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Reckzeh, Kristian

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LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

DECIPHERING THE PATHOGENESIS OF ACUTE MYELOID LEUKEMIA



LUND UNIVERSITY
Faculty of Medicine

Kristian Reckzeh

2012

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On the cover: Wright-Giemsa staining of AML. © AFIP Atlas of Tumor Pathology, with permission.

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Abstract

Acute myeloid leukemia (AML) is a malignant disorder of the blood system. Hematopoietic stem cells (HSC) supply and maintain this system by differentiating via intermediates into lineage-restricted progenitors that strongly proliferate to keep up with the high turn-over of mature blood cells. In AML, the mechanisms controlling differentiation and proliferation of myeloid cells are disturbed leading to the accumulation of undifferentiated cells that interfere with the production of normal blood cells.

Mutations of the transcription factor C/EBP α have been observed in 10 percent in AML with normal cytogenetics. In addition, internal tandem duplications (ITD) of FLT3 are frequently observed alterations in AML and coincide with mutations of C/EBP α .

The effects of FLT3-ITD cooperation with C/EBP α mutations in AML are not fully understood. To address this, knockin mouse strains harboring different Cebpa mutations and Flt3-ITD were used to generate an AML mouse model. This model demonstrated a block at the transition from pre-granulocyte-macrophage progenitor (pGMP) to GMP due to disrupted C/EBP α function. The cooperative effect of FLT3-ITD is composed of enhancing the generation of leukemia-initiating GMPs and activation of STAT5 targets. In in vitro studies it was demonstrated that FLT3-ITD reduces the cytokine-requirements for cell growth and that leukemic cells harboring FLT3-ITD are more sensitive to inhibition of the FLT3 pathway in vitro. To address the impact of FLT3-ITD gene dosage and loss of Flt3 wild type allele in vivo the Flt3-ITD knockin mouse was crossed to the Flt3 receptor knockout mouse. These studies demonstrated that the myeloproliferative phenotype was FLT3-ITD dosage-dependent and independent of FL. In summary, the data presented provide deeper insights into oncogene cooperation and FLT3-ITD dosage in AML.

Abbreviations

AGM	Aorta-gonad-mesonephros
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AML1	Acute myeloid leukemia 1 (RUNX1)
ANG-1	Angiopoietin 1
ATP	Adenosine triphosphate
bHLH	basic helix-loop-helix motif
bZIP	basic region leucine zipper
BM	Bone marrow
BMI-1	B-lymphoma Mo-MLV insertion region 1
BRM	Basic region mutation
CB	Cord blood
CBF	Core-binding factor
CDDO	2-Cyano-3,12-dioxooleana-1,9-dien-28-oic acid
CDK	cyclin-dependent kinase
C/EBP α	CCAAT/enhancer binding protein alpha
CFC	Colony-forming cell
CFU-S	Colony-forming unit spleen
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
CN	Copy number
DNA	Desoxyribonucleic acid
E2F	E2-binding factor
eIF	eukaryotic translation initiation factor
ERK	Extracellular signal-regulated kinase
ESC	Embryonic stem cell
FAB	French-American-British
FACS	Fluorescence activated cell sorting
FL	FLT3 ligand
FLT3	FMS-like tyrosine kinase receptor 3
GATA-1	GATA binding protein 1
GMP	Granulocyte-macrophage progenitor
G-CSF	Granulocyte-colony stimulating factor
G-CSFR	G-CSF receptor
GM-CSF	Granulocyte-macrophage-colony stimulating factor

GM-CSFR	GM-CSF receptor
GTP	Guanosine triphosphate
GvHD	Graft-versus-host disease
GvL	Graft-versus-leukemia
HLA	Human leukocyte antigen
HOXB4	Homeobox gene B4
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
ICM	Inner cell mass
IL	Interleukin
IPSC	Induced pluripotent stem cell
ITD	Internal tandem duplication
JAK	Janus kinase
JM	Juxtamembrane domain
KIT	SCF receptor
LEF-1	lymphoid enhancer binding factor 1
LMO2	Lim-domain only 2
LMPP	Lymphoid-primed multipotent progenitor
LOH	Loss of heterozygosity
LT	Long-term
MAPK	Mitogen-activated protein kinase
MASI	Mutant allele-specific imbalance
M-CSF	Macrophage-colony stimulating factor
M-CSFR	M-CSF receptor
MDS	Myelodysplastic syndrome
MEP	Megakaryocyte-erythrocyte progenitor
MLL	Mixed lineage-leukemia
MPN	Myeloproliferative neoplasm
MPP	Multipotent progenitor
MSC	Mesenchymal stem cell
MYC	Myelocytomatosis oncogene
NK cell	Natural killer cell
ORF	Open reading frame
PB	Peripheral blood
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol 3-kinase
PU.1	SPI1
RAS	Rat sarcoma
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
RUNX1	Runt-related transcription factor 1

SCF	Stem cell factor
SCL	Stem cell leukemia
SCN	Severe congenital neutropenia
SDF-1	Stromal cell-derived factor 1
SFFV	Spleen focus forming virus
SH2	Src-homology 2
SHIP	SH2-domain-containing inositol phosphatase
SLAM	Signaling lymphocyte activation molecule
SPI1	SFFV proviral integration 1
ST	Short-term
STAT5	Signal transducer and activator of transcription 5
SWI/SNF	Switch/sucrose nonfermentable
TAD1	Transactivation domain 1
TAL-1	T-cell acute lymphocytic leukemia 1
TBP	TATA-box binding protein
TF	Transcription factor
TFIIB	Transcription initiation factor IIB
TGF- β	Transforming growth factor beta
TI	Tyrosine kinase inhibitory domain
TKD	Tyrosine kinase domain
TNF- α	Tumor necrosis factor alpha
TPO	Thrombopoietin
TRIB2	Tribbles homolog 2
VCAM1	Vascular cell adhesion molecule 1
WBC	White blood cell count
WHO	World Health Organization
wt	wild-type

List of Publications

This thesis is based on the articles listed below and are referred to in the text by their roman numerals (I-III).

- I Reckzeh K, Bereshchenko O, Mead AJ, Rehn M, Kharazi S, Jacobsen SE, Nerlov C, Cammenga J. *Molecular and cellular effects of oncogene cooperation in a genetically accurate AML mouse model*. **Leukemia** **2012**. Feb 9. doi: 10.1038/leu.2012.37. [Epub ahead of print].
- II Reckzeh K, Ohlsson E, Fock V, Cammenga J. *Cytokine requirements and oncogene addiction in a mouse model for AML with FLT3-ITD*. Manuscript.
- III Kharazi S, Mead A, Mansour A, Hultquist A, Boiers C, Luc S, Buza-Vidas N, Ma Z, Ferry H, Atkinson D, Reckzeh K, Masson K, Cammenga J, Rönstrand L, Arai F, Suda T, Nerlov C, Sitnicka E, Jacobsen SE. *Impact of gene dosage and loss of the wild type allele on FLT3 internal tandem duplication induced expansion of myeloid primed multipotent progenitor cells*. **Blood** **2011**; 118(13): 3613-3621.

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Additional publications during PhD training:

Reckzeh K, Cammenga J. *Molecular mechanisms underlying deregulation of C/EBPalpha in acute myeloid leukemia*. **Int J Hematol.** 2010; 91, 557-568. Review.

Rehn M, Olsson A, Reckzeh K, Diffner E, Carmeliet P, Landberg G, Cammenga J. *Hypoxic induction of vascular endothelial growth factor regulates murine hematopoietic stem cell function in the low-oxygenic niche*. **Blood.** 2011; 118(6):1534-1543.

Preface

This is my thesis! It took six years of studying, reading, experimenting and commuting to get to this point. I am happy and proud to finally have come so far. I was very privileged to work at this institution, the Lund Stem Cell Center, where scientists from different research areas come together and perform research of impact and importance. Particularly, the *Division of Molecular Medicine and Gene Therapy* has been a dynamic place of creative interaction and scientific inspiration. I was very fortunate to receive so much invaluable help and support from colleagues, friends and family during this time. With this thesis I want to summarize my work on the role of two factors involved in regulation of normal and malignant hematopoiesis which is the process of blood formation in adult bone marrow. The proteins I have studied contain important functions in pathways controlling proliferation and differentiation and appear to be frequently altered in malignant hematopoiesis.

This thesis also wants to introduce this field to a broader audience, since many people who are not scientists have followed me with interest over the years. During this time I have always tried to explain what I was doing, but usually I failed. So, it is my last attempt. Listen carefully, this time!

The thesis gives you an overview on the hematopoietic system, will briefly discuss the concept of hematopoietic stem cells and introduce acute myeloid leukemia (AML), one of many malignant hematopoietic disorders. Finally, it presents a summary of my studies and a discussion of the results.

The above mentioned proteins C/EBP α and FLT3 are frequently altered by mutations, which change their function and eventually lead to development of AML. To learn more about the mechanisms involved in leukemia initiation we designed a mouse model that allows us to study the molecular and cellular background of this disease. Ultimately, our findings may help discovering better therapy and cure for AML.

Parts of the text and figures in this thesis have previously been published in a review article about the transcription factor C/EBP α in the *International Journal of Hematology* (Reckzeh and Cammenga, 2010).

Introduction

Stem Cells

The term *stem cell* defines a cell of undifferentiated state with the properties 1) to indefinitely self-renew by dividing into cells of identical state and properties and 2) to differentiate into all mature, specialized cells existent in a specific tissue.

Stem cells are distinguished developmentally into *embryonic* stem cells (ESC) and somatic or *adult* stem cells (Figure 1). ESCs are derived from the inner cell mass (ICM) of an early embryo and have the potential to differentiate into all tissues of the organism (*pluripotent*). While adult stem cells are virtually found in all tissues of an adult organism, their potential to differentiate is restricted to the specific tissue or organ. An example of a tissue-specific stem cell is the hematopoietic stem cell (HSC) which supplies the blood system and differentiates into multiple cell types found in blood (*multipotent*).

Because of their potential to differentiate into multiple tissues and capacity to self-renew, stem cell therapies have been proposed for regenerative medicine and tissue replacement after injury or disease. However, the isolation of human ESCs includes destruction of the embryo which raises ethical controversies utilizing these cells for research or treatment of diseases.

The generation of induced pluripotent stem cells (iPSC) from somatic cells has been an important advance in stem cell research because it does not involve the ‘use’ of embryos and creates cells with practically the same properties as ESCs (Takahashi and Yamanaka, 2006; Yu et al., 2007). Before ESCs or iPSCs can effectively be used in the treatment of patients a couple of drawbacks need to be resolved. The indefinite growth of stem cells carries the risk of cancer. Embryonic stem cells transplanted to a recipient would form teratomas.

The use of adult stem cells is limited by the low number that can be isolated from the specific tissue and stem cell expansion *in vitro* is still stuck at a preliminary stage. Moreover, unless the stem cells are patient-derived, immunological differences between donor and recipient would always require lifelong immuno-suppressive medication. Hence, the discovery to induce pluripotency in a somatic cell from a patient opened new opportunities. However, recipes to safely guide an unspecialized, replicating cell towards functional tissue are not yet available.

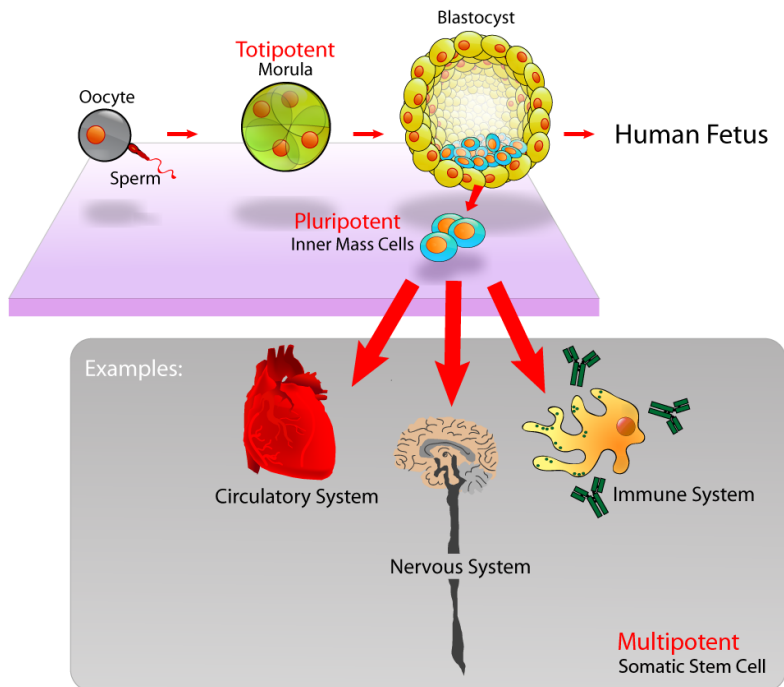


Figure 1. Differentiation potential of embryonic and somatic stem cells. Pluripotent embryonic stem cells derived from the inner cell mass of the blastocyst can give rise to all cell types of the primary germ layers including ectoderm (e.g. spine, peripheral nerves and brain), mesoderm (e.g. muscles, blood) and endoderm (e.g. thymus, lungs, digestive organs). Restricted to a specific tissue, somatic stem cells are able to replenish dying cells and repair damages. © Mike David Jones, with permission.

Stem cell therapy has been proposed for the treatment of many degenerative disorders such as Alzheimer and Parkinson, cardiovascular diseases, diabetes and various forms of cancer. So far however, only hematopoietic stem cell transplantation (HSCT) is used routinely in the clinic. Here, transplantation of HSCs is supposed to replace the defective hematopoietic system of the host. However, HSCT also comes with risks and side effects.

Hence, to make stem cell therapy more applicable, it requires a better understanding of the underlying mechanisms and factors controlling differentiation and self-renewal of stem cells.

All experiments in this thesis are carried out in mice and therefore all data and discussion refers to murine hematopoiesis unless otherwise indicated.

Hematopoiesis

The hematopoietic system is probably one of the best studied tissues and the HSC serves in many ways as a model for adult stem cells of other tissues.

The process of blood formation to continuously replace short-lived mature blood cells is referred to as hematopoiesis (Orkin and Zon, 2008). Mature blood cells carry out a variety of tasks and can be categorized into specific cell lineages according to their function (Table 1). Red blood cells or erythrocytes are specialized to transport oxygen. Platelets derived from megakaryocytes prevent excessive bleeding from injury by clotting. White blood cells (WBC) include lymphocytes (B- and T-cells), natural killer (NK) cells, granulocytes (neutrophils, eosinophils and basophils), macrophages which protect from infections, and pathogens and build up the immune system of the individual.

Most cell types of these blood lineages are short-lived compared to the lifespan of the individual and need to be replenished constantly. In human adults the hematopoietic system produces therefore around 10^{12} new blood cells per day at steady state (Ogawa, 1993). To maintain a balanced output of mature cells and to react to increased demands for specific cell types under stress like bleedings or disease, proliferation and differentiation processes are subject to strict control mechanisms. Defects in these processes causing an imbalance result in various hematopoietic disorders.

Table 1. Cell types, their function and cell count in human peripheral blood.

Cell type	Function	Count per μl Blood
Red cells (erythrocytes)	Transport of O_2 and CO_2	$4\text{-}6 \times 10^6$
Platelets (thrombocytes)	Blood clotting	$0.15\text{-}0.4 \times 10^6$
B-cells (B lymphocytes)	Antibody production	$0.14\text{-}0.8 \times 10^3$
T-cells (T lymphocytes)	Elimination of virus-infected cells and regulation of the immune system	$0.5\text{-}1.8 \times 10^3$
NK cells	Elimination of tumor and virus-infected cells	$0.08\text{-}0.4 \times 10^3$
Macrophages	Phagocytosis of bacteria, fungi and neutrophils	$0.2\text{-}0.9 \times 10^3$
Neutrophils	Phagocytosis of microorganisms and pathogens	$2.1\text{-}6 \times 10^3$
Eosinophils	Parasite destruction	$0.03\text{-}0.3 \times 10^3$
Basophils	Histamine release	$0.01\text{-}0.1 \times 10^3$

Hematopoietic Stem Cells

The hematopoietic system is supplied and replenished by rare, self-renewing HSCs capable of maintaining homeostasis throughout life residing in the bone marrow (BM) of adult mammals (Morrison et al., 1995). The definition of an HSC includes the following

properties: (1) HSCs are multipotent and should therefore give rise to all hematopoietic lineages; (2) HSCs are able to reconstitute hematopoiesis in an ablated recipient; (3) HSCs are able to give rise to HSCs, i.e. they possess self-renewal capacity.

History

First evidence for cells with these properties was provided in 1961 when it was demonstrated that colonies of hematopoietic cells emerged in the spleen of lethally irradiated recipient mice after BM transplantation (Till and McCulloch, 1961). These nodules of proliferating hematopoietic cells were termed CFU-S (colony-forming unit spleen). Following experiments demonstrated that CFU-S contain rare cells able to produce different other hematopoietic cells and to give rise to new colonies (Becker et al., 1963; Siminovitch et al., 1963), which are considered properties of a stem cell. However, later it was suggested that an even more primitive HSC (preCFU-S) colonizes the BM to give rise to cells capable of colonizing the spleen (Hodgson and Bradley, 1979; Schofield, 1978). These experiments were just the beginning of the identification of HSCs, and the HSC-defining criteria proposed by Till and McCulloch still remain valid.

Later, by tracking myeloid and lymphoid cells in primary and secondary recipients carrying identical retroviral markers different groups provided evidence for the existence of a cell in BM that has multi-lineage potential, can self-renew and functionally repopulate the blood system of an irradiated recipient (Dick et al., 1985; Keller et al., 1985; Keller and Snodgrass, 1990). However, isolation and functional examination of an untouched HSC required the identification of characteristic HSC markers and the development of techniques such as fluorescence activated cell sorting (FACS). Enormous efforts have been taken to find single markers specific for HSCs. However, prospective isolation of HSCs still involves several cell surface markers. Major work in this regard was carried out by Osawa and colleagues who demonstrated the clonal nature of HSCs by serial transplantation of single cells, identified as a part of a rare population in the BM, which gave sustained multi-lineage engraftment (Osawa et al., 1996). The identification and employment of cell surface receptors of the SLAM family has improved and simplified the ability to rigorously isolate HSCs from progenitors (Kiel et al., 2005).

Hierarchy

Hematopoiesis is described as a process where rare HSCs constitute the top of a hierarchy of intermediate subpopulations that successively develop into large numbers of mature cells of all lineages (Figure 2) (Weissman, 2000).

The HSC compartment is a heterogeneous population containing cells with long-term (lifespan) and short-term (8 weeks) self-renewal capacity and multipotent progenitors

(LT-, ST-HSCs and MPPs) without self-renewal. However, all these subpopulations carry the potential to give rise to all blood lineages (Morrison and Weissman, 1994).

It is generally believed that in a first pivotal step, differentiation of the HSC results in lineage-committed progenitors with either lymphoid or myeloid potential defined as common lymphoid progenitor (CLP) and common myeloid progenitor (CMP). In murine hematopoiesis two distinct progenitor populations with exclusive myeloid or lymphoid potential have been identified (Akashi et al., 2000; Kondo et al., 1997).

B-, T- and NK-cells are derived from the CLP through intermediate generation of lineage restricted progenitors. The CMP generates the granulocyte-macrophage progenitor (GMP) and megakaryocyte/ erythroid progenitor (MEP) through which mature granulocytes, macrophages, megakaryocytes/platelets and erythrocytes are formed. The development from lineage-committed progenitors to mature cells progresses through a number of intermediates which potential have not been completely identified. However, with being more and more lineage-restricted these intermediates become increasingly proliferating to produce the amount of blood cells needed.

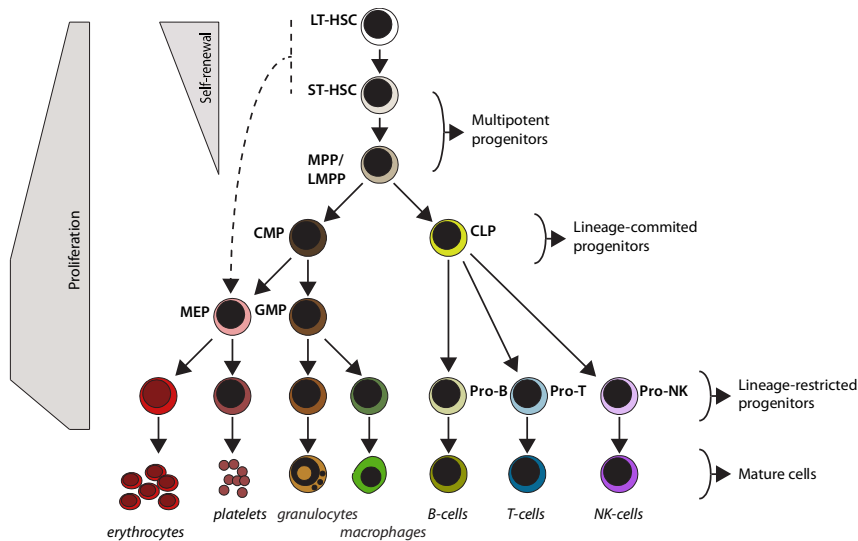


Figure 2. Schematic overview of the hematopoietic system. Self-renewing hematopoietic stem cells give rise to subsets of intermediates becoming more and more lineage-restricted. At the same time, proliferation of these progenitors increases to meet the immense needs of mature cells.

While it is widely accepted that a single HSC has the capacity to differentiate into all other hematopoietic cell types, the differentiation paths towards mature cells are not completely resolved and remain controversial. Adolfsson et al. (2005) questioned the view of MPPs differentiating into CMP and CLP subpopulations by postulating that a first lineage commitment step would already take place directly after the HSC level

resulting in a multipotent progenitor with lymphoid and myeloid lineage potential (LMPP) lacking megakaryocyte/erythroid potential. In this model, the MEP originates directly from the HSC pool (Adolfsson et al., 2005). However, this view has been challenged by Forsberg et al. (2006) who were able to show that lineage separation occurs downstream of non-self-renewing MPPs (Boyer et al., 2011; Forsberg et al., 2006). New insights to the debate of which path HSCs take to differentiate into committed progenitors were provided by Pronk et al. (2007) who isolated myeloid progenitor subfractions with distinct lineage potential according to their expression of CD150, Endoglin (CD105) and CD41 (Pronk et al., 2007). This study presents a novel set of intermediates and suggests that there are more ways an HSC can take.

Clinical application of hematopoietic stem cells

The field of BM transplantation was pioneered by Donnall Thomas and colleagues in the 1950s when BM cells were for the first time transfused to patients that had received irradiation and chemotherapy (Thomas et al., 1957). It had been observed that mice could be rescued receiving BM transfusions after lethal irradiation (Lorenz et al., 1951), and later this approach had also shown curative results in mice with leukemia (Barnes et al., 1956) opening the field of BM transplantation as it is used today routinely in clinical practice for treatment of hematological malignancies. However, the attempts of human application were generally met with a total lack of success except for a few transplants involving identical twins. The understanding of the human leukocyte antigen (HLA) system led to the ability to select compatible sibling donors and resulted in the first successful long-term survivors in 1960s (Bach et al., 1968; Van Rood et al., 1958). In the 1970s patients were given allogeneic, HLA-matched transplants for leukemia after failure of all other treatment and although the survival rates were poor some patients were cured (Buckner et al., 1970). During the following decades improved control of infections (introduction of ganciclovir), the use of peripheral blood (PB) or cord blood (CB) as the source of HSCs, and the ability to select donors other than family members resulted in wide application of HSC transplantation (Thomas, 2000).

Although allogeneic HSC transplants are matched to the donor, small immunological differences between donor cells and the recipient's tissue can cause a complication called graft-versus-host-disease (GvHD). Donor T-cells recognize the recipient's body tissues as foreign and start to fight them. The symptoms of GvHD range from mild to severe including abdominal pain, diarrhea, hair loss, hepatitis, skin rash, and can lead to fatal infections. On the other hand, allogeneic BM transplantations in leukemia patients have been associated with lower risk of disease relapse. This phenomenon is also known as graft-versus-leukemia (GvL) and arises as a result of an immune reaction of the grafted donor T-cells against the diseased BM of the recipient (Baron et al., 2005). This lower rate of relapse accounts for the increased success rate of allogeneic transplants compared

to transplants from identical twins, and indicates that allogeneic BM transplantation is a form of immunotherapy. GvL is the major benefit of transplants that do not employ the highest immuno-suppressive regimens. However, GvL is mainly beneficial in hematopoietic malignancies with slow progress such as chronic leukemia and less effective in rapidly growing acute leukemias.

The use of HSC transplantation is not limited to hematological malignancies but also widely applicable to treat other hematological disorders such as immune deficiencies and hemoglobin disorders. In addition, the application of HSCs in gene therapy has been proposed to correct monogenetic hematopoietic disorders where HSCs are collected from a patient and the deficient gene is corrected by retroviral gene transfer followed by replacing the host's hematopoietic system with those transduced HSCs (Karlsson, 1991; Woods et al., 2002). Although the principals of this approach have been proven and patients with severe combined immuno-deficiencies have been successfully treated, major concerns exist regarding retroviral insertion mutagenesis and require future improvements in vector design and gene transfer strategies. However, in many cases gene therapy is the final approach for the patient due to lack of alternative treatment (Baum et al., 2004).

From the beginning, aspirated BM was the source of HSCs, but today HSCs are less invasively harvested from PB after mobilization with growth factors such as granulocyte-stimulating factor (G-CSF) or a combination of cyclophosphamide and G-CSF, and from umbilical CB which has been shown to be less immuno-responsive to HLA-mismatch and therefore associated with a lower risk of GvHD. The amount of harvested HSCs from cord blood is however often too low for successful treatment of adults, and the *ex vivo* expansion of HSCs prior transplantation has therefore been a major goal in stem cell research over the last two decades. However, attempts to expand HSCs *in vitro* while maintaining self-renewal and long-term repopulating capacity have met with limited success. Recent studies have identified potential in Notch ligand, aryl hydrocarbon receptor antagonists or pleiotrophin to expand human HSCs *in vitro* (Boitano et al., 2010; Delaney et al., 2010; Himgburg et al., 2010).

Self-Renewal and HSC Fates

The HSC's ability to self-renew is a prerequisite in order to meet the immense needs of mature blood cells over a lifespan period. *Self-renewal* of the HSC occurs by either undergoing symmetric cell division giving rise to two new identical daughter cells with the same properties as the original HSC (resulting in HSC expansion) or dividing asymmetrically into one new daughter cell sustaining HSC properties and another daughter cell that starts to differentiate into more restricted, multipotent progenitors (HSC maintenance). Herein, asymmetry could either be achieved by intrinsic cell-fate

determinants (divisional asymmetry) or by exposure of the two daughter cells to different extrinsic signals (environmental asymmetry).

While populating the fetal liver during embryonic development HSCs divide symmetrically and expand the HSC pool, whereas during steady-state adult hematopoiesis the number of HSCs remains relatively constant. It is believed that HSCs are widely non-dividing or *quiescent* under steady-state conditions to maintain the HSC pool and life-lasting hematopoietic homeostasis (Cheshier et al., 1999; Domen and Weissman, 1999; Wagers et al., 2002). In addition, as every other cell, HSCs can go into programmed cell-death (*apoptosis*). Finally, migrating to other anatomical sites in or outside the BM is also considered a fate option of HSCs (Figure 3).

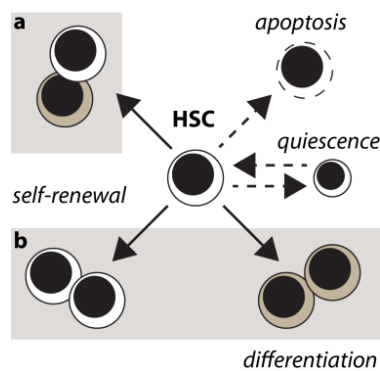


Figure 3. HSC fate options. HSCs divide asymmetrically (a) giving rise to a new HSC and a differentiating cell or symmetrically (b) yielding either two HSCs or two differentiating daughter cells. HSCs can furthermore undergo apoptosis or become quiescent.

Regulation of Hematopoietic Stem Cells

Because of the high turnover of cells in the hematopoietic system and to be able to act rapidly in response to acute blood loss or infection the equilibrium of HSC self-renewal, quiescence and differentiation must be strictly regulated. The mechanisms regulating HSCs and their fate options involve multiple, highly orchestrated pathways that determine the cell cycle status and gene expression profiles. In general, cell-extrinsic and cell-intrinsic factors may be distinguished. Extrinsic factors in the surrounding environment called the hematopoietic niche signaling through receptors on the cell surface can influence HSC behavior as well as transcription factors regulating gene expression inside the HSC.

The hematopoietic stem cell niche

HSCs reside in specialized anatomical sites (*niches*) in the BM of adult mammals. These sites include the surface along the endosteum and areas nearby vascular sinusoids found throughout the marrow (Ehninger and Trumpp, 2011). This microenvironment involves interaction with other cell types such as osteoblasts, stromal fibroblasts and endothelial cells, which provide cytokines and growth factors. The hematopoietic niches are believed to be less oxygenated (hypoxic), a condition promoting HSC maintenance by protecting vital HSCs from damaging effects of reactive oxygen species (Suda et al., 2011). The concept of stem cell niches as highly organized places controlling HSC function was firstly proposed more than 30 years ago (Schofield, 1978), and today it is clear that the niche plays a pivotal role in regulating HSC fate together with cell autonomous mechanisms (Lymperi et al., 2010).

Extrinsic factors

The different cell types found in the hematopoietic niche provide growth factors and cytokines that affect HSC and their behavior. Cells contained in the niche express several factors important for HSC function including membrane-bound stem cell factor (SCF; binds to the KIT receptor), thrombopoietin (TPO), a ligand for the MPL receptor, erythropoietin (EPO) and G-CSF (Arai et al., 2009; Buza-Vidas et al., 2006; Krantz, 1991; Yang et al., 1998). The *fms-like tyrosine kinase receptor* (FLT3) ligand is expressed in stromal fibroblasts of the BM microenvironment and synergizes with other hematopoietic growth factors to promote proliferation of primitive hematopoietic precursors (Lyman et al., 1995; Stirewalt and Radich, 2003).

An important role has been recently addressed to nestin-positive mesenchymal stem cells (MSC) which were found in close proximity to HSCs (Mendez-Ferrer et al., 2010). These cells express factors crucial for HSC maintenance including stromal cell-derived factor 1 (SDF-1 also named CXCL12), SCF, angiopoietin-1 (ANG-1), IL-7, vascular cell adhesion molecule 1 (VCAM1), and osteopontin (OPN)(Ehninger and Trumpp, 2011).

There are several other factors that have been demonstrated to act negatively on HSCs. The secreted transforming growth factor beta (TGF- β) protein has been shown to repress HSC activity by signaling through the SMAD pathway and subsequent regulation of the cell cycle (Blank and Karlsson, 2011). The tumor necrosis factor alpha (TNF- α) was demonstrated to negatively regulate HSCs *in vitro* and recently a suppressive role for TNF- α on HSCs *in vivo* was identified in TNF- α -deficient mice (Bryder et al., 2001; Pronk et al., 2011).

Intrinsic factors

Transcription factors (TF) are internal signals that govern gene expression and regulate HSC fate. Whether gene expression is directly determined by the influence of extrinsic factors (instructive model) or through stochastic variation of internal signals in an environment with extrinsic factors allowing certain key decisions (permissive model) remains not fully understood. However, it is now believed that HSC fate decisions rely more on stochastic mechanisms while committed progenitors may be higher influenced by extrinsic signals (Enver et al., 1998).

The number of TFs involved in specification of HSCs during ontogeny and fate regulation during adult hematopoiesis has grown large and only a few can be discussed here.

In embryonic hematopoiesis, it has been shown that the bHLH factor SCL/tal-1 (Stem cell leukemia/T-cell acute lymphocytic leukemia 1) and its associated partner, the Lim-domain only 2 (LMO2) are essential for specification of a HSC precursor from the hemangioblast (Kim and Bresnick, 2007). HSCs are initially found in the aorta-gonad-mesonephros (AGM) region and dorsal arteries of the mid-gestation mouse embryo (de Bruijn et al., 2000). At this stage, the runt domain containing DNA-binding subunit of the core-binding factor (CBF) RUNX1 (AML1) is required for the emergence and function of HSCs (Okuda et al., 1996; Wang et al., 1996). The mixed lineage-leukemia gene (MLL) and HOXB4 have been identified as regulators of HSC maturation during early hematopoiesis (Ernst et al., 2004). HOXB4, a member of the homeobox domain TFs was shown to have a dramatic effect on HSC self-renewal. Retroviral overexpression of HOXB4 demonstrated great expansion of HSCs *ex vivo* (Antonchuk et al., 2002).

Once adult HSCs have arisen and moved to the bone marrow several of the TFs previously required for emergence and formation of HSCs may no longer be required for continuous survival and self-renewal. However, there are a few factors known to be involved in regulation of adult HSC self-renewal and quiescence. BMI-1, a member of the Polycomb group proteins that regulate HOX proteins, was identified as an important transcriptional regulator of self-renewal (Iwama et al., 2004; Lessard and Sauvageau, 2003). Another important factor for HSC integrity is Gfi-1, a zinc-finger transcriptional repressor that regulates the cell cycle inhibitor p21 (Hock et al., 2004; Zeng et al., 2004).

In addition to TFs like SCL and RUNX1 that are involved in almost all hematopoietic lineages there are factors that have lineage-specific expression patterns. Their disruption does usually only affect a single or a small number of related lineages. Important TFs for the development and differentiation of myeloid lineages are PU.1, C/EBP α and GATA-1. The latter is highly expressed in MEPs and to a lesser extent in HSCs and CMPs while GATA-1 expression is required for definitive erythropoiesis (McDevitt et al., 1997).

The level of PU.1 expression is relatively low in HSCs, CMPs and CLPs compared to B-cells whereas its expression is high in mature myeloid cells (Scott et al., 1994). *Pu.1* knock-out mice lack mature myeloid and B-cells probably due to an early block of transition from multipotent progenitors (MPPs) to lineage-restricted CMPs and CLPs. Interestingly, PU.1 deficient mice (PU.1 level at 10% of wt mice) develop erythroid cells and have a normal MEP compartment supporting the model of MEPs being the direct progeny of HSCs (Rosenbauer et al., 2006).

The transcription factor C/EBP α is an example of a lineage-specific TF. It is expressed by HSCs, myeloid progenitors, and granulocytes. It is indispensable for the maturation of neutrophil and eosinophil granulocytes as C/EBP α knockout mice lack mature granulocytes without affecting other hematopoietic lineages.

Because TFs regulate the specification and emergence of HSCs and subsequently govern hematopoiesis by controlling production and differentiation of HSCs, it is conceivable that disorders of the hematopoietic system often involve alterations of the regulatory network of TFs. In hematopoietic malignancies TFs are often involved in chromosomal translocations or become targets of somatic mutations that disrupt their normal function (Orkin and Zon, 2008).

The Role of C/EBP α in Hematopoiesis

The *CCAAT/enhancer binding protein alpha* (C/EBP α) is one of six members of a subgroup of basic region leucine zipper (bZIP) TF proteins (Landschulz et al., 1988). They share structurally related N-terminal transactivation domains whereas the basic DNA-binding region and C-terminal leucine rich dimerization domain are highly conserved throughout all members (Ramji and Foka, 2002).

C/EBPs are able to form homo- and heterodimers with other C/EBPs as well as with transcription factors of other families via their leucine zipper domain and can subsequently bind to DNA via the adjacent located basic amino acid residues. The exact distance between the basic region and leucine zipper domain is crucial for proper binding to DNA. Alteration by nucleotide insertions or deletions in the DNA-binding domain therefore impairs or limits the function of C/EBPs (Miller et al., 2003). Several C/EBPs appear in functionally different isoforms by expression from multiple translational initiation sites and alternative splicing.

Structure and function of C/EBP α .

The intronless *CEBPA* gene is located on chromosome 19q13.1 and encodes 2783 base pairs. C/EBP α is expressed in various cell types including hepatocytes, adipocytes, enterocytes, keratinocytes, lung epithelial cells, mammary gland cells and hematopoietic cells. In these cells, C/EBP α protein-dimers directly activate transcription from lineage-specific gene promoters. The C/EBP α protein was originally isolated from rat liver nuclei capable of interacting with the CCAAT homology present in many gene promoters and viral enhancers (Landschulz et al., 1988). Deletion of the *Cebpa* gene in mice leads to insufficient storage of hepatic glycogen and death from hypoglycemia shortly after birth. Expression of enzymes important in glycogen synthesis are decreased or delayed, and hepatocytes fail to accumulate lipids properly demonstrating the need of C/EBP α for the establishment and maintenance of energy homeostasis in neonates (Wang et al., 1995). At the same time C/EBP α knock-out mice lack white adipose tissue, mature neutrophil and eosinophil granulocytes indicating a role in differentiation of hematopoietic and non-hematopoietic tissues (Zhang et al., 1997).

The mRNA encoding C/EBP α contains alternative translation initiation sites resulting in two major protein isoforms: the fully translated protein C/EBP α (42 kDa; p42), and an N-terminal truncated protein C/EBP α (30 kDa; p30), lacking a transactivation domain important for anti-mitotic activity (Lin et al., 1993). The open reading frame (ORF) used for translation of the two isoforms harbors three AUG (AUG_{1,2} and AUG₃) start codons. Additionally, there is another translation start site further upstream in a different reading frame. Translation initiation from AUG_{1,2} results in full-length translation of p42 whereas translation from the out of frame start site is prematurely terminated making the ribosome read over AUG_{1,2} and re-initiate at AUG₃ to translate a truncated p30 protein (Kaufman, 2004).

The shorter p30 protein lacks the first 117 amino-acids of full-length C/EBP α (Figure 4). This transactivation domain (TAD1) is important for direct interaction with the TATA-box binding protein (TBP) and another transcription initiation factor (TFIIB), both essential components of the RNA polymerase II transcription complex (Nerlov and Ziff, 1995). Notably, the transactivation domain is also crucial for growth arrest by E2F-mediated regulation of the level of c-myc expression (Johansen et al., 2001; Lin et al., 1993; Porse et al., 2001). The truncated p30 isoform inhibits p42 C/EBP α -mediated transactivation of target genes. This inhibition occurs by formation of heterodimers that have impaired DNA-binding and transactivation ability compared to p42 C/EBP α homodimers (Cleaves et al., 2004).

The protein isoforms share another transactivation domain (TAD2) that has been shown to interact with the cyclin-dependent kinases CDK2/4, the SWI/SNF chromatin remodeling complex and the CDK inhibitor 1 (p21) (Wang et al., 2001). Both proteins contain a basic DNA-binding domain and a leucine zipper (bZIP) required for

dimerization and interaction with other TFs in the C-terminus. Highly conserved, non-DNA-binding residues in this region interact with E2F. Mutation of these residues results in loss of C/EBP α -E2F interaction leading to dysplasia of neutrophil granulocytes and hypoplasia of white adipose tissues (Porse et al., 2001).

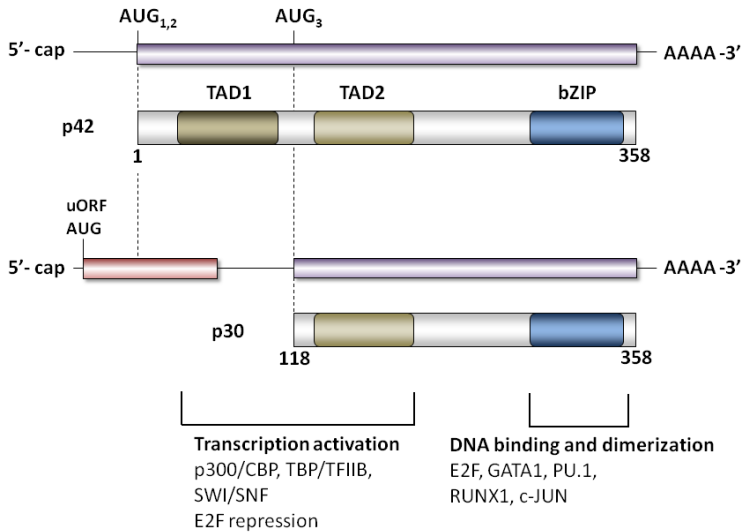


Figure 4. Functional distinct C/EBP α isoforms. Two major proteins (42 kDa, p42 and 30 kDa, p30) with specific functional properties are naturally translated from C/EBP α mRNA containing three AUG start codons (AUG_{1,2} and AUG₃) within the main open reading frame (ORF) and a weak AUG start site in a small upstream ORF (uORF). Translation initiation is modulated by activity of eIF2 α / eIF4E. The full-length protein consists of 358 amino acids while the N-terminal truncated protein lacks the first 117 amino acids. The region that is unique for p42 contains a transactivation domain (TAD1). Both isoforms share another transactivation domain (TAD2) and the basic leucine zipper (bZIP) DNA binding domain.

C/EBP α in normal and malignant hematopoiesis.

The TF C/EBP α plays a crucial role in the process of myeloid lineage commitment and granulocyte differentiation. Its expression is required throughout granulopoiesis, and mature neutrophil and eosinophil granulocytes are absent in mice deficient of C/EBP α while other hematopoietic lineages including monocytes remain unaffected. C/EBP α regulates the expression of many myeloid genes, including genes encoding growth factor receptors (granulocyte colony-stimulating factor receptor, G-CSFR; macrophage colony-stimulating factor receptor, M-CSFR; granulocyte-macrophage colony-stimulating factor receptor, GM-CSFR) (Ramji and Foka, 2002).

C/EBP α is expressed in hematopoietic stem and progenitor cells (Figure 5) and is involved in HSC regulation as C/EBP α deficient HSCs demonstrate enhanced reconstitution abilities and self-renewal compared to wt HSCs (Zhang et al., 2004). However, C/EBP α deficient HSCs have lost the potential to form GMP and its progeny whereas enforced expression of C/EBP α in MPPs induces myeloid differentiation at the cost of erythroid development demonstrating the ability to take action in cell fate decisions and hence the importance of C/EBP α at developmental stages before CMP and GMP level (Heath et al., 2004; Suh et al., 2006).

An essential prerequisite of C/EBP α is coordinating growth arrest and cell differentiation. Overexpression of p42 is strongly inhibiting cell growth *in vitro*. Several mechanisms have been proposed how C/EBP α mediates growth arrest and differentiation [for review see (Nerlov, 2004)]. Widely accepted is the ability of C/EBP α to repress *c-myc* via protein-protein interaction with E2F. However, C/EBP α promotes growth arrest in a cell-specific manner; therefore different mechanisms may apply in different tissues.

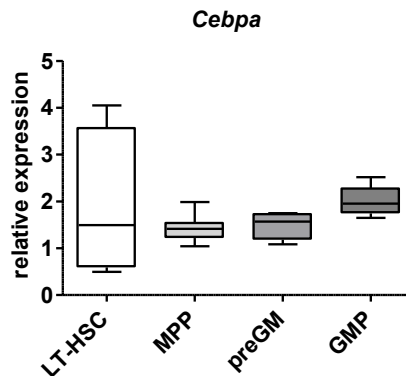


Figure 5. Relative expression of *Cebpa* in myeloid progenitors and HSCs. Q-PCR was carried out on sorted cell populations of *B6SJL* mice using Fluidigm. Expression of *Cebpa* is shown relatively to expression of *Hprt*. (LT-HSC as LSKCD150⁺CD48⁻; MPP as LSKCD150⁺CD48⁺; preGM as LKCD41⁻CD16/32^{low/-}; CD150⁺CD105⁺; GMP as LKCD41⁻CD16/32⁺CD150⁻ (n=4-8).

Due to its function in cell cycle arrest and neutrophil differentiation *CEBPA* mutations were proposed to be involved in leukemogenesis and today it is clear that *CEBPA* mutations are present in 7%–10% of human AML and are mostly associated with a normal karyotype. The mutation patterns observed are generally biallelic and almost always combine an N-terminal frameshift mutation on one allele with a C-terminal in-frame insertion/deletion mutation on the other allele (Pabst and Mueller, 2007). N-terminal mutations prevent expression of the p42 protein isoform and favor the expression of the p30 isoform, whereas C-terminal mutations disrupt DNA binding and

dimerization of both isoforms (Figure 6). The mouse models that studied C/EBP α function in normal and malignant hematopoiesis are summarized in Table 2.

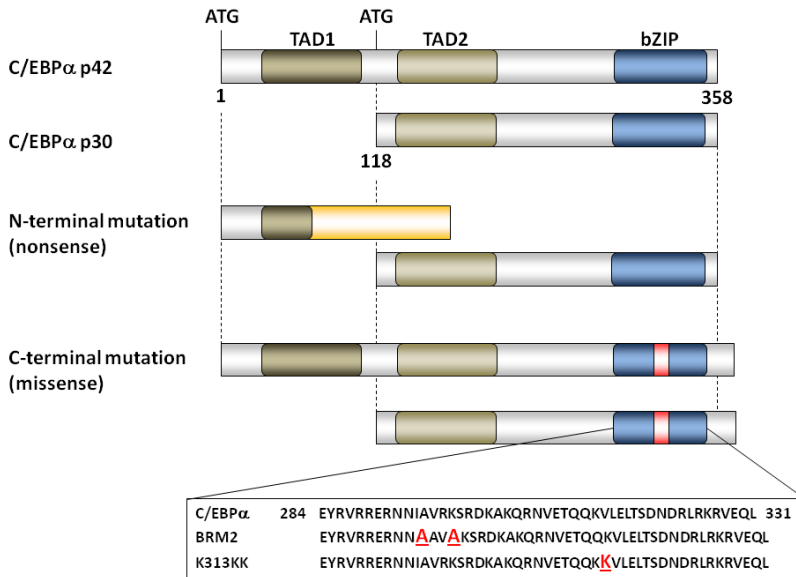


Figure 6. Mutations of the *CEBPA* gene in AML. C/EBP α mutations observed in acute myeloid leukemia (AML) frequently occur as biallelic mutations and cluster predominantly in the N- and C-terminal region of either allele. Mutations within the first 300 base pairs lead to a frameshift or premature stop codon (yellow bar) eliminating p42 expression without affecting p30 translation. C-terminal insertions or deletions (red letters) disrupt the bZIP domain and affect both p42 and p30 C/EBP α . Mutations in the bZIP domain have been studied in the BRM2 (I294A, K297A) and K313KK mouse models.

Pabst and colleagues firstly identified mutations leading to N-terminal truncation and expression of the p30 C/EBP α protein (Pabst et al., 2001). Initially, as it was thought to be the most frequent leukemogenic event the effect of p30 C/EBP α was studied. Overexpression of p30 C/EBP α in human CD34⁺ cord blood cells showed a remarkable inhibition of myeloid and erythroid differentiation (Schwieger et al., 2004).

Kirstetter and colleagues studied mice heterozygous or homozygous for a similar mutation in the *Cebpa* locus resulting in exclusive expression of p30 C/EBP α from the targeted allele (L allele; *Cebpa*^{L/+}) (Kirstetter et al., 2008). Strikingly, *Cebpa*^{L/+} exhibit 4-fold more p30 than p42 C/EBP α , a finding also observed in AML patients with the N-terminal mutation. However, the mice did not display alterations in hematopoietic tissues or granulocyte differentiation. Hence, although reduced DNA-binding activity by p30/p42 heterodimers may be involved in disturbed granulocytic differentiation, further decreased C/EBP α function is required for leukemogenesis. Solely, total abrogation of p42 C/EBP α translation in *Cebpa*^{L/L} mice uncoupled cell-cycle control and terminal

differentiation resulting in aberrant granulopoiesis and progression to AML with full penetrance. In summary, p30 C/EBP α accounts for loss of growth control but sustain crucial myeloid commitment to the GMP level. The importance of GMP formation for AML development is supported by the finding that C/EBP α deficient hematopoietic chimera lack GMPs due to a block in transition from CMP to GMP and do not develop AML. Also, retroviral expression of BCR-ABL in C/EBP α null background resulted in erythroid leukemia rather than AML/myeloid blast-crisis of CML (Wagner et al., 2006; Zhang et al., 2004).

Table 2. Models investigating C/EBP α function in normal and malignant hematopoiesis.

Model	C/EBP α function	Hematopoietic phenotype	Reference
Cebpa ^{-/-}	Null	No GMPs or mature granulocytes; enhanced HSC self-renewal; no AML	(Zhang et al., 1997)
C/EBP α -BRM2*	Binding to E2F disrupted, otherwise normal DNA-binding	Transplantable MPN; transformation of myeloid cells; no AML	(Porse et al., 2001)
C/EBP α -p30*	Enforced p30 expression, normal bZIP domain	Myeloid lineage commitment; transplantable AML; gene expression profile shared with MLL-AF9 AML	(Kirstetter et al., 2008)
C/EBP α -K313KK*	Disrupted bZIP domain	Expansion of pre-leukemic HSCs; impaired myeloid lineage commitment; no GMPs; transplantable AML	(Bereshchenko et al., 2009)
C/EBP α -K313KK/p30	Enforced p30 expression / disrupted bZIP domain	Expansion of pre-leukemic HSCs; myeloid lineage commitment; accelerated AML development	(Bereshchenko et al., 2009)

*no phenotype in heterozygous mice.

Porse and colleagues studied repression of E2F-mediated transcription by deleting the region in the bZIP domain of C/EBP α (BRM2) that interacts with E2F in vivo. However, this mutation does not affect DNA-binding or induction of C/EBP α target genes. BRM2 homozygous mice developed a transplantable disorder of the granulocytic lineage with expansion of myeloid progenitor populations indicating a role of C/EBP α mediated E2F repression in controlling cell cycle and proliferation of myeloid progenitors (Porse et al., 2005). However, although transformation of myeloid cells

occurred in this mouse model, clinical symptoms of AML such as thrombocytopenia, anemia and elevated white blood count were absent.

These observations support the role of C/EBP α as a myeloid specific tumor suppressor but also demonstrate that E2F repression alone is not sufficient to induce AML. Therefore, targeting DNA-binding function of C/EBP α was the next step to investigate. An in-frame insertion of lysine at position 314 (K313KK; K allele; *Cebpa*^{K/+}) in the *Cebpa* locus was engineered by Bereshchenko et al. mimicking a common C-terminal mutation in AML (Bereshchenko et al., 2009). Combining different hypomorphic *Cebpa* alleles (L/L, K/K and K/L), this study investigated the potential of *CEBPA* mutations to initiate AML. The presence of the K allele promoted expansion and proliferation of HSCs and MPPs prior to development of AML as K/K and K/L mice exhibited less quiescent and increased numbers of HSCs and MPPs. Myeloid differentiation was more profoundly blocked in K/K and K/L mice (no GMPs) than in L/L mice (normal GMP levels). However, K/K mice in some cases developed even erythroleukemia instead of AML. This phenotype has otherwise been observed in C/EBP α deficient hematopoietic chimera. Further, whereas all combinations led to AML the K/L combination of both mutations accelerated disease progression and mice of that setup developed AML fastest. In conclusion, the predominant biallelic *CEBPA* mutations affecting the C-terminus and the N-terminus in human AML obviously meet exactly the level of C/EBP α function that is needed for myeloid lineage commitment but prevents terminal granulocyte differentiation leading to the development of AML.

Regulation of C/EBP α

C/EBP α has been found to be regulated at a number of levels, including gene transcription, translation, protein-protein interactions and phosphorylation mediated changes in DNA-binding activity, activation potential and nuclear localization.

Repression of C/EBP α transcription by CEBPA promoter silencing has been demonstrated in AML patients without mutations in the CEBPA gene (Wouters et al., 2007). The transcription factor LEF-1 (lymphoid enhancer binding factor 1) has been reported to be suppressed in myeloid cells exhibiting a block in differentiation at a promyelocytic stage in patients with severe congenital neutropenia (SCN). LEF-1 activates the CEBPA promoter and its expression in SCN patient cells restored C/EBP α levels and induced myeloid differentiation (Skokowa et al., 2006).

Leukemic fusion genes involving CBF family members such as AML1-MDS-EV11 in t(3;21) AML or CBF β -MYH11 in inv(16) are involved in repression of C/EBP α at the post-transcriptional level (Helbling et al., 2004; Helbling et al., 2005). The RNA-binding protein calreticulin is highly activated in t(3;21) AML. Calreticulin binds GC-rich structures of C/EBP α mRNA and inhibits its translation. The same inhibitory

mechanism of C/EBP α mRNA translation was shown for the C/EBP α -MYH11 fusion protein resulting from inv(16). Similarly, in blasts from patients with CML myeloid blast crisis, inhibition of translation of C/EBP α mRNA is caused by hnRNP-E2, another RNA-binding protein. HnRNP-E2 is induced by BCR-ABL via a MAPK-dependent mechanism. Down-regulation of hnRNP-E2 by the BCR-ABL inhibitor imatinib restores both C/EBP α protein expression and granulocytic differentiation of those blasts (Chang et al., 2007; Perrotti et al., 2002).

Ribosomal scanning for translation initiation sites depends on activity of the alpha subunit of the eukaryotic translation initiation factor 2 (eIF-2 α) that is regulated by eIF2 kinases (Calkhoven et al., 2000). The eIF-2 α acts as an attenuator of protein synthesis initiation. Interestingly, mice with a homozygous mutation at the eIF-2 α phosphorylation site (S51A) die after birth due to defective gluconeogenesis. In addition, homozygous mutant embryos and neonates display a deficiency in pancreatic beta cells (Scheuner et al., 2001). This phenotype is surprisingly similar to that of C/EBP α null mice. Hence, by regulating the ratio between p42 and p38 C/EBP α activity of eIF-2 α and the process of translation initiation [for review see (Kaufman, 2004)] may be involved in leukemogenesis. Experimental evidence supporting this hypothesis is limited although it has been demonstrated that eIF-4E is aberrantly expressed in AML (Assouline et al., 2009). CDDO (2-Cyano-3,12-dioxooleana-1,9-dien-28-oic acid), increased the ratio between p42 and p38 C/EBP α isoforms by dephosphorylation of eIF-2 α and phosphorylation of eIF-4E. In addition, CDDO had the potential to differentiate primary blasts from patients with AML (Koschmieder et al., 2007).

There is growing evidence for deregulation of C/EBP α function at the protein level also being involved in leukemogenesis. Various mechanisms affecting phosphorylation, ubiquitination and sumoylation of C/EBP α protein have been described. Tribbles homolog 2 (TRIB2), a downstream target of NOTCH1 has been identified in AML. It associates with and inhibits C/EBP α by enhancing proteasomal degradation of C/EBP α protein (Keeshan et al., 2006). Phosphorylation of C/EBP α at Serine 21 led to a conformational change in C/EBP α protein dimers turning their transactivation domains further apart. Mediated by extracellular signal-regulated kinase 1/2 (ERK-1/2), phosphorylation of C/EBP α inhibited granulocytic differentiation and favors monocytic development (Ross et al., 2004). Activated FLT3 signaling affecting ERK1/2 in human AML was shown to inhibit C/EBP α function by the same mechanism (Radomska et al., 2006).

FLT3 Signaling in Hematopoiesis

The FLT3 (CD135) receptor, also known as fetal liver kinase 2 (FLK2) (Rosnet et al., 1991) belongs to the receptor tyrosine kinase (RTK) type III family which also includes the M-CSFR (CD115) (Coussens et al., 1986), KIT (CD117) (Yarden et al., 1987) as well as the platelet-derived growth factor receptors alpha (PDGFR α) and beta (PDGFR β) (Claesson-Welsh et al., 1989; Yarden et al., 1986). All members of this RTK group play a crucial role in proliferation, survival and differentiation of hematopoietic cells: KIT and FLT3 are both required for the survival, proliferation and differentiation of early hematopoietic progenitors, M-CSFR is important for growth and differentiation of macrophages and PDGFR β is believed to play a role in megakaryopoiesis (Reilly, 2003).

The receptor FLT3 is expressed on early myeloid and lymphoid progenitors, but not in erythroid cells, megakaryocytes or mast cells (Stirewalt and Radich, 2003). In fact, FLT3 surface expression is believed to distinguish an early commitment step from self-renewing, uncommitted murine HSCs to MPPs which have started to differentiate (Adolfsson et al., 2001).

Structure and function of FLT3

FLT3 is the receptor for the FLT3 ligand (FL). It contains five immunoglobulin-like domains in the extracellular ligand-binding domain (LBD). The human *FLT3* gene is located on chromosome 13q12 and encodes a protein of 993 amino acid with 85% sequence-homology compared to mouse *Flt3* (Rosnet et al., 1993). A transmembrane domain spans the region through the cell membrane while the intracellular domain contains the juxtamembrane domain (JM), the tyrosine kinase 1 domain (TKD1), tyrosine kinase inhibitory domain (TI) and the tyrosine kinase 2 domain (TKD2) (Figure 7). The receptor appears in two forms: the mature and membrane-bound N-glycosylated form (150 kDa) and the immature, mannose-rich form of 130 kDa (Stirewalt and Radich, 2003).

The FLT3 receptor remains as an unphosphorylated inactivated protein monomer until binding of FL induces a conformational change allowing receptor dimerization and subsequently activation of the intracellular tyrosine kinase that phosphorylates several sites of the intracellular domain. This leads ultimately to activation of downstream effectors (Stirewalt and Radich, 2003). The activated and phosphorylated FLT3-FL complex is rapidly internalized and degraded similarly to the rapid internalization and turnover of other RTK type III receptors like KIT. Serum levels of FL are normally low in homeostasis. However, FL can be released upon low white-blood-cell (WBC) counts as a result of hematopoietic disease or chemotherapy.

FL is expressed in many hematopoietic cell types as soluble form or membrane-bound, which are both biologically active (Lyman et al., 1995). Whether these two isoforms contain distinct functions remains to be investigated (Lyman and Jacobsen, 1998). Disruption of the *Fl* gene reduced the number of LMPPs, and myeloid and lymphoid progenitors (Boiers et al., 2010; McKenna et al., 2000; Sitnicka et al., 2002) highlighting the important role of FLT3 signaling in hematopoiesis. Moreover, complementary signaling of FLT3 and IL-7 has been demonstrated to be indispensable for fetal and adult lymphopoiesis (Jensen et al., 2008; Sitnicka et al., 2003).

Stimulation of early myeloid progenitors *in vitro* by FL without the addition of other growth factors promoted monocytic differentiation without a distinct proliferative effect. However, FL stimulation in combination with IL-3, SCF and G-CSF resulted in a more vigorous proliferation (Rusten et al., 1996).

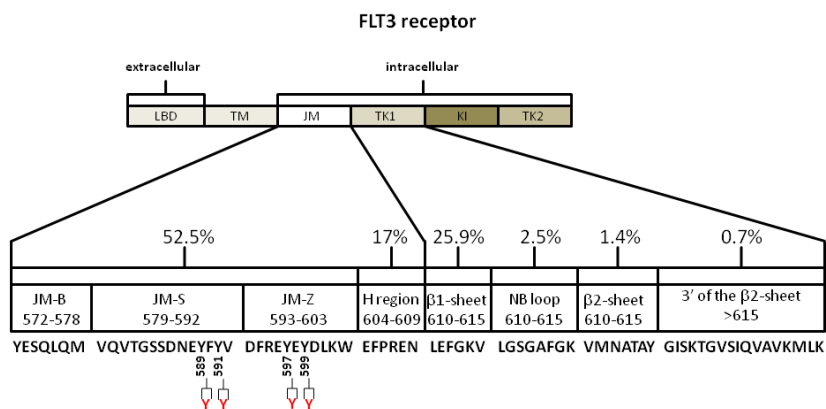


Figure 7. FLT3 receptor domains and sites frequently mutated. About 70 percent of mutations observed in AML are located in the juxtamembrane domain (JM), whereas the remaining 30 percent target the tyrosine kinase 1 domain (TK1).

The FLT3 signaling pathway

The FLT3 signaling cascade has not been completely described. However, it has been shown that binding of the FL triggers the *phosphatidylinositol 3-kinase* (PI3K), a regulatory protein of phospholipid metabolism and the *rat sarcoma* (RAS) pathway which is involved in cell proliferation and apoptosis (Figure 8). The activated receptor can bind to the *Src-homology 2* (SH2) domain of the p85 subunit of PI3K and with SH2-containing sequence proteins (SHC) that are involved in phospholipid and proliferative pathways (Zhang and Broxmeyer, 1999). In addition, FLT3 binds to GTPase-activating

protein, important regulatory protein of the RAS pathway (Dosil et al., 1993; Rottapel et al., 1994).

The FLT3 receptor has also been found to be associated with SH2-domain-containing inositol phosphatase (SHIP), which might act as a negative regulator of proliferation by competitively binding SHCs that otherwise activate RAS (Marchetto et al., 1999; Zhang et al., 1999). The activation of FLT3 involves also modulation of STAT5A indicating the involvement of the *Janus kinase / signal transducer and activator of transcription* (JAK/STAT) pathway in FLT3 signaling (Zhang et al., 2000).

The signaling pathway data must be interpreted with caution since most of these experiments were carried out in either mouse models or human cell lines artificially expressing FLT3. In addition, the expression pattern of FLT3 differs in mouse and human. Although being species cross-reactive, FL exists in different isoforms in mouse and human, whose biological relevance is unknown. Further, it is unclear whether the different isoforms are tissue-specifically expressed (Stirewalt and Radich, 2003). In conclusion, FLT3 pathways seem to be highly species and tissue specific, which indicates that it might be difficult to generalize the above findings to normal human hematopoietic cells (Dosil et al., 1993; Zhang et al., 1999).

FLT3 mutations in acute myeloid leukemia

Due to the fact of being a strong promoter of cell proliferation and survival the implication of FLT3 signaling in malignant hematopoiesis appears obvious. In fact, FLT3 is highly expressed in almost all AML subtypes, B-cell acute lymphoblastic leukemia (ALL), a few T-cell ALLs and CMLs (Gilliland and Griffin, 2002). In addition, genomic alterations of the *FLT3* gene occur in up to 30 percent of all AMLs making it one of the most frequently targeted genes in AML.

The most common form is an internal tandem duplication (ITD) in exon 14 and 15 due to in-frame insertions or deletions varying in size between 3 and more than 400 base pairs (Nakao et al., 1996). Until recently, it was believed that ITD mutations occur exclusively in the JM domain but newer investigations show that approximately 70 percent of ITD mutations localize in this region whereas the remaining 30 percent are in fact found in the TKD1 domain (Breitenbuecher et al., 2009). Cell line studies utilizing retroviral transduction have shown that FLT3-ITD promote ligand-independent dimerization and constitutive activation of the FLT3 receptor leading to cytokine-independent cell growth (Kiyoi et al., 1998). Whether FL can further stimulate the activity of FLT3 is likely dependent on the ratio of wt versus activated receptor. This ratio is important because FLT3-ITD can form homodimers or heterodimers with wt FLT3 and may affect the degree of autophosphorylation (Kiyoi et al., 2002). Experiments have not fully elucidated this question (Gilliland and Griffin, 2002).

The second most common type of *FLT3* mutations is a missense point mutation in exon 20 of the TKD substituting an aspartic acid to tyrosine (D835Y). Other mutations in codon 835, such as deletions, have also been described, but these are far less common (Schnittger et al., 2002). The kinase domain is inactive until dimerization and a conformational change allows access of adenosine triphosphate (ATP) and substrate. The D835Y mutation targets the so-called activation-loop leading to phosphorylation of a specific tyrosine residue within the loop and causing the loop to change to an activated configuration which in term allows access to the kinase (Yamamoto et al., 2001). Activation loop mutations have also been reported for other RTKs (Gilliland and Griffin, 2002).

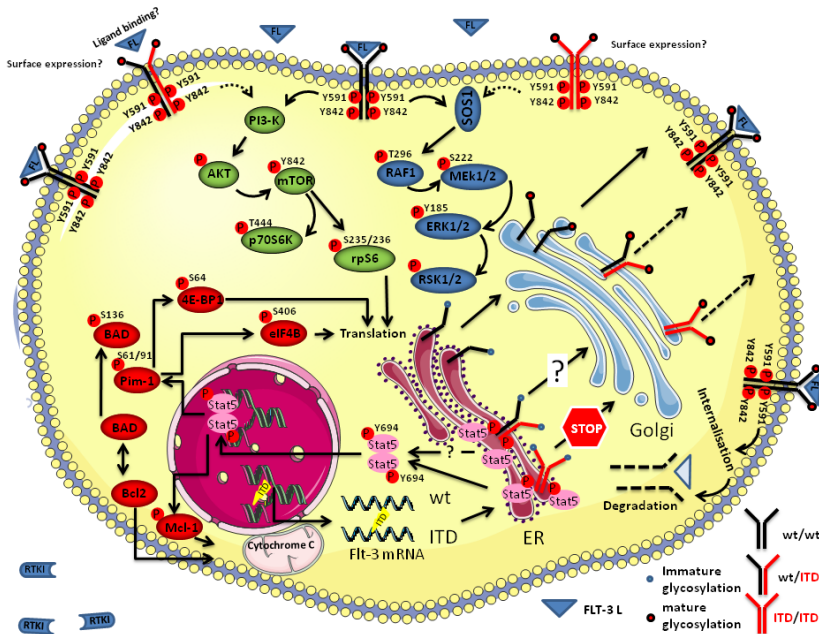


Figure 8. Cellular localization and signaling of wt FLT3 and FLT3-ITD. The wt FLT3 receptor is expressed on the cell surface after intracellular maturation and glycosylation. Upon FLT3 ligand binding the receptor dimerizes and autophosphorylates activating its kinase domains which in term activate downstream targets. The activated receptor is rapidly internalized and subsequently degraded. The properties of the wt/ITD FLT3 receptor in regards to cellular localization and signaling are much less defined. Mutant and wt FLT3 mRNA is transported to the endoplasmic reticulum (ER) and translated into protein. The immature glycosylated 130 kDa form of FLT3-ITD is retained in the ER and activates the STAT5 pathway while wt FLT3 is transported to the Golgi apparatus where complex glycosylation occurs followed by surface expression. Activation of STAT5 by FLT3-ITD leads to transcription of genes like BAD etc via Pim-1 while wt FLT3 signals through PI3K and MAPK pathways.

Both ITD and activation loop mutations result in constitutive activation of the FLT3 kinase and downstream targets including STAT5, RAS/MAPK, and PI3K/AKT (Fenski et al., 2000; Yamamoto et al., 2001). FLT3-ITD was shown to confer factor-independent growth to the murine hematopoietic cell lines 32D and Ba/F3 that are dependent on IL-3 for growth. In contrast, wt FLT3, even in the presence of FL, did not confer factor-independent growth to these cells (Hayakawa et al., 2000; Mizuki et al., 2000; Mizuki et al., 2003).

The molecular mechanisms responsible for different signaling of mutated FLT3 receptor versus wt receptor are not completely understood (see also Figure 8). In addition, whether ITD and TKD mutations of FLT3 result in activation of the same signaling pathways is still in dispute. However, recent attention has been drawn to the fact that wt FLT3 is predominantly occurring as the mature, complexly glycosylated 150 kDa form whereas FLT3-ITD is retained in the endoplasmic reticulum (ER) and therefore not as much glycosylated (Koch et al., 2008). Recent studies have demonstrated that aberrant localization of the mutated FLT3 receptor in the ER led to activated STAT5 while the wt receptor located in the cell membrane signals mainly via the ERK and PI3K pathway (Choudhary et al., 2009; Schmidt-Arras et al., 2009). In accordance with the experimental data showing FLT3-ITD entrapment in the ER is the observation that surface expression of FLT3-ITD is decreased or absent depending on the mutational status (Hu et al., 2004).

Different mouse models using various approaches have been generated to study the molecular background of mutant FLT3 in hematopoietic malignancies. Kelly et al. (2002) could show that expression of FLT3-ITD using a murine transduction/transplantation model leads to an oligoclonal MPN with a relatively short latency (Kelly et al., 2002). Interestingly, this disease was not transplantable into secondary recipients. Using the same experimental system, Grundler et al. (2005) showed that FLT3-ITD and FLT3-TKD mutations induce different diseases with different latency and that this is due to a difference in the ability of the two mutations to phosphorylate STAT5 (Grunder et al., 2005).

More genetically accurate models were generated by using knockin and conditional knockin of a human ITD sequence into the normal *Flt3* locus guaranteeing correct timing and level of expression of FLT3-ITD. Li et al. concentrated mainly on the heterozygous mice while Lee et al. analyzed FLT3-ITD homozygous mice (Lee et al., 2007; Li et al., 2008). Both models showed a myeloproliferative disease and as expected, the myeloproliferative phenotype was more pronounced in mice having two copies of FLT3-ITD.

Acute Myeloid Leukemia

The term *leukemia* (from ancient greek: *leukós haima*, “white blood”) was originally described by Rudolf Virchow (Virchow, 1856). Leukemia is characterized by high numbers of non-functional, immature white blood cells in peripheral blood and bone marrow.

Deregulated hematopoiesis ultimately leading to leukemia is a consequence of acquisition of genetic and epigenetic changes in blood stem and progenitor cells, which otherwise produce the huge numbers of mature, red and white blood cells. These so-called blast cells have lost their ability to differentiate and response to normal regulation of proliferation and survival, and progressively displace normal blood cells in the BM with the result of fatal infections, bleedings, and infiltration of other organs (Tenen, 2003). Depending on which blood cell lineage is affected, leukemia is subdivided into lymphoid or myeloid leukemia. Furthermore, chronic and acute leukemia are distinguished by the amount of blast cells in the BM. The occurrence of more than 20 percent blasts in the BM is considered as acute leukemia.

Epidemiology

Acute myeloid leukemia (AML) is a complex malignant disease and its great genetic heterogeneity makes this disorder exceptionally challenging in the clinic. AML is the most common acute leukemia with a prevalence of 3.8 per 100000 adults and increasing to 18 in 100000 adults older than 65 years (Deschler and Lubbert, 2006).

During the past decades, refinements in diagnosis of AML subtypes and advances in therapeutic approaches have improved the situation for patients with AML. However, still most people suffering from AML will die of their disease or complications related to therapy.

Clinical aspects

AML is diagnosed as a result of a complete blood count showing decreased numbers of red blood cells (anemia), platelets (thrombocytopenia) and neutrophil granulocytes (neutropenia) whereas the total white blood count are most commonly increased (leukocytosis) due to an accumulation of leukemic blast cells. For identification of blasts an examination of peripheral blood analysis can be carried out, but definitive diagnosis requires a BM sample, which is analyzed morphologically by microscopy and flow cytometry to diagnose the presence of leukemic blasts and to differentiate from other

types of leukemia. In addition, cytogenetic analysis is carried out to identify chromosomal abnormalities and translocations which can have prognostic significance.

The early signs of AML are often vague and non-specific including fever, loss of appetite and weight loss. However, the patient shows symptoms due to the replacement of mature, functional blood cells by leukemic blasts: lack of neutrophils increases the susceptibility to infections; anemia causes fatigue and short breath while low platelet numbers lead to bleedings and easy bruising.

Treatment of AML consists primarily of chemotherapy and can be divided into *induction therapy* and *consolidation therapy*. Induction therapy aims at reducing the number of leukemic cells to a minimum (complete remission) while the goal of consolidation therapy is to eliminate any residual, undetectable disease. The standard induction chemotherapy regimen consists of *cytarabine* for seven consecutive days and *anthracycline* for three days (Abeloff, 2004). Despite the variety of underlying cytogenetics of the disease, this protocol induces complete remission in up to 70 percent of all patients (Bishop, 1997).

After complete remission, leukemic cells still remain in numbers too small to be detected and would cause a relapse in short time unless consolidation therapy is given. Consolidation therapy is individualized based on the patient's cytogenetic status and prognostic factors as well as general health and age. For *inv(16)*, *t(8;21)*, and *t(15;17)* AMLs (good prognosis), patients will typically undergo an additional 3–5 courses of intensive chemotherapy (Abeloff, 2004). For patients with high-risk cytogenetics, underlying myelodysplastic syndrome (MDS), or therapy-related AML (bad prognosis, high risk of relapse), allogeneic stem cell transplantation is usually recommended if the patient has a suitable donor. The best consolidation therapy for intermediate-risk AML (normal cytogenetics) is less clear and depends on the specific situation, including the age and overall health of the patient, the patient's personal values, and whether a suitable stem cell donor is available (Appelbaum et al., 2000). Hematopoietic stem cell transplantation is becoming the standard care for young patients with intermediate risk AML and is considered if a patient relapses.

Classification

The diversity of myeloid malignancies and in particular acute myeloid leukemia in terms of phenotype and prognostic outcome required a classification to select the right therapeutic approach and improve treatment. Originally, AML was classified into groups depending on the maturation status of the blast cells. The so-called French-American-British (FAB) nomenclature (Bennett et al., 1976) divided AML into 8 subtypes M0 to M7. However this classification did not incorporate underlying genetic abnormalities or clinical outcome. Recent advances in the understanding of the genetic basis of AML have provided new insights with regards to genetics and clinical features of AML and the

newer WHO classification attempts to define homogenous subgroups including prognostic and therapeutic relevance (Table 3).

Table 3. WHO classification of AML and related neoplasms including underlying genetic abnormalities. AML with mutated *NPM1* or *CEBPA* are recognized as provisional entities. AML with mutated *FLT3* is not distinguished separately.

Acute myeloid leukemia and related neoplasms
Acute myeloid leukemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); <i>RUNX1-RUNX1T1</i>
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); <i>CBFB-MYH11</i>
APL with t(15;17)(q22;q12); <i>PML-RARA</i>
AML with t(9;11)(p22;q23); <i>MLLT3-MLL</i>
AML with t(6;9)(p23;q34); <i>DEK-NUP214</i>
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); <i>RPN1-EVT1</i>
AML (megakaryoblastic) with t(1;22)(p13;q13); <i>RBM15-MKLI</i>
AML with mutated <i>NPM1</i>
AML with mutated <i>CEBPA</i>
AML with mutated <i>FLT3</i> (no WHO entity)
Acute myeloid leukemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
Pure erythroid leukemia
Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm

Molecular pathology of AML

The fundamental features of AML include inappropriate proliferation in the absence of normal growth signals, acquired self-renewal, escape from programmed cell death, block of differentiation, aberrant cell cycle control, genomic instability and multi-organ dissemination of leukemic cells. These properties (Table 4) may be directly linked to genetic lesions and individually amenable to therapy, but it is clear that many of them result from integration of multiple alterations in the leukemic cell (Licht and Sternberg, 2005).

Table 4. Malignant properties of AML and the underlying lesions (Licht and Sternberg, 2005).

Property	Molecular lesion
Differentiation block	Fusion transcription factors; Retinoic acid receptor α : PML-RAR α , PLZF-RAR α , Core binding factor: RUNX1-MTG8, CBF β -MYH11, RUNX1- EVI1, MLL-fusions, Hox gene fusions and overexpression, Point mutation of transcription factors PU.1, C/EBP α , RUNX1, GATA-1.
Autonomous cell proliferation	Activating mutations: FLT3, RAS, c-KIT, c-FMS.
Escape from apoptosis	AKT pathway activation following RTK activation leads to Bad deactivation, p53 mutations in AML of the elderly, p53 dysregulation by fusion proteins, NPM1 mutations, Bcl2 overexpression, Survivin (IAP) overexpression.
Increased self-renewal	β -catenin mutations, activation of Wnt-catenin pathway by fusion transcription factors, Activated RTK pathways cooperate to induce self-renewal.
Loss of cell cycle control	p53 dysfunction, loss of Rb, p15 and p16 cyclin-dependent kinase gene methylation.
Dissemination	TNF secretion by leukemic blasts stimulates endothelium. Increased selectin, cadherin and integrin expression encourage adhesion and egress through vessels.

However, although oncogenes like receptor tyrosine kinases and classical tumor-suppressor genes (like RB and p53) are also mutated in AML, experimental evidence suggests that hematopoiesis-specific TFs are the *gatekeepers* of AML (Cammenga, 2005). Disruption of these gatekeepers interferes with hematopoietic differentiation and subsequent apoptosis of cells. The disrupted TFs in AML can be classified into three

different groups: (1) lineage-specific TFs, which play pivotal roles in the differentiation process of a specific lineage such as C/EBP α , a key regulator of granulopoiesis; PU.1, instrumental in determining cell fate into the myeloid lineage; GATA-1, an important regulator of erythro- and megakaryopoiesis, and the retinoic acid receptor alpha (RAR α); (2) TFs that are expressed in multiple lineages, and which are probably more aptly designated as transcriptional organizers such as the core binding factor (CBF) family, and the C2H2 zinc-finger TF (EVI-1); and (3) TFs that are generally thought to orchestrate global changes in transcriptional control during tissue development such as the HOX genes. Deregulation of the function of these gatekeeper TFs may affect the normally short-lived myeloid progenitors that undergo rapid proliferation before they differentiate into functional, mature cells and lead to a prolonged phase of proliferation due to a block in differentiation. In this phase, acquisition of additional cooperating lesions puts the progenitor at a high risk to transform and turn into a leukemic cell (Rosenbauer and Tenen, 2007).

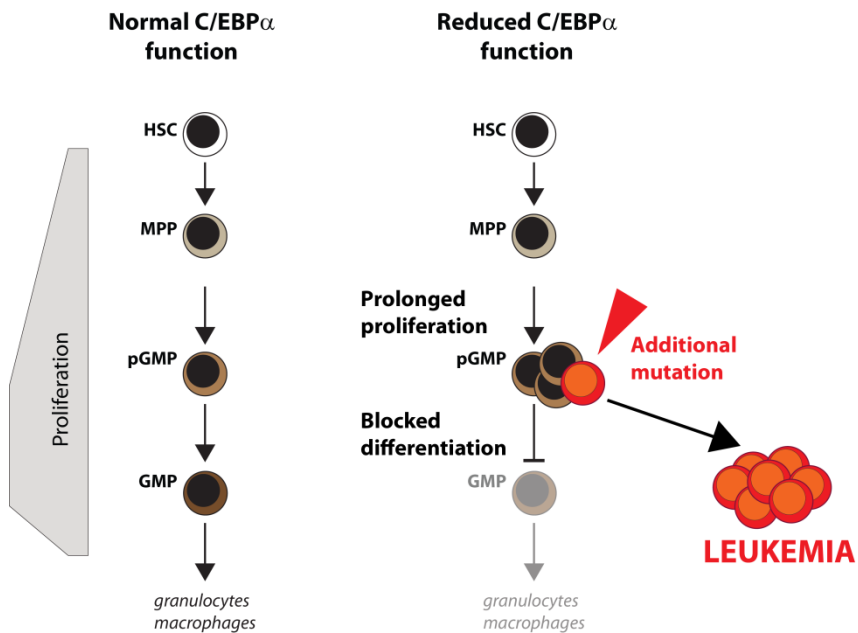


Figure 9. Reduced transcription factor function contributing to leukemia.

Transcription factors (TF) such as C/EBP α control differentiation by activating myeloid gene programs and inducing cell cycle arrest. Due to reduced TF function highly proliferating progenitors do not enter the differentiation program and acquire additional mutations, preferentially those mutations that confer a growth or survival advantage such as FLT3-ITD (Rosenbauer and Tenen, 2007).

In the model of reduced TF function contributing to leukemia (Figure 9) additional alterations occur affecting normal proliferation and survival to ultimately transform leukemic cells (Rosenbauer and Tenen, 2007). In term, Gilliland and Griffin (2002) postulated a two-hit model in leukemogenesis where at least two genetic changes take place: one initial mutation affecting differentiation, and secondary, mutations conferring a proliferative or survival advantage. This idea has arisen from the observation that FLT3-ITD causes an MPN but is not capable of inducing AML (Castilla et al., 1999; Gilliland and Griffin, 2002; Lee et al., 2007; Li et al., 2008).

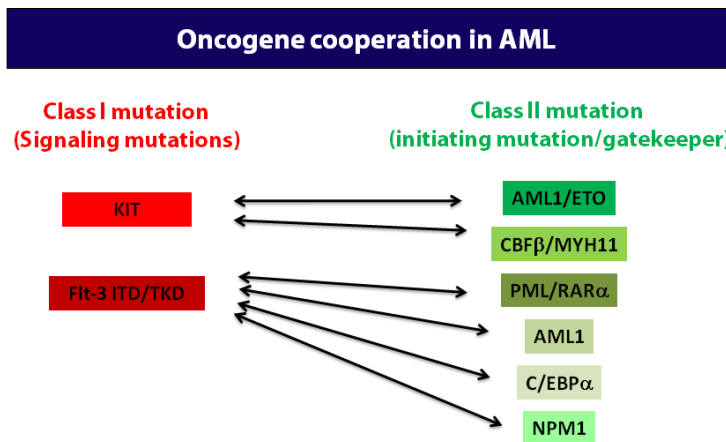


Figure 10. Oncogene cooperation in AML. Growing body of evidence suggests that signaling mutations cooperate with specific partners in leukemogenesis. Alterations of the same class are absent/rarely observed in AML. In addition, translocations including t(8;21) and inv(16) cluster together with KIT alterations whereas t(15;17), point mutations in *AML1* and *CEBPA* coincide with FLT3 activating mutations (Haferlach, 2008).

However, FLT3 mutations frequently occur in conjunction with other gene rearrangements. For example, FLT3-ITDs have been infrequently reported in patients with t(8;21) and inv(16), but are most commonly associated with mutant NPM1 and t(15;17). The translocations result in expression of fusion genes including the AML1-ETO, CBFβ-SMMHC, and PML-RARα, respectively. There are convincing data that these fusion genes are not sufficient to cause leukemia, but do contribute to impaired differentiation of hematopoietic cells (Gilliland and Griffin, 2002).

Growing experimental evidence and clinical observations point to specific association of signaling mutations cooperating with initiating gatekeeper mutations (Figure 10) (Castilla et al., 1999; Haferlach, 2008; Okuda et al., 1998; Westendorf et al., 1998). The hypothesis has important implications in approach to novel therapies of AML, in that molecular targeting of both FLT3-ITD and mutations involving transcription factors

may improve outcome in AML. Therefore, it is of great interest to design mouse models that as accurate as possible reflect the genetic background of AML. These models would allow us to study the molecular and cellular effects of oncogene cooperation during onset and progression of AML and subsequently help to identify new approaches in AML treatment.

Focus of the Present Investigation

The pathogenesis of AML is a complex process involving the disruption of multiple regulatory mechanisms due to acquisition of genetic alterations in hematopoietic stem and progenitor cells.

The role of the transcription factor C/EBP α in normal and malignant hematopoiesis has been intensively studied and specific mutations of the *CEBPA* gene modulating its function have been causally linked to development of AML. In addition, ITD mutations of the FLT3 receptor tyrosine kinase are a common scheme in hematopoietic malignancies, especially in AML. It has been proposed that the consecutively acquired genetic changes collaborate, but how the functional changes of these factors cooperate in leukemogenesis is still poorly understood.

The studies presented in this thesis have been aimed at deciphering the pathogenesis of AML by investigating the molecular and cellular effects of C/EBP α and FLT3 mutations before onset and towards progression to AML in a mouse model.

Specific Aims and Questions

Do FLT3-ITD and C/EBP α cooperate in AML pathogenesis? What are the molecular and cellular effects of cooperation of biallelic C/EBP α mutations and FLT3-ITD in a genetically accurate mouse model for human AML?

Does FLT3-ITD reduce the cytokine requirements of leukemic cells and substitute for cytokine dependent growth *in vitro*? Does FLT3 inhibition limit leukemic cell growth *in vitro* and *in vivo*? Do increased levels of FLT3 ligand impair the efficacy of FLT3 inhibition?

What are the hematopoietic consequences of acquisition of *Flt3*-ITD homozygosity/hemizyosity in adult mice *in vivo*? Which role plays FLT3 ligand in the myeloproliferative phenotype of *Flt3*^{ITD/ITD} mice?

Summary of Results

Paper I Molecular and cellular effects of oncogene cooperation in a genetically accurate AML mouse model

Based on three knock-in mouse strains a genetically accurate AML mouse model was designed to investigate recurrent C/EBP α mutations and FLT3-ITD collaborating in leukemogenesis. In opposite to biallelic *Cebpa* mutations, monoallelic *Cebpa* mutations in cooperation with *Flt3*-ITD were not sufficient to initiate AML. Hematopoietic chimeras with biallelic *Cebpa* mutations showed a profound block in differentiation of granulocytes regardless of the mutational status of *Flt3* and developed AML with full penetrance. However, additional *Flt3*-ITD shortened the latency of AML development. Leukemic mice showed an expansion of phenotypic GMPs in the BM which were able to initiate leukemia upon transplantation into secondary recipients confirming LIC potential in a myeloid committed progenitor cell.

As a result of disrupted C/EBP α function, myeloid differentiation was halted at the transition from pGMP to GMP. In addition, a higher frequency of MegE progenitors (MkP, pMegE, pCFU-E and CFU-E) and an increased pool of hematopoietic stem and progenitor cells (LSK cells including LT-HSCs and MPPs) was observed prior onset of AML. In hematopoietic chimeras harboring the additional *Flt3*-ITD the effect of biallelic *Cebpa* mutations on MegE progenitors was reduced and led to higher frequency of MPPs, pGMPs and GMPs. Gene expression analysis revealed a deregulated GMP population that had partially or fully left the myeloid differentiation path and acquired HSC-like self-renewal and enhanced survival. The cooperative effect of *Flt3*-ITD is composed of an additional proliferative signal via activation of STAT5 target genes and enhancing the generation of leukemia-initiating GMPs.

Paper II Cytokine requirements and oncogene addiction in a mouse model for AML with FLT3-ITD

The previously described AML mouse model was utilized in this study to investigate the effect of FLT3 inhibitors on leukemic cell growth and more generally clarify whether leukemic cells are growth dependent of the activated FLT3 pathway. Continuous leukemic cell growth *in vitro* required colony-formation in semisolid medium supplemented with SCF and IL-3. Further, in suboptimal IL-3 concentration leukemic

cells heterozygous for FLT3-ITD demonstrated higher colony-forming cell (CFC) capacity compared to leukemic cells with undisturbed FLT3 signaling. In the presence of the FLT3 inhibitors CEP-701 (Lestaurtinib, Cephalon, Inc.) or AC-220 (Quizartinib, Ambit Bioscience, Inc.), CFC capacity was reduced in an inhibitor dosage-dependent manner. However, a higher sensitivity of leukemic cells harboring FLT3-ITD could be demonstrated. To test the FLT3-inhibitory effect *in vivo* recipients transplanted with leukemic cells were treated with CEP-701 by subcutaneous injections once daily over a period of 2 weeks and monitored for AML development. There was no difference in survival observed between treated and untreated recipients. However, it remained unclear if plasma levels of CEP-701 were high enough to inhibit FLT3 activation.

Paper III Impact of gene dosage and loss of the wild type allele on FLT3 internal tandem duplication induced expansion of myeloid primed multipotent progenitor cells

Based on cross-breeding of $Flt3^{ITD/ITD}$ knock-in mice with $Flt3^{+/+}$, $Flt3^{-/-}$ and $Fl^{-/-}$ (FLT3 ligand knockout) mice, this study aimed to specifically address the impact of FLT3-ITD gene dosage, loss of $Flt3$ wild type allele and the role of FLT3 ligand on the myeloproliferative disease induced by FLT3-ITD in adult mice. It has been previously shown that mice develop an MPN-like disorder and that the phenotype was FLT3-ITD dosage-dependent (Lee et al., 2007). In comparison to $Flt3^{ITD/+}$ mice it was demonstrated that the MPN phenotype in $Flt3^{ITD/ITD}$ mice was paralleled by an enhanced cellularity, expansion of myeloid progenitors as well as monomyelocytic cells and overexpression of STAT5 target genes. This phenotype was also found in hemizygous $Flt3^{ITD/-}$ mice, although at less pronounced levels. Moreover, the manifestation of the MPN in $Flt3^{ITD/ITD}$ mice was FLT3 ligand independent. Further, the stronger myeloid phenotype in $Flt3^{ITD/-}$ mice compared to $Flt3^{ITD/+}$ mice could not be linked to the lack of wt FLT3 interaction with FLT3 ligand as $Flt3^{ITD/+}$ mice did not show enhanced MPN in the absence of FLT3 ligand. In summary, this study demonstrated that the enhanced MPN phenotype of $Flt3^{ITD/ITD}$ mice compared to $Flt3^{ITD/+}$ mice is attributable to a FL-independent FLT3-ITD dosage effect.

General Discussion

The pathology of AML is of great molecular heterogeneity. These molecular alterations disrupt the regulation of cell proliferation, differentiation, self-renewal, survival, cell cycle checkpoint control, DNA repair and chromatin stability, and cell dissemination (see Table 4). Insights into the pathways that are affected in AML has come from three different approaches: cytogenetic identification and molecular cloning of recurrent chromosomal rearrangements, identification of retroviral integration sites in murine models of AML and screening for mutations in genes known to be important regulators of hematopoiesis (Cammenga, 2005). Subsequently, the identified factors were studied in a variety of *in vitro* and *in vivo* models including retroviral transduction in cell lines or primary cells followed by transplantation, transgenic mice, knockin and knockout models. However, apart from making the disease accessible to experimentation otherwise impossible in human patients the main contribution of these models has been to our understanding of leukemogenesis as a multistep process. Nevertheless, the clinical relevance was quite limited (McCormack et al., 2005).

Genetically accurate mouse models of AML investigating the relationship between two or more cooperating oncogenes are missing. The model presented here utilized knockin mouse strains and demonstrated cooperation of a FLT3-ITD mutation with commonly observed C/EBP α mutations (**Paper I**). While disruption of the C/EBP α TF function introduced a myeloid differentiation block at the transition from pGMP to GMP, FLT3-ITD enhanced the generation of leukemia-initiating GMPs and conferred an additional proliferative signal via activation of STAT5 target genes which accelerated progression to AML. Due to expression from the original promoter preserving accurate levels and regulation of oncogene expression the design of this model is as genetically accurate as possible and allows furthermore the investigation of molecular and cellular processes prior transformation and onset of leukemia.

The inhibition of FLT3 signaling has been proposed to be a potential target in the fight against AML. However, the identification of RTK inhibitors was previously based on IL-3 dependent cell lines transfected with FLT3-ITD or AML cell lines over expressing FLT3-ITD (Levis et al., 2002; Pratz et al., 2009; Smith et al., 2004). None of these studies actually resemble the normal levels and ratios between wt and mutant FLT3 observed in primary cells in mouse or human AML. This discrepancy presumably accounts partly for the failure of significant numbers of new drugs late in clinical trials and points out how essential it is that accurate murine AML models are developed to exploit more specific, targeted therapeutics (Fathi and Levis, 2011; McCormack et al., 2005). Although, as every mouse model has to deal with pathway discrepancies (proven

for FLT3 and ligand) between mouse and human species (Kikushige et al., 2008), the model presented here could serve as a new *in vivo* platform to test the potential of new drugs before they enter expensive clinical trials. It was used in the ongoing study (**Paper II**) that tested Lestaurtinib (CEP-701) and Quizartinib (AC-220) for their potential to inhibit activated FLT3 signaling and growth of leukemic cells *in vitro* and *in vivo*. Although final data is still missing this study demonstrated that much higher concentrations of CEP-701 and AC-220 are needed to achieve similar FLT3-ITD inhibition as in the originally studies with cytokine-dependent cell lines.

The second part of this study was focused on clinical observations regarding the FLT3 receptor (**Paper III**). FLT3-ITD is associated with high relapse rate and poor overall survival in AML. The clinical outcome is furthermore determined by the allelic ratio of wt FLT3 and FLT3-ITD. Variations in the allelic ratio are a result of mutant allele-specific imbalance (MASI) either due to copy number-neutral loss of heterozygosity (CN-LOH) or mutant allele amplification. While the impact of *Flt3*-ITD dosage on disease phenotype has been modeled *in vivo*, less is known about the effect when the second wt allele is lost (Lee et al., 2007). However, loss of wt FLT3 and acquisition of FLT3-ITD homozygosity upon disease progression was shown to be associated with a worse outcome (Gale et al., 2008). Whether this outcome is impaired by an FLT3-ITD dosage effect or loss of the wt allele remains unclear. Previous studies demonstrated hetero-complexes between wt FLT3 and FLT3-ITD resulting in tyrosine phosphorylation of the wt receptor (Kiyoi et al., 2002) suggesting aberrant activation of wt FLT3 by FLT3-ITD.

To address this complex field and the hematopoietic consequences homo- and hemizygous *Flt3*-ITD mice were generated and bred against *Fl* knockout mice (McKenna et al., 2000). Using these models it was demonstrated that the MPN phenotype in *Flt3*-ITD homozygous mice was paralleled by an enhanced cellularity, expansion of myeloid progenitors as well as monomyelocytic cells and overexpression of STAT5 target genes. This phenotype was also found in hemizygous *Flt3*^{ITD/-} mice, although at less pronounced levels. The data presented here suggests that both gene dosage of FLT3-ITD and the loss of wt FLT3 contributes to the phenotype in the respective mouse strains. In addition, the degree of the myeloproliferative disease in *Flt3*^{ITD/ITD} mice was independent of FL. The FLT3-ITD knockin model used here closely reflects the event of acquiring an activating FLT3 mutation, expressed under the control of the endogenous promoter. However, the impact of FLT3-ITD dosage, loss of wt allele and impact of FL would be more relevant, if tested in an AML model setup presented in Paper I as FLT3-ITD mutations are typically occurring secondary to mutations disrupting a hematopoietic gatekeeper.

Svensk Sammanfattning

Leukemi är en cancersjukdom i blod och benmärg som karakteriseras av en störd blodbildning. Vid normal blodbildning delar sig ett begränsat antal omogna blodceller så kallade blod stamceller (eller hematopoietiska stamceller) och mognar till funktionella blodceller. Genom att dela sig utan att mogna har stamcellerna även potential till att förnya sig själva. När nya stamceller och mogna celler samtidigt bildas kan en kontinuerlig blodproduktion upprätthållas genom livet. Då leukemi uppstår mognar blodcellerna inte fullt ut, vilket leder till en anhopning av omogna celler med brist av funktionella blodceller som följd. Ett fåtal leukemiska stamceller tros ligga bakom den maligna expansionen. Konventionell leukemibehandling minskar mängden omogna celler men förefaller inte vara verksamt på de leukemiska stamcellerna. De leukemiska stamceller som blir kvar efter behandling förmodas vara den bakomliggande orsaken till återfall. Akut myeloisk leukemi (AML) är en typ av leukemi där man känner till flera genetiska förändringar (mutationer) som förekommer i de maligna blodcellerna. För att bättre kunna förstå uppkomst av AML har vi upprättat en musmodell för human AML genom att föra in tre vanligt förekommande mutationer i det genetiska materialet hos musens blodceller. Vår modell tillåter molekylära och cellulära studier både före och under manifestationen av sjukdomen. Jämfört med tidigare modeller representerar vår modell orsaken till leukemien på ett bättre sätt.

Våra resultat och de framtida studier de kommer att leda till kan bidra till nya framtida terapier i kampen mot AML. Dessutom kan musmodellen användas för att studera innovativa och oprövade läkemedel.

Deutsche Zusammenfassung

Leukämie ist eine Krebserkrankung im Blut und Knochenmark, die durch eine gestörte Blutbildung charakterisiert ist. Bei normaler Blutbildung teilt sich eine Anzahl weniger unreifer Blutzellen, sogenannter Blutstammzellen und reift zu funktionellen Blutzellen heran. Stammzellen können sich selbst erneuern indem sie sich teilen ohne dabei zu reifen. Durch die kontinuierliche Neubildung von unreifen Stammzellen und reifen Blutzellen ist es möglich die Blutproduktion ein Leben lang aufrechtzuerhalten. Bei einer Leukämieerkrankung reifen die Blutzellen nicht vollständig, wodurch es zu einer Anhäufung von unreifen Zellen und einem Mangel an funktionellen, gereiften Blutzellen kommt. Man nimmt an, dass eine geringe Zahl leukämischer Stammzellen die maligne Vermehrung unreifer Zellen verursacht. Konventionelle Behandlungsmethoden versuchen diese Zellen zu vernichten und im besten Fall ganz zu beseitigen. Sie haben jedoch häufig keine oder geringe Wirkung auf die leukämischen Stammzellen. Die Zellen, welche durch die Therapy nicht beseitigt werden, verursachen häufig einen Rückfall. Akute myeloische Leukämie (AML) ist eine akute Form von Leukämie, in der eine Anzahl genetischer Veränderungen (Mutationen) identifiziert sind. Um die Kausalität zwischen genetischen Veränderungen und der Leukämieentstehung zu zeigen sowie die Entstehung von AML besser zu verstehen, haben wir ein Mausmodell für humane AML entwickelt, indem wir drei häufig vorkommende Mutationen in das genetische Material von Blutzellen der Maus eingeführt haben. Dieses Modell ermöglicht es uns, die molekulären und zellulären Zusammenhänge vor und während der Manifestation der Krankheit zu studieren. Im Vergleich zu früheren Modellen spiegelt unser Modell die leukemie-verursachenden Bedingungen besser wider.

Diese Ergebnisse und weiterführende Studien helfen möglicherweise neue Therapieansätze im Kampf gegen AML zu finden. Darüber hinaus erlaubt uns das Mausmodell neue und bisher ungeprüfte Medikamente zu testen.

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Appendix

Paper I

Paper II

Paper III