IDENTIFICATION OF ADDITIONAL GENETIC

ALTERATIONS IN RUNX1 RELATED LEUKEMIAS

BINDYA JACOB

(B.Sc (Hons), NUS)

A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICINE

NATIONAL UNIVERSITY OF SINGAPORE

2007/2008

Acknowledgements

Table of Contents

Summary

The *RUNX1/AML1* gene is a key regulator of hematopoiesis and it is the most frequently mutated gene in human leukemia. Loss-of-function of RUNX1 predisposes cells to leukemia, and with the acquisition of cooperating genetic alterations, the cells become fully leukemogenic. Conditional deletion of *Runx1* in adult mice results in an increase of hematopoietic stem/progenitor cells which may serve as the target cell pool for leukemia. However, in most cases, *Runx1* knockout mice do not develop spontaneous leukemia due to the phenomenon called "stem cell exhaustion". Bone marrow transplantation experiments showed that *Runx1* knockout stem cell maintenance was compromised, resulting in progressively decreasing contribution of *Runx1* knockout stem cells to blood cell production. The development of leukemia from *Runx1* knockout stem cells harboring property of exhaustion may therefore require accumulation of additional genetic alterations that prevent exhaustion. I employed retroviral insertional mutagenesis on conditional *Runx1* knockout mice to identify additional genetic alterations that cooperate with loss-of-function of Runx1 in leukemogenesis.

Runx1 knockout mice infected with MoMuLV retrovirus showed shorter latency of leukemia onset than wild type littermates. Majority of the *Runx1* knockout mice developed early onset leukemia with myeloid features while majority of the wild type mice developed T-cell leukemia or lymphoma with varying onset time. This indicates that *Runx1* knockout status drives myeloid tropism despite T- lymphotropism of MoMuLV virus. 710 retroviral integration sites were obtained using inverse PCR techniques from 63 *Runx1-/-* mice and 52 WT mice. From *Runx1* knockout series, 15 known and 5 novel common integration sites were identified. The locus that was most

frequently affected in *Runx1* knockout mice was the *Gfi1/ Evi5* locus and majority of the mice with integrations at this locus showed early onset leukemia with myeloid features.

Gfi1 is a stem-cell factor and Evi5 is known to be a cell cycle regulator whose overexpression leads to a delay in mitotic entry. Quantitative real-time PCR results showed that *Evi5* was preferentially overexpressed due to integrations at the *Gfi1/Evi5* locus, without much change in *Gfi1* levels. Experiments were carried out on *Runx1* knockout and wild type bone marrow cells retrovirally overexpressing *GFI1* or *EVI5,* to study rescue of exhaustion and synergy with *Runx1* knockout status in maintaining stem cells. *In vitro* experiments such as long term culture of stem cells showed clear synergy between loss of function of Runx1 and overexpression of *EVI5*, but not *GFI1*. Results from *in vivo* bone marrow transplantation experiments also demonstrated similar synergy. *EVI5* overexpression maintained increased number of *Runx1* knockout stem cells by preventing their exhaustion in recipient mice. The mechanism of *Runx1* knockout stem cell exhaustion and rescue by *EVI5* seems to be niche dependant since *Runx1* knockout cells expressed lower levels of critical niche interaction factor, CXCR4 and CD49b which may result in impaired interaction with the stem-cell niche. Defective homing and niche interacting ability of *Runx1* knockout bone marrow cells was confirmed by homing assay. Overexpression of *EVI5* in *Runx1* knockout cells restored normal levels of CXCR4 and CD49b; and at the same time upregulated critical stem cell and antiapoptotic genes such as *Bmi1*, *p21* and *Bcl*-2, thereby maintaining an expanded pool of aberrant *Runx1* knockout stem cells in the niche which may act as targets of further oncogenic hits. Finally, *EVI5* was also found to be overexpressed in 44% of human RUNX1 related leukemia patients, acute myeloid leukemia M2 subtype with t (8; 21).

Index of tables

Index of figures

List of abbreviations

AGM	Aorta-Gonad-Mesonephros
AML	Acute myeloid leukemia
BM	Bone Marrow
BMT	Bone Marrow Transplantation
C/EBPα	CCAAT/enhancer binding protein α
CAFC	Cobblestone area forming cells
CAR	CXCL12 abundant reticular cells
CBF	Core binding factor
CFU	Colony forming unit - culture
CIS	Common integration site
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
CSF	Colony stimulating factor
EGFP	Enhanced green fluorescence protein
FAB	French-American-British
FACS	Fluorescence activated cell sorting
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GMP	Granulocyte monocyte progenitor
HAT	Histone acetyl transferase
HDAC	Histone deacetylases
HSC	Hematopoietic stem cell

IL	Interleukin
IPCR	Inverse PCR
KSL	c-Kit+ Stem cell antigen 1+ Lineage-
LTC-IC	Long term culture initiating cell
LT-HSC	Long term hematopoietic stem cell
LTR	Long terminal repeat
M-CSF	Macrophage colony stimulating factor
MEP	Megakaryocyte erythrocyte progenitor
MoMuLV	Moloney Murine Leukemia Virus
MPP	Multipotent progenitor
PB	Peripheral Blood
PEBP2	Polyomavirus enhancer binding protein 2
pIpC	poly Inosine poly Cytidine
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RBC	Red blood cells
RIM	Retroviral insertional mutagenesis
RIS	Retroviral integration site
SDF-1	Stromal cell derived factor - 1
SNO	Spindle shaped, N-cadherin+ , Osteoblast cells
ST-HSC	Short term hematopoietic stem cell
TAD	Transcription activation domain
TLE	Transducin-like enhancer
WBC	White blood cells
WT	Wild type
YAP	Yes-associated protein

Publications List

1. **Stem cell exhaustion due to Runx1 deficiency is prevented by Evi5 activation in leukemogenesis**.

   Jacob B, Osato M, Yamashita N, Taniuchi I, Littman D, Asou N, Ito Y

   Manuscript in preparation

2. **Haploinsufficiency of *Runx1/AML1* leads to hypersensitivity to granulocyte colony-stimulating factor (G-CSF)**

   Jacob B, Osato M, MotodaL, YokomizoT, Yanagida M, Ogawa M, Nishikawa S, Shigesada K, Ito Y

   Manuscript in preparation

3. **Runx1 Protects Hematopoietic Stem/progenitor Cells from Oncogenic Insult.**

   Motoda L, Osato M, Yamashita N, **Jacob B**, Chen LQ, Yanagida M, Ida H, Wee HJ, Sun AX, Taniuchi I, Littman D, Ito Y.

   Stem Cells. 2007 Dec;25(12):2976-86

4. **Increased dosage of Runx1/AML1 acts as a positive modulator of myeloid leukemogenesis in BXH2 mice**

   Yanagida M, Osato M, Yamashita N, Liqun H, **Jacob B**, Wu F, Cao X, Nakamura T, Yokomizo T, Takahashi S, Yamamoto M, Shigesada K, Ito Y.

   Oncogene. 2005 Jun 30;24(28):4477-85

Chapter 1 – Introduction

1.1 Hematopoiesis

The term hematopoiesis refers to the formation and development of the cells of the blood. Vertebrate hematopoiesis traditionally has been divided into an early or primitive phase and a late or definitive phase. Primitive hematopoiesis produces only a restricted range of blood cell types, including primitive nucleated erythrocytes and macrophages. Definitive hematopoiesis is multilineage hematopoiesis that gives rise to all lineages of blood cells that populate the organism. Primitive blood cells, which populate the early embryo, have properties that diverge from those of their definitive counterparts. Thus, two waves of hematopoiesis are required for various physiological activities that are differentially mediated by the embryo at various phases of development.

1.1.1 Hematopoiesis during development

In the human embryo, primitive hematopoiesis resides at first in the yolk sac outside the embryo. Nucleated erythroid cells arise in the aggregates of blood cells in the yolk sac, called blood islands and circulate through the embryo supplying oxygen and nutrients to the developing tissues. Pluripotent hematopoietic stem cells arise from within the embryo in a region described as the aorta-gonad-meso-nephros (AGM) region between 25 and 35 days post coitus (Godin et al., 1995; Huyhn et al., 1995; Medvinsky et al., 1993; Tavian et al., 1996). As the embryo develops, definitive hematopoiesis appears in the fetal liver at approximately 5 weeks of gestation (Migliaccio et al., 1986) and it remains the primary site of hematopoiesis until mid-gestation. Around the 20th week of gestation,

hematopoiesis is established in the bone marrow (BM). Progressively, hepatic hematopoiesis decreases and the BM becomes the main site for formation of the blood cells (Golfier et al., 1999; Golfier et al., 2000) **(Figure1.1)**. After birth, BM is the only site of blood formation. However, maturation, activation, and some proliferation of lymphoid cells occur in secondary lymphoid organs (spleen, thymus, and lymph nodes). The liver and spleen may resume their hematopoietic function under pathologic conditions, called extramedullary hematopoiesis (Marshall and Thrasher, 2001).

In mice, the process of hematopoiesis follows similar developmental steps with primitive hematopoiesis taking place in the yolk sac and definitive hematopoiesis in the fetal liver of the embryo and BM of adults. Primitive hematopoiesis starts at embryonic day 7.5 (E7.5) at blood islands in the yolk sac. Around embryonic day 8.5, definitive hematopoietic progenitor cells which are multipotent and capable of lymphoid and myeloid differentiation are found in the AGM region. Isolated AGM cultured *in vitro* demonstrated that this region is a source of hematopoietic stem cells (Dzierzak and Medvinsky, 1995; Yokomizo et al., 2001). These immature cells begin to circulate following the onset of cardiovascular function and migrate to the developing fetal liver by E10, which serves as the site for definitive hematopoiesis that starts around E12. The liver serves as the predominant site of hematopoiesis until just before birth when the spleen and BM compartments become seeded with circulating stem cells. From that point on, the BM serves as the primary site of hematopoiesis.
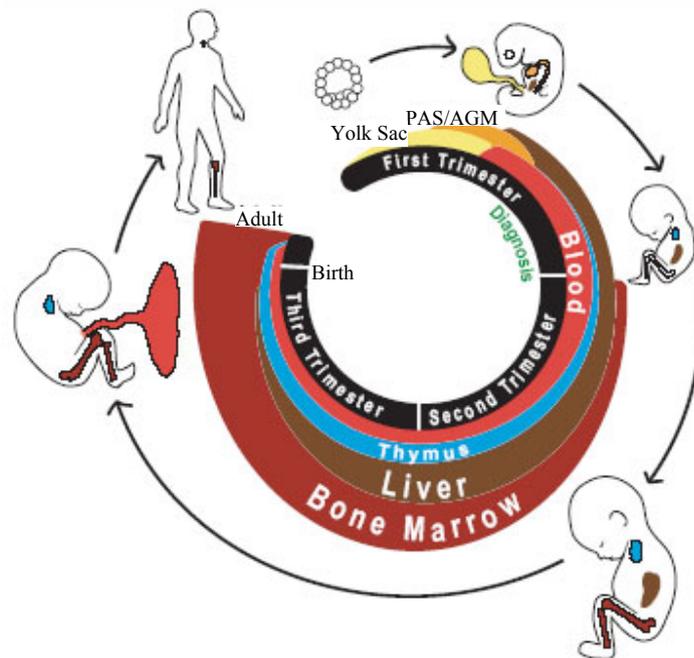
AGM, aorta-gonad-mesonephros; PAS, Para-aortic splanchnopleure

Figure 1.1: Steps and sites of hematopoiesis in humans during development (www.medscape.com).

1.1.2 Multilineage hematopoiesis

Every functional specialized mature blood cell is derived from a rare population of cells in the BM known as the hematopoietic stem cells (HSC). These stem cells represent a self-renewing population of cells that have the potential to generate progenitor cells that differentiate and become committed to a particular blood cell lineage. A single stem cell is capable of completely restoring the hematopoietic process. Two properties define these cells. First, they can generate more HSC, through a process of self-renewal. Second, they have the potential to differentiate into various progenitor cells that eventually commit to further maturation along specific pathways. The end result of these events is the

continuous production of sufficient, but not excessive, numbers of hematopoietic cells of all lineages. The pluripotent HSC can undergo a decision to either self renew or differentiate into committed progenitor cells. Once the process of differentiation is triggered, HSC generate progenitor cells, namely common lymphoid progenitor (CLP) and common myeloid progenitor (CMP) (Ling and Dzierzak, 2002; Ogawa, 1993; Akashi et al., 2000; Orkin, 2000; Kondo et al., 2003). These cells are committed to a given cell lineage; nevertheless, they are highly proliferative and undergo several successive stages of differentiation till they terminally differentiate into mature non dividing progeny that make up specific blood cell types. The CMP gives rise to myeloid and erythroid lineage through granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythroid progenitors (MEPs). GMPs differentiate into granulocytes including neutrophils, eosinophils, basophils; and monocytes which further differentiate into macrophages. MEPs differentiate into megakaryocytes/platelets and erythrocytes (**Figure1.2**). The myeloid lineage is involved in various functions such as innate immunity, adaptive immunity and blood clotting.

The CLP gives rise to the lymphoid lineage, namely T, B and NK cells which form the cornerstone of the adaptive immune system. Lymphocyte progenitors leave the BM and mature in lymphoid organs, including the thymus, lymph nodes, and spleen; these provide specialized microenvironments for the expression of factors that move lymphocytes along their distinctive pathways of differentiation. B-cell development to the stage of the mature B lymphocyte is completed within the BM. Further differentiation into plasma cells or memory B-cells does not occur until the mature (but naïve) B lymphocyte encounters specific antigen. T-cell development to the stage of precursor
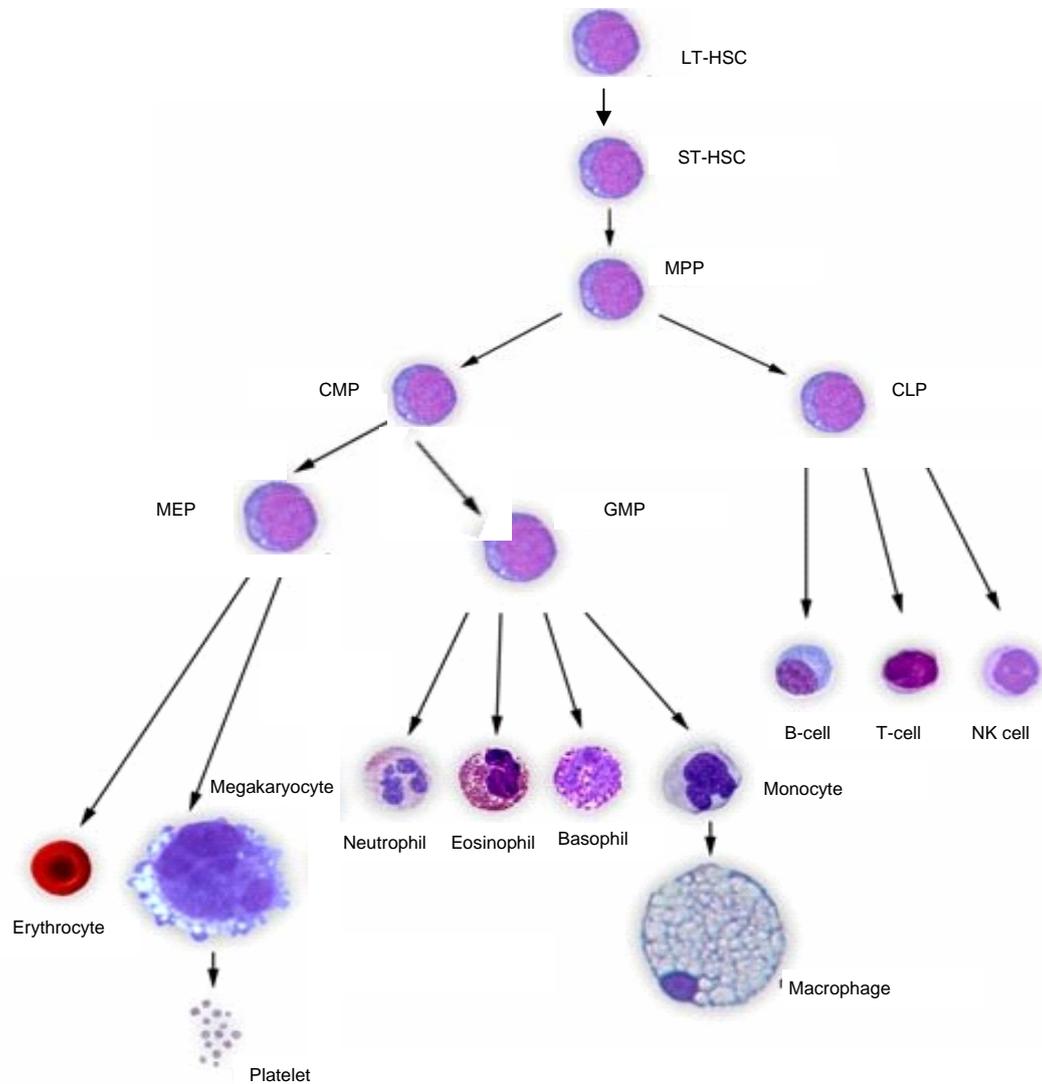
HSC, hematopoietic stem cell; LT, long term; ST, short term; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte erythrocyte progenitor; GMP, granulocyte monocyte progenitor.

Figure 1.2: Hematopoiesis differentiation chart. Maturation patterns of myeloid and lymphoid cells into their respective lineage.
(Modified http://daley.med.harvard.edu/ assets/Willy/Willy_Frames4.htm)

T lymphocyte occurs within the BM. The precursor T lymphocytes then go to the thymus to complete maturation. When mature T lymphocytes leave the thymus, they are mature, (but naïve) Tc (T cytotoxic lymphocytes) or Th (T helper lymphocytes). Further differentiation does not occur until the mature T-cells encounter antigen (presented to the T-cell in association with MHC proteins on 3 types of antigen presenting cells: macrophages, B-cells and dendritic cells) (Schwarz and Bhandoola, 2006).

1.1.3 Hematopoietic stem cell niche

HSC usually reside in a highly specialized microenvironment called the stem cell niche that produces essential factors to maintain a pool of HSC that provides the appropriate numbers of mature blood cells throughout life. Most primitive HSC are thought to be in a quiescent state in these niches and regulation of HSC is largely dependant on their interaction with the niche. The niche serves as both a means of preserving and protecting stem cells from potentially depleting stimuli such as apoptotic and differentiation stimuli; and as a means of protecting the host from the potential adverse effects of excessive stem cell activity. However, stem cells must be periodically activated to produce progenitor cells that are committed to produce mature cell lineages. Thus, maintaining a balance of stem cell quiescence and activity is the hallmark of a functional niche. The niche therefore produces signals for the localization, expansion and constraint of stem cells (Moore and Lemischka, 2006; Wilson and Trumpp, 2006).

HSC have a defined spatial organization in the BM cavity, with the most-primitive cells being located in stem cell niches near the endosteum of the bone — the layer of connective tissue that lines the medullary cavity of a bone. The endosteum is

lined with osteoblasts (bone generating cells) which are thought to secrete or activate a variety of factors such as angiopoietin-1 and CXCL12 (chemokine ligand 12) that regulate the maintenance or numbers of HSC in the BM (Arai et al., 2004; Calvi et al., 2003; Zhang et al., 2003). Especially, SNO cells (spindle shaped, N-cadherin+, osteoblast cells) fulfill the function of niche cells on the endosteum of BM (Zhang et al., 2003). The second niche for HSC is the sinusoidal niche located in the vascular network (the sinusoids) of the BM and spleen, with two thirds of the HSC localized at this niche (Kiel et al., 2005), especially attached to CXCL12 abundant reticular cells or CAR cells. CXCL12 is also known as SDF-1 (stromal cell derived factor) and its main receptor is CXCR4 which is found on HSC (Peled et al., 1999; Sugiyama et al., 2006). High amounts of SDF-1 is secreted by both the CAR cells in the sinusoidal niche and the osteoblast cells lining the endosteal niche to which most of the HSC are attached. Thus, interaction of SDF-1 with its receptor CXCR4 found on HSC is essential for the interaction of the HSC with its niche, both endosteal and sinusoidal (Kollet et al., 2006; Sugiyama et al., 2006). Interrupting this localization of stem cells to the niche impairs engraftment or retention of normal HSC in the BM, preventing these cells from self-renewing and contributing to blood formation (Sugiyama et al., 2006). Collectively, all the genetic and functional data indicate that the SDF-1–CXCR4 pathway is crucial and probably most important for retention and maintenance of adult HSC. In addition to CXCR4, other cell-surface receptors expressed on HSC and several cell-surface adhesion molecules, including selectins and integrins, are involved in stem cell homing, localization and retention in the niche (Lapidot and Petit, 2002; Lapidot et al., 2005). For

example, β1-integrin-deficient HSC fail to migrate to the BM after transplantation (Potocnik et al., 2000).

The stem cells behave in a dynamic manner and often leave the BM (mobilization), circulate in the blood and return to endosteal niche or sinusoidal niche (homing). The release of HSC from their niche is observed during homeostasis, when a small number of HSC are constantly released into the circulation (Wright et al., 2001). Although their precise physiological role remains unclear, they might provide a rapidly accessible source of HSC to repopulate areas of injured BM (Lapidot and Petit, 2002). Alternatively, circulating HSC might be a secondary consequence of permanent bone remodeling that causes constant destruction and formation of HSC niches, therefore requiring frequent re-localization of HSC which are on the lookout for empty niche. Transplanted HSC also have the capacity to home back to and lodge in stem cell niche in recipients. The stem cell pool is tightly controlled in the body and it is essential that the circulating stem cells or transplanted stem cells have their homing and niche interacting machinery intact so as to find a new niche and maintain their stem cell properties. Defects in this machinery could lead to loss of stem cells in the body as is seen in *CXCR4* conditional knockout mice (Sugiyama et al., 2006).

1.1.4 Growth factors important for hematopoiesis

Hematopoietic stem and progenitor cell commitment depends upon the acquisition of responsiveness to certain growth factors. A large number of cytokines that turn on and off transcriptional regulators of blood cell fate at the appropriate times have been identified. Based on their function, one can distinguish stem cell factors that promote maintenance

of HSC (such as SCF) (Nishikawa et al., 2001), multilineage colony stimulating factors (CSF) that act on several lineages (for example GM-CSF or IL-3) and lineage-specific factors (such as G-CSF for granulocytes, M-CSF for monocytes or EPO for erythrocytes) (Barreda et al., 2004; Richmond et al., 2005). The CSF act in a stepwise manner inducing proper maturation of blood cells. IL-3 (multi-CSF) acts early, possibly even at the level of the pluripotent stem cell, to induce formation of the myeloid progenitors. GM-CSF acts at a slightly later stage, and induces formation of granulocyte and monocyte progenitors. M-CSF and G-CSF act still later to promote the formation of monocytes and granulocytic cells, respectively. The other category of growth factors are the interleukins. Interleukins are present at extremely low concentrations and have biological activity at concentrations as low as 10^{-12} M. They are produced by various sources of blood and stromal cells and mediate various functions **(Table 1.1)**.

Hematopoiesis is a continuous process throughout adulthood and production of mature blood cells equals their loss. The process of hematopoiesis is tightly regulated; however, due to genetic alterations in stem/progenitor cells, the balance between proliferation and differentiation of stem/progenitor cells is affected leading to accumulation of white blood cells in the body which are usually dysfunctional. This leads to the disease state known as leukemia.

	Major source	Major effects
IL-1	Macrophages	Stimulation of T-cells and antigen-presenting cells. B-cell growth and antibody production. Promotes hematopoiesis (blood cell formation).
IL-2	Activated T-cells	Proliferation of activated T-cells.
IL-3	T lymphocytes	Growth of blood cell precursors.
IL-4	T-cells and mast cells	B-cell proliferation. IgE production.
IL-5	T-cells and mast cells	Eosinophil growth.
IL-6	Activated T-cells	Synergistic effects with IL-1 or TNFα.
IL-7	Thymus and BM stromal cells	Development of T-cell and B-cell precursors.
IL-8	Macrophages	Chemo attracts neutrophils.
IL-9	Activated T-cells	Promotes growth of T-cells and mast cells.
IL-10	Activated T-cells, B-cells and monocytes	Inhibits inflammatory and immune responses.
IL-11	Stromal cells	Synergistic effects on hematopoiesis.
IL-12	Macrophages, B-cells	Promotes T_H1 cells while suppressing T_H2 functions
IL-13	T_H2 cells	Similar to IL-4 effects
IL-15	Epithelial cells and monocytes	Similar to IL-2 effects.
IL-16	CD8 T-cells	Chemoattracts CD4 T-cells.
IL-17	Activated memory T-cells	Promotes T-cell proliferation.
IL-18	Macrophages	Induces IFNγ production.

Table 1.1: Major source and effects of various types of interleukins
(http://www.web-books.com/MoBio/Free/Ch2G1.htm).

1.2 Leukemia

Leukemia is characterised by an accumulation of abnormal or dysfunctional blood cells, leading to suppression of normal hematopoiesis, including production of normal red blood cells (RBC), white blood cells (WBC) and platelets. In parallel with the understanding of normal hematopoiesis has come a recognition that hematopoietic stem/progenitor cell dysregulation is involved in leukemogenesis. The progression to leukemia, especially acute leukemia, involves accumulation of at least two or more mutational events that lead to enhancement of stem cell proliferation or acquisition of stem cell behavior by a progenitor cell, coupled with maturation inhibition. Leukemia can be classified into distinct types according to the clinical manifestation (acute or chronic), and the property of leukemic cells, particularly, the lineage (myeloid or lymphoid) and the maturity.

Chronic leukemia — It is distinguished by the excessive build up of relatively mature, but abnormal, blood cells. Early in the disease, the people with chronic leukemia may not have many symptoms, but chronic leukemia gets worse progressively. It causes symptoms as the number of leukemic cells in the blood rises. Typically taking months to years to progress, the cells are produced at a much higher rate than normal cells, resulting in many abnormal white blood cells in the blood over time.

Acute leukemia — It is characterized by the rapid growth of immature blood cells. The blood cells are very abnormal and cannot carry out their normal functions. The number of abnormal cells increases rapidly and the crowding makes the BM unable to produce healthy blood cells. Immediate treatment is required in acute leukemias due to the rapid

progression and accumulation of the malignant cells, which then spill over into the bloodstream and spread to other organs of the body. If left untreated, the patient will die within months or even weeks. The types of leukemia are also grouped by the type of white blood cell that is affected. Leukemia can arise in lymphoid or myeloid cells.

1.3 Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder of hematopoietic progenitor/precursor cells and the most common hematological malignancy. In normal hematopoiesis, the myeloid progenitor gradually matures into a mature myeloid cell. However, in AML, the myeloid progenitor accumulates genetic changes which maintain the cell in its immature state and prevent differentiation (Fialkow, 1976). Such mutations alone do not cause leukemia; however, when such a differentiation arrest is combined with other mutations which affect genes controlling proliferation, the result is the uncontrolled growth of an immature clone of cells, leading to the clinical entity of AML (Fialkow et al., 1991). Specific cytogenetic abnormalities can be found in many patients with AML and the types of chromosomal abnormalities often have prognostic significance. The chromosomal translocations encode abnormal fusion proteins, usually involving transcription factors whose altered properties may cause the differentiation arrest. The clinical signs and symptoms of AML result from the fact that, as the leukemic clone of cells grows, it tends to displace or interfere with the development of normal blood cells in the BM. This leads to anemia, and thrombocytopenia.

Much of the diversity and heterogeneity of AML stems from the fact that leukemic transformation can occur at a number of different steps along the differentiation

pathway (Bonnet and Dick, 1997). Modern classification schemes for AML recognize that the characteristics and behavior of the leukemic cell (and the leukemia) may depend on the stage at which differentiation was halted. Based on the extent of differentiation, AML can be further classified into subtypes. The majority of the literature on leukemia is using the French-American-British (FAB) classification, which was mainly based on the morphology of the abnormal cells, although immunophenotyping is needed to confirm a few specific subtypes (**Table 1.2**).

1.3.1 The genetic basis for development of AML

A number of risk factors for AML have been documented including exposure to ionizing radiations, organic solvents such as benzene and chemotherapeutic agents. The molecular basis of this disease needs to be elucidated so as to develop effective targeted therapies to kill the leukemic clones specifically. Two major types of genetic events have been described that are crucial for leukemic transformation: (1) alterations in myeloid transcription factors governing hematopoietic differentiation and (2) activating mutations of signal transduction intermediates (Steelman et al., 2004; de Koning et al., 1998). These processes are highly interdependent, since the molecular events changing the transcriptional control in hematopoietic progenitor cells modify the composition of signal transduction molecules available for growth factor receptors, while the activating mutations in signal transduction molecules induce alterations in the activity and expression of several transcription factors that are crucial for normal myeloid differentiation.

13

FAB Subtype	Description	Comments
M0	Undifferentiated	Myeloperoxidase negative; myeloid markers positive
M1	Myeloblastic with maturation	Some evidence of granulocytic differentiation
M2	Myeloblastic with maturation	Maturation at or beyond the promyelocytic stage of differentiation; can be divided into those with and without t(8;21) RUNX1-ETO fusion protein
M3	Promyelocytic	APL; most cases have t(15;17) PML-RARα or another translocation involving RARα
M4	Myelomonocytic	
M4Eo	Myelomonocytic with BM eosinophilia	Characterized by inversion of chromosome 16 involving CBFβ/PEBP2β
M5	Monocytic	
M6	Erythroleukemia	
M7	Megakaryoblastic	GATA1 mutations in those associated with Down's syndrome

AML1, Acute Myeloid Leukemia 1/ RUNX1; APL, Acute promyelocytic leukemia; PML, Promyelocytic leukemia; RAR-α, Retinoic acid receptor α.

Table 1.2: French-American-British (FAB) classification of AML (Bennett et al., 1976). Modified from Tenen D.G, 2003.

A number of studies have pointed to the dominant role of transcription factors usually involved in normal hematopoiesis, in the pathogenesis of AML. The evidence for this comes from two separate areas of studies. Chromosome studies have established that translocations/inversions of transcription factors are the most common cytogenetic defects in AML. Cloning of chromosome breakpoints has shown that genes involved in the chromosome abnormalities are hematopoietic transcription factors, the functional loss of which results in the disruption of myeloid differentiation. In a number of AML cases

that do not show chromosomal translocations, mutations have been found in the coding regions of hematopoietic transcription factors. Thus, it can be concluded that the most common genetic mechanism that is associated with AML is the deregulation of a transcription factor due to mutations or chromosomal translocations (Tenen D.G, 2003).

1.4 Transcription factors

A transcription factor is defined by its ability to bind DNA and modulate the expression of its target genes. It usually contains three regions: the DNA-binding domain, the multimerisation domain and the effector domain, which modulates activation or repression of transcription (Semenza G L, 1998). Transcription factors do not generally act alone. They interact with other proteins in the context of a protein complex. Their transactivation and DNA binding activities are cooperatively enhanced by these interactions. Transcription factors play a major role in the regulation of gene expression and the distinct combinations of transcription factors expressed in each cell of an organism need to be regulated spatially and temporally. The alteration of a transcription factor's functions or expression patterns usually results in a severe phenotype as illustrated by transcription factor deficient mice, which are often embryonic lethal or harbor dramatic developmental defects. The action of a transcription factor can be altered by mutations either in the transcription factor sequence itself or in its cis-regulatory elements.

Germ-line point mutations in transcription factors, while rare, are observed in approximately 10% of genetic disorders for which the responsible gene is known (Jimenez-Sanchez et al., 2001). The majority of these mutations affect embryonic

development, demonstrating the importance of these proteins in early development. Somatic mutations in transcription factors are also often observed in cancer, especially in leukemia. These mutations include both point mutations and various chromosomal abnormalities. As mentioned before, many of the translocations involved in leukemia target transcription factors. It was shown recently that 38% and 44% of the genes involved in chromosomal abnormalities, associated with hematopoietic and solid tumors respectively code for regulators of transcription (Mitelman et al., 2004). Transcription factor mutations can have 3 consequences. There can be a gene dosage effect resulting in haploinsufficiency of the transcription factor function, or the mutant can act as a dominant negative and interfere with the wild type transcription factor (Semenza G L, 1998). Gain of function mutants can also be generated, especially if the mutation is in an inhibitory domain of the protein.

1.4.1 Transcription factors in hematopoiesis and leukemia

Important information about the role of transcription factors in hematopoiesis has been obtained from studies involving either targeted disruption or overexpression of these factors **(Table 1.3)**. Hematopoietic transcription factors include factors such as RUNX1/AML1, SCL and GATA2 which are involved in formation of almost all lineages, and differentiation factors, such as GATA1, PU.1 and CCAAT/enhancer binding protein-α (C/EBPα), which usually affects only a single or small number of related lineages. Disruption of *RUNX1/AML1* or *SCL* during development affects formation of the entire blood cell lineage, because these transcription factors function during development of HSC.

The *RUNX1/AML1* gene is a key regulator of hematopoiesis involved in definitive hematopoiesis during development and in differentiation of adult HSC. It is also the most frequently mutated gene in human leukemia. The role of *RUNX1/AML1* gene in hematopoiesis and leukemia is the focus of this thesis and it will be discussed in detail in the next section.

GATA1 was the first 'lineage-specific' transcription factor to be described, and its role in the development of erythroid and megakaryocytic lineages has been elucidated in a number of studies (Shivdasani and Orkin, 1996; Orkin, 2000). GATA1 participates in the differentiation of CMPs to megakaryocyte/erythroid progenitors (MEPs) and not GMPs. This role is supported by studies involving targeted disruption of regulatory elements that resulted in selective loss of erythroid development (Shivdasani et al., 1997). The relative expression levels of GATA1 is critical for normal differentiation and a study reported that every pediatric patient that was analysed — with acute megakaryoblastic leukemia associated with Down's syndrome — harbored mutations in *GATA1*, whereas other M7 AML samples did not (Wechsler et al., 2002) (**Table 1.4**).

PU.1 and *C/EBPα* are the 2 genes important in myeloid lineage development. In normal myelopoiesis, PU.1 seems to have two well-defined functions. The first is to mediate an early role in the development of a multipotential myeloid precursor, by promoting HSC differentiation. The second is a later role in the development of monocytes/macrophages (DeKoter et al., 1998; Anderson et al., 1999). In mice, Pu.1 is absolutely required for the development of macrophages and B-cells, and disruption of

17

Transcription factor	Site of expression	Hematopoietic phenotype in knockout mice and conditional knockout mice
RUNX1	Hematopoietic cells, nervous tissue, skeletal muscle, reproductive tissue	Knockout: lack of all definitive hematopoiesis Conditional knockout: impaired megakaryocytic maturation, defective B-cell and T-cell development, myeloid proliferation
SCL	Hematopoietic cells ('hemangioblasts', HSC, MPPs, erythrocytes and megakaryocytes, endothelial cells, brain tissue	Knockout: complete absence of yolk sac hematopoiesis, lack of angiogenesis Conditional knockout: decreased erythrocytes and megakaryocytes, impaired ST-HSC, normal LT-HSC
PU.1	Hematopoietic cells (HSC, CMPs, CLPs, GMPs, monocytes, granulocytes and B-cells)	Knockout: lack of mature myeloid and B-cells Conditional knockout: block prior to CMP and CLP stages, increased granulopoiesis, defective HSC
CEBPα	Hematopoietic cells (HSC, CMPs, GMPs, granulocytes), liver, adipose tissue	Knockout: lack of GMPs and granulocytes, impaired monocytes, increased immature myeloid cells Conditional knockout: same as knockout mice, plus increased HSC self-renewal
IRF8/ ICSBP1	Hematopoietic cells (B-cells, macrophages, dendritic cells and stimulated T-cells)	Knockout: increased susceptibility to viral infections, increased granulocytic cells, CML-like disease
GFI1	Sensory epithelial cells in the inner ear, neuroendocrine cells of the lungs, neutrophils, B and T-cells, HSC	Knockout: reduction in earliest lymphoid progenitors, complete block in late neutrophil maturation, defective HSC
CEBPε	Preferentially in myeloid and lymphoid cells	Knockout: abnormal late neutrophil maturation, block in eosinophil development, defective macrophage function

C/EBP, CCAAT/enhancer binding protein; CLP, common lymphoid progenitor; CML, chronic myeloid leukemia; CMP, common myeloid progenitor; GFI1, growth factor independent 1; GMP, granulocyte/monocyte progenitor; IRF8, interferon-regulatory factor 8; LT-HSC, long-term hematopoietic stem cell; MPP, multipotential progenitor; PU.1, transcription factor encoded by SPI1; RUNX1, Runt-related transcription factor 1; SCL, stem-cell leukemia factor; ST-HSC, short-term hematopoietic stem cell.

Table 1.3: Transcription factors involved in normal hematopoiesis- expression, and knockout phenotypes (Rosenbauer and Tenen, 2007).

Pu.1 also leads to delayed development of granulocytes and T-cells (Scott et al., 1994; McKercher et al., 1996). PU.1 regulates almost all myeloid genes, including the receptors for GM-CSF, M-CSF and G-CSF. *PU.1* mutations have been detected in 7% of 126 AML patients (Mueller et al., 2002). In general, the mutations were found in either the most immature FAB subtype (M0), myelomonocytic or monocytic (M4 or M5), or erythroleukemia (M6) — consistent with the normal role of PU.1 in hematopoiesis (**Table 1.4**).

In contrast to PU.1, C/EBPα has a more specific function in granulopoiesis and is required for development of granulocytes. Non-conditional targeted disruption of *C/EBP*α results in a selective early block in granulocyte maturation, without affecting other hematopoietic lineages, including monocytes (Zhang et al., 1997). Analysis of adult hematopoiesis in conditional *C/EBP*α knockout models shows a block at the CMP to GMP stage. Loss of *C/EBP*α affects expression of the G–CSF receptor, but not the receptors for GM–CSF or M–CSF (Iwama et al., 1998). Mutations in *C/EBP*α gene have been found with an approximate frequency of 7–9% in all AML patients (Preudhomme et al., 2002; Pabst et al., 2001; Gombart et al., 2002) (**Table 1.4**).

Transcription Factor	Mutations and effects	Frequency in AML	FAB Subtype
RUNX1–ETO t(8;21)	RUNX1 DNA-binding domain fused to the transcriptional corepressor ETO; downregulates expression or activity of RUNX1, PU.1 and C/EBPα	12-15%	M2
CBFβ–MYH11 (inv16)	Inversion of breaks in chromosome 16; joins CBFβ with the myosin gene MYH11	8-10%	$M4_{Eo}$
PML–RARα t(15;17)	PML fused to RARα; blocks myeloid transcription factors (such as C/EBPα and PU.1)	6-7%	M3
MLL fusions t11q23	MLL fused with one of 30 distinct partner proteins; believed to dysregulate *HOX* genes	4-7%	Diverse pattern of myeloid and lymphoid leukemias
RUNX1	Missense, nonsense or frameshift mutations (often biallelic); clustered within the Runt domain	9%	M0 (most)
PU.1	Mutations decrease heterodimer formation and DNA binding*; PU.1 activity downregulated by RUNX1–ETO, P ML–RARα and FLT3–ITD	<7%	M0, M4, M5, M6
C/EBPα	Amino-terminal dominant negative; carboxyterminal loss of DNA binding	7-9%	M1, M2 (most), M4
GATA1	Amino-terminal dominant negative	100% in AMKL associated with Down's syndrome	M7 with Down's syndrome

*Japanese cohort only. AML, Acute Myeloid Leukemia; AMKL, acute megakaryoblastic leukemia; CBFβ, core-binding factor-β; C/EBPα, CCAAT/enhancer binding protein-α; FAB, French–American–British; FLT3, FMS-related tyrosine kinase 3; GATA1, GATA-binding protein 1; HOX, homeobox; ITD, internal tandem duplication; MLL, mixed lineage leukemia; MYH11, myosin heavy chain 11; PML, promyelocytic leukemia; PU.1, transcription factor encoded by SPI1; RARα, retinoic acid receptor-α; RUNX1, Runt-related transcription factor 1.

Table 1.4: Hematopoietic transcription factors altered in AML
(Rosenbauer and Tenen, 2007).

1.5 Transcription factor RUNX1/AML1

1.5.1 Runt domain transcription factors

The *RUNX* genes belong to a small family of heterodimeric transcription factors that control critical cell fate decisions in a number of different cell lineages (Downing et al., 2000; Speck et al., 1999). This family is composed of two subunits: a DNA-binding α subunit (*RUNX* genes) and a non DNA-binding β subunit. The Runt domain, specific to the RUNX family of proteins, was first identified in *Drosophila*, which has 4 genes (Runt, *Lozenge, RunxA and RunxB*) (Rennert et al., 2003) coding for the α subunit, and 2 genes (*Brother* and *Big Brother*) for the β subunit. In contrast, mammals have three genes coding for the α subunit, *RUNX1, RUNX2*, and *RUNX3* and only one for the β subunit, *PEBP2β/CBFβ* (**Figure 1.3**).

PEBP2/CBF

α subunit   β subunit

Mammals   **Mammals**
Runx1/AML1   PEBP2β / CBFβ
Runx2/CBFA1
Runx3/PEBP2αC

Drosophila   **Drosophila**
Runt   Brother
lozenge   Big brother
CG15455
CG1379

Pu/TACCPuC
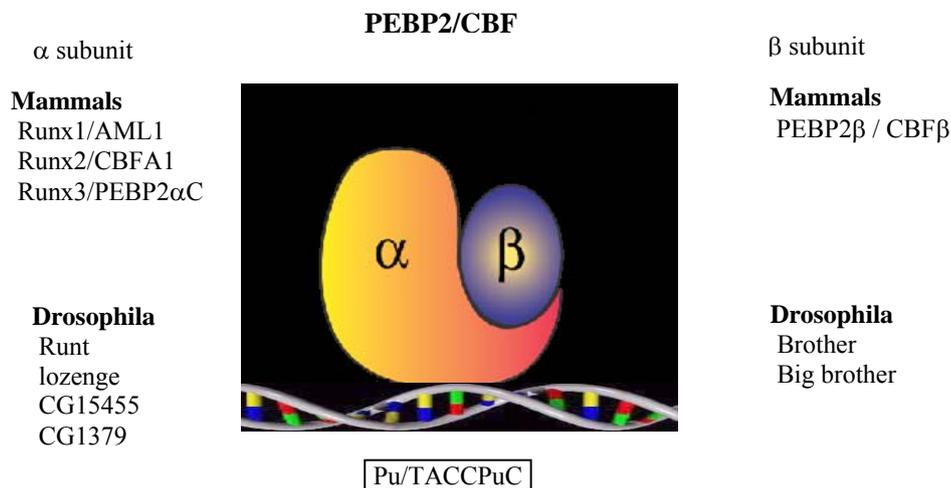
Figure 1.3: *RUNX1/AML1* encodes an α subunit of the Runt domain transcription factor, PEBP2/CBF.

The human genes coding for α subunits have a number of alternative names **(Table 1. 5)**. Since *RUNX1* was first identified in chromosomal rearrangements observed in patients with leukemia, it was also called *Acute Myeloid Leukemia 1* (AML 1) (Miyoshi et al., 1991). At the same time, *RUNX2* gene was identified as the gene that codes for a protein that regulates the transcription of the mouse polyomavirus and thus was called Polyomavirus enhancer binding protein 2 (*PEBP2α*) (Satake et al., 1989). It was also called *CBFα* because it was identified from the core binding factor (CBF) complex that binds to the core site of murine leukemia viruses (Wang et al., 1993). The official nomenclature from the Human Gene Nomenclature Committee (http;//www.gene.ucl.ac.uk/nomenclature/) renamed the genes *RUNX1-3* and these names will be used throughout this thesis (van Wijnen et al., 2004).

RUNX1	AML1	CBFα2	PEBP2αB
RUNX2	AML3	CBFα1	PEBP2αA
RUNX3	AML2	CBFα3	PEBP2αC

Table 1.5: Alternative names of RUNX transcription factors

The three α subunits are required in different biological systems, but they share many common features. They recognize the same DNA-binding site in the promoter region of their target genes (Pu/TACCPuC) and all of them heterodimerize with the β subunit, through the Runt domain. Their protein sequences are highly conserved with

more than 90% identity in the Runt domain. Moreover, all RUNX proteins have PPxY motif, a domain for the binding of WW domain-containing proteins, such as Yes-associated protein (YAP), within their transcription activation domain (TAD). Furthermore, they share a distinct five amino acid sequence, VWRPY, at the C-terminus. VWRPY motif was shown to bind to a transcriptional repressor called Transducin-like enhancer (TLE), the mammalian homolog of *Groucho* in *Drosophila*, which recruits histone deacetylases (HDAC) to repress transcription **(Figure 1.5)**.

The genomic loci of the three mammalian *RUNX* genes are structurally highly conserved, in addition to their protein homology. *RUNX3*, which is the smallest α subunit, has the fewest number of exons, which are all conserved in *RUNX1* and *RUNX2*. The genes downstream of *RUNX2* and *RUNX3* are paralogues of *CLIC6* and *DSCR1*, which are found downstream of the *RUNX1* gene. Finally, all three α subunits use 2 distinct promoters, distal (P1) and proximal (P2) **(Figure 1.4)** (Levanon and Groner, 2004). Hence, these genes are probably derived from duplication of a common ancestor.
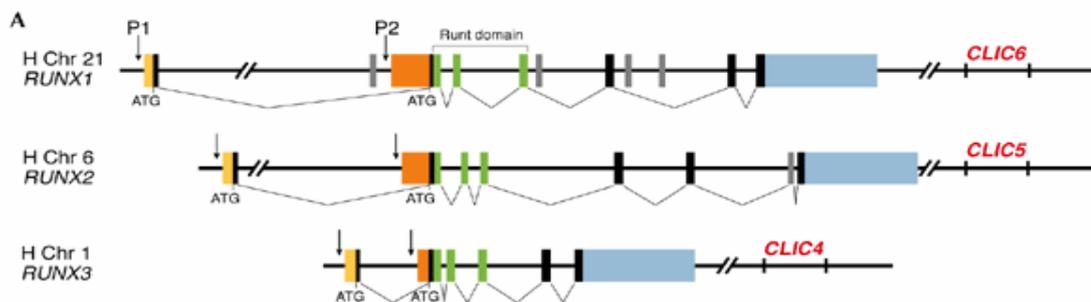
Figure 1.4: RUNX genomic loci. Common exons are shown in similar colors. Exons in the RUNT domain are shown in green. 5'UTR are in yellow for the P1 promoter and in orange for the P2 promoter. 3'UTR are in blue. Neighboring genes are indicated. (Modified from Levanon & Groner, 2004).

Interestingly, the three proteins are rarely expressed in similar cells (Levanon et al., 2001), suggesting that they have distinct functions and their expression is spatially and temporally regulated. The strongest evidence for this came from knockout mouse studies which showed that the three genes are, indeed, involved in distinct systems. Runx1 is required for definitive hematopoiesis as shown by the *Runx1* knockout mice, which lack fetal liver hematopoiesis and show hemorrhaging (Wang et al., 1996; Okuda et al., 1996); Runx2 is required for bone formation and the differentiation of osteoblasts as illustrated by the *Runx2* knockout mice, which show a lack of ossification and die soon after birth because of severe respiratory defects due to absence of rib cage (Otto et al., 1997; Komori et al., 1997); Runx3 is involved in the development of the nervous system (Inoue et al., 2002; Levanon et al., 2002), spine and thymocytes (Taniuchi et al., 2002; Woolf et al., 2003). *Runx3* knockout mice show hyperproliferation of the gastric mucosa (Li et al., 2002); and limb ataxia due to defective dorsal root ganglion neurons.

Germ-line mutations in both *RUNX1* and *RUNX2* genes are also responsible for human disorders. *RUNX1* is mutated in a familial platelet disorder (FPD-AML) and *RUNX2* is mutated in cleidocranial dysplasia (Mundlos et al., 1997). Finally, all three *RUNX* genes play an important role in tumor development. The involvement of *RUNX1* in leukemia is well known and will be discussed later; *RUNX2* overexpression predisposes cells to T-cell lymphomas (Vaillant et al., 2002); and both hemizygous deletions and hypermethylation of the *RUNX3* promoter have been identified in human gastric cancer (Li et al., 2002).

1.5.2 RUNX1: Gene and Protein

The human *RUNX1* gene is mapped to chromosome 21q22.12 and spans 260 kb of genomic sequence. It contains 8 exons. The Runt domain, which is the most important functional domain, contains 128 amino acids, spanning from the end of exon 3 to exon 5 (**Figure 1.4**, green exons). The Runt domain is essential for the DNA-binding activity of the protein as well as the heterodimerization with PEBP2β/CBFβ (Ito, 2008). Although the β subunit itself does not bind to DNA, it enhances DNA-binding affinity of α subunit (Ogawa et al., 1993) and also protects the α subunit from ubiquitination and degradation (Huang et al., 2001). A second domain called the transactivation domain (TAD), which is rich in proline, serine and threonine, is found in many RUNX1 isoforms (**Figure 1.5**). The TAD is essential for transactivation activity of the protein. Isoforms without TAD cannot transactivate target genes; on the other hand, these isoforms have been shown to suppress transactivation by competing with the full length RUNX1 proteins for DNA binding (Tanaka et al., 1995). Both the Runt and the TAD domains are involved in protein interactions (**Figure 1.5**). The RUNX1 protein also contains two regions essential for its nuclear localisation; a nuclear localisation signal (NLS), which is present at the end of the Runt domain; and the nuclear matrix targeting signal (NMTS) in the C-terminal part of the protein, which is responsible for the interaction of the protein with the nuclear matrix (Zeng et al., 1997).
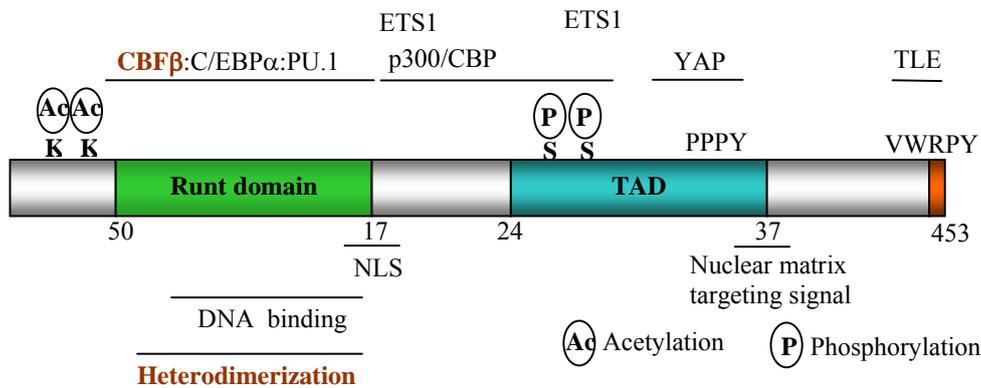
NLS, nuclear localization signal; TAD, transcription activation domain

Figure 1.5: RUNX1 domains and interactions. A diagram of RUNX1 protein with main functional domains, interacting proteins and sites of phosphorylation and acetylation (modified from Ito, 2004).

1.5.3. Regulation of RUNX1 expression

RUNX1 is regulated at the transcription and translational levels, resulting in a very accurate spatial and temporal expression pattern. There are two promoters, proximal (P2) and distal (P1), found in all 3 *RUNX* genes. They are spaced approximately 160 kilobases apart in *RUNX1* and give rise to mRNAs with different 5'UTR and proteins with different N-terminal ends. A large number of different transcripts with distinct expression patterns are generated by the combination of different N-terminal ends and many alternative splicing events (Corsetti and Calabi, 1997). Though studies are still ongoing to identify activators and repressors that bind to *RUNX1* regulatory regions, a few binding sites have already been identified. Binding sites for the RUNX transcription factors themselves, conserved in human and mouse, are present at the beginning of the P1 5'UTR (Drisi et al., 2002). This suggests that RUNX proteins can autoregulate themselves by feedback mechanism. Binding sites for other transcription factors such as PU.1 and c-Myb that

have been shown to interact with RUNX1 are also present in the *RUNX1* promoter (Levanon et al., 1996).

RUNX1 is also regulated at the translational and post-translational levels. The extracellular signal-regulated kinase (ERK), a member of the MAPK family, phosphorylates RUNX1 on two serine residues at the beginning of the TAD **(Figure 1.5)** (Tanaka et al., 1996). This phosphorylation enhances the transactivation ability of RUNX1, but does not seem to affect its DNA-binding affinity. Phosphorylation of RUNX1 is thought to disrupt the interaction between RUNX1 and the co-repressor of transcription, Sin3A, activating the transactivation ability of RUNX1. Phosphorylation is also important for the turnover of the protein as the interaction with Sin3A protects RUNX1 from degradation. Finally, phosphorylation of RUNX1 also plays a role in the subnuclear localisation of the protein to the nuclear matrix (Imai et al., 2004). p300 has been shown to acetylate two Lysine residues (24 and 43), present N-terminal to the Runt domain (**Figure 1.5**), which leads to increased DNA binding affinity of RUNX1. Acetylation of these two residues also increases the transactivation activity of RUNX1, but does not affect heterodimerization with CBFβ/PEBP2β (Yamaguchi et al., 2004).

Finally, a negative regulatory region that regulates DNA-binding activity and also dimerization with CBFβ/PEBP2β is found in the long RUNX1 isoforms. The conformation of these regions can change by interaction with other transcription factors, thus allowing interactions with DNA. For example, the interaction between ETS-1 and RUNX1 leads to reciprocal stimulation of their DNA affinity and activation of their transactivation function by changing their 3D structure, leaving the DNA-binding domain unprotected and free for binding (Kim et al., 1999).

1.5.4. Transcriptional activity of RUNX1

RUNX1 recruits protein complexes to the promoters and enhancers of its target genes and thus acts as an organizing factor. RUNX1 function is context dependent based on the level of expression of cell-specific RUNX1 isoforms, the availability of co-factors and the signals triggering posttranslational modifications. Its function is also promoter-specific as RUNX1 depends on other binding sites present in the regulatory regions of its target genes. Through these combinational mechanisms, RUNX1 can act as an activator of transcription as well as a repressor.

1.5.4.1 Activation of transcription

RUNX1, by itself, has only a small effect on the transcriptional level of its target genes. RUNX1 works in synergy with other transcription factors and co-activators to enable efficient activation of the transcription of its target genes (Mao et al., 1999; Petrovick et al., 1998).The majority of proteins that have been shown to interact with RUNX1 are involved in the hematopoietic system. Some of them are the hematopoietic lineage-specific factors, illustrating the cell-specific action of RUNX1. For example, C/EBPα is expressed in myeloid cells, while ETS-1 is expressed only in lymphoid cells, and PU.1 in both lineages. The well-known co-activators of RUNX1 are CREB (cAMP response element binding protein) and core binding protein (CBP)/p300, which has histone acetyltransferase (HAT) activity. CBP/p300 is important for active chromatin remodeling by histone acetylation and acts as protein bridges between the sequence-specific

transcription factors, such as RUNX1, and the basic transcriptional machinery on the promoter of the target gene, thereby enhancing its transcription.

1.5.4.2. Repression of transcription

RUNX1 protein can also act as a transcriptional repressor (Durst and Hiebert, 2004). The mechanisms of repression, which are cell and promoter specific, are direct repression and gene silencing. Direct repression is a reversible mechanism, whilst gene silencing is a long term mechanism as it is maintained during cell division. In the case of CD4 gene silencing, for example, both mechanisms are used at two different developmental stages. RUNX1 is required for an active repression of CD4 in double negative thymocytes, whilst RUNX3 and probably RUNX1 are required for the establishment of epigenetic silencing of CD4 in CD8+ single positive thymocytes (**Figure 1.6**) (Taniuchi et al., 2002).
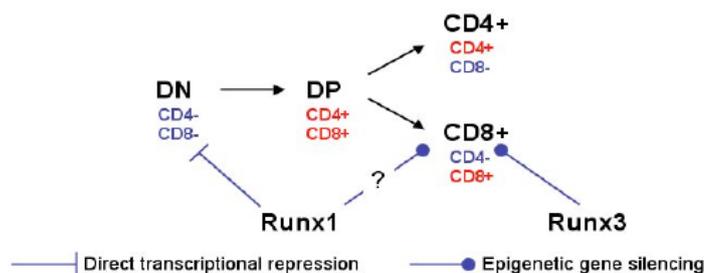
Figure 1.6: *CD4* repression / silencing
Runx1 binds the *CD4* silencer and is required to repress transcription in immature double-negative (DN) thymocytes. Runx3 establishes *CD4* epigenetic silencing in CD4-CD8+ cytotoxic T-cells, probably with Runx1 involvement (Durst and Hiebert, 2004).

Direct repression

In order to repress transcription, RUNX1 transcriptional complexes can recruit repressors such as TLE/Groucho at the C-terminal end of the protein via the VWRPY motif (Levanon et al., 1998) and Sin3A downstream of the Runt domain (**Figure 1.5**). Rather than directly binding DNA, TLE/Groucho and Sin3A interact with transcription factors and recruit histone deacetylases in order to repress transcription. Knock-in mouse studies have demonstrated that the motif VWRPY is not essential for the development of hematopoietic cells (Nishimura et al., 2004) but is necessary for other functions such as thymocyte development. This illustrates again the cell-specific activity of RUNX1.

Gene silencing

RUNX1 can also directly interact with HDAC proteins, which promote gene silencing. RUNX1 has been shown to interact strongly with HDAC1, 3 and 9 but only weakly with HDAC2, 5 and 6 *in vitro*. RUNX1 is also involved in gene silencing through the recruitment of SUV39H1 (Durst & Hiebert, 2004) **(Table 1.6)**.

1.5.5 *Target genes of RUNX1*

A number of genes have already been described as target genes of RUNX1. The majority of these studies have focused on specific genes known to be involved in the hematopoietic system. However, they are mainly based on *in vitro* studies, and whether their expression is really transcriptionally regulated by RUNX1 *in vivo* remains elusive. **Table 1.7** lists some of target genes of RUNX1 reported to date. They include growth factors, receptors, and cell surface molecules, signaling molecules, transcription activators and others.

Interacting proteins	Description	Clinical leukemia association
Activation		
ETS1	TF; ETS family	
ELF4 (MEF)	TF; ETS family	
SPI1 (PU.1)	TF; ETS family	Point mutation in 7% of AML
ELF2 (NERF2)	TF; ETS family	
CEBPA	TF	Point mutation in AML
Pax5	TF; paired box gene	t(9;12) Pax5-ETV6/TEL (ALL)
MITF	TF; bHLH-Zip family	
ZNFN1A1 (Ikaros)	TF; zinc finger	t(3;7) Bcl6-Ikaros; high-level expression in ALL infants
JUN (AP1)	TF	
GATA1	TF	Point mutations in DS - AMKL
ATF2 (CREBP1)	Cyclic AMP	
LEF1	Architectural protein	
THOC4 (ALY)	Protein interaction	
CREBBP (CBP)	Transcriptional adaptor; HAT	t(8;16) CBP-MOZ
SMAD3	Signaling molecule	
MYST3 (MOZ)	Histone acetyl transferase	t(8;16) CBP-MOZ
Repression		
TLE1,2 (Groucho)	Protein interaction	
Sin3A	Histone deacetylase	
NR2F6 (Ear2)	Nuclear hormone receptor family	
ELF2 (NERF 1a)	TF; ETS family	
HDAC1,3,9	Histone deacetylases	
SUV39H1	Histone methyltransferase	

DS, Down's syndrome; AMKL, Acute megakaryocytic leukemia; HAT, Histone acetyl transferase; TF, Transcription factor

Table1.6: RUNX1 interacting proteins. The association of the interacting proteins with leukemia development is indicated.

Gene	Description	Affected cell type
Growth factors		
GM-CSF	Granulocyte-macrophage colony stimulating factor	granulocytes, macrophages, eosinophils & progenitors
IL-3	Interleukin-3	mature/immature myeloid / lymphoid
Receptors		
M-CSFR	Macrophage colony stimulating factor receptor	macrophages, monocytes / progenitors
TCR α, β, γ, δ	T-cell receptors α, β, γ, δ chain	T-cell
Surface molecules		
CD11a	CD11a integrin	T-cells, macrophages, neutrophils
CD36	CD36 antigen; cell surface glycoprotein	macrophages, monocytes
Signal molecules		
BLK	B-lymphocyte specific tyrosine kinase	B-cells
p21^{CIP1}	Cyclin dependent kinase inhibitor 1A	myeloid / lymphoid
Bcl-2	B- cell CLL/Lymphoma 2; prevent apoptosis	myeloid / lymphoid
p14ARF	Cyclin dependent kinase inhibitor 2A	myeloid / lymphoid
CCND3	Cyclin D3	myeloid / lymphoid
Transcription activators		
Fos	Osteosarcoma viral oncogene homologue; AP-1 component	
Others		
MPO	Myeloperoxidase	Myeloid
MMCP6	Mast cell protease 6	mast cells
ELA2	Neutrophil elastase	Myeloid
GZMB	Granzyme B	NK cells, cytotoxic T-cells

Table 1.7: Targets of Runx1 regulation (modified from Peterson and Zhang, 2004).

1.5.6 Role of Runx1 in hematopoiesis

Runx1 knockout mice have largely illustrated the role of Runx1 in hematopoiesis. In wild type mice, *Runx1* expression is seen as early as E8.5 at all sites from which definitive hematopoietic cells emerge later such as the yolk sac, the umbilical arteries and the AGM region (North et al., 1999). In *Runx1* knockout embryos, there is a complete absence of definitive fetal liver hematopoiesis though primitive hematopoiesis is not impaired **(Figure 1.7)**. *Runx1* knockout embryos are unable to generate definitive hematopoietic cells from the endothelial cells in the AGM region (Yokomizo et al., 2001) and they die at E12.5 (Okuda et al., 1996; Wang et al., 1996). Hemorrhaging is also seen in the *Runx1* knockout embryos. The areas where hemorrhages occur are very specific and correspond to areas of high expression of *Runx1*. Cellular necrosis precedes hemorrhaging, suggesting a developmental defect in the endothelial cells of the central nervous system capillaries (Wang et al., 1996).

E12.5

Runx1+/+   *Runx1-/-*
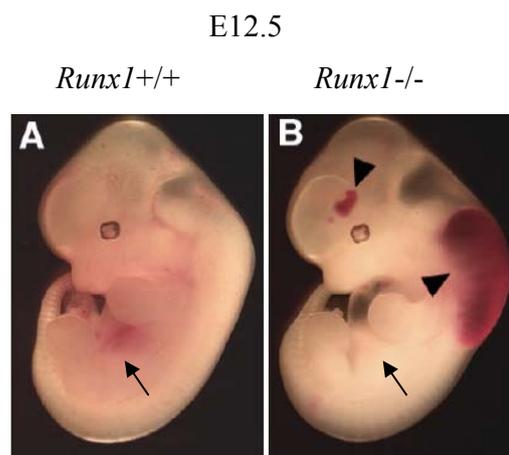
Figure 1.7: *Runx1* knockout embryos lack definitive hematopoiesis
Runx1 knockout mice die at E12.5 due to absence of fetal lever hematopoiesis (arrow). Arrowhead shows areas of hemorrhaging.

Conditional knockout mice have recently demonstrated the role of Runx1 in adult mouse hematopoiesis (Growney et al., 2005; Ichikawa et al., 2004; Putz et al., 2006; Taniuchi et al., 2002; Motoda et al., 2007). *Runx1*-excised adult mice have an expanded, immunophenotypically defined HSC compartment. These mice survive for more than a year, indicating that there is long-term HSC activity in these animals. This finding is surprising in light of the hematopoietic defect observed in *Runx1* knockout embryos, which leads to the conclusion that Runx1 may be dispensable for maintenance of HSC in adult hematopoiesis. On the other hand, Runx1 is required for efficient lymphoid maturation at multiple stages of differentiation. There is a significant reduction in the number of mature peripheral blood (PB) B-cells and BM derived B-cell precursors in primary *Runx1*-excised mice which suggests a significant block in B-cell maturation. In the T-cell lineage, there is a specific block in T-cell maturation during the transition from the DN2 (CD44+CD25+) to the DN3 (CD44-CD25+) stage.   In contrast to the pronounced inhibition of the lymphoid lineage, excision of *Runx1* does not inhibit maturation of the myeloid lineage. *Runx1*-excised mice demonstrate expansion of the myeloid lineage by several phenotypic and functional criteria, with no evidence of a block in myeloid development. Runx1 loss has no apparent effect on the erythroid lineage, but it does have a significant effect on megakaryocytic maturation. Runx1 is required for normal maturation of the megakaryocyte lineage but not for the establishment of this lineage, leading to an accumulation of megakaryoblasts in *Runx1*-excised mice. Thus, Runx1 deficient mice have a higher number of stem/progenitor cell fractions, show abnormal differentiation of megakaryocytes leading to accumulation of megakaryoblasts and thrombocytopenia, and accumulation of immature lymphocytes cells due to defective

T- and B-cell development (**Figure 1.8**). However, the lack of Runx1 in adult mice does not impair the production of erythrocytes and granulocytes.

▲ Affected in RUNX leukemia

▲ Affected in conditional *Runx1* knockout mouse
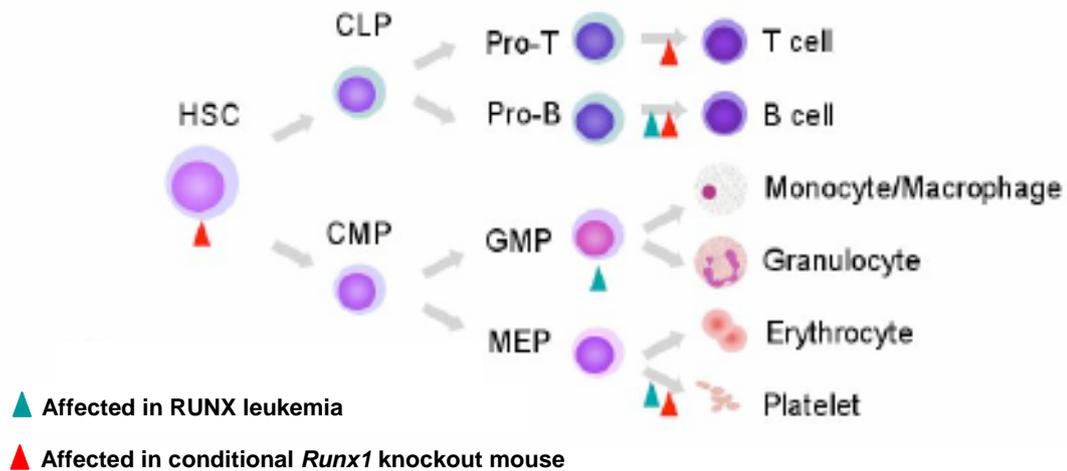
Figure 1.8: Adult hematopoiesis and affected lineages due to Runx1 deficiency (Ichikawa et al., 2004).

Ichikawa *et al.* did not report the development of leukemia or other disease phenotypes in their conditional *Runx1* knockout model. Another conditional knockout mouse model developed by Taniuchi *et al* also did not show any spontaneous leukemia phenotypes. Growney *et. al.* showed a mild myeloproliferative phenotype in *Runx1* excised mice, with mild expansion of myeloid cells in the BM and spleen. Nevertheless, there was no leukemia development in these mice. Putz *et al*. also showed the moderate myeloproliferation with splenomegaly in their *Runx1* conditional knockout model and some of the mice progressed to develop lymphoma at late stages of adulthood.

1.5.7 RUNX leukemia

The *RUNX1* gene is the most frequently mutated gene in human leukemias and genetic alterations of this gene are found in approximately 30% of all human AML. The genetic alterations of the *RUNX1* gene could be either because of chromosomal translocations resulting in fusion protein products with RUNX1 or mutations in the *RUNX1* gene itself.

1.5.7.1 Chromosomal Translocations

The PEBP2/CBF complex is one of the most frequent targets of chromosomal translocations associated with human leukemia and account for ⁓20–25% of adult AML. *RUNX1* is involved in many translocations and its co-factor *PEBP2β/CBFβ* is altered in an inversion of chromosome 16 associated with the AML M4Eo subtype (Liu et al., 1993) **(Figure 1.9)**. One of the most frequent translocations of the *RUNX1* gene is the t(8;21) that fuses *RUNX1* to the *Eight twenty-one (ETO)* gene (Licht, 2001). This translocation is found in 40% of patients with the AML M2 leukemia subtype. All molecularly defined *RUNX1*-translocations are found in leukemias of the myeloid lineage except for the *TEL-RUNX1* translocation, which is only found in the lymphoid lineage (Loh et al., 1998) (**Table 1.8**).

After translocation, a normal copy of the gene is still present in the affected cells since the translocation usually affects only one copy of the translocated gene. The fusion proteins resulting from the translocations can contribute to leukemia pathogenesis by a variety of mechanisms including:

- A dominant-negative effect of the fusion proteins on RUNX1 and/or its fusion partner
- A gain-of-function due to the fusion itself

- Haploinsufficiency of wild type RUNX1 and/or its fusion partner

- A combination of these mechanisms

Nevertheless, studies in mice suggested that the translocations are not sufficient for the development of leukemia and additional mutations are required (Rhoades et al., 2000).
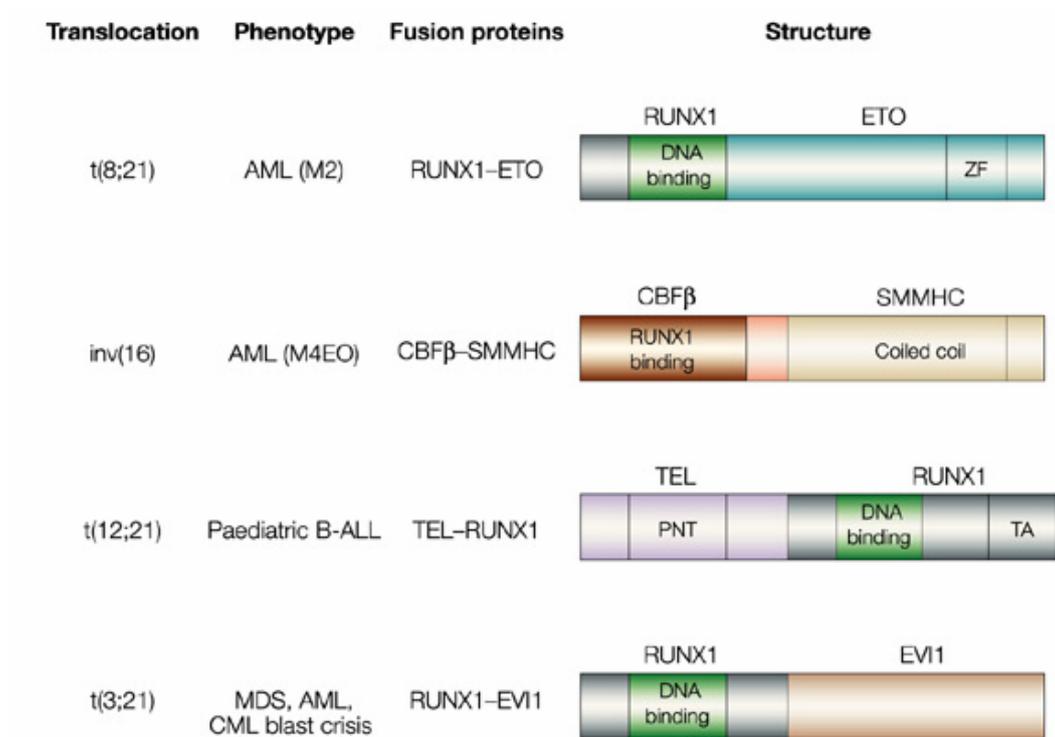
PNT, pointed domain; TA, transactivation domain; ZF, zinc finger

Figure 1.9: CBF fusion genes that are associated with leukemia
(Speck and Gilliland, 2002)

1.5.7.2   Somatic point mutations

Point mutations in the *RUNX1* gene have been identified in various sporadic myeloid malignancies including various AML subtypes, atypical CML and MDS (Osato et al., 2001; Osato et al., 1999). Majority of the mutations fall on the Runt domain and none of the typical leukemia-associated translocations are present in these leukemias. Monoallelic mutations in *RUNX1* may predispose myeloid hematopoietic stem/progenitor cells to the development of a hematological malignancy, but the affected lineage and consequent type of malignancy depends upon which genes subsequently sustain additional somatic mutations. Indeed the phenotypes of leukemic cells with monoallelic mutations are varied, suggesting the occurrence of additional genetic events in a number of different molecules. Biallelic *RUNX1* point mutations have also been observed and are tightly linked to the AML M0 subtype (**Table 1.8**) (Osato et al., 1999; Preudhomme et al., 2000). The AML M0 subtype is AML with minimal differentiation and shows accumulation of immature myeloid cells or blasts. AML M0 very frequently carries cytogenetic abnormalities such as -5/5q- or -7/7q- deletions and or complex karyotype and shows poor prognosis. *RUNX1* proteins derived from point mutants are proved to be non-functional as a result of which biallelic mutations may result in blocked differentiation and continuous growth of stem/progenitor cells at the earliest stage of myeloid differentiation, thus contributing to accumulation of immature cells.

1.5.7.3 Familial leukemia

Germ-line monoallelic *RUNX1* mutations have been identified in pedigrees with an autosomal dominant familial platelet disorder (FPD/AML; Song et al., 1999). Individuals

with the disease develop thrombocytopenia and show a propensity to develop AML. Because one pedigree harbors a complete deletion of one of the *RUNX1* alleles, it has been suggested that haploinsufficiency of RUNX1 is responsible for this disease (Song et al., 1999). Our laboratory has established the mouse model for FPD/AML using the BXH2 mouse strain, which carries an ecotropic retrovirus that infects neonates through the milk. BXH2-*Runx1+/-* mice show shorter latency of leukemia onset, suggesting that *Runx1+/-* status makes the mouse more leukemia prone (Yamashita et al., 2005).

Type	FAB Subtype	Phenotype	Associated genetic defects
AML	M0	Undifferentiated	Biallelic *RUNX1* mutations
	M1	Myeloblastic with maturation	
	M2	Myeloblastic with maturation	t(8;21) *RUNX1-ETO*
	M3	Promyelocytic	t(15;17) *PML-RARα*
	M4	Myelomonocytic	
	M4Eo	Myelomonocytic with BM eosinophilia	inv(16) *CBFβ-MYH11*
	M5	Monocytic	
	M6	Erythroleukemia	
	M7	Megakaryoblastic	*GATA1* mutation in DS patients
ALL	B Lymphocytic	B-cell leukemia	t(21;12) *TEL-RUNX1*
	T Lymphocytic	T-cell leukemia	
CML		Myelocytic	t(9;22) *BCR-ABL*
CLL		Lymphocytic	
MDS			

Table 1.8: Description of selected leukemia subtypes and associated genetic defect.

1.5.7.4 Increased RUNX1 gene dosage

Down syndrome (DS) patients with a constitutional trisomy 21, have a 10-20 fold increased risk of developing leukemia compared to the non-DS population. DS children account for approximately 2% and 13% of children diagnosed with ALL and AML respectively. The most frequent AML subtype observed in these children is the AML M7 subtype (megakaryocytic). The etiological role of trisomy 21 in leukemogenesis could be due to increased proliferation of leukemic progenitor cells due to overexpression of a tumorigenic protein coded by one human chromosome 21 gene, such as *RUNX1* (Osato and Ito, 2005). Furthermore, transgenic mice overexpressing *Runx1* under the control of the *GATA-1* promoter developed megakaryocytic leukemia as observed in DS patients (Yanagida et al., 2005), suggesting indeed that increased *RUNX1* gene dosage might be responsible for the leukemia predisposition observed in DS patients. Interestingly, trisomy 21 is also among the most commonly observed acquired chromosomal abnormalities in leukemic BM, particularly in ALL (Berger, 1997). Extra copies of RUNX1 (2-8 copies) due to tandem repetition of chromosome 21 or the presence of a non-constitutional chromosome 21 were also observed in several patients with acute lymphoblastic leukemia (ALL) (Ferro et al., 2004; Mikhail et al., 2002). Together, an increase of *RUNX1* dosage may also contribute to leukemogenesis.

1.5.7.5 Multistep development of RUNX leukemias

Various genetic alterations mentioned above such as translocations and point mutations in the *RUNX1* gene lead to loss-of-function of RUNX1 which results in hematopoietic abnormalities to some extent. However, a series of mouse models designed to recapitulate

genetic alterations in human leukemias such as *RUNX1-ETO* conditional knock-in mice or majority of the *Runx1* conditional knock-out mice models failed to develop spontaneous leukemia (Growney et al., 2005; Ichikawa et al., 2004; Higuchi et al., 2002). Moreover, chimeric genes involving *RUNX1* are detected even in healthy volunteers (Basecke et al., 2002; Mori et al., 2002). Studies of children with t(12;21) translocation, having a chimeric gene *TEL-RUNX1*, showed that the translocation sometimes occurs *in utero* and that leukemia develops only after up to 9 years of latency (Ford et al., 1998).

Such ample evidence points to the model of multi-step leukemogenesis in which *RUNX1* alteration *per se* does not readily result in leukemia but additional genetic changes, or "second hit", are required for full-blown leukemia **(Figure 1.10)**. The affected lineage and consequent type of leukemia, following a *RUNX1* mutation, may depend upon which genes are somatically mutated as secondary hits (Michaud et al., 2003). To identify the additional genetic alterations that cooperate with *RUNX1* abnormality, our group has applied retroviral insertional mutagenesis in our experimental system.

1.6 Retroviral insertional mutagenesis (RIM)

RIM is a powerful tool to identify oncogenes and tumor suppressors that are important for carcinogenesis. In our laboratory, RIM has been used to study the effect of *Runx1* alteration on leukemogenesis and to identify cooperative genetic alterations in RUNX1 related leukemias.
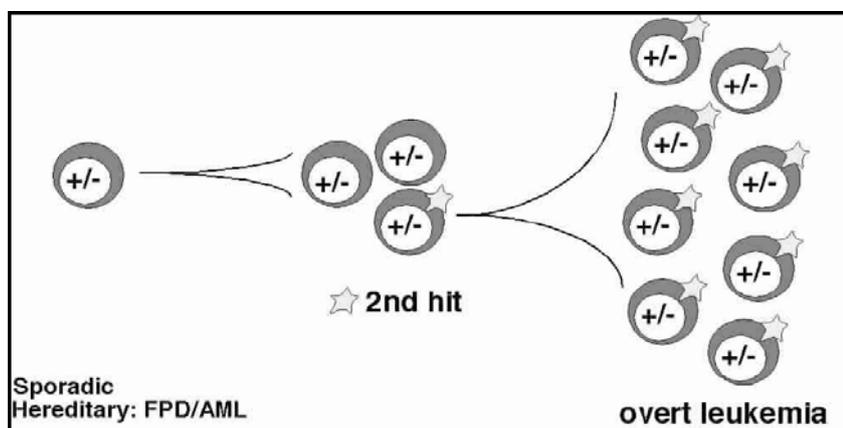
Figure 1.10: Secondary hit is required for full blown RUNX leukemia.
Mutations in *RUNX1* gene only predispose for the development of leukemia and secondary mutations are necessary for development of full blown leukemia (Reproduced from Osato et al., 2001).

1.6.1. Mechanism of RIM

Oncogenic retroviruses are generally divided into two categories.

(1) The acute transforming retroviruses induce polyclonal tumors within 2-3 weeks after infection. Their viral genes are replaced with transduced cellular gene, or a part of a gene, that is responsible for the oncogenic activity.

(2) The slow-transforming retroviruses cause mono- or oligoclonal tumors after a latency period of several months. They do not carry viral oncogenes and their capacity to induce

neoplasms is based on the ability of their provirus to integrate in the host DNA, and mutate or transcriptionally activate flanking cellular genes. Insertions can occur over a broad distance (approximately 300 kb) upstream or downstream of many targeted loci, and the provirus can be in the same transcriptional orientation as the gene or in the opposite orientation (Jonkers and Berns, 1996). In this case, there are basically three mechanisms by which expression of host genes is affected by retroviral integration (**Figure 1.11**).

(a) Activation by promoter insertion:

When a provirus integrates upstream of a proto-oncogene in the same transcriptional orientation, the promoter and enhancer elements in the proviral long terminal repeats (LTRs) can direct increased levels of proto-oncogene expression. In this case, one of the LTRs is fused with the targeted proto-oncogene.

(b) Transcriptional enhancement:

Many proviruses are inserted upstream of target genes or downstream from target genes in either orientation. These proviruses seem to increase gene expression by placing the gene under the influence of strong enhancer elements within the retroviral U3 region. Occasionally, the proviruses are separated from the gene by as much as 300 kb. Transcriptional enhancement is probably the most frequent mechanism of gene activation by insertional mutagenesis, although the precise mechanism remains unclear.

(c) Protein truncation by transcription termination:

The integration of a provirus into the transcription unit can have adverse effects on the protein product. If viral insertion disrupts coding domains, the protein sequence can be completely mutated in such a way that an aberrant gene product with abnormal biologic

activity is produced. There are a few cases whereby viral integration inside a gene also results in activation of gene due to production of a constitutively active truncated protein such as activating integrations in the *Notch* gene (Feldman et al., 2000).

1.6.2. The identification of oncogenes or tumor suppressors by RIM

Somatic and clonal integration of retroviruses in the host genome may enhance proto-oncogene expression or inactivate tumor suppressor genes. A cell that has such integrations acquires growth advantage, and is then clonally selected to become tumorigenic. In these cases, the oncogenes/tumor suppressors responsible for tumorigenesis can be identified by using proviral DNA as 'a molecular tag'. High throughput cloning of retroviral integration sites (RIS) has been accomplished by employing the inverse polymerase chain reaction (IPCR) technique, and database mapping of RIS by using the mouse genome database (Yanagida et al., 2005; Suzuki et al., 2002). These studies have enabled the identification of multiple retroviral insertion sites in each tumor, and it could clarify cooperative genetic interactions in tumorigenesis, as retrovirally induced leukemias/lymphomas usually contain multiple integrations of proviral genomes within a single tumor clone. Practically, retroviral insertions have been achieved either in mouse strains with high endogenous retrovirus expression, such as BXH2 strain, or in the mice infected by replication competent retroviruses, such as Moloney Murine Leukemia Virus (MoMuLV) (Nakamura, 2005; Jonkers and Berns, 1996).

Cooperative genetic alterations might be identified using a simple insertional mutagenesis model. However, a more efficient device is needed to clarify cooperative

genes for particular genes. RIM on genetically engineered animal models that are predisposed to tumor formation is therefore a good method for identification of cooperative genes of the primary genetic defect in these animals. When the cells, which already have primary genetic abnormality, receive additional genetic alterations by retroviral insertion and develop cancer, then the integration serves to add specific cooperative genetic changes, or "second hit", for the primary alterations (Nakamura, 2005; Yanagida et al., 2005; Yamashita et al., 2005). In other words, a gene can be defined as a putative cooperating gene if it is more frequently targeted in mice with specific genetic background compared to wild type under the same retroviral insertional stress. I have applied this RIM method on *Runx1* conditional knockout mice models to identify genes cooperating with Runx1 deficient status in leukemogenesis.
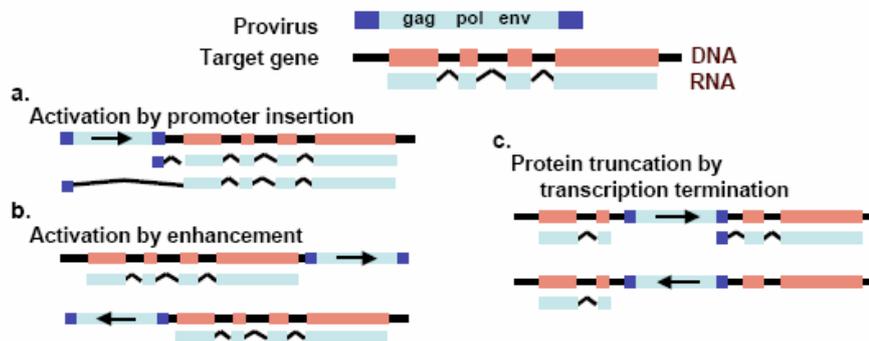
Fig. 1.11: RIM of host genes
Structural features of an integrated provirus and different modes of proviral activation/inactivation of a hypothetical target gene. Colored boxes on DNA represent exons and their transcripts are depicted below on RNA. (Modified from Jonkers and Berns, 1996)

1.7. Aims of the project

It is clear that loss-of-function of RUNX1 contributes to the pathogenesis of RUNX1 related leukemia, as discussed in the previous section. On the other hand, it is equally obvious that *RUNX1* alteration alone is insufficient for development of full blown leukemia and these alterations probably only result in a preleukemic state.

This leads us to the first question to decipher the mechanism of RUNX1 related leukemia. Why is loss-of-function of RUNX1 preleukemic and not completely leukemogenic? The conditional *Runx1* knockout mice provide an excellent model to answer this question. The *Runx1* gene is knocked out in the mice after they reach adulthood and therefore, these mice probably recapitulate the state of loss-of-function of RUNX1 in humans. The first aim of my project is to gain an understanding of why loss-of-function of Runx1 is only preleukemic and does not result in full blown leukemia, using hematopoietic cells from BM of conditional *Runx1* knockout mice.

The next question is how does loss-of-function of Runx1 finally result in leukemia? The general consensus is that additional genetic alterations are required for leukemia development. Therefore, the second aim of my project is to identify genetic alterations that may cooperate with Runx1 deficiency in leukemogenesis, especially genetic alterations that help to overcome the inherent defect in Runx1 deficient cells that prevent them from developing full blown leukemia. To address this question, I employed RIM on conditional *Runx1* knockout mice.

The final aim is to verify the cooperation between Runx1 deficient status and identified genetic alterations in the initiation and/or progression to leukemia and gain some mechanistic understanding of this cooperation.

Chapter 2 - Materials and Methods

Generation of mice

The mice harboring *Runx1* alleles flanked by loxP sites were generated by Ichiro Taniuchi *et a*l (2002). *Runx1*$^{F/+}$ mice were backcrossed against C57BL/6 mice for 3 generations, and then intercrossed to obtain *Runx1*$^{F/F}$ mice. They were crossed with interferon-inducible Mx-Cre (myxovirus resistance 1 gene promoter driven Cre recombinase) transgenic mice (Kuhn et al., 1995), a gift from Dr. K. Rajewsky, to generate *Runx1*$^{F/F}$— Tg(*Mx1*-Cre) mice. *Runx1*F/F—Tg(*Mx1*-Cre) mice were then mated to *Runx1*$^{F/F}$ mice to generate *Runx1*$^{F/F}$—Tg(*Mx1*-Cre) and *Runx1*$^{F/F}$ littermates for various experiments. In *Runx1*$^{F/F}$—Tg(*Mx1*-Cre) mice, exon 4 encoding part of Runt domain on both alleles can be deleted by conditionally expressed Cre-recombinase.

For RIM experiments, *Runx1*$^{F/F}$—Tg(*Mx1*-Cre) mice were mated with *Runx1*$^{F/F}$ mice and progenies were intraperitoneally injected with Moloney Murine Leukemia Virus (MoMuLV) 3 days after birth. They were genotyped by PCR at the age of one month, using their tail genomic DNA extracted by Direct PCR Lysis Reagents (Viagen Biotech, USA) as template. One month after birth, mice were subjected to 600 μg of polyinosinic-polycytidylic acid (pIpC; Sigma, USA) intraperitoneal injection on seven alternate days for excision of the floxed alleles with greater than 90% efficiency (Growney et al., 2005). For other experiments, to obtain conditional *Runx1* knockout and wild type littermate control BM, progenies of *Runx1*$^{F/F}$—Tg(*Mx1*-Cre) and *Runx1*$^{F/F}$ parents were genotyped after a month and subjected to pIpC treatment as described above at one month of age or later when required. pIpC treated mice were checked for the

deleted *Runx1* alleles by PCR using their PB DNA one month after the final pIpC injection. All mice were maintained in Biological Resource Center (BRC), Biopolis, Singapore and all animal experiments followed the strict guidelines set by National Advisory Committee for Laboratory Animal Research (NACLAR).

Hematological analysis

Retrovirus injected conditional *Runx1* knockout mice and wild type littermates were monitored for the development of leukemia by examining their health condition and by weekly checking of complete blood cell count (CBC) by an automatic hematology analyzer (Celltac alpha MEK-6358, Nihon Kohden, Japan). Severely moribund mice, often with a rapid elevation of leukocyte count, were euthanized and subjected to necropsy. Abnormalities in hematopoietic tissues were recorded as follows: enlargement of the thymus, liver, and spleen, and swelling of lymph nodes. Leukemic cells from the PB, BM and spleen were subjected to May-Giemsa staining. Immunophenotypic analysis of leukemic cells was carried out by flow cytometry. The following antibodies (Pharmingen, San Diego, CA, USA) were used: PE – conjugated Mac1 (M1/70), c-Kit (2B8), Fas (MFL3), CD3(145-2C11), CD8 (53-6.7), TCRδ (GL3) (Caltag Labs, Burlingame, CA, USA), CD44 (IM7), CD61 (2C9.G2), Ter119 (TER-119) and FITC – conjugated Gr1 (RB6-8C5), CD34 (RAM34), CD4 (RM4-5), TCRβ (H57-597), CD25 (7D4), B220 (RA3-6B2), CD19 (1D3), CD41 (MWReg30) and CD71 (C2). low cytometry analysis was performed using a fluorescence activated cell sorting (FACS) vantage instrument with the Cellquest program (Becton Dickson, USA) after addition of 2 mg/ml of propidium iodide (PI; Sigma) to exclude dead cells.

Identification of retroviral integration sites by inverse PCR

In all, 5 μg of genomic DNA extracted from mouse leukemic cells from the spleen or thymus was digested by *Bst*YI and self circularized overnight by T4 DNA ligase (New England Biolabs, Inc., MA, USA) at 16°C. Then, 5' and 3' integration flanking fragments were amplified individually by iPCR. The first PCRs were performed with AccuPrime Taq (Invitrogen life technologies, Carlsbad, CA, USA) employing an initial preheating step at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 60°C for 1 min and 68°C for 3 min. The second PCRs were performed by rTaq (Promega, Madison, WI, USA) employing an initial preheating step at 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 68°C for 2 min. The amplified PCR products were cloned by the TA cloning method using the plasmid pGEM-T vector (Pharmingen Co, Madison, WI, USA) and subjected to cycle sequencing (Applied Biosystems, Foster City, CA, USA) using the M13-Reverse primer. The position mapping of the RIS on the mouse chromosome was performed by BLAT searching of the UCSC Genome Bioinformatics database (http://genome.ucsc.edu). The definition of a common integration site (CIS) was the same in the retroviral-tagged cancer gene database (RTCGD) (http://RTCGD.ncifcrf.gov); each window size is 100, 50, or 30 kb for CIS with four (or more), three, or two insertions, respectively, in each model (Akagi et al., 2004).

Plasmid construction

The MIG retroviral vector (MSCV-IRES-GFP) was used for cloning human *GFI1*, *EVI5* and *EVI1*. The MIG vector is composed of MSCV (murine stem cell virus) LTRs (long terminal repeat), IRES (internal ribosome entry site) and *EGFP* (enhanced green

fluorescent protein) gene. Human *EVI5* and *GFI1* fragments were amplified by PCR method using Accuprime Taq (Invitrogen, USA) and template complementary DNAs (cDNA) from U937 cell line. The PCR was performed using gene specific primers complimentary to the ends of the gene with *EcoR*I recognition sequence attached to forward primer and *Not*I recognition sequence attached to reverse primer. PCR cycling conditions included preheating step at 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, 58°C for 40 sec and 68°C for 3 min, and a final extension step at 68°C for 10 min. The PCR products were purified by QIAquick PCR Purification Kit (QIAGEN, Germany), digested with *EcoR*I and *Not*I and cloned into similarly digested MIG vector using standard protocol. The sequence of each clone was verified by cycle sequencing (Applied Biosystems). Human *EVI1* construct was a gift from Dr. K. Moroshita. The *EVI1* gene was subcloned into the MIG vector after restriction digestion from the original construct. All the amplified vector plasmids were purified by EndoFree Plasmid Maxi kit (QIAGEN GmbH, Germany).

Packaging cell line and retroviral transduction

Phoenix-Eco packaging cell line was kindly provided by Dr. G. P. Nolan. The cells were selected by Hygromycin B (0.36 units/ml) and diphtheria toxin (1 mg/ml) (Calbiochem, San Diego, CA, USA) in low glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA) and 1% antibiotic-antimycotic (Gibco) for one to two weeks. Two 100 cm dishes of selected cells were transiently transfected with 120 μg of each constructed plasmid using Fugene (Roche, Mannheim, Germany). The medium was changed to high glucose DMEM 12

hours after the transfection. The supernatant containing retrovirus was collected twice, at 36 hours and 60 hours after transfection and centrifuged at 8000 rpm (revolutions per minute) at 4°C for 12 hours and resuspended in 1 ml of Minimum Essential Medium alpha Medium (αMEM) (Gibco) containing 10% FBS and 1% antibiotic-antimycotic. 500 µl of virus solution was added together with 25-50 µg/ml of Retronectin (TAKARA, Japan) to 1-2.5 x 10^6 BM cells pre-cultured under cytokine stimulation as described below. Two rounds of spin infection of target cells were performed at 2000 rpm at 30°C for two hours, one after each harvest of retrovirus. Subsequently the plate was re-cultured at 37°C. The virus transfected BM cells were harvested 24 hours after the second spin infection (i.e. 48 hours after the first spin infection) for further experimental use.

Bone marrow cells collection

For the experiments using BM cells, the host mice were injected with 2.5 mg of 5-FU (five fluorouracil; Sigma) per 500 µl of phosphate buffered saline (PBS) per mouse five days prior to the BM collection to get the best yield of stem/progenitor cell fraction. BM cells were collected from four limbs of each mouse by flushing media through the marrow of the bones. The RBC were first lysed by lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM Na2EDTA), remaining BM cells washed with cold PBS and resuspended in αMEM supplemented with 10% FBS and 1% antibiotic-antimycotic. In the experiments using retrovirally transfected BM cells, 1-2.5 x 10^6 cells were cultured in 1.5 ml of αMEM supplemented with 10% FBS, 1% antibiotic-antimycotic and 10 ng/ml recombinant murine interleukin-3 (IL-3), 20 ng/ml interleukin-6 (IL-6) and 10 ng/ml stem cell factor (SCF) (all cytokines are from Pepro Tech EC Ltd, USA) per well of a 6

well plate. BM cells were preincubated for 24 hours before the first spin infection of retrovirus.

Bone marrow transplantation (BMT)

BM cells collected from pIpC treated $Runx1^{F/F}$—Tg($Mx1$-Cre) and $Runx1^{F/F}$ mice were transfected with MIG vector containing gene of interest. After one day of culture following spin infection, the cells were collected in αMEM media and 5×10^5 cells were transplanted into sublethally (6 Gy) irradiated recipient C57BL/6 mice around 8 weeks old which were given acid water one week prior to irradiation. GFP+ cells were not selected after the transduction protocol, so transplants consisted of a mixture of transduced and nontransduced cells. Transplanted recipient mice were monitored at least once a week and at several points after transplantation, blood was drawn from the retro-orbital plexus and total cell numbers were counted. The reconstitution ability of donor cells was also assessed at the same time points by flow cytometric analysis of GFP+ cells in PB.

For serial BMT experiments, initial steps were similar to that described above. A mixture of 5×10^5 transfected and non transfected BM cells were transplanted into sublethally (8 Gy) irradiated primary recipients. At an average of 4 months after primary transplantation, the primary recipients were sacrificed and BM cells extracted. BM cells were processed to eliminate RBCs by lysing, following which 5×10^5 cells were immediately transplanted into sublethally (8 Gy) irradiated secondary recipients. Survival, PB counts and percentage of GFP positive cells in the blood were monitored at regular intervals after transplantation.

***In vivo* homing assay**

BM cells from pIpC treated *Runx1*^F/F^—Tg(*Mx1*-Cre) and *Runx1*^F/F^ mice were isolated from hind/forelimbs by flushing, followed by RBC lysis. The BM cells were stained with 2.0 μM carboxy fluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes/Invitrogen, Carlsbad, CA) at a concentration of 10^6 cells/ml for 15 min at 37°C, washed, and further incubated at 37°C for 30 min in cell culture medium, according to manufacturer's instructions. 5 x 10^6 stained cells were then transplanted into lethally irradiated (10 Gy) recipient mice. 16 hours after transplantation, BM cells were isolated from recipients and directly analyzed on a FACS vantage.

Flow cytometric analysis

For flow cytometric analyses, BM cells either directly collected from mice or harvested after viral transfection, were preincubated with mouse serum for 15 min on ice before staining. The antibody reaction was carried out in the mouse serum for additional 15 min on ice. All the labeled monoclonal antibodies were purchased from BD PharMingen, USA. For c-Kit single staining and analysis/sorting, PE-conjugated anti-c-Kit (2B8) was used. For KSL CD34 fraction analysis, the following antibodies were used: PE-conjugated anti-Gr1 (RB6-8C5), Mac-1 (M1/70), Ter119 (TER-119), CD4 (RM4-5), CD3 (145-2C11), CD8 (53-6.7), B220 (RA3-6B2), IL7Rα (SB/199), APC-conjugated anti-c-Kit (2B8), APC-Cy7 conjugated anti-Sca-1 (E13-161.7) and FITC-conjugated CD34 (RAM34). Flow cytometric analysis was performed using FACS vantage instrument with the Cellquest program after addition of 2 mg/ml of propidium iodide to exclude dead cells.

Long-term culture-initiating cell (LTC-IC) assay

BM cells collected from pIpC treated $Runx1^{F/F}$—Tg($Mx1$-Cre) and $Runx1^{F/F}$ were transfected with mock MIG vector or MIG vector containing gene of interest and sorted for c-Kit+GFP+ fraction using FACS vantage. 10^4 sorted cells were cultured on OP9 stromal cells per well in a 6-well plate in 2 ml of αMEM media supplemented with 10% FBS, 1% antibiotic-antimycotic and 10 ng/ml recombinant murine IL-3, 100 ng/ml G-CSF, 10 ng/ml SCF and 10 ng/ml EPO (Pepro Tech EC Ltd). Twice a week, 500 μl of medium with original concentration of cytokines was supplemented into each well. Every fourteen days, the floating cells were collected and the well was refilled with fresh cytokines/medium. On day 30, OP9 stromal cells together with adherent hematopoietic cells were trypsinized and harvested, resuspended in 1 ml of αMEM containing 10% FBS and 1% antibiotic-antimycotic. The harvested cell suspension was replated in a new well and was incubated at 37°C for 30 min to 45 min. This procedure allows the OP9 cells to adhere to the bottom of the culture dish first, so that floating hematopoietic cells can be separately collected. These floating hematopoietic cells collected from each well were then subjected to colony-forming unit-culture (CFU-C) assays and another round of long term culture on OP9 stromal cells for 30 days.

Colony-forming unit-culture (CFU-C) assay

10^4 cells were cultured in 35-mm dishes in either duplicate or triplicate in 1 ml Methocult M3131 methylcellulose medium (StemCell Tec., Canada) containing 1% antibiotic-antimycotic supplemented with 10 ng/ml recombinant murine IL-3, 10 ng/ml SCF, 100 ng/ml G-CSF and 10 ng/ml EPO. The dishes were cultured at 37°C. Colony formation

(one colony consists of more than 30 cells) was scored on day 10. For serial replating assays, colonies were harvested in cold PBS, centrifuged at 1500 rpm for 5 min to collect cells and colony assay was repeated using 10^4 cells for next round.

Luciferase Assay

The luciferase reporter plasmid PGL3-CXCR4 promoter (860 bp)-luc was a gift from Dr. Wilhelm Krek. The reporter plasmid (0.2 μg) and the effector plasmids, pEF-RUNX1 (0.2 μg), pEF-R174Q (0.2 μg) and pEF-PEBP2β (0.1 μg) were transfected into HL60 cells by a nonliposomal transfection reagent, FuGENE6 (Roche). The transfected cells were incubated for 48 hours and assayed for luciferase activity as previously described (Osato et al., 1999).

Quantitative real-time PCR (qRT-PCR)

The cKit+GFP+ fraction of BM cells transduced with MIG vector was sorted by FACS directly into TRIZOL LS Reagent (Invitrogen, USA). RNA extraction was performed following the manufacturers instruction. cDNA was synthesized by Expand Reverse Transcriptase (Roche Molecular Biochemicals) and subjected to qRT-PCR. The real-time PCR was performed using ABI prism 7900HT (Applied Biosystems) with an initial step of 95°C for 10 min followed by 40 cycles of 94°C for 15 sec and 60°C for 1 min. The specific primers and TaqMan probes were purchased from Applied Biosystems. All the reactions were performed in triplicate. Relative quantity of expression was calculated by SDS software ver.2.2.2 using Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as an endogenous internal control.

Cytospin preparation

$5 - 10 \times 10^4$ cells per well were cytospun using a cytocentrifuge (Cytospin4, Thermo Shandon, USA) on a slide. The slides were first stained with May-grunwald stain solution (May-grunwald solution (BDH, UK) : Methyl Alcohol = 1:3) for 5 min. After running off the solution, the slides were stained with Giemsa stain solution (Giemsa solution (BDH): phosphate buffer (pH 6.4, KH2PO4 6.63 g, Na2HPO4 2.56 g / 1000 ml) = 1:20) for 30 min at room temperature and washed with running water, dried.

Chapter 3 - Results

Runx1 knockout stem/progenitor cell expansion is followed by stem cell exhaustion

Loss-of-function of RUNX1 is frequently seen in human AML, especially in adults. However, _RUNX1_ alteration alone is considered to be preleukemic rather than fully leukemogenic. Conditional deletion of _Runx1_ in adult mice provides a good animal model system to understand the consequence of loss-of-function in hematopoiesis and leukemogenesis. Of special interest is the effect of _Runx1_ alteration on the hematopoietic stem/progenitor cell compartment since this population of cells seems to be the main target of oncogenic events that could give rise to leukemia. Hence, analyses of the short term and long term effects of Runx1 deficiency in hematopoietic stem/progenitor cells of conditional _Runx1_ knockout mice could shed some light on preleukemic effects of loss-of-function of Runx1 and additional steps that are required for full blown leukemogenesis.

Runx1 null BM cells generated by Cre-recombinase-mediated knock-out of _Runx1_ (referred to as _Runx1-/-_ in this thesis) show an increase in hematopoietic stem/progenitor cell fraction (Growney et al., 2005; Ichikawa et al., 2004; Putz et al., 2006) . There is more than two fold increase of the c-Kit+Sca1+Lin- (KSL) stem cell fraction and c-Kit+Sca1-Lin- progenitor cell fraction in _Runx1-/-_ mice, compared to wild type littermates (referred to as _Runx1+/+_ in this thesis). Despite the increased number of stem cells, Growney _et al_. reported that _Runx1-/-_ BM cells are outcompeted by simultaneously transplanted wild type BM cells in competitive repopulation assay, indicating that _Runx1-/-_ cells are compromised in reconstituting hematopoiesis in the recipient mice.

I observed similar results in our laboratory. Recipient mice were transplanted with BM cells from *Runx1-/-* and *Runx1+/+* mice transfected with retroviral vector expressing EGFP as a surrogate marker. Contribution of donor *Runx1+/+* and *Runx1-/-* cells to hematopoiesis in recipients was monitored periodically by checking the percentage of GFP positive cells in the PB, hereafter referred to as GFP chimerism. *Runx1+/+* BM cells showed stable contribution to hematopoiesis in recipients throughout while *Runx1-/-* BM cells showed decreasing contribution progressively. Six weeks after transplantation, the GFP chimerism in PB of the recipients that received *Runx1+/+* cells was similar to that of the recipients of *Runx1-/-* cells with a mean value of 32.1% and 23.6% respectively in a cohort of 6 recipient mice. After 40 weeks, the mean GFP chimerism in recipients of *Runx1+/+* cells remained the same, at 32.5%; however, the GFP chimerism in the recipients of *Runx1-/-* cells progressively decreased and by 40 weeks after transplantation, it was significantly lower than that in the recipients of *Runx1+/+* BM cells, with a mean of 13.1% **(Figure 2.1)**.

By 2 years after transplantation, the GFP chimerism in PB of recipients of *Runx1-/-* cells had dropped even more significantly and the difference between GFP chimerism of recipients of *Runx1-/-* cells and *Runx1+/+* cells was more pronounced **(Figure 2.2A)**. There was also a concomitant decrease in absolute number of immature (ckit+Lin-) *Runx1-/-* cells in the BM of the recipients, indicating lower number of *Runx1-/-* stem cells, which may explain the decreasing reconstitution of hematopoiesis **(Figure 2.2B)**. This suggests that *Runx1-/-* stem cells undergo stem cell exhaustion.
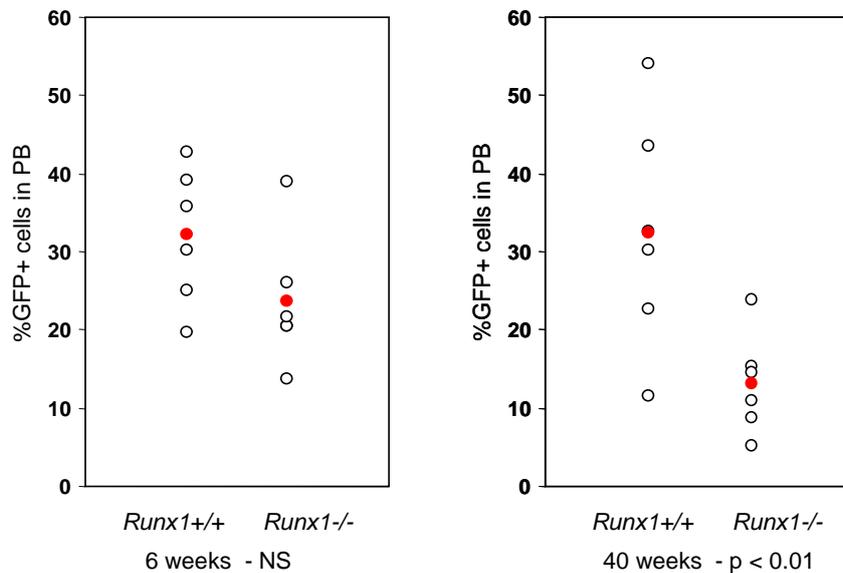
Figure 2.1: *Runx1-/-* stem cells are impaired in long term reconstitution of hematopoiesis
GFP chimerism in PB of recipients of *Runx1+/+* (n=6) and *Runx1-/-* (n=6) cells 6 and 40 weeks after transplantation. Each open circle represents data from an individual mouse and closed red circle is the average of a cohort. Stastical difference using unpaired student-t test are given at the bottom. NS, not significant.

However, surprisingly, colony assay of immature (c-Kit+Lin-GFP+) *Runx1-/-* and *Runx1+/+* cells from the BM of recipient mice showed that they were functional even 2 years after transplantation. In fact, immature *Runx1-/-* cells formed higher number of colonies than immature *Runx1+/+* cells, similar to the observations soon after the conditional deletion of *Runx1* gene by pIpC injection **(Figure 2.2C)**. This indicates that immature *Runx1-/-* cells maintain their inherent properties of increased proliferation even after long periods of time, suggesting that stem cell exhaustion of *Runx1-/-* cells may not be due to alterations in cell intrinsic machinery.
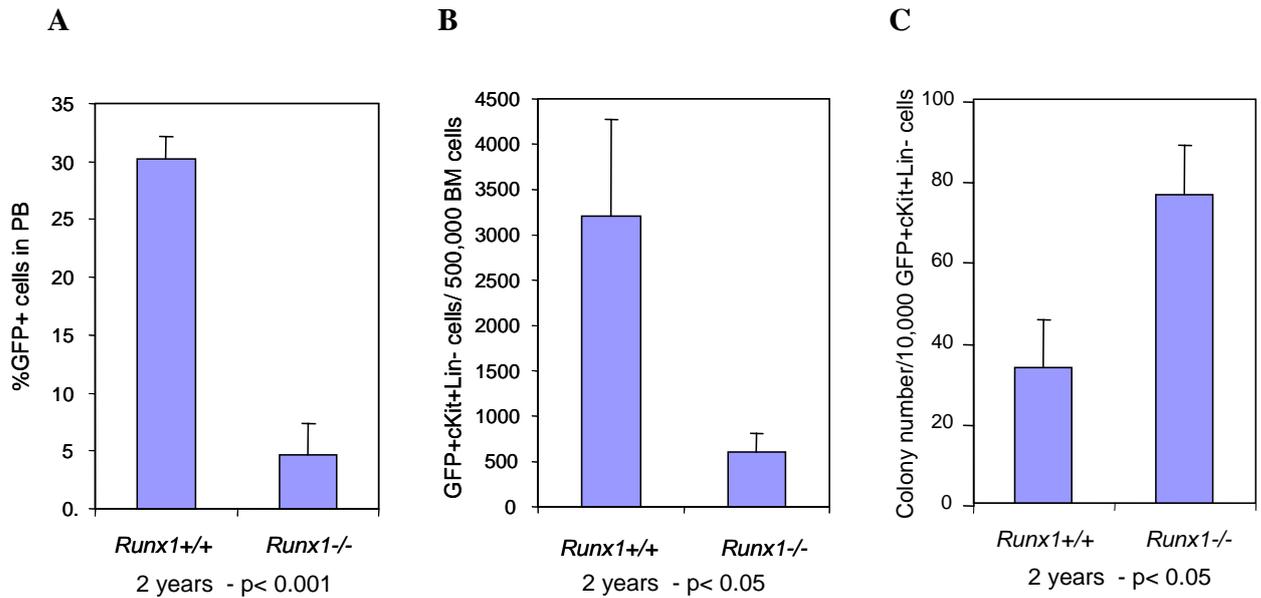
Figure 2.2: Immature *Runx1-/-* cell numbers decrease progressively, resulting in lower reconstitution of hematopoiesis, but they form higher number of colonies
Analysis of recipients of *Runx1+/+* and *Runx1-/-* BM cells 2 years after transplantation - Graphs showing (A) percentage of GFP+ cells in PB; (B) numbers of GFP+cKit+Lin- cells in BM; and (C) colony assay of GFP+cKit+Lin- cells from the recipient BM. Stastical difference using Mann-Whitney U test are given at the bottom

Serial transplantation experiment was then carried out to confirm if the *Runx1-/-* stem cells undergo exhaustion and are impaired in long term reconstitution of hematopoiesis. *Runx1+/+* and *Runx1-/-* BM cells were transfected with MIG vector and transplanted into sublethally irradiated (8 Gy) primary recipients. GFP chimerism was monitored for an average of 4 months in these recipients. Following that, two to three primary recipients with similar GFP chimerism were sacrificed; and BM cells were transplanted into 10 sublethally irradiated (8 Gy) secondary recipients. 6 out of 10 recipients of *Runx1-/-* cells died within three months of secondary transplantation, while all the recipients of control *Runx1+/+* cells are alive to date **(Figure 2.3)**. This result

60

indicates that *Runx1-/-* stem cells are impaired in reconstituting hematopoiesis in the secondary recipients and majority of them die due to pancytopenia; unlike *Runx*1+/+ stem cells which are capable of reconstituting hematopoiesis in the secondary recipients. The serial transplantation results confirm the fact that *Runx1-/-* stem cells undergo exhaustion.
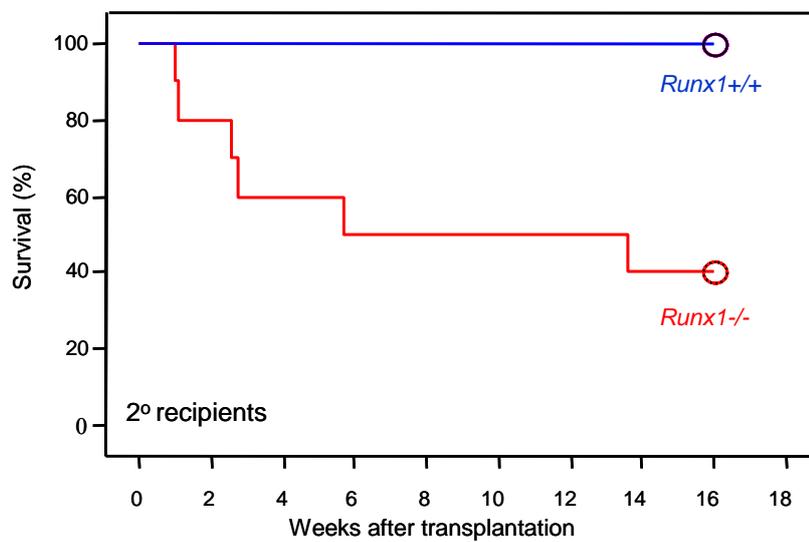
Figure 2.3: High mortality in secondary recipients of *Runx1-/-* BM cells
Kaplan-Meier survival curves of secondary recipients of mock MIG vector transfected *Runx1+/+* (blue line; n=10) and *Runx1-/-* (red line; n=10) BM cells, 4 months after primary transplantation. Circles represent end point of analysis.

BMT of cells from aged *Runx1-/-* and *Runx1+/+* mice also lends support to the above observations. BM cells obtained from *Runx1*$^{F/F}$— Tg(*Mx1*-Cre) mice 6 months after conditional deletion of *Runx1* by pIpC injection and similarly treated wild type, *Runx1*$^{F/F}$, littermates were transplanted into sublethally irradiated (8 Gy) recipients (n = 4

to 5) and GFP chimerism of PB was assessed at selected time points. The GFP chimerism in PB of recipients of *Runx1-/-* cells (average of 38.2%) was significantly lower than that of the recipients of *Runx1+/+* cells (average of 61.3%) right from the first time point which was 4 weeks after transplantation **(Figure 2.4)**. Lower GFP chimerism of *Runx1-/-* cells was also seen during subsequent time points such as 6 and 8 weeks after transplantation. In few of the recipient mice that were sacrificed and analyzed 2 months after transplantation, the GFP chimerism in BM was also significantly lower in the recipients of *Runx1-/-* cells than *Runx1+/+* cells. This indicates that in aged *Runx1-/-* mice, the stem/progenitor cells are already slowly undergoing the process of exhaustion as a result of which, they cannot repopulate the recipient mice like stem cells from *Runx1+/+* mice or younger *Runx1-/-* mice.

We further investigated the frequency of long term (LT) and short term (ST) HSC in *Runx1+/+* and *Runx1-/-* KSL fraction by checking CD34 expression. Only CD34- LT-HSC, but not CD34+ ST-HSC, amongst KSL fraction are believed to be quiescent and capable of long term reconstitution of hematopoiesis (Osawa et al., 1996). Consistent with our data indicating stem cell exhaustion, there was a shift in *Runx1-/-* KSL cells towards CD34+ ST-HSC fraction while *Runx1+/+* KSL cells showed both CD34- (LT-HSC) and CD34+ (ST-HSC) peaks **(Figure 1D)**. The average percentage (n=4) of CD34- cells was 50.5% in *Runx1+/+* KSL cells and 27.1% in *Runx1-/-* KSL cells **(Figure 2.5)**.
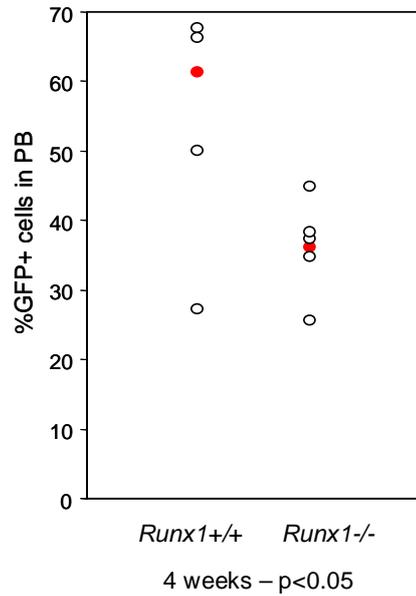
Figure 2.4: Early defects in hematopoietic reconstitution by aged *Runx1-/-* cells
Graph showing reconstitution of recipients by BM cells from aged *Runx1+/+* (n=4) and
*Runx1-/- (*n=5) mice four weeks after transplantation. Each open circle represents data
from an individual mouse and closed red circle is the average of a cohort.
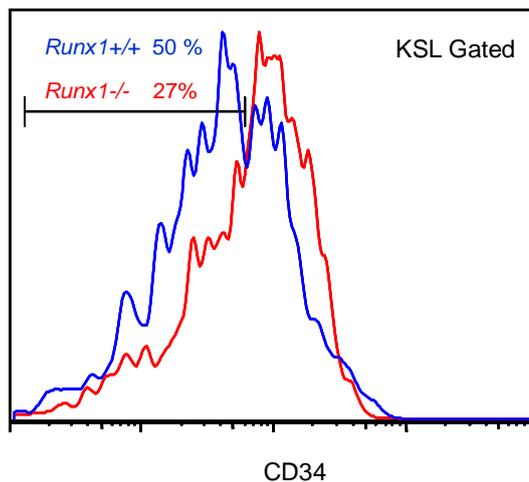
Figure 2.5: Quiescent LT-HSC are reduced in *Runx1-/-* mice
Expression of CD34 in KSL gated fraction of *Runx1+/+* (blue) and *Runx1-/-* (red) BM
cells. One representative result out of 4 is shown.

All the above results taken together show that though there is an initial expansion of *Runx1-/-* stem/progenitor cells as has been previously reported by various groups, the expansion seems to be transient as this is followed by *Runx1-/-* stem cell exhaustion. The decreasing contribution of *Runx1-/-* stem cells in primary recipients and the inability to reconstitute hematopoiesis in secondary recipients confirm this phenomenon of stem cell exhaustion. Thus, although *Runx1* alteration leads to a transient increase in stem/progenitor cell numbers the development of leukemia from these aberrant stem cells may require accumulation of additional genetic alterations that prevent stem cell exhaustion and maintain the aberrant cells. To this end, I used RIM on conditional *Runx1* knockout mice to identify additional genetic alterations that cooperate with loss-of-function of Runx1 in leukemogenesis.

Runx1-/- mice are more susceptible to leukemia development than wild type mice

I employed RIM to induce leukemia in *Runx1-/-* mice and control *Runx1+/+* littermates to study susceptibility of these mice to leukemia and to fish out candidate cancer genes involved in Runx1 related leukemia with loss-of-function of Runx1. Lymphotropic murine leukemia virus, MoMuLV, which can integrate randomly into the host genome, was injected into *Runx1-/-* mice and wild type littermates. The retroviral insertion into host genome may result in activation of host oncogenes or disruption of tumor suppressors as a consequence of which mice develop leukemia/lymphoma. Interestingly, *Runx1-/-* mice showed significantly shorter latency of tumor development than wild type littermates (**Figure 3.1**).
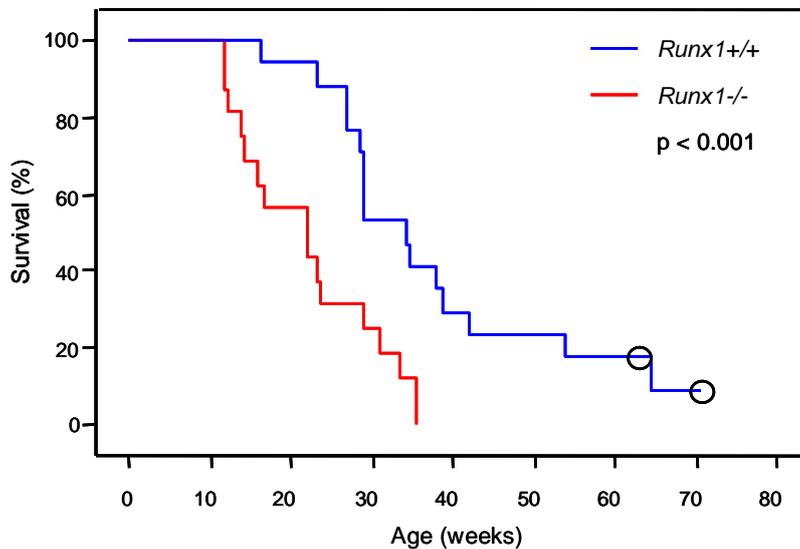
Figure 3.1: *Runx1-/-* **mice show higher incidence and earlier onset of tumor**
Kaplan Meier survival curves of *Runx1+/+* (blue line; n=17) and *Runx1-/-* (red line; n=16) mice injected with MoMuLV retrovirus. Kaplan–Meier method showed significant difference between the two genotypes (p<0.001, Mantel-Cox test). Open circles represent censored cases.

When the mice became moribund, they were sacrificed and subjected to necropsy. PB cell counts were measured and abnormalities in hematopoietic tissues were recorded as follows: enlargement of the thymus, liver, and spleen, and swelling of lymph nodes. Based on necropsy, the cancer disease was divided into leukemia or lymphoma cases. Leukemia cases showed elevated blood cell counts and only splenomegaly with normal thymus/lymph nodes, while lymphoma cases showed normal or mildly elevated blood counts and enlarged thymus/lymph node (**Figure 3.2**).
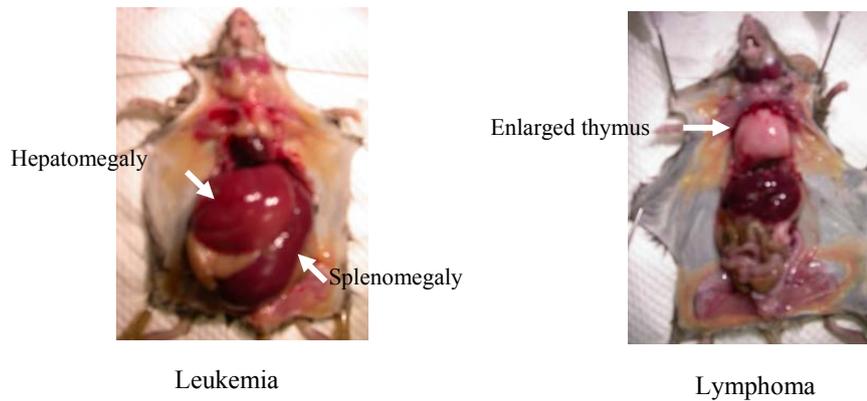
Figure 3.2: Necropsy of mice with leukemia or lymphoma
Leukemia mice usually show enlarged spleen and liver while lymphoma mice usually show enlarged thymus. Enlarged spleen may also be found in some lymphoma cases.

Immunophenotypic analysis was done using FACS analysis to check for the presence of cell surface markers for immature cells, myeloid cells, and T- and B-cells. Leukemic cells from the PB, BM and spleen were subjected to May-Giemsa staining for morphological analyses. Based on combination of leukocyte counts, necropsy, immunophenotype and morphological analyses, tumors were classified into the following 4 groups **(Figure 3.3)**

1. Group 1 - Leukemia with myeloid features alone; shows elevated blood cell count, splenomegaly, myeloid cell morphology and expression of myeloid antigens. Leukemia onset is within 24 weeks after birth, early onset.

2. Group 2 - Leukemia with T-cell and myeloid features; shows elevated blood cell counts, splenomegaly and immature/myeloid/T-cell morphology. At least majority of these leukemias are biphenotypic as they express high percentage of both myeloid and T-cell antigens. Leukemia onset is within 16 weeks after birth, early onset.

3. Group 3 - Leukemia with T-cell features alone; shows elevated blood cell counts, splenomegaly, slight enlargement of thymus in some cases, T-cell morphology and expression of T-cell antigens. Leukemia onset can be early (<28 weeks) or late (>28 weeks).

4. Group 4 – Lymphoma; shows normal or mildly elevated blood cell counts and significant enlargement of thymus and/or lymph node due to accumulation of T-cells and expression of T-cell antigens. Onset of lymphoma can be early (<28 weeks) or late (>28 weeks).

Majority of the *Runx1-/-* mice developed early onset leukemia with myeloid features that fell into group 1 and 2 or early onset leukemia/lymphoma that fell into group 3 and 4. Most of the *Runx1+/+* mice developed T-cell leukemia/lymphoma that belonged to group 3 and 4, with varying onset times **(Figure 3.3)**. This indicates that *Runx1* knockout status drives myeloid tropism despite the strong T-lymphotropism of MoMuLV virus. Some of the group 1 and 2 leukemias recapitulated human RUNX1 related leukemias with accumulation of immature blasts (as seen in AML M0) or accumulation of myeloid cells with slight differentiation (as seen in AML M2) **(Figure 3.4)**.

The fact that *Runx1-/-* mice showed significantly earlier onset and higher incidence of leukemias with myeloid features (group 1 and 2) indicates that they are more susceptible to leukemia development and the retroviral integration sites (RIS) in these mice could cooperate with *Runx1-/-* status in the development of leukemia with myeloid features. Some of these genetic alterations may aid in leukemia progression by preventing exhaustion of *Runx1-/-* stem cells and maintaining the abnormal cells in the individual mice till they become completely leukemogenic. Identification and analysis of the viral
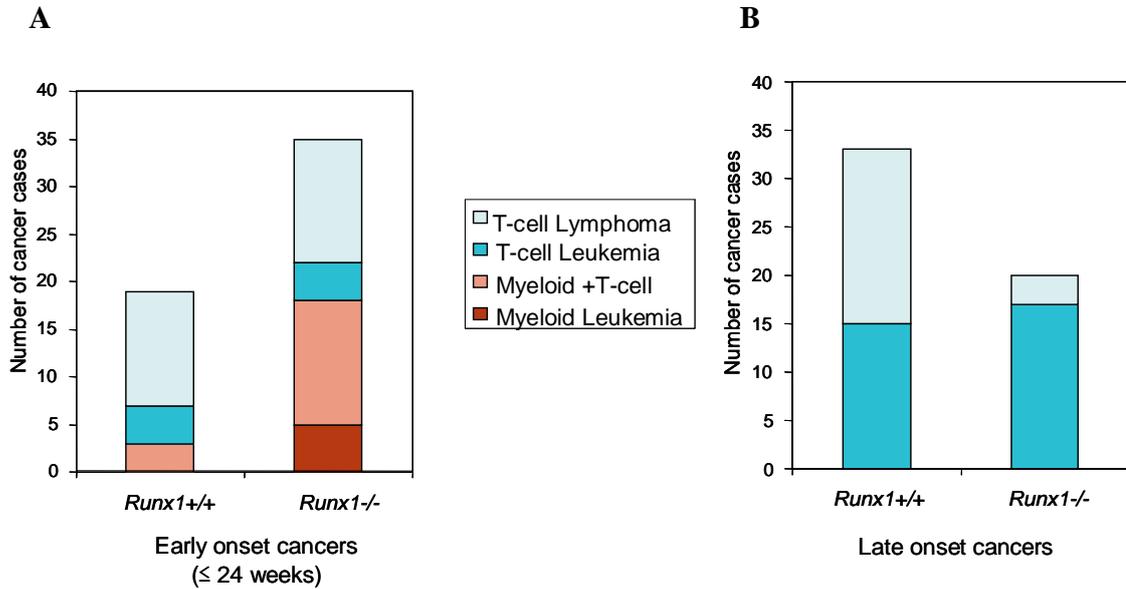
Figure 3.3: *Runx1-/- mice develop early onset leukemia with myeloid features*
(A) Graph showing frequency of different types of leukemia/lymphoma, groups 1-4, in early onset (≤ 24 weeks) cancers of *Runx1+/+* (n=19) and *Runx1-/-* (n=34) mice. (B) Graph showing frequency of leukemia or lymphoma cases in late onset cancers of *Runx1+/+* (n=33) and *Runx1-/-*(n=20) mice. In total, n=52 for *Runx1+/+* mice and n=54 for *Runx1-/-* mice.
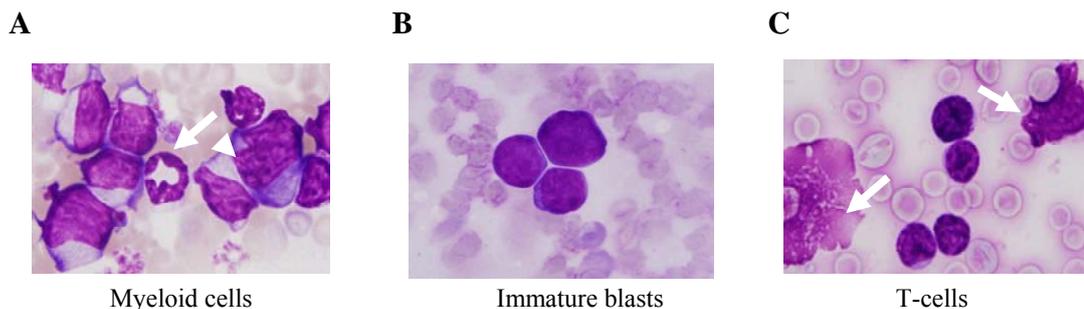
Figure 3.4: Morphology of *Runx1-/-* leukemic cells recapitulates human leukemias
Morphology of cells from PB of representative leukemic case from (A) Group 1 showing granulocytes (arrow) and monocytes (arrowhead) (B) Group 2 showing immature blasts and (C) Group 3 showing T-cells and ghost cells (arrows) which are frequently seen in T-cell malignancy.

integration sites may shed some light on genetic alterations that cooperate with loss-of-function of Runx1 by preventing stem cell exhaustion or other mechanisms, thus aiding the progression of Runx1 deficient cells from preleukemic state to full blown leukemia.

Stemness related genes are preferentially affected in *Runx1-/-* mice

An individual tumor (leukemia/lymphoma) induced by MoMuLV virus had 4 to 10 retroviral integration sites, suggesting that by the time a mouse develops leukemia, sufficient number of genes are activated or inactivated that cooperate with each other or the background (*Runx1-/-* status) to induce full blown leukemia/lymphoma. To identify the retroviral integration sites (RIS) in the *Runx1-/-* and *Runx1+/+* mice, the genomic DNAs extracted from their leukemic cells were subjected to an iPCR method after *Bst*YI digestion (Yanagida et al., 2005). I found 710 integration flanking sequences (tags) in 63 *Runx1-/-* mice and 52 *Runx1+/+* mice. These sequences were mapped to the mouse genome to identify the chromosomal location of the sequence and to identify candidate genes at that locus. 20 loci were affected more than once by retroviral integrations in *Runx1-/-* or *Runx1+/+* mice and these are referred to as CIS which are considered to be near candidate leukemogenic genes **(Table 2)**. The relative locations of these integration sites were compared to the tags from the publicly available retroviral-tagged cancer gene database (RTCGD) (Akagi et al., 2004). This comparison revealed that 15 of the CIS correspond to previously known loci where retroviral integration occurred more than once. In addition, five CIS that have been identified only by our study and hence have been designated as Slis (Singapore leukemia integration site) were classified as novel CIS **(Table 2)**.

Classification of RIS	Chromosome Number [a]	Gene [b]	*Runx1 +/+* n=52	*Runx1 -/-* n=63
Known CIS (15)[c]				
	5	**Gfi1/Evi5**	**2**	**11 (3)[d]**
	15	**Myc**	**3**	**11**
	17	**Ccnd3**	**2**	**6**
	7	RRas2	4	5
	10	Ahi1/Myb	7	3
	2	Rasgrp1	2	2
	3	**Evi1**	**0**	**5**
	6	Ccnd2	0	3
	12	Nmyc	0	3
	17	Pim1	0	3
	2	Bcas1	0	2
	5	Bcl7a	0	2
	7	Sema4b	0	2
	12	Jundm2	0	2
	11	Ikaros	0	2
Novel CIS (5)[c]	X	SlisX	0	4
	3	Slis6	0	3
	5	Slis7	0	2
	16	Slis9	0	2
	5	Mad1l1	0	2

CIS, common integration site; RIS, retroviral integration site

Table 2: Classification of RIS identified in *Runx1+/+* and *Runx1-/-* leukemias
[a] The genomic positions of the RIS were determined according to BLAT searching of the UCSC Genome Bioinformatics database. [b] Candidate genes in the vicinity of the RIS are shown. Number in parentheses indicates [c] number of known and novel CIS and [d] number of integrations inside the *Evi5* gene. The bold rows indicate the particularly interesting CIS that are discussed in the text.

The CIS that have been repeatedly identified in the same cancer model are assumed to be more significant candidate genes with regard to the pathogenesis of the specific type of cancer in question. It is of special interest to note the genes that were hit with high frequency in *Runx1-/-* mice, but not affected or affected with lower frequency in *Runx1+/+* mice, because they may be specifically involved in leukemogenesis of *Runx1-/-* mice. Integrations at the *Gfi1/Evi5* locus were found in 11 out of 63 *Runx1-/-* mice analyzed while only 2 *Runx1+/+* mice out of 52 showed integrations at this locus (**Figure 4.1**; purple and blue arrows respectively). More than half the integrations, 8 out of 13, were in between the *Gfi1* and *Evi5* genes, 3 were inside the *Evi5* gene, one inside *Gfi1* and one downstream of *Gfi1*. 7 of the *Runx1-/-* leukemia cases with integrations at the *Gfi1/Evi5* locus belonged to group 1 and 2 which showed early onset leukemia with myeloid features. Thus, 7 out of the 18 *Runx1-/-* leukemia cases (approximately 40%) in group 1 and 2 had integrations at *Gfi1/Evi5* locus. The remaining 4 mice with integrations at *Gfi1/Evi5* locus could not be classified due to unavailability of morphology and immunophenotype data, but they could belong to group1 and 2 since they showed early onset of leukemia and there was no enlargement of thymus/lymph node (**Table 3**).

Gfi1 (Growth Factor Independent 1), is a well known factor involved in stem cell maintenance. In the absence of Gfi1, stem cells lose their repopulating ability and cannot maintain normal stem cell functions (Hock et al., 2004). Evi5 was shown to be a cell cycle regulator required for stabilizing key proteins such as Emi1 which inhibits Anaphase Promoting Complex (APC) and prevents premature entry of cells into mitosis (Eldridge et al., 2006). The role of Evi5 in stemness has not been investigated to date. In order to clarify which of the two genes, *Gfi1* or *Evi5* may be altered by integrations at the

Gfi1/Evi5 locus in *Runx1-/-* mice, I carried out expression check using qRT-PCR on cDNA from 6 of the available leukemic samples from group 1 and 2 with integrations at this locus and 3 control samples with no integration at this locus. *Evi5* overexpression was seen in all affected *Runx1-/-* cases with integration outside the gene, compared to the three control cases (**Figure 4.2**). Moreover, expression of *Evi5* increased proportionately as the distance between the viral integration site and the *Evi5* gene decreased, indicating specific, integration site dependant alteration of *Evi5* expression. Integrations inside the *Evi5* gene did not alter expression of the gene. In contrast to *Evi5, Gfi1* expression was not significantly affected by viral integrations in most leukemia cases. They showed similar expression levels of *Gfi1* as the three controls (**Figure 4.2**).

Out of the integrations that were present only in *Runx1-/-* mice and not in *Runx1+/+* mice, the most frequent was at *Evi1* locus, seen in 5 *Runx1-/-* mice out of 63. *Evi1* also has an established role in promoting stemness and it regulates stem cell proliferation through GATA2 expression (Yuasa et al., 2005). Since *Runx1-/-* stem cells undergo exhaustion, they would require genetic alterations in a gene involved in stem cell maintenance for leukemia development. Coincidentally, the most frequent integrations in *Runx1-/-* leukemia cases were near known stem cell genes such as *Gfi1* and *Evi1* and cell cycle regulator *Evi5* which may play a role in stemness. These integrations could result in overexpression of these genes which may cooperate with *Runx1-/-* status in initiation and/or progression to leukemia. Hence, I decided to carry out further studies of cooperation between *Runx1-/-* status and overexpression of *Gfi1, Evi5* and *Evi1* in the maintenance of aberrant *Runx1-/-* stem cells that may give rise to leukemia.
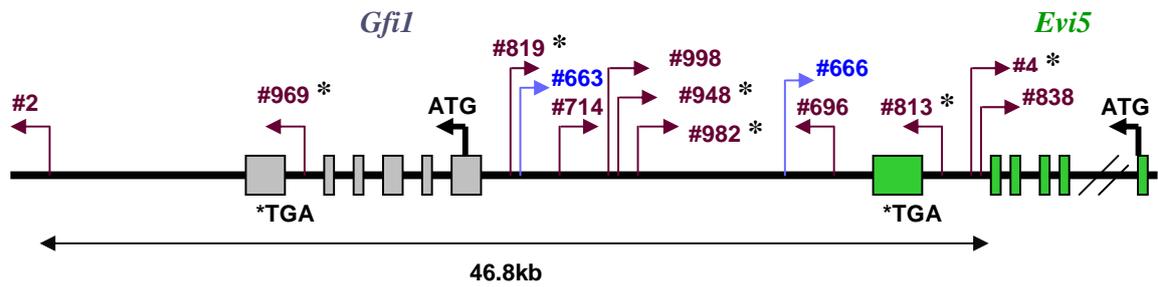
Figure 4.1: Viral integrations at *Gfi1/Evi5* locus frequently seen in *Runx1-/-* mice
Schematic diagram of retroviral integration sites in *Runx1+/+* (blue) and *Runx1-/-* (purple) leukemias. Numbers are unique to each leukemic mouse. Thin bent arrows represent the retrovirus integration and its direction of integration. The two genes *Gfi1* (grey) and *Evi5* (green) span from their start sites (ATG) to stop sites (TGA) with shaded boxes representing exons. * Leukemia cases in which expression of *Gfi1* and *Evi5* was checked using real time PCR (refer to next figure).
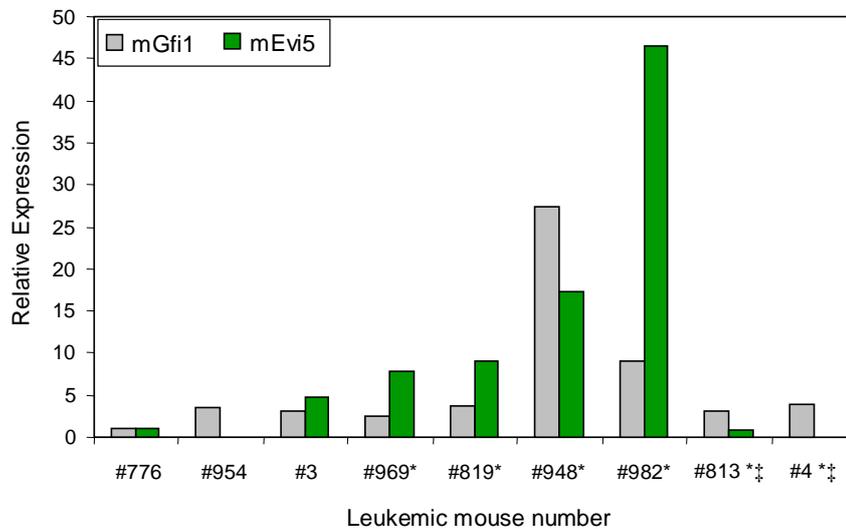
Figure 4.2: Integrations at *Gfi1/Evi5* locus result in overexpression of *Evi5*
QRT-PCR analysis of *Gfi1* and *Evi5* expression in PB of leukemic samples harboring integrations at *Gfi1/Evi5* locus (*), integrations within the *Evi5* gene (‡) and 3 control samples with no integrations at this locus. Data are represented as fold change relative to control sample # 776.

	Tumor ID	Genotype	Stem cell **	Proliferation**	Tumor supressor**	Novel
Group 1 Myeloid Leukemia	#696	*Runx1-/-*	Evi5	cMyc		
	#708	*Runx1-/-*	Evi1	Pik3cd	Mapk9 (Jnk)	Slis6
	#714	*Runx1-/-*	Evi5	Cyclin D3, Cyclin D2	Dab2	
	#807	*Runx1-/-*		Ncoa2	Ing4	
	#966	*Runx1-/-*	Evi1	Il6st		
Group 2 Myeloid+T-cell Leukemia	#641	*Runx1-/-*		Cyclin D3, Cyclin D2	Sh3md2, Stag1	Slis7
	#770	*Runx1-/-*		Cyclin D3, Sema4d	Gadd45	Slis6
	#775	*Runx1-/-*		cMyc		
	#779	*Runx1-/-*		cMyc	Nkd1	Slis7
	#813	*Runx1-/-*	Evi5			SlisX
	#819	*Runx1-/-*	Evi5			
	#821	*Runx1-/-*				
	#948	*Runx1-/-*	Evi5	Nmyc		
	#969	*Runx1-/-*	Evi5, Evi1	Nmyc, Cyclin D1	Mad1l1	
	#972	*Runx1-/-*				
	#974	*Runx1-/-*	Lmo2	cMyc, Cyclin D3, Lef1		
	#982	*Runx1-/-*	Evi5		Stag1	SlisX
	#691‡	*Runx1-/-*				
	#663	*Runx1+/+*	Evi5	Pip5k2a		
	#690	*Runx1+/+*	Lmo2		Foxp1	
	#693	*Runx1+/+*		Tnfrsf191	Tspan32	
Unclassified *	#2	*Runx1-/-*	Evi5	Nmyc, Sept9, Pim2		
	#4	*Runx1-/-*	Evi5, Hes1	Il2		SlisX
	#838	*Runx1-/-*	Evi5			
	#998	*Runx1-/-*	Evi5			

Table 3: Cooperating genetic changes in leukemic mice in group 1 and 2
Classification of genes near CIS (black) and RIS (violet) identified in this study that correspond to known CIS from RTCGD database; and genes near RIS from this study (blue) which may have a role in oncogenesis, based on their ** known or predicted function. Underlined genes have retroviral integration inside the gene. * Unclassified leukemia cases which could belong to group 1 or 2; they showed early onset of disease, no enlargement of thymus/lymph node, and no FACS data. ‡ RIS information is not available for this sample.

However, other genes identified in the retroviral mutagenesis screen could also cooperate with *Runx1-/-* status in leukemogenesis through alternate ways. Integrations near *c-Myc*, *Cyclin D2* and *Cyclin D3* genes were also more frequent in *Runx1-/-* mice. *c-Myc* is a well known protooncogene that causes uncontrolled proliferation of cells when overexpressed. *Cyclin D2* and *D3* are G1 cyclins and dysregulation of their expression could lead to abnormal cycling of cells. In most leukemic cases, overlapping CIS as well as RIS near genes which may play a role in leukemogenesis were found which may represent second and third hits in multi-step leukemogenesis. A comprehensive list of interesting genes near CIS or RIS in group 1 and 2 leukemia cases is given in **Table 3**.

Overexpression of *EVI5* cooperates with *Runx1-/-* status in long term maintenance of aberrant stem/progenitor cells *in vitro*

The CIS genes chosen for studies of cooperation with *Runx1-/-* status were *Gfi1*, *Evi5*, and *Evi1*, due to high frequency of viral integrations near these genes in *Runx1-/-* leukemias. Moreover, they were suspected to prevent exhaustion of *Runx1-/-* stem cells due to their known function in stemness and thus contribute to Runx leukemogenesis. In order to study the cooperation between *Runx1-/-* status and overexpression of these genes, in the maintenance of aberrant *Runx1-/-* stem cells, I employed *in vitro* assays using BM from *Runx1-/-* and control *Runx1+/+* littermates transfected with retroviral MIG vector carrying *EVI5*, *GFI1* or *EVI1*. To assess the effect of overexpression of these candidate oncogenes in immature hematopoietic cells, the cKit+ fraction of transfected cells was isolated by FACS and subjected to the experiments.

Serial replating colony assay was first carried out to assess the ability of cKit+ immature cells to form colonies in successive rounds of replating, referred to as round 1 and round 2. In round 1 of colony assay, mock vector transfected *Runx1-/-* cells showed significantly higher colonies than *Runx1+/+* cells. However, after replating, in round 2, these cells showed very few colony numbers **(Figure 5.1)**. On the other hand, *Runx1-/-* cells transfected with *EVI5* and *EVI1* showed fewer numbers of colonies than mock transfected cells in round 1 of colony assay, but they formed a higher number of colonies after replating, in round 2, than mock transfectants. On *Runx1+/+* background*, EVI5, EVI1* and mock transfectants formed similar number of colonies in round 1; and in round 2, they formed significantly lower number of colonies. *GFI1* ovexpression resulted in no colonies on *Runx1+/+* background and very few colonies on *Runx1-/-* background right from first round of colony assay. In round 2, colony numbers were still very few. In terms of synergy between transfected genes and *Runx1-/-* status in maintenance of stem/progenitor cells, *EVI5* showed highest synergy because *EVI5* transfected *Runx1-/-* cells formed highest number of colonies in round 2. *EVI1* overexpression showed a mild synergy **(Figure 5.1)**. These results indicate that *Runx1-/-* cells proliferate abnormally as shown by the higher capability of colony formation of these cells in round 1. Overexpression of genes such as *EVI5* helps to prevent this abnormal proliferation and maintain stem/progenitor cells that are capable of giving rise to new colonies.
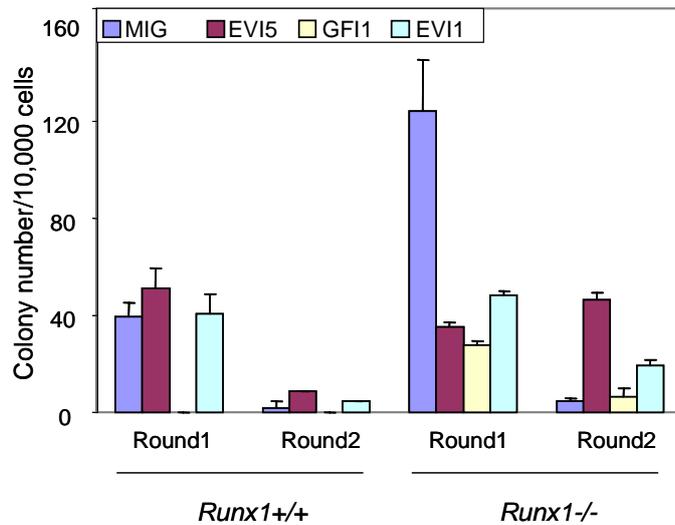
Figure 5.1: *EVI5* **overexpression shows highest synergy with** *Runx1-/-* **status in serial replating colony assay**
Colony assay of *Runx1+/+* and *Runx1-/-* BM cells transfected with mock vector, *EVI5, GFI1* and *EVI1*. Transfected (GFP+), immature (c-Kit+) cells were used for round 1 of colony assay and cells harvested from colonies after 10 days were used for round 2. Experiment was done in triplicates and the results are representative of 3 independent experiments.

LTC-IC assay was performed to further confirm the synergy between overexpression of *EVI5* and *Runx1-/-* status in stem cell maintenance. The number of colony forming cells scored after more than 30 days culture of transfected *Runx1-/-* and *Runx1+/+* stem/progenitor cells (GFP+ c-Kit+) on OP9 stromal cells, which support the maintenance of HSC *in vitro*, is considered to reflect the self-renewal capacity of stem cells. The plating efficiency (colony number) of *Runx1-/-* cells with overexpression of *EVI5* gene was much higher than that of mock transfected *Runx1-/-* cells or *EVI5* transfected *Runx1+/+* cells, suggesting that *Runx1-/-* cells overexpressing *EVI5* maintain a higher number of stem cells **(Figure 5.2A and B)**. Replating of cells after 30 days of

culture on new OP9 stromal cells and counting cobblestone area-forming cells (CAFC) 3 to 4 days later further strengthened this observation because *Runx1-/-* cells carrying *EVI5* showed a high number of CAFC, significantly higher than *Runx1-/-* cells transfected with mock vector or *EVI5* overexpressing *Runx1+/+* cells (**Figure 5.2C**). Furthermore, colony assay and CAFC assay after 30 more days of culture of replated cells (total 60 days after initial transfection) still showed a high number of colonies and CAFC in *Runx1-/-* cells overexpressing *EVI5* as compared to other combinations. *GFI1* and *EVI1* transfected Runx1+/+ or Runx1-/- cells did not show any colony or CAFC after 30 days of LTC (**Figure 5.2A and C**).

Morphological analyses of cells after 30 and 60 days of LTC revealed the presence of a large number of immature cells with characteristically large nucleus and basophilic cytoplasm in *Runx1-/-* cells overexpressing *EVI5*. *Runx1-/-* cells transfected with mock vector and *Runx1+/+* cells overexpressing EVI5 showed completely differentiated mast cell morphology after both 30 days and 60 days of LTC (**Figure 5.2D**).

Taken together, it is clear that the overexpression of *EVI5* strongly cooperates with *Runx1-/-* status in maintenance and proliferation of stem cells *in vitro*, rather than overexpression of *GFI1* or *EVI5*. Furthermore, *Runx1-/-* status and *EVI5* alteration have to coexist for robust stem cell maintenance and either of these alterations alone cannot sustain the continued maintenance of stem cells. Hence, it is interesting to further verify if these 2 genetic alterations can also cooperate *in vivo* and maintain *Runx1-/-* stem cells without exhaustion and whether *EVI5* overexpression can rescue the progressively impaired reconstitution of hematopoiesis by *Runx1-/-* stem cells.

A

B

C

D
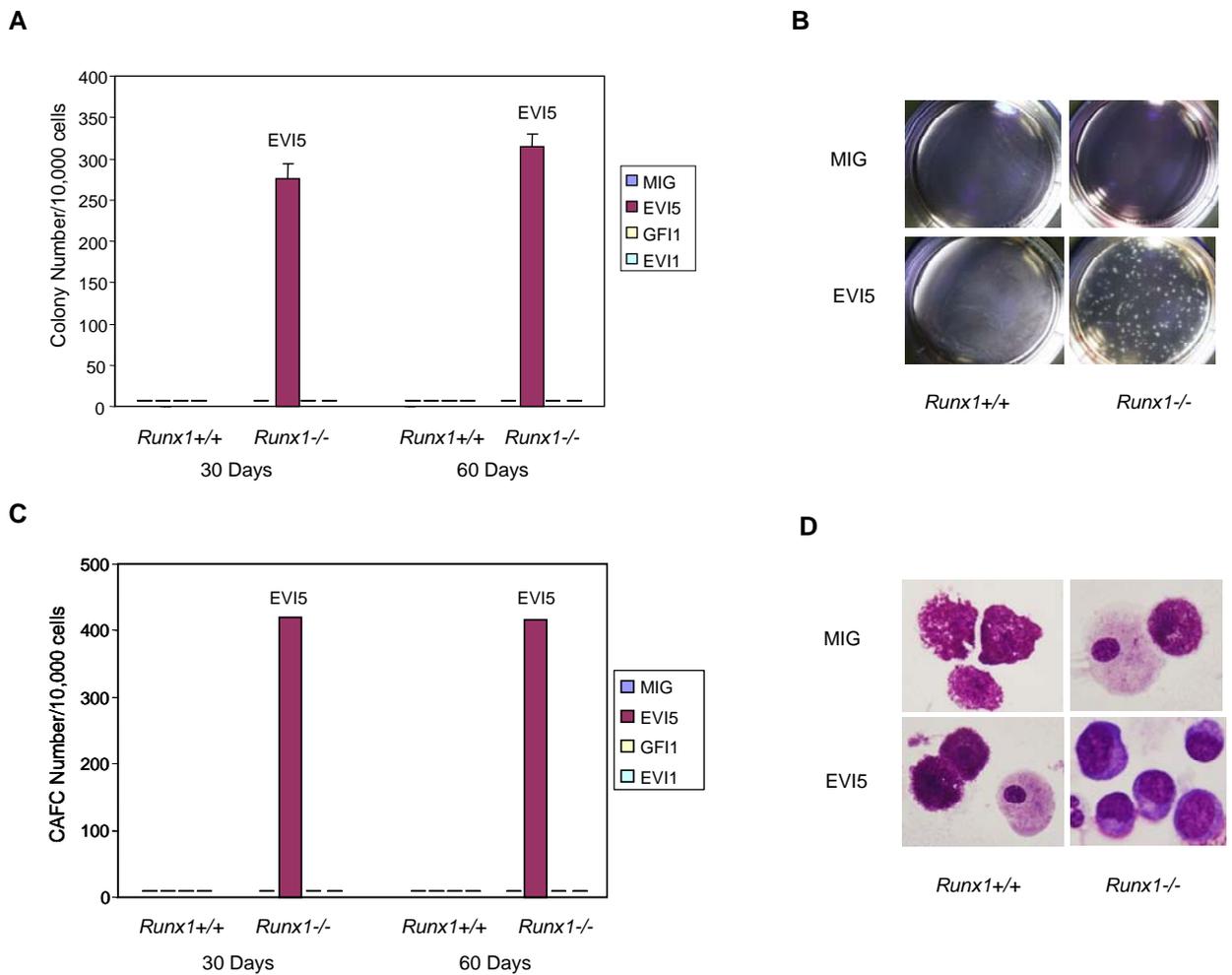
Figure 5.2: *EVI5* **overexpression and** *Runx1-/-* **status synergize in long term maintenance of stem cells**

Graphical representation of (A) colony assay and (B) CAFC assay of immature cells from *Runx1+/+* and *Runx1-/-* BM transfected with mock MIG vector, *EVI5, GFI1* and *EVI1,* after 30 and 60 days of long term culture. Pictures of (C) Colonies and (D) Morphology of cells after 30 days of long term culture.

Overexpression of EVI5 prevents exhaustion of *Runx1-/-* stem cells *in vivo*

In order to assess the *in vivo* effect of overexpressing *EVI5* in *Runx1-/-* cells, I transplanted *Runx1+/+* and *Runx1-/-* BM cells transfected with *EVI5* gene into sublethally irradiated (6 Gy) recipient mice. The percentage of GFP positive cells in PB was checked at regular intervals after transplantation to assess the reconstitution ability of *Runx1+/+* and *Runx1-/-* stem cells overexpressing *EVI5*. Recipients of *Runx1+/+* cells overexpressing *EVI5* showed stable GFP chimerism throughout, from 6 weeks to 30 weeks after transplantation, at an average of 20 to 25% **(Figure 6.1)**. This is similar to previously described GFP chimerism of recipients of mock transfected *Runx1+/+* cells. However, the GFP chimerism of mice transplanted with *Runx1-/-* cells overexpressing *EVI5* increased progressively, with a mean value of 25% at 6 weeks and 50% at 30 weeks **(Figure 6.1)**. This is in contrast to the results seen after transplantation of mock vector transfected *Runx-/-* and *Runx1 +/+* cells described earlier where the contribution of *Runx1-/-* cells to PB of recipient mice decreased progressively **(Figure 2.1)**, probably due to stem cell exhaustion. Thus, *EVI5* seems to cooperate with *Runx1-/-* status *in vivo* also by preventing stem cell exhaustion and maintaining an expanded population of aberrant *Runx1-/-* stem cells.

Serial transplantation experiments were also repeated to check if *EVI5* overexpression in *Runx1-/-* cells could rescue the defects in long term repopulating abilities of *Runx1-/-* stem cells. Similar to previous experimental setting, both *Runx1+/+* and *Runx1-/-* BM cells were transfected with retrovirus carrying *EVI5* gene; GFP chimerism was monitored for average of 4 months in these recipients following which BM cells were transplanted into four sublethally irradiated secondary recipients. Contrary

to the previous results where majority of the recipients of *Runx1-/-* cells died within three months of secondary transplantation **(Figure 2.3)**, all the recipients of *Runx1-/-* cells overexpressing *EVI5* are alive to date, indicating that these stem cells are still capable of reconstituting hematopoiesis in recipients **(Figure 6.2)**. Altogether, it can be concluded that *EVI5* overexpression can prevent *Runx1-/-* stem cell exhaustion and render them capable of reconstituting hematopoiesis in secondary recipients, thus ensuring the survival of the mice.

Evi5 overexpression and *Runx1-/-* status cooperate *in vivo* also to maintain aberrant *Runx1-/-* stem cells which are capable of long term repopulating abilities. In fact, the increasing chimerism in PB of recipients transplanted with these cells shows that *EVI5* overexpression can not only rescue *Runx1-/-* stem cell exhaustion, but it can also maintain an expanded pool of target *Runx1-/-* stem cells which has a higher probability of acquiring further mutations that could make them completely leukemogenic. These results validate the high frequency of retroviral integrations, seen near the *Evi5* gene in *Runx1-/-* mice, which result in overexpression of *Evi5*.
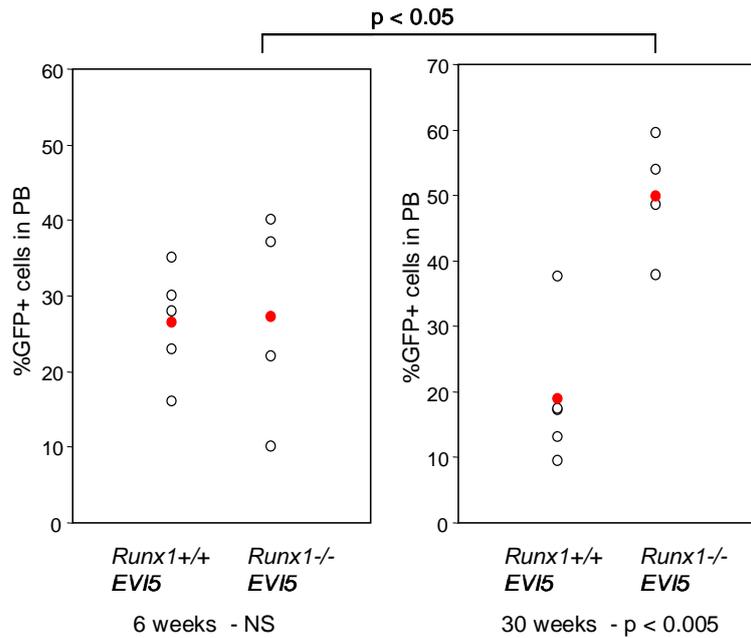
Figure 6.1: *EVI5* **overexpression rescues** *Runx1-/-* **stem cell exhaustion** *in vivo*
GFP chimerism in recipients of *Runx1+/+* (n=5) and *Runx1-/-* (n=4) BM cells transfected with *EVI5,* 6 and 30 weeks after transplantation. Each open circle represents data from an individual mouse and closed red circle is the average of a cohort. Stastical difference using unpaired student-t test are given at the bottom and on top.
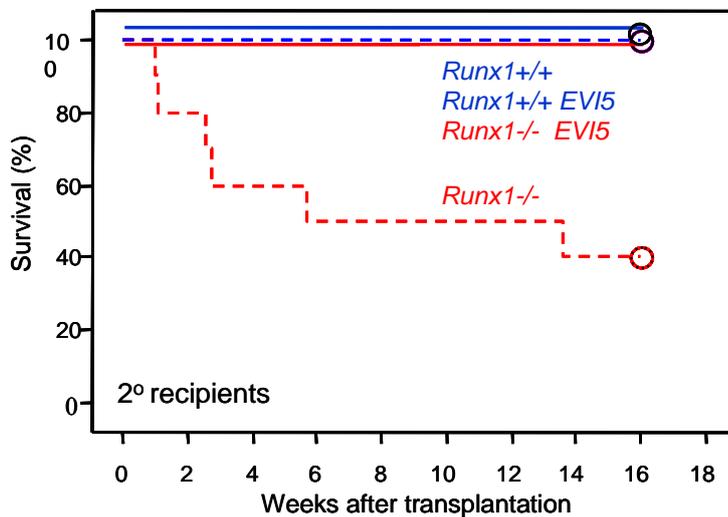
Figure 6.2: *EVI5* **rescues** *Runx1-/-* **stem cell exhaustion in secondary recipients**
Kaplan-Meier survival curves of secondary recipients of *Runx1+/+* (blue) and *Runx1-/-* (red) BM cells transfected with mock MIG vector (dashed line) or MIG vector carrying *EVI5* (solid line), 4 months after primary transplantation. Circles represent end point of analysis.

Mechanism of cooperation between *Runx1-/-* status and *EVI5* overexpression

Before addressing the mechanism of cooperation between *EVI5* overexpression and *Runx1-/-* status, it is important to understand why *Runx1-/-* stem cell exhaustion occurs. The reason for this could be cell intrinsic factors or stem cell niche related factors. Since immature *Runx1-/-* cells still maintained their hyperproliferative ability 2 years after transplantation **(Figure 2.2C)**, intrinsic changes may not be responsible for stem cell exhaustion.   Hence, impaired stem cell interaction with the niche could be a reason for *Runx1-/-* stem cell exhaustion as interaction of stem cells with its niche is important for maintaining the integrity and self-renewal properties of stem cells.

I investigated the possibility of impaired interaction of *Runx1-/-* stem cells with the niche and whether it could be rescued by *EVI5* overexpression. FACS analysis of a panel of niche related factors in cKit+GFP+ cells from transfected *Runx1+/+* and *Runx1-/-* BM cells revealed that one of the most important molecules for interaction with the stem cell niche, CXCR4, was down regulated in *Runx1-/-* cells **(Table 4)**. CXCR4 expression is critical for stem cells to respond to the SDF-1α ligand secreted by the stem cell niche, thus enabling the stem cells to home to their niche and interact with it, thereby maintaining their stem cell properties. Normal level of CXCR4 expression was restored after overexpression of *EVI5* in *Runx1-/-* cells. CD49b, which is an α2 integrin required for adhesion to the stem cell niche, was also downregulated in *Runx1-/-* cells and expression restored to normal after overexpression of *EVI5* in these cells **(Table 4)**.

The downregulation of *CXCR4* in immature *Runx1-/-* cells was confirmed by qRT-PCR **(Figure 7.1A)**. CXCR4 expression was also downregulated in wild type immature BM cells transfected with the dominant negative chimera gene *RUNX1-ETO*,

indicating that stem cell exhaustion due to altered niche interaction may also take place in human RUNX1 deficient cells (**Figure 7.1B**). This result also indicates the importance of intact Runx1 transcriptional machinery in maintaining normal levels of *CXCR4* expression. Analysis of the promoter region of human *CXCR4* gene revealed the presence of 2 RUNX binding sites 28 bp and 90 bp upstream of the transcriptional start site. Therefore, we checked if *CXCR4* gene expression is directly regulated by RUNX1, using the luciferase reporter system. A luciferase reporter containing *CXCR4* promoter fragment 860 bp upstream of the transcription start site was activated more than 20 fold in the presence of RUNX1, when introduced into HL60 leukemic cell line. However, the DNA binding defective mutant of RUNX1, R174Q, could not transactivate the *CXCR4* promoter (**Figure 7.2**). This indicates that RUNX1 regulates transactivation of *CXCR4* in a DNA binding dependent manner.

To confirm the defective niche interaction of *Runx1-/-* HSC due to reduced expression of CXCR4 and CD49b, we assessed the homing capacity of *Runx1-/-* and control *Runx1+/+* BM cells to the stem cell niche in the BM, using an *in vivo* homing assay. 5×10^6 BM cells from *Runx1-/-* and *Runx1+/+* littermates were stained with a fluorescent dye (CFSE) and injected into lethally irradiated (10 Gy) recipient mice. The frequency of *Runx1-/-* and *Runx1+/+* cells present in the recipient BM was assessed by flow cytometry 16 hours after transplantation. We found that *Runx1-/-* cells traffic to the BM with significantly reduced efficiency relative to *Runx1+/+* cells (**Figure 7.3**). This result suggests that *Runx1-/-* HSC are impaired in their ability to home and attach to the stem cell niche in the BM.

Niche factor	Runx1+/+ Mock (%)	Runx1-/- Mock (%)	Runx1+/+ EVI5 (%)	Runx1-/- EVI5 (%)
CXCR4	**52**	**41**	**62**	**60**
CD49b	**39**	**24**	**38**	**34**
CD49d	99	99	99	99
CD49e	99	99	99	100
CD11a	98	100	99	100
CD18	98	100	100	100
CD44	100	100	100	100
CD29	99	100	100	100

Table 4: *Runx1-/-* cells express lower levels of some niche interacting molecules whose expression is restored by overexpression of *EVI5*.
Expression of niche interacting molecules on immature (cKit+) *Runx1-/-* and *Runx1+/+* cells transfected with mock vector or *EVI5* (GFP+). Experiment was done in duplicate.

A

B
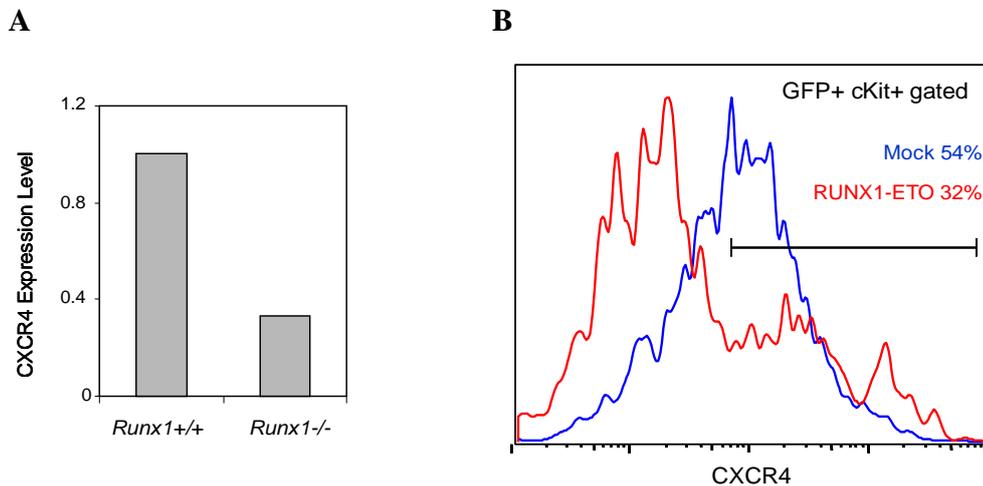
Figure 7.1: CXCR4 expression is reduced under Runx1 deficient conditions
(A) QRT-PCR analysis of expression of *CXCR4* in immature (cKit+) *Runx1+/+* and *Runx1-/-* BM cells. **(B)** Expression of CXCR4 in cKit+GFP+ cells from wild type BM cells transfected with mock MIG vector (blue) or *RUNX1-ETO* (red). One representative result out of 2 experiments is shown.
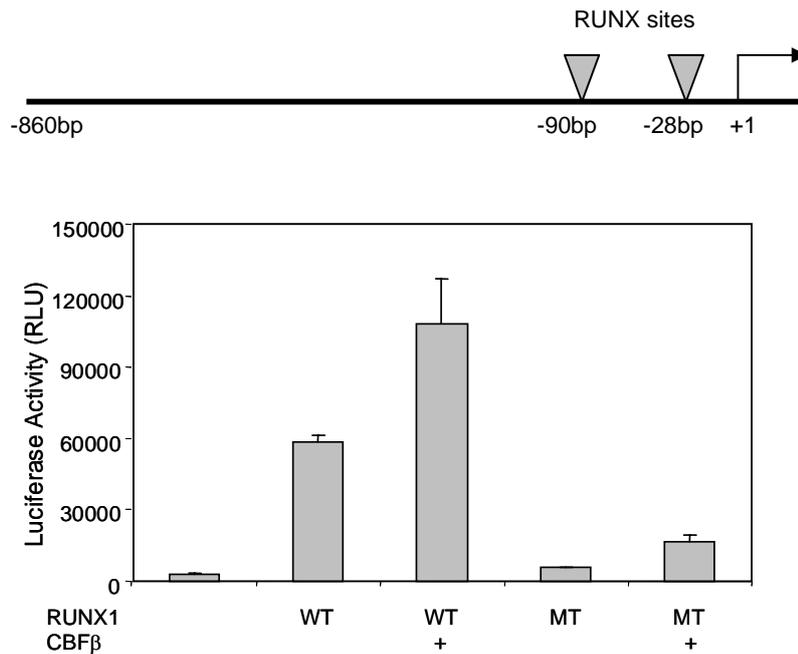
Figure 7.2: *CXCR4* is a direct transcriptional target of RUNX1
Schematic diagram of promoter region of human *CXCR4*, 860 bp upstream of transcription start site, containing 2 RUNX binding sites (grey arrowheads); Luciferase assay showing transcriptional activity of wild type RUNX1 (WT) or its mutant form R174Q (MT) with (+) /without PEBP2β, on *CXCR4* promoter.
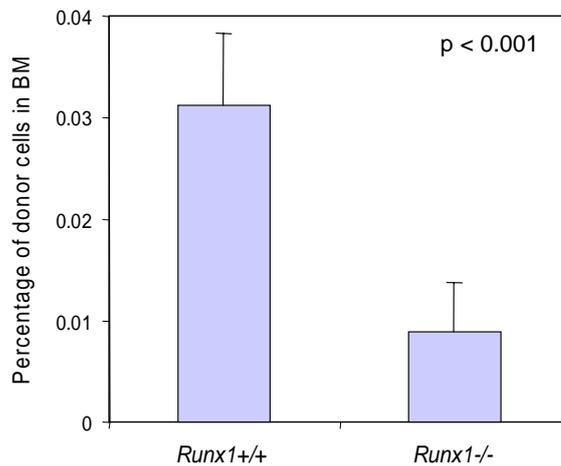
Figure 7.3: *Runx1-/-* BM cells are defective in homing to the stem cell niche
Graph showing percentage of CFSE stained *Runx1+/+* or *Runx1-/-* BM cells found in the recipient BM (n = 4 and 6 respectively), 16 hours after transplantation. Stastical difference using unpaired student-t test is given at the top right corner.

Decrease in levels of CXCR4 and CD49b in *Runx1-/-* stem cells results in impaired interaction with the niche as a result of which these cells may lose their stem cell properties, resulting in a gradual decrease in stem cell numbers. *EVI5* overexpression may rescue defects in niche interaction of *Runx1-/-* HSC and attenuate stem cell exhaustion. In addition, there may be cell intrinsic mechanism also that contributes to the strong synergy seen between *Runx1-/-* status and *EVI5* overexpression. In order to find these cell intrinsic factors, expression of several genes involved in stem cell function and apoptosis were checked in immature (c-Kit+) cell fraction of mock or *EVI5* transfected (GFP+) BM cells from *Runx1+/+* and *Runx1-/-* mice, by qRT-PCR. Among the candidate genes tested, *Bmi-1*, important for self-renewal of normal and cancer stem cells (Iwama et al., 2004); *p21*, essential for stem cell quiescence (Cheng et al., 2000); and the anti-apoptotic gene, *Bcl2* which is negatively regulated by Runx family (Klampfer et al., 1996; Abe et al., 2005), were overexpressed in *Runx1-/-* cells, and the expression of these genes was significantly enhanced by overexpression of *EVI5* (data not shown). Thus, activation of *Bmi1, p21* and *Bcl-2,* together with rescue of interaction of *Runx1-/-* stem cells with the niche by *EVI5* overexpression can explain to some extent the mechanism of synergy between *Runx1-/-* status and *EVI5* overexpression in long term maintenance and expansion of aberrant stem/progenitor cells.

EVI5 is overexpressed in 44% of human RUNX leukemia patients examined

In order to check if *EVI5* overexpression synergizes with loss-of-function of RUNX1 in human RUNX1 related leukemia patients, I carried out qRT-PCR on cDNA from BM or PB of patient samples showing AML M2 carrying RUNX1-ETO fusion protein or AML

M4 patient samples carrying PEBP2β-SMMHC fusion protein. Both these fusion proteins are more commonly found RUNX1 alterations. They are dominant negative factors and cause loss-of-function of RUNX1. cDNA from BM of couple of patients who had undergone complete remission was used as control. Indeed, very significant overexpression of *EVI5* was seen in 4 out of 9 (44%) AML M2 patients examined. Even though some of the AML M4 patients showed 2 to 3 fold overexpression as compared to control samples, the overexpression was not as significant as what was seen in the 4 AML M2 cases (**Figure 8**). Therefore, in human RUNX1 related leukemia cases, especially in AML M2 carrying RUNX1-ETO, *EVI5* overexpression may synergize with loss-of-function of RUNX1 in leukemia development.
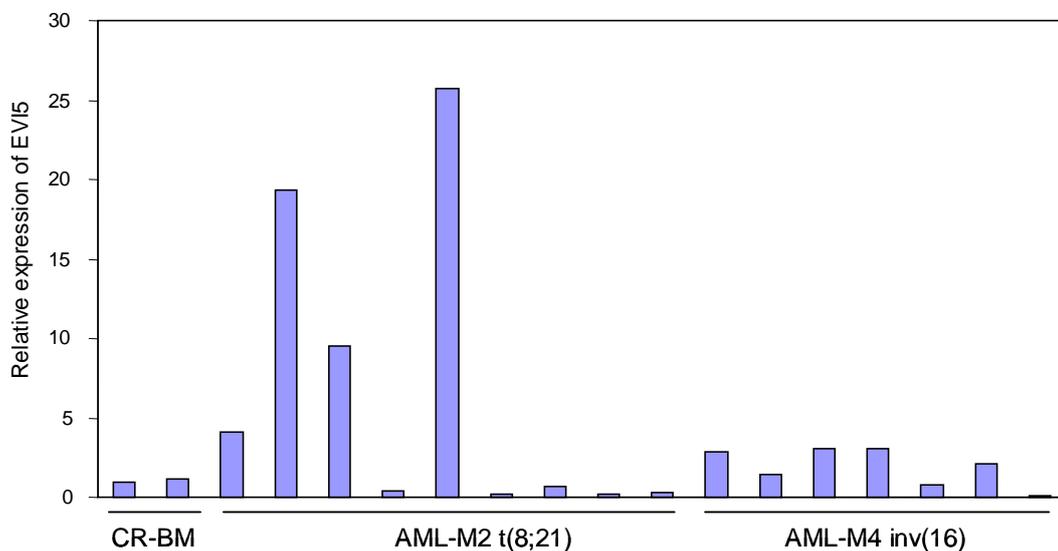
Figure 8: *EVI5* is overexpressed in human RUNX1 related leukemia with t(8;21)
Real time RT-PCR analysis of *EVI5* expression in human RUNX1 related leukemia samples, AML M2 with t(8;21) resulting in RUNX1-ETO fusion protein and AML M4 with inv(16) resulting in PEBP2β-SMMHC fusion protein. Data are represented as fold change relative to BM samples undergoing complete remission (CR-BM).

Chapter 4 - Discussion

Loss-of-function of RUNX1 has been implicated in approximately 30% of human AML as well as in childhood ALL. Despite the prevalence of RUNX1 loss-of-function mutants or dominant negative fusion proteins which impair the function of wild type RUNX1, the *RUNX1* alteration *per se* does not cause leukemia. Rather, cells with loss-of-function of RUNX1 remain preleukemic and only with acquisition of additional hits, they become fully leukemic. In recent years, leukemia has been viewed to originate from leukemia stem cells (LSC), a small hematopoietic subpopulation with enhanced self-renewal capacity. LSC can arise from normal HSC which has undergone genetic mutations that gives it a proliferative advantage together with the inherent property of self-renewal. LSC could also arise from immature progenitor cells, which are capable of active proliferation, when they acquire mutations that revert them back to stem cell like cells which have self-renewal properties.

One of the important points in studies of the role of *RUNX1* in leukemogenesis is to understand how loss-of-function of RUNX1 is involved in the generation and progression of a leukemia initiating cell which could give rise to LSC. Loss-of-function of RUNX1 by itself may not be able to give rise to a LSC clone, as is obvious from experiments that show that mutations in *RUNX1* are present in healthy individuals and transgenic or knock-in mice carrying dominant negative fusion proteins do not develop spontaneous leukemia. Nevertheless, studies done on conditional *Runx1* knockout mice have revealed that *Runx1* excision results in accumulation of immature stem/progenitor cells, with numbers that are approximately 2 to 3 fold higher than wild type cells. Also,

Runx1 deficiency alters the properties of hematopoietic stem/progenitor cells, probably due to increased expression of critical genes such as *Bmi1* and *HoxB4* which are involved in self-renewal of stem cells and *Bcl-2* which is an anti-apoptotic factor. Hence, Runx1 deficient cells have enhanced self-renewal properties and greater resistance to apoptosis than wild type cells (Motoda et al., 2007). Together, this may explain how loss-of-function of Runx1 predisposes cells to leukemia because it supports the accumulation of aberrant stem/progenitor cells that have a growth advantage and could potentially become LSC. Interestingly, the observation that Runx1 deficiency concomitantly increases the expression of *Bmi-1* in mice is further supported by the previous report that describes higher *BMI-1* expression in AML M0 subtype of leukemia in which *RUNX1* often harbors biallelic loss-of-function mutations (Sawa et al., 2005).

The next intriguing question is how does loss-of-function of RUNX1 make cells preleukemic without further progression to full blown leukemia. Why is it that the aberrant stem/progenitor cell fraction, with RUNX1 deficiency, does not undergo extensive proliferation and give rise to leukemia? Based on our experimental results, the reason for this seems to be exhaustion of *Runx1-/-* stem cells. Despite the initial increase in stem/progenitor cell fraction in *Runx1-/-* mice, the immature cell numbers reduce progressively and after a certain period of time the stem/progenitor cell numbers in *Runx1-/-* mice are much lower than those in wild type mice. The evidence for this mainly comes from analysis of recipients of BMT of *Runx1-/-* and control *Runx1+/+* cells. This experimental setting could be more representative of actual physiological setting rather than studying *Runx1-/-* mice themselves, because in actual leukemia cases, before the initiating event, most of the cells in the body are normal wild type cells. Then,

presumably, one or few cells get a genetic alteration resulting in loss-of-function of RUNX1 and these cells turn into possible leukemia initiating cells. This is recapitulated by transplantation of *Runx1-/-* cells into wild type recipients. *Runx1* conditional knockout mice themselves may not be as ideal to study the development of leukemia since almost all the cells in the mouse, including niche for HSC, lack functional Runx1.

The reconstitution of PB in recipient mice by donor *Runx1-/-* cells decreases progressively whereas reconstitution by *Runx1+/+* cells remains constant throughout. Furthermore, 2 years after transplantation, absolute number of immature *Runx1-/-* cells is significantly lower than immature *Runx1+/+* cells in the recipient BM. Since the immature cell population contains the stem/progenitor fraction, we can conclude that there is a decrease in number of *Runx1-/-* stem/progenitor cells over time which may result in declining contribution to hematopoiesis. Therefore, the decrease of *Runx1-/-* cells in PB may not be due to differentiation block of *Runx1-/-* stem/progenitor cells, but rather due to a progressive decline in *Runx1-/-* stem cell numbers or stem cell exhaustion. Serial transplantation experiments also further supported the fact that *Runx1-/-* stem cell exhaustion occurs because they are incapable of reconstituting hematopoiesis in secondary recipients and more than half of the recipients die due to pancytopenia. This is a true test of donor stem cell maintenance and functionality because only real long term HSC can reconstitute hematopoiesis in secondary recipients when transplanted from primary recipients, especially after long intervals. Short term stem cells or progenitors are not capable of such hematopoietic reconstitution of secondary recipients. Also, at each step of BMT, there is a decrease in stem cell numbers. The fact that majority of secondary recipients of *Runx1-/-* cells die within three months of transplantation indicates

that *Runx1-/-* stem cells are not maintained in primary recipients or in other words undergo stem cell exhaustion as a result of which when transplanted into sublethally irradiated secondary recipients, they cannot reconstitute hematopoiesis and the secondary recipients die due to lack of adequate hematopoiesis. The decreased ratio of quiescent CD34- LT-HSC and increased ratio of cycling CD34+ ST-HSC and progenitors amongst KSL stem cell fraction in *Runx1-/-* mice may further help to explain the initial increase of stem/progenitor cell compartment followed by stem cell exhaustion.

The phenomenon of stem cell exhaustion may explain why loss-of-function of Runx1 is only preleukemic and not completely leukemogenic. Cells with Runx1 deficiency may be able to expand abnormally in the beginning, providing a target pool of abnormal stem/progenitor cells for subsequent mutations that can maintain the abnormal clones and confer further proliferative advantage to cause full blown leukemia. In the absence of such cooperative genetic alterations, *Runx1-/-* stem/progenitor cells may slowly undergo exhaustion, without any leukemia development. In order for the preleukemic *Runx1-/-* cells to progress into leukemic cells, probably one of the most important cooperation would be with a genetic alteration that prevents exhaustion of *Runx1-/-* stem/progenitor cells and maintains the abnormal clones in the body till they accumulate enough genetic alterations for them to turn into LSC clones that can give rise to full blown leukemia.

I employed RIM to identify such genetic abnormalities that may cooperate with loss-of-function of Runx1 in the development of leukemia. *Runx1-/-* mice injected with retrovirus showed shorter latency and higher incidence of leukemia than wild type littermates. This result is consistent with the preleukemic effect of *Runx1-/-* status which

results in accelerated leukemia development after acquisition of additional oncogenic hits. Since conditional knockout mice lack functional Runx1, they are probably in a more susceptible state to develop leukemia due to accumulation of abnormal stem/progenitor cells. Even though, in general, *Runx1-/-* mice developed leukemia earlier than wild type littermates, there were variations in the onset time of leukemia as well as the type of cancer in both *Runx1-/-* and wild type mice. The murine cancers induced by MoMuLV virus were classified into early and late onset cases with early onset referring to less than 6 months after birth and late onset to later than that. Further, they were classified into 4 different groups based on the kind of leukemia or lymphoma seen in the mice as described in the results section. MoMuLV virus is strongly lymphotropic and predominantly induces the formation of lymphoid leukemia/ lymphoma which is underlined by the fact that in my study also almost all wild type mice developed T-cell leukemia/lymphoma. On the contrary, majority of the *Runx1-/-* leukemias fell into groups 1 and 2 which were entirely comprised of early onset leukemias with myeloid features. This indicates a more dominant contribution of *Runx1-/-* status in the leukemogenesis of these cases as loss-of-function of Runx1 is usually myelotropic. Thus, I decided to focus on leukemia cases in groups 1 and 2 for two main reasons; firstly, the genes affected by retrovirus integration in these leukemias may show a strong cooperation with *Runx1-/-* status which is probably the reason why these mice develop leukemia earlier than other mice and secondly, since these gene alterations cooperate with *Runx1-/-* status in causing leukemia with myeloid features, they may be more relevant to human RUNX1 related leukemias which are mainly AML **(Table 3)**. In fact, morphological analysis of leukemic cells from some *Runx1-/-* mice in groups 1 and 2 recapitulated the immature myeloid

morphology seen in AML M0 (which sometimes harbors biallelic mutation of *Runx1*) and slightly differentiated myeloid morphology seen in other subtypes such as AML M2 and M4 (which are associated with fusion genes of *RUNX1* or its partner *PEBP2β*, such as *RUNX1-ETO* and *PEBP2β-SMMHC* respectively).

Identification of retroviral insertion sites in mice leukemias by iPCR revealed a large number of integration sites in both *Runx1-/-* and *Runx1+/+* mice. I was specifically interested in CIS that occur with high frequency in *Runx1-/-* mice since they would obviously be strong candidates for cooperating genetic alterations in Runx leukemogenesis. Interestingly, from the IPCR screen, stem cell related and cell cycle related genes turned out to be preferentially affected in *Runx1-/-* mice. This corroborates my hypothesis that gene alterations of a stemness related gene is a likely candidate for cooperation with *Runx1-/-* status as it would help to prevent *Runx1-/-* stem cell exhaustion. Many cell cycle proteins also play a role in stemness and stem cell maintenance because HSC self-renewal properties are tightly linked to their cell cycle.

The prime example of such a CIS is the *Gfi1/Evi5* locus where integrations were seen in 11 *Runx1-/-* leukemia cases, majority belonging to groups 1 and 2. Integrations at this locus were seen only in two *Runx1+/+* leukemia cases. This is a strong indication that when *Runx1-/-* status and integrations at *Gfi1/Evi5* locus coexist, the mice develop aggressive leukemia quite early and these leukemias show myeloid features despite the retrovirus being T-lymphotropic. On the other hand, when these alterations do not coexist, that is, when *Runx1-/-* mice have integrations at other loci, the onset time of leukemia as well as the leukemic phenotype can vary. This strongly implies cooperation between *Runx1-/-* status and genetic alterations resulting from integrations at *Gfi1/Evi5* locus in

development of early onset leukemia with myeloid features. Real time PCR analysis of expression levels of *Gfi1* and *Evi5* to identify the one which is more susceptible to viral integrations at this locus revealed the preferential and specific overexpression of *Evi5* over *Gfi1* in most of the affected leukemic samples, without much change in *Gfi1* levels. This implies that *Evi5* overexpression could be the cooperating genetic alteration in early onset *Runx1-/-* leukemias with myeloid features carrying integrations at *Gfi1/Evi5* locus. This was surprising because *Gfi1* overexpression was expected to play a synergistic role with *Runx1-/-* status by preventing exhaustion of *Runx1-/-* stem cells since *Gfi1* is known to be involved in maintaining self-renewal properties of stem cells (Hock et al., 2004). On the other hand, *Evi5* has not been implicated in stemness and its role in stem cells or other hematopoietic compartments is not known. The known role of *Evi5* is in cell cycle control where it acts by preventing premature entry of cells into mitosis by stabilizing the *Emi1* protein which inhibits the APC complex till the cells are ready to enter mitosis (Eldridge et al., 2006). Nevertheless, further confirmatory assays of synergy were performed for both *Gfi1* and *Evi5* in the Runx1 deficient and control wild type background.

Before discussing the confirmation of synergy in the physiological setting, it is interesting to take a look at the other CIS that have been affected with higher frequency in *Runx1-/-* mice compared to control *Runx1+/+* mice (**Table 2**). Along with the *Gfi1/Evi5* locus, the most frequent integrations in *Runx1-/-* mice were seen near the *c-Myc* oncogene. 4 out of 11 mice with integrations at this locus also fell into groups 1 and 2. The strongest evidence for co-operation between overexpression of *c-Myc* gene and *Runx1-/-* status comes from previously published reports. *c-Myc* is a well established

oncogene; and an oncogenic stimulus in a cell results primarily in hyperproliferation, but can also induce detrimental effects. For instance, *c-Myc* and oncogenic *Ras* have been well described to induce apoptosis and premature senescence in primary cells (Braig et al., 2005). All these effects, oncogene-induced apoptosis, senescence, and differentiation are currently well recognized as a vital fail-safe mechanism to restrict the malignant transformation, particularly in the initial development of cancer. It has been shown that this response of cellular fail-safe mechanism to overexpression of *c-Myc* is attenuated by overexpression of *Runx2* in lymphomas (Blyth et al., 2006). This is because ectopic expression of *Runx2* leads to a preneoplastic state defined by an accumulation of cells with an immature phenotype and a low proliferative rate. The overexpression of *c-Myc* is enough to induce proliferation while *Runx2* overexpression in turn prevents apoptotic responses which may kill the abnormally proliferative malignant clones with *c-Myc* overexpression. Motoda *et al.* (2007) showed a similar cooperation between Runx1 insufficiency (which also leads to accumulation of immature cells with block in cellular fail-safe machinery such as apoptosis, senescence and differentiation) and overexpression of the *Ras* oncogene that results in overt proliferation of cells, turning them into malignant clones. Thus, it is highly possible that the cooperation of *Runx1-/-* status and *c-Myc* overexpression follows a similar mechanism whereby *Runx1-/-* status provides a target population of abnormal cells that can attenuate the fail-safe mechanism when *c-Myc* oncogene is overexpressed, resulting in continued survival and expansive proliferation of these abnormal clones, resulting in leukemia. The changes in stem/progenitor cells due to *Runx1* impairment may contribute to leukemogenesis by

preventing the leukemia initiating clone from being eliminated by the fail-safe mechanism normally triggered by oncogenic stimuli.

The third gene that is more frequently hit in *Runx1-/-* mice is *Evi1* gene. This gene is also a known stem cell regulator that is frequently upregulated in leukemias. During the last few years, there has been a renewed interest in the role of *EVI1* in leukemia since there seems to be a strong correlation between detection of *EVI1* in the BM of patients and poor clinical outcome. It has also long been known that the murine *Evi1* locus is a preferential integration site of ecotropic retroviruses and that the integration leads to myeloid tumors in selected strains of susceptible mice (Mucenski et al., 1988). A recent report however indicates that while there is still a significant integration preference at this site in non-susceptible mice, in these animals the activation of *Evi1* does not induce leukemia but it rather leads to a nonmalignant clonal expansion in long-term hematopoietic progenitors (Kustikova et al., 2005). There have also been reports of *Evi1* being important for proliferation of stem cells and its overexpression leading to myelodysplastic syndrome (Yuasa et al., 2005; Buonamici et al., 2004). The fact that *Evi1* overexpression can maintain stem/progenitor clones over long periods of time makes it quite a prime candidate that may prevent exhaustion of *Runx1-/-* stem cells. Considering the genes that are altered by high frequency of viral integrations in *Runx1-/-* leukemias and the known properties and functions of these genes, altogether, there could be 2 distinct mechanisms of cooperation between *Runx1-/-* status and genetic alterations identified by RIM **(Figure 9)**.

As described above, there is a high frequency of stem cell related genes affected in *Runx1-/-* mice and this is consistent with our hypothesis that overexpression of stem

cell related genes may rescue exhaustion of *Runx1-/-* stem cells and maintain them in the body till they acquire additional hits and become completely leukemogenic. For example, *Gfi1/Evi5* locus was predominantly affected in *Runx1-/-* mice where *Gfi1* is a bona fide stem cell related gene. *Evi1* is another stem cell related gene preferentially affected in *Runx1-/-* cases. Moreover there are other stem cell related genes such as *Hes1* and *Lmo2* which have been affected in *Runx1-/-* mice albeit only once. From an overview of the total number of *Runx1-/-* leukemias and wild type leukemias harboring integrations near stem cell related genes, it can be safely deduced that stem cell related genes are much more preferentially affected in *Runx1-/-* mice.

For further studies of cooperation between identified genes and *Runx1-/-* status, I chose genes near some of the common integration sites, based on frequency of integration and their possible function in stemness, namely *GFI1, EVI5* and *EVI1*. Surprisingly, *EVI5* overexpression showed highest synergy with *Runx1-/-* status in long term maintenance of stem cells as compared to *GFI1* and *EVI1,* both of which are well known to function in stem cell maintenance. *EVI5* showed synergy with *Runx1-/-* status in short term experiments such as colony replating assay as well as in long term maintenance of stem/progenitor cells on OP9 stromal cells in LTC-IC assay. In round 1 of colony assay of sorted c-Kit+GFP+ cells, mock transfected *Runx1-/-* cells showed much higher colony numbers than *Runx1+/+* cells. This is consistent with the increase in stem/progenitor cell fraction seen in *Runx1-/-* mice. However, when round 1 colonies were harvested and replated in round 2, both *Runx1+/+* and *Runx1-/-* transfectants showed either no colonies or very few colonies, probably due to inability to maintain immature cells. On the other hand, overexpression of *EVI5* and the stemness related gene *EVI1* in *Runx1+/+* cells

A

Runx1-/- stem/progenitor cell   Transient expansion of stem/progenitor cells   Stem cell exhaustion and differentiation   No Leukemia

B

Runx1-/- stem/progenitor cell   Overexpression of stem cell related gene such as *EVI5*   Expanded pool of aberrant cells maintained   ★ Additional hits

Aberrant cells acquire further hits and become leukemogenic

C

Runx1-/- stem/progenitor cell   Overexpression of strong oncogene such as *cMyc*   Cells overcome fail-safe mechanism, resulting in uncontrolled proliferation and leukemia

Figure 9: Schematic representation of (A) *Runx1-/-* stem cell exhaustion; leukemia development by cooperation between (B) *Runx1-/-* status and overexpression of stem cell related genes and (C) *Runx1-/-* status and overexpression of strong oncogenes such as *cMyc*.

showed similar colony number as mock transfectants in round 1 and round 2, whereas their overexpression in *Runx1-/-* cells showed lower number of colonies than mock transfectants in round 1. This suppression may be a mode of action for stem cell related genes which try to prevent excess proliferation that could lead to further differentiation and instead try to push the cells towards a more immature status that gives it long term survival properties. Indeed, colony assay results from round 2 showed higher number of colonies in *Runx1-/-* cells transfected with *EVI1* and *EVI5* compared to other combinations. This indicates the maintenance of *Runx1-/-* cells in an immature state by *EVI1* and *EVI5,* probably more effectively by *EVI5* since its overexpression in *Runx1-/-* cells showed the highest synergy in colony formation after replating, in round 2. Overexpression of *GFI1* resulted in significant suppression of colony formation of *Runx1+/+* and *Runx1-/-* cells in round 1. Although this behavior of *GFI1* is consistent with earlier reports describing that overexpression of *GFI1* results in an initial suppression of proliferation, even after replating, colony numbers were very low. This leads to the conclusion that even though in the absence of *Gfi1,* there is a defect in stem cell maintenance, as is clear from *Gfi1* knockout mice studies; ectopic expression of *GFI1* cannot maintain immature status of *Runx1-/-* cells under these conditions.

The above observations are further strengthened by the results from LTC-IC assay. Mock transfected *Runx1+/+* and *Runx1-/-* cKit+GFP+ cells could not be maintained on OP9 stromal cultures even for 30 days. These cells showed terminal differentiation after 30 days of culture on stromal cells. Nevertheless, out of the three genes studied for cooperation with *Runx1-/-* status, *EVI5* overexpression showed the strongest synergy in long term maintenance of stem cells. *Runx1-/-* stem/progenitor cells transfected with

EVI5 could be maintained on OP9 cells for at least 60 days in culture and these long surviving cells retained stem cell properties as evidenced by the presence of high numbers of CAFC, colony forming cells and immature morphology. Usually, only long term stem cells can be maintained for such long periods of time on stromal cultures while short term stem cells or progenitors and other committed cells undergo terminal differentiation. This means that *EVI5* overexpression strongly synergizes with *Runx1-/-* status in maintaining the long term stem cells. On the other hand, *EVI5* transfected wild type cells were incapable of maintaining stem cells and also underwent terminal differentiation. This shows very specific cooperation between *EVI5* overexpression and *Runx1-/-* status. Surprisingly, such specific cooperation was not seen for other genes such as *EVI1* and *GFI1* even though they are known to be involved in stem cell maintenance. Taken together, these results suggest that *EVI5* overexpression helps to maintain *Runx1-/-* stem cells for long periods of time, probably by preventing stem cell exhaustion.

In vivo BMT experiments further strengthened this conclusion. In contrast to recipients of mock transfected *Runx1-/-* cells which showed decreasing percentage of GFP+ cells in PB progressively, recipients of *EVI5* transfected *Runx1-/-* cells showed increasing GFP chimerism. This strongly indicates that indeed, *EVI5* overexpression rescues *Runx1-/-* stem cell exhaustion and in fact helps to maintain an expanded, increasing pool of these aberrant stem cells. Recipients of *EVI5* overexpressing wild type cells showed similar chimerism as mock transfected wild type cells with a slight decrease in GFP chimerism over time. This again underlines the specific synergy between *EVI5* overexpression and Runx1 deficiency, whereby both genetic alterations have to coexist to effectively maintain stem cells. Serial transplantation assays further confirmed the above

results and in contrast to the higher number of secondary recipients of *Runx1-/-* cells that died within one month, all the secondary recipients of *EVI5* overexpressing *Runx1-/-* cells survived with a significant percentage of GFP+ cells in their PB. This implies that *EVI5* overexpression helps to maintain Runx1 deficient stem cells in primary recipients over long periods of time and they are capable of reconstituting hematopoiesis in secondary recipients, leading to enhanced survival. Thus, both *in vitro* and *in vivo* experiments give clear evidence that *EVI5* synergizes with *Runx1-/-* status strongly and specifically to rescue exhaustion of *Runx1-/-* stem cells and maintain an expanded pool of these aberrant cells.

Retroviral integrations were seen at the *Gfi1/Evi5* locus in 2 wild type mice also during RIM experiments. However, in the confirmatory assays, there was not any significant effect due to overexpression of *EVI5* in wild type cells. The reason for this discrepancy could be either of the following. In wild type leukemias, *Gfi1* expression may be altered by retrovirus insertion because under different conditions such as disease phenotype or background, different genes are preferentially expressed due to insertions at the same site. Another possibility is that in the wild type background, *Gfi1* or *Evi5* overexpression may be cooperating with other genetic alterations in the same tumor in leukemia progression; which may not be accurately represented in our cooperation verification studies where we focus only on the cooperation between the gene overexpression and the background genotype.

The next step is to gain an insight into the mechanism by which *EVI5* overexpression could rescue *Runx1-/-* stem cell exhaustion. In order to address questions about mechanisms of cooperation, it is important to understand why exactly *Runx1-/-*

stem cell exhaustion occurs. Once I have the answer to this question, I can examine how *EVI5* overexpression rectifies this problem of stem cell exhaustion. *Runx1-/-* stem cell exhaustion could occur due to cell intrinsic factors or factors involved in the interaction with the stem cell niche. Based on experimental data, the *Runx1-/-* immature cells seem to maintain their intrinsic properties throughout without much change in functionality. Colony assay results of immature *Runx1-/-* cells, two years after transplantation, show similar propensity as *Runx1-/-* cells from young mice to form higher number of colonies than immature *Runx1+/+* cells. This indicates that there is not much change in inherent or intrinsic properties of *Runx1-/-* cells and even 2 years after transplantation; they maintain their higher proliferation capability. Therefore, compromised interaction with the stem cell niche may be a main reason for *Runx1-/-* stem cell exhaustion. *In vivo* homing assay of *Runx1+/+* and *Runx1-/-* cells confirmed this hypothesis because when equal number of labelled BM cells was transplanted into recipient mice, the recipients of *Runx1-/-* cells showed lower percentage of donor cells in the BM 16 hours after transplantation, which confirmed impaired homing efficiency of *Runx1-/-* cells.

A number of molecules expressed by HSC and interacting molecules or chemicals in the niche are important for homing and niche interactions of HSC. A comprehensive list of niche interaction factors were checked in *Runx1+/+* and *Runx1-/-* BM cells transfected with mock vector or *EVI5*. One of the main niche interacting factors that was downregulated in immature cKit+*Runx1-/-* cells was CXCR4. Overexpression of *EVI5* in *Runx1-/-* cells restored normal levels of CXCR4. During steady-state homeostasis, CXCR4 is expressed by hematopoietic cells and its ligand, the chemokine SDF-1 (CXCL12) is expressed in the BM niche. In fact, cells expressing high levels of SDF-1 in

both the endosteal niche and the sinusoidal niche are reported to be the real niche cells to which the HSC attach. Hence, the CXCR4-SDF-1 interaction is very important for stem cell homing and niche interaction, and results in stem cells migrating to and taking up residence in the niche, thus regulating stem cell survival and self-renewal. Blockage of this interaction by G-CSF (that reduces expression levels of SDF-1) or other agents results in increased mobilization of stem cells and decreased retention in the niche. Another niche interacting factor, CD49b which is an α2 integrin required for adhesion to the stem cell niche was also downregulated in *Runx1-/-* cells and expression restored to normal after overexpression of *EVI5* in these cells. Even though the other niche interacting molecules examined did not show any differences in the samples analyzed, defects in these 2 factors, especially CXCR4, may be enough to impair the interaction of *Runx1-/-* stem cells with their niche. Together, *Runx1-/-* stem cell exhaustion may be due to impaired niche interaction of *Runx1-/-* stem cells.

Impaired niche interaction may also help to explain why *Runx1-/-* stem cell exhaustion takes place very gradually. Initially, after transplantation, due to high number of cells transplanted and expansion of *Runx1-/-* stem/progenitor cells, there may be enough number of *Runx1-/-* HSC which home to and interact with the niche; and remain functional and reconstitute hematopoiesis. However, during homeostasis, these cells often leave their original niche and go into circulation. Once that happens, the circulating *Runx1-/-* HSC may be compromised in homing to and interacting appropriately with another niche due to impaired expression of CXCR4 and CD49b (**Figure 10A and B**). Alternatively, since the circulating stem cells are made up of a mixture of normal HSC with intact Runx1 and aberrant HSC which lack Runx1 and express lower levels of

CXCR4 and CD49b, HSC with *Runx1* alteration could be outcompeted by normal wild type HSC in establishing adequate interaction with the niche (**Figure 10C**). This may lead to defective self-renewal of *Runx1-/-* stem cells, leading to loss of stem cells. Since the number of circulating stem cells is a small fraction of the actual stem cell pool, and because a few of the *Runx1-/-* stem cells may actually be able to reestablish niche interaction, decrease in number of stem cells may not be noticeable till sufficient number of stem cells have left their niche, entered circulation and lost their stem cell properties due to impaired niche interaction. Thus, this mechanism satisfactorily explains the stem cell exhaustion of *Runx1-/-* stem cells, since it happens gradually and progressively, over a long interval. Overexpression of *EVI5* may rescue this niche interaction by restoring higher levels of CXCR4 and CD49b, making sure that the circulating, aberrant *Runx1-/-* stem cells home back and interact with their niche, thus maintaining leukemia initiating clones that could give rise to LSC.

There may also be additional mechanisms which could explain the synergy between *EVI5* overexpression and Runx1 deficiency. The known function of *EVI5* in cell cycle control could also contribute to this synergy. Overexpression of *EVI5* leads to stabilization of *Emi1* which in turn may block the APC complex, preventing mitosis of these cells. After prolonged mitotic arrest, cells may die, resulting in decreasing number

A *Runx1+/+* HSCs

Quiescent
LT-HSC
KSL CD34⁻

ST-HSC
KSL CD34⁺

B *Runx1-/-* HSCs

Detached HSCs
ST-HSCs

C

Mixture of circulating HSCs

Niche

Bone marrow

Blood stream

	Niche cell		CXCR4
	Runx1+/+ HSC		SDF-1
	Runx1+/+ progenitor		Other niche factor
	Runx1-/- HSC		Receptor of niche factor
	Runx1-/- progenitor		
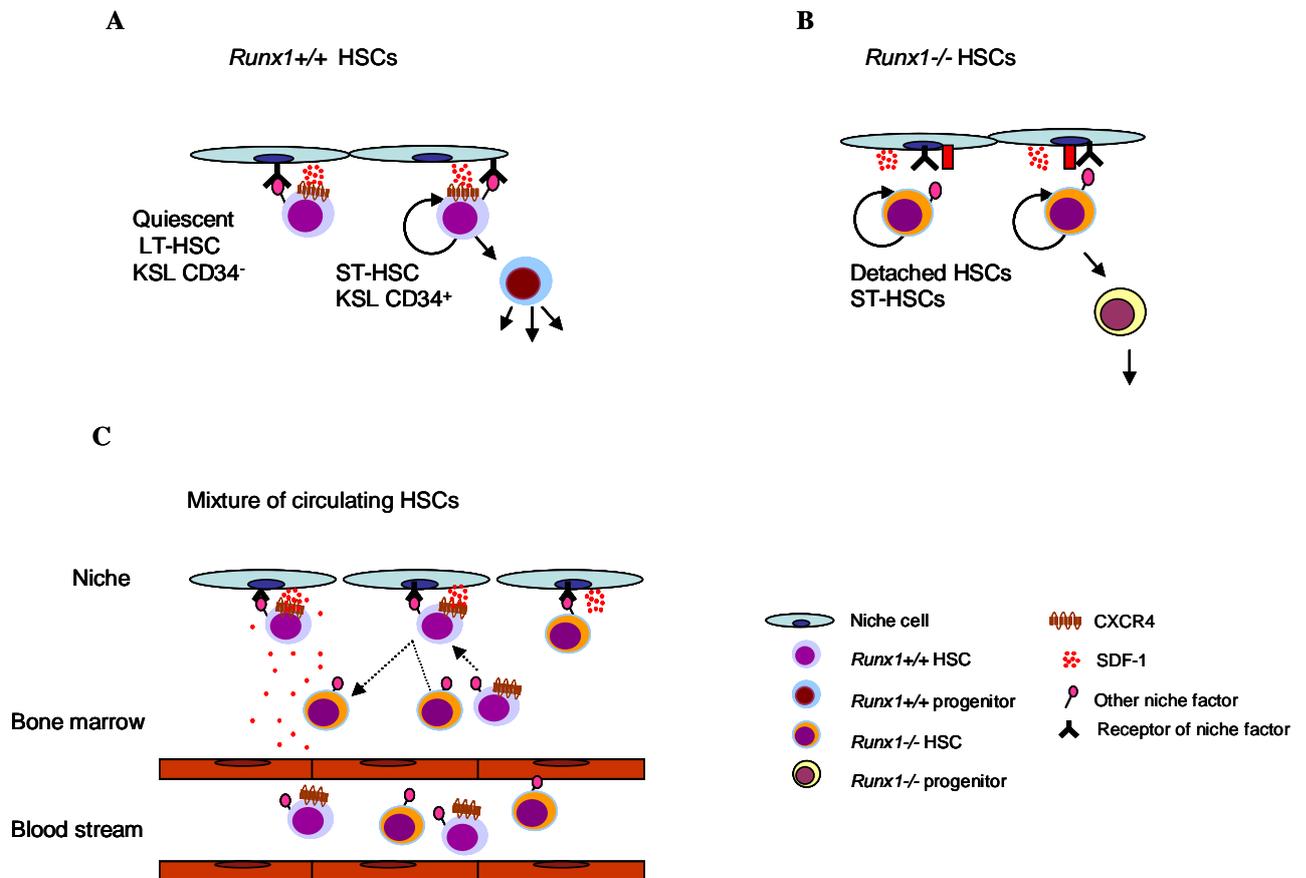
Figure 10: Schematic representation of mechanism by which impaired interaction of *Runx1-/-* stem cells with HSC niche results in *Runx1-/-* stem cell exhaustion.

of *EVI5* transfectants progressively. This may explain the slight decrease of GFP chimerism seen in recipients of *Runx1+/+* cells overexpressing *EVI5*. Applying the same scenario to *Runx1-/-* cells overexpressing *EVI5*, these cells should undergo mitotic arrest due to overexpression of *EVI5*. However, these aberrant cells may not die despite prolonged mitotic arrest due to overexpression of anti-apoptotic genes. Consistent with this idea, expression studies of mock and *EVI5* transfected *Runx1+/+* and *Runx1-/-* cells showed that genes involved in self-renewal such as *Bmi1*, and anti-apoptotic genes such

as *Bcl-2* were upregulated in *Runx1-/-* cells and their expression was further enhanced by overexpression of *EVI5*. Thus, *EVI5* overexpression may maintain *Runx1-/-* cells in an immature state in part by controlling their proliferation rate, preventing excessive proliferation and differentiation and at the same time giving them survival advantage. Hence, overexpression of *Bcl-2* and *Bmi1* could be the cell intrinsic factors that play a role in the cooperation between Runx1 deficiency and *EVI5* overexpression in maintenance of an expanded pool of aberrant stem cells that could give rise to LSC.

Seeing the results from mouse studies whereby I identified and confirmed the overexpression of *EVI5* as a genetic alteration that could cooperate with Runx1 deficiency in leukemogenesis, I next investigated human RUNX1 related leukemia patient samples. Around 40% of the AML M2 patients carrying the RUNX1-ETO fusion protein, which inhibits the wild type RUNX1 in a dominant negative fashion, showed very significant upregulation of *EVI5* expression. This recapitulates the mouse *Runx1-/-* leukemia scenario whereby approximately 40% of the mice which developed leukemia with myeloid features had integrations at the *Gfi1/Evi5* locus, while the others had integrations at other loci, especially near *c-Myc*. Thus *EVI5* overexpression may cooperate with loss-of-function of RUNX1 in a significant proportion of human RUNX1 related leukemia patients. However, there are other key players also that cooperate in RUNX leukemogenesis, especially mitogens such as receptor tyrosine kinases including *c-KIT* and oncogenic *RAS* mutants that have been previously reported and possibly *MYC*. And some of these genetic alterations may even overlap with each other and act as second and third hits in RUNX1 related leukemia.

In summary, loss-of-function of Runx1 results in a preleukemic state without development of full blown leukemia, despite enhanced proliferation of cells after deletion of *Runx1*. This is probably due to stem cell exhaustion of Runx1 deficient cells. Hence, for leukemogenesis, subsequent oncogenic hits have to take place before the *Runx1* altered stem cells undergo complete exhaustion. The second hit is most likely to be a stem cell related gene that can maintain the aberrant *Runx1-/-* cells for long periods of time till they acquire additional hits which would make them leukemogenic. Since exhaustion of *Runx1-/-* stem cells is a very gradual process, these aberrant cells persist in the body for long periods of time and there is adequate time for a second hit to occur before complete stem cell exhaustion takes place. This may be a critical point for leukemogenesis because rapid proliferation accompanied by exhaustion of aberrant stem cells may not confer the required time window for subsequent cooperating alterations and the stem cells may undergo complete exhaustion without being able to progress to leukemia. For example, deficiency of Pten, which is a well known tumor supressor, leads to transient expansion of stem cells. However, Pten deficient mice undergo rapid stem cell exhaustion with defects in HSC numbers and repopulating abilities obvious within one to three months after conditional deletion of *Pten* gene (Zhang et al., 2006). This rapid exhaustion of Pten deficient stem cells may be the reason why mutations in this gene are very rare in human leukemias because the aberrant cells may not have enough time to acquire additional cooperating genetic alterations before they are completely exhausted and eradicated from the individual despite the initial stem cell expansion. On the other hand, Runx1 deficient stem cells, though aberrant, seem to persist in the body

long enough to acquire additional genetic alterations which make them leukemic and this could explain the high frequency of *RUNX1* mutations that are found in human leukemias.

Evi5 has been identified as a potential second hit whose overexpression could cooperate with loss-of-function of Runx1 in leukemia initiation/progression, using mouse leukemia model generated by RIM. Overexpression of *Evi5* helps to maintain *Runx1-/-* stem cells *in vitro* and *in vivo,* thus increasing the chances for development of RUNX1 related leukemia. This cooperation between Runx1 deficiency and *Evi5* overexpression appears to be due to restoration of niche interaction properties of *Runx1-/-* stem cells by *Evi5* along with overexpression of critical stem cell and anti-apoptotic factors such as *Bmi1* and *Bcl2* respectively. *EVI5* overexpression is also seen in significant proportion of human RUNX1 related leukemia patient samples carrying *RUNX1-ETO* fusion gene. Thus, *EVI5* overexpression seems to be a very strong cooperating genetic alteration with loss-of-function of RUNX1 in leukemogenesis. However, the recipient mice transplanted with *Runx1-/-* cells overexpressing *EVI5* did not develop leukemia even one year after BMT, even though the stem cell exhaustion was definitely rescued. Further genetic changes, such as strong mitogenic stimuli, are considered to be required for overt leukemia. Indeed, overexpression of oncogenes such as *N-Myc*, *c-Myc* or *D* type *cyclins* that promote cell proliferation were concurrently seen in 5 out of 8 *Runx1-/-* leukemia cases carrying integration outside *Evi5* gene in the RIM study **(Table 3)**. In human RUNX1 related leukemia, similar mitogenic events such as activating mutations in receptor tyrosine kinases including *c-KIT* and *RAS* have been previously reported (Motoda et al., 2007; Speck and Gilliland, 2002). In fact, out of the 4 human AML M2

cases carrying *RUNX1-ETO* which showed overexpression of *EVI5,* three cases had concurrent activating mutations in *c-KIT* or *FLT3*.

Understanding the mechanism of RUNX1 related leukemia and elucidation of cooperating genetic alterations is for the ultimate purpose of developing specific drugs that target only aberrant cells, especially LSC, without affecting normal cells in order to achieve complete eradication of leukemic clone. Further studies are required to gain deeper mechanistic insights into cooperation between *RUNX1* alterations, overexpression of *EVI5* and other mitogenic stimuli to understand pathways that would provide easy, specific targets for drug design.

References

Abe,N., Kohu,K., Ohmori,H., Hayashi,K., Watanabe,T., Hozumi,K., Sato,T., Habu,S., and Satake,M. (2005). Reduction of Runx1 transcription factor activity up-regulates Fas and Bim expression and enhances the apoptotic sensitivity of double positive thymocytes. J Immunol *175*, 4475-4482.

Akagi,K., Suzuki,T., Stephens,R.M., Jenkins,N.A., and Copeland,N.G. (2004). RTCGD: retroviral tagged cancer gene database. Nucleic Acids Res *32*, D523-D527.

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

Anderson,K.L., Smith,K.A., Perkin,H., Hermanson,G., Anderson,C.G., Jolly,D.J., Maki,R.A., and Torbett,B.E. (1999). PU.1 and the granulocyte- and macrophage colony-stimulating factor receptors play distinct roles in late-stage myeloid cell differentiation. Blood *94*, 2310-2318.

Arai,F., Hirao,A., Ohmura,M., Sato,H., Matsuoka,S., Takubo,K., Ito,K., Koh,G.Y., and Suda,T. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the BM niche. Cell *118*, 149-161.

Barreda,D.R., Hanington,P.C., and Belosevic,M. (2004). Regulation of myeloid development and function by colony stimulating factors. Dev. Comp Immunol. *28*, 509-554.

Basecke,J., Cepek,L., Mannhalter,C., Krauter,J., Hildenhagen,S., Brittinger,G., Trumper,L., and Griesinger,F. (2002). Transcription of AML1/ETO in BM and cord blood of individuals without acute myelogenous leukemia. Blood *100*, 2267-2268.

Bennett,J.M., Catovsky,D., Daniel,M.T., Flandrin,G., Galton,D.A., Gralnick,H.R., and Sultan,C. (1976). Proposals for the classification of the acute leukemias. French-American-British (FAB) co-operative group. Br. J. Hematol. *33*, 451-458.

Berger,R. (1997). Acute lymphoblastic leukemia and chromosome 21. Cancer Genet. Cytogenet. *94*, 8-12.

Blyth,K., Vaillant,F., Hanlon,L., Mackay,N., Bell,M., Jenkins,A., Neil,J.C., and Cameron,E.R. (2006). Runx2 and MYC collaborate in lymphoma development by suppressing apoptotic and growth arrest pathways in vivo. Cancer Res. *66*, 2195-2201.

Bonnet,D. and Dick,J.E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat. Med. *3*, 730-737.

Braig,M., Lee,S., Loddenkemper,C., Rudolph,C., Peters,A.H., Schlegelberger,B., Stein,H., Dorken,B., Jenuwein,T., and Schmitt,C.A. (2005). Oncogene-induced senescence as an initial barrier in lymphoma development. Nature *436*, 660-665.

Buonamici,S., Li,D., Chi,Y., Zhao,R., Wang,X., Brace,L., Ni,H., Saunthararajah,Y., and Nucifora,G. (2004). EVI1 induces myelodysplastic syndrome in mice. J Clin Invest *114*, 713-719.

Calvi,L.M., Adams,G.B., Weibrecht,K.W., Weber,J.M., Olson,D.P., Knight,M.C., Martin,R.P., Schipani,E., Divieti,P., Bringhurst,F.R., Milner,L.A., Kronenberg,H.M., and Scadden,D.T. (2003). Osteoblastic cells regulate the hematopoietic stem cell niche. Nature *425*, 841-846.

Cheng,T., Rodrigues,N., Shen,H., Yang,Y., Dombkowski,D., Sykes,M., and Scadden,D.T. (2000). Hematopoietic stem cell quiescence maintained by p21cip1/waf1. Science *287*, 1804-1808.

Corsetti,M.T. and Calabi,F. (1997). Lineage- and stage-specific expression of Runt box polypeptides in primitive and definitive hematopoiesis. Blood *89*, 2359-2368.

de Koning,J.P., Soede-Bobok,A.A., Schelen,A.M., Smith,L., van,L.D., Santini,V., Burgering,B.M., Bos,J.L., Lowenberg,B., and Touw,I.P. (1998). Proliferation signaling and activation of Shc, p21Ras, and Myc via tyrosine 764 of human granulocyte colony-stimulating factor receptor. Blood *91*, 1924-1933.

DeKoter,R.P., Walsh,J.C., and Singh,H. (1998). PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors. EMBO J. *17*, 4456-4468.

Downing,J.R., Higuchi,M., Lenny,N., and Yeoh,A.E. (2000). Alterations of the AML1 transcription factor in human leukemia. Semin. Cell Dev. Biol. *11*, 347-360.

Drissi,H., Pouliot,A., Stein,J.L., van Wijnen,A.J., Stein,G.S., and Lian,J.B. (2002). Identification of novel protein/DNA interactions within the promoter of the bone-related transcription factor Runx2/Cbfa1. J. Cell Biochem. *86*, 403-412.

Durst,K.L. and Hiebert,S.W. (2004). Role of RUNX family members in transcriptional repression and gene silencing. Oncogene *23*, 4220-4224.

Dzierzak,E. and Medvinsky,A. (1995). Mouse embryonic hematopoiesis. Trends Genet. *11*, 359-366.

Eldridge,A.G., Loktev,A.V., Hansen,D.V., Verschuren,E.W., Reimann,J.D., and Jackson,P.K. (2006). The evi5 oncogene regulates cyclin accumulation by stabilizing the anaphase-promoting complex inhibitor emi1. Cell *124*, 367-380.

Feldman,B.J., Hampton,T., and Cleary,M.L. (2000). A carboxy-terminal deletion mutant of Notch1 accelerates lymphoid oncogenesis in E2A-PBX1 transgenic mice. Blood *96*, 1906-1913.

Ferro,M.T., Hernaez,R., Sordo,M.T., Garcia-Sagredo,J.M., Garcia-Miguel,P., Fernandez,G.M., Lopez,J., Villalon,C., Vallcorba,I., Cabello,P., and San,R.C. (2004). Chromosome 21 tandem repetition and AML1 (RUNX1) gene amplification. Cancer Genet. Cytogenet. *149*, 11-16.

Fialkow,P.J. (1976). Clonal origin of human tumors. Biochim. Biophys. Acta *458*, 283-321.

Fialkow,P.J., Janssen,J.W., and Bartram,C.R. (1991). Clonal remissions in acute nonlymphocytic leukemia: evidence for a multistep pathogenesis of the malignancy. Blood *77*, 1415-1417.

Ford,A.M., Bennett,C.A., Price,C.M., Bruin,M.C., Van Wering,E.R., and Greaves,M. (1998). Fetal origins of the TEL-AML1 fusion gene in identical twins with leukemia. Proc. Natl. Acad. Sci. U. S. A *95*, 4584-4588.

Godin,I., Dieterlen-Lievre,F., and Cumano,A. (1995). Emergence of multipotent hemopoietic cells in the yolk sac and paraaortic splanchnopleura in mouse embryos, beginning at 8.5 days postcoitus. Proc Natl Acad Sci U S A *92*, 773-777.

Golfier,F., Barcena,A., Cruz,J., Harrison,M., and Muench,M. (1999). Mid-trimester fetal livers are a rich source of CD34+/++ cells for transplantation. BM Transplant. *24*, 451-461.

Golfier,F., Barcena,A., Harrison,M.R., and Muench,M.O. (2000). Fetal BM as a source of stem cells for in utero or postnatal transplantation. Br. J Hematol. *109*, 173-181.

Gombart,A.F., Hofmann,W.K., Kawano,S., Takeuchi,S., Krug,U., Kwok,S.H., Larsen,R.J., Asou,H., Miller,C.W., Hoelzer,D., and Koeffler,H.P. (2002). Mutations in the gene encoding the transcription factor CCAAT/enhancer binding protein alpha in myelodysplastic syndromes and acute myeloid leukemias. Blood *99*, 1332-1340.

Growney,J.D., Shigematsu,H., Li,Z., Lee,B.H., Adelsperger,J., Rowan,R., Curley,D.P., Kutok,J.L., Akashi,K., Williams,I.R., Speck,N.A., and Gilliland,D.G. (2005). Loss of Runx1 perturbs adult hematopoiesis and is associated with a myeloproliferative phenotype. Blood *106*, 494-504.

Higuchi,M., O'Brien,D., Kumaravelu,P., Lenny,N., Yeoh,E.J., and Downing,J.R. (2002). Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia. Cancer Cell *1*, 63-74.

Hock,H., Hamblen,M.J., Rooke,H.M., Schindler,J.W., Saleque,S., Fujiwara,Y., and Orkin,S.H. (2004). Gfi-1 restricts proliferation and preserves functional integrity of hematopoietic stem cells. Nature *431*, 1002-1007.

Huang,G., Shigesada,K., Ito,K., Wee,H.J., Yokomizo,T., and Ito,Y. (2001). Dimerization with PEBP2beta protects RUNX1/AML1 from ubiquitin-proteasome-mediated degradation. EMBO J. *20*, 723-733.

Huyhn,A., Dommergues,M., Izac,B., Croisille,L., Katz,A., Vainchenker,W., and Coulombel,L. (1995). Characterization of hematopoietic progenitors from human yolk sacs and embryos. Blood *86*, 4474-4485.

Ichikawa,M., Asai,T., Saito,T., Seo,S., Yamazaki,I., Yamagata,T., Mitani,K., Chiba,S., Ogawa,S., Kurokawa,M., and Hirai,H. (2004). AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. Nat. Med. *10*, 299-304.

Imai,Y., Kurokawa,M., Yamaguchi,Y., Izutsu,K., Nitta,E., Mitani,K., Satake,M., Noda,T., Ito,Y., and Hirai,H. (2004). The corepressor mSin3A regulates phosphorylation-induced activation, intranuclear location, and stability of AML1. Mol. Cell Biol. *24*, 1033-1043.

Inoue,K., Ozaki,S., Shiga,T., Ito,K., Masuda,T., Okado,N., Iseda,T., Kawaguchi,S., Ogawa,M., Bae,S.C., Yamashita,N., Itohara,S., Kudo,N., and Ito,Y. (2002). Runx3 controls the axonal projection of proprioceptive dorsal root ganglion neurons. Nat. Neurosci. *5*, 946-954.

Ito,Y. (2008). RUNX genes in development and cancer: regulation of viral gene expression and the discovery of RUNX family genes. Adv. Cancer Res *99*, 33-76.

Iwama,A., Oguro,H., Negishi,M., Kato,Y., Morita,Y., Tsukui,H., Ema,H., Kamijo,T., Katoh-Fukui,Y., Koseki,H., van Lohuizen,M., and Nakauchi,H. (2004). Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. Immunity. *21*, 843-851.

Iwama,A., Zhang,P., Darlington,G.J., McKercher,S.R., Maki,R., and Tenen,D.G. (1998). Use of RDA analysis of knockout mice to identify myeloid genes regulated in vivo by PU.1 and C/EBPalpha. Nucleic Acids Res. *26*, 3034-3043.

Jimenez-Sanchez,G., Childs,B., and Valle,D. (2001). Human disease genes. Nature *409*, 853-855.

Jonkers,J. and Berns,A. (1996). Retroviral insertional mutagenesis as a strategy to identify cancer genes. Biochim. Biophys. Acta *1287*, 29-57.

Kiel,M.J., Iwashita,T., Yilmaz,O.H., and Morrison,S.J. (2005). Spatial differences in hematopoiesis but not in stem cells indicate a lack of regional patterning in definitive hematopoietic stem cells. Dev Biol *283*, 29-39.

Kim,W.Y., Sieweke,M., Ogawa,E., Wee,H.J., Englmeier,U., Graf,T., and Ito,Y. (1999). Mutual activation of Ets-1 and AML1 DNA binding by direct interaction of their autoinhibitory domains. EMBO J. *18*, 1609-1620.

Klampfer,L., Zhang,J., Zelenetz,A.O., Uchida,H., and Nimer,S.D. (1996). The AML1/ETO fusion protein activates transcription of BCL-2. Proc Natl Acad Sci U S A *93*, 14059-14064.

Kollet,O., Dar,A., Shivtiel,S., Kalinkovich,A., Lapid,K., Sztainberg,Y., Tesio,M., Samstein,R.M., Goichberg,P., Spiegel,A., Elson,A., and Lapidot,T. (2006). Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. Nat Med. *12*, 657-664.

Komori,T., Yagi,H., Nomura,S., Yamaguchi,A., Sasaki,K., Deguchi,K., Shimizu,Y., Bronson,R.T., Gao,Y.H., Inada,M., Sato,M., Okamoto,R., Kitamura,Y., Yoshiki,S., and Kishimoto,T. (1997). Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell *89*, 755-764.

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

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

Kustikova,O., Fehse,B., Modlich,U., Yang,M., Dullmann,J., Kamino,K., von Neuhoff,N., Schlegelberger,B., Li,Z., and Baum,C. (2005). Clonal dominance of hematopoietic stem cells triggered by retroviral gene marking. Science *308*, 1171-1174.

Lapidot,T., Dar,A., and Kollet,O. (2005). How do stem cells find their way home? Blood *106*, 1901-1910.

Lapidot,T. and Petit,I. (2002). Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. Exp Hematol. *30*, 973-981.

Levanon,D., Bernstein,Y., Negreanu,V., Ghozi,M.C., Bar-Am,I., Aloya,R., Goldenberg,D., Lotem,J., and Groner,Y. (1996). A large variety of alternatively spliced and differentially expressed mRNAs are encoded by the human acute myeloid leukemia gene AML1. DNA Cell Biol. *15*, 175-185.

Levanon,D., Bettoun,D., Harris-Cerruti,C., Woolf,E., Negreanu,V., Eilam,R., Bernstein,Y., Goldenberg,D., Xiao,C., Fliegauf,M., Kremer,E., Otto,F., Brenner,O., Lev-Tov,A., and Groner,Y. (2002). The Runx3 transcription factor regulates development and survival of TrkC dorsal root ganglia neurons. EMBO J. *21*, 3454-3463.

Levanon,D., Brenner,O., Negreanu,V., Bettoun,D., Woolf,E., Eilam,R., Lotem,J., Gat,U., Otto,F., Speck,N., and Groner,Y. (2001). Spatial and temporal expression pattern of

Runx3 (AML2) and Runx1 (AML1) indicates non-redundant functions during mouse embryogenesis. Mech. Dev. *109*, 413-417.

Levanon,D., Goldstein,R.E., Bernstein,Y., Tang,H., Goldenberg,D., Stifani,S., Paroush,Z., and Groner,Y. (1998). Transcriptional repression by AML1 and LEF-1 is mediated by the TLE/Groucho corepressors. Proc. Natl. Acad. Sci. U. S. A *95*, 11590-11595.

Levanon,D. and Groner,Y. (2004). Structure and regulated expression of mammalian RUNX genes. Oncogene *23*, 4211-4219.

Li,Q.L., Ito,K., Sakakura,C., Fukamachi,H., Inoue,K., Chi,X.Z., Lee,K.Y., Nomura,S., Lee,C.W., Han,S.B., Kim,H.M., Kim,W.J., Yamamoto,H., Yamashita,N., Yano,T., Ikeda,T., Itohara,S., Inazawa,J., Abe,T., Hagiwara,A., Yamagishi,H., Ooe,A., Kaneda,A., Sugimura,T., Ushijima,T., Bae,S.C., and Ito,Y. (2002). Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell *109*, 113-124.

Licht,J.D. (2001). AML1 and the AML1-ETO fusion protein in the pathogenesis of t(8;21) AML. Oncogene *20*, 5660-5679.

Ling,K.W. and Dzierzak,E. (2002). Ontogeny and genetics of the hemato/lymphopoietic system. Curr. Opin. Immunol. *14*, 186-191.

Liu,P., Tarle,S.A., Hajra,A., Claxton,D.F., Marlton,P., Freedman,M., Siciliano,M.J., and Collins,F.S. (1993). Fusion between transcription factor CBF beta/PEBP2 beta and a myosin heavy chain in acute myeloid leukemia. Science *261*, 1041-1044.

Loh,M.L., McLean,T.W., Buckley,J.D., Howells,W., Gilliland,D.G., and Smith,F.O. (1998). Lack of TEL/AML1 fusion in pediatric AML: further evidence for lineage specificity of TEL/AML1. Leuk. Res. *22*, 461-464.

Mao,S., Frank,R.C., Zhang,J., Miyazaki,Y., and Nimer,S.D. (1999). Functional and physical interactions between AML1 proteins and an ETS protein, MEF: implications for the pathogenesis of t(8;21)-positive leukemias. Mol. Cell Biol. *19*, 3635-3644.

Marshall,C.J. and Thrasher,A.J. (2001). The embryonic origins of human hematopoiesis. Br. J Hematol. *112*, 838-850.

McKercher,S.R., Torbett,B.E., Anderson,K.L., Henkel,G.W., Vestal,D.J., Baribault,H., Klemsz,M., Feeney,A.J., Wu,G.E., Paige,C.J., and Maki,R.A. (1996). Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. EMBO J. *15*, 5647-5658.

Medvinsky,A.L., Samoylina,N.L., Muller,A.M., and Dzierzak,E.A. (1993). An early pre-liver intraembryonic source of CFU-S in the developing mouse. Nature *364*, 64-67.

Michaud,J., Scott,H.S., and Escher,R. (2003). AML1 interconnected pathways of leukemogenesis. Cancer Invest *21*, 105-136.

Migliaccio,G., Migliaccio,A.R., Petti,S., Mavilio,F., Russo,G., Lazzaro,D., Testa,U., Marinucci,M., and Peschle,C. (1986). Human embryonic hemopoiesis. Kinetics of progenitors and precursors underlying the yolk sac----liver transition. J Clin Invest *78*, 51-60.

Mikhail,F.M., Serry,K.A., Hatem,N., Mourad,Z.I., Farawela,H.M., El Kaffash,D.M., Coignet,L., and Nucifora,G. (2002). AML1 gene overexpression in childhood acute lymphoblastic leukemia. Leukemia *16*, 658-668.

Mitelman,F., Johansson,B., and Mertens,F. (2004). Fusion genes and rearranged genes as a linear function of chromosome aberrations in cancer. Nat. Genet. *36*, 331-334.

Miyoshi,H., Shimizu,K., Kozu,T., Maseki,N., Kaneko,Y., and Ohki,M. (1991). t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. Proc. Natl. Acad. Sci. U. S. A *88*, 10431-10434.

Moore,K.A. and Lemischka,I.R. (2006). Stem cells and their niches. Science *311*, 1880-1885.

Mori,H., Colman,S.M., Xiao,Z., Ford,A.M., Healy,L.E., Donaldson,C., Hows,J.M., Navarrete,C., and Greaves,M. (2002). Chromosome translocations and covert leukemic clones are generated during normal fetal development. Proc. Natl. Acad. Sci. U. S. A *99*, 8242-8247.

Motoda,L., Osato,M., Yamashita,N., Jacob,B., Chen,L.Q., Yanagida,M., Ida,H., Wee,H.J., Sun,A.X., Taniuchi,I., Littman,D., and Ito,Y. (2007). Runx1 Protects Hematopoietic Stem/progenitor Cells from Oncogenic Insult. Stem Cells.

Mueller,B.U., Pabst,T., Osato,M., Asou,N., Johansen,L.M., Minden,M.D., Behre,G., Hiddemann,W., Ito,Y., and Tenen,D.G. (2002). Heterozygous PU.1 mutations are associated with acute myeloid leukemia. Blood *100*, 998-1007.

Mundlos,S., Otto,F., Mundlos,C., Mulliken,J.B., Aylsworth,A.S., Albright,S., Lindhout,D., Cole,W.G., Henn,W., Knoll,J.H., Owen,M.J., Mertelsmann,R., Zabel,B.U., and Olsen,B.R. (1997). Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. Cell *89*, 773-779.

Nakamura,T. (2005). Retroviral insertional mutagenesis identifies oncogene cooperation. Cancer Sci *96*, 7-12.

Nakamura,T. (2005). Retroviral insertional mutagenesis identifies oncogene cooperation. Cancer Sci *96*, 7-12.

Nichols,J. and Nimer,S.D. (1992). Transcription factors, translocations, and leukemia. Blood *80*, 2953-2963.

Nishikawa,M., Tahara,T., Hinohara,A., Miyajima,A., Nakahata,T., and Shimosaka,A. (2001). Role of the microenvironment of the embryonic aorta-gonad-mesonephros region in hematopoiesis. Ann. N. Y. Acad. Sci. *938*, 109-116.

Nishimura,M., Fukushima-Nakase,Y., Fujita,Y., Nakao,M., Toda,S., Kitamura,N., Abe,T., and Okuda,T. (2004). VWRPY motif-dependent and -independent roles of AML1/Runx1 transcription factor in murine hematopoietic development. Blood *103*, 562-570.

North,T., Gu,T.L., Stacy,T., Wang,Q., Howard,L., Binder,M., Marin-Padilla,M., and Speck,N.A. (1999). Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. Development *126*, 2563-2575.

Ogawa,E., Inuzuka,M., Maruyama,M., Satake,M., Naito-Fujimoto,M., Ito,Y., and Shigesada,K. (1993b). Molecular cloning and characterization of PEBP2 beta, the heterodimeric partner of a novel Drosophila Runt-related DNA binding protein PEBP2 alpha. Virology *194*, 314-331.

Ogawa,M. (1993a). Differentiation and proliferation of hematopoietic stem cells. Blood *81*, 2844-2853.

Okuda,T., van,D.J., Hiebert,S.W., Grosveld,G., and Downing,J.R. (1996). AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell *84*, 321-330.

Orkin,S.H. (2000). Diversification of hematopoietic stem cells to specific lineages. Nat. Rev. Genet. *1*, 57-64.

Osato,M., Asou,N., Abdalla,E., Hoshino,K., Yamasaki,H., Okubo,T., Suzushima,H., Takatsuki,K., Kanno,T., Shigesada,K., and Ito,Y. (1999). Biallelic and heterozygous point mutations in the Runt domain of the AML1/PEBP2alphaB gene associated with myeloblastic leukemias. Blood *93*, 1817-1824.

Osato,M. and Ito,Y. (2005). Increased dosage of the RUNX1/AML1 gene: a third mode of RUNX1 related leukemia? Crit Rev. Eukaryot. Gene Expr. *15*, 217-228.

Osato,M., Yanagida,M., Shigesada,K., and Ito,Y. (2001). Point mutations of the RUNX1/AML1 gene in sporadic and familial myeloid leukemias. Int. J. Hematol. *74*, 245-251.

Osawa,M., Hanada,K., Hamada,H., and Nakauchi,H. (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science *273*, 242-245.

Otto,F., Thornell,A.P., Crompton,T., Denzel,A., Gilmour,K.C., Rosewell,I.R., Stamp,G.W., Beddington,R.S., Mundlos,S., Olsen,B.R., Selby,P.B., and Owen,M.J. (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell *89*, 765-771.

Pabst,T., Mueller,B.U., Zhang,P., Radomska,H.S., Narravula,S., Schnittger,S., Behre,G., Hiddemann,W., and Tenen,D.G. (2001). Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. Nat. Genet. *27*, 263-270.

Peled,A., Petit,I., Kollet,O., Magid,M., Ponomaryov,T., Byk,T., Nagler,A., Ben Hur,H., Many,A., Shultz,L., Lider,O., Alon,R., Zipori,D., and Lapidot,T. (1999). Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science *283*, 845-848.

Petrovick,M.S., Hiebert,S.W., Friedman,A.D., Hetherington,C.J., Tenen,D.G., and Zhang,D.E. (1998). Multiple functional domains of AML1: PU.1 and C/EBPalpha synergize with different regions of AML1. Mol. Cell Biol. *18*, 3915-3925.

Potocnik,A.J., Brakebusch,C., and Fassler,R. (2000). Fetal and adult hematopoietic stem cells require beta1 integrin function for colonizing fetal liver, spleen, and BM. Immunity. *12*, 653-663.

Preudhomme,C., Sagot,C., Boissel,N., Cayuela,J.M., Tigaud,I., De,B.S., Thomas,X., Raffoux,E., Lamandin,C., Castaigne,S., Fenaux,P., and Dombret,H. (2002). Favorable prognostic significance of CEBPA mutations in patients with de novo acute myeloid leukemia: a study from the Acute Leukemia French Association (ALFA). Blood *100*, 2717-2723.

Preudhomme,C., Warot-Loze,D., Roumier,C., Grardel-Duflos,N., Garand,R., Lai,J.L., Dastugue,N., MacIntyre,E., Denis,C., Bauters,F., Kerckaert,J.P., Cosson,A., and Fenaux,P. (2000). High incidence of biallelic point mutations in the Runt domain of the AML1/PEBP2 alpha B gene in Mo acute myeloid leukemia and in myeloid malignancies with acquired trisomy 21. Blood *96*, 2862-2869.

Putz,G., Rosner,A., Nuesslein,I., Schmitz,N., and Buchholz,F. (2006). AML1 deletion in adult mice causes splenomegaly and lymphomas. Oncogene *25*, 929-939.

Rennert,J., Coffman,J.A., Mushegian,A.R., and Robertson,A.J. (2003). The evolution of Runx genes I. A comparative study of sequences from phylogenetically diverse model organisms. BMC. Evol. Biol. *3*, 4.

Rhoades,K.L., Hetherington,C.J., Harakawa,N., Yergeau,D.A., Zhou,L., Liu,L.Q., Little,M.T., Tenen,D.G., and Zhang,D.E. (2000). Analysis of the role of AML1-ETO in leukemogenesis, using an inducible transgenic mouse model. Blood *96*, 2108-2115.

Richmond,T.D., Chohan,M., and Barber,D.L. (2005). Turning cells red: signal transduction mediated by erythropoietin. Trends Cell Biol. *15*, 146-155.

Rosenbauer,F. and Tenen,D.G. (2007). Transcription factors in myeloid development: balancing differentiation with transformation. Nat Rev Immunol *7*, 105-117.

Satake,M., Ibaraki,T., Yamaguchi,Y., and Ito,Y. (1989). Loss of responsiveness of an AP1-related factor, PEBP1, to 12-O-tetradecanoylphorbol-13-acetate after transformation of NIH 3T3 cells by the Ha-ras oncogene. J. Virol. *63*, 3669-3677.

Sawa,M., Yamamoto,K., Yokozawa,T., Kiyoi,H., Hishida,A., Kajiguchi,T., Seto,M., Kohno,A., Kitamura,K., Itoh,Y., Asou,N., Hamajima,N., Emi,N., and Naoe,T. (2005). BMI-1 is highly expressed in M0-subtype acute myeloid leukemia. Int. J. Hematol. *82*, 42-47.

Schwarz,B.A. and Bhandoola,A. (2006). Trafficking from the BM to the thymus: a prerequisite for thymopoiesis. Immunol. Rev. *209*, 47-57.

Scott,E.W., Simon,M.C., Anastasi,J., and Singh,H. (1994). Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science *265*, 1573-1577.

Semenza G L. Transcription factors and human diseases. 1998.
Ref Type: Generic

Shivdasani,R.A., Fujiwara,Y., McDevitt,M.A., and Orkin,S.H. (1997). A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. EMBO J. *16*, 3965-3973.

Shivdasani,R.A. and Orkin,S.H. (1996). The transcriptional control of hematopoiesis. Blood *87*, 4025-4039.

Song,W.J., Sullivan,M.G., Legare,R.D., Hutchings,S., Tan,X., Kufrin,D., Ratajczak,J., Resende,I.C., Haworth,C., Hock,R., Loh,M., Felix,C., Roy,D.C., Busque,L., Kurnit,D., Willman,C., Gewirtz,A.M., Speck,N.A., Bushweller,J.H., Li,F.P., Gardiner,K., Poncz,M., Maris,J.M., and Gilliland,D.G. (1999). Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukemia. Nat. Genet. *23*, 166-175.

Speck,N.A. and Gilliland,D.G. (2002). Core-binding factors in hematopoiesis and leukemia. Nat Rev Cancer *2*, 502-513.

Speck,N.A., Stacy,T., Wang,Q., North,T., Gu,T.L., Miller,J., Binder,M., and Marin-Padilla,M. (1999). Core-binding factor: a central player in hematopoiesis and leukemia. Cancer Res. *59*, 1789s-1793s.

Steelman,L.S., Pohnert,S.C., Shelton,J.G., Franklin,R.A., Bertrand,F.E., and McCubrey,J.A. (2004). JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. Leukemia *18*, 189-218.

Sugiyama,T., Kohara,H., Noda,M., and Nagasawa,T. (2006). Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in BM stromal cell niches. Immunity. *25*, 977-988.

Suzuki,T., Shen,H., Akagi,K., Morse,H.C., Malley,J.D., Naiman,D.Q., Jenkins,N.A., and Copeland,N.G. (2002). New genes involved in cancer identified by retroviral tagging. Nat. Genet. *32*, 166-174.

Tanaka,T., Kurokawa,M., Ueki,K., Tanaka,K., Imai,Y., Mitani,K., Okazaki,K., Sagata,N., Yazaki,Y., Shibata,Y., Kadowaki,T., and Hirai,H. (1996). The extracellular signal-regulated kinase pathway phosphorylates AML1, an acute myeloid leukemia gene product, and potentially regulates its transactivation ability. Mol. Cell Biol. *16*, 3967-3979.

Taniuchi,I., Osato,M., Egawa,T., Sunshine,M.J., Bae,S.C., Komori,T., Ito,Y., and Littman,D.R. (2002). Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. Cell *111*, 621-633.

Tavian,M., Coulombel,L., Luton,D., Clemente,H.S., Dieterlen-Lievre,F., and Peault,B. (1996). Aorta-associated CD34+ hematopoietic cells in the early human embryo. Blood *87*, 67-72.

Vaillant,F., Blyth,K., Andrew,L., Neil,J.C., and Cameron,E.R. (2002). Enforced expression of Runx2 perturbs T cell development at a stage coincident with beta-selection. J. Immunol. *169*, 2866-2874.

van Wijnen,A.J., Stein,G.S., Gergen,J.P., Groner,Y., Hiebert,S.W., Ito,Y., Liu,P., Neil,J.C., Ohki,M., and Speck,N. (2004). Nomenclature for Runt-related (RUNX) proteins. Oncogene *23*, 4209-4210.

Wang,Q., Stacy,T., Binder,M., Marin-Padilla,M., Sharpe,A.H., and Speck,N.A. (1996). Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. Proc. Natl. Acad. Sci. U. S. A *93*, 3444-3449.

Wang,S., Wang,Q., Crute,B.E., Melnikova,I.N., Keller,S.R., and Speck,N.A. (1993). Cloning and characterization of subunits of the T-cell receptor and murine leukemia virus enhancer core-binding factor. Mol. Cell Biol. *13*, 3324-3339.

Wechsler,J., Greene,M., McDevitt,M.A., Anastasi,J., Karp,J.E., Le Beau,M.M., and Crispino,J.D. (2002). Acquired mutations in GATA1 in the megakaryoblastic leukemia of Down syndrome. Nat. Genet. *32*, 148-152.

Wilson,A. and Trumpp,A. (2006). Bone-marrow hematopoietic-stem-cell niches. Nat Rev Immunol *6*, 93-106.

Woolf,E., Xiao,C., Fainaru,O., Lotem,J., Rosen,D., Negreanu,V., Bernstein,Y., Goldenberg,D., Brenner,O., Berke,G., Levanon,D., and Groner,Y. (2003). Runx3 and Runx1 are required for CD8 T cell development during thymopoiesis. Proc. Natl. Acad. Sci. U. S. A *100*, 7731-7736.

Wright,D.E., Wagers,A.J., Gulati,A.P., Johnson,F.L., and Weissman,I.L. (2001). Physiological migration of hematopoietic stem and progenitor cells. Science *294*, 1933-1936.

Yamaguchi,Y., Kurokawa,M., Imai,Y., Izutsu,K., Asai,T., Ichikawa,M., Yamamoto,G., Nitta,E., Yamagata,T., Sasaki,K., Mitani,K., Ogawa,S., Chiba,S., and Hirai,H. (2004). AML1 is functionally regulated through p300-mediated acetylation on specific lysine residues. J. Biol. Chem. *279*, 15630-15638.

Yamashita,N., Osato,M., Huang,L., Yanagida,M., Kogan,S.C., Iwasaki,M., Nakamura,T., Shigesada,K., Asou,N., and Ito,Y. (2005). Haploinsufficiency of Runx1/AML1 promotes myeloid features and leukemogenesis in BXH2 mice. Br. J. Hematol. *131*, 495-507.

Yanagida,M., Osato,M., Yamashita,N., Liqun,H., Jacob,B., Wu,F., Cao,X., Nakamura,T., Yokomizo,T., Takahashi,S., Yamamoto,M., Shigesada,K., and Ito,Y. (2005). Increased dosage of Runx1/AML1 acts as a positive modulator of myeloid leukemogenesis in BXH2 mice. Oncogene *24*, 4477-4485.

Yokomizo,T., Ogawa,M., Osato,M., Kanno,T., Yoshida,H., Fujimoto,T., Fraser,S., Nishikawa,S., Okada,H., Satake,M., Noda,T., Nishikawa,S., and Ito,Y. (2001). Requirement of Runx1/AML1/PEBP2alphaB for the generation of hematopoietic cells from endothelial cells. Genes Cells *6*, 13-23.

Yuasa,H., Oike,Y., Iwama,A., Nishikata,I., Sugiyama,D., Perkins,A., Mucenski,M.L., Suda,T., and Morishita,K. (2005). Oncogenic transcription factor Evi1 regulates hematopoietic stem cell proliferation through GATA-2 expression. EMBO J *24*, 1976-1987.

Zeng,C., van Wijnen,A.J., Stein,J.L., Meyers,S., Sun,W., Shopland,L., Lawrence,J.B., Penman,S., Lian,J.B., Stein,G.S., and Hiebert,S.W. (1997). Identification of a nuclear matrix targeting signal in the leukemia and bone-related AML/CBF-alpha transcription factors. Proc. Natl. Acad. Sci. U. S. A *94*, 6746-6751.

Zhang,D.E., Zhang,P., Wang,N.D., Hetherington,C.J., Darlington,G.J., and Tenen,D.G. (1997). Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. Proc. Natl. Acad. Sci. U. S. A *94*, 569-574.

Zhang,J., Grindley,J.C., Yin,T., Jayasinghe,S., He,X.C., Ross,J.T., Haug,J.S., Rupp,D., Porter-Westpfahl,K.S., Wiedemann,L.M., Wu,H., and Li,L. (2006). PTEN maintains hematopoietic stem cells and acts in lineage choice and leukemia prevention. Nature *441*, 518-522.

Zhang,J., Niu,C., Ye,L., Huang,H., He,X., Tong,W.G., Ross,J., Haug,J., Johnson,T., Feng,J.Q., Harris,S., Wiedemann,L.M., Mishina,Y., and Li,L. (2003). Identification of the hematopoietic stem cell niche and control of the niche size. Nature *425*, 836-841.