype	No. samples	TPO responders		TPO no responders	
		wild type	RTK mt	Wild type	RTK mt
NN	40	4	4	20	12
Inv16	4	0	0	3	1
t(8;21)	7	5	2	0	0
t(11q23;x)	9	0	1	4	4
t(15;17)	2	1	0	0	1
Other	21	5	1	8	7
Total	83	15	8	35	25

Note: normal karyotype (NN), samples with (w RTK mt) or without (wild type) receptor tyrosine kinase mutations.

Table 3.2.

Distribution of *FLT3, NRAS, KRAS,* and *KIT* mutations in the 7 t(8;21)-positive AML

Sample	FLT3-ITD	FLT3-TKD	NRAS	KRAS	KIT-exon 8	KIT-exon17	Fold Inc
6372	P	N	N	N	N	P	2.26
2262	N	N	N	N	N	N	3.82
5357	P	N	N	N	N	N	6.30
6457	N	N	N	N	N	N	6.86
5283	N	N	N	N	N	N	8.30
2243	N	N	N	N	N	N	8.75
2267	N	N	N	N	N	N	57.00

Note: Positive (P) or negative (N) for the presence of mutation; Fold increase proliferation in response to cytokine TPO (Fold Inc.).

Tables 3.1, 3.2. Distribution of mutations in commonly mutated genes (*KIT, FLT3, NRAS, KRAS*) in AML samples. Table 3.1: Number of AML samples with or without mutations in the listed genes is shown. The AML samples are sorted according to their karyotype (rows) and grouped into TPO-sensitive and TPO non-sensitive (columns). Table 3.2: Presence (P) or absence (N) of common mutations in the listed genes is shown (columns) for each individual t(8;21)-positive AML sample (rows). The rightest column indicates fold increase in proliferation of the cells upon stimulation with THPO.

transplanted with cells that expressed *Mpl* and either *RUNX1-ETO* or *RUNX1-ETO9a* developed leukemia with a median latency of 50 days post transplantation and full penetrance (Figure 3.2B, red and green lines). Conversely, mice transplanted with MIG-R1E/MID did not develop leukemia up to 24 weeks (experimental end point), and mice transplanted with MIG-R1E9a/MID presented a delayed leukemia (median latency 140 days; Figure 3.2B, blue and black lines). Control mice transplanted with cells expressing MID-MPL or MID remained healthy over the experiment. Furthermore, 8×10^4 GFP$^+$hCD4$^+$ immature cells from RUNX1-ETO/MID and RUNX1-ETO/Mpl moribund mice transplanted into sublethally irradiated recipients induced leukemia with a latency of 25 days and full penetrance (Figure 3.2C, blue and purple lines).

Time-course analysis of peripheral blood from MID-Mpl mice revealed the gradual increase of erythroid progenitors with hCD4$^+$c-kit$^-$Ter119^{low} immunophenotype. These cells peaked at 6 weeks after transplantation, and gradually decreased to be undetectable by 24 weeks (Figure 3.2D, E). Transplantation of bone marrow from these mice did not replicate the phenotype indicating that the observed disease is not leukemia, but rather an erythroproliferative disorder (Figure 3.2C, green line).

The pathology of leukemia development was comparable among *RUNX1-ETO* groups. The leukemic mice from the *RUNX1-ETO9a/MIG, RUNX1-ETO9a/Mpl* and *RUNX1-ETO/Mpl* groups exhibited splenomegaly with average spleen weights of 1.1 g, 0.2g and 0.21g respectively (Figure 3.3A) when compared to wild type spleens (0.07 g), and the splenic architecture of white and red pulp was disrupted (Figure 3.3B). The peripheral blood of sick mice revealed a consistent increase in white blood cell count

A.
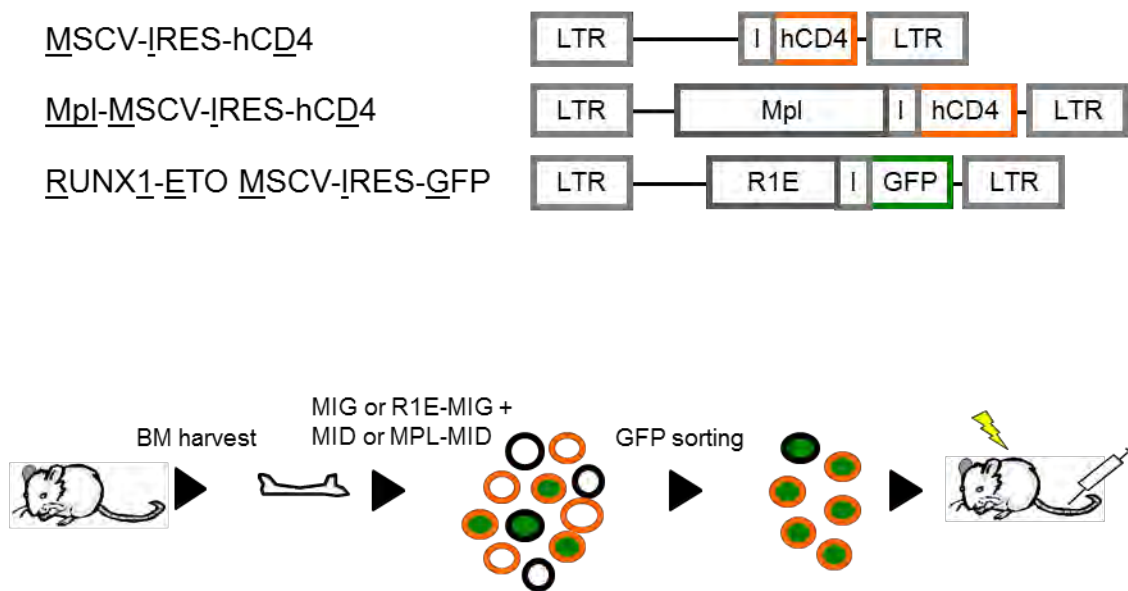
Figure 3.2. MPL cooperates with RUNX1-ETO in leukemia development in mice. A. Experimental Design. Top: Diagram depicting retroviral constructs used in the transplantation experiment. Bottom: Cartoon depicting the transplantation assay. Bone marrow cells (circles) harvested from wild type mice are co-transduced with retroviruses carrying the *Mpl* gene (orange) and *RUNX1-ETO* (green). Double positive cells are sorted and transplanted into irradiated recipient mice.

D.

E.
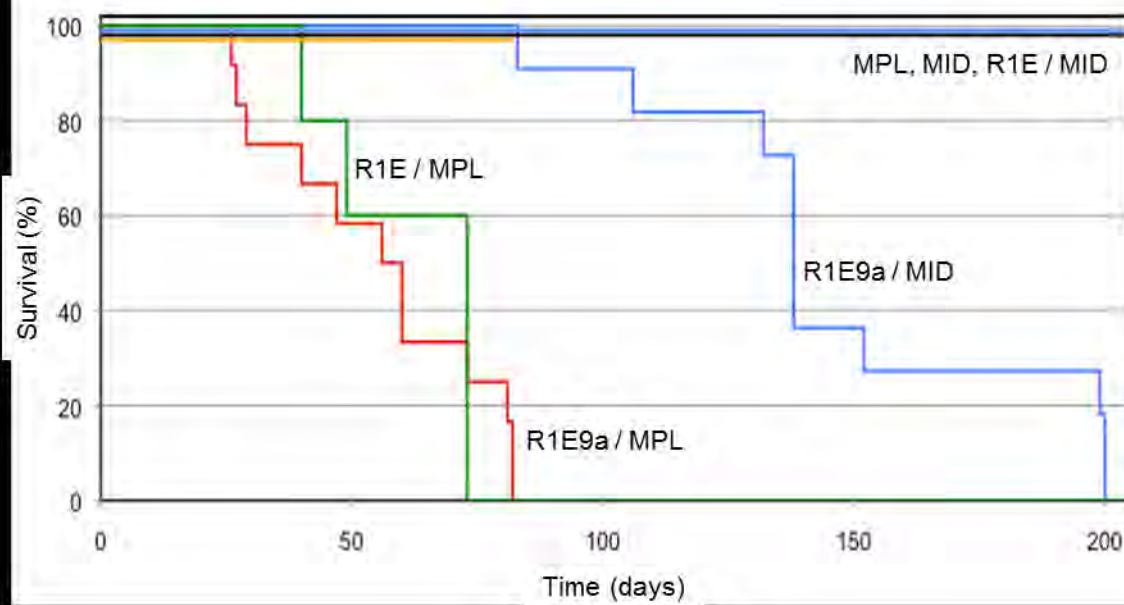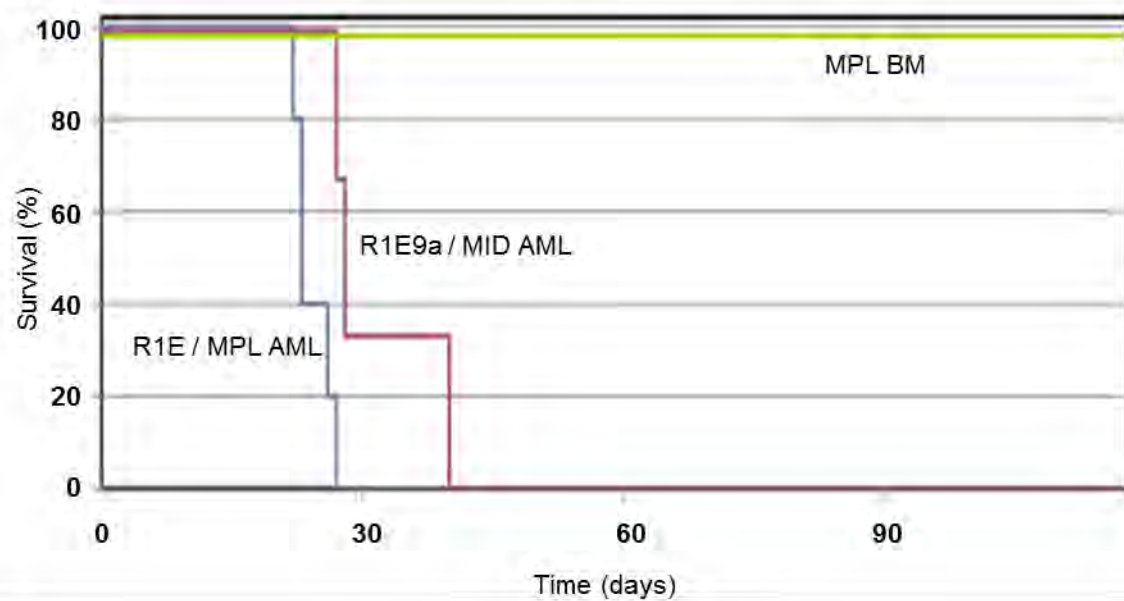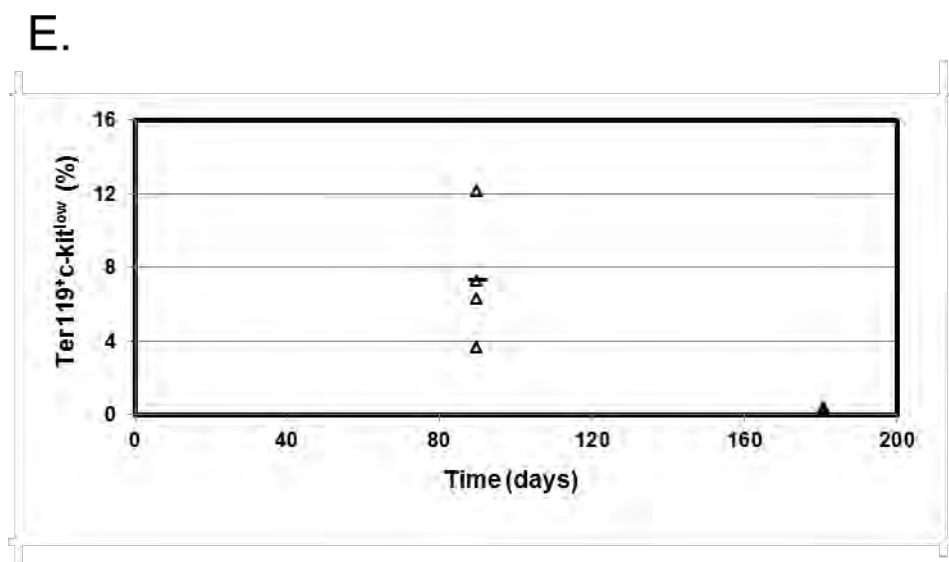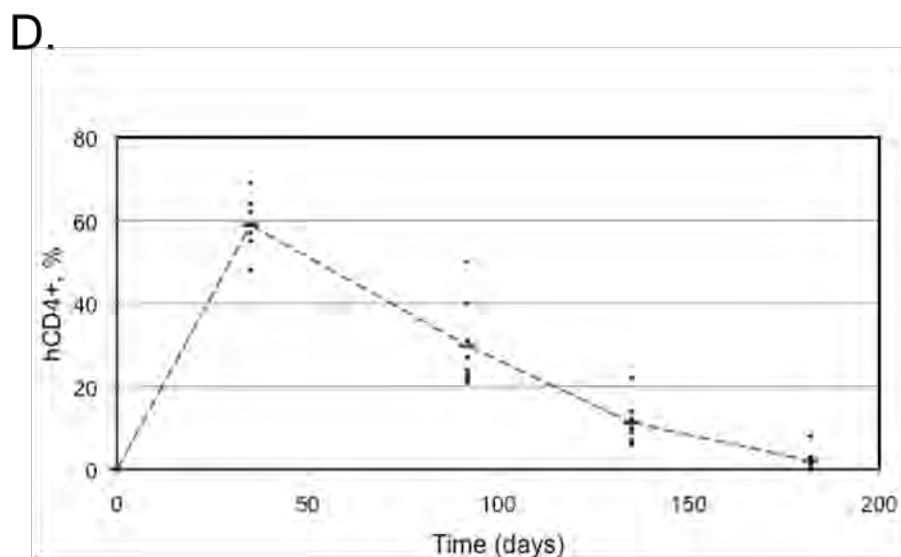
Figure 3.2 (cont.). MPL cooperates with RUNX1-ETO in leukemia development in mice. D. Time-course analysis of cells expressing exogenous Mpl (hCD4+ cells) in PB of recipient mice expressing Mpl alone. Each dot represents percentage of hCD4+ cells in PB of individual mouse, lines represent average percentage of hCD4+ cells. E. Time-course analysis of percentage of abnormal Ter119+c-kit^{low} cells in PB of recipient mice expressing Mpl alone. Each square represents percentage of Ter119+c-kit^{low} cells in PB of individual mouse, lines represent average percentage of the cells.

(WBC counts > 18 ± 7 x 10^6 cells/ml; normal WBC count = 4x10^6 cells/ml), and

increased presence of immature leukocyte morphologies (Figure 3.3C, D). Flow

cytometry analysis of peripheral blood revealed that leukemic cells carrying *RUNX1-ETO*

and *MPL* (GFP$^+$hCD4$^+$) were predominantly immature c-kit$^+$ cells that lacked lineage

specific markers for differentiated leukocytes (Ter119, Mac1, Gr1, B220, and CD3;

Figure 3.3E). A significant fraction of cells in bone marrow and peripheral blood

expressed both markers for *RUNX1-ETO* and *Mpl* ($20 - 40\%$ of the cell populations).

Similar pathology scenario was observed in *RUNX1-ETO* leukemic cells expressing *Mpl*

or MID vector (Figure 3.3A, B, C, D, F), suggesting that the pathology of leukemia is

marked by the fusion protein. Together, these studies show that expression of wild type

Mpl receptor induces transient erythroid expansion and that in the presence of RUNX1-

ETO acts as an oncogenic signal in the development of AML in mice.

Transcription factor PlagL2 regulates endogenous Mpl expression

As part of this thesis, I participated in a project that investigated role of PlagL2

transcription factor in *Cbfb-MYH11* AML. The results of this study were published

(Landrette et al., 2011). Previously, Dr. Castilla"s group has shown that *Plag1* and

PlagL2 cooperate with *Cbfb-MYH11* in development of AML (Landrette et al., 2005). In

this study we have shown by quantitative reverse transcription-PCR analysis that the

level of *Mpl* transcript was increased over 100-fold 48 h after transduction of

hematopoietic progenitor cells with retroviral constructs expressing human *PLAGL2*, or
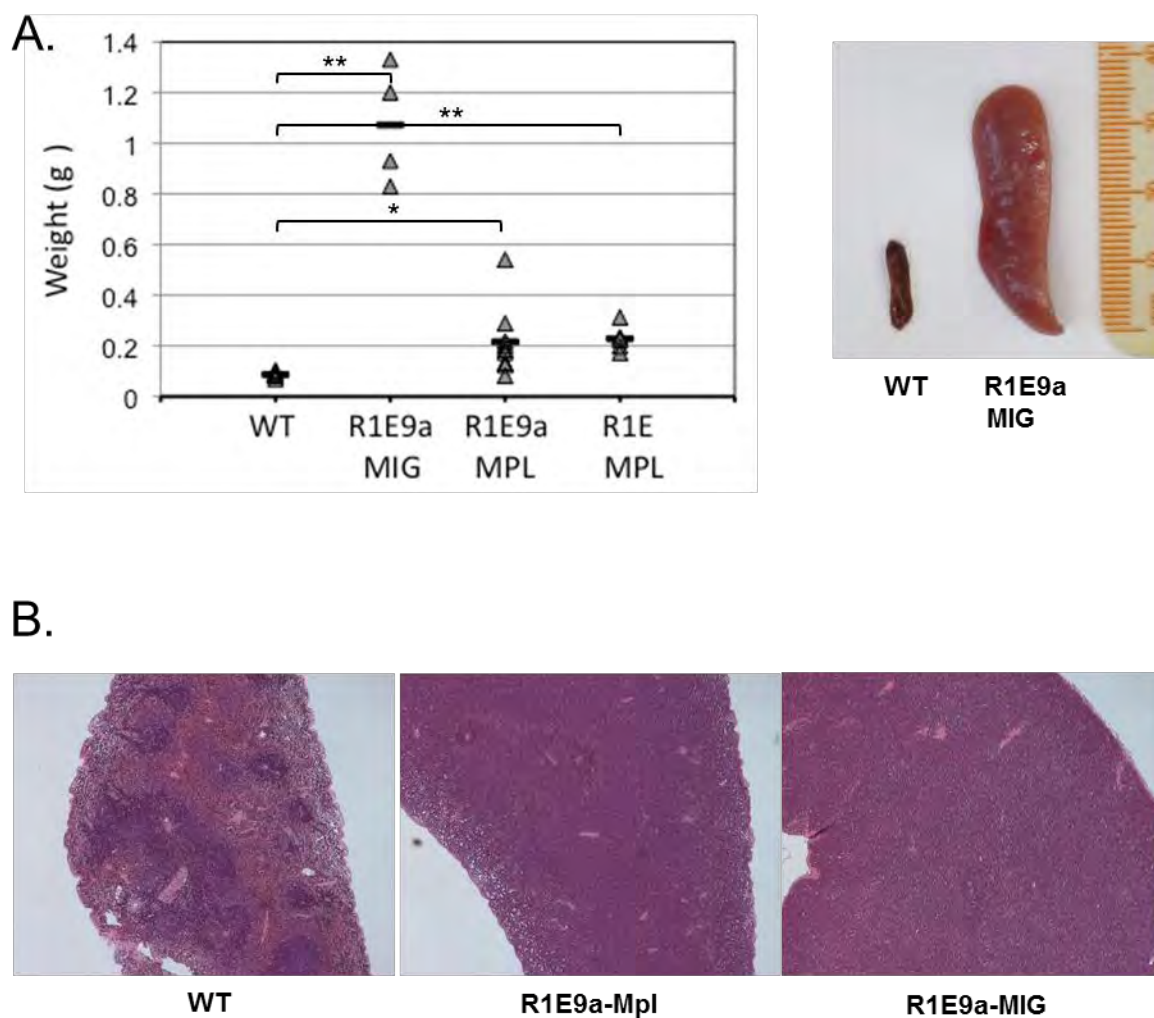
Figure 3.3. Pathology of *RUNX1-ETO/MPL* myeloid leukemia. A. Analysis of infiltration of leukemic cells into the spleen. Left: quantification of spleen weight from wild type mice (n=6), *RUNX1-ETO9a*/MIG AML (n=4), *RUNX1-ETO9a/MPL* (n=7), and *RUNX1-ETO/MPL* (n=5). The Student's t-test was used to determine the statistical differences between various experimental and control groups. p =0.01 (*), or p < 0.002 (**). Right: representative spleens from wild type (WT) and *RUNX1-ETO9a*/MIG (R1E9a/MIG) mice. B. Representative cross section of normal spleen (WT), and leukemic spleens from *RUNX1-ETO9a/MPL* (R1E9a/MPL) and *RUNX1-ETO9a*/MIG (R1E9a/MIG) mice.

C.

D.

R1E-MPL

R1E-MIG
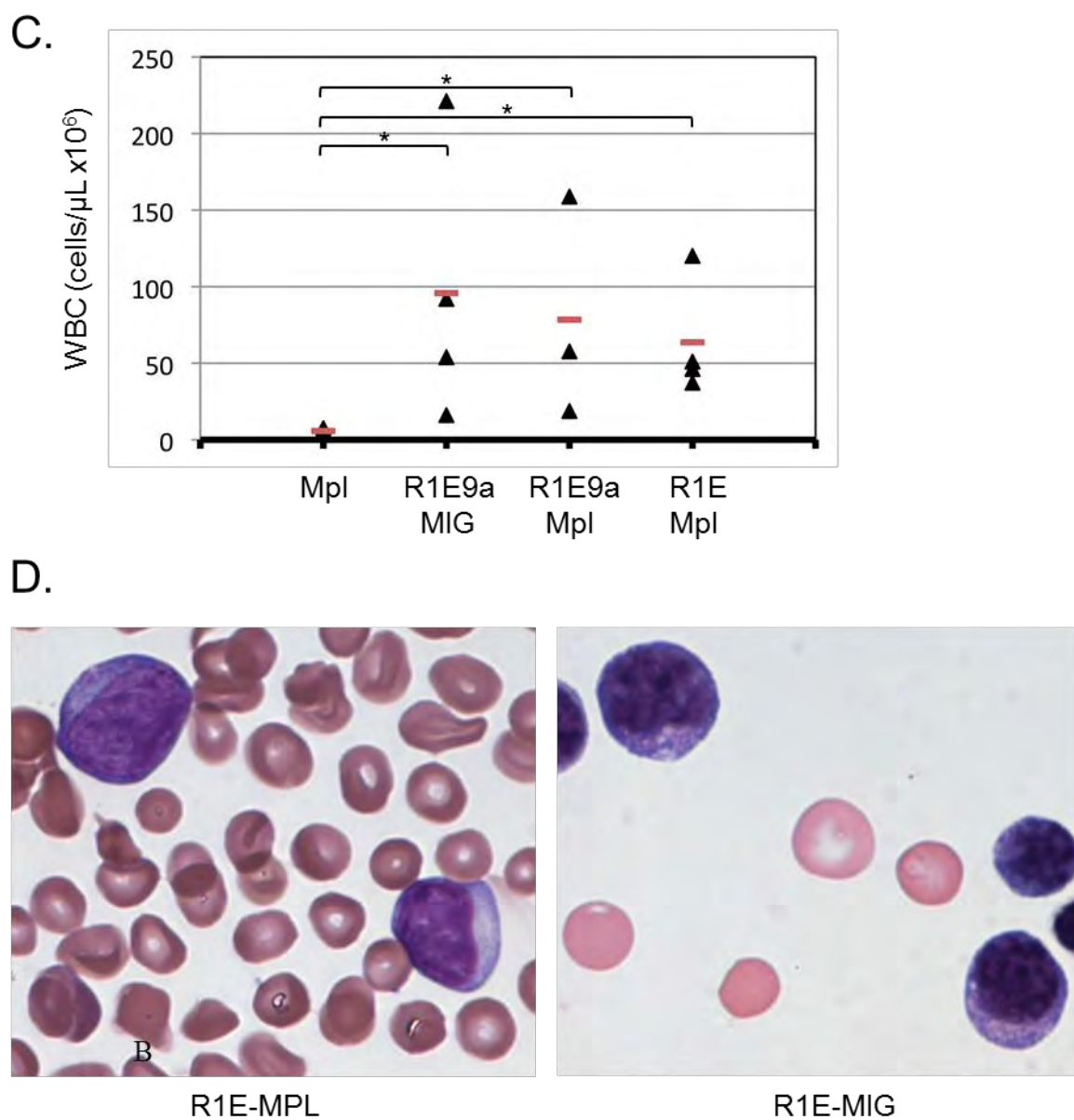
Figure 3.3 (cont.). Pathology of *RUNX1-ETO/MPL* myeloid leukemia. C. Quantification of peripheral blood white blood cells from recipient mice expressing Mpl, R1E9a/MIG, R1E9a/Mpl, and R1E/MPL. Individual values (triangle) and mean (line) are represented. . The Student's t-test was used to determine the statistical differences between various experimental and control groups. p < 0.05 (*). D. Representative immature cells detected in peripheral blood of R1E/Mpl (left) and R1E/MIG) groups.

E.
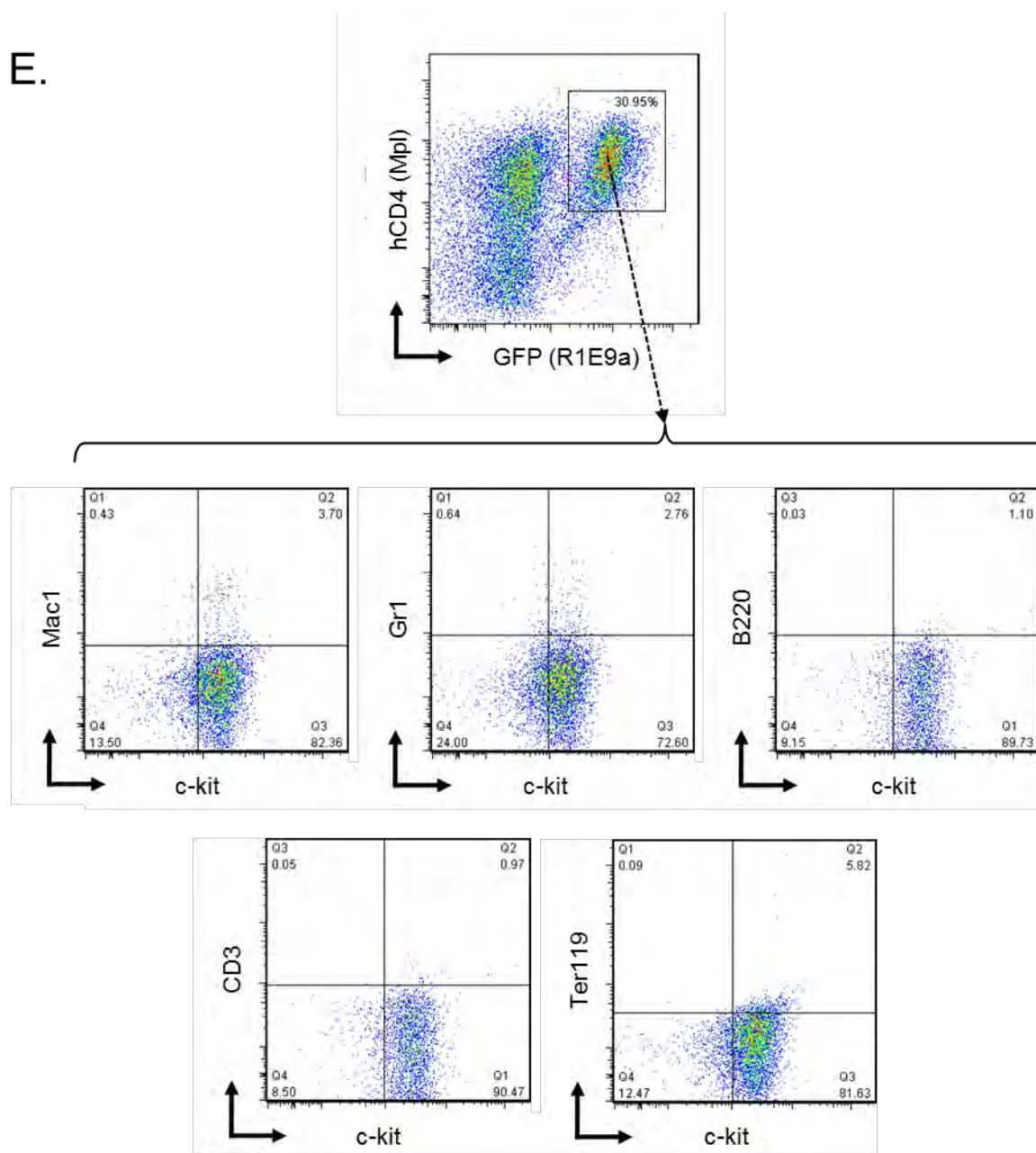
Figure 3.3 (cont.). Pathology of *RUNX1-ETO/MPL* myeloid leukemia. E. Representative flow cytometry analysis of peripheral blood from R1E9a/Mpl leukemic mice. Double positive GFP$^+$hCD4$^+$ are enriched with immature c-kit-positive cells and negative for lineage markers Mac1, Gr1, B220, CD3 and Ter119.

F.
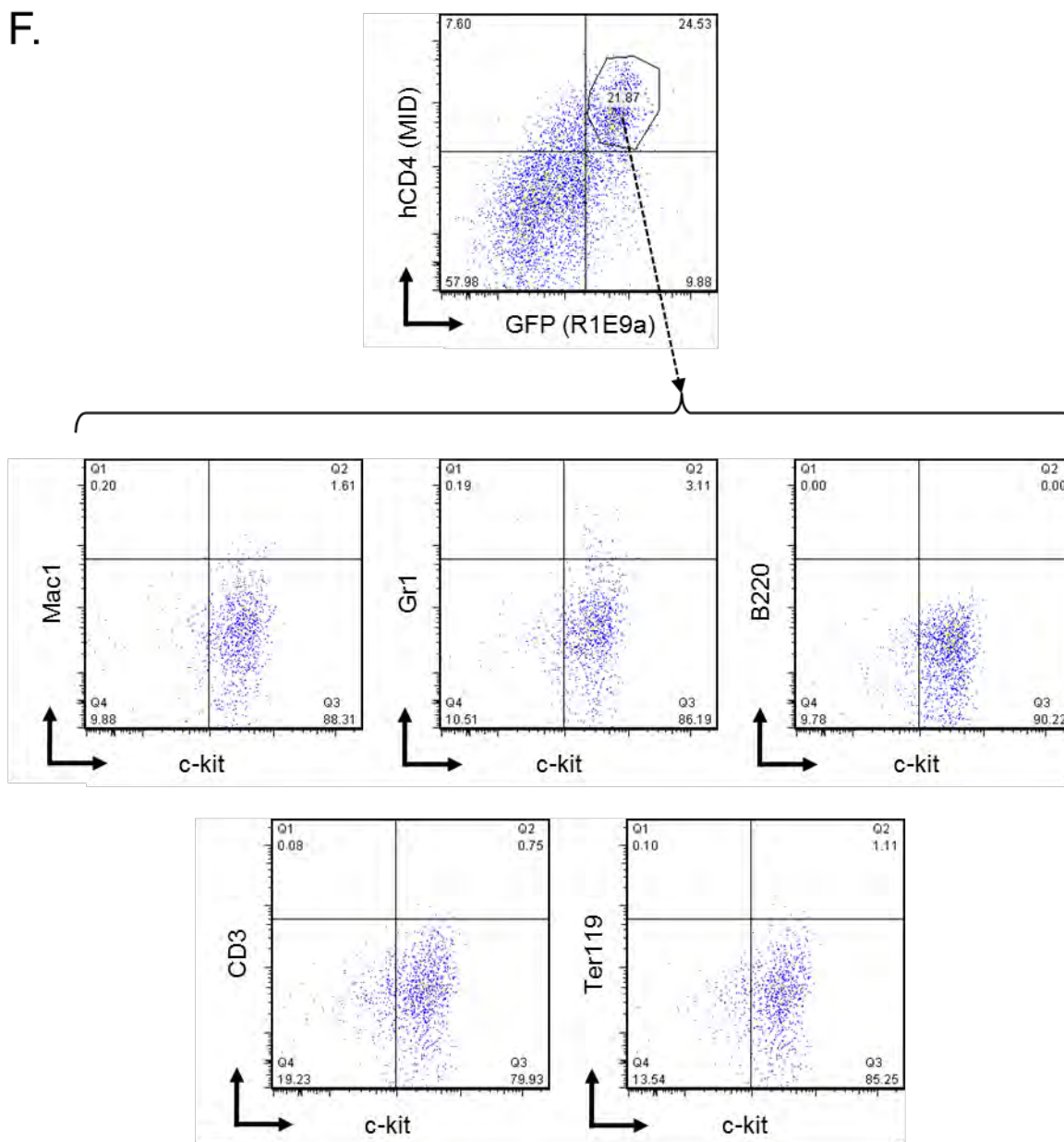
Figure 3.3 (cont.). Pathology of *RUNX1-ETO/MPL* myeloid leukemia. F. Representative flow cytometry analysis of peripheral blood from R1E9a/MID leukemic mice. Double positive GFP+hCD4+ are enriched with immature c-kit-positive cells and negative for lineage markers Mac1, Gr1, B220, CD3 and Ter119.

with mock control (Figure 3.4A, lanes 1 and 2). A similar increase (30- to 500-fold) was

observed in *Cbfb-MYH11* AML cells expressing *PLAGL2* when compared with *Cbfb-*

MYH11 AML cells not expressing *PLAGL2* (Figure 3.4A, lanes 3–10). In addition, the

levels of Mpl receptor in membrane were markedly increased in AML cells expressing

PLAGL2 using flow cytometry (Figure 3.4B).

We have also identified 3 conserved PLAG consensus binding sites in *Mpl*

proximal promoter that determine PLAGL2 binding and *Mpl* regulation (Figure 3.5A).

To test whether PLAGL2 could directly activate *Mpl* expression, we tested PLAGL2

responsiveness of a luciferase reporter carrying the *Mpl* proximal promoter in NIH3T3

cells. PLAGL2 activated the reporter over 30-fold relative to control (Figure 3.5B, lanes

1 and 2). The introduction of previously described point mutations (Voz et al., 2000) in

PLAG-binding sites 1 (m1) and 2 (m2) resulted in a two- and five-fold reduction in

luciferase activity, respectively (Figure 3.5B, columns 3-6), indicating that these sites are

critical for PLAGL2-induced *Mpl* transcription. Conversely, mutation of the third PLAG

site (m3) did not change significantly the PLAGL2 responsiveness, suggesting that this

site may not participate in the *Mpl* promoter activity (Figure 3.5B, columns 7 and 8).

Importantly, mutation of all three PLAG-binding sites totally obliterated PLAGL2

responsiveness of the reporter, confirming that binding of PLAGL2 to the promoter is

necessary for transcriptional activation (Figure 3.5B, columns 9 and 10). To validate

PLAGL2 binding to these sites, we performed electrophoretic mobility shift assays using

oligonucleotides covering each of the three Mpl-binding sites (o) and a combination of

labeled oligonucleotides with cold competitor (c) or mutated competitor (m)
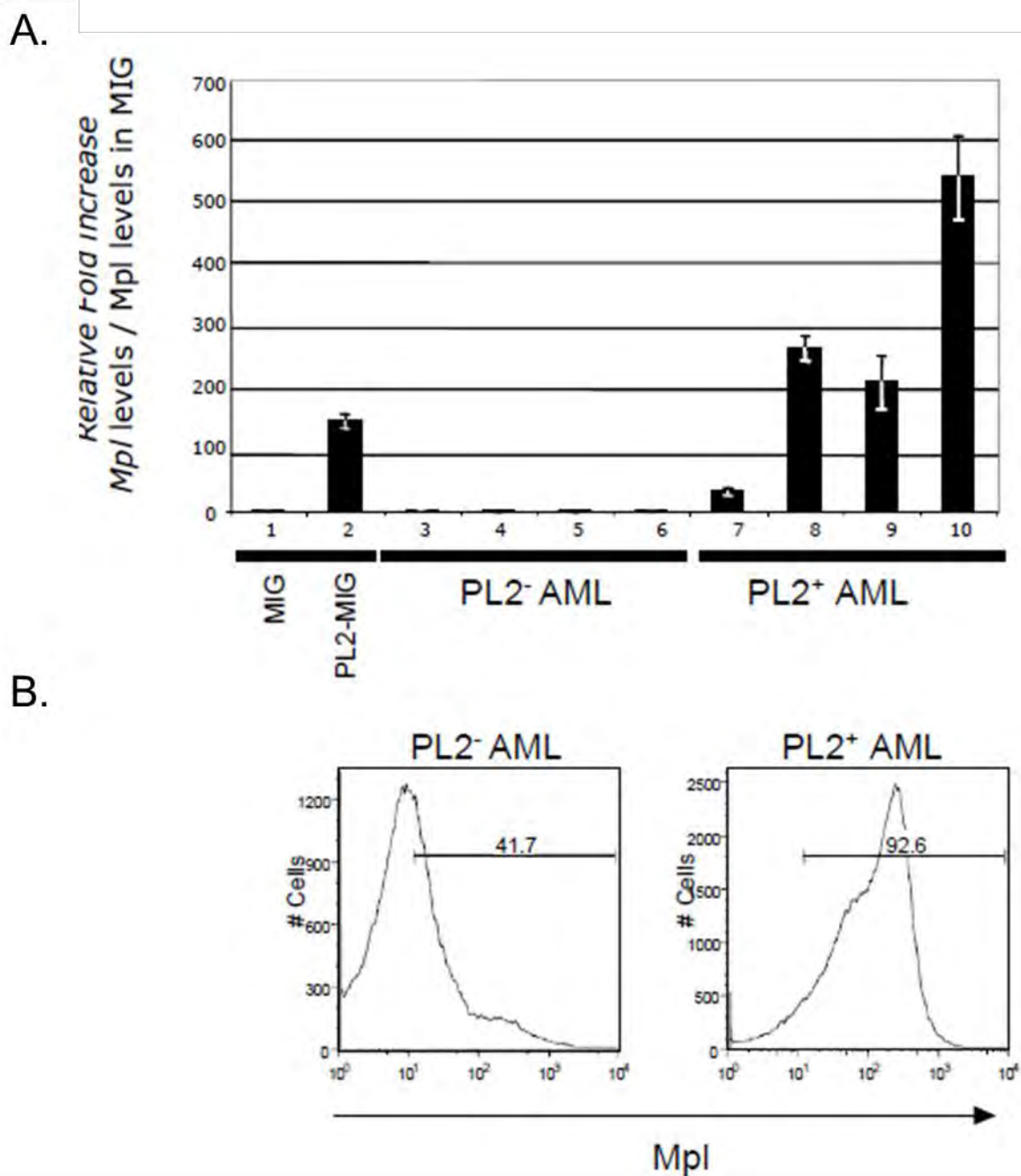
Figure 3.4. Regulation of *Mpl* expression by PLAGL2. A. Relative *Mpl* transcript levels of Cbfb-MYH11^{+} hematopoietic progenitors transduced with MIG (lane 1) or MIG-PLAGL2 (lane 2) retrovirus, and of PLAGL2-negative (PL2^{-}; lanes 3–6) and PLAGL2-positive (PL2^{+}; lanes 7–10) Cbfb-MYH11 AML samples using quantitative reverse transcription-PCR analysis. B. Expression of Mpl receptor in AML cells with non-detectable PLAGL2 expression (left panel) or expressing PLAGL2 (right panel) using fluorescence-activated cell sorting analysis. Percentage of cells expressing Mpl is shown relative to negative control.

A.

B.
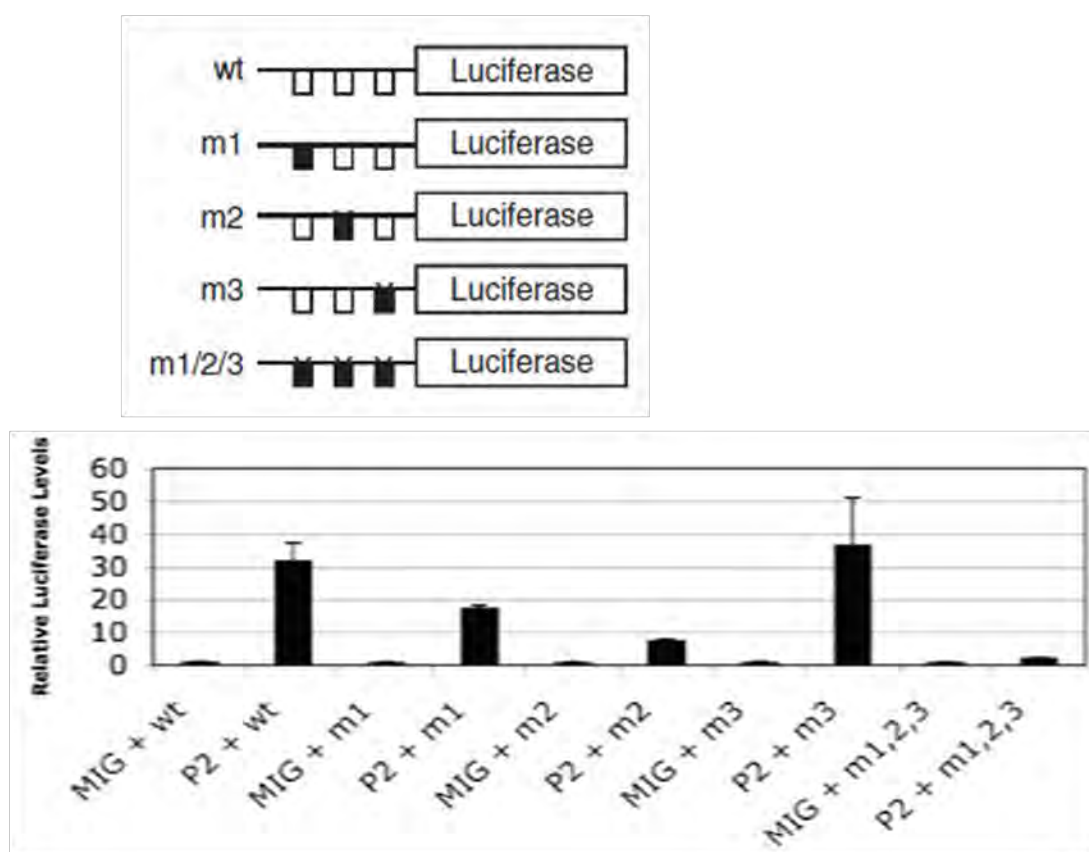
Figure 3.5. PLAGL2 regulates *Mpl* by binding to PLAG-specific binding sites in *Mpl* promoter. A. ClustalW sequence alignment of the *Mpl* proximal promoter (-189 to +3 from translation start site) including (from top to bottom): human, monkeys, mouse, dog, horse and cow. The PLAGL2 consensus binding sites GRGGC$_{(6-8)}$RGGK (P1 and P2) and a third site (P3) with an (N)$_9$ linker are shown in boxes. Arrows indicate orientation of binding site. B. Analysis of PLAGL2 activation of the *Mpl* proximal promoter using luciferase reporters in NIH3T3 cells. The luciferase reporters with 300 bp of the *Mpl* proximal promoter (wt) and respective mutants ablating one or more sites (m1, m2, m3, m1, 2, 3) are illustrated on the top panel. Fold activation relative to wt reporter and no PLAGL2 (MIG + wt) of each construct with (PL2) or without (MIG) PLAGL2 is shown on the bottom panel.

C.
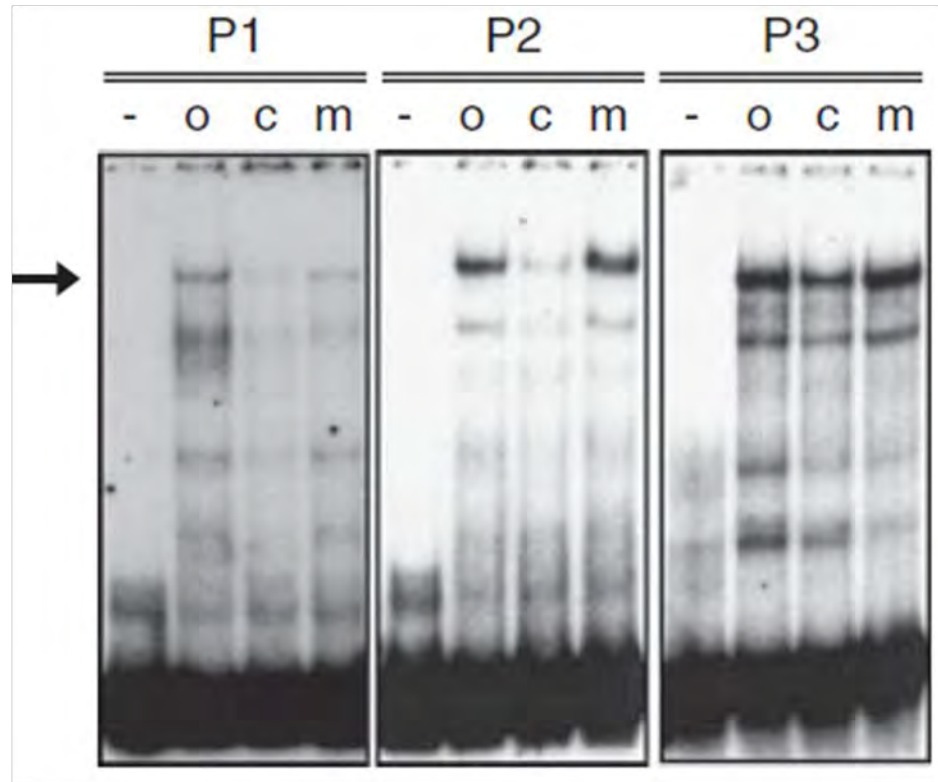
Figure 3.5 (cont.). PLAGL2 regulates *Mpl* by binding to PLAG-specific binding sites in *Mpl* promoter. C. Electrophoretic mobility shift assay of PLAGL2 consensus sites P1, P2 and P3 in the Mpl proximal promoter, using labeled wild-type oligo (o), unlabeled oligo (c) or labeled oligo with point mutation in the core box of the consensus PLAGL2 site (m). Arrow indicates PLAGL2:DNA binding.

oligonucleotides (Figure 3.5C). These assays show that P1 and P2 sites bind to PLAGL2 with specificity, but P3 site binding is poorly competed with cold competitor. These results suggest that PLAGL2 can directly activate the *Mpl* proximal promoter using the P1 and P2-binding sites.

Finally, we showed that *Cbfb-MYH11/PlagL2* AML cells are hypersensitive to thrombopoietin exhibiting hyperphosphorylation of downstream effectors of Mpl signaling pathway (Jak2, Stat5, Erk1/2, Akt) upon stimulation with Thpo, and that *Mpl* also cooperates with *Cbfb-MYH11* in AML development *in vivo* (data not shown (Landrette et al., 2011)).

Overexpression of Mpl-regulating PLAGL2 cooperates with RUNX1-ETO in leukemia

Considering that the retrovirus-induced overexpression of *Mpl* may create unspecific activation of cellular pathways, we indirectly tested the ability of the endogenous *Mpl* upregulation to induce leukemia through retroviral overexpression of the transcription factor PlagL2, an activator of *Mpl* transcript expression in hematopoietic cells (Landrette et al., 2011).

Bone marrow cells co-transduced with MIG-R1E and MID-PlagL2 (MID-PL2) retroviruses were transplanted into isogenic irradiated recipients (Figure 3.6A). Recipient mice carrying RUNX1-ETO/PlagL2 developed leukemia with a median latency of 11 weeks (range: 8 to 17 weeks; n = 12) and full penetrance. In contrast, only 18% (2/11) of RUNX1-ETO/MID transplanted recipients developed leukemia after longer latency
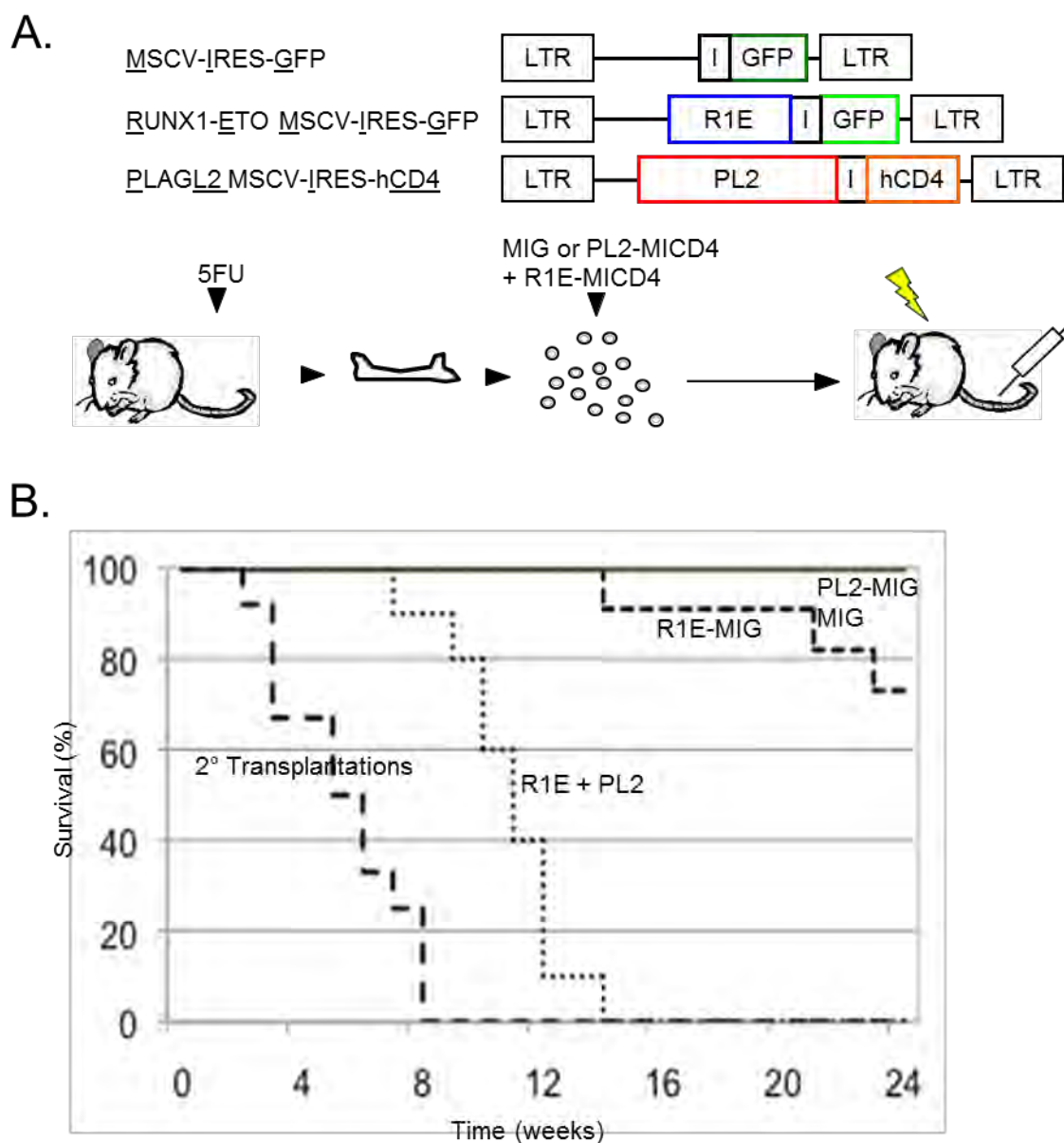
Figure 3.6. PLAGL2 induces AML in cooperation with *RUNX1-ETO* in mice. A. Experimental Design. Top: Schematic representation of retroviral constructs used in the transplantation assay. Bottom: Design of transplantation assay. Bone marrow cells (circles) harvested from wild type mice are co-transduced with retroviruses carrying the *PLAGL2* gene (red) and *RUNX1-ETO* (blue), and transplanted into irradiated recipient mice. B. Kaplan Meyer plot depicting survival curve of mice transduced with *RUNX1-ETO* and *PLAGL2* (dotted line; n=11), *RUNX1-ETO* and MID, (short dash line; n=12), and secondary transplantations of *R1E/PL2* leukemia (long dash line; n=12). Experimental endpoint was 24 weeks. The long-rank test was used to determine the statistical differences between the R1E-MIG and R1E9a + PL2 groups survival, p < 0.0001.

C.
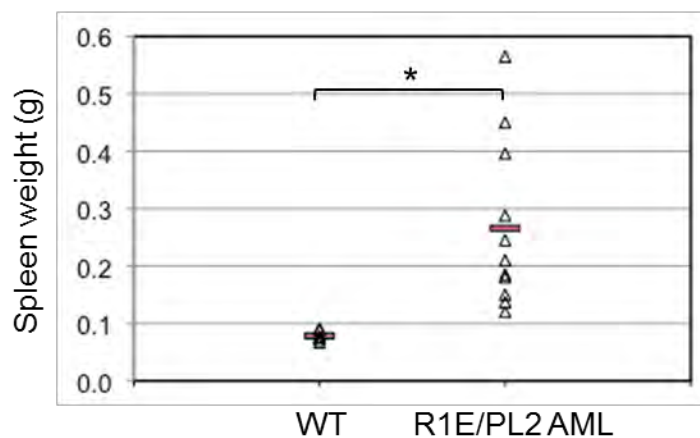
D.
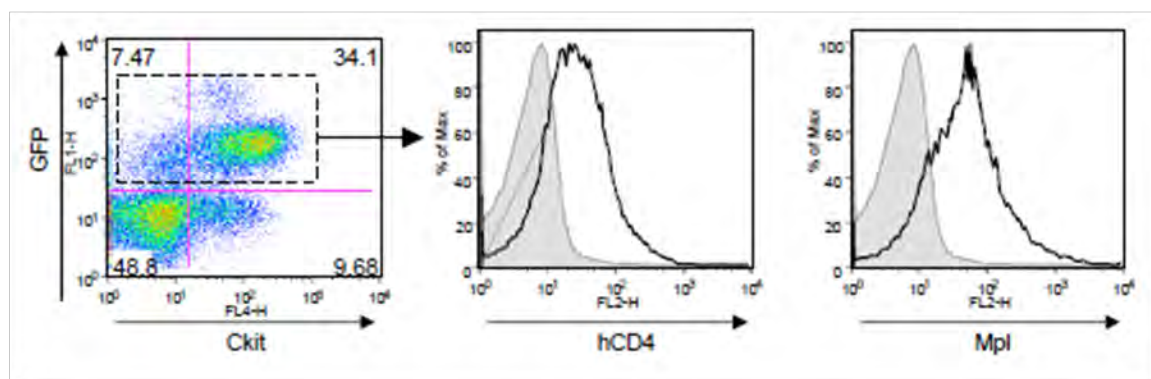
Figure 3.6 (cont.). PLAGL2 induces AML in cooperation with *RUNX1-ETO* in mice. C. Quantification of spleen weight from normal (WT) and *R1E/PL2* leukemic mice. Individual values (triangles) and the mean of group weights (red line) are shown. The Student's t-test was used to determine the statistical differences between WT and R1E/PL2 groups. p = 0.019 (*). D. Flow cytomery analysis showing PLAGL2 (hCD4), Mpl receptor expression in c-kit+GFP+ peripheral blood from *R1E/PL2* leukemia.

E.
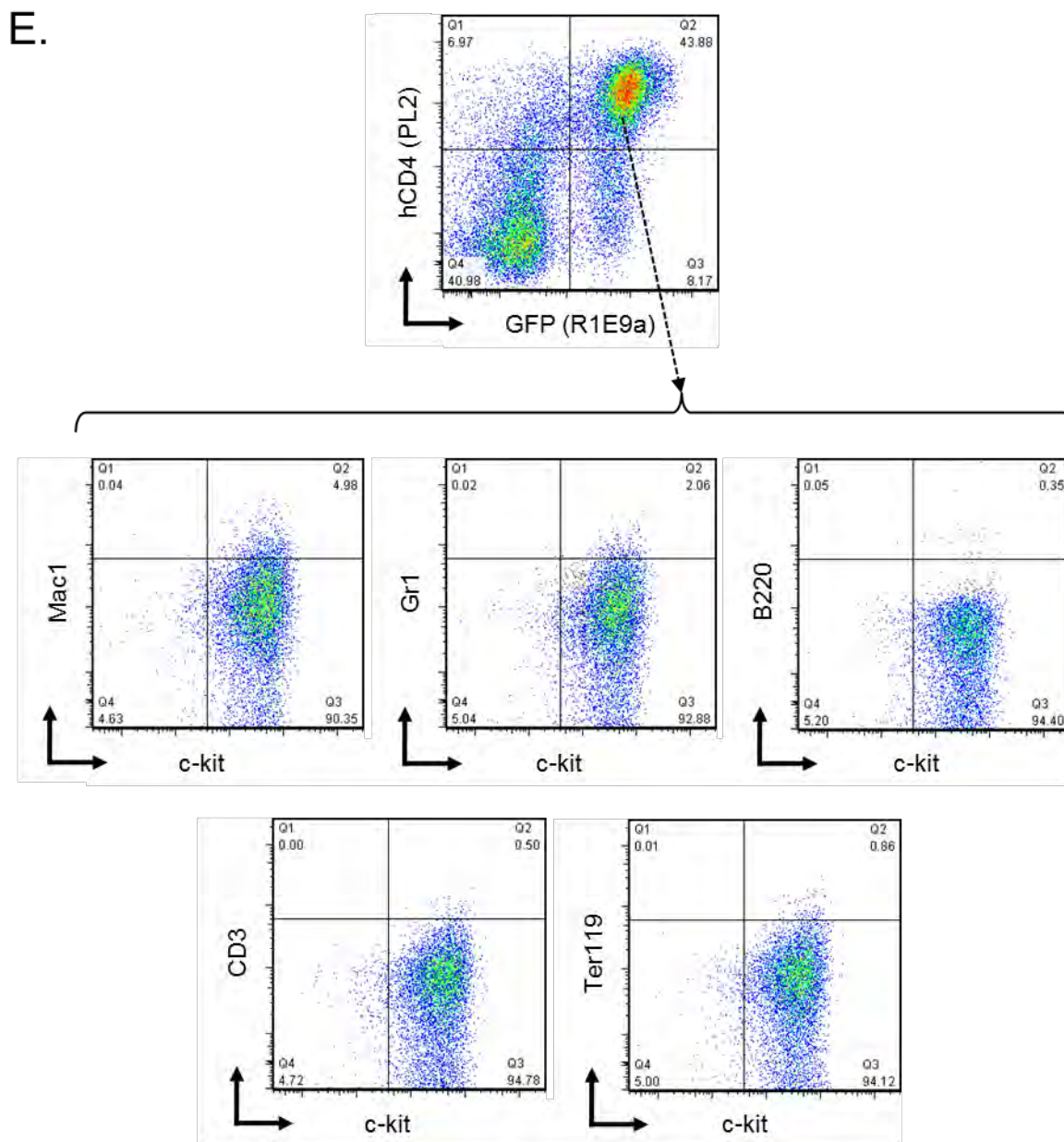
Figure 3.6 (cont.). PLAGL2 induces AML in cooperation with *RUNX1-ETO* in mice. E. Representative flow cytometry analysis of peripheral blood from R1E9a/PL2 leukemic mice. Double positive GFP⁺hCD4⁺ are enriched with immature c-kit-positive cells and negative for lineage markers Mac1, Gr1, B220, CD3 and Ter119.

F.
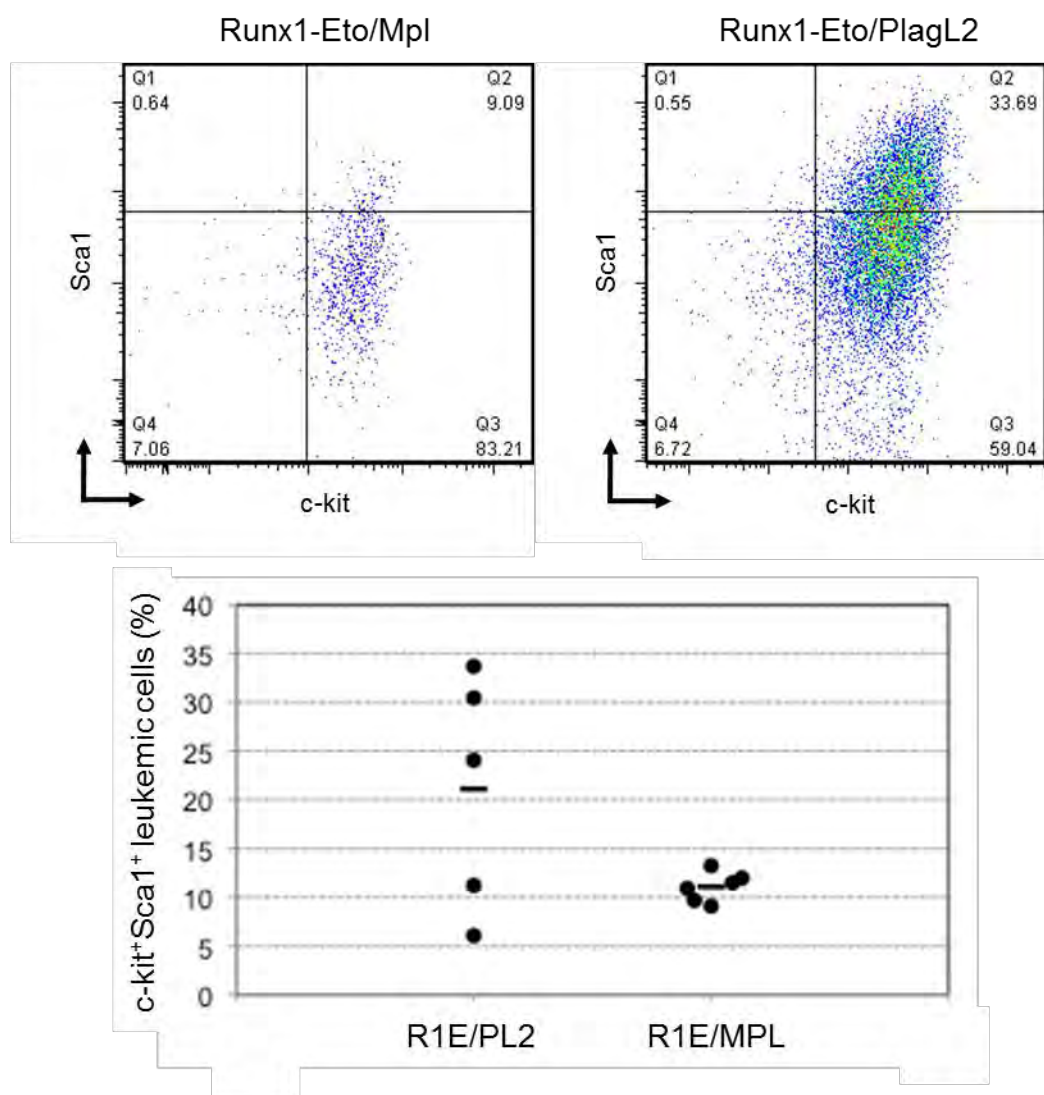
Figure 3.6 (cont.). PLAGL2 induces AML in cooperation with *RUNX1-ETO* in mice. F. Flow cytometry analysis of early progenitor markers c-kit and Sca1 in *R1E/PL2* and *R1E/MPL* leukemic cells. Representative samples (left panels) and quantitative representation (right graph).

(median latency=20 weeks) and incomplete penetrance (Figure 3.6B). Control groups

including MID or PlagL2 alone did not develop leukemia. The phenotype of the leukemia

was similar to that observed in RUNX1-ETO/MPL AMLs. These included efficient

secondary transplantation of RUNX1-ETO/PlagL2 leukemic cells into secondary

transplants (Figure 3.6B, long dash lines) and splenomegaly (Figure 3.6C). The leukemic

cells in peripheral blood were GFP$^+$hCD4$^+$, consistent with expression of *RUNX1-ETO*

and *PlagL2*, and expressed the Mpl receptor in the membrane (Figure 3.6D). The

immunophenotype of the leukemic cells was c-kit$^+$Lin$^-$, although a fraction of samples

showed Gr1^{low}Mac1^{low} markers (Figure 3.6E). In addition, a fraction of leukemic cells in

both RUNX1-ETO/MPL and RUNX1-ETO/PlagL2 groups presented c-kit$^+$Sca1$^+$

markers, a signature of early progenitor blasts (Figure 3.6F). Together, these results show

that *Mpl* expression induces a transient erythroid progenitor expansion, but that in the

presence of *RUNX1-ETO* is an oncogenic signal that induces acute myeloid leukemia.

Mpl expression is not regulated by RUNX1-ETO in mouse progenitor and leukemic cells

Considering that *Mpl* expression is frequently increased in t(8;21) AML samples and that

Mpl has been reported as a direct CBF target in hematopoietic stem cells and

megakaryocytes (Heller et al., 2005; Satoh et al., 2008b), it is possible that RUNX1-ETO

could directly upregulate *Mpl* transcript expression. To test this hypothesis we measured

Mpl expression by quantitative reverse transcriptase PCR (qRT-PCR) in bone marrow

cells transduced with MIG, MIG-R1E, or MID-PL2 retroviruses, and sorted for GFP or

A.
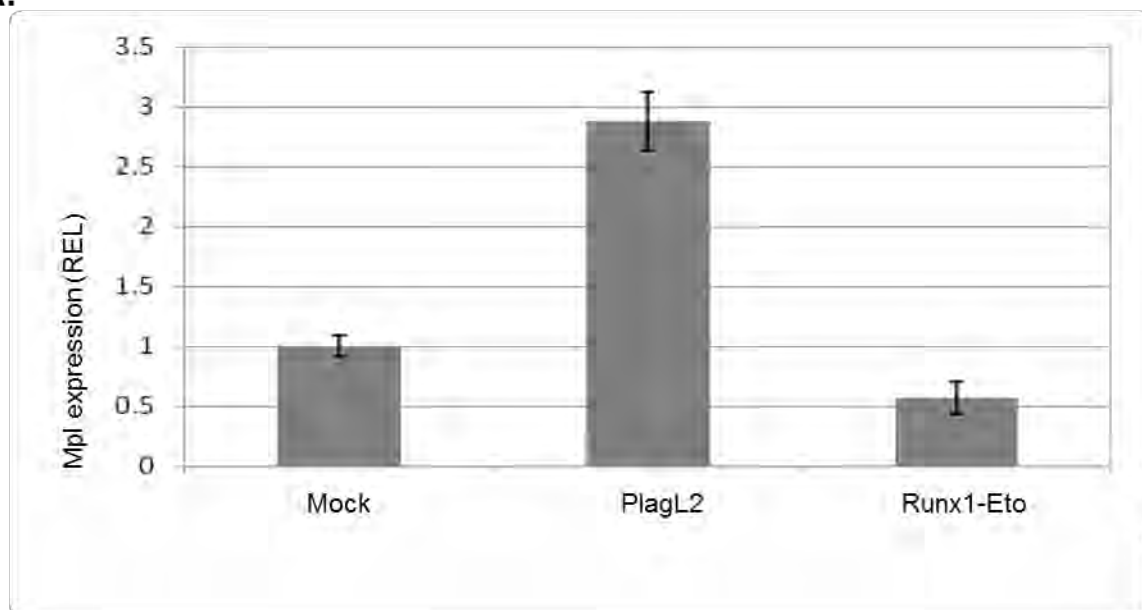
B.
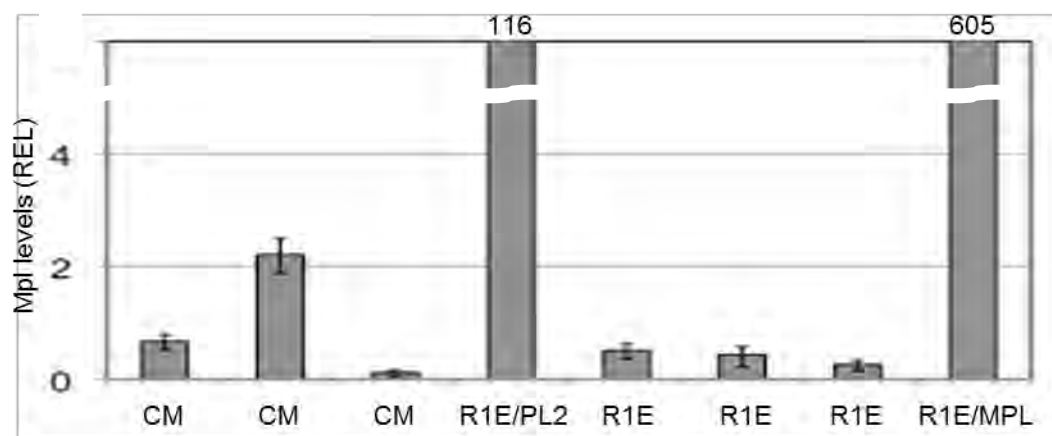
Figure 3.7. The levels of *Mpl* transcript are not affected by RUNX1-ETO expression. Relative expression levels of *Mpl* transcript using quantitative RT-PCR of A. cDNA from bone marrow progenitors transduced with MIG vector, MID-PlagL2 (PL2), or MIG-R1E (R1E), and normalized to MIG sample; and B. leukemic cells carrying CBFB-MYH11 fusion gene (CM), R1E/PL2, R1E/MID (R1E), or R1E/MPL, normalized to average of CM samples. Experiments were performed in triplicate.

hCD4 markers. The *Mpl* levels were similar between MIG and RUNX1-ETO groups, and increased 3 fold in bone marrow cells transduced with PLAGL2 (Figure 3.7A). The expression of *Mpl* was also tested on AML cells expressing RUNX1-ETO/MIG, RUNX1-ETO/PLAGL2 and RUNX1-ETO/MPL, and compared to AML cells carrying the *CBFB-MYH11* fusion gene (CM). The *Mpl* levels in RUNX1-ETO AML cells were comparable to controls, and increased markedly in AML cells expressing PLAGL2 or Mpl (Figure 3.7B). Together, these results suggest that *Mpl* levels are not induced by RUNX1-ETO in hematopoietic progenitors and leukemic cells.

THPO/MPL signaling activates survival pathway in leukemic cells expressing Mpl

The activation of THPO/MPL signaling in hematopoietic progenitors includes the phosphorylation of JAK2 and antiapoptotic and proliferative response via activation of PI3K/AKT, JAK2/STAT5, and MAPK/ERK pathways (Kaushansky, 2009b). In order to determine the downstream signal activation by Mpl expressing leukemic cells we tested phosphorylation levels of Jak2, Stat3/5, Akt1, and Erk1/2 in RUNX1-ETO/PlagL2 (expressing endogenous Mpl) and control RUNX1-ETO/MIG leukemic cells. The RUNX1-ETO/PlagL2 leukemic cells showed THPO hypersensitivity as phosphorylation of Jak2, Stat5, ERK1/2 and Akt1 was detected at low THPO concentrations (5 ng/mL THPO), and saturated at 25 ng/mL (Figure 3.8A). These results suggest that Mpl-expressing leukemic cells may expand by a hypersensitive signaling downstream of Mpl.

If Mpl expression elicits survival and proliferative response in RUNX1-ETO

leukemic cells, their expansion and cell-death in culture with THPO as single cytokine should be THPO dependent. The RUNX1-ETO/MPL leukemic cells were cultured for 48 hours in serum-free medium to test the effect of THPO addition in cell number and apoptosis. The levels of apoptosis and proliferation in AML cells expressing RUNX1-ETO but not MPL were unaltered in presence of THPO, but in RUNX1-ETO/MPL leukemic cells the apoptosis was increased by 20% in the absence of ligand, and cell number reduced by 80% (Figure 3.8B, C). Cell cycle analysis failed to reveal a significant change in proliferation rate (data not shown). To identify the mechanism underlying the THPO/Mpl mediated survival advantage in RUNX1-ETO/MPL leukemic cells, the apoptosis assay was repeated using cells preincubated with the PI3K-inhibitor LY294002 or the MEK-inhibitor PD98059. The group treated with LY294002 significantly blocked THPO survival signal while those with PD98059 remained sensitive to THPO signaling (Figure 3.8B, C), thus indicating that PI3K and not MEK function in the survival signaling.

Since RUNX1-ETO/MPL cells showed functional PI3K signaling and a hypersensitive Akt/mTor pathway, the role of the Akt/mTor pathway was tested *in vivo* by analyzing the engraftment efficiency of the leukemic cells using the mTor inhibitor rapamycin. Recipient mice were transplanted with 1×10^5 leukemic cells and administered with daily injections of rapamycin or vehicle. Mice treated with vehicle succumbed with AML with a median latency of 30 days (range 28 to 38 days), while rapamycin-treated mice showed significant delay in disease latency (P < 0.0001, long-rank test) and incomplete penetrance (Figure 3.8D). These results suggest that the hyperactive

A.
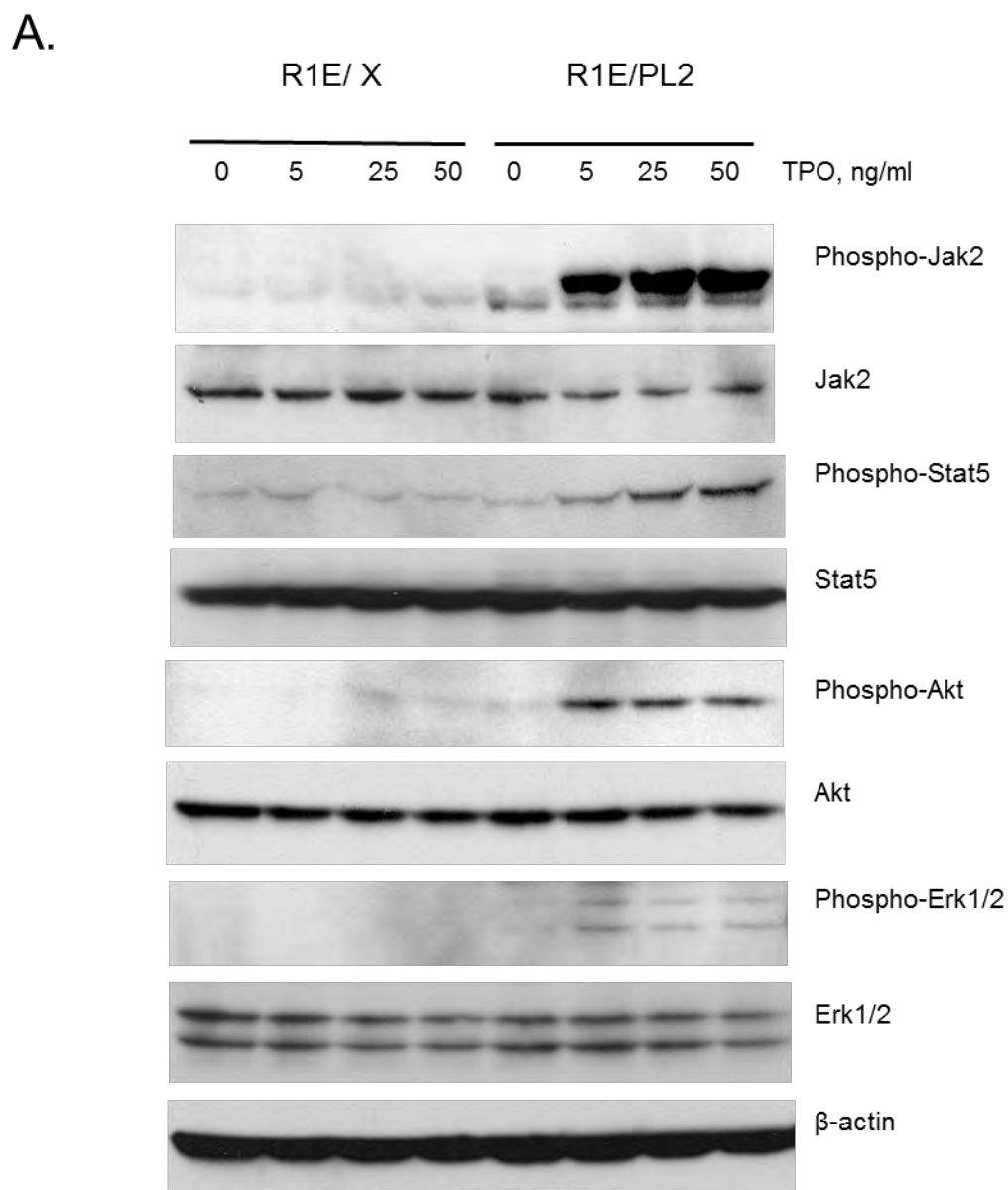
Figure 3.8. AML cells expressing RUNX1-ETO and Mpl are hypersensitive to THPO signal. A.
Western blot of signaling proteins activated by Mpl. RUNX1-ETO positive AML cells expressing
PLAGL2 (R1E/PL2) or not (R1E/X) were stimulated with 0, 5, 25, or 50 ng/mL THPO after serum
starvation. Expression of phospho-Jak2, Jak2, phospho-Stat5, Stat5, phospho-Akt1, Akt1, phospo-Erk1/2
and Erk1/2 was tested by immunoblot analysis

B.

C.
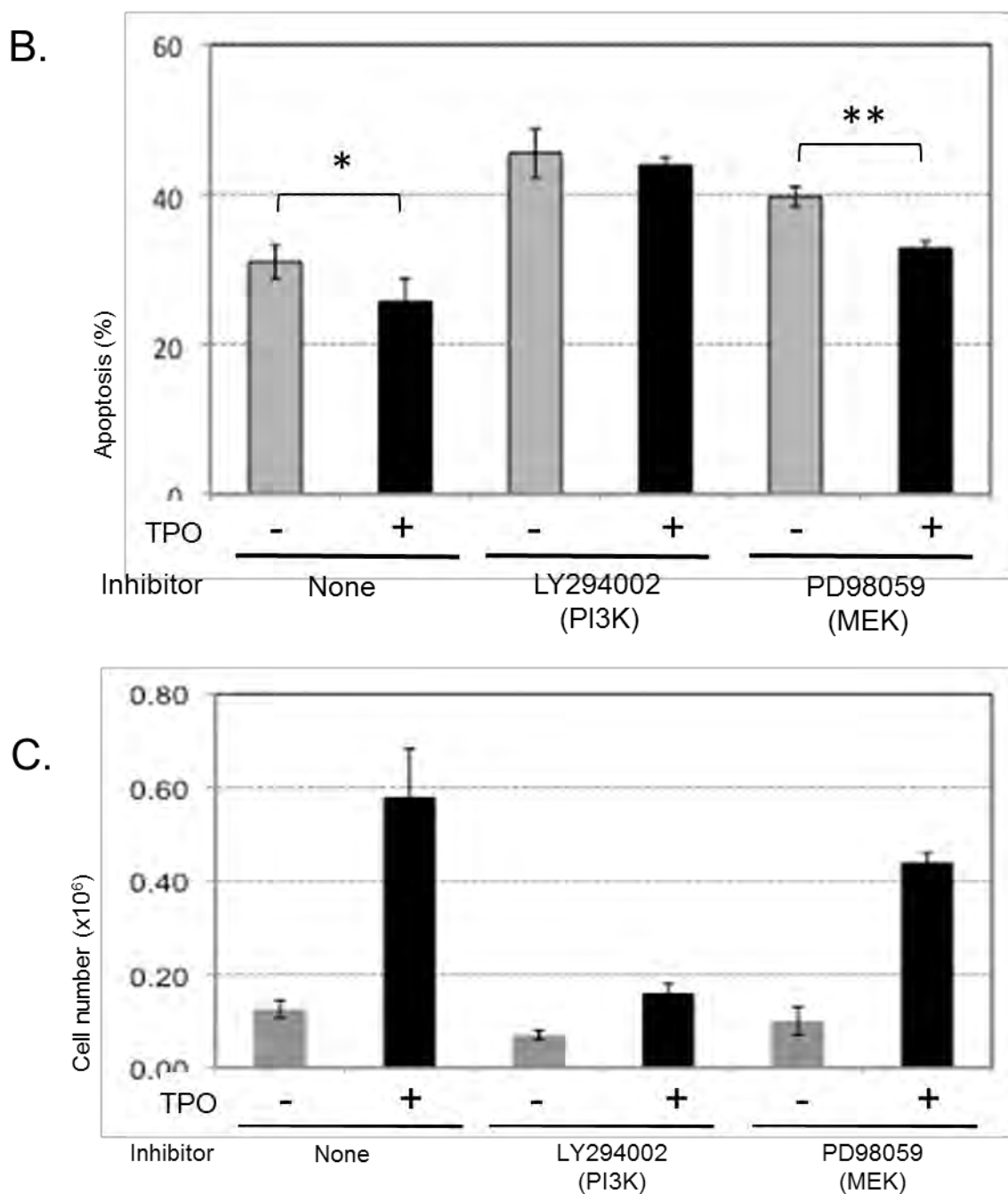
Figure 3.8 (cont.) AML cells expressing RUNX1-ETO and Mpl are hypersensitive to THPO signal. (B) Apoptosis analysis and © estimation of cell number of R1E/MPL leukemic cells estimated after 48 hours of culture in presence of PBS (gray) or THPO (black), with pretreatment of no inhibitor (none) or inhibitors for PI3K and MEK. Experiments were performed in quadruplicate. The Student's t-test was used to determine the statistical differences between various experimental and control groups. p =0.001 (*), or p < 0.0001 (**).

D.
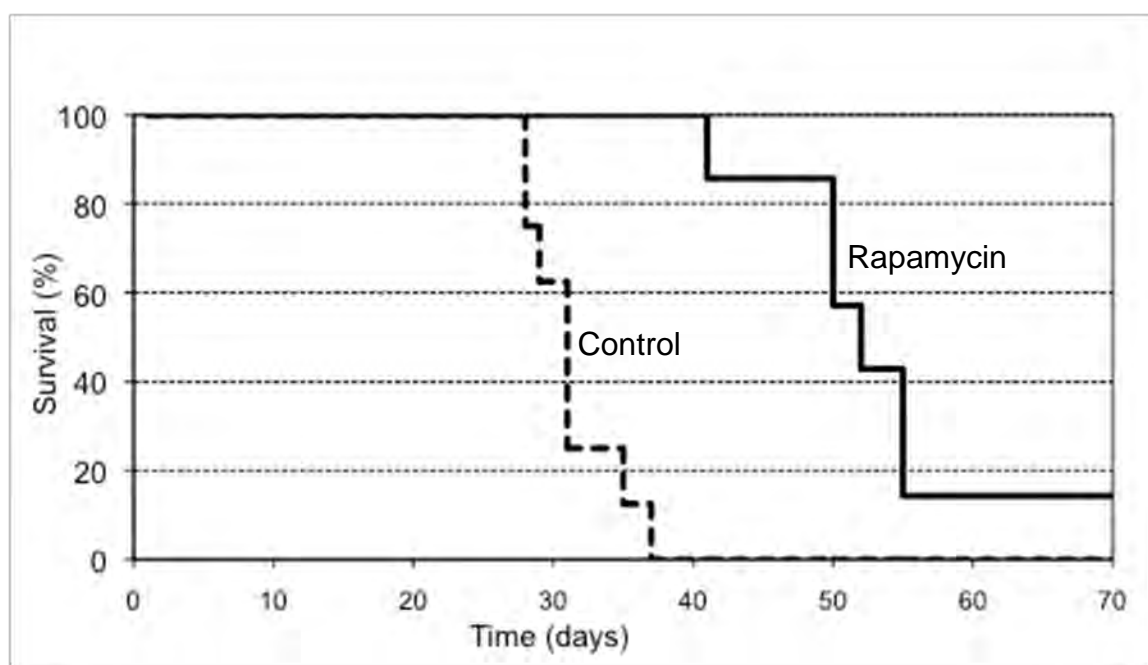
Figure 3.8 (cont.). AML cells expressing RUNX1-ETO and Mpl are hypersensitive to THPO signal.
D. Kaplan Meier survival curve of transplantations of R1E/PL2 AML cells in mice injected with mTOR inhibitor rapamycin (solid line, n=8) as compared to vehicle injected controls (dashed line, n=8). The long-rank test was used to determine the statistical differences between the treated and untreated groups survival, p < 0.0001.

Akt1/mTor signaling is an important component on the MPL-mediated survival of AML cells.

Discussion

The regulation of cytokine signaling moderates proliferation, differentiation and survival of hematopoietic stem and progenitor cells. Components of these pathways are prime targets of mutations in leukemia initiation, compromising their proliferative and survival functions (Scholl et al., 2008b). Patients with AML frequently present mutations in genes encoding components of the receptor tyrosine kinase pathways. In this study we show that increased expression of the wild type thrombopoetin receptor MPL acts as an oncogenic mutation, activating proliferative and survival signals. These signals cooperate with the differentiation block caused by the fusion gene *RUNX1-ETO* to induce leukemia.

The expression analysis of a large panel of AMLs in our study shows that MPL expression is present in a fraction of samples across cytogenetic groups as previously reported (Kaushansky, 1998; Matsumura et al., 1995). However, we find that most t(8;21) AMLs express moderate to high Mpl levels and proliferate in response to THPO ligand. Importantly, this bias was also observed when comparing leukemic cells of similar morphology within the M2 FAB subtype. The unexpected finding that Mpl transcript levels were unchanged in mouse hematopoietic progenitor and leukemic cells expressing RUNX1-ETO, strongly suggests that Mpl expression is not a direct target of the fusion protein in mouse bone marrow hematopoietic progenitors. Alternatively, it is

likely that MPL and RUNX1-ETO function may effectively synergize to induce

leukemia. The RUNX1-ETO preleukemic cells accumulate in the bone marrow as

myeloid progenitors unable to differentiate and with increased self-renewal function

(Yuan et al., 2001b), and accumulate additional mutations that transforms them into full

blown leukemia. These leukemia progenitors may be selected for increased MPL levels

by their response to THPO, therefore promoting leukemia transformation.

The MPL gene can present oncogenic mutations or be expressed at higher levels

in myeloproliferative disorders (Kaushansky, 2003), and abnormal MPL function can

induce expansion of erythroid progenitors. Overexpression of MPL in mouse

hematopoietic progenitors promotes proliferation of erythroid progenitors (Yan et al.,

1999). Consistent with these observations, we find a significant and transient erythroid

expansion in mice transplanted with cells overexpressing MPL. This expansion, however,

was not observed when MPL was co-transduced with RUNX1-ETO. Instead, cells

remained in bone marrow and emerged as leukemic cells in both mouse models

(RUNX1-ETO/MPL and RUNX1-ETO/PLAGL2), suggesting that the combined

expression preempts the MPD phenotype and induces leukemia.

The t(8;21) AML cells co-express the full-length RUNX1-ETO and the

alternative spliced (and more aggressive) RUNX1-ETO9a isoform (Yan et al., 2006a).

We find that both isoforms efficiently induce leukemia in cooperation with MPL. Further,

the pathology of leukemia is similar in retrovirally expressed *Mpl* and PLAGL2-activated

Mpl, indicating that constitutive Mpl expression at moderate levels is sufficient for

leukemia development. In the simplistic but valid model of a multistep pathogenesis of

AML, these "class-I" mutations provide proliferative and survival advantage and cooperate with "class II" mutations, such as *RUNX1-ETO,* which inhibit differentiation and apoptosis (Kelly and Gilliland, 2002b). Our results implicate MPL expression as a class-I oncogenic signal able to initiate leukemia in mice and expand murine and human leukemic cells.

Activation of MPL elicits proliferative and pro-survival functions through activation of PI3K/AKT/mTOR/FOXO, RAS/MAPK/MEK/ERK, and STAT5/Bcl-xL cascades (Kaushansky, 2009b). The MPL receptor lacks intrinsic tyrosine kinase activity, contrary to other members of the cytokine receptor superfamily. Activation of Jak2 is critical for Mpl phosphorylation and activation. The phosphorylation of Jak2 was rapidly increased upon incubation with low THPO concentrations. Furthermore, RUNX1-ETO/Mpl leukemic cells showed a hypersensitivity to THPO on the phosphorylation of AKT1, ERK1/2, and STAT5 factors. The inhibition of PI3K pathway (but not MEK/ERK) abrogated the THPO mediated survival, and suggests that the PI3K/AKT/mTOR pathway plays a critical function in RUNX1-ETO/MPL leukemia. Furthermore, this finding was confirmed *in vivo* as treatment with the mTor inhibitor rapamycin significantly delayed leukemia development in secondary transplants. In addition to PI3KAKT/mTor, the THPO/MPL survival response probably results from interaction between downstream effectors of other pathways.

Considering that the RUNX1-ETO/MPL blasts seem to be "addicted" to TPO ligand for proliferation and survival in human and mouse leukemias, inhibitors of PI3K/AKT may hold promising therapeutic value if able to target the leukemia initiating

cells (L-ICs). The challenge will likely reside on identifying drugs that show high efficacy inducing apoptosis of L-ICs rather than reducing the other cells of the leukemic cell population. These may reside in the stem cell-like (lin⁻c-kit⁺Sca1⁺) compartment found to be expanded in RUNX1-ETO AMLs (de Guzman et al., 2002b; Peterson et al., 2007), including the RUNX1-ETO/MPL and RUNX1-ETO/PLAGL2.

Mutations in MPL are frequently found in several hematopoietic malignancies, but rarely in AML. Our studies show that expression of wild type MPL creates a hypersensitive signaling pathway, and support the idea that the hypersensitive MPL signaling causes an oncogenic response when co-expressed with a cooperating mutation such as RUNX1-ETO. Although this study focused on the role of MPL in AML cells carrying the t(8;21) translocation, it is reasonable to assume that the oncogenic function of upregulated MPL on initiation and maintenance of leukemia can play a role in other AML subtypes. Therefore, the therapeutic value of targeting MPL-mediated survival signals could be extended to other AML cases expressing MPL.

CHAPTER IV

Discussion

In Chapters II and III I discussed specific results that were obtained by us and compared the novel data with what was previously known. In this Chapter, I will discuss the results from a more global perspective, with focus on their relevance in frame with the general field of AML. I will also explore immediate future questions that are raised by these studies.

Cooperation of *miR-17-92* miRNA cluster with *Cbfb-MYH11* in AML development

MiR-17-92 in leukemia

The study of miRNA expression profiling in large panels of human AML samples provide a start-point for the identification of candidate miRNAs with roles in leukemia development, and as markers of AML subtypes (Jongen-Lavrencic et al., 2008; Li et al., 2008). For example, the *miR-126/126** was found to be upregulated in CBF leukemias with t(8;21) and inv(16), when compared to other AML samples, and was shown to promote proliferation and inhibit apoptosis in the AML cells *in vitro* by targeting a tumor suppressor gene Polo-like kinase 2 (PLK2) (Li et al., 2008). The *miR-126/126** was

shown to regulate vascular formation by targeting Spred1 and PIK3R (Zou et al., 2011), and megakaryocytic differentiation by targeting Myb in zebrafish (Grabher et al., 2011). In addition, *miR-126* was recently reported to participate in gastric carcinogenesis by repressing SRY-box 2 (SOX2) (Otsubo et al., 2011). Its functional role in CBF AML has not been studied, but from the abovementioned studies it would be expected that *miR-126* may promote proliferation of leukemic cells through activation of AKT and ERK pathways.

The *miR-29* was not shown to directly cooperate with CBF fusion genes either, but its role in a regulatory feedback loop with c-Kit RTK in c-KIT$^+$ AMLs indicated a strong possibility that *miR-29* may be playing a role in CBF AMLs with *c-KIT* oncogenic mutations. Treatment of *c-KIT*-transfected FDCP-1 cells with inhibitors of the feedback loop effectors (Sp1/NFκB complex) and overexpression of *miR-29 in vitro* resulted in decreased proliferation and clonogenic activity of the cells. Moreover, inhibiting the feedback loop in a xenograft mouse model of c-KIT$^+$ leukemia increased latency of the disease, further suggesting its role in c-Kit$^+$ AML (Liu et al., 2010a). The *miR-29* family may play a specific role in CBF AMLs because the CBF fusion proteins suppress expression of C/EBPα, which is an activator of *miR-29a/b* expression (Eyholzer et al., 2010). Suppression of the *miR-29a/b* in the AML cells could result in increased c-KIT expression and signaling. Indeed, according to a study of Lück et al., more than 10% of human CBF AMLs without mutations in *KIT* or its known regulators, have a gene expression profile similar to that of CBF AMLs that carry c-KIT activating mutations (Lück et al., 2010). The loss-of-function of *miR-29* could potentially explain this result,

so a study of *miR-29* expression in CBF AMLs with wild type KIT may reveal a role of *miR-29* in CBF AML.

Another example of potential miRNA involvement in CBF AML is the function of *miR-221/222*, which are shown to downregulate c-KIT expression in erythropoiesis (Felli et al., 2005). They are also shown to be direct targets of RUNX1 fusion proteins and to be downregulated in human CBF leukemias with high c-KIT expression, probably due to direct repression with the CBF fusions. However, cooperation between oncogenic CBF fusion proteins and loss of *miR-221/222* in AML development was not tested (Brioschi et al., 2010).

To date, there are no reports that would implicate *miR-17-92* cluster in CBF leukemia, although it is shown to be involved in pathogenesis of several other leukemias. Specifically, *miR-17-92* is consistently overexpressed in MLL-associated AMLs (Jongen-Lavrencic et al., 2008; Li et al., 2008; Wong et al., 2010), when compared to other AMLs. Moreover, MLL fusions have high affinity to *miR-17-92* promoter and directly upregulate *miR-17-92* expression (Mi et al., 2010). Role of *miR-19* and the whole cluster in B- and T-cell lympocytic leukemias is also well documented (He et al., 2005; Inomata et al., 2009; Lenz et al., 2008; Mavrakis et al., 2010; Mu et al., 2009; Olive et al., 2009; Wang et al., 2006).

Our study for the first time links *miR-17-92* with *Cbfb-MYH11*-associated AML, defining an example of a miRNA involved in CBF AML pathogenesis, and introducing *miR-17-92* as a player in CBF leukemias. Importantly, this is the first study that proves direct cooperation between a miRNA and a CBF fusion protein *in vivo*, unlike previous

reports that contained only correlative data. Also our study, combined with data from other groups, indicates that *miR-17-92* is a pan-leukemic oncogene that cooperates with variety of oncogenes in all types of leukemias derived from different progenitor compartments (lymphoid, myeloid, mixed lineages and erythroid).

Role of individual miRNAs in Cbfb-MYH11-related AML

It has been shown previously that *miR-17-92* can provide an advantage to cancer cells by increasing survival and/or proliferation (Chow et al., 2010; He et al., 2005; Hong et al., 2010; Matsubara et al., 2007; Mu et al., 2009; Olive et al., 2009; Takakura et al., 2008; Yu et al., 2010b). Here we found a pro-survival effect in leukemic cells dependent on *miR-17* and *miR-20a* function. To date, the establishment of a molecular mechanism of this effect in *Cbfb-MYH11* AML is evolving, and our studies point at *miR-17* family and not at *miR-19* family as CBF oncomiRs. This is contrary to the findings reported in B-cell lymphoma model that identified Pten as the main target of *miR-19* that was involved in tumorigenicity and the anti-apoptotic effect (Olive et al., 2009).

The *miR-17* and *miR-19* families of miRNAs are co-expressed from the same clusters (*miR-17-92* and *miR-106a-363*), and have been shown to regulate expression of both common and specific targets. Probably, the best known common target is the tumor suppressor PTEN, and over 185 other putative common targets are predicted by miRNA target algorithms, including BCL2L11, CCND1, CCND2. This regulation raises several questions. Does the regulation depend on cell type and is it synergistic? Are both miRNA families relevant to cancer development, or only one family plays a role in the process in

a given cancer type? Studies that have characterized individual role of miRNAs

concluded that either *miR-17* or *miR-19* families play main role in cancer development,

but not together (Cloonan et al., 2008; Matsubara et al., 2007; Olive et al., 2009; Sun et

al., 2010; Yu et al., 2010b). The results from our study suggest that the same may be true

in *Cbfb-MYH11* AML, as only *miR-17/20a* miRNAs provided survival capacity in mouse

leukemic cells. One of the questions that remains unanswered by our study is the specific

molecular function of *miR-17* family in CBF AML. Top-down approaches studying *miR-17*-specific targets (such as MEKK2, MAPK9, DUSP2, and BCL2L11), or bottom-up

approaches studying the effect of *miR-17* family miRs on the phosphorylation or protein

levels of pro- and anti-apoptotic proteins are currently being pursued.

It is also possible that some of the functional effects of *miR-19a/b* on the AML

cells were not detected due to limitations of the *in vitro* settings or the antagomir-based

experiments. Experimental data suggest that proliferation effects may be difficult to

detect in cells that were previously cryopreserved (Peter Valk, personal communication).

The ultimate readout of individual miRNA roles in AML development in cooperation

with Cbfβ-SMMHC will come from cooperation studies with single miRNAs in the

mouse model.

Activation of PI3K/Akt pathway in the AML cells by miR-17-92

The miRNAs may regulate a single miRNA target responsible for an observed phenotype

(Molitoris et al., 2011; Sun et al., 2010; Wang, 2008; Wong et al., 2010; Zhang et al.,

2011). In other cases, miRNAs could cause relatively small effects on potentially

hundreds of targets, so that each individual change may not be a determining cause of a functional effect.

Our study shows that *miR-17-92* downregulates Pten protein levels in mouse *Cbfb-MYH11/miR-17-92* AML blasts. Considering that Pten is a common target of both *miR-17* and *miR-19* family miRs and the fact that Bad-phosphorylation (pBad) levels are increased, these data strongly suggest that these miRs may be implicated in Bad-mediated survival functions. The mechanism of increased Bad-phosphorylation is not yet clear, but it underscores the hypothesis that multiple survival pathways may be altered by these miRNAs. For example, downregulation of Pten by both *miR-17* and *miR-19* miRNA families resulted in moderate pBad increase, but may not have sufficiently shifted the pro- and anti-apoptotic signals balance to determine the *miR-17/20a* anti-apoptotic effect. Some other regulators of apoptosis (e.g. Bim and E2F1), when targeted by *miR-17* family, could contribute to cell survival, but only in the context of increased pBad. To address some of these remaining questions, the Pten put-back and shRNA-mediated knock-down experiments in the context of individual miRNA silencing could dissect the role of Pten targeting by each miRNA family. Additionally, analysis of expression and regulation of other apoptotic regulators (including those not targeted directly by the miRNAs, e.g. Bcl-xL and Bcl2) would allow us to determine what particular signals contribute to the anti-apoptotic effect of *miR-17*, but not *miR-19* family.

Role of miR-17-92 in wild type and cancer cells

Upregulation of *miR-17-92* has been shown to play an oncogenic role in multiple cell types. Also, *miR-17-92* is implicated in normal regulation of differentiation, survival and cell cycle in a number of cell types. It remains unclear whether (1) *miR-17-92* normal function in a particular cell type is related to its oncogenic effect in these cells or (2) the oncogenic function is completely unrelated to its normal functions in the cell type. Previously we saw an example that can be interpreted as an illustration of the first possibility. The *miR-17-92* is involved in myeloid differentiation at late stages of myeloid maturation (Fontana et al., 2007), whereas it blocks B-cell development at pro-B stage (Ventura et al., 2008). In our study, in the leukemic setting with Cbfβ-SMMHC, expression of *miR-17-92* did not affect block of myeloid differentiation by the fusion protein (by blast morphology and FACS), yet, it led to earlier block of B-cell differentiation in the *Eµ-Myc* B-cell lymphoma model (He et al., 2005). Interestingly, in MLL fusions-associated AMLs, *miR-17-92* enhances a block in myeloid differentiation, presumably due to the fact that MLL fusions block myeloid differentiation at later stages where *miR-17-92* is involved (Wong et al., 2010). Based on these examples, it is plausible that *miR-17-92* effect on differentiation of leukemic cells depends on its normal function. If the cluster has no function in differentiation of a lineage before the stage where it is blocked by a cooperating oncogene, the overexpression of the cluster does not affect the differentiation of the cells. On the contrary, if the *miR-17-92* normal function in differentiation of a particular cell type is before a differentiation block provided by a

cooperating oncogene, overexpression of *miR-17-92* affects differentiation of the leukemic cells.

Emerging data from different cancer cell types suggest that *miR-17-92* may have distinct functions in normal and tumor cells. The overexpression of *miR-17-92* in normal lungs resulted in impaired differentiation and increased proliferation (Lu et al., 2007). However, in lung cancer the function of *miR-17-92* was determined to be anti-apoptotic (Matsubara et al., 2007). Another example comes from a study of human B-cell lymphoma cell lines. The overexpression of *miR-17-92* in Raji cells provides an anti-apoptotic response, while its overexpression in SUDHL4 cells promotes proliferation of the cells by inhibiting p21 expression (Inomata et al., 2009). This example suggests that within the same cancer type, gene expression context provided by other oncogenes dictates function of *miR-17-92* in the cells, rather than its normal functions in the cell type determining its oncogenic activity.

While in most cases *miR-17-92* functions as an oncogene, it has been also shown to function as a tumor suppressor in breast cancer cells (Hossain et al., 2006). Therefore, even the role of *miR-17-92* as an oncogene or (in rare cases) as a tumor suppressor seems to be determined by the cell context, suggesting that *miR-17-92* signaling by itself is not a direct oncogenic signal, but is complex and ambiguous, and can provide a functional readout only working in a cancer cell context determined by other signals.

Oncogenic role of miR-17-92 cluster in the context of CBFB-MYH11 expression

As described previously, *CBFB-MYH11* is considered a class II oncogenic mutation that provides a block in differentiation, but fails to transform the cells due to absence of self-renewal and/or pro-survival signals. Therefore, it is often found in AMLs to be associated with class I mutations that confer such signals. In this study we have shown that *miR-17-92* is one of the possible class I mutations that cooperate with *Cbfb-MYH11* in a mouse model of AML. The mechanism of this cooperation is suggested to be due to an anti-apoptotic effect elicited by *miR-17-92* in the AML cells. The suggested cooperation between *Cbfb-MYH11* and *miR-17-92* fits the two-hit hypothesis of AML development. Whether tertiary mutations are necessary for the cooperation to occur (for example, mutations that would increase proliferation of the cells), is an open question that can be addressed by identification of CISs for the *miR-17-92*-expressing retroviruses and by determination of clonality of the AMLs. Relatively long latency of the primary AML development and high variability in expression of tested *miR-17-92* targets suggest that the acquisition of additional mutations during leukemogenesis is likely.

The molecular mechanism for the observed cooperation remains unclear. The upregulation of the PI3K/Akt pathway may be one of them, but it may not be upregulated strongly enough to explain the cooperation in this particular cellular context. Similar effects of *miR-17* and *miR-19* families on regulation of Bad phosphorylation, yet different effect of the two miRNA families on apoptosis suggest that additional molecular pathways play a role. Due to hundreds of potential miRNA targets and post-

transcriptional mode of their regulation by the miRNAs, it is hard to identify all relevant changes caused by the *miR-17-92* cluster in the AML cells. We did not find significant deregulation of Rb2, Runx1, E2f1, Mcl-1 and Bim in the *Cbfb-MYH11*/*miR-17-92* AMLs. Yet, other possibilities remain, including Jnk2, Mekk2, MAPK regulator Dusp2 that were also shown to be involved in apoptosis regulation. Careful analysis of regulation of these and other proteins in the $Cbfb^{+/MYH11}$/*miR-17-92* AML cells by the *miR-17-92* cluster and the effect of such regulation on apoptosis and, potentially, other cellular mechanisms is one of the future directions necessary for a deeper understanding of the observed role of *miR-17-92* in the *Cbfb-MYH11*-associated AMLs.

Upregulation of wild type Mpl cooperates with RUNX1-ETO in AML development

Wild type RTK functions are different from mutant RTK functions

Oncogenic mutations in RTKs have been shown to be involved in many cancer types, including AML (Boissel et al., 2006b; Care et al., 2003; Shih et al., 2008). Here we show for the first time that upregulation of a wild type RTK that does not bear mutations can also serve as a second hit in AML development. This result is novel because to our knowledge previous studies determined the role of mutated RTKs in AML development (Brandts et al., 2005; Kohl et al., 2005; Mizuki et al., 2000; Ning et al., 2001b; Schessl et al., 2005a; Wang et al., 2011b), while overexpression of wild type RTKs was found, but

functional role of this overexpression was not properly assessed (Drexler, 1996; Muroi et al., 1998; Rosnet et al., 1996; Turner et al., 1996; Wang et al., 1989).

Functional studies show that signaling downstream of mutated RTKs may be qualitatively different from wild type RTK-induced signaling, and not just mimic wild type signaling with the only difference of having higher activation levels of the same effectors and ligand independence. For example, wild type FLT3 receptor does not induce STAT5 phosphorylation and activation upon stimulation with FLT3 ligand, while FLT3-ITD mutant strongly induces STAT5 activity, and the STAT5 activity was shown to be functionally relevant to oncogenic signaling of FLT3-ITD *in vitro* (Choudhary et al., 2007; Hayakawa et al., 2000; Masson et al., 2009; Mizuki et al., 2000; Spiekermann et al., 2003). Therefore, a finding that an overexpressed wild type RTK is involved in cancer is distinct from identification of a mutant RTK involvement in cancer.

The aforementioned possible difference in wild type and mutant RTK signaling together with our finding of wild type Mpl cooperation with CBF fusion proteins in AML, raises a question that is relevant to the biology of human AML. A significant fraction of acute megakaryoblastic leukemias carry activating mutations of MPL (most commonly mutations in the amino acid W515 residing in the JM domain) (Hussein et al., 2009a), yet they are not detected in other AML cases, including CBF fusions-associated AML. This may seem to contradict our results that suggest that MPL upregulation cooperates with RUNX1-ETO fusion and, importantly, is found upregulated in a large fraction of AML in general and in t(8;21) AML in particular. Of note, *JAK2* activating mutations are readily found particularly in CBF AML (Dohner et al., 2006; Illmer et al.,

2007; Schnittger et al., 2007b), making the absence of *MPL* mutations in AML even more puzzling. A possible explanation would imply differences in signaling from upregulated wild type and constitutively activated mutant MPL. For example, MPL W515 mutants fail to increase phosphorylation of STAT3 without thrombopoietin induction (Staerk et al., 2006). Such changes in signaling could result in a different overall effect on oncogenic cells and prevent cooperation of the mutant MPL with other AML-associated oncogenes, for example, by inducing myeloid differentiation (Staerk et al., 2006). Analysis of cooperation between mutant MPL and CBF fusions in AML *in vivo* would allow to determine possible functional differences between upregulated wild type and mutant MPL.

The described finding may be relevant to strategies of leukemia treatment. Most common ways of subclassification of leukemic cases currently rely on search for mutations in known oncogenes involved in a particular type of cancer. The example of a role of wild type Mpl in AML illustrates the importance of assessing actual expression levels of genes rather than sole reliance on mutation search. In this aspect, microarray profiling studies in leukemia may be a valuable complementing strategy in diagnosis and classification of leukemia (at least in cases where known mutations were not found).

Different second hits, same pathways

In order to develop AML, CBF fusions require presence of additional mutations often called "second hits." A number of the second hits have been found either by studying mutations in human AML or by searching for cooperating genes in mouse models. As

described earlier, majority of the second hit mutations found in human AML activate PI3K/Akt, Jak/Stat and Ras/Raf/Mek/Erk pathways or are players in these pathways. Our data presents other examples of this consistent pattern. All three pathways are activated in the *RUNX1-ETO*/*Mpl* mouse leukemic cells, and functional importance of PI3K/Akt pathway for AML development was shown *in vivo*. *In vitro* data also indicates that PI3K and Mek activity plays an anti-apoptotic role in the cells. Importantly, majority of other studies suggesting activation of the PI3K/Akt, Mek/Erk and Jak/Stat pathways in AML is correlative and comes from studying of human AML samples (Haferlach et al., 2010; Neubauer et al., 2008; Tamburini et al., 2007; Xu et al., 2003). Our data on Mpl cooperation, on the other hand, provides a direct biochemical link between the pathways activity and AML development in a mouse model and primary human AML. This underscores previous findings and makes PI3K/AKT and other MPL-activated pathways promising targets for AML treatment.

Upregulation of Mpl by PlagL2

We have previously shown that PlagL2 upregulates endogenous Mpl expression (Landrette et al., 2011). In this study we show that PLAGL2 cooperates with RUNX1-ETO in AML development, upregulates Mpl expression and its signaling pathways. However, we did not directly show that Mpl is necessary and sufficient for oncogenic activity of PLAGL2 in the presence of RUNX1-ETO and/or Cbfβ-SMMHC. We attempted to address this question using the Mpl blocking antibody AMM2 (Arai et al., 2009) in secondary transplantation assays of RUNX1-ETO/PLAGL2 AML cells.

Unfortunately, we were unable to complete the study due to the severe anemia and HSC mobilization/depletion caused by Mpl blockade. This question is being addressed currently in our lab using two alternative approaches. First, by testing whether RUNX1-ETO and PLAGL2 can induce leukemia in BM transplantation assays when co-transduced with *Mpl* shRNA-expressing constructs. Second, we are testing whether RUNX1-ETO and PLAGL2 can induce leukemia using *Mpl*-null BM cells.

Oncogenic role of Mpl in the context of RUNX1-ETO expression

We have shown that wild type Mpl cooperates with Cbfβ-SMMHC (Landrette et al., 2011) and with RUNX1-ETO in development of AML *in vivo*. We have also determined that in the context of both CBF fusion proteins expression the wild type Mpl activates PI3K/Akt, Jak/Stat and MAPK8 pathways and provides pro-survival and (in human AML samples) a pro-proliferative signals. These results fit the two-hit model of AML development and characterize Mpl overexpression as a potent class I cooperating event.

We have shown that inhibition of PI3K/Akt pathway components or Mek *in vitro* alleviated the anti-apoptotic or pro-proliferative signaling of MPL in the human and mouse AML cells. We demonstrated that inhibition of mTOR effector of PI3K/AKT pathway *in vivo* delayed development of AML *in vivo*, further supporting the idea that activation of PI3K/AKT signaling is important for AML progression. Finally, we demonstrated that a large fraction of t(8;21) human AML samples overexpress MPL and are sensitive to THPO. Taken together, these results suggest a possible clinical relevance of inhibition of MPL and its downstream signaling to treatment of CBF-associated AML.

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