LUND UNIVERSITY

Functional Screens Identify Vulnerabilities in Acute Leukemia

Ramakrishnan, Ramprasad

2020

Document Version:
Publisher's PDF, also known as Version of record

[Link to publication](#)

Citation for published version (APA):
Ramakrishnan, R. (2020). *Functional Screens Identify Vulnerabilities in Acute Leukemia*. [Doctoral Thesis (compilation), Department of Laboratory Medicine]. Lund University, Faculty of Medicine.

Total number of authors:
1

Functional screens identify vulnerabilities in acute leukemia

Ramprasad Ramakrishnan

LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended on Friday, 18th of September 2020 at 13.00 in
Segerfalksalen, BMC A10, Sölvegatan 19, Lund

Main supervisor

Marcus Järås, PhD

Associate professor, Lund University, Lund, Sweden

Faculty opponent

Julian Walfridsson, PhD

Assistant professor, Karolinska Institute, Stockholm, Sweden

Organization LUND UNIVERSITY Faculty of Medicine Department of Laboratory Medicine Lund Division of Clinical Genetics Author: Ramprasad Ramakrishnan	**Document name:** DOCTORAL DISSERTATION
	Date of issue 2020-09-18
	Sponsoring organization

Title: Functional screens identify vulnerabilities in acute leukemia

Abstract

Acute leukemia refers to a group of aggressive hematological malignancies of myeloid and lymphoid lineages termed acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) respectively. Acute leukemia is characterized by the presence of underlying genetic aberrations which alter the biology of normal hematopoietic cells resulting in the accumulation of immature abnormally differentiated blast cells. In this thesis, we have used advanced molecular techniques to identify vulnerabilities in acute leukemia.

In **paper I**, we performed an *in vivo* CRISPR-Cas9 screen targeting cell surface genes in murine AML stem cells and showed that CXCR4 is a top cell surface regulator of AML cell growth and survival. Notably, loss of CXCR4 signaling in leukemia cells leads to oxidative stress and differentiation *in vivo*. In contrast, the CXCR4 ligand CXCL12 is dispensable for leukemia development in recipient mice.

To identify key regulators of AML, in **paper II**, we performed an *ex vivo* cytokine screen on arrayed molecularly barcoded murine AML cells with a competitive *in vivo* read-out of their leukemia-initiating capacity. We identified TNFSF13 as a positive regulator of leukemia-initiating cells. We confirmed that TNFSF13 supports leukemia initiation under physiological conditions using *Tnfsf13$^{-/-}$* mice. We further showed that TNFSF13 suppresses apoptosis and promotes AML cell proliferation in an NF-κB dependent manner.

DUX4-rearranged BCP-ALL is a recently identified molecular subtype characterized by the expression of the *IGH-DUX4* fusion gene. With the aim of identifying biological dependencies of this subtype, in **paper III**, we performed a genome-wide CRISPR-Cas9 screen in the NALM6 cell line, driven by the *IGH-DUX4* fusion gene, and two control cell lines. We showed that *FNIP1*, *IRF4* and *SYNCRIP* are selectively important for the growth and survival of NALM6 cells and that their expression is under the control of the *IGH-DUX4* fusion gene. While the deletion of FNIP1 led to the enrichment of transcriptional signatures associated with metabolic dysregulation, loss of IRF4 resulted in upregulation of genes involved in differentiation and apoptosis of NALM6 cells. Moreover, disruption of SYNCRIP caused downregulation of the TGFβ-SMAD signaling pathways in NALM6 cells.

In **paper IV**, we explored the immune-mediated anti-leukemic activity of the cytokine interleukin 4 (IL4) in a murine AML model. Overexpression of IL4 in AML cells resulted in a strong anti-leukemic effect accompanied by an expansion of macrophages in the bone marrow and spleen of the recipient mice. Depletion of macrophages *in vivo* eliminated the antileukemic effect of IL4. In addition, IL4 directly activates murine macrophages resulting in enhanced phagocytosis of AML cells *in vitro*. Interestingly, IL4 also induced *Stat6*-dependent upregulation of CD47 in AML cells thereby inhibiting phagocytosis. Consistent with this finding, IL4 stimulation combined with CD47 blockade enhanced macrophage-mediated phagocytosis of AML cells.

Taken together, the studies included in this thesis employed high-throughput functional screens using CRISPR-Cas9 and molecular barcoding techniques to identify key regulators of AML cells. These findings improve our understanding of the disease and may translate into the development of new therapies for acute leukemia.

Key words: Acute leukemia, CRISPR, CXCR4, CXCL12, TNFSF13, DUX4, Interleukin 4, Leukeima stem cell, Macrophage	
Classification system and/or index terms (if any)	
Supplementary bibliographical information	**Language:** English
ISSN and key title: 1652-8220	**ISBN:** 978-91-7619-953-4
Recipient's notes	**Number of pages:** 86	Price
	Security classification	

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature   Date 2020-08-12

Functional screens identify vulnerabilities in acute leukemia

Ramprasad Ramakrishnan

LUND
UNIVERSITY

2020

Division of Clinical Genetics

Department of Laboratory Medicine, Lund

Faculty of Medicine, Lund University

கற்றது கைமண் அளவு, கல்லாதது உலகளவு

- அவ்வையார்

Known is a mere handful, unknown is the size of the world

- Avvaiyar[1]

---

[1] Avvaiyar - Female Tamil poet from 10th century AD

Table of Contents

Original papers

This thesis is based on the following original papers:

Paper I

Ramakrishnan, R., Peña-Martínez, P., Agarwal, P., Rodriguez-Zabala, M., Chapellier, M., Högberg, C., Eriksson, M., Yudovich, D., Shah, M., Ehinger, M., Nilsson, B., Larsson, J., Hagström-Andersson, A., Ebert, B. L., Bhatia, R., & Järås, M. (2020). **CXCR4 Signaling Has a CXCL12-Independent Essential Role in Murine MLL-AF9-Driven Acute Myeloid Leukemia**. *Cell Reports*, *31*(8), 107684

Paper II

Chapellier, M., Peña-Martínez, P., Ramakrishnan, R., Eriksson, M., Talkhoncheh, M. S., Orsmark-Pietras, C., Lilljebjörn, H., Högberg, C., Hagström-Andersson, A., Fioretos, T., Larsson, J., & Järås, M. (2019). **Arrayed molecular barcoding identifies TNFSF13 as a positive regulator of acute myeloid leukemia-initiating cells.** *Haematologica*, 104(10), 2006–2016

Paper III

Ramakrishnan, R., Rodriguez-Zabala, M., Lilljebjörn, H., Askmyr, M., Rissler, M., Järås, M., and Fioretos, T. **A genome-wide CRISPR screen identifies key regulators of DUX4-rearranged BCP-ALL.** *Manuscript*

Paper IV

Peña-Martínez, P., Ramakrishnan, R., Högberg, C., Jansson, C., Gisselsson-Nord, D., & Järås, M. **IL4 has a dual role in regulating phagocytosis of murine leukemia cells.** *Manuscript*

Papers not included in the thesis:

Eriksson, M., Peña-Martínez, P., Ramakrishnan, R., Chapellier, M., Högberg, C., Glowacki, G., Orsmark-Pietras, C., Velasco-Hernández, T., Lazarević, V. L., Juliusson, G., Cammenga, J., Mulloy, J. C., Richter, J., Fioretos, T., Ebert, B. L., & Järås, M. (2017). **Agonistic targeting of TLR1/TLR2 induces p38 MAPK-dependent apoptosis and NF-κB dependent differentiation of AML cells**. *Blood Advances*, 1(23), 2046–2057

Peña-Martínez, P., Eriksson, M., Ramakrishnan, R., Chapellier, M., Högberg, C., Orsmark-Pietras, C., Richter, J., Andersson, A., Fioretos, T., & Järås, M. (2018). **Interleukin 4 induces apoptosis of acute myeloid leukemia cells in a Stat6-dependent manner**. *Leukemia*, 32(3), 588–596

Hyrenius-Wittsten, A., Pilheden, M., Sturesson, H., Hansson, J., Walsh, M. P., Song, G., Kazi, J. U., Liu, J., Ramakrishan, R., Garcia-Ruiz, C., Nance, S., Gupta, P., Zhang, J., Rönnstrand, L., Hultquist, A., Downing, J. R., Lindkvist-Petersson, K., Paulsson, K., Järås, M., Gruber, T. A., Ma, J., Hagström-Andersson, A. K. (2018). **De novo activating mutations drive clonal evolution and enhance clonal fitness in KMT2A-rearranged leukemia**. *Nature Communications*, 9(1), 1770

Abbreviations

aCML	Atypical chronic myeloid leukemia
ADCC	Antibody dependent cell cytotoxicity
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ATO	Arsenic trioxide
ATRA	All-*trans*-retinoic acid
BCP-ALL	B-cell precursor acute lymphoblastic leukemia
CAR	Chimeric antigen receptor
Cas	CRISPR-associated
CBF	Core binding factor
CEL	Chronic eosinophilic leukemia
CHIP	Clonal hematopoiesis of indeterminate potential
CLL	Chronic lymphocytic leukemia
CLP	Common lymphoid progenitors
CML	Chronic myelogenous leukemia
CMML	Chronic myelomonocytic leukemia
CMP	Common myeloid progenitors
CNL	Chronic neutrophilic leukemia
CNS	Central nervous system
COL	Cell of origin of leukemia
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CXCL12	CXC-chemokine ligand 12
dCas9	dead-Cas9
DECL	DNA-encoded chemical libraries
DNMT3A	DNA methyl transferase 3A
DOT1L	DOT1-like histone lysine methyltransferase
DUX4	Double-homeobox 4
ECCITE-seq	Expanded CRISPR-compatible CITE-seq
EGIL	European group for the immunological characterization of leukemias
ELN	European leukemia network
EMP	Erythro-myeloid progenitors
ERG	ETS-related gene
ESA	Erythropoiesis stimulating agents
ET	Essential thrombocythemia
FAB	French-American-British
FISH	Fluorescence *in situ* hybridization
FLT3	FMS-like tyrosine kinase 3
FLT3-ITD	FLT3-internal tandem duplication
FLT3-TKD	FLT3-tyrosine kinase domain
FSHD	Facioscapulohumeral muscular dystrophy
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMP	Granulocyte-macrophage progenitors
HDAC	Histone deacetylase

HDC	Histamine dihydrochloride
HDR	Homology-directed repair
HMA	Hypomethylating agents
HSC	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
HSPC	Hematopoietic stem and progenitor cells
IL15	Interleukin 15
IL2	Interleukin 2
IL4	Interleukin 4
JMML	Juvenile myelomonocytic leukemia
LIC	Leukemia initiating cell
LMPP	Lymphoid-primed multipotent progenitors
LSC	Leukemia stem cells
MDS	Myelodysplastic syndromes
MDS/MPN	Myelodysplastic/Myeloproliferative neoplasms
MDS/MPN-RS-T	MDS/MPN with ring sideroblasts and thrombocytosis
MEP	Megakaryocyte-erythroid progenitors
MHC	Major histocompatibility complex
MLN	Mature lymphoid neoplasms
MM	Multiple myeloma
MM6	Monomac 6
MPN	Myeloproliferative neoplasms
MPP	Multi-potent progenitor
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
NK	Natural killer
NOD	Non-obese diabetic
NSG	NOD/SCID/IL2Rγ^{null}
NSG-β2m^{null}	NSG - beta2-microglobulin^{null}
ORF	Open reading frame
PAF	Polymerase associated factor
PAM	Protospacer adjacent motif
PDX	Patient-derived xenograft
PMF	Primary myelofibrosis
pTEFb	Positive transcription elongation factor b
PV	Polycythemia vera
RNAi	RNA interference
SCF	Stem cell factor
SCID	Severe combined immunodeficient
scRNA-seq	Single cell RNA sequencing
sgRNA	Single guide RNA
shRNA	Short hairpin RNA
SNP	Single nucleotide polymorphism
T-ALL	T-cell acute lymphoblastic leukemia
TALEN	Transcription activator-like effector nucleases
TAM	Tumor associated macrophages
tracrRNA	Trans-activating CRISPR RNA
WBC	White blood cell
WHO	World health organization
ZFN	Zinc finger nucleases

Populärvetenskaplig sammanfattning

Blod är en viktig komponent i nästan alla multicellulära organismer och en symbol för livet. Blod transporterar näringsämnen och syre till de olika organen i kroppen och tar bort restprodukter från dem. Blodet är också viktigt för att upprätthålla vår kroppstemperatur och för att bekämpa infektioner. Blodceller tillverkas i benmärgen som finns i våra ben. I benmärgen finns ett litet antal celler, så kallade stamceller, som har förmågan att både kopiera sig själva och producerar alla mogna blodceller. En genomsnittlig människa producerar cirka 200 miljarder blodceller per dag. Baserat på deras färg delas blodcellerna upp i röda blodceller och vita blodceller. De klassificeras också i myeloida celler och lymfoida celler baserat på var de finns eller mognar.

Under normala omständigheter är produktionen av blodceller felfri. Men ibland inträffar onormala genetiska förändringar i stamcellerna vilket leder till en okontrollerad tillväxt av omogna blodceller som resulterar i blodcancer. Akut leukemi är en gruppbeteckning som anger de mest aggressiva cancerformerna som uppkommer från vita blodceller. Beroende på om leukemin är av myeloid eller lymfoid typ, kategoriseras den i akut myeloid leukemi (AML) respektive akut lymfoid leukemi (ALL). Både AML och ALL drivs av en mängd olika genetiska avvikelser som förändrar de grundläggande egenskaperna hos normala stamceller och därmed förvandlar dem till leukemistamceller. Leukemiceller återfinns främst i benmärgen och regleras av ett molekylärt nätverk som består av ett antal gener, proteiner, metaboliter etc. För att förstå funktionerna hos dessa celler krävs identifiering av nyckelelementen i detta nätverk, och deras roll i leukemiceller. Det övergripande syftet med denna avhandling är att identifiera sårbarheter i akut leukemi. För att uppnå detta undersökte vi ett antal gener och proteiner med banbrytande molekylära tekniker och utvärderade deras förmåga att påverka tillväxten av leukemiceller.

I den första studien (**artikel I**) använde vi CRISPR, en såkallad gensax, för att ta reda på vilka gener som är kritiska för leukemistamcellerna i AML. CRISPR är en speciell typ av gensax som kan användas för att effektivt stänga av gener. Genom CRISPR stängde vi samtidigt av cirka 100 gener i leukemistamceller och studerade effekten på leukemicellernas tillväxt i en musmodell. Vi identifierade att genen CXCR4 är absolut nödvändig för leukemicellerna. När denna gen stängdes av mognade leukemistamcellerna ut till celler med begränsad livslängd.

Detta berodde delvis på oxidativ stress som involverar uppbyggnad av gifter som produceras som en biprodukt i cellerna när syre omvandlas till energi. Vi fann också att interaktionen mellan proteinerna CXCL12 och CXCR4 inte var nödvändig för leukemistamcellerna. Detta var förvånande eftersom denna interaktion är nödvändig för normal produktion av blodceller. Således identifierade vi en viktig skillnad mellan funktionen av normala blodstamceller och leukemiceller. Detta fynd kan vara användbart vid utveckling av nya terapier för AML.

I nästa studie (**artikel II**) sökte vi efter cytokiner som påverkar leukemi-initierande förmåga hos AML-celler. Cytokiner är proteinmolekyler som fungerar som kemiska budbärare mellan celler och påverkar deras funktioner. Vi använde en innovativ teknik där leukemistamcellerna var markerade med DNA-streckkoder. Denna teknik möjliggjorde att vi samtidigt kunde utvärdera hur cirka 100 cytokiner påverkade leukemicellerna. Genom detta tillvägagångssätt fann vi att en av cytokinerna, TNFSF13, förbättrade den leukemi-initierande förmågan hos AML-cellerna genom att öka deras tillväxt.

I **artikel III** sökte vi efter gener som är kritiska för en subtyp av ALL som drivs av genen DUX4. Vi använde återigen CRISPR, denna gång för att stänga av samtliga gener i ALL cellerna och studera effekten på celltillväxt. Vi fann att tre gener: FNIP1, IRF4 och SYNCRIP, var avgörande för överlevnaden av leukemiceller i denna subtyp av ALL. Ytterligare studier pågår för att förstå via vilka mekanismer dessa gener påverkar leukemicellerna.

Vi hade tidigare identifierat att interleukin 4 (IL4) inhiberar AML-celler i en musmodell. Den exakta mekanismen bakom den negativa effekten av IL4 är dock inte känd. I **artikel IV** fann vi att IL4 aktiverar makrofager som sedan attackerar och dödar AML-celler. Makrofager är en typ av immunceller som vanligtvis äter skadade celler och bakterier. Intressant nog fann vi också att IL4 delvis skyddar leukemicellerna från de attackerande makrofagerna genom att öka nivåerna av CD47, ett protein på ytan av AML-celler. Blockering av CD47 tillsammans med IL4-stimulering ökade makrofagernas dödande av AML-celler. Dessa fynd avslöjar den komplexa roll som IL4 spelar i AML.

Sammantaget har studierna som ingår i denna avhandling använt flera av de senaste molekylära teknikerna för att identifiera nyckelfaktorer som reglerar akut leukemi. Denna nya kunskap förbättrar vår förståelse av sjukdomen och kan leda till utvecklingen av nya behandlingsmetoder.

Popular science summary

Blood is a vital component of almost all multicellular organisms and is often a symbol for life itself. Blood transports nutrients and oxygen to the different organs in our body and removes waste materials from them. It is also important for maintaining our body temperature and for fighting against infections. Blood cells are made in a spongy tissue located inside some of our bones called bone marrow. In the bone marrow, a small number of parent cells called stem cells are present. These stem cells make copies of themselves and also produce all mature blood cells. An average human body produces about 200 billion blood cells per day. Based on their color, blood cells are divided into red and white blood cells. They are also classified into myeloid and lymphoid cells based on their place of maturation and prevalence.

Under normal circumstances, the production of blood cells is error-free. Occasionally, abnormal genetic changes occur in the stem cells which leads to an uncontrolled growth of immature blood cells resulting in blood cancer. Acute leukemia is a collective term denoting the aggressive cancers of white blood cells. Depending on whether the cancerous white blood cells are of myeloid or lymphoid type, it is categorized into acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) respectively. Both AML and ALL are driven by a wide variety of genetic abnormalities which alter the fundamental characteristics of normal immature blood cells thereby turning them into leukemia stem cells. Leukemia cells also reside in the bone marrow and are regulated by a molecular network consisting of a number of genes, proteins, metabolites etc. Identifying the key elements of this network and their role in leukemia cells could help us understand how these cells are regulated. The overarching aim of this thesis is to identify vulnerabilities in acute leukemia. In order to achieve this, we used cutting edge molecular techniques to assess the functional role of a number of genes and proteins in leukemia cells.

In the first study (**Paper I**), we performed a CRISPR screen to identify genes that are essential for leukemia stem cells in AML. CRISPR is a special type of gene scissors that can be used to turn-off genes effectively. Through CRISPR, we simultaneously turned off about 100 genes in leukemia stem cells and studied the effect on leukemia cell growth in a mouse model. We identified the gene CXCR4 to be essential for leukemia stem cells. When this gene was turned off, the leukemia stem cells matured into cells with limited lifespan and eventually died. This was partly due to oxidative stress which involves the build-up of toxins produced as a

by-product in the cells when oxygen is converted into energy. We also found that the interaction between the proteins CXCL12 and CXCR4 was not necessary for leukemia stem cells. This was surprising because this interaction is essential for normal blood stem cells. Thus, we identified a key difference between the functioning of normal and leukemia stem cells. This finding could be useful for designing new therapies for AML.

In the next study (**Paper II**), we searched for cytokines that affect the leukemia-initiating capacity of AML cells. Cytokines are protein molecules that act as chemical messengers between cells and affect their functions. We used an innovative technique in which the leukemia stem cells were marked with DNA barcodes. This technique allowed us to simultaneously assess the individual effect of about 100 cytokines on the leukemia stem cells. By using this approach, we found that one of the cytokines, TNFSF13 enhanced the leukemia initiating-capacity of AML cells by reducing the cell death and increasing the cell growth.

In **paper III**, we searched for genes that are critical for a subtype of ALL driven by the gene *DUX4*. We again used the special gene scissor, CRISPR and turned off all the genes in the ALL cells simultaneously and studied its effect on leukemia cell growth. We found three genes, *FNIP1*, *IRF4* and *SYNCRIP* to be essential for the survival of leukemia cells of this subtype of ALL. Further studies are ongoing to decipher the mechanisms by which these genes affect the leukemia cells.

We had previously identified interleukin 4 (IL4), a cytokine to be harmful for AML cells in a mouse model. However, the exact mechanism behind the negative effect of IL4 is not known. In **paper IV**, we examined if this negative effect was mediated by the immune cells. We found that IL4 activates macrophages which then attacks and kills AML cells. Macrophages are a type of immune cells that usually eat damaged cells and pathogens such as bacteria. Interestingly, we also found that IL4 partially protects the leukemia cells from the attacking macrophages by increasing the levels of CD47, a protein on the surface of AML cells. Blocking of CD47 along with IL4 stimulation mitigated the protective effect and increased the killing of AML cells by macrophages. These findings reveal the complex role played by IL4 in AML.

Collectively, the studies included in this thesis have employed several of the latest molecular techniques to identify key factors that regulate acute leukemia. The new knowledge created through these studies improves our understanding of the disease and may translate into the development of new therapies.

Hematopoiesis

Blood is a unique tissue in the human body that has fascinated the inquisitive minds for ages. According to the Greek philosopher Aristotle (384-322 BC), blood is a homogenous fluid produced in the heart and is distributed all over the body to nourish the different organs and to induce their growth [1, 2]. Between the 17th to the 19th century AD, just two types of blood cells were known based on their color under a microscope - red blood cells and white blood cells [3]. Modern medical science has come a long way from these rudimentary understandings of blood and has identified several types of specialized blood cells and their diverse functions such as transport of oxygen and nutrients to every cell in the body, defense against pathogens, coagulation etc. The process of generation of new blood cells is termed hematopoiesis (from Greek; *haimato* - blood, *poiein* - to make). The primary site of hematopoiesis in adults is the medullary cavity of the bone marrow. In addition, extramedullary hematopoiesis can take place in the spleen [4] and lungs [5].

The human body is estimated to have about 2.7×10^{13} blood cells amounting to ~90% of all cells in the body [6]. The turnover of blood cells in an average human adult is about 1 trillion cells per day [7]. Moreover, during severe physiological stress such as infections or blood loss, even more blood cells are produced. In order to maintain the number and diversity of the blood cells, hematopoiesis is tightly regulated. By the end of 20th century, it was well established that all of the mature blood cells are derived from a common pool of cells termed hematopoietic stem cells (HSC) through a hierarchical differentiation process.

Hematopoietic lineages and differentiation

About a dozen different types of mature blood cells have been identified. They are broadly classified into two types – myeloid and lymphoid cells based on their site of origin and prevalence. Myeloid (from Greek; *muelos* - marrow) cells comprise of granulocytes, monocytes, erythrocytes and megakaryocytes all of which originate and mature in the bone marrow. As the name suggests, granulocytes have large cytoplasmic granules that contain enzymes and are classified into four types – basophils, eosinophils, neutrophils and mast cells. Lymphoid cells are predominantly found in the lymphatic (from Latin; *lympha* - clear water) circulation and include B-cells, T-cells and Natural Killer (NK) cells. While mature B-cells and

NK-cells are produced in the bone marrow, immature T-cells migrate to the thymus where they undergo maturation. Both myeloid and lymphoid cells arise from progenitor populations which in turn are derived from HSCs through complex regulations of differentiation.
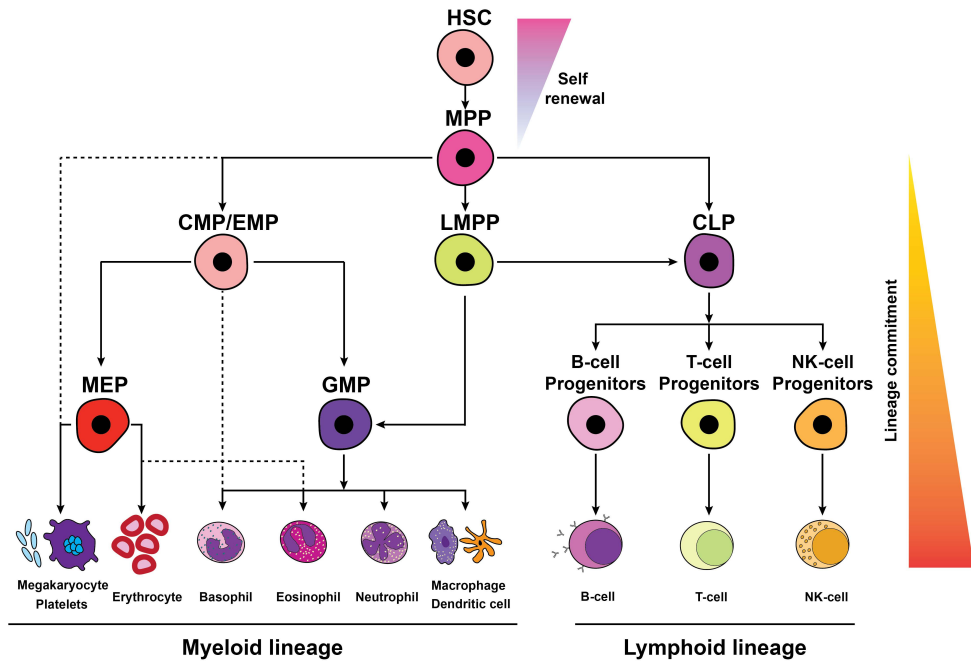
Figure 1. Hematopoietic hierarchical tree
Mature blood cells of myeloid and lymphoid lineages are produced through a hierarchical differentiation process with the HSCs at the apex and progenitor populations in the middle which are progressively committed to their corresponding lineages. Dotted lines indicate the recently proposed changes to the hierarchical tree.

The classical model of hematopoietic lineage commitment is depicted as a hierarchical tree involving successive binary fate decisions as HSCs differentiate through progenitor populations into mature blood cells (**Figure 1**) [8]. HSCs which are at the top of the hierarchy are defined based on two essential characteristics – long-term self-renewal and multipotency, i.e., the capacity to contribute to all types of mature blood cells. HSCs lose their self-renewal capacity while differentiating into multi-potent progenitor (MPP) populations which have reduced capacity to reconstitute the entire hematopoietic system in the long-term [9]. Subsequently, MPPs differentiate into committed progenitors namely common myeloid progenitors (CMP) [10], common lymphoid progenitors (CLP) [11] and lymphoid-primed multipotent progenitors (LMPP) [12].

CMPs have traditionally been considered as an oligopotent population, which is the only source of myeloid progenitors – granulocyte-macrophage progenitors (GMP) and megakaryocyte-erythroid progenitors (MEP). Recently, LMPPs with predominantly lymphoid developmental capacities have also been shown to give rise to GMPs suggesting that the segregation of myeloid and lymphoid lineages do not occur early in the hierarchical differentiation tree as previously thought [13]. Moreover, the capacity of CMP to produce a true GMP population is being questioned as they fail to differentiate into monocytes and neutrophils. Therefore, a suggestion that has been put forward is to rename CMPs as erythro-myeloid progenitors (EMP) which generate erythrocytes, basophils, eosinophils and megakaryocytes [14]. Megakaryocyte progenitor cells are derived from MEPs which further differentiate into megakaryocytes and platelets. However, it has been reported that megakaryocyte progenitor cells also arise directly from MPPs [15]. In the lymphoid compartment, CLPs that are derived from MPPs or LMPPs further differentiate to produce NK-cells, B-cells and T-cells.

The model of the hierarchical hematopoietic differentiation has been developed based on two key experimental pillars – i) immunophenotype-based cell purification, ii) *in vitro* and *in vivo* clonal assays. Every population in the hematopoietic system is defined by its immunophenotype -i.e., a unique profile of cell surface markers that can be used to purify the cells. Subsequently, the differentiation potential of these purified populations is assessed either by *in vitro* colony forming assays or by *in vivo* transplantation assays into murine models. For example, CMPs isolated based on their immunophenotype generate myeloid, erythroid or megakaryocytic colonies in a colony forming assay. In contrast, GMPs only give rise to myeloid colonies and MEPs produce erythroid or megakaryocytic colonies. Based on these observations, a model was proposed in which CMPs differentiate into GMPs and MEPs [10]. However, this interpretation is based on a key assumption, that the cells purified based on the immunophenotype are functionally homogenous. During the past decade, several studies have established that both the HSC pool and the progenitor populations are functionally heterogenous [14, 16, 17]. Moreover, the assessment of the differentiation potential of cells transplanted into immune-deficient mice does not reflect the fundamental properties of unperturbed native hematopoiesis. Using genetic labelling, it has been shown that in an unperturbed system, hematopoiesis is mainly driven by multipotent progenitors and not HSCs [18, 19]. Due to these limitations, the validity of the current model of the hierarchical hematopoietic differentiation is being challenged.

During the last decade, there has been an explosion of single cell RNA sequencing (scRNA-seq) studies [20-25] in the hematopoietic system that has offered a snapshot of the expression state of cells at a particular time point. These studies have proposed the idea that hematopoietic differentiation is in a continuum rather than in distinct differentiation stages [26]. For example, the HSC/MPP compartment in the upper tier of the hematopoietic tree has been proposed to be characterized by a

continuous differentiation landscape [27] as opposed to distinct subpopulations [28]. Moreover, transcriptional priming towards different lineages are initiated earlier than first anticipated, already at the MPP stage [27]. Similarly, profiling of myeloid progenitors using scRNA-seq has revealed tremendous heterogeneity, identifying 18 subpopulations with different degrees of lineage priming [29].
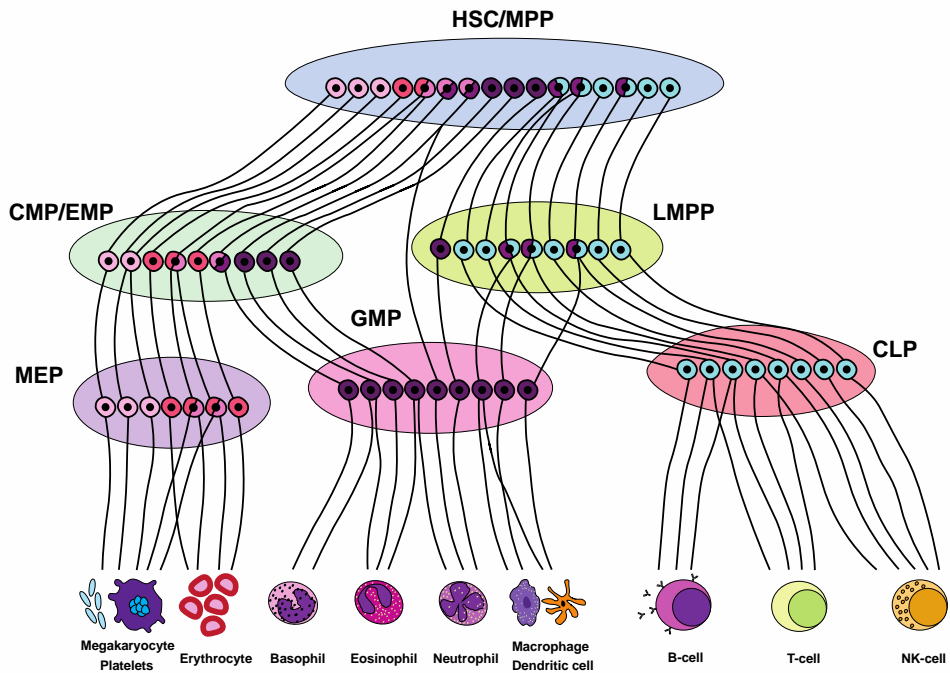
Figure 2. Alternative model of hematopoietic hierarchy
HSCs and the progenitor populations are highly heterogenous in their capacity to differentiate into specific lineages. Lineage choices are made early in the hematopoietic hierarchy and the cells follow different trajectories of differentiation to become mature cells (adapted from Laurenti, et al., [30]).

scRNA-seq studies in the HSC/MPP compartment have identified three major trajectories in the differentiation landscape – lymphoid, erythroid and granulocytic/monocytic lineages [31]. Analysis of the progenitor populations revealed that multiple types of mature cells arises from more than one trajectory [22, 27]. These recent findings have led to the proposal of a new representation of the hierarchical organization of hematopoiesis which appreciates the heterogeneity of the cells, prevalence of early lineage choices and diversity in possible routes of differentiation [30] (**Figure 2**).

4

Regulation of hematopoiesis

For maintenance of a robust hematopoietic system, fate decisions such as self-renewal vs differentiation, quiescence vs proliferation, survival vs death and lineage choices are tightly regulated in HSCs and other progenitor populations. These fate choices are guided both by external stimuli and cell intrinsic factors which affect the molecular circuitries of the cells. External factors that regulate hematopoiesis include cytokines, chemokines, extracellular matrix and membrane-bound signaling molecules provided by the microenvironment where the HSCs reside. The internal factors include transcription factors, signaling modulators, epigenetic modifiers and cell cycle regulators [32].

Hematopoietic niche

HSCs are proposed to be localized mainly in a hypoxic bone marrow microenvironment [33] termed perivascular niche, where they reside adjacent to sinusoidal blood vessels [34-36]. The major factors that promote the maintenance of HSCs in the bone marrow are stem cell factor (SCF) [37, 38], CXC-chemokine ligand 12 (CXCL12) [39, 40] and thrombopoietin [41]. Perivascular mesenchymal stromal cells and endothelial cells are the main sources of SCF and CXCL12 [42, 43] in the bone marrow niche. Other cell types in the niche that regulate HSCs are osteoblasts [44], osteoclasts [45], schwann cells [46], macrophages [47], and megakaryocytes [48]. Apart from HSC, osteoblasts and perivascular mesenchymal stromal cells also affect lymphoid progenitors [42]. During times of hematopoietic stress such as infection [49], pregnancy [4] or other hematological disorders [50], HSCs are mobilized from the bone marrow and colonize tissues – predominantly spleen and liver leading to extramedullary hematopoiesis [51].

Lineage choices

Transcription factors play a key role in the differentiation of HSCs and progenitors into specific lineages. Stochastic fluctuations in the expression of transcription factors or environmental cues such as cytokines have been proposed to control the lineage choice [52]. For example, expression of *GATA1* and *SPI1* is mutually exclusive during erythroid and myeloid differentiation, respectively. Moreover, *GATA1* and *SPI1* have been shown to repress each other and to activate themselves via a positive feedback loop, favoring a stochastic model of lineage commitment [53]. However, this model has been challenged by a recent study which suggests that the lineage choice is made at an earlier stage and the transcription factors merely execute the differentiation of the cell [54].

Maintenance of lineage choice is achieved by the sustained expression of lineage-specific transcription factors in the committed cells and their progeny. Positive autoregulation of a transcription factor while inhibiting opposing factors leads to stability of lineage commitment. The "GATA switch" is a classic example of this phenomenon where early progenitors express the transcription factor *GATA2* that induces *GATA1* expression which in turn represses *GATA2* and activates its own expression, thereby pushing cells towards the erythropoietic lineage [55].

Apart from transcription factors, cytokines play a vital role in lineage choices, proliferation, maturation, survival and activation of cells in the hematopoietic system [56]. For example, ectopic upregulation of granulocyte-macrophage colony stimulating factor (GM-CSF) receptor has been shown to instruct the conversion of CLPs from the lymphoid to myeloid lineage [57]. Similarly, expansion of lymphoid and myeloid progenitors can be induced by high levels of the FMS-like tyrosine kinase 3 (FLT3) ligand [58]. Collectively, cytokines instruct the lineage commitment of blood cells thereby regulating hematopoiesis.

Epigenetic modifiers are emerging as important regulators of hematopoiesis apart from transcription factors and cytokines. Epigenetic modifications such as histone acetylation and methylation of cytosine residues modulates chromatin accessibility and transcription of genes involved in lineage commitment [59]. For example, loss of DNA methyl transferase 3A (DNMT3A) results in impaired differentiation and clonal expansion of HSCs, causing a pre-leukemic state [60] (see *Pre-leukemia to overt leukemia*).

Hematological malignancies

Hematological malignancies represent a heterogenous group of neoplasms that are characterized by the abnormal production of blood cells. Under normal conditions, key characteristics of hematopoietic stem and progenitor cells (HSPC) such as self-renewal, differentiation and proliferation are well regulated in order to ensure efficient hematopoiesis. Occasionally, unintended genetic and epigenetic changes occur in these cells which dysregulates these characteristics resulting in the accumulation of abnormally differentiated blood cells that are dysfunctional. Interestingly, some of the initiating genetic lesions are not by themselves sufficient to cause an overt disease but lead to clonal hematopoiesis [61].

The genetic changes that lead to malignant transformation of normal HSPCs include gene mutations, chromosomal translocations and other structural abnormalities such as aneuploidy, copy number variations etc. Over the past two decades, next generation sequencing (NGS) technologies have been instrumental in identifying a number of these genetic aberrations. Several of these mutations have been used for diagnosis, risk stratification, selection of treatment regimen and to predict the prognosis of the patients. Similar to normal hematopoiesis, hematological malignancies are also broadly classified into myeloid and lymphoid malignancies based on the lineage of origin of the neoplasia. Additionally, they are classified into acute and chronic malignancies based on the rate of progression of the disease. Taking into account the diversity of the hematological malignancies, the world health organization (WHO) has made a classification system for these diseases [62-64] (**Figure 3**).

Myeloid malignancies

Myeloid malignancies comprise of four sub-categories - myeloproliferative neoplasms (MPN), myelodysplastic syndromes (MDS), myelodysplastic/myeloproliferative neoplasms (MDS/MPN) and acute myeloid leukemia (AML), all of which are caused by genetic lesions resulting in clonal proliferation of defective blood cells of myeloid lineage (**Figure 3**). Genetic mutations associated with myeloid malignancies belong to five main classes: transcription factors (e.g. *CEBPA*, *IKZF1*), tumor suppressors (e.g. *TP53*),
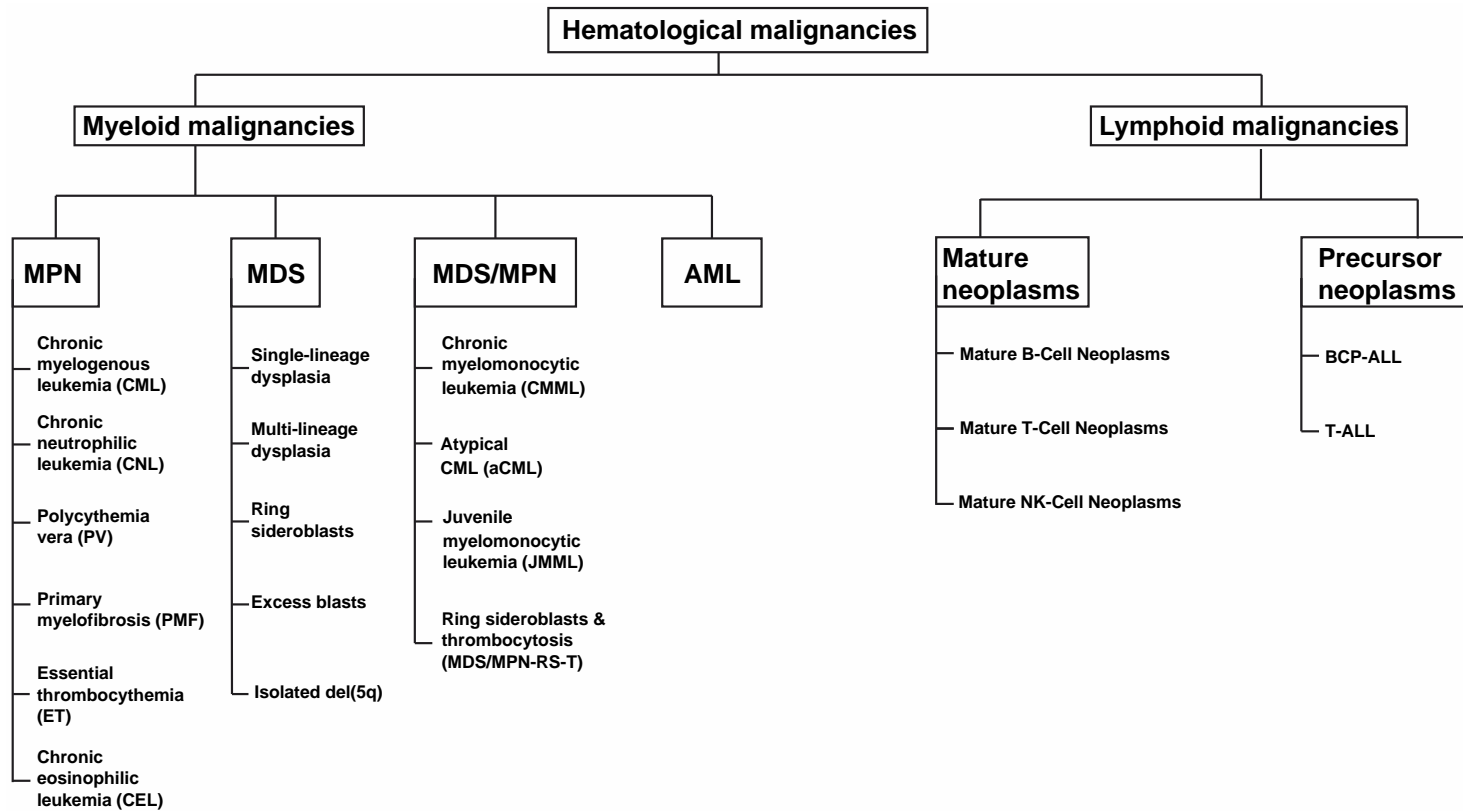
Figure 3. Major hematological malignancies
Flowchart depicting the major subtypes of myeloid and lymphoid malignancies included in the WHO classification of hematological malignancies. The subtypes of AML and BCP-ALL are discussed in detail in the following chapters.

8

signaling pathways (e.g. *FLT3*, *RAS*), epigenetic regulators (e.g. *DNMT3A*, *EZH2*) and components of the spliceosome (e.g. *SF3B1*, *SRSF2*) [65]. Understanding the complex mutational landscape of these diseases has been instrumental in improving risk stratification and prognosis of the patients.

MPN is characterized by a relatively slow clonal expansion of hematopoietic progenitors and increased proliferation of mature myeloid cells in the bone marrow [66]. MPN is associated with thrombotic and hemorrhagic events and an increased risk of transformation into secondary AML (sAML) [67]. MPN is further classified into chronic myelogenous leukemia (CML), chronic neutrophilic leukemia (CNL), polycythemia vera (PV), primary myelofibrosis (PMF), essential thrombocythemia (ET) and chronic eosinophilic leukemia (CEL) [63]. Genetic aberrations that lead to constitutive activation of signaling cascades and cytokine-independent proliferation are hallmarks of MPN. For example, CML is characterized by a reciprocal t(9;22)(q34;q11) translocation occurring in HSCs leading to the formation of the *BCR/ABL1* fusion gene, which encodes for a constitutively active tyrosine kinase that promotes cell proliferation. Development of tyrosine kinase inhibitors have revolutionized the treatment of CML patients allowing for a near-normal life [68].

MDS is a heterogenous disease characterized by morphological dysplasia of myeloid cells and inefficient hematopoiesis resulting in cytopenias [63]. Approximately one-third of MDS patients progress to high risk MDS and sAML [69]. The founding genetic event in MDS is believed to occur in HSCs resulting in clonal hematopoiesis of indeterminate potential (CHIP) (see *Pre-leukemia to overt leukemia*). The transformation from CHIP to MDS is suggested to involve a complex interplay between epigenetic changes in the HSCs, dysregulation of the bone marrow microenvironment and acquisition of additional driving mutations [70]. Due to the heterogeneity of the disease, a risk-adapted treatment strategy is adopted. Allogenic HSC transplantation is the only curative option which is offered based on the availability of donors and fitness of patients. Other noncurative treatments include administration of erythropoiesis stimulating agents (ESA), hypomethylating agents (HMA), immunosuppressants and red blood cell transfusions, aimed at improving cytopenias and quality of life [71].

Myeloid neoplasms that exhibit clinical and morphological characteristics overlapping with both MDS and MPN along with ineffective hematopoiesis are classified as myelodysplastic/myeloproliferative neoplasms (MDS/MPN). MDS/MPN includes five distinct subtypes - chronic myelomonocytic leukemia (CMML), atypical chronic myeloid leukemia (aCML), juvenile myelomonocytic leukemia (JMML) and MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T) [63]. Many of the somatic mutations and cytogenetic abnormalities found in MDS and MPN are also found in MDS/MPN [72]. The current treatment options for MDS/MPN comprise of risk- and symptom- based therapies co-opted from other myeloid malignancies [73].

AML is an aggressive clonal disorder characterized by the accumulation of immature abnormally differentiated myeloid blast cells. It is a heterogenous disease with several different subtypes classified based on their biological and prognostic characteristics. As three of the studies (**Papers I, II & IV**) included in this thesis focus on AML, it is discussed in detail in a separate chapter (see *Acute myeloid leukemia*).

Lymphoid malignancies

Lymphoid malignancies are broadly classified into mature and precursor neoplasms based on the differentiation stage of the malignant lymphoid cells (**Figure 3**). As the name suggests, mature lymphoid neoplasms (MLN) are characterized by the clonal expansion of mature lymphocytes and are classified based on the type of lymphocyte involved into mature T-cell neoplasms, mature NK-cell neoplasms, mature B-cell neoplasms. Among these, mature B-cell neoplasms accounts for the majority of the cases and comprise of a heterogenous group of disorders driven by different genetic aberrations [74]. The WHO classification of lymphoid malignancies lists as many as 41 mature B-cell neoplasms [64]. Some of the predominant subtypes of mature B-cell neoplasms include chronic lymphocytic leukemia (CLL), multiple myeloma (MM), diffuse large B-cell lymphoma, and follicular lymphoma.

Precursor lymphoid neoplasms are characterized by the accumulation of abnormally differentiated lymphoblasts and are historically referred as acute lymphoblastic leukemia (ALL). ALL is the most common form of cancer among children [75]. Depending on whether B- or T-lymphoblasts are involved, ALL is further classified into T-cell acute lymphoblastic leukemia (T-ALL) or B-cell precursor acute lymphoblastic leukemia (BCP-ALL) respectively. Both classes of ALL comprise of multiple subtypes which are stratified based on the genetic lesions such as structural chromosomal alterations, DNA copy number alterations and gene mutations that contribute towards leukemia development [76]. Treatment of childhood ALL is considered a success story with an overall survival rate of ~80% and with certain subtypes approaching a cure rate of ~98% [77, 78]. However, survival and outcomes of adult ALL cases (18-60 years of age) continues to remain poor and is about 35% [79].

BCP-ALL accounts for about 85% of ALL cases [80]. One of the studies (**Paper III**) in this thesis focuses on BCP-ALL and is therefore discussed in detail in a separate chapter (see *B-cell precursor acute lymphoblastic leukemia*).

Leukemogenesis

Acute leukemia is a collective term referring to a group of aggressive hematological malignancies of either myeloid or lymphoid lineage and is characterized by a rapid clonal expansion of blast cells with impaired differentiation. The evolutionary path of leukemogenesis starting with the acquisition of the first somatic mutation eventually leading to the development of overt leukemia is poorly understood. A detailed genetic analysis of acute leukemia using NGS technology has revealed a complex clonal architecture with multiple driver and cooperating mutations, coexistence of multiple subclones and their evolution over time [81, 82]. Moreover, the varied response of different subclones to therapies adds another layer of complexity to the disease. A clear understanding of the process of leukemogenesis in AML and BCP-ALL would be beneficial in devising effective therapies.

Rise of leukemia

Somatic mutations in human genome are estimated to occur at a rate of ~0.06 – 1.47 x 10^{-9} mutations per base pair per cell division [83]. The functional effect of a particular mutation depends on the type of mutation, the cell type and the genomic region in which the mutation occurs. Many of the mutations are functionally irrelevant and do not affect the fitness of the cells. However, some mutations will alter the fundamental characteristics of the cells such as self-renewal, differentiation or proliferation, which eventually may result in a neoplasm. A widely accepted model for the development of acute leukemia is that the occurrence of the first genetic lesion in specific hematopoietic cells results in a pre-leukemic state and the subsequent acquisition of additional mutations leads to the leukemic transformation.

Pre-leukemia to overt leukemia

As we age, there is an accumulation of mutations in different cell types. In the hematopoietic cells, several recurrent mutations have been shown to drive clonal hematopoiesis which is characterized by the overrepresentation of blood cells derived from a single clone containing the mutation. This is probably due to the
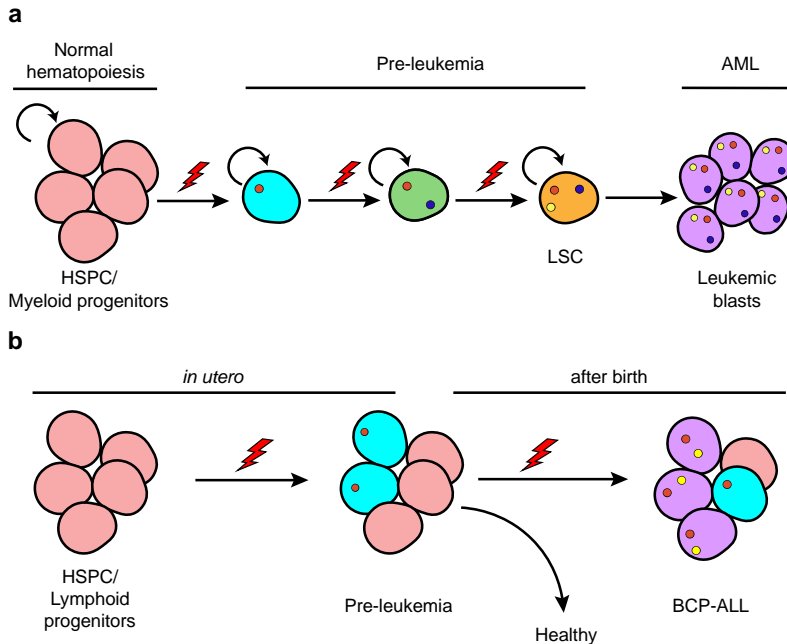
Figure 4. Development of acute leukemia
Schematic pictures depicting the transformation of normal hematopoietic cells into a pre-leukemic state and subsequently into overt leukemia in **(a)** AML and **(b)** BCP-ALL. Each colored dot represents a unique mutation. The curved arrow in (a) represents the self-renewal capacity of the cells.

acquisition of mutations in HSCs that provide them with a growth advantage [84]. As clonal hematopoiesis may or may not transform into a hematological malignancy, it is labeled as CHIP. CHIP refers to the existence of a cancer-associated variant in the hematopoietic cells in the absence of malignancy but is associated with an increased risk of progressing into malignancies [85, 86]. A majority of mutations associated with CHIP occur in the genes encoding epigenetic modifiers, such as *DNMT3A* and *TET2*. Other genes frequently mutated in CHIP include *ASXL1*, *JAK2*, *SF3B1* and *TP53* [87]. Many of the genes mutated in CHIP have also been identified as recurrent mutations in MDS and AML [88-91]. Therefore, CHIP has been postulated as a pre-leukemic state which could progress into MDS and subsequently into AML (**Figure 4a**). The committed progenitor populations of the pre-leukemic cells are susceptible to additional somatic mutations such as *NPM1* and *FLT3* [92-94] which then leads to leukemic transformation. In population-based cohorts, CHIP was associated with a ~10-fold increased relative risk of the development of hematological malignancies over several years of follow-up [84].

Relative to AML, BCP-ALL is more common among children where the occurrence of clonal hematopoiesis is rare suggesting that there are other mechanisms of leukemogenesis in pediatric leukemias. A number of genetic lesions associated with BCP-ALL have been shown to arise *in utero* [95-99]. As secondary mutations are required for the leukemic transformation of most of the subtypes, some of these prenatal genetic lesions are hypothesized to produce a pre-leukemic state (**Figure 4b**) [100]. The secondary genetic events often target genes critical for lymphoid development such as *RUNX1*, *IKZF1* and *PAX5* [101]. The mechanisms by which prenatal pre-leukemia transforms into postnatal BCP-ALL is unclear. Since only a part of the carriers of the pre-leukemic lesions suffers secondary mutations and subsequent leukemic transformation, it has been hypothesized that environmental factors such as infections are involved in the acquisition of the secondary mutations [102, 103]. Dysregulation of immune cells, particularly T-helper cells and NK-cells by infections has been proposed to play a major role in the leukemic transformation [104, 105]. Interestingly, *MLL*-rearranged BCP-ALL (official name *KMT2A*) have been found to occur already in newborns [106] and is the only leukemia-related genetic lesion in some patients [107, 108] suggesting that the fusion itself is sufficient for leukemia onset.

Cell of origin

The beginning of leukemia can be traced back to a normal hematopoietic cell termed cell of origin of leukemia (COL) in which the first initiating genetic aberration occurs. In AML, HSPCs and myeloid progenitors have been shown to be the COL through transplantation based murine leukemia models [109]. However, only HSPCs are suggested to be the COL of human AML due to the presence of somatic mutations in pre-leukemic HSPCs that lead to CHIP and subsequently into AML [110]. In BCP-ALL, the COL varies between HSCs and committed lymphoid progenitors according to the underlying genetic lesion. For example, *BCR-ABL1* fusions can originate either from HSCs or from a B-cell progenitor depending on the isoform of *BCR-ABL1* [111]. It is important to note that identification of COL is generally challenging as the initiating genetic lesions can alter its properties such that the cell is reprogrammed into a different stage of differentiation [112].

Acute leukemia is sustained by a population of cells termed leukemia stem cells (LSC). LSCs are functionally defined as the cells with the ability to maintain and repopulate leukemia. They share the characteristic of self-renewal with normal HSCs. LSCs are also termed as leukemia initiating cells (LIC) as they can initiate leukemia when transplanted into a host. The use of the term "LIC" has been contentious as it has also been occasionally used to denote COL. Since additional mutations are often required for leukemic transformation, LSC/LIC may not always be the COL and is important to clarify the context in which these terms are used.

In AML, LSCs were initially thought to be a small population that shares immunophenotypic characteristics with normal HSCs [113, 114]. However, with refinements in the immunophenotypic definition of HSCs, LSCs were proposed to be present in non-HSC progenitor stages [115, 116]. Subsequently, LSCs have been shown to closely mirror normal LMPPs and GMPs suggesting that LSCs acquire abnormal self-renewal potential and maintains a hierarchical structure [117]. LSCs give rise to cells that lack the self-renewal capacity and the ability to terminally differentiate termed blast cells. Transcriptome analysis of LSCs revealed the presence of a "stemness" signature which is associated with adverse prognosis in AML [118]. In BCP-ALL, several studies have shown that the ability of leukemia cells to engraft immunodeficient mice is not restricted to specific immunophenotypes suggesting a stochastic stem cell model according to which most of the leukemia cells have the ability to propagate the disease [119-121].

Clonal heterogeneity and evolution

Accumulation of genetic alterations in the neoplastic cells is one of the defining features of cancer. Acquisition of a mutation that confers a cell with a selective growth advantage results in clonal expansion thereby creating a population of cells termed as a clone which carries the mutation. With the accumulation of additional mutations, many subclones are created which compete with each other and with normal cells over resources in the tissue microenvironment. Thus, cancer progression is essentially a process of mutational diversification and clonal evolution [122]. Different models of clonal evolution in tumors have been proposed [123]. Linear clonal evolution involves the stepwise accumulation of mutations that confer a strong selective advantage to progeny clones which replace the parent clones in full selective sweep [124]. By contrast, branching evolution involves the coexistence of multiple subclones, each with their selective growth advantages that compete for ascendency [125].

Due to the mobile nature of leukemia cells as opposed to the fixed tissue architecture of solid tumors, they can undergo more cellular mixing and a homogenous population through a linear clonal evolution could have been expected [126]. However, several genomic studies of acute leukemia have shown the presence of a heterogenous mixture of clonal populations suggesting a branching clonal evolution [127-132]. Thus, the eventual composition of the leukemic clones is determined by the competition between the different mutant populations.

Acute myeloid leukemia

Epidemiology

The incidence of AML per 100,000 people in USA is estimated to be ~4.3 [133]. In Sweden, about 350 AML cases are diagnosed every year (www.cancerfonden.se) and the overall prevalence is estimated to be 13.7 per 100,000 people [134]. AML is more common in the elderly with a median age of 72 years and a peak in incidence at 80-84 years of age [134]. About 25% of the patients diagnosed with AML have a previous hematological disease [135].

Classification

AML is a heterogenous disease encompassing a wide range of variations in cell morphology, molecular profile, response to treatment and prognosis. A robust classification system of the disease is necessary to gain insights into biology of the leukemia cells and to develop effective therapies for the specific subtypes. An early effort to have a standardized classification system for AML was undertaken by French-American-British (FAB) co-operative group in 1976. This classification was entirely based on the morphology of the leukemia cells in the bone marrow and peripheral blood taking into account the degree of maturation and the direction of differentiation towards one or more mature cell lines [136]. During the 1980s, the FAB-classification of AML was revised to include additional subtypes and modified diagnostic criteria to account for the stage of impaired differentiation of the leukemia cells in the patient sample [137, 138] (**Table 1**). Although morphological heterogeneity of AML is taken into consideration in the FAB-classification, it does not always reflect the diversity of genetic lesion in the disease.

Over the past two decades, the WHO has proposed and revised a new classification system for AML based on the morphology, presence of specific genetic abnormalities, therapy-responses and prior history of myeloid malignancy [63, 139, 140] (**Table 1**). The recurrent genetic abnormalities included in the WHO-classification system primarily involves structural changes. However, ~50% of AML patients present with normal karyotype and exhibit variable clinical response to conventional chemotherapy indicating disease heterogeneity within the group

[141]. Advancements in sequencing technologies have led to the identification of a number of recurring mutations in AML patient samples [88, 142-144]. The present WHO-classification system does not include several of these mutations. Therefore, a new system of classification based on the genomic mutation profile has been proposed that can better address the heterogeneity and risk stratification of AML [144].

Table 1. FAB and WHO classifications of AML

FAB Classification

M0	Minimally differentiated AML
M1	AML without maturation
M2	AML with maturation
M3	Acute promyelocytic leukemia
M4	Acute myelomonocytic leukemia
M5	Acute monoblastic/monocytic leukemia
M6	Acute erythroleukemia
M7	Acute megakaryoblastic leukemia

WHO classification

AML with recurrent genetic abnormalities	Genetic abnormality
AML with t(8;21)(q22;q22.1)	*RUNX1-RUNX1T1* fusion gene
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22)	*CBFB-MYH11* fusion gene
AML with t(15;17)(q22;q21)	*PML-RARA* fusion gene
AML with t(9;11)(p21.3;q23.3)	*MLL-AF9* fusion gene
AML with t(6;9)(p23;q34.1)	*DEK-NUP214* fusion gene
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)	*MECOM* activation or *RPN1-EVI1* fusion gene
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3)	*RBM15-MKL1* fusion gene
AML with mutated *NPM1*	*NPM1* loss of function mutations
AML with biallelic mutations of *CEBPA*	*CEBPA* dominant-negative mutations
AML with *BCR-ABL1* (provisional entity)	*BCR-ABL1* fusion gene
AML with mutated *RUNX1* (provisional entity)	*RUNX1* loss of function mutations
AML with myelodysplasia related changes	
Therapy-related myeloid neoplasms	
AML not otherwise specified	

16

Genetic aberrations in AML

Cytogenetic abnormalities

About five decades ago, the first somatic chromosomal abnormality involving a balanced translocation t(8;21) was identified in AML [145]. Since then, a number of cytogenetic aberrations have been identified which are found in nearly half of the newly diagnosed adult AML patients [141]. Karyotyping serves as the primary diagnostic tool to identify the recurrent cytogenetic abnormalities which include chromosomal deletions, inversions, aneuploidy and translocations. Based on the number of cytogenetic aberrations, the AML cases are classified into normal karyotype and complex karyotype ($\geq$ 3 abnormalities).

Some of the major chromosomal translocations include t(8;21)(q22;q22.1), t(15;17)(q22;q21), inv(16)(p13.1q22)/t(16;16)(p13.1;q22) and 11q23 abnormalities which together are found in ~29% of adult AML patients [146]. AML arising due the t(15;17)(q22;q21) translocation is characterized by a rapid expansion of abnormal promyelocytes and is termed acute promyelocytic leukemia [147]. This translocation results in the expression of the *PML-RARA* fusion gene which drives the leukemic transformation by blocking differentiation through transcriptional regulation of its target genes. Both t(8;21)(q22;q22) and inv(16)(p13.1q22)/t(16;16)(p13.1;q22) affect the proteins involved in the core binding factor (CBF) transcription complex and hence are termed CBF-AML [148]. The t(8;21)(q22;q22.1) rearrangement is characterized by *RUNX1-RUNX1T1* (official name – *AML1-ETO*) fusion gene that encodes a protein which acts as a dominant negative regulator of RUNX1 target genes [149, 150]. However, the translocation by itself is insufficient to cause leukemia and secondary cooperative mutations are likely required [151]. Such additional mutations include activating mutations in *KRAS*, *NRAS*, *ASXL1*, *KIT*, *PTPN11* and/or loss-of-function mutations in *NF1,* which are found in about two-thirds of t(8;21)(q22;q22.1) AML patients [152-154]. Abnormalities in chromosome 16 includes inversion (16)(p13.1q22) and translocation (16;16)(p13.1;q22), both resulting in the formation of the *CBFB-MYH11* fusion gene that alters the transcriptional profile of the cells leading to leukemic transformation. The inv(16) is more common than t(16;16) and is often accompanied by aneuploidies such as +22, +8, del(7q) and +21 [155].

MLL gene (official name *KMT2A*), a H3K4 methyltransferase is a key epigenetic regulator playing a critical role in the emergence of fetal HSCs and maintenance of adult HSPC [156, 157]. 11q23 abnormalities involve chromosomal translocations resulting in in-frame fusions of the *MLL* gene to more than 100 different partner genes [158]. However, six fusion partners – *AF9*, *ENL*, *AF10*, *ELL*, *PTD* and *AF6* constitute ~83% of the *MLL*-rearranged AML cases [158]. The distribution of the different *MLL*-rearrangements varies with age with *AF10* and *AF9* being the most

common *MLL*-fusion partners in infant and pediatric AML cases, respectively. Although the proteins encoded by the different *MLL*-fusion genes have diverse functions, they can be classified into two groups based on their localization, either in the cytoplasm or nucleus of the cells. Cytoplasmic fusion partners include *ESP15*, *GAS7*, *SH3GL1*, *AFDN* and *FOXO4* which have been shown to be important for the dimerization of MLL which contributes to leukemic transformation [159]. Nuclear proteins constitute the majority of the *MLL*-fusion partners and are involved in the regulation of transcriptional elongation via recruitment of polymerase associated factor (PAF) complex, the DOT1-like histone lysine methyltransferase (DOT1L) complex and positive transcription elongation factor b (pTEFb) complex that leads to deregulated transcription [160-164]. DOT1L is also a methyltransferase that catalyzes H3K79 dimethylation which is associated with actively transcribed genes. Through its interactions with MLL-fusion protein, DOT1L mediates H3K79 methylation on the regulatory regions of later *HOXA* cluster genes including *HOXA9*. This results in a distinct gene expression signature [165] with elevated expression of *HOXA* cluster genes and the *HOX* cofactor *MEIS1* which has been shown to be essential for leukemia development [166, 167].

Mutational landscape

The first whole cancer genome was sequenced in 2008 from an AML patient [168]. Since then, several hundred AML genomes have been sequenced by NGS which has resulted in the emergence of the mutational landscape of AML [88, 144, 169]. The most common genetic change in AML involves mutations in the *NPM1* gene amounting for 27% of adult AML cases [144]. NPM1 is a nuclear protein playing a critical role in ribosome biogenesis, DNA repair and regulation of apoptosis. The mutations result in the aberrant localization of the NPM1 protein in the cytosol which contributes to leukemogenesis [170].

Other recurrent mutations found in AML can be classified into several groups based on their functional role. These include mutations in activated signaling pathway components, epigenetic/chromatin modifiers, cohesin complex, RNA splicing factors, tumor suppressor and transcription factors (**Table 2**). The most commonly mutated gene involved in signaling pathways is *FLT3,* which encodes a tyrosine kinase that acts as a cytokine receptor for the FLT3 ligand. *FLT3* mutations can occur either as *FLT3*-internal tandem duplication (*FLT3*-ITD) in the juxtamembrane region or as point mutations in the tyrosine kinase domain (*FLT3*-TKD) resulting in constitutive activation of the tyrosine kinase. This results in the activation of RAS, MAPK and STAT5 signaling pathways which leads to increased proliferation [171]. Other genes mutated in signaling pathway components include *c-KIT*, *KRAS*, *NRAS* and other kinases [88]. Mutations in genes encoding epigenetic and chromatin modifiers are usually the dominant clones in AML patients suggesting that they are

Table 2. Recurrent gene mutations in AML

Mutation category	Genes
Activated signaling pathway components	*FLT3, NRAS, KRAS, PTPN11, NF1, KIT, CBL*
Epigenetic/chromatin modifiers	*DNMT3A, TET2, IDH1, IDH2*-R140, *IDH2*-R172, WT1, *ASXL1, MLL-PTD, PHF6, ASXL2, BCOR, EZH2*
Cohesin complex	*RAD21, SMC1A, SMC3, STAG1, STAG2*
Splicesome	*SRSF2, SF3B1, U2AF1, ZRSR2*
Tumor suppressor	*TP53*
Transcription factors	*RUNX1, CEBPA*
Other	*NPM1*

founding mutations [144]. These include mutations in *DNMT3A*, *TET2*, *ASXL1*, *IDH1* and *IDH2* which independently results in the dysregulation of DNA and histone methylation. The cohesin complex plays a key role in mediating sister chromatid cohesion during mitosis. Apart from that, it is also involved in DNA damage repair and transcriptional regulation. Mutations in the genes encoding the cohesin complex such as *STAG1* and *STAG2* results in the deregulation of cohesin mediated gene regulation [172]. Commonly mutated genes involved in RNA splicing are *SF3B1*, *U2AF1*, *SRSF2* and *ZRSR2* which are also frequently found in MDS patients suggesting that they progressed into secondary AML [173]. Mutations in the transcription factors *CEBPA* and *RUNX1* together accounts for ~14% of AML cases [144] and leads to a block in myeloid differentiation. Many of the recurrent mutations mentioned above were found to co-occur suggesting that they are cooperating genetic lesions. Most prominent co-occurring mutations are in *DNMT3A*, *NPM1* and *FLT3*.

AML microenvironment

The bone marrow microenvironment plays an important role in AML development as it does in normal hematopoiesis (see *Hematopoietic niche*) [174]. Genetic lesions that occur in non-hematopoietic niche cells can contribute to the leukemic transformation. For example, constitutive activation of Wnt signaling pathway in murine osteoblast cells due to an activating mutation in *β-catenin* (official name *Ctnnb1*) has been shown to initiate an AML-like disease with common chromosomal aberrations [175]. Alternatively, AML cells can also remodel the bone marrow niche to support disease progression. This is achieved through the dysregulation of signaling in the niche cells resulting in aberrant cytokine secretion that supports the expansion of AML cells. For example, the secretion of CXCL12 by the mesenchymal stromal cells derived from the bone marrow of AML patients is markedly lower compared to that of healthy subjects. This results in an overall

reduction of CXCL12 levels in the bone marrow plasma of AML patients which could contribute to the impaired maintenance of normal HSCs [176]. A corollary of this finding is that the AML cells could be less dependent on CXCL12 for the disease progression. In line with this, in **article I**, we show that CXCL12 expression in the bone marrow is dispensable for AML development in a murine model. Apart from these changes, the bone marrow microenvironment has also been shown to protect LSCs from chemotherapy thereby contributing to therapy resistance and disease relapse [177].

Murine models of AML

Much of what we know about the mechanism of action of different genetic alterations associated with AML is through *in vivo* mouse models. They are also an integral part of drug development pipelines by providing a platform to test different pharmaceutical compounds prior to clinical trials. The existing mouse models of AML can be broadly categorized into three groups – carcinogen induced AML models, xenograft models and genetically engineered models [178]. Carcinogens such as chemicals, radiation and murine viruses have been used for random mutagenesis in murine hematopoietic cells leading to leukemia development [179-181]. Although these models have been instrumental in the identification of proto-oncogenes and development of anti-leukemic therapies, they do not fully phenocopy human AML.

AML patient-derived xenograft (PDX) mouse models are generated by the transplantation of leukemia cells from AML patients into mice which engraft in their bone marrow and mimic the human disease. Successful engraftment and repopulation of patient cells into mice depends on factors such as the availability of niche space, intensity of murine immune responses against the graft and the presence of supportive cross-reactive signals for human cells in the mice. In order to reduce the rejection of the transplantation, mice with different levels of immunodeficiencies such as nude, severe combined immunodeficient (SCID), non-obese diabetic (NOD)/SCID and NOD/SCID/IL2Rγ^{null} (NSG) mice have been used [182]. In addition, ablation of mouse hematopoietic cells in the bone marrow through irradiation or use of *c-Kit* mutant mice [183] creates open niches for transplanted human cells to home and engraft. To further support the growth of human cells, NSG mice expressing three human cytokines – IL3, GM-CSF, SCF has been developed (NSG-S mice) which allows for higher engraftment of AML patient cells [184]. Additionally, NSG strains with mutated major histocompatibility complex (MHC) class I and class II beta2-microglobulin (NSG-β2m^{null}) [185] have been developed which reduces the immune reactivity of human cells against host tissue. Irrespective of the manipulations in the host, efficient expansion of PDX also depends on the underlying genetic aberrations in the AML cells [186].

Genetically engineered mouse models include transgenic mice with constitutive or conditional expression of *PML-RARA* [187], *RUNX1-RUNX1T1* [188], *MLL-AF9* [189], *MLL-ENL* [190] or cooperating mutations such as *NPM1* + *FLT3*-ITD/*NRAS-G12D* [191, 192], *MLL-PTD* + *FLT3*-ITD [193], *TET2* + *FLT3*-ITD [194] in hematopoietic lineage cells. Genome editing techniques such as CRISPR/Cas9 and TALEN have been used to generate mouse models containing AML specific mutations [195] or cytogenetic abnormalities [196, 197]. Genetically engineered mouse models have also been generated through adaptive transfer of hematopoietic cells that are virally expressing AML-associated fusion genes such as *MLL-ENL* [198], *MOZ-TIF2* [199] or *MLL-AF9* [200].

The *MLL-AF9* driven AML mouse model is well characterized and has been used extensively in this thesis. In this model, AML is initiated in committed GMPs through retroviral introduction of *MLL-AF9* and the leukemia cells can be serially propagated in mice. The transformed leukemia cells possess a gene expression signature associated with self-renewal indicating the progression of the disease from committed progenitor to LSCs. Moreover, a portion of the murine gene signature associated with self-renewal of leukemia cells is found in the human *MLL-AF9* rearranged AML [200]. Serial propagations of the leukemia cells in recipient mice results in reduced disease latency possibly due to the enrichment of leukemia initiating cells *in vivo* [201]. For example, transplantation of 1×10^6 quaternary transplant cells results in a fully developed AML along with splenomegaly in the mice in ~2-3 weeks. The short disease latency and the recapitulation of many of the AML characteristics makes it a suitable model for studying AML [200, 202].

Despite extensive efforts in the development of different AML mouse models, they do not completely recapitulate the complexity of human AML. Nevertheless, they are valuable in studying AML pathology and development of therapies for AML. With advancements in genome editing techniques, it is expected that newer mouse strains will be developed which better models the disease.

Clinical aspects

Diagnosis

Early symptoms of the early stages of AML include fever, lethargy, fatigue, frequent infections and unusual bleedings. Diagnosis of AML is performed based on morphology, immunophenotyping, cytogenetics and gene mutations in the cells according to the recommendations from the European leukemia network (ELN) [146]. Except for AML§ with t(15;17), t(8;21), inv(16)/t(16;16), a blood blast count of ≥20% is required for the diagnosis of AML. Immunophenotyping is performed

Table 3. Risk stratification of AML patients according to the underlying genetic abnormalities

Risk category	Genetic abnormality
Favorable	t(8;21)(q22;q22.1)
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22)
	Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low}
	Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD^{high}
	Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low}
	t(9;11)(p21.3;q23.3)
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1)
	t(v;11q23.3)
	t(9;22)(q34.1;q11.2)
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)
	−5 or del(5q); −7; −17/abn(17p)
	Complex karyotype, monosomal karyotype
	Wild-type *NPM1* and *FLT3*-ITD^{high}
	Mutated *RUNX1*
	Mutated *ASXL1*
	Mutated *TP53*

to assess the expression levels of specific lineage markers for establishing the diagnosis of AML and distinguishing it from mixed-phenotype acute leukemia [203]. Subsequently, cytogenetic analysis is performed to identify any of the recurrent chromosomal abnormalities. Additionally, screening for mutations in *NPM1*, *CEBPA*, *RUNX1*, *FLT3*, *TP53* and *ASXL1* genes is performed for establishing the subtype and the prognosis of the AML cases.

Risk stratification

According to the ELN recommendations [146], AML cases are risk stratified based on the underlying genetic abnormality and their response to standard therapy (**Table 3**). With increased numbers of AML genomes sequenced, patterns of co-occurring mutations have been identified which could be used to further refine the risk stratification. For example, co-occurrence of *FLT3*-ITD, *NPM1* and *DNMT3A* mutations has a worse prognosis compared to mutations in just *FLT3*-ITD and *NPM1*. Similarly, mutations in *IDH2* or *DNMT3A* do not by themselves have prognostic information, but their co-occurrence results in a worse prognosis [144].

Therapies

Although AML is a heterogenous disease with several molecular subtypes, the treatment regimen is surprisingly similar involving induction chemotherapy followed by consolidation chemotherapy for the majority of the patients. Colloquially termed "7+3" regimen, the induction therapy involves administration of cytarabine for 7 days along with short infusions of an anthracycline, usually daunorubicin on each of the first 3 days [204]. While the use of such high doses of chemotherapy is aimed at achieving complete remission (less than 5% blasts in the bone marrow) by targeting rapidly dividing AML blast cells, it also exerts toxic effects on normal cells and is therefore not suitable for elderly patients. In such patients, non-intensive treatments such as hypomethylating agents, for example, azacytidine, low dose cytarabine or cytostatic drugs such as hydroxyurea are administered [146].

In the absence of additional treatments, the great majority of patients relapse after achieving complete remission following induction therapy [205]. Therefore, appropriate post-remission consolidation therapy is necessary. Consolidation therapy usually involves administration of additional intermediate-dose chemotherapy and allogeneic hematopoietic stem cell transplantation (HSCT) i.e., transplantation of HSCs from the bone marrow of a healthy matching donor. Although allogenic HSCT has a strong anti-leukemic effect, it is associated high risk of non-relapse treatment related mortality due to graft versus host disease and is therefore recommended only for intermediate- and adverse- risk patients [146].

Even after receiving standard therapy, the relapse rate is ~50% in AML patients < 60 years of age and is as high as ~85% in older patients [206]. Such high relapse rates in AML is often attributed to the inability to target quiescent LSCs using standard chemotherapy. Moreover, AML cells may also acquire additional mutations that confer them with resistance to additional chemotherapy [127]. Other possible mechanisms by which AML cells acquire chemoresistance include increased anti-apoptotic signaling through upregulation of BCL2 [207] and drug efflux [208].

Apart from standard therapy, targeted therapies have been developed for specific subgroups of AML. The use of all-*trans*-retinoic acid (ATRA) and arsenic trioxide (ATO) in the treatment of patients diagnosed with acute promyelocytic leukemia characterized by the *PML-RARA* rearrangement has been highly effective achieving a remission rate of >90 % [209]. ATRA/ATO combination therapy degrades *PML-RARA* fusion protein and results in the differentiation of the leukemia cells [210]. Recently, several new targeted therapies have been approved for the treatment of AML patients. These include a drug conjugated anti-CD33 antibody (gemtuzumab ozogamicin), FLT3 inhibitors (midostaurin, gilteritinib), IDH1/IDH2 inhibitors (Enasidenib, Ivosidenib) and a BCL2 inhibitor (Venetoclax) [211]. Venetoclax in combination with hypomethylating agents and low dose cytarabine has been

approved for treating AML patients who are previously untreated and unfit for intensive chemotherapy. This combination has shown promising results for patients in this group who lack effective treatment options.

Immunotherapy

Successful usage of allogeneic HSCT to induce a graft versus leukemia effect shows that AML cells are susceptible targets of donor immune cells, specifically NK- and T-cells [212]. Autologous immune cells have also been shown to target AML cells when stimulated with cytokines [213]. Thus, it is clear that immune cells have the ability to target AML cells which is the fundamental principle behind immunotherapy. Immunotherapies can be based either on boosting the patient's own immune system with cytokines or vaccines or by conferring immunity by adoptive T-cell therapy, NK-cell therapy or by monoclonal antibodies.

Cytokines are key regulatory factors of leukemic cells and have also been shown to activate immune cells of AML patients. Administration of interleukin 2 (IL2) supplemented with histamine dihydrochloride (HDC) has been shown to activate NK- and T-cells and be efficacious in AML patients [214]. Interleukin 15 (IL15) has also been shown to enhance autologous NK-cell cytotoxicity in pre-clinical studies [215]. In **paper IV** of this thesis, we elucidate the complex regulatory role of interleukin 4 (IL4) on macrophages. Thus, it is clear that cytokines regulate immune cells and could potentially be developed as immunotherapy. Therefore, it is worth investigating the role of other cytokines in immune regulation.

Immune evasion in AML is mediated by the expression of cell surface proteins on the leukemia cells that binds to inhibitory receptors on immune cells. A classic example of this phenomenon is the upregulation of CD47 by AML cells which inhibits phagocytosis by providing a "don't eat me" signal to macrophages. Blocking of CD47 on AML cells using monoclonal antibodies has been shown to enhance phagocytosis of leukemia cells in pre-clinical studies [216].

AML cells can also be targeted by directing monoclonal antibodies against cell surface receptors that are upregulated selectively on AML cells. Gemtuzumab ozogamicin is a drug conjugated antibody targeting CD33 which is upregulated on AML cells. Upon binding to CD33, the antibody-drug conjugate is internalized by the leukemia cells resulting in cell death [217]. IL1RAP [218], CD123 [219], CLL-1 [220] and TIM-3 [221] are some of the other cell surface receptors that have been identified as therapeutic targets of AML using monoclonal antibodies. Upon binding the target cells, the monoclonal antibodies could elicit anti-leukemic activity by modulating the signaling mediated through the receptor as well as recruit immune cells through the fragment crystallizable (Fc) region leading to antibody dependent cell cytotoxicity (ADCC) [222]. Thus, the use of monoclonal antibodies has strong anti-leukemic effect due to the dual mechanism of inhibition of AML cells.

B-cell precursor acute lymphoblastic leukemia

Epidemiology

BCP-ALL is the most common type of pediatric cancer with about 75% of cases occurring between 2-5 years of age [223, 224]. In children younger than 14 years of age, the age-adjusted incidence of BCP-ALL in Sweden between 1989 and 2001 was 3.36 – 3.59/100,000 children with a slightly higher predominance in boys [225].

Classification

Based on the morphology of the leukemic blasts, ALL was first classified into L1, L2 and L3 subtypes according to FAB classification [136, 226]. A major limitation of this system of classification was that these subtypes did not distinguish BCP-ALL from T-ALL. Subsequently, the European group for the immunological characterization of leukemias (EGIL) proposed a revised classification of BCP-ALL into B-I (pro-B), B-II (common-B) and B-III (pre-B) ALL based on the immunophenotype corresponding to the differentiation stage of the leukemic blasts [227]. With the identification of a number of cytogenetic aberrations and gene mutations associated with BCP-ALL, WHO has proposed a molecular classification system based on recurrent genetic abnormalities (**Table 4**) [63]. Until recently, ~20% of the BCP-ALL cases were still unclassified due to the lack any known genetic aberration. Deep genomic characterization of these cases has led to the identification of several new oncogenic subtypes [228] inclusion of which would further refine the current WHO classification system.

Genetic abnormalities

The established molecular subtypes of BCP-ALL are defined by the presence of aneuploidies – high hyperdiploid (50-67 chromosomes) and hypodiploidy (less than 44 chromosomes) or fusion genes – *BCR-ABL1*, *IL3-IGH*, *ETV6-RUNX1* (also

Table 4. WHO classification of BCP-ALL

Category	Genetic abnormality
BCP-ALL with recurrent genetic abnormalities	
BCP-ALL with t(9;22)(q34.1;q11.2)	*BCR-ABL1* fusion gene
BCP-ALL with t(v;11q23.3)	*MLL*-rearranged fusion gene
BCP-ALL with t(12;21)(p13.2;q22.1)	*ETV6-RUNX1* fusion gene
BCP-ALL with hyperdiploidy	Hyperdiploidy
BCP-ALL with hypodiploidy	Hypodiploidy
BCP-ALL with t(5;14)(q31.1;q32.3)	*IL3-IGH* fusion gene
BCP-ALL with t(1;19)(q23;p13.3)	*TCF3-PBX1* fusion gene
BCP-ALL with *BCR-ABL1*–like	Translocations involving kinases - *ABL1, ABL2, PGFRB, NTRK3, TYK2, CSF1R, JAK2*
BCP-ALL with iAMP21	Amplification of a portion of chromosome 21 resulting in 5 or more copies of *RUNX1*
BCP-ALL not otherwise specified	

termed *TEL-AML1)*, *TCF3-PBX1* (also termed *E2A-PBX1*) or *MLL*-rearrangements. Additionally, two more subtypes - *BCR-ABL1*-like and iAMP21 have been provisionally included in the classification system [63]. Among the different molecular subtypes, high hyperdiploidy and *ETV6-RUNX1* together accounts for more than half of pediatric BCP-ALL cases while the other subtypes individually constitute less than 10% (**Figure 5**) [228].

The genetic aberrations in ALL have been shown to deregulate transcription, resulting in distinct gene expression patterns [229, 230]. For example, the t(12;21)(p13;q22) translocation characterized by the formation of the ETV6-RUNX1 fusion protein, alters the transcriptional profile by inhibiting the expression of RUNX1 target genes through the recruitment of a transcriptional co-repressor complex with histone deacetylase (HDAC) activity [231, 232]. This altered transcription profile affects the differentiation capacity of the cells as RUNX1 has been shown to be critical for normal hematopoiesis [233, 234]. Similarly, the t(1;19)(q23;p13.3) translocation characterized by the TCF3-PBX1 fusion protein results in the aberrant activation of PBX1 target genes [235]. Additionally, the fusion protein represses the expression of TCF3 target genes leading to uncontrolled cell cycle progression [236]. The genetic abnormalities of these established subtypes are assumed to be the initiating events that are accompanied by secondary genetic aberrations, which contribute to disease evolution and progression. These mutations occur in genes such as *IKAROS*, *PAX5*, *EBF1*, *ARIDB5*, *CEBPE*, *CDKN2A*, many of which encode key transcriptional regulators of B-cell development [237].
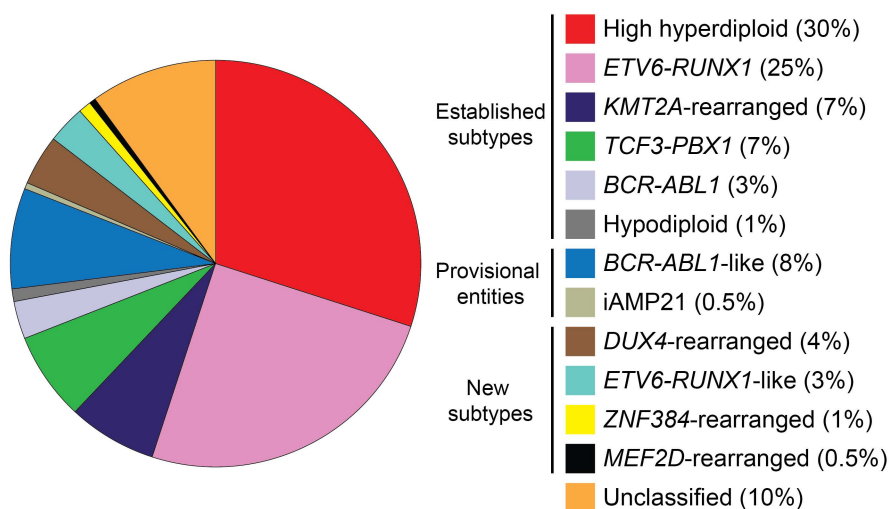
Figure 5. Molecular subtypes of pediatric BCP-ALL
Cytogenetic abnormalities are a hallmark of pediatric BCP-ALL and are used to stratify them into molecular subtypes with unique gene expression profiles. The data presented in the pie chart is adapted from Lilljebjörn et al [238].

Recent studies of BCP-ALL cases using high-resolution sequencing techniques have led to the identification of additional molecular subtypes with recurrent chromosomal rearrangements and distinct gene expression profiles [238-241]. Double-homeobox 4 (*DUX4*)-rearranged BCP-ALL is one of these new subtypes which constitutes ~ 4-5% of all pediatric BCP-ALL cases (**Figure 5**). As a newly identified subtype, *DUX4*-rearranged BCP-ALL is yet to be fully characterized.

DUX4-rearranged BCP-ALL

DUX4 is a transcription factor which is normally expressed during human embryonic development from oocyte to the 4-cell stage to initiate zygotic genome activation [242]. It is epigenetically silenced thereafter during the rest of the development and in somatic tissues. The reactivation of *DUX4* expression in skeletal muscle leads to the degradation of the muscle cells resulting in facioscapulohumeral muscular dystrophy (FSHD) [243]. Apart from BCP-ALL, *DUX4*-rearrangements resulting in aberrant expression of DUX4 have also been reported in Ewing-like sarcoma [244] and rhabdomyosarcoma [245].

DUX4 is present in 11-100 copies on each allele in the subtelomeric D4Z4 repeat region on chromosome 4q and 10q. The leukemia-initiating event in *DUX4*-rearranged BCP-ALL is a chromosomal translocation which leads to the insertion of *DUX4* within the *IGH* locus in chromosome 14. This results in the expression of

a chimeric IGH-DUX4 protein in which the C-terminus of DUX4 is truncated which leads to transcriptional deregulation and subsequent differentiation arrest in the late pro-B/pre-B-cell stage of B-cell development [246]. Although *IGH* translocations are common driver events in several malignancies of lymphoid lineage [247-249], the precise contribution of *IGH* locus in *DUX4*-rearranged BCP-ALL is currently unclear. Wild-type DUX4 is a transcription factor that contains a double-homeobox domain with transactivation capacity. Although IGH-DUX4 shares a similar DNA binding mechanism with the wild-type DUX4, the transactivation capacity of *IGH-DUX4* is markedly reduced due to the truncation of the C-terminal domain [250]. Consistent with this, IGH-DUX4 exhibits a distinct transcription profile compared to wild-type DUX4 [251].

A large number of *DUX4*-rearranged BCP-ALL cases also exhibit heterozygous intragenic ETS-related gene (*ERG)* deletions [238, 239]. *ERG* encodes a transcription factor that is essential for B-cell development and *ERG* deletions have previously been identified as a molecular subtype of BCP-ALL [252, 253]. Interestingly, IGH-DUX4 has also been shown to induce the expression of a C-terminal truncated isoform of ERG with a non-canonical transcription start site termed ERGalt. ERGalt acts as a dominant negative inhibitor of wild-type ERG and has the potential of leukemic transformation in mice [239]. Thus, ERG deregulation seems to cooperate with the *DUX4*-rearrangement in disease progression.

Models of BCP-ALL

Studies of BCP-ALL have been greatly facilitated by the establishment of cell lines derived from patient samples. More than 150 individual BCP-ALL cell lines have been reported in the literature [254]. These include cell lines such as MHH-CALL2, REH, 697, TOM1, SEM and NALM6 each representing one of the distinct molecular subtypes of BCP-ALL. Although the NALM6 cell line was established about four decades ago [255] and has been extensively studied as a pre-B ALL cell line, the underlying genetic abnormality, a *DUX4*-rearrangement, was identified only a few years ago [240]. The use of cell lines as models to study BCP-ALL has several advantages such as monoclonality, ease of manipulation and can be grown *in vitro* in large numbers. However, cell lines do not fully reflect the disease as their physiology, phenotype and other features are altered due to adaptation to *in vitro* growth conditions.

Apart from cell lines, *in vivo* mouse models provide insights into disease processes and act as preclinical platforms for testing of therapies. The classical approach to develop mouse models of different hematological malignancies is to overexpress the oncogene of interest in normal HSPCs of mice [256]. However, development of *in vivo* mouse models of BCP-ALL using this approach has largely been

unsuccessful. For example, mouse models generated by overexpression of *ETV6-RUNX1* in murine bone marrow cells lead to the accumulation of early progenitors in the B-cell compartment but do not result in leukemic transformation [257-259]. Notably, additional cooperating mutations caused due to low-dose irradiation along with the loss of *Cdkn2a* induced leukemic transformation [260]. These findings demonstrate that the expression of the *ETV6-RUNX1* fusion gene by itself is not sufficient for the development of BCP-ALL which is in line with the clinical observations [96]. Similarly, transgenic mice generated by the overexpression of *TCF3-PBX1* in lymphoid cells developed T-cell ALL instead of B-cell ALL [261]. Subsequently, two mouse models of *TCF3-PBX1* BCP-ALL have been developed either by expression of *TCF3-PBX1* specifically in B-cell lineage cells [262] or in bone marrow cells of T-cell deficient mice [263]. Additionally, mouse models for several other subtypes of BCP-ALL have been developed with varying levels of success [256]. One of the common features in the majority of these mouse models is the long latency periods with the expression of single leukemia associated aberration which makes the studies difficult and time-consuming. Engineering additional cooperating mutations in these models using advanced genome editing techniques could result in models with a more rapid manifestation of the disease.

Clinical aspects

Diagnosis

Common symptoms of BCP-ALL include B-symptoms (fever, weight loss, night sweats), easy bleedings, fatigue, dyspnea and infections [264]. Other symptoms include anemia, thrombocytopenia and leukopenia. The primary diagnostic criteria for BCP-ALL is the presence of 20% of lymphoblasts in the bone marrow during morphological analysis. Additionally, cytogenetic analysis is routinely performed to identify the common chromosomal abnormalities except for t(12;21)(p13;q22) translocation (*ETV6-RUNX1)* which is detected using fluorescence *in situ* hybridization (FISH) assay [140]. Eosinophilia in the peripheral blood is a marker of t(5;14)(q31;q32) (*IL3-IGH*) translocation [265] and is included in the diagnostic criteria of this subtype. Apart from this, cerebrospinal fluid analysis is performed to investigate central nervous system (CNS) involvement in the disease.

Risk stratification

Different ALL study groups around the world have risk stratified BCP-ALL patients into standard-risk, intermediate-risk and high-risk groups according to their specific criteria. Broadly, the risk stratification is based on major prognostic factors which

includes age, white blood cell (WBC) count, cytogenetic abnormalities, immunophenotype and treatment responses. Increasing age is associated with poor prognosis of BCP-ALL with patients over the age of 60 having poor long-term survival [266]. Similarly, patients with cytogenetic abnormalities such as hypodiploidy, *MLL*-rearrangement or *BCR-ABL1* are classified in the high-risk category due to their poor prognosis.

Therapies

The standard treatment of BCP-ALL is one of the most effective risk-based treatment regimens with high overall survival. Although there are small differences in the standard treatment protocols adopted by different ALL study groups, they broadly consist of a multidrug regimen that is administered in three phases – induction, consolidation and maintenance with an overall duration of about 2 to 3 years [178]. The drugs administered in the induction phase are vincristine, corticosteroids, asparaginase and anthracycline and lasts for 4-6 weeks. About 95% of the patients achieve complete remission following the completion of induction therapy. Allogenic HSCT is sometimes performed upon achieving complete remission. The consolidation phase usually includes a combination of chemotherapeutic drugs that are not used in the induction phase to minimize drug resistance. The duration and intensity of consolidation therapy depends on the risk group of the patients and usually lasts for 6 to 9 months. Maintenance chemotherapy is the final and the longest phase of standard treatment regimen lasting for about 2 years and involves administration of low intensity chemotherapy and anti-metabolite therapy. Additionally, therapy is directed towards the brain both as a prophylaxis and for patients with a clinical CNS disease. Despite achieving complete remission, about 10-20% of pediatric BCP-ALL patients relapse after standard therapy and the treatment options for relapsed BCP-ALL are limited [267].

In addition to the standard treatment, several targeted therapies have been developed for the treatment of BCP-ALL patients. Antibody-based immunotherapies - Rituximab [268], inotuzumab ozagomycin [269, 270] and blinatumomab [271] targeting CD20, CD22 and CD19 respectively have improved outcomes for specific groups of patients who do not respond to standard therapy. Other successful immunotherapeutic strategy in BCP-ALL is the development of chimeric antigen receptor (CAR) T-cells that combines the specificity provided by monoclonal antibodies and the cytotoxicity provided by engineered autologous T-cells. CAR T-cell therapy targeting CD19 has been highly effective in relapsed/refractory BCP-ALL patients with 70-97% of patients achieving complete remission [272].

CRISPR/Cas9 mediated genome engineering

Our ability to manipulate the DNA through genome engineering is instrumental in deciphering the functions of different coding genes and non-coding regions of the genome. Early efforts to edit the genome of eukaryotic cells was through homologous recombination [273, 274]. Although homologous recombination was a revolutionary technique of its time, it was labor-intensive, time consuming and had poor editing efficiencies. Subsequently, two programmable nucleases - zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN) were developed both of which include sequence-specific DNA-binding domains attached to a non-specific DNA cleavage module inducing targeted double-strand breaks [275, 276]. Although ZFNs and TALENs can be used to make precise genome editing, both these techniques have several disadvantages which include low editing efficiency, off-target effects, and high costs. The latest addition to the list of programmable nucleases is CRISPR/Cas9, which has revolutionized the field of genome engineering due to its high efficiency, low costs and being more user-friendly than previous technologies.

CRISPR/Cas – an adaptive immune system in prokaryotes

About three decades ago, short palindromic repeat sequences with unknown function were identified in several bacterial and archaea genomes [277-279]. Subsequently, these repeats were named as clustered regularly interspaced short palindromic repeats (CRISPR) and several CRISPR-associated (*Cas*) genes close to the CRISPR loci were identified [280]. Interestingly, the spacer sequences between the repeats were homologous to genetic elements in bacteriophages and conjugative plasmids [281]. The presence of these specific sequences in the genome of phages prevent them from infecting bacteria containing the corresponding spacer sequences [282]. A key study revealed the presence of an inheritable adaptive immune system in bacteria against phages by showing that the *Cas* genes mediated the degradation of the viral genome. The degraded protospacer DNA sequences were integrated as new spacer sequences in the CRISPR locus of the bacterial genome [283]. The

spacer units are expressed as short guide CRISPR RNAs (crRNA) which are used to silence invading pathogens containing foreign nucleic acids in a sequence specific manner [284].

Based on the sequences and organization of the CRISPR repeats and CRISPR/Cas loci, the CRISPR/Cas systems are classified into three major types; type I, II and III [285]. The type II CRISPR/Cas system exists exclusively in bacteria and is characterized by the presence of Cas9, a protein with endonuclease activity. Cas9 contains HNH and RuvC-like nuclease domains each of which cleaves one of the strands of DNA. In *Streptococcus pyogenes,* a trans-activating CRISPR RNA (tracrRNA) binds to mature crRNA through base pairing resulting in a duplex structure [286]. This RNA-duplex directs the Cas9 endonuclease to cleave the invading phage-derived DNA at specific sites. The target loci is determined by sequence complementarity between the target protospacer DNA and the crRNA and a motif adjacent to the protospacer referred as the protospacer adjacent motif (PAM) [287]. This results in the degradation of the target DNA thereby conferring *Streptococcus pyogenes* with immunity against certain phages.

CRISPR/Cas9 system as a genome editing tool

Successful engineering of the type II CRISPR/Cas system of *Streptococcus pyogenes* into an RNA programmable nuclease was a major innovation in the field of genome editing. This involved the fusion of the tracrRNA and customized crRNA to form a chimeric RNA termed single guide RNA (sgRNA), which provides specificity for the Cas9 nuclease to cleave the target DNA [287] (**Figure 6a**). Subsequently, the CRISPR/Cas9 system was applied on mammalian cells to create double strand breaks in the DNA in a site-specific manner [288, 289]. There are two major pathways for repairing double-strand DNA breaks – Non-homologous end joining (NHEJ) and Homology-directed repair (HDR) (**Figure 6b**). The usage of the two DNA repair pathways depends on the organism, cell type and phase of the cell cycle. NHEJ-mediated repair of double-strand break is more common in vertebrate cells compared to less complex eukaryotes [290]. The process of NHEJ is error-prone and can result in the introduction of insertions/deletions (indels). HDR mediated DNA repair can be used to perform more precise genome edits such as point mutations or insertion of pieces of DNA. This is achieved through recombination of the target locus with exogenous donor DNA template. Apart from Cas9, variants of the Cas12 nuclease has also been engineered for genome editing applications [291-293].
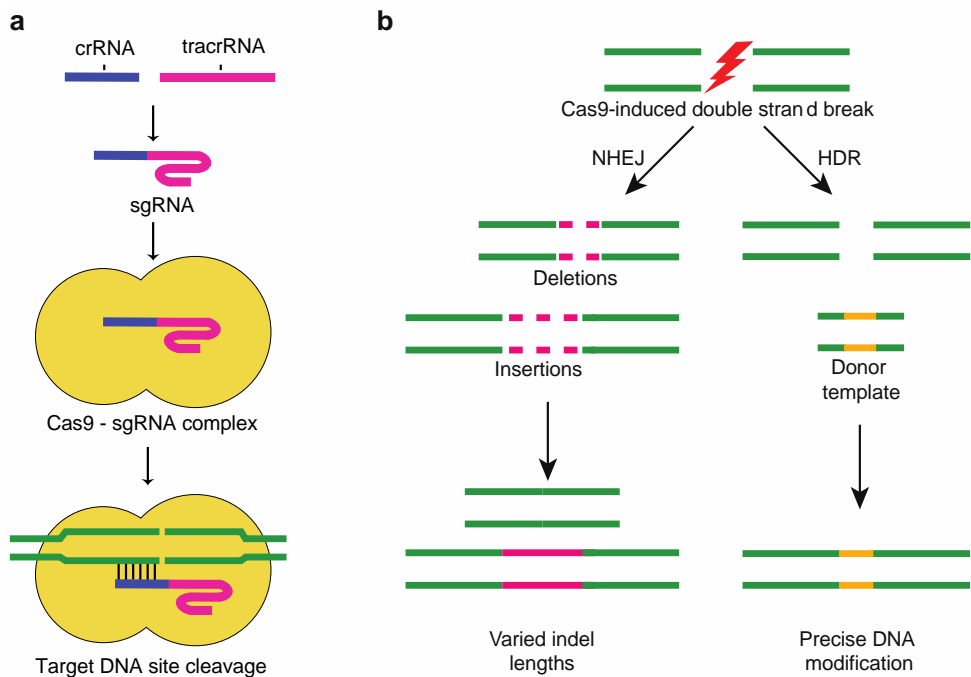
Figure 6. CRISPR/Cas9 genome editing tool
a) The CRISPR/Cas9 system has been engineered by the fusion of crRNA and tracrRNA resulting in a synthetic sgRNA which combines with the Cas9 protein to form the Cas9-sgRNA complex. In this complex, the sgRNA guides the Cas9 nuclease to the target the DNA site to induce a double strand break. **b)** NHEJ and HDR are the two DNA repair mechanisms that could be triggered following the cleavage by Cas9.

Applications of the CRISPR/Cas9 system

Due to the versatility and efficacy of the CRISPR/Cas9 system, it has a wide range of applications. The most common use of the CRISPR/Cas9 technology is the disruption of the open reading frame of coding genes thereby inhibiting their protein expression. In this process, random mutations are introduced as indels through NHEJ-mediated repair at the site of the double-strand break. Alternatively, specific mutations such as single nucleotide polymorphism (SNP) can be introduced through HDR-mediated repair. The CRISPR/Cas9 system can also be used to introduce large deletions or to engineer chromosomal rearrangements [196, 294] by simultaneous cleavage of DNA at two genomic locations. Apart from these, CRISPR/Cas9-mediated gene knockout can also be performed in a multiplexed manner in high-throughput loss-of-function screens [295, 296]. In this thesis, we have used the CRISPR/Cas9 technique both for simple gene knockout and to perform *in vivo* and *in vitro* functional screens (**Papers I**, **III**).

33

To utilize the DNA binding capacity of the CRISPR/Cas9 system without its nuclease activity, a mutant form of Cas9, termed dead-Cas9 (dCas9) was engineered, which lacks the ability to induce double-strand breaks [297]. Tethering of various protein effectors to dCas9 opened up a new set of applications for the CRISPR/Cas9 technique. These include modulation of gene expression by fusing dCas9 to transcriptional repression or activation domains [298]. CRISPR/dCas9 can also be used to induce epigenetic changes [299], study chromatin interactions [300] and imaging specific genomic loci [301] by tethering appropriate effector proteins to dCas9.

Functional screens

In humans and other complex organisms, biological function is determined at the cellular level through different biological processes including cell division, proliferation, differentiation, apoptosis, nutrient transport etc. These processes are mediated by different entities such as genes, proteins and other molecules. A major challenge in understanding the cellular function of these entities is to identify their roles in different biological processes. Functional screens are used to determine the effect of a number of entities in specific biological processes by assaying the change in phenotypes associated with these processes [302, 303]. As the read-out of a functional screen is based on the phenotype of interest, functional screens are often termed as phenotypic screens. In general, cell-based functional screens involve two main steps – i) cell perturbation and ii) assessment of change in phenotype.

Cell perturbation

In cell biology, perturbation refers to the alteration of the functioning of a cell through external means [304]. A common method of perturbation in functional screens is by treatment of cells with molecules such as chemical compounds, peptides and cytokines. For example, **in paper II**, we have performed a functional screen by treating murine AML cells with a library of cytokines. Alternatively, cell perturbation can also be achieved through modulation of gene expression which has been useful in establishing links between individual genes and specific biological phenomena [303]. The two common forms of genetic screens are gain-of-function and loss-of-function screens. Gain-of-function screens involve the ectopic expression of genes by introducing a library of corresponding cDNAs or open reading frames (ORF) into cells [305]. Loss-of-function genetic screens have traditionally been performed by inhibiting the expression of genes through RNA interference (RNAi). Although RNAi is a useful tool for inhibiting gene expression, it has major limitations in terms of its off-target effects and low knock-down efficiencies [306]. Currently, loss-of-function genetic screens are often performed using CRISPR/Cas9 genome editing technique due to its low off-target effects and higher inhibition of gene expression compared to RNAi. In line with this, we have performed CRISPR/Cas9 mediated loss-of-function screens in **papers I & III**.

Assessment of change in phenotype

The choice of the phenotype to be assessed depends on the type of cell perturbation and the biological question that is being addressed using the functional screens. Cell viability is the most common phenotype assessed in functional screens as it allows for the identification of entities that modulate cell growth. This strategy has been particularly effective in identifying active compounds or genes that are critical for the survival of cancer cells [307, 308]. **In papers I & III**, we have assessed cell viability as a measure of gene functionality. Cell viability could also be used as a surrogate phenotypic read-out to address more complex biological questions. For example, **in paper II**, we have established an *ex vivo* functional cytokine screen where the assessment of leukemia cell viability *in vivo* is a surrogate phenotypic read-out of the effect of cytokines on the leukemia initiating capacity of the cells. Other common phenotypes assayed in functional screens include proliferation, apoptosis, cell cycle, cell signaling and differentiation [303].

Arrayed vs pooled functional screens

Cell-based functional screens are typically performed either in an arrayed format or in a pooled format. In arrayed well-by-well format, a single known perturbation is applied to the cells in each well and the phenotype of interest is assayed in individual wells. Although it allows for the assessment of complex and multi-parametric read-outs, an arrayed format is usually expensive, labor intensive and requires specialized equipment for liquid and plate handling to increase reproducibility. By contrast, pooled screens are rapid, cost-effective and allows for high-throughput analysis [309]. In pooled screens, cell perturbations are performed using molecular barcoded reagents which allow for multiplexing of the assay (see *Barcoded-pooled samples*).

Molecular barcodes

One of the important aspects of any assay is the throughput it offers, i.e., the number of attributes that can be simultaneously measured in the assay. Advancements in automation of laboratory hardware such as robotic liquid handling systems have allowed for multiplexed approaches which increase the throughput of the assays thereby reducing the time and cost. Similarly, recent innovations with the molecular barcoding technology has enabled the application of molecular tags that greatly increase the throughput of assays with a molecular read-out [310]. The molecular tags act as unique identifiers which can be used to label individual cells, cell lineages, macromolecules and pooled samples. Commonly used molecular tags are either short DNA oligonucleotides detected by NGS or fluorochromes detected by their spectral emission. Due to the limitations of spectral overlap, fluorochromes offer lower multiplexing capabilities compared to DNA oligonucleotides.

Barcoding of individual cells

A high degree of cellular heterogeneity exists both in healthy and diseased tissues [311, 312]. However, most of our current genomic studies have focused on analyzing bulk tissue samples consisting of a large number of cells. Although effective in assessing the collective effects in these population-averaged datasets, cell-to-cell variations are difficult to resolve. Single-cell sequencing offers a solution to overcome this limitation and provides a better overview of the cellular heterogeneity. DNA barcoding has been instrumental in the development of high-throughput single cell sequencing assays. Advancements in droplet microfluidics technology allow for the capture and barcoding of individual cells in liquid drops [313]. This greatly increases the number of individual cells that can be analyzed simultaneously thereby improving throughput of the sequencing assays.

DNA barcoding has also improved the throughput of lineage tracing, i.e., identification of the progeny of the cells of interest. This has traditionally relied on methods such as direct observations through microscopy [314], radioactive labels [315], dyes [316] and fluorescent markers [317]. A major drawback of these methods is that only a limited number of lineages can be traced in parallel. This limitation has been overcome through molecular barcoding where insertion of unique DNA barcodes into the genome of the cells ensures that it will be transmitted

to the next generation after cell division. Lentiviral DNA barcodes have been used to study cell lineages in the hematopoietic system *in vivo* [318-320].

Barcoded macromolecules

Molecular barcoding techniques can be used to label macromolecules such as antibodies or chemical compounds. Conjugating antibodies to DNA barcodes enable the simultaneous measurement of transcription and translation of individual cells in methods such as cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) [321], RNA expression and protein sequencing (REAP-seq) [322] and cell hashing [323]. Here, barcoded antibodies bind to cell surface proteins and the cells are subsequently captured for scRNA-seq. The DNA barcodes attached to the antibody allows for the coupling of the immunophenotype to the transcriptome of individual cells.

Screening of chemical compound libraries is performed to identify binding molecules to protein targets of pharmaceutical interest. This process is limited by large library sizes and logistical issues. DNA-encoded chemical libraries (DECL) has enabled the screening of libraries consisting of millions of chemical compounds at moderate costs [324]. DECLs are collections of compounds that are individually conjugated to DNA barcodes thereby enabling multiplexing.

Barcoded pooled samples

Despite the significant reduction in the cost for NGS in recent years, it still remains an expensive assay. Molecular barcoding offers a multiplexing strategy wherein samples from different origins are barcoded and pooled prior to sequencing [325]. While processing the sequencing data, the origin of each sequencing read can be identified by its DNA barcode allowing the demultiplexing of the samples. This considerably reduces the sequencing cost and processing time per sample.

Molecular barcoding also allows for the development of pooled competitive cell growth assays. Pooled loss-of-function short hairpin RNA (shRNA) or CRISPR/Cas9 screens are classic examples of a molecular barcoded competitive cell growth assay [326]. Here, the cells are transduced with retro- or lentiviral shRNAs or sgRNAs which gets integrated in the genome and functions both as a means of genetic perturbation as well as a barcode. Thus, cells with unique molecular barcodes and genetic perturbations are made to compete with each other for cell growth. The change in representation of the barcodes (shRNA or sgRNA)

assessed by NGS is a measure of the effect of the corresponding genetic perturbation on cell growth.

Molecular barcoding could also be used to increase the throughput of functional screens involving non-genetic perturbations. For example, **in paper II**, we have assessed the *ex vivo* effect of a library of cytokines on the leukemia initiating capacity of AML cells *in vivo*. One of the technical challenges in addressing this biological question is that a large number of experimental animals are required to get a meaningful *in vivo* read-out. Therefore, we used molecular barcoding to multiplex the assay. Unlike lentiviral shRNA/sgRNA, cytokines cannot be used as a molecular barcode. Therefore, the classical pooled approach used in the genetic screens could not be applied. In order to overcome this limitation, we designed a hybrid functional screening assay by combining *ex vivo* cytokine stimulation of barcoded AML cells in an arrayed format with a competitive *in vivo* read-out of leukemia initiating capacity of AML cells in a pooled format. Through this multiplexing strategy, the effect of a large number of cytokines on AML cells could be assessed using a relatively small number of experimental animals.

Present investigation

Leukemia has been known to human-kind as a blood disorder since the times of the ancient Greeks in the 4th - 5th century BC [327]. Our understanding of leukemia in general and acute leukemia in particular has improved over time which can be classified in chronological order as the morphological era, the cytogenetic era and the molecular era. Each of these eras have been driven by major technological advancements. The advent of the microscope in the 17th - 18th century was the beginning of the morphological era which provided an opportunity to visually characterize the leukemia cells for the first time. The cytogenetic era commenced with the development of techniques such as karyotyping during the last century which allowed for the visualization of chromosomes. This facilitated the characterization of leukemia as a genetic disease and together with morphological data allowed for classification of the disease into distinct entities. The last two decades have heralded the dawning of the molecular era in which NGS technologies have been instrumental in characterizing the diversity of genetic aberrations in leukemia. Additionally, the recent development of an array of tools for genetic manipulation has revolutionized our ability to investigate the complex molecular regulation of leukemia cells.

As the acute forms of leukemia are associated with poor survival, especially among the elderly population, there is a need to identify new and more effective therapies. To achieve this, it is critical to decipher the molecular networks that sustain acute leukemia. In this thesis, we apply cutting-edge molecular tools such as CRISPR/Cas9 and molecular barcoding in functional screens combined with massively parallel sequencing to identify and characterize key molecular regulators of acute leukemia.

Objectives

The specific objectives of the individual studies are as follows:

Paper I - Identification of cell surface receptors critical for the survival of AML stem cells *in vivo.*

Paper II - Identification of cytokines that positively regulate AML stem cells.

Paper III - Identification of key dependencies of *DUX4*-rearranged BCP-ALL.

Paper IV - Deciphering the immune-dependent anti-leukemic role of IL4 in AML.

Results and Discussion

Paper I

CXCR4 Signaling Has a CXCL12-Independent Essential Role in Murine *MLL-AF9*-Driven Acute Myeloid Leukemia

AML is propagated by LSCs that reside in the bone marrow and are resistant to standard chemotherapy [93]. LSCs modulate the bone marrow microenvironment which in turn regulates the LSCs through signaling cues [174]. This interaction between the LSCs and the microenvironment is mediated by cell surface receptors on LSCs. In order to identify cell surface receptors that are biologically important for the survival of LSCs under physiological conditions, we performed a pooled *in vivo* CRISPR/Cas9 screen targeting 96 cell surface genes that are upregulated in murine *MLL-AF9* LSCs. We identified CXCR4 as the top positive cell surface regulator of AML cell growth and survival *in vivo*.

CXCR4 expression is critical for normal hematopoiesis and plays a key role in the maintenance of HSCs in the bone marrow niche [328]. Although high CXCR4 expression is associated with poor prognosis of AML patients [329], its functional role in AML has remained elusive [330, 331]. Therefore, we selected CXCR4 for follow-up studies. CRISPR-mediated disruption of *Cxcr4* resulted in a strong depletion of murine *MLL-AF9* AML cells *in vivo* but only had a mild effect on the cells *in vitro*. Interestingly, there was no negative effect in the homing of the AML cells to the bone marrow due to the loss of CXCR4 expression as CXCR4 has previously been shown to be critical for the homing of normal HSCs to the bone marrow [332]. However, loss of CXCR4 signaling resulted in the activation of p38 MAPK and NF-κB signaling pathways accompanied by increased oxidative stress and differentiation. This suggests that CXCR4 signaling is dispensable for homing of leukemia cells to the bone marrow but is essential for the maintenance of AML cells *in vivo*.

CXCL12 is the main ligand for CXCR4 and is expressed by several cell types in the bone marrow. Particularly, CXCL12 expression in endothelial cells and mesenchymal progenitor cells has been shown to be critical for the retention of HSPCs in the bone marrow [42, 333]. However, it is unclear whether CXCL12 also regulates AML cells in the bone marrow. Using transgenic mice with *Cxcl12* deletion either globally or specifically in endothelial cells and mesenchymal progenitor cells, we showed that CXCL12 expression in the bone marrow is dispensable for murine *MLL-AF9* AML development. These findings indicate that unlike normal HSPCs, AML cells are less dependent on these niches for disease development.

To further validate the role of CXCR4 signaling in AML development, we generated *Cxcr4* variants with mutations in codons critical either for down-stream signaling or for CXCL12 binding. Using these variants, we showed that CXCR4 signaling but not CXCL12 binding is critical for murine *MLL-AF9* AML development. Additionally, we showed that two other known ligands of CXCR4, MIF and UBIQUITIN, do not regulate AML cells in a CXCR4-dependent manner. This suggests that CXCR4 provides baseline signaling independent of ligand stimulation which supports AML development *in vivo.*

In conclusion, we performed an *in vivo* CRISPR/Cas9 screen targeting cell surface receptors and identified key dependencies of murine *MLL-AF9* AML cells. Our study reveals a critical role of CXCR4 signaling independent of CXCL12 stimulation in AML cells by protecting them from oxidative stress and differentiation (**Figure 7**).

In vivo CRISPR drop-out Screen

CRISPR sgRNA library

Functional selection

Cxcr4 identified as the top regulator of AML cells in vivo

CXCR4 signaling independent of CXCL12 is essential for AML development

CXCR4

MLL-AF9 AML cells

Oxidative stress
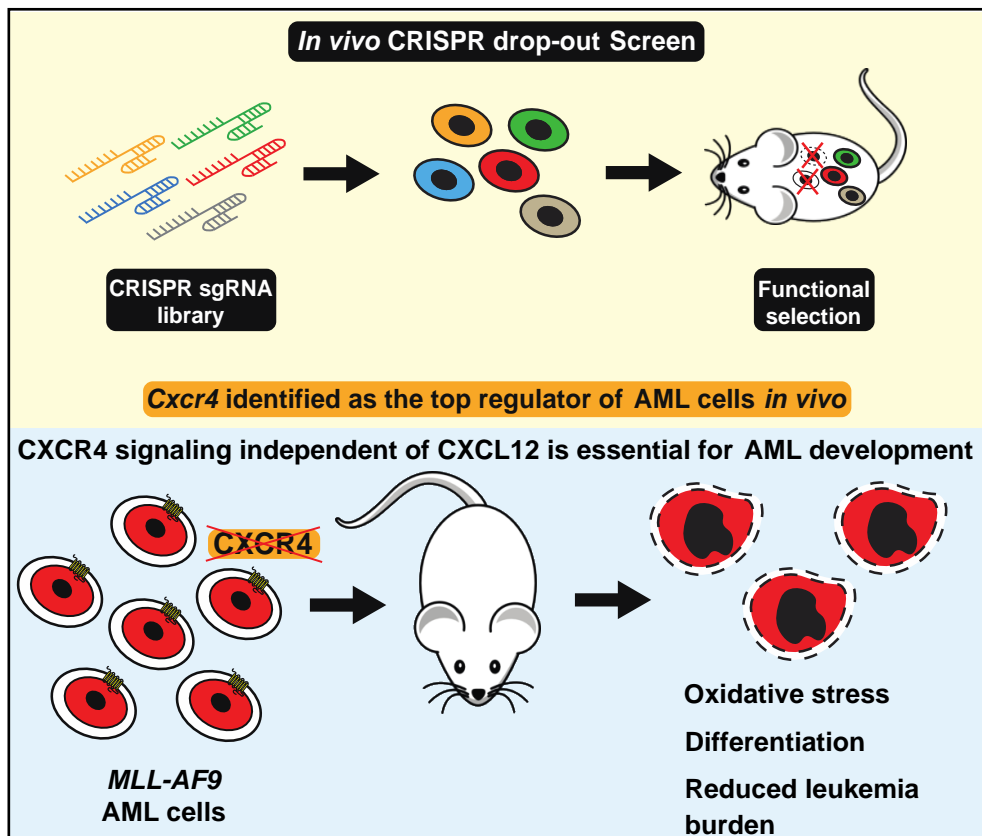
Differentiation

Reduced leukemia burden

Figure 7. Graphical abstract of paper I (adapted from Ramakrishnan et al., [334]).

Paper II

Arrayed molecular barcoding identifies TNFSF13 as a positive regulator of acute myeloid leukemia-initiating cells

One of the key characteristics of AML is the modification of the bone marrow niche. This includes the dysregulation of cytokines which contribute to the selective growth and survival of LSCs [174]. Cytokines such as CCL3, IL6, TGFβ have previously been shown to play critical roles in different hematological malignancies [335-337]. One of the challenges in screening for regulators of LSCs in the niche is the low-throughput of *in vivo* assays. Using arrayed molecular barcoding, we developed a high-throughput *ex vivo* cytokine screen which allowed us to assess the effect of multiple cytokines on the leukemia cells in a competitive manner with an *in vivo* read-out of leukemia-initiating capacity. With this approach, we studied the effect of 114 murine cytokines on the leukemia-initiating capacity of murine *MLL-AF9* AML cells and identified TNFSF13 as a top positive regulator of LSCs.

TNFSF13 has been shown to have a pro-tumor effect in several solid cancers [338-340] and B-cell malignancies [341-343]. Although elevated levels of TNFSF13 have been reported in AML patients [344], TNFSF13 has not been previously been associated with myelopoiesis or LSCs. Therefore, TNFSF13 was selected for follow-up studies. Measurement of TNFSF13 levels in healthy control and leukemic mice showed that although TNFSF13 was present at physiologically relevant levels in both the groups, it was secreted predominantly by normal myeloid cells and not by AML cells, suggesting that mature myeloid cells support murine *MLL-AF9* AML cell growth by secretion of TNFSF13. Characterization of the hematopoietic cells of *Tnfsf13$^{-/-}$* mice revealed a significant reduction in GMP cells, accompanied by lower numbers of monocytes. Notably, TNFRSF17, one of the receptors of TNFSF13, was highly expressed in monocytes relative to other hematopoietic progenitors in wild-type mice. Taken together, our data suggests that TNFSF13 supports normal myelopoiesis by binding to TNFRSF17.

Next, we assessed the role of extrinsic TNFSF13 in leukemia initiation and progression *in vivo* by retroviral overexpression of *MLL-AF9* in c-Kit$^+$ *Tnfsf13$^{-/-}$* bone marrow cells followed by serial transplantations into *Tnfsf13$^{+/+}$* or *Tnfsf13$^{-/-}$* recipient mice. We found a significant reduction in the leukemia burden and increased survival in the *Tnfsf13$^{-/-}$* recipient group. TNFSF13 has previously been shown to elicit an anti-apoptotic effect in B-cell malignancies [345, 346]. Consistent with this, we also observed that TNFSF13 promoted the AML cells by suppressing apoptosis and promoting cell cycle progression. Collectively, our data suggests that TNFSF13 supports leukemia initiation and maintenance *in vivo*.

Additionally, TNFSF13 stimulated the proliferation of several human myeloid leukemia cell lines. Notably, TNFSF13 promoted monomac 6 (MM6) cells by suppressing apoptosis which was consistent with our observation in murine c-Kit$^+$

AML cells. The pro-leukemic effect of TNFSF13 on MM6 cells was mediated through its receptor TNFRSF17 and was dependent on the activation of the NF-κB signaling pathway. This is in line with studies showing that TNFSF13 activates NF-κB signaling in B-cell malignancies [342, 343]. Taken together, these findings demonstrate that TNFSF13 also supports the growth of human AML cells in an NF-κB dependent manner.

In summary, we developed an *ex vivo* screening technique using arrayed molecular barcoding and identified a previously unknown role of TNFSF13 as a positive regulator of AML cells (**Figure 8**). This screening methodology can be extended to other types of *ex vivo* screens where a multiplexed *in vivo* read-out of stem-cell functionality is required.
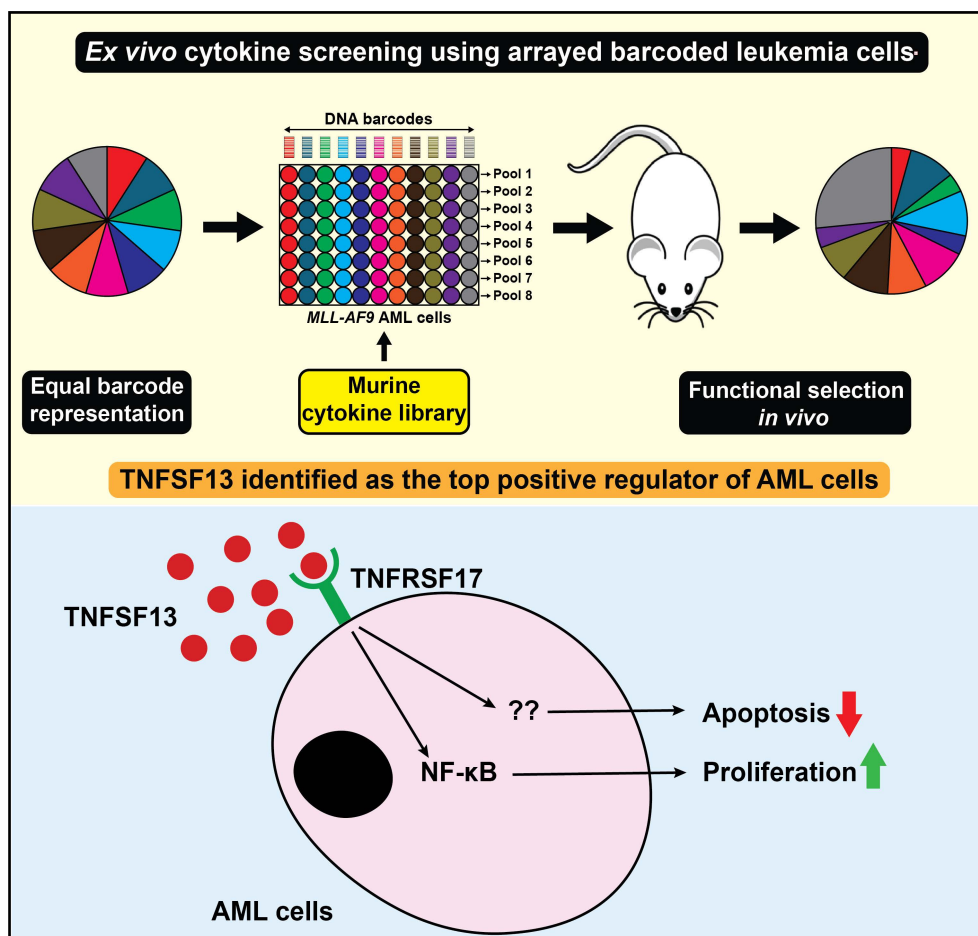

Figure 8. Graphical abstract of paper II.

Paper III

A genome-wide CRISPR screen identifies key regulators of DUX4-rearranged BCP-ALL

DUX4-rearranged BCP-ALL is characterized by the aberrant expression of DUX4 in B-cell progenitors, leading to leukemic transformation [238-240]. As a newly identified subtype of BCP-ALL, the molecular mechanisms by which DUX4 drives leukemia progression is currently unknown. To identify key dependencies of *DUX4*-rearranged BCP-ALL, we performed genome-wide CRISPR screens in the NALM6 cell line harboring the *IGH-DUX4* rearrangement along with two non-*DUX4*-rearranged BCP-ALL reference cell lines, 697 and REH. By comparing the depletion of sgRNAs in NALM6 relative to 697 and REH, we generated a ranked gene list of positive regulators of *DUX4*-rearranged BCP-ALL.

To identify the genes regulated by DUX4 among the top hits in the screen, we silenced *DUX4* expression in NALM6 cells using shRNA and performed RNA sequencing. Among the top ranked genes in the screen, we identified *FNIP1*, *IRF4* and *SYNCRIP* as differentially expressed. Notably, *FNIP1* and *IRF4* were upregulated due to the knock-down of *DUX4* suggesting that DUX4 either directly or indirectly inhibits these genes. In contrast, *SYNCRIP* expression was downregulated upon *DUX4* knock-down, indicating that DUX4 is a positive regulator of *SYNCRIP*.

FNIP1 is a metabolic regulator which has also been shown to be critical for B-cell development [347, 348]. Transcriptome analysis of *FNIP1*-disrupted NALM6 cells revealed an enrichment of gene sets associated with mTOR, FOXO and ROS signaling pathways, all of which are associated with cellular metabolism [349-351]. We also observed an enrichment of genes related to the B-cell receptor signaling pathway. Collectively, these findings suggest that loss of *FNIP1* results in metabolic dysregulation and affects B-cell signaling in *DUX4*-rearranged BCP-ALL cells.

IRF4 is a transcription factor that is a well-known regulator of lymphopoiesis [352, 353] and has previously been associated with CLL and multiple myeloma [354, 355]. Disruption of *IRF4* resulted in upregulation of genes associated with apoptosis, the ROS signaling pathway and B-cell differentiation, suggesting that *IRF4* regulates multiple functions in the leukemia cells that are critical for their survival.

SYNCRIP is a ubiquitously expressed RNA-binding protein that plays a critical role in RNA processing [356, 357]. We observed a downregulation of genes associated with RNA processing and protein translation upon loss of *SYNCRIP* expression in NALM6 cells. In addition, we observed a downregulation of the TGFβ-SMAD signaling pathway. SYNCRIP has previously been shown to interact with MSI2 and regulate AML stem cells [358], as well as positively regulate the expression of TGFβR1 and SMAD3 in hematopoietic stem cells [359]. Hence, we speculate that

DUX4 induces SYNCRIP expression that interacts with MSI2 leading to activation of the TGFβ-SMAD signaling pathway, which could be responsible for the block in B-cell differentiation.

Taken together, we have performed a genome-wide CRISPR screen and identified *FNIP1*, *IRF4* and *SYNCRIP* as key regulators of *DUX4*-rearranged BCP-ALL (**Figure 9**). Further studies are ongoing to validate these findings at the protein level and in primary patient samples.
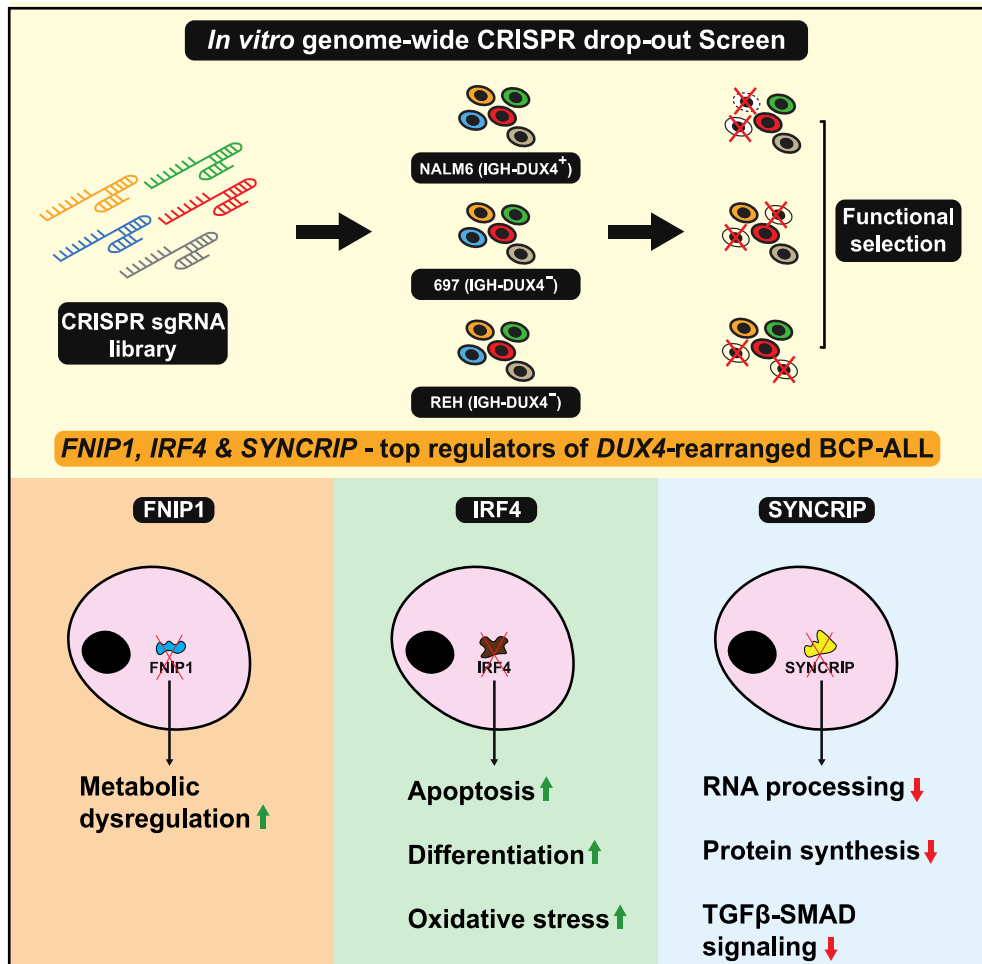

Figure 9. Graphical abstract of paper III.

Paper IV

IL4 has a dual role in regulating phagocytosis of murine leukemia cells

IL4 is a pleiotropic cytokine which has been shown to have both pro- and anti-tumor effects and its precise role in cancer is unclear [360]. In a cytokine screen, we previously identified IL4 as a negative regulator of AML that induces apoptosis in the leukemia cells [361]. As IL4 is also known to regulate several immunological processes [362, 363] under physiological conditions, we hypothesized that the anti-leukemic effect of IL4 is partially mediated by immune cells.

To test this hypothesis, we transplanted *MLL-AF9* AML cells overexpressing IL4 into immunocompetent wild-type mice and two strains of immunodeficient recipient mice, NOD/SCID and NSG. We observed an increase in leukemia burden in the immunodeficient mice, indicating a critical role for immune cells in the anti-leukemic effect of IL4. Interestingly, the immunocompetent mice transplanted with constitutive IL4-expressing AML cells also died despite having low levels of leukemia cells in the bone marrow. In addition, we observed an expansion of F4/80[+] macrophages in the bone marrow and spleen of these mice and depletion of macrophages *in vivo* resulted in the elimination of the antileukemic effect of IL4. Notably, the macrophage expansion was accompanied by a reduction in white blood cells, red blood cells and platelets in the peripheral blood. Constitutive expression of IL4 in mice has previously been associated with excessive phagocytosis, decreased blood cell counts and increased mortality [364, 365]. Combined, these findings suggest that although elevated IL4 levels reduce the leukemia burden by activation of macrophages, it also triggers excessive phagocytosis resulting in bone marrow failure.

Next, we studied the effect of IL4 on macrophages by performing RNA sequencing of macrophages with or without IL4 stimulation both *in vitro* and *in vivo*. We observed that IL4 stimulation resulted in the upregulation of several genes associated with activation of macrophages derived from monocytes that is distinct from tumor associated macrophages (TAM). While TAMs which are classically associated to an M2 phenotype are known to suppress the immune system and thereby have an anti-tumor effect, other types of macrophages play a critical role in tumor immune surveillance through phagocytosis [366]. In addition, gene signatures associated with phagocytosis, inflammation and immune activation were enriched in macrophages harvested from mice in the IL4 group. Consistent with these findings, we observed an increased phagocytosis due to IL4 stimulation of murine macrophages in an *in vitro* phagocytosis assay. However, stimulation of human macrophages with human IL4 resulted in reduced phagocytosis of human AML cell lines. This result is in line with previous findings showing that IL4 differentiated human monocytes into anti-inflammatory, alternatively activated macrophages [367]. Thus, our data indicates that while IL4 stimulation boosts murine

phagocytosis due to the expansion of monocyte-derived macrophages, it suppressed phagocytosis by human macrophages.

CD47 is the main inhibitory receptor for macrophages and its upregulation protects AML cells from phagocytosis [368]. Since IL4 stimulation resulted in selective elimination of AML cells by macrophages through phagocytosis, we hypothesized that IL4 downregulates the expression of CD47 on AML cells. In contrast, we found that IL4 induces CD47 expression on AML cells in a STAT6 dependent manner. The IL4-mediated upregulation of CD47 resulted in reduced phagocytosis of AML cells. Interestingly, IL4 also upregulated *CD47* transcript expression on normal c-Kit[+] bone marrow cells, suggesting that this is a regulatory mechanism that possibly has evolved to protect endogenous cells from phagocytosis in environments with high IL4 levels. Consistent with our finding, blocking of CD47 on AML cells combined with IL4 stimulation of macrophages resulted in increased phagocytosis of AML cells.

In summary, we show that IL4 has opposing roles in murine phagocytosis with direct activation of macrophages thereby boosting phagocytosis while also upregulating CD47 expression on AML cells thereby inhibiting phagocytosis (**Figure 10**).
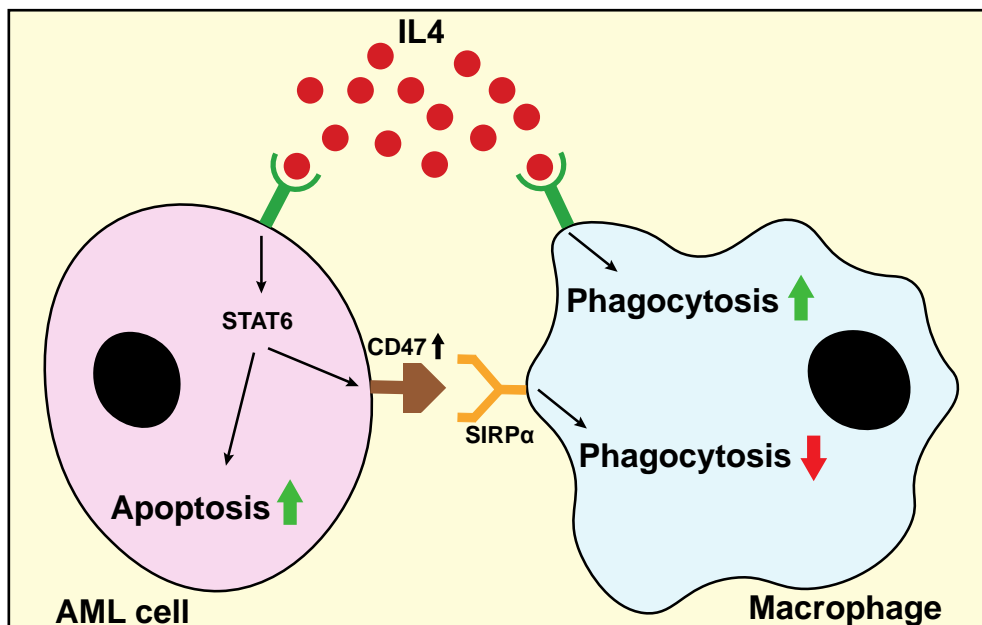

Figure 10. Graphical abstract of paper IV.

Conclusions and future perspectives

Hematological malignancies are driven by different genetic aberrations which alter the normal biology of blood cells resulting in their uncontrolled growth. Identification and characterization of factors that regulate leukemia cells in a physiological context contributes towards a better understanding of their altered biology. In this thesis, we have employed advanced molecular techniques and identified several key regulators of acute leukemia.

The CXCR4-CXCL12 axis is an example of a tumor-microenvironment interaction that is critical in several types of cancers [369] and has been assumed to be important for AML biology as well. However, we have uncovered a previously unknown critical role for CXCR4-signaling independent of CXCL12 in the maintenance of AML stem cells *in vivo* (**Paper I**). These findings suggest that therapeutic strategies targeting CXCR4 signaling in AML cells could be more effective than just the disruption of the CXCR4-CXCL12 axis. A major limitation of this study is that the experiments were performed only using the *MLL-AF9* AML mouse model. It remains to be studied whether our findings also extend to other types of AML.

In **papers II & IV**, we deciphered important roles of the cytokines TNFSF13 and IL4 in the regulation of AML cells. Although we demonstrated that TNFSF13 positively regulates AML cells in a NF-κB dependent manner, we observed that TNFSF13 is also required for normal myelopoiesis. This indicates that there could be challenges in the development of anti-TNFSF13 therapies. However, elevated serum levels of TNFSF13 have been reported in AML patients and shown to be associated with chemoresistance of AML cells [344, 370]. Therefore, TNFSF13 could potentially be utilized as a prognostic marker in AML. For IL4, we revealed the complex regulatory role it plays on murine AML cells and macrophages. IL4 induces murine phagocytosis of AML cells through macrophage activation resulting in a negative regulation of AML cells. At the same time, IL4 inhibits murine phagocytosis by upregulating the expression of CD47 in AML cells resulting in a positive regulation of AML cells. The net effect of IL4 on AML cell survival is determined by the equilibrium of these opposing signals. In a previous study, we have shown that IL4 also induces apoptosis in both murine and human AML cells [361]. Here, we find that while IL4 activates murine macrophages which are the main effector cells of its anti-leukemic activity, it inhibits human macrophages. Therefore, the therapeutic potential of IL4 in AML patients is limited. Nevertheless, the concept of using cytokines that activate macrophages combined with the inhibition of immune checkpoint proteins such as CD47 could potentially be developed as a therapeutic approach for leukemia.

The ongoing work in **paper III** aims to the characterize the dependencies of *DUX4*-rearranged BCP-ALL. Identification of FNIP1 and IRF4 as regulators of *DUX4*-rearranged BCP-ALL is of particular interest as both have been shown to be critical

for normal B-cell development. However, transcription of these genes is also inhibited by DUX4 suggesting that the expression levels of FNIP1 and IRF4 need to be at a moderate level for the maintenance of *DUX4*-rearranged BCP-ALL. In contrast, we demonstrate that DUX4 positively regulates SYNCRIP in *DUX4*-rearranged BCP-ALL and therefore is either a direct or indirect downstream target of DUX4 that drives leukemia. Further studies are ongoing to explore the mechanism of action of these genes in *DUX4*-rearranged BCP-ALL. Given that DUX4 is a transcription factor with potential binding sites to the regulatory regions of a large number of genes, there are probably multiple pathways by which DUX4 drives BCP-ALL. It is possible that some of the genes regulated by DUX4 are also critical for other types of BCP-ALL. However, these genes could not be identified in this study as the hits from the CRISPR screen were filtered to identify genes specific for the survival of *DUX4*-rearranged BCP-ALL.

The unifying aspect of **papers I-III** in this thesis is the use of high-throughput functional screens featuring CRISPR/Cas9 and molecular barcoding technologies on leukemia cells to identify factors that are critical either for the growth and survival of leukemia cells. The read-out of these screens was based on the change in growth and survival of the cells being assayed. Although effective, this screening strategy does not take into consideration the cellular heterogeneity. Moreover, the initial screens do not provide information about the mechanistic basis for the functional role of the identified factors. With the advancements in single-cell genomics, it is now possible to perform high-throughput functional screens at single cell resolution with a multi-modal read-out. One such innovation is the recently developed screening method termed Expanded CRISPR-compatible CITE-seq (ECCITE-seq) which combines the CRISPR/Cas9 technology with cell hashing, scRNA-seq and CITE-seq techniques [371]. Here, the read-out includes both the transcriptome and cell surface proteins of the assayed single cells, making it a powerful technique to address interesting research questions. For example, therapies have been developed targeting immune checkpoints on the surface of cancer cells. However, the genes and the regulatory networks that control the expression of these genes is currently unclear. Through ECCITE-seq, it would be possible to screen for the genes that modulate the expression of the immune checkpoint genes using the read-out at the transcript and protein level. Apart from this, advanced molecular barcoding techniques could also be used to perform other phenotypic screens with multi-modal read-outs.

In conclusion, this thesis demonstrates the power of high-throughput functional screens to identify key regulators of leukemia cells. The new knowledge generated through this work enhances our understanding of the vulnerabilities of acute leukemia, findings that may translate into new therapies.

Acknowledgements

ఎందరో మహానుభావులు అందరికీ వందనములు

-    త్యాగరాజు

There are so many great people, salutations to all of them

-    Saint Thyagaraju[2]

My PhD years have truly been an enriching life experience. I take this opportunity to express my heartfelt gratitude to everyone who have helped me in this magnificent journey.

First and foremost, I would like to thank my supervisor, Marcus. You are an inspiring scientist, an epitome of positivity and a great human being. I cherish the freedom and the space you have provided me to grow as a researcher. I am truly blessed to have worked with you.

My co-supervisor, Thoas, for accepting me as a shared PhD student and letting me be part of an exciting project. You have inspired me to always look at the big picture and not be afraid of trying new things.

Our collaborators in Sweden, Jonas Larsson and David Yudovich for your contributions in this thesis.

Our collaborators in USA, Ravi Bhatia and Puneet, for being gracious hosts when I visited your lab.

My team, it has been great working with such wonderful people. Pablo, I truly respect your scientific mind and temperament and have no doubts that you will become an excellent scientist. Thank you for all the help throughout these years. Maria, you bring so much warmth to our group and always ready to help. Hope you have enjoyed the first half of your PhD; but the best is yet to come. Somadri, a short conversation with you can cheer anyone up even on a rough day. Thank you for spreading the joy around. Kishan, thanks for helping me out when my hands were full. Katrin, I am amazed by the amount of work you do for all of us. You are a rare find! Marion, you have always been so nice to me even though I was competing with you to get recruited by Marcus ☺. I treasure the memories of all the fun we have had, especially when I "saved" you and Helene from the moose. Calle H, Mia, thank you for being a great support during the start of my PhD. You really made me feel at home in those initial days. I would also like to specially thank everyone in our team for helping me out with mouse work. And all the mice that were sacrificed for this thesis.

Fioretos group, collaborating with you all has been a great pleasure and a learning experience for me. Henrik, thank you for always being there to discuss and clarify my not-so-smart bioinformatic questions. Marianne, you are an endearing person and talking to you always makes me happy. Thank you for all your encouragement to improve my Swedish. Thanks to Maria Askmyr, Helena Å, Calle S, Noelia, Rasmus and Christina for all the help during these years.

Hagström group, Anna, your thorough approach towards science is inspiring. Thank you for all the guidance you have provided for my projects. Mattias, the deep conversations we have about almost

---

[2] Thyagaraju – 18th century Telugu poet/saint. He starts his famous poem "Entharo mahanubhavulu" with these iconic words.

anything under the sun are always stimulating. I can't wait to infect you with another earworm Tamil song. Also, thanks for being an unpaid advisor to me ☺. Ton, you have shown me that one could be a great researcher and have fun at the same time.

My fellow PhDs, Elsa, Axel, Niklas, Jakob, Sofia, Karim, Anders and Naveen, for all the fun we had making strange movies which somehow turned out to be funny. Thanks for being nice co-passengers in this journey. Saskia, start training for lundalöppet already! (P.S- I still run just once a year ☺). Hanna, Louise, Larissa, Natalie and Valeria, Good luck with your PhD and have fun while doing it.

Special thanks to all the technical staff and senior scientists, Linda M, Jenny N, Caroline J, Andrea, Eleanor, Minjun, Qirui, Helena S, Linda HM, Jenny K, Kristina, Geffroy. The labs won't be functioning without your support and Anette, for fixing everything. Thank you for being there for us.

Amazing PIs of Clinical Genetics. Together, you have created a rich environment to do great science. Kudos to all of you! Of course, thanks to Felix for being the shining light guiding us all.

My mentors before PhD, Kunal Mukhopadhyay, Rajib Bandopadhyay, Smrithi Srivastava from BIT Ranchi, Suresh Kumar Rayala, R. Baskar from IIT Madras, Kedarnath Sastry, Nagaraj Govindappa, Suma Srinivas, Nandini Pradeep, Aparajita Dubey from Biocon, Willem Voncken from Maastricht, Susana chuva desousa lopez from Leiden. Thanks to all of you that I have come this far.

All my friends here in Sweden and all around the world, thank you all for being there for me ☺

என் குடும்பத்திற்கு,

தந்தை மகற்காற்றும் நன்றி அவையத்து முந்தி இருப்பச் செயல் என்ற குறளுக்கு ஏற்ப படிக்க வைத்து என்னை முன்னேற்றிய அப்பாவுக்கு நன்றி. வெற்றி பெரும் ஒவ்வொரு ஆணுக்குப் பின்னாலும் ஒரு பெண் இருப்பாள் என்ற பழமொழி உண்டு. என் பின்னாலோ நான்கு பெண்கள். நான் பிறந்ததிலிருந்து இன்று வரை என்னை நேசித்து எந்நாளும் ஊக்கப்படுத்தும் அம்மாவுக்கு இந்தப் புத்தகம் சமர்ப்பணம். என் முன்னேற்றத்தில் அயராமல் அக்கறை செலுத்தும் ஜானகி அக்காவின் அன்பிற்கு ஈடு இணையே இல்லை.

மனைவி அமைவதெல்லாம் இறைவன் கொடுத்த வரம் என்பர். என்னுடைய வரம், என் மனைவி ஐஸ்வரியா! என்னை நிமிர்ந்து நிர்க்கச் செய்யும் வேர் நீ. உன் துணை இருந்தால் இன்னொரு PhD செய்யவும் தயங்க மாட்டேன். செய்யட்டுமா? ☺

இறுதியாக, என்னைக் கலி தீர்த்தே உலகில் ஏற்றம் புரிய வரும் என் சின்னஞ்சிறு கிளி! உன்னைக் காணக் கண் ஆயிரம் வேண்டாமோ!

கற்றதனால் ஆய பயனென்கொல் வாலறிவன்
நற்றாள் தொழாஅர் எனின்

- திருவள்ளுவர்

References

1. Jori A. Aristotle on the function of blood in the processes of life. Med Secoli. 2005;17(3):603-25.
2. Shoja MM, Tubbs RS, Loukas M, Ardalan MR. The Aristotelian account of "heart and veins". Int J Cardiol. 2008;125(3):304-10.
3. Hajdu SI. A note from history: The discovery of blood cells. Ann Clin Lab Sci. 2003;33(2):237-8.
4. Inra CN, Zhou BO, Acar M, Murphy MM, Richardson J, Zhao Z, et al. A perisinusoidal niche for extramedullary haematopoiesis in the spleen. Nature. 2015;527(7579):466-71.
5. Lefrancais E, Ortiz-Munoz G, Caudrillier A, Mallavia B, Liu F, Sayah DM, et al. The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. Nature. 2017;544(7648):105-9.
6. Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLoS Biol. 2016;14(8):e1002533.
7. Ogawa M. Differentiation and proliferation of hematopoietic stem cells. Blood. 1993;81(11):2844-53.
8. Morrison SJ, Uchida N, Weissman IL. The biology of hematopoietic stem cells. Annu Rev Cell Dev Biol. 1995;11:35-71.
9. Majeti R, Park CY, Weissman IL. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell Stem Cell. 2007;1(6):635-45.
10. Manz MG, Miyamoto T, Akashi K, Weissman IL. Prospective isolation of human clonogenic common myeloid progenitors. Proc Natl Acad Sci U S A. 2002;99(18):11872-7.
11. Galy A, Travis M, Cen D, Chen B. Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. Immunity. 1995;3(4):459-73.
12. Adolfsson J, Mansson R, Buza-Vidas N, Hultquist A, Liuba K, Jensen CT, et al. Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell. 2005;121(2):295-306.
13. Mansson R, Hultquist A, Luc S, Yang L, Anderson K, Kharazi S, et al. Molecular evidence for hierarchical transcriptional lineage priming in fetal and adult stem cells and multipotent progenitors. Immunity. 2007;26(4):407-19.
14. Gorgens A, Radtke S, Mollmann M, Cross M, Durig J, Horn PA, et al. Revision of the human hematopoietic tree: granulocyte subtypes derive from distinct hematopoietic lineages. Cell Rep. 2013;3(5):1539-52.
15. Sanjuan-Pla A, Macaulay IC, Jensen CT, Woll PS, Luis TC, Mead A, et al. Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. Nature. 2013;502(7470):232-6.
16. Yamamoto R, Morita Y, Ooehara J, Hamanaka S, Onodera M, Rudolph KL, et al. Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. Cell. 2013;154(5):1112-26.
17. Psaila B, Barkas N, Iskander D, Roy A, Anderson S, Ashley N, et al. Single-cell profiling of human megakaryocyte-erythroid progenitors identifies distinct megakaryocyte and erythroid differentiation pathways. Genome Biol. 2016;17:83.
18. Busch K, Klapproth K, Barile M, Flossdorf M, Holland-Letz T, Schlenner SM, et al. Fundamental properties of unperturbed haematopoiesis from stem cells in vivo. Nature. 2015;518(7540):542-6.
19. Sun J, Ramos A, Chapman B, Johnnidis JB, Le L, Ho YJ, et al. Clonal dynamics of native haematopoiesis. Nature. 2014;514(7522):322-7.
20. Zheng GX, Terry JM, Belgrader P, Ryvkin P, Bent ZW, Wilson R, et al. Massively parallel digital transcriptional profiling of single cells. Nat Commun. 2017;8:14049.
21. Dahlin JS, Hamey FK, Pijuan-Sala B, Shepherd M, Lau WWY, Nestorowa S, et al. A single-cell hematopoietic landscape resolves 8 lineage trajectories and defects in Kit mutant mice. Blood. 2018;131(21):e1-e11.

22. Tusi BK, Wolock SL, Weinreb C, Hwang Y, Hidalgo D, Zilionis R, et al. Population snapshots predict early haematopoietic and erythroid hierarchies. Nature. 2018;555(7694):54-60.

23. Kowalczyk MS, Tirosh I, Heckl D, Rao TN, Dixit A, Haas BJ, et al. Single-cell RNA-seq reveals changes in cell cycle and differentiation programs upon aging of hematopoietic stem cells. Genome Res. 2015;25(12):1860-72.

24. Olsson A, Venkatasubramanian M, Chaudhri VK, Aronow BJ, Salomonis N, Singh H, et al. Single-cell analysis of mixed-lineage states leading to a binary cell fate choice. Nature. 2016;537(7622):698-702.

25. Hay SB, Ferchen K, Chetal K, Grimes HL, Salomonis N. The Human Cell Atlas bone marrow single-cell interactive web portal. Exp Hematol. 2018;68:51-61.

26. Watcham S, Kucinski I, Gottgens B. New insights into hematopoietic differentiation landscapes from single-cell RNA sequencing. Blood. 2019;133(13):1415-26.

27. Rodriguez-Fraticelli AE, Wolock SL, Weinreb CS, Panero R, Patel SH, Jankovic M, et al. Clonal analysis of lineage fate in native haematopoiesis. Nature. 2018;553(7687):212-6.

28. Cabezas-Wallscheid N, Klimmeck D, Hansson J, Lipka DB, Reyes A, Wang Q, et al. Identification of regulatory networks in HSCs and their immediate progeny via integrated proteome, transcriptome, and DNA methylome analysis. Cell Stem Cell. 2014;15(4):507-22.

29. Paul F, Arkin Y, Giladi A, Jaitin DA, Kenigsberg E, Keren-Shaul H, et al. Transcriptional Heterogeneity and Lineage Commitment in Myeloid Progenitors. Cell. 2015;163(7):1663-77.

30. Laurenti E, Gottgens B. From haematopoietic stem cells to complex differentiation landscapes. Nature. 2018;553(7689):418-26.

31. Nestorowa S, Hamey FK, Pijuan Sala B, Diamanti E, Shepherd M, Laurenti E, et al. A single-cell resolution map of mouse hematopoietic stem and progenitor cell differentiation. Blood. 2016;128(8):e20-31.

32. Rieger MA, Schroeder T. Hematopoiesis. Cold Spring Harb Perspect Biol. 2012;4(12).

33. Spencer JA, Ferraro F, Roussakis E, Klein A, Wu J, Runnels JM, et al. Direct measurement of local oxygen concentration in the bone marrow of live animals. Nature. 2014;508(7495):269-73.

34. Crane GM, Jeffery E, Morrison SJ. Adult haematopoietic stem cell niches. Nat Rev Immunol. 2017;17(9):573-90.

35. Nombela-Arrieta C, Pivarnik G, Winkel B, Canty KJ, Harley B, Mahoney JE, et al. Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. Nat Cell Biol. 2013;15(5):533-43.

36. Acar M, Kocherlakota KS, Murphy MM, Peyer JG, Oguro H, Inra CN, et al. Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. Nature. 2015;526(7571):126-30.

37. Czechowicz A, Kraft D, Weissman IL, Bhattacharya D. Efficient transplantation via antibody-based clearance of hematopoietic stem cell niches. Science. 2007;318(5854):1296-9.

38. Barker JE. Sl/Sld hematopoietic progenitors are deficient in situ. Exp Hematol. 1994;22(2):174-7.

39. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity. 2006;25(6):977-88.

40. Tzeng YS, Li H, Kang YL, Chen WC, Cheng WC, Lai DM. Loss of Cxcl12/Sdf-1 in adult mice decreases the quiescent state of hematopoietic stem/progenitor cells and alters the pattern of hematopoietic regeneration after myelosuppression. Blood. 2011;117(2):429-39.

41. Qian H, Buza-Vidas N, Hyland CD, Jensen CT, Antonchuk J, Mansson R, et al. Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. Cell Stem Cell. 2007;1(6):671-84.

42. Ding L, Morrison SJ. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. Nature. 2013;495(7440):231-5.

43. Mendez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature. 2010;466(7308):829-34.

44. Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature. 2003;425(6960):841-6.

45. Kollet O, Dar A, Shivtiel S, Kalinkovich A, Lapid K, Sztainberg Y, et al. Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. Nat Med. 2006;12(6):657-64.

46. Yamazaki S, Ema H, Karlsson G, Yamaguchi T, Miyoshi H, Shioda S, et al. Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. Cell. 2011;147(5):1146-58.

47. Winkler IG, Sims NA, Pettit AR, Barbier V, Nowlan B, Helwani F, et al. Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. Blood. 2010;116(23):4815-28.

48. Bruns I, Lucas D, Pinho S, Ahmed J, Lambert MP, Kunisaki Y, et al. Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. Nat Med. 2014;20(11):1315-20.

49. Baldridge MT, King KY, Boles NC, Weksberg DC, Goodell MA. Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. Nature. 2010;465(7299):793-7.

50. O'Malley DP. Benign extramedullary myeloid proliferations. Mod Pathol. 2007;20(4):405-15.

51. Koch CA, Li CY, Mesa RA, Tefferi A. Nonhepatosplenic extramedullary hematopoiesis: associated diseases, pathology, clinical course, and treatment. Mayo Clin Proc. 2003;78(10):1223-33.

52. Kato H, Igarashi K. To be red or white: lineage commitment and maintenance of the hematopoietic system by the "inner myeloid". Haematologica. 2019;104(10):1919-27.

53. Wolff L, Humeniuk R. Concise review: erythroid versus myeloid lineage commitment: regulating the master regulators. Stem Cells. 2013;31(7):1237-44.

54. Hoppe PS, Schwarzfischer M, Loeffler D, Kokkaliaris KD, Hilsenbeck O, Moritz N, et al. Early myeloid lineage choice is not initiated by random PU.1 to GATA1 protein ratios. Nature. 2016;535(7611):299-302.

55. Grass JA, Boyer ME, Pal S, Wu J, Weiss MJ, Bresnick EH. GATA-1-dependent transcriptional repression of GATA-2 via disruption of positive autoregulation and domain-wide chromatin remodeling. Proc Natl Acad Sci U S A. 2003;100(15):8811-6.

56. Rieger MA, Schroeder T. Instruction of lineage choice by hematopoietic cytokines. Cell Cycle. 2009;8(24):4019-20.

57. Kondo M, Scherer DC, Miyamoto T, King AG, Akashi K, Sugamura K, et al. Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. Nature. 2000;407(6802):383-6.

58. Tsapogas P, Swee LK, Nusser A, Nuber N, Kreuzaler M, Capoferri G, et al. In vivo evidence for an instructive role of fms-like tyrosine kinase-3 (FLT3) ligand in hematopoietic development. Haematologica. 2014;99(4):638-46.

59. Hu D, Shilatifard A. Epigenetics of hematopoiesis and hematological malignancies. Genes Dev. 2016;30(18):2021-41.

60. Jeong M, Park HJ, Celik H, Ostrander EL, Reyes JM, Guzman A, et al. Loss of Dnmt3a Immortalizes Hematopoietic Stem Cells In Vivo. Cell Rep. 2018;23(1):1-10.

61. Steensma DP, Bejar R, Jaiswal S, Lindsley RC, Sekeres MA, Hasserjian RP, et al. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. Blood. 2015;126(1):9-16.

62. Jurlander J. Hematological Malignancies, Leukemias and Lymphomas. In: Schwab M, editor. Encyclopedia of Cancer. Berlin, Heidelberg: Springer Berlin Heidelberg; 2011. p. 1640-4.

63. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127(20):2391-405.

64. Swerdlow SH, Campo E, Pileri SA, Harris NL, Stein H, Siebert R, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. Blood. 2016;127(20):2375-90.

65. Murati A, Brecqueville M, Devillier R, Mozziconacci MJ, Gelsi-Boyer V, Birnbaum D. Myeloid malignancies: mutations, models and management. BMC Cancer. 2012;12:304.

66. Vannucchi AM, Harrison CN. Emerging treatments for classical myeloproliferative neoplasms. Blood. 2017;129(6):693-703.

67. Noor SJ, Tan W, Wilding GE, Ford LA, Barcos M, Sait SN, et al. Myeloid blastic transformation of myeloproliferative neoplasms--a review of 112 cases. Leuk Res. 2011;35(5):608-13.

68. Hochhaus A, Larson RA, Guilhot F, Radich JP, Branford S, Hughes TP, et al. Long-Term Outcomes of Imatinib Treatment for Chronic Myeloid Leukemia. N Engl J Med. 2017;376(10):917-27.

69. Steensma DP. Myelodysplastic Syndromes: Diagnosis and Treatment. Mayo Clin Proc. 2015;90(7):969-83.

70. Sperling AS, Gibson CJ, Ebert BL. The genetics of myelodysplastic syndrome: from clonal haematopoiesis to secondary leukaemia. Nat Rev Cancer. 2017;17(1):5-19.

71. Platzbecker U. Treatment of MDS. Blood. 2019;133(10):1096-107.

72. Meggendorfer M, Jeromin S, Haferlach C, Kern W, Haferlach T. The mutational landscape of 18 investigated genes clearly separates four subtypes of myelodysplastic/myeloproliferative neoplasms. Haematologica. 2018;103(5):e192-e5.

73. Kuykendall AT, Padron E. Treatment of MDS/MPN and the MDS/MPN IWG International Trial: ABNL MARRO. Curr Hematol Malig Rep. 2019;14(6):543-9.

74. Onaindia A, Medeiros LJ, Patel KP. Clinical utility of recently identified diagnostic, prognostic, and predictive molecular biomarkers in mature B-cell neoplasms. Mod Pathol. 2017;30(10):1338-66.

75. Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. N Engl J Med. 2006;354(2):166-78.

76. Iacobucci I, Mullighan CG. Genetic Basis of Acute Lymphoblastic Leukemia. J Clin Oncol. 2017;35(9):975-83.

77. Hunger SP, Mullighan CG. Acute Lymphoblastic Leukemia in Children. N Engl J Med. 2015;373(16):1541-52.

78. Gaynon PS, Angiolillo AL, Carroll WL, Nachman JB, Trigg ME, Sather HN, et al. Long-term results of the children's cancer group studies for childhood acute lymphoblastic leukemia 1983-2002: a Children's Oncology Group Report. Leukemia. 2010;24(2):285-97.

79. Bassan R, Hoelzer D. Modern therapy of acute lymphoblastic leukemia. J Clin Oncol. 2011;29(5):532-43.

80. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. N Engl J Med. 2004;350(15):1535-48.

81. Zhang X, Lv D, Zhang Y, Liu Q, Li Z. Clonal evolution of acute myeloid leukemia highlighted by latest genome sequencing studies. Oncotarget. 2016;7(36):58586-94.

82. Oshima K, Khiabanian H, da Silva-Almeida AC, Tzoneva G, Abate F, Ambesi-Impiombato A, et al. Mutational landscape, clonal evolution patterns, and role of RAS mutations in relapsed acute lymphoblastic leukemia. Proc Natl Acad Sci U S A. 2016;113(40):11306-11.

83. Lynch M. Rate, molecular spectrum, and consequences of human mutation. Proc Natl Acad Sci U S A. 2010;107(3):961-8.

84. Jaiswal S, Ebert BL. Clonal hematopoiesis in human aging and disease. Science. 2019;366(6465).

85. Genovese G, Kahler AK, Handsaker RE, Lindberg J, Rose SA, Bakhoum SF, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. N Engl J Med. 2014;371(26):2477-87.

86. Jaiswal S, Fontanillas P, Flannick J, Manning A, Grauman PV, Mar BG, et al. Age-related clonal hematopoiesis associated with adverse outcomes. N Engl J Med. 2014;371(26):2488-98.

87. Bowman RL, Busque L, Levine RL. Clonal Hematopoiesis and Evolution to Hematopoietic Malignancies. Cell Stem Cell. 2018;22(2):157-70.

88. Cancer Genome Atlas Research N, Ley TJ, Miller C, Ding L, Raphael BJ, Mungall AJ, et al. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N Engl J Med. 2013;368(22):2059-74.

89. Papaemmanuil E, Gerstung M, Malcovati L, Tauro S, Gundem G, Van Loo P, et al. Clinical and biological implications of driver mutations in myelodysplastic syndromes. Blood. 2013;122(22):3616-27; quiz 99.

90. Nangalia J, Massie CE, Baxter EJ, Nice FL, Gundem G, Wedge DC, et al. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. N Engl J Med. 2013;369(25):2391-405.

91. Couronne L, Bastard C, Bernard OA. TET2 and DNMT3A mutations in human T-cell lymphoma. N Engl J Med. 2012;366(1):95-6.

92. Corces-Zimmerman MR, Hong WJ, Weissman IL, Medeiros BC, Majeti R. Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. Proc Natl Acad Sci U S A. 2014;111(7):2548-53.

93. Shlush LI, Mitchell A, Heisler L, Abelson S, Ng SWK, Trotman-Grant A, et al. Tracing the origins of relapse in acute myeloid leukaemia to stem cells. Nature. 2017;547(7661):104-8.

94. Shlush LI, Zandi S, Mitchell A, Chen WC, Brandwein JM, Gupta V, et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. Nature. 2014;506(7488):328-33.

95. Ford AM, Bennett CA, Price CM, Bruin MC, Van Wering ER, Greaves M. Fetal origins of the TEL-AML1 fusion gene in identical twins with leukemia. Proc Natl Acad Sci U S A. 1998;95(8):4584-8.

96. Wiemels JL, Cazzaniga G, Daniotti M, Eden OB, Addison GM, Masera G, et al. Prenatal origin of acute lymphoblastic leukaemia in children. Lancet. 1999;354(9189):1499-503.

97. Yagi T, Hibi S, Tabata Y, Kuriyama K, Teramura T, Hashida T, et al. Detection of clonotypic IGH and TCR rearrangements in the neonatal blood spots of infants and children with B-cell precursor acute lymphoblastic leukemia. Blood. 2000;96(1):264-8.

98. Cazzaniga G, van Delft FW, Lo Nigro L, Ford AM, Score J, Iacobucci I, et al. Developmental origins and impact of BCR-ABL1 fusion and IKZF1 deletions in monozygotic twins with Ph+ acute lymphoblastic leukemia. Blood. 2011;118(20):5559-64.

99. Ford AM, Ridge SA, Cabrera ME, Mahmoud H, Steel CM, Chan LC, et al. In utero rearrangements in the trithorax-related oncogene in infant leukaemias. Nature. 1993;363(6427):358-60.

100. Hein D, Borkhardt A, Fischer U. Insights into the prenatal origin of childhood acute lymphoblastic leukemia. Cancer Metastasis Rev. 2020;39(1):161-71.

101. Mullighan CG, Goorha S, Radtke I, Miller CB, Coustan-Smith E, Dalton JD, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature. 2007;446(7137):758-64.

102. Greaves MF. Speculations on the cause of childhood acute lymphoblastic leukemia. Leukemia. 1988;2(2):120-5.

103. Kinlen LJ, Clarke K, Hudson C. Evidence from population mixing in British New Towns 1946-85 of an infective basis for childhood leukaemia. Lancet. 1990;336(8715):577-82.

104. Babor F, Manser AR, Fischer JC, Scherenschlich N, Enczmann J, Chazara O, et al. KIR ligand C2 is associated with increased susceptibility to childhood ALL and confers an elevated risk for late relapse. Blood. 2014;124(14):2248-51.

105. Burgler S, Nadal D. Pediatric precursor B acute lymphoblastic leukemia: are T helper cells the missing link in the infectious etiology theory? Mol Cell Pediatr. 2017;4(1):6.

106. Gill Super HJ, Rothberg PG, Kobayashi H, Freeman AI, Diaz MO, Rowley JD. Clonal, nonconstitutional rearrangements of the MLL gene in infant twins with acute lymphoblastic leukemia: in utero chromosome rearrangement of 11q23. Blood. 1994;83(3):641-4.

107. Andersson AK, Ma J, Wang J, Chen X, Gedman AL, Dang J, et al. The landscape of somatic mutations in infant MLL-rearranged acute lymphoblastic leukemias. Nat Genet. 2015;47(4):330-7.

108. Greaves M. When one mutation is all it takes. Cancer Cell. 2015;27(4):433-4.

109. Chopra M, Bohlander SK. The cell of origin and the leukemia stem cell in acute myeloid leukemia. Genes Chromosomes Cancer. 2019;58(12):850-8.

110. Abelson S, Collord G, Ng SWK, Weissbrod O, Mendelson Cohen N, Niemeyer E, et al. Prediction of acute myeloid leukaemia risk in healthy individuals. Nature. 2018;559(7714):400-4.

111. Castor A, Nilsson L, Astrand-Grundstrom I, Buitenhuis M, Ramirez C, Anderson K, et al. Distinct patterns of hematopoietic stem cell involvement in acute lymphoblastic leukemia. Nat Med. 2005;11(6):630-7.

112. Vicente-Duenas C, Romero-Camarero I, Cobaleda C, Sanchez-Garcia I. Function of oncogenes in cancer development: a changing paradigm. EMBO J. 2013;32(11):1502-13.

113. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature. 1994;367(6464):645-8.

114. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3(7):730-7.

115. Miyamoto T, Weissman IL, Akashi K. AML1/ETO-expressing nonleukemic stem cells in acute myelogenous leukemia with 8;21 chromosomal translocation. Proc Natl Acad Sci U S A. 2000;97(13):7521-6.

116. Weissman I. Stem cell research: paths to cancer therapies and regenerative medicine. JAMA. 2005;294(11):1359-66.

117. Goardon N, Marchi E, Atzberger A, Quek L, Schuh A, Soneji S, et al. Coexistence of LMPP-like and GMP-like leukemia stem cells in acute myeloid leukemia. Cancer Cell. 2011;19(1):138-52.

118. Ng SW, Mitchell A, Kennedy JA, Chen WC, McLeod J, Ibrahimova N, et al. A 17-gene stemness score for rapid determination of risk in acute leukaemia. Nature. 2016;540(7633):433-7.

119. le Viseur C, Hotfilder M, Bomken S, Wilson K, Rottgers S, Schrauder A, et al. In childhood acute lymphoblastic leukemia, blasts at different stages of immunophenotypic maturation have stem cell properties. Cancer Cell. 2008;14(1):47-58.

120. Rehe K, Wilson K, Bomken S, Williamson D, Irving J, den Boer ML, et al. Acute B lymphoblastic leukaemia-propagating cells are present at high frequency in diverse lymphoblast populations. EMBO Mol Med. 2013;5(1):38-51.

121. Elder A, Bomken S, Wilson I, Blair HJ, Cockell S, Ponthan F, et al. Abundant and equipotent founder cells establish and maintain acute lymphoblastic leukaemia. Leukemia. 2017;31(12):2577-86.

122. Merlo LM, Pepper JW, Reid BJ, Maley CC. Cancer as an evolutionary and ecological process. Nat Rev Cancer. 2006;6(12):924-35.

123. Davis A, Gao R, Navin N. Tumor evolution: Linear, branching, neutral or punctuated? Biochim Biophys Acta Rev Cancer. 2017;1867(2):151-61.

124. Bozic I, Antal T, Ohtsuki H, Carter H, Kim D, Chen S, et al. Accumulation of driver and passenger mutations during tumor progression. Proc Natl Acad Sci U S A. 2010;107(43):18545-50.

125. Greaves M, Maley CC. Clonal evolution in cancer. Nature. 2012;481(7381):306-13.

126. Landau DA, Carter SL, Getz G, Wu CJ. Clonal evolution in hematological malignances and therapeutic implications. Leukemia. 2014;28(1):34-43.

127. Ding L, Ley TJ, Larson DE, Miller CA, Koboldt DC, Welch JS, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature. 2012;481(7382):506-10.

128. Paguirigan AL, Smith J, Meshinchi S, Carroll M, Maley C, Radich JP. Single-cell genotyping demonstrates complex clonal diversity in acute myeloid leukemia. Sci Transl Med. 2015;7(281):281re2.

129. Li AH, Rosenquist R, Forestier E, Lindh J, Roos G. Detailed clonality analysis of relapsing precursor B acute lymphoblastic leukemia: implications for minimal residual disease detection. Leuk Res. 2001;25(12):1033-45.

130. Notta F, Mullighan CG, Wang JC, Poeppl A, Doulatov S, Phillips LA, et al. Evolution of human BCR-ABL1 lymphoblastic leukaemia-initiating cells. Nature. 2011;469(7330):362-7.

131. Gawad C, Koh W, Quake SR. Dissecting the clonal origins of childhood acute lymphoblastic leukemia by single-cell genomics. Proc Natl Acad Sci U S A. 2014;111(50):17947-52.

132. Welch JS, Ley TJ, Link DC, Miller CA, Larson DE, Koboldt DC, et al. The origin and evolution of mutations in acute myeloid leukemia. Cell. 2012;150(2):264-78.

133. Shallis RM, Wang R, Davidoff A, Ma X, Zeidan AM. Epidemiology of acute myeloid leukemia: Recent progress and enduring challenges. Blood Rev. 2019;36:70-87.

134. Juliusson G, Abrahamsson J, Lazarevic V, Antunovic P, Derolf A, Garelius H, et al. Prevalence and characteristics of survivors from acute myeloid leukemia in Sweden. Leukemia. 2017;31(3):728-31.

135. Juliusson G, Antunovic P, Derolf A, Lehmann S, Mollgard L, Stockelberg D, et al. Age and acute myeloid leukemia: real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry. Blood. 2009;113(18):4179-87.

136. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. Br J Haematol. 1976;33(4):451-8.

137. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Ann Intern Med. 1985;103(4):620-5.

138. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. Criteria for the diagnosis of acute leukemia of megakaryocyte lineage (M7). A report of the French-American-British Cooperative Group. Ann Intern Med. 1985;103(3):460-2.

139. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood. 2002;100(7):2292-302.

140. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009;114(5):937-51.

141. Grimwade D, Hills RK, Moorman AV, Walker H, Chatters S, Goldstone AH, et al. Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. Blood. 2010;116(3):354-65.

142. Patel JP, Gonen M, Figueroa ME, Fernandez H, Sun Z, Racevskis J, et al. Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. N Engl J Med. 2012;366(12):1079-89.

143. Dohner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. N Engl J Med. 2015;373(12):1136-52.

144. Papaemmanuil E, Gerstung M, Bullinger L, Gaidzik VI, Paschka P, Roberts ND, et al. Genomic Classification and Prognosis in Acute Myeloid Leukemia. N Engl J Med. 2016;374(23):2209-21.

145. Rowley JD. Identificaton of a translocation with quinacrine fluorescence in a patient with acute leukemia. Ann Genet. 1973;16(2):109-12.

146. Dohner H, Estey E, Grimwade D, Amadori S, Appelbaum FR, Buchner T, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 2017;129(4):424-47.

147. Warrell RP, Jr., de The H, Wang ZY, Degos L. Acute promyelocytic leukemia. N Engl J Med. 1993;329(3):177-89.

148. Goyama S, Mulloy JC. Molecular pathogenesis of core binding factor leukemia: current knowledge and future prospects. Int J Hematol. 2011;94(2):126-33.

149. Miyoshi H, Kozu T, Shimizu K, Enomoto K, Maseki N, Kaneko Y, et al. The t(8;21) translocation in acute myeloid leukemia results in production of an AML1-MTG8 fusion transcript. EMBO J. 1993;12(7):2715-21.

150. Melnick A, Carlile GW, McConnell MJ, Polinger A, Hiebert SW, Licht JD. AML-1/ETO fusion protein is a dominant negative inhibitor of transcriptional repression by the promyelocytic leukemia zinc finger protein. Blood. 2000;96(12):3939-47.

151. Hatlen MA, Wang L, Nimer SD. AML1-ETO driven acute leukemia: insights into pathogenesis and potential therapeutic approaches. Front Med. 2012;6(3):248-62.

152. Radtke I, Mullighan CG, Ishii M, Su X, Cheng J, Ma J, et al. Genomic analysis reveals few genetic alterations in pediatric acute myeloid leukemia. Proc Natl Acad Sci U S A. 2009;106(31):12944-9.

153. Micol JB, Duployez N, Boissel N, Petit A, Geffroy S, Nibourel O, et al. Frequent ASXL2 mutations in acute myeloid leukemia patients with t(8;21)/RUNX1-RUNX1T1 chromosomal translocations. Blood. 2014;124(9):1445-9.

154. Faber ZJ, Chen X, Gedman AL, Boggs K, Cheng J, Ma J, et al. The genomic landscape of core-binding factor acute myeloid leukemias. Nat Genet. 2016;48(12):1551-6.

155. Marcucci G, Mrozek K, Ruppert AS, Maharry K, Kolitz JE, Moore JO, et al. Prognostic factors and outcome of core binding factor acute myeloid leukemia patients with t(8;21) differ from those of patients with inv(16): a Cancer and Leukemia Group B study. J Clin Oncol. 2005;23(24):5705-17.

156. McMahon KA, Hiew SY, Hadjur S, Veiga-Fernandes H, Menzel U, Price AJ, et al. Mll has a critical role in fetal and adult hematopoietic stem cell self-renewal. Cell Stem Cell. 2007;1(3):338-45.

157. Jude CD, Climer L, Xu D, Artinger E, Fisher JK, Ernst P. Unique and independent roles for MLL in adult hematopoietic stem cells and progenitors. Cell Stem Cell. 2007;1(3):324-37.

158. Meyer C, Burmeister T, Groger D, Tsaur G, Fechina L, Renneville A, et al. The MLL recombinome of acute leukemias in 2017. Leukemia. 2018;32(2):273-84.

159. So CW, Lin M, Ayton PM, Chen EH, Cleary ML. Dimerization contributes to oncogenic activation of MLL chimeras in acute leukemias. Cancer Cell. 2003;4(2):99-110.

160. Erfurth F, Hemenway CS, de Erkenez AC, Domer PH. MLL fusion partners AF4 and AF9 interact at subnuclear foci. Leukemia. 2004;18(1):92-102.

161. Mueller D, Bach C, Zeisig D, Garcia-Cuellar MP, Monroe S, Sreekumar A, et al. A role for the MLL fusion partner ENL in transcriptional elongation and chromatin modification. Blood. 2007;110(13):4445-54.

162. Mohan M, Herz HM, Takahashi YH, Lin C, Lai KC, Zhang Y, et al. Linking H3K79 trimethylation to Wnt signaling through a novel Dot1-containing complex (DotCom). Genes Dev. 2010;24(6):574-89.

163. Yokoyama A, Lin M, Naresh A, Kitabayashi I, Cleary ML. A higher-order complex containing AF4 and ENL family proteins with P-TEFb facilitates oncogenic and physiologic MLL-dependent transcription. Cancer Cell. 2010;17(2):198-212.

164. Muntean AG, Tan J, Sitwala K, Huang Y, Bronstein J, Connelly JA, et al. The PAF complex synergizes with MLL fusion proteins at HOX loci to promote leukemogenesis. Cancer Cell. 2010;17(6):609-21.

165. Armstrong SA, Staunton JE, Silverman LB, Pieters R, den Boer ML, Minden MD, et al. MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. Nat Genet. 2002;30(1):41-7.

166. Li Z, Luo RT, Mi S, Sun M, Chen P, Bao J, et al. Consistent deregulation of gene expression between human and murine MLL rearrangement leukemias. Cancer Res. 2009;69(3):1109-16.

167. Wong P, Iwasaki M, Somervaille TC, So CW, Cleary ML. Meis1 is an essential and rate-limiting regulator of MLL leukemia stem cell potential. Genes Dev. 2007;21(21):2762-74.

168. Ley TJ, Mardis ER, Ding L, Fulton B, McLellan MD, Chen K, et al. DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. Nature. 2008;456(7218):66-72.

169. Tyner JW, Tognon CE, Bottomly D, Wilmot B, Kurtz SE, Savage SL, et al. Functional genomic landscape of acute myeloid leukaemia. Nature. 2018;562(7728):526-31.

170. Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med. 2005;352(3):254-66.

171. Kelly LM, Liu Q, Kutok JL, Williams IR, Boulton CL, Gilliland DG. FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model. Blood. 2002;99(1):310-8.

172. Mazumdar C, Shen Y, Xavy S, Zhao F, Reinisch A, Li R, et al. Leukemia-Associated Cohesin Mutants Dominantly Enforce Stem Cell Programs and Impair Human Hematopoietic Progenitor Differentiation. Cell Stem Cell. 2015;17(6):675-88.

173. Cazzola M, Della Porta MG, Malcovati L. The genetic basis of myelodysplasia and its clinical relevance. Blood. 2013;122(25):4021-34.

174. Schepers K, Campbell TB, Passegue E. Normal and leukemic stem cell niches: insights and therapeutic opportunities. Cell Stem Cell. 2015;16(3):254-67.

175. Kode A, Manavalan JS, Mosialou I, Bhagat G, Rathinam CV, Luo N, et al. Leukaemogenesis induced by an activating beta-catenin mutation in osteoblasts. Nature. 2014;506(7487):240-4.

176. Ge J, Hou R, Liu Q, Zhu R, Liu K. Stromal-derived factor-1 deficiency in the bone marrow of acute myeloid leukemia. Int J Hematol. 2011;93(6):750-9.

177. Tabe Y, Konopleva M. Role of Microenvironment in Resistance to Therapy in AML. Curr Hematol Malig Rep. 2015;10(2):96-103.

178. Almosailleakh M, Schwaller J. Murine Models of Acute Myeloid Leukaemia. Int J Mol Sci. 2019;20(2).

179. Kawasaki Y, Hirabayashi Y, Kaneko T, Kanno J, Kodama Y, Matsushima Y, et al. Benzene-induced hematopoietic neoplasms including myeloid leukemia in Trp53-deficient C57BL/6 and C3H/He mice. Toxicol Sci. 2009;110(2):293-306.

180. Peng Y, Borak TB, Bouffler SD, Ullrich RL, Weil MM, Bedford JS. Radiation leukemogenesis in mice: loss of PU.1 on chromosome 2 in CBA and C57BL/6 mice after irradiation with 1 GeV/nucleon 56Fe ions, X rays or gamma Rays. Part II. Theoretical considerations based on microdosimetry and the initial induction of chromosome aberrations. Radiat Res. 2009;171(4):484-93.

181. Cmarik J, Ruscetti S. Friend Spleen Focus-Forming Virus Activates the Tyrosine Kinase sf-Stk and the Transcription Factor PU.1 to Cause a Multi-Stage Erythroleukemia in Mice. Viruses. 2010;2(10):2235-57.

182. Theocharides AP, Rongvaux A, Fritsch K, Flavell RA, Manz MG. Humanized hemato-lymphoid system mice. Haematologica. 2016;101(1):5-19.

183. Cosgun KN, Rahmig S, Mende N, Reinke S, Hauber I, Schafer C, et al. Kit regulates HSC engraftment across the human-mouse species barrier. Cell Stem Cell. 2014;15(2):227-38.

184. Wunderlich M, Chou FS, Link KA, Mizukawa B, Perry RL, Carroll M, et al. AML xenograft efficiency is significantly improved in NOD/SCID-IL2RG mice constitutively expressing human SCF, GM-CSF and IL-3. Leukemia. 2010;24(10):1785-8.

185. Kollet O, Peled A, Byk T, Ben-Hur H, Greiner D, Shultz L, et al. beta2 microglobulin-deficient (B2m(null)) NOD/SCID mice are excellent recipients for studying human stem cell function. Blood. 2000;95(10):3102-5.

186. Culen M, Kosarova Z, Jeziskova I, Folta A, Chovancova J, Loja T, et al. The influence of mutational status and biological characteristics of acute myeloid leukemia on xenotransplantation outcomes in NOD SCID gamma mice. J Cancer Res Clin Oncol. 2018;144(7):1239-51.

187. Brown D, Kogan S, Lagasse E, Weissman I, Alcalay M, Pelicci PG, et al. A PMLRARalpha transgene initiates murine acute promyelocytic leukemia. Proc Natl Acad Sci U S A. 1997;94(6):2551-6.

188. Yuan Y, Zhou L, Miyamoto T, Iwasaki H, Harakawa N, Hetherington CJ, et al. AML1-ETO expression is directly involved in the development of acute myeloid leukemia in the presence of additional mutations. Proc Natl Acad Sci U S A. 2001;98(18):10398-403.

189. Stavropoulou V, Kaspar S, Brault L, Sanders MA, Juge S, Morettini S, et al. MLL-AF9 Expression in Hematopoietic Stem Cells Drives a Highly Invasive AML Expressing EMT-Related Genes Linked to Poor Outcome. Cancer Cell. 2016;30(1):43-58.

190. Ugale A, Norddahl GL, Wahlestedt M, Sawen P, Jaako P, Pronk CJ, et al. Hematopoietic stem cells are intrinsically protected against MLL-ENL-mediated transformation. Cell Rep. 2014;9(4):1246-55.

191. Vassiliou GS, Cooper JL, Rad R, Li J, Rice S, Uren A, et al. Mutant nucleophosmin and cooperating pathways drive leukemia initiation and progression in mice. Nat Genet. 2011;43(5):470-5.

192. Mupo A, Celani L, Dovey O, Cooper JL, Grove C, Rad R, et al. A powerful molecular synergy between mutant Nucleophosmin and Flt3-ITD drives acute myeloid leukemia in mice. Leukemia. 2013;27(9):1917-20.

193. Zorko NA, Bernot KM, Whitman SP, Siebenaler RF, Ahmed EH, Marcucci GG, et al. Mll partial tandem duplication and Flt3 internal tandem duplication in a double knock-in mouse recapitulates features of counterpart human acute myeloid leukemias. Blood. 2012;120(5):1130-6.

194. Shih AH, Jiang Y, Meydan C, Shank K, Pandey S, Barreyro L, et al. Mutational cooperativity linked to combinatorial epigenetic gain of function in acute myeloid leukemia. Cancer Cell. 2015;27(4):502-15.

195. Heckl D, Kowalczyk MS, Yudovich D, Belizaire R, Puram RV, McConkey ME, et al. Generation of mouse models of myeloid malignancy with combinatorial genetic lesions using CRISPR-Cas9 genome editing. Nat Biotechnol. 2014;32(9):941-6.

62

196.   Reimer J, Knoss S, Labuhn M, Charpentier EM, Gohring G, Schlegelberger B, et al. CRISPR-Cas9-induced t(11;19)/MLL-ENL translocations initiate leukemia in human hematopoietic progenitor cells in vivo. Haematologica. 2017;102(9):1558-66.

197.   Schneidawind C, Jeong J, Schneidawind D, Kim IS, Duque-Afonso J, Wong SHK, et al. MLL leukemia induction by t(9;11) chromosomal translocation in human hematopoietic stem cells using genome editing. Blood Adv. 2018;2(8):832-45.

198.   Lavau C, Szilvassy SJ, Slany R, Cleary ML. Immortalization and leukemic transformation of a myelomonocytic precursor by retrovirally transduced HRX-ENL. EMBO J. 1997;16(14):4226-37.

199.   Huntly BJ, Shigematsu H, Deguchi K, Lee BH, Mizuno S, Duclos N, et al. MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. Cancer Cell. 2004;6(6):587-96.

200.   Krivtsov AV, Twomey D, Feng Z, Stubbs MC, Wang Y, Faber J, et al. Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. Nature. 2006;442(7104):818-22.

201.   Miller PG, Al-Shahrour F, Hartwell KA, Chu LP, Jaras M, Puram RV, et al. In Vivo RNAi screening identifies a leukemia-specific dependence on integrin beta 3 signaling. Cancer Cell. 2013;24(1):45-58.

202.   Somervaille TC, Cleary ML. Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia. Cancer Cell. 2006;10(4):257-68.

203.   Bene MC, Nebe T, Bettelheim P, Buldini B, Bumbea H, Kern W, et al. Immunophenotyping of acute leukemia and lymphoproliferative disorders: a consensus proposal of the European LeukemiaNet Work Package 10. Leukemia. 2011;25(4):567-74.

204.   Lichtman MA. A historical perspective on the development of the cytarabine (7days) and daunorubicin (3days) treatment regimen for acute myelogenous leukemia: 2013 the 40th anniversary of 7+3. Blood Cells Mol Dis. 2013;50(2):119-30.

205.   Cassileth PA, Harrington DP, Hines JD, Oken MM, Mazza JJ, McGlave P, et al. Maintenance chemotherapy prolongs remission duration in adult acute nonlymphocytic leukemia. J Clin Oncol. 1988;6(4):583-7.

206.   Burnett A, Wetzler M, Lowenberg B. Therapeutic advances in acute myeloid leukemia. J Clin Oncol. 2011;29(5):487-94.

207.   Campos L, Rouault JP, Sabido O, Oriol P, Roubi N, Vasselon C, et al. High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. Blood. 1993;81(11):3091-6.

208.   Wuchter C, Leonid K, Ruppert V, Schrappe M, Buchner T, Schoch C, et al. Clinical significance of P-glycoprotein expression and function for response to induction chemotherapy, relapse rate and overall survival in acute leukemia. Haematologica. 2000;85(7):711-21.

209.   McCulloch D, Brown C, Iland H. Retinoic acid and arsenic trioxide in the treatment of acute promyelocytic leukemia: current perspectives. Onco Targets Ther. 2017;10:1585-601.

210.   Wang ZY, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. Blood. 2008;111(5):2505-15.

211.   Lai C, Doucette K, Norsworthy K. Recent drug approvals for acute myeloid leukemia. J Hematol Oncol. 2019;12(1):100.

212.   Barrett AJ. Understanding and harnessing the graft-versus-leukaemia effect. Br J Haematol. 2008;142(6):877-88.

213.   Lowdell MW, Ray N, Craston R, Corbett T, Deane M, Prentice HG. The in vitro detection of anti-leukaemia-specific cytotoxicity after autologous bone marrow transplantation for acute leukaemia. Bone Marrow Transplant. 1997;19(9):891-7.

214.   Brune M, Castaigne S, Catalano J, Gehlsen K, Ho AD, Hofmann WK, et al. Improved leukemia-free survival after postconsolidation immunotherapy with histamine dihydrochloride and interleukin-2 in acute myeloid leukemia: results of a randomized phase 3 trial. Blood. 2006;108(1):88-96.

215.   Szczepanski MJ, Szajnik M, Welsh A, Foon KA, Whiteside TL, Boyiadzis M. Interleukin-15 enhances natural killer cell cytotoxicity in patients with acute myeloid leukemia by upregulating the activating NK cell receptors. Cancer Immunol Immunother. 2010;59(1):73-9.

216.   Liu J, Wang L, Zhao F, Tseng S, Narayanan C, Shura L, et al. Pre-Clinical Development of a Humanized Anti-CD47 Antibody with Anti-Cancer Therapeutic Potential. PLoS One. 2015;10(9):e0137345.

217.   Godwin CD, Gale RP, Walter RB. Gemtuzumab ozogamicin in acute myeloid leukemia. Leukemia. 2017;31(9):1855-68.

218. Askmyr M, Agerstam H, Hansen N, Gordon S, Arvanitakis A, Rissler M, et al. Selective killing of candidate AML stem cells by antibody targeting of IL1RAP. Blood. 2013;121(18):3709-13.

219. Jin L, Lee EM, Ramshaw HS, Busfield SJ, Peoppl AG, Wilkinson L, et al. Monoclonal antibody-mediated targeting of CD123, IL-3 receptor alpha chain, eliminates human acute myeloid leukemic stem cells. Cell Stem Cell. 2009;5(1):31-42.

220. Jiang YP, Liu BY, Zheng Q, Panuganti S, Chen R, Zhu J, et al. CLT030, a leukemic stem cell-targeting CLL1 antibody-drug conjugate for treatment of acute myeloid leukemia. Blood Adv. 2018;2(14):1738-49.

221. Kikushige Y, Shima T, Takayanagi S, Urata S, Miyamoto T, Iwasaki H, et al. TIM-3 is a promising target to selectively kill acute myeloid leukemia stem cells. Cell Stem Cell. 2010;7(6):708-17.

222. Schurch CM. Therapeutic Antibodies for Myeloid Neoplasms-Current Developments and Future Directions. Front Oncol. 2018;8:152.

223. Pui CH, Robison LL, Look AT. Acute lymphoblastic leukaemia. Lancet. 2008;371(9617):1030-43.

224. Inaba H, Greaves M, Mullighan CG. Acute lymphoblastic leukaemia. Lancet. 2013;381(9881):1943-55.

225. Hjalgrim LL, Rostgaard K, Schmiegelow K, Soderhall S, Kolmannskog S, Vettenranta K, et al. Age- and sex-specific incidence of childhood leukemia by immunophenotype in the Nordic countries. J Natl Cancer Inst. 2003;95(20):1539-44.

226. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. The morphological classification of acute lymphoblastic leukaemia: concordance among observers and clinical correlations. Br J Haematol. 1981;47(4):553-61.

227. Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A, et al. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia. 1995;9(10):1783-6.

228. Lilljebjorn H, Fioretos T. New oncogenic subtypes in pediatric B-cell precursor acute lymphoblastic leukemia. Blood. 2017;130(12):1395-401.

229. Yeoh EJ, Ross ME, Shurtleff SA, Williams WK, Patel D, Mahfouz R, et al. Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. Cancer Cell. 2002;1(2):133-43.

230. Ross ME, Zhou X, Song G, Shurtleff SA, Girtman K, Williams WK, et al. Classification of pediatric acute lymphoblastic leukemia by gene expression profiling. Blood. 2003;102(8):2951-9.

231. Hiebert SW, Sun W, Davis JN, Golub T, Shurtleff S, Buijs A, et al. The t(12;21) translocation converts AML-1B from an activator to a repressor of transcription. Mol Cell Biol. 1996;16(4):1349-55.

232. Guidez F, Petrie K, Ford AM, Lu H, Bennett CA, MacGregor A, et al. Recruitment of the nuclear receptor corepressor N-CoR by the TEL moiety of the childhood leukemia-associated TEL-AML1 oncoprotein. Blood. 2000;96(7):2557-61.

233. Wang Q, Stacy T, Binder M, Marin-Padilla M, Sharpe AH, Speck NA. Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. Proc Natl Acad Sci U S A. 1996;93(8):3444-9.

234. Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell. 1996;84(2):321-30.

235. Lu Q, Wright DD, Kamps MP. Fusion with E2A converts the Pbx1 homeodomain protein into a constitutive transcriptional activator in human leukemias carrying the t(1;19) translocation. Mol Cell Biol. 1994;14(6):3938-48.

236. Aspland SE, Bendall HH, Murre C. The role of E2A-PBX1 in leukemogenesis. Oncogene. 2001;20(40):5708-17.

237. Campos-Sanchez E, Toboso-Navasa A, Romero-Camarero I, Barajas-Diego M, Sanchez-Garcia I, Cobaleda C. Acute lymphoblastic leukemia and developmental biology: a crucial interrelationship. Cell Cycle. 2011;10(20):3473-86.

238. Lilljebjorn H, Henningsson R, Hyrenius-Wittsten A, Olsson L, Orsmark-Pietras C, von Palffy S, et al. Identification of ETV6-RUNX1-like and DUX4-rearranged subtypes in paediatric B-cell precursor acute lymphoblastic leukaemia. Nat Commun. 2016;7:11790.

239. Zhang J, McCastlain K, Yoshihara H, Xu B, Chang Y, Churchman ML, et al. Deregulation of DUX4 and ERG in acute lymphoblastic leukemia. Nat Genet. 2016;48(12):1481-9.

240. Yasuda T, Tsuzuki S, Kawazu M, Hayakawa F, Kojima S, Ueno T, et al. Recurrent DUX4 fusions in B cell acute lymphoblastic leukemia of adolescents and young adults. Nat Genet. 2016;48(5):569-74.

241. Liu YF, Wang BY, Zhang WN, Huang JY, Li BS, Zhang M, et al. Genomic Profiling of Adult and Pediatric B-cell Acute Lymphoblastic Leukemia. EBioMedicine. 2016;8:173-83.

242. De Iaco A, Planet E, Coluccio A, Verp S, Duc J, Trono D. DUX-family transcription factors regulate zygotic genome activation in placental mammals. Nat Genet. 2017;49(6):941-5.

243. Lemmers RJ, van der Vliet PJ, Klooster R, Sacconi S, Camano P, Dauwerse JG, et al. A unifying genetic model for facioscapulohumeral muscular dystrophy. Science. 2010;329(5999):1650-3.

244. Kawamura-Saito M, Yamazaki Y, Kaneko K, Kawaguchi N, Kanda H, Mukai H, et al. Fusion between CIC and DUX4 up-regulates PEA3 family genes in Ewing-like sarcomas with t(4;19)(q35;q13) translocation. Hum Mol Genet. 2006;15(13):2125-37.

245. Sirvent N, Trassard M, Ebran N, Attias R, Pedeutour F. Fusion of EWSR1 with the DUX4 facioscapulohumeral muscular dystrophy region resulting from t(4;22)(q35;q12) in a case of embryonal rhabdomyosarcoma. Cancer Genet Cytogenet. 2009;195(1):12-8.

246. Tian L, Shao Y, Nance S, Dang J, Xu B, Ma X, et al. Long-read sequencing unveils IGH-DUX4 translocation into the silenced IGH allele in B-cell acute lymphoblastic leukemia. Nat Commun. 2019;10(1):2789.

247. Russell LJ, Enshaei A, Jones L, Erhorn A, Masic D, Bentley H, et al. IGH@ translocations are prevalent in teenagers and young adults with acute lymphoblastic leukemia and are associated with a poor outcome. J Clin Oncol. 2014;32(14):1453-62.

248. Ott G, Rosenwald A, Campo E. Understanding MYC-driven aggressive B-cell lymphomas: pathogenesis and classification. Hematology Am Soc Hematol Educ Program. 2013;2013:575-83.

249. Fonseca R, Debes-Marun CS, Picken EB, Dewald GW, Bryant SC, Winkler JM, et al. The recurrent IgH translocations are highly associated with nonhyperdiploid variant multiple myeloma. Blood. 2003;102(7):2562-7.

250. Dong X, Zhang W, Wu H, Huang J, Zhang M, Wang P, et al. Structural basis of DUX4/IGH-driven transactivation. Leukemia. 2018;32(6):1466-76.

251. Tanaka Y, Kawazu M, Yasuda T, Tamura M, Hayakawa F, Kojima S, et al. Transcriptional activities of DUX4 fusions in B-cell acute lymphoblastic leukemia. Haematologica. 2018;103(11):e522-e6.

252. Mullighan CG, Miller CB, Su X, Radtke I, Dalton J, Song G, et al. ERG Deletions Define a Novel Subtype of B-Progenitor Acute Lymphoblastic Leukemia. Blood. 2007;110(11):691-.

253. Harvey RC, Mullighan CG, Wang X, Dobbin KK, Davidson GS, Bedrick EJ, et al. Identification of novel cluster groups in pediatric high-risk B-precursor acute lymphoblastic leukemia with gene expression profiling: correlation with genome-wide DNA copy number alterations, clinical characteristics, and outcome. Blood. 2010;116(23):4874-84.

254. Matsuo Y, Drexler HG. Establishment and characterization of human B cell precursor-leukemia cell lines. Leuk Res. 1998;22(7):567-79.

255. Hurwitz R, Hozier J, LeBien T, Minowada J, Gajl-Peczalska K, Kubonishi I, et al. Characterization of a leukemic cell line of the pre-B phenotype. Int J Cancer. 1979;23(2):174-80.

256. Jacoby E, Chien CD, Fry TJ. Murine models of acute leukemia: important tools in current pediatric leukemia research. Front Oncol. 2014;4:95.

257. Fischer M, Schwieger M, Horn S, Niebuhr B, Ford A, Roscher S, et al. Defining the oncogenic function of the TEL/AML1 (ETV6/RUNX1) fusion protein in a mouse model. Oncogene. 2005;24(51):7579-91.

258. Morrow M, Horton S, Kioussis D, Brady HJ, Williams O. TEL-AML1 promotes development of specific hematopoietic lineages consistent with preleukemic activity. Blood. 2004;103(10):3890-6.

259. Tsuzuki S, Seto M, Greaves M, Enver T. Modeling first-hit functions of the t(12;21) TEL-AML1 translocation in mice. Proc Natl Acad Sci U S A. 2004;101(22):8443-8.

260. Li M, Jones L, Gaillard C, Binnewies M, Ochoa R, Garcia E, et al. Initially disadvantaged, TEL-AML1 cells expand and initiate leukemia in response to irradiation and cooperating mutations. Leukemia. 2013;27(7):1570-3.

261. Dedera DA, Waller EK, LeBrun DP, Sen-Majumdar A, Stevens ME, Barsh GS, et al. Chimeric homeobox gene E2A-PBX1 induces proliferation, apoptosis, and malignant lymphomas in transgenic mice. Cell. 1993;74(5):833-43.

262. Duque-Afonso J, Feng J, Scherer F, Lin CH, Wong SH, Wang Z, et al. Comparative genomics reveals multistep pathogenesis of E2A-PBX1 acute lymphoblastic leukemia. J Clin Invest. 2015;125(9):3667-80.

263. Bijl J, Sauvageau M, Thompson A, Sauvageau G. High incidence of proviral integrations in the Hoxa locus in a new model of E2a-PBX1-induced B-cell leukemia. Genes Dev. 2005;19(2):224-33.

264. Alvarnas JC, Brown PA, Aoun P, Ballen KK, Barta SK, Borate U, et al. Acute Lymphoblastic Leukemia, Version 2.2015. J Natl Compr Canc Netw. 2015;13(10):1240-79.

265. Meeker TC, Hardy D, Willman C, Hogan T, Abrams J. Activation of the interleukin-3 gene by chromosome translocation in acute lymphocytic leukemia with eosinophilia. Blood. 1990;76(2):285-9.

266. Rowe JM. Prognostic factors in adult acute lymphoblastic leukaemia. Br J Haematol. 2010;150(4):389-405.

267. Raetz EA, Bhatla T. Where do we stand in the treatment of relapsed acute lymphoblastic leukemia? Hematology Am Soc Hematol Educ Program. 2012;2012:129-36.

268. Thomas DA, O'Brien S, Faderl S, Garcia-Manero G, Ferrajoli A, Wierda W, et al. Chemoimmunotherapy with a modified hyper-CVAD and rituximab regimen improves outcome in de novo Philadelphia chromosome-negative precursor B-lineage acute lymphoblastic leukemia. J Clin Oncol. 2010;28(24):3880-9.

269. Kantarjian HM, DeAngelo DJ, Stelljes M, Martinelli G, Liedtke M, Stock W, et al. Inotuzumab Ozogamicin versus Standard Therapy for Acute Lymphoblastic Leukemia. N Engl J Med. 2016;375(8):740-53.

270. Bhojwani D, Sposto R, Shah NN, Rodriguez V, Yuan C, Stetler-Stevenson M, et al. Inotuzumab ozogamicin in pediatric patients with relapsed/refractory acute lymphoblastic leukemia. Leukemia. 2019;33(4):884-92.

271. Nagorsen D, Kufer P, Baeuerle PA, Bargou R. Blinatumomab: a historical perspective. Pharmacol Ther. 2012;136(3):334-42.

272. Gardner RA, Finney O, Annesley C, Brakke H, Summers C, Leger K, et al. Intent-to-treat leukemia remission by CD19 CAR T cells of defined formulation and dose in children and young adults. Blood. 2017;129(25):3322-31.

273. Smithies O, Gregg RG, Boggs SS, Koralewski MA, Kucherlapati RS. Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. Nature. 1985;317(6034):230-4.

274. Thomas KR, Folger KR, Capecchi MR. High frequency targeting of genes to specific sites in the mammalian genome. Cell. 1986;44(3):419-28.

275. Smith J, Bibikova M, Whitby FG, Reddy AR, Chandrasegaran S, Carroll D. Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. Nucleic Acids Res. 2000;28(17):3361-9.

276. Miller JC, Tan S, Qiao G, Barlow KA, Wang J, Xia DF, et al. A TALE nuclease architecture for efficient genome editing. Nat Biotechnol. 2011;29(2):143-8.

277. Mojica FJ, Diez-Villasenor C, Soria E, Juez G. Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. Mol Microbiol. 2000;36(1):244-6.

278. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. J Bacteriol. 1987;169(12):5429-33.

279. Mojica FJ, Ferrer C, Juez G, Rodriguez-Valera F. Long stretches of short tandem repeats are present in the largest replicons of the Archaea Haloferax mediterranei and Haloferax volcanii and could be involved in replicon partitioning. Mol Microbiol. 1995;17(1):85-93.

280. Jansen R, Embden JD, Gaastra W, Schouls LM. Identification of genes that are associated with DNA repeats in prokaryotes. Mol Microbiol. 2002;43(6):1565-75.

281. Bolotin A, Quinquis B, Sorokin A, Ehrlich SD. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. Microbiology. 2005;151(Pt 8):2551-61.

282. Mojica FJ, Diez-Villasenor C, Garcia-Martinez J, Soria E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J Mol Evol. 2005;60(2):174-82.

283. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, et al. CRISPR provides acquired resistance against viruses in prokaryotes. Science. 2007;315(5819):1709-12.

284. Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, et al. Small CRISPR RNAs guide antiviral defense in prokaryotes. Science. 2008;321(5891):960-4.

285. Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, et al. Evolution and classification of the CRISPR-Cas systems. Nat Rev Microbiol. 2011;9(6):467-77.

286. Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, et al. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature. 2011;471(7340):602-7.

287. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012;337(6096):816-21.

288. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, et al. RNA-guided human genome engineering via Cas9. Science. 2013;339(6121):823-6.

289. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013;339(6121):819-23.

290. Sonoda E, Hochegger H, Saberi A, Taniguchi Y, Takeda S. Differential usage of non-homologous end-joining and homologous recombination in double strand break repair. DNA Repair (Amst). 2006;5(9-10):1021-9.

291. Liu JJ, Orlova N, Oakes BL, Ma E, Spinner HB, Baney KLM, et al. CasX enzymes comprise a distinct family of RNA-guided genome editors. Nature. 2019;566(7743):218-23.

292. Strecker J, Jones S, Koopal B, Schmid-Burgk J, Zetsche B, Gao L, et al. Engineering of CRISPR-Cas12b for human genome editing. Nat Commun. 2019;10(1):212.

293. Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell. 2015;163(3):759-71.

294. Vanden Bempt M, Demeyer S, Mentens N, Geerdens E, De Bock CE, Wlodarska I, et al. Generation of the Fip1l1-Pdgfra fusion gene using CRISPR/Cas genome editing. Leukemia. 2016;30(9):1913-6.

295. Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelson T, et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. Science. 2014;343(6166):84-7.

296. Wang T, Wei JJ, Sabatini DM, Lander ES. Genetic screens in human cells using the CRISPR-Cas9 system. Science. 2014;343(6166):80-4.

297. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, et al. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell. 2013;152(5):1173-83.

298. Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell. 2013;154(2):442-51.

299. Thakore PI, Black JB, Hilton IB, Gersbach CA. Editing the epigenome: technologies for programmable transcription and epigenetic modulation. Nat Methods. 2016;13(2):127-37.

300. Fujita T, Fujii H. Efficient isolation of specific genomic regions and identification of associated proteins by engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) using CRISPR. Biochem Biophys Res Commun. 2013;439(1):132-6.

301. Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, Li GW, et al. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. Cell. 2013;155(7):1479-91.

302. Eggert US. The why and how of phenotypic small-molecule screens. Nat Chem Biol. 2013;9(4):206-9.

303. Tochitani S, Hayashizaki Y. Functional screening revisited in the postgenomic era. Mol Biosyst. 2007;3(3):195-207.

304. Wang R-S. Perturbation. In: Dubitzky W, Wolkenhauer O, Cho K-H, Yokota H, editors. Encyclopedia of Systems Biology. New York, NY: Springer New York; 2013. p. 1680-1.

305. Yang X, Boehm JS, Yang X, Salehi-Ashtiani K, Hao T, Shen Y, et al. A public genome-scale lentiviral expression library of human ORFs. Nat Methods. 2011;8(8):659-61.

306. Liberali P, Snijder B, Pelkmans L. Single-cell and multivariate approaches in genetic perturbation screens. Nat Rev Genet. 2015;16(1):18-32.

307. Moffat JG, Rudolph J, Bailey D. Phenotypic screening in cancer drug discovery - past, present and future. Nat Rev Drug Discov. 2014;13(8):588-602.

308. Mullenders J, Bernards R. Loss-of-function genetic screens as a tool to improve the diagnosis and treatment of cancer. Oncogene. 2009;28(50):4409-20.

309. Costa JR, Bejcek BE, McGee JE, Fogel AI, Brimacombe KR, Ketteler R. Genome Editing Using Engineered Nucleases and Their Use in Genomic Screening. In: Sittampalam GS, Grossman A, Brimacombe K, Arkin M, Auld D, Austin CP, et al., editors. Assay Guidance Manual. Bethesda (MD)2004.

310. Binan L, Drobetsky EA, Costantino S. Exploiting Molecular Barcodes in High-Throughput Cellular Assays. SLAS Technol. 2019;24(3):298-307.

311. Bedard PL, Hansen AR, Ratain MJ, Siu LL. Tumour heterogeneity in the clinic. Nature. 2013;501(7467):355-64.

312. Pollen AA, Nowakowski TJ, Shuga J, Wang X, Leyrat AA, Lui JH, et al. Low-coverage single-cell mRNA sequencing reveals cellular heterogeneity and activated signaling pathways in developing cerebral cortex. Nat Biotechnol. 2014;32(10):1053-8.

313. Macosko EZ, Basu A, Satija R, Nemesh J, Shekhar K, Goldman M, et al. Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. Cell. 2015;161(5):1202-14.

314. Sulston JE, Schierenberg E, White JG, Thomson JN. The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev Biol. 1983;100(1):64-119.

315. Axelrod D. Carbocyanine dye orientation in red cell membrane studied by microscopic fluorescence polarization. Biophys J. 1979;26(3):557-73.

316. Serbedzija GN, Bronner-Fraser M, Fraser SE. A vital dye analysis of the timing and pathways of avian trunk neural crest cell migration. Development. 1989;106(4):809-16.

317. Fink J, Andersson-Rolf A, Koo BK. Adult stem cell lineage tracing and deep tissue imaging. BMB Rep. 2015;48(12):655-67.

318. Mazurier F, Gan OI, McKenzie JL, Doedens M, Dick JE. Lentivector-mediated clonal tracking reveals intrinsic heterogeneity in the human hematopoietic stem cell compartment and culture-induced stem cell impairment. Blood. 2004;103(2):545-52.

319. Kristiansen TA, Jaensson Gyllenback E, Zriwil A, Bjorklund T, Daniel JA, Sitnicka E, et al. Cellular Barcoding Links B-1a B Cell Potential to a Fetal Hematopoietic Stem Cell State at the Single-Cell Level. Immunity. 2016;45(2):346-57.

320. Lu R, Neff NF, Quake SR, Weissman IL. Tracking single hematopoietic stem cells in vivo using high-throughput sequencing in conjunction with viral genetic barcoding. Nat Biotechnol. 2011;29(10):928-33.

321. Stoeckius M, Hafemeister C, Stephenson W, Houck-Loomis B, Chattopadhyay PK, Swerdlow H, et al. Simultaneous epitope and transcriptome measurement in single cells. Nat Methods. 2017;14(9):865-8.

322. Peterson VM, Zhang KX, Kumar N, Wong J, Li L, Wilson DC, et al. Multiplexed quantification of proteins and transcripts in single cells. Nat Biotechnol. 2017;35(10):936-9.

323. Stoeckius M, Zheng S, Houck-Loomis B, Hao S, Yeung BZ, Mauck WM, 3rd, et al. Cell Hashing with barcoded antibodies enables multiplexing and doublet detection for single cell genomics. Genome Biol. 2018;19(1):224.

324. Zimmermann G, Neri D. DNA-encoded chemical libraries: foundations and applications in lead discovery. Drug Discov Today. 2016;21(11):1828-34.

325. Smith AM, Heisler LE, St Onge RP, Farias-Hesson E, Wallace IM, Bodeau J, et al. Highly-multiplexed barcode sequencing: an efficient method for parallel analysis of pooled samples. Nucleic Acids Res. 2010;38(13):e142.

326. Sims D, Mendes-Pereira AM, Frankum J, Burgess D, Cerone MA, Lombardelli C, et al. High-throughput RNA interference screening using pooled shRNA libraries and next generation sequencing. Genome Biol. 2011;12(10):R104.

327. Thomas X. First contributors in the history of leukemia. World J Hematol. 2013;2(3):62-70.

328. Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature. 1998;393(6685):595-9.

329. Spoo AC, Lubbert M, Wierda WG, Burger JA. CXCR4 is a prognostic marker in acute myelogenous leukemia. Blood. 2007;109(2):786-91.

330. Tavor S, Petit I, Porozov S, Avigdor A, Dar A, Leider-Trejo L, et al. CXCR4 regulates migration and development of human acute myelogenous leukemia stem cells in transplanted NOD/SCID mice. Cancer Res. 2004;64(8):2817-24.

331. Monaco G, Konopleva M, Munsell M, Leysath C, Wang RY, Jackson CE, et al. Engraftment of acute myeloid leukemia in NOD/SCID mice is independent of CXCR4 and predicts poor patient survival. Stem Cells. 2004;22(2):188-201.

332. Lapidot T, Kollet O. The essential roles of the chemokine SDF-1 and its receptor CXCR4 in human stem cell homing and repopulation of transplanted immune-deficient NOD/SCID and NOD/SCID/B2m(null) mice. Leukemia. 2002;16(10):1992-2003.

333. Greenbaum A, Hsu YM, Day RB, Schuettpelz LG, Christopher MJ, Borgerding JN, et al. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. Nature. 2013;495(7440):227-30.

334. Ramakrishnan R, Pena-Martinez P, Agarwal P, Rodriguez-Zabala M, Chapellier M, Hogberg C, et al. CXCR4 Signaling Has a CXCL12-Independent Essential Role in Murine MLL-AF9-Driven Acute Myeloid Leukemia. Cell Rep. 2020;31(8):107684.

335. Krause DS, Fulzele K, Catic A, Sun CC, Dombkowski D, Hurley MP, et al. Differential regulation of myeloid leukemias by the bone marrow microenvironment. Nat Med. 2013;19(11):1513-7.

336. Reynaud D, Pietras E, Barry-Holson K, Mir A, Binnewies M, Jeanne M, et al. IL-6 controls leukemic multipotent progenitor cell fate and contributes to chronic myelogenous leukemia development. Cancer Cell. 2011;20(5):661-73.

337. Schepers K, Pietras EM, Reynaud D, Flach J, Binnewies M, Garg T, et al. Myeloproliferative neoplasia remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche. Cell Stem Cell. 2013;13(3):285-99.

338. Garcia-Castro A, Zonca M, Florindo-Pinheiro D, Carvalho-Pinto CE, Cordero A, Gutierrez del Fernando B, et al. APRIL promotes breast tumor growth and metastasis and is associated with aggressive basal breast cancer. Carcinogenesis. 2015;36(5):574-84.

339. Wang R, Guo Y, Ma H, Feng L, Wang Q, Chen X, et al. Tumor necrosis factor superfamily member 13 is a novel biomarker for diagnosis and prognosis and promotes cancer cell proliferation in laryngeal squamous cell carcinoma. Tumour Biol. 2016;37(2):2635-45.

340. Hahne M, Kataoka T, Schroter M, Hofmann K, Irmler M, Bodmer JL, et al. APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth. J Exp Med. 1998;188(6):1185-90.

341. Chiu A, Xu W, He B, Dillon SR, Gross JA, Sievers E, et al. Hodgkin lymphoma cells express TACI and BCMA receptors and generate survival and proliferation signals in response to BAFF and APRIL. Blood. 2007;109(2):729-39.

342. Endo T, Nishio M, Enzler T, Cottam HB, Fukuda T, James DF, et al. BAFF and APRIL support chronic lymphocytic leukemia B-cell survival through activation of the canonical NF-kappaB pathway. Blood. 2007;109(2):703-10.

343. Tai YT, Acharya C, An G, Moschetta M, Zhong MY, Feng X, et al. APRIL and BCMA promote human multiple myeloma growth and immunosuppression in the bone marrow microenvironment. Blood. 2016;127(25):3225-36.

344. Bolkun L, Lemancewicz D, Jablonska E, Szumowska A, Bolkun-Skornicka U, Ratajczak-Wrona W, et al. The impact of TNF superfamily molecules on overall survival in acute myeloid leukaemia: correlation with biological and clinical features. Ann Hematol. 2015;94(1):35-43.

345. Kern C, Cornuel JF, Billard C, Tang R, Rouillard D, Stenou V, et al. Involvement of BAFF and APRIL in the resistance to apoptosis of B-CLL through an autocrine pathway. Blood. 2004;103(2):679-88.

346. Moreaux J, Legouffe E, Jourdan E, Quittet P, Reme T, Lugagne C, et al. BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone. Blood. 2004;103(8):3148-57.

347. Baba M, Keller JR, Sun HW, Resch W, Kuchen S, Suh HC, et al. The folliculin-FNIP1 pathway deleted in human Birt-Hogg-Dube syndrome is required for murine B-cell development. Blood. 2012;120(6):1254-61.

348. Park H, Staehling K, Tsang M, Appleby MW, Brunkow ME, Margineantu D, et al. Disruption of Fnip1 reveals a metabolic checkpoint controlling B lymphocyte development. Immunity. 2012;36(5):769-81.

349. Panieri E, Santoro MM. ROS homeostasis and metabolism: a dangerous liason in cancer cells. Cell Death Dis. 2016;7(6):e2253.

350. Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. Cell. 2017;169(2):361-71.

351. Yadav RK, Chauhan AS, Zhuang L, Gan B. FoxO transcription factors in cancer metabolism. Semin Cancer Biol. 2018;50:65-76.

352. Matsuyama T, Grossman A, Mittrucker HW, Siderovski DP, Kiefer F, Kawakami T, et al. Molecular cloning of LSIRF, a lymphoid-specific member of the interferon regulatory factor family that binds the interferon-stimulated response element (ISRE). Nucleic Acids Res. 1995;23(12):2127-36.

353. Klein U, Casola S, Cattoretti G, Shen Q, Lia M, Mo T, et al. Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. Nat Immunol. 2006;7(7):773-82.

354. Asslaber D, Qi Y, Maeding N, Steiner M, Denk U, Hopner JP, et al. B-cell-specific IRF4 deletion accelerates chronic lymphocytic leukemia development by enhanced tumor immune evasion. Blood. 2019;134(20):1717-29.

355. Shaffer AL, Emre NC, Lamy L, Ngo VN, Wright G, Xiao W, et al. IRF4 addiction in multiple myeloma. Nature. 2008;454(7201):226-31.

356. Mizutani A, Fukuda M, Ibata K, Shiraishi Y, Mikoshiba K. SYNCRIP, a cytoplasmic counterpart of heterogeneous nuclear ribonucleoprotein R, interacts with ubiquitous synaptotagmin isoforms. J Biol Chem. 2000;275(13):9823-31.

357. Weidensdorfer D, Stohr N, Baude A, Lederer M, Kohn M, Schierhorn A, et al. Control of c-myc mRNA stability by IGF2BP1-associated cytoplasmic RNPs. RNA. 2009;15(1):104-15.

358. Vu LP, Prieto C, Amin EM, Chhangawala S, Krivtsov A, Calvo-Vidal MN, et al. Functional screen of MSI2 interactors identifies an essential role for SYNCRIP in myeloid leukemia stem cells. Nat Genet. 2017;49(6):866-75.

359. Park SM, Deering RP, Lu Y, Tivnan P, Lianoglou S, Al-Shahrour F, et al. Musashi-2 controls cell fate, lineage bias, and TGF-beta signaling in HSCs. J Exp Med. 2014;211(1):71-87.

360. Li Z, Chen L, Qin Z. Paradoxical roles of IL-4 in tumor immunity. Cell Mol Immunol. 2009;6(6):415-22.

361. Pena-Martinez P, Eriksson M, Ramakrishnan R, Chapellier M, Hogberg C, Orsmark-Pietras C, et al. Interleukin 4 induces apoptosis of acute myeloid leukemia cells in a Stat6-dependent manner. Leukemia. 2018;32(3):588-96.

362. Paul WE. History of interleukin-4. Cytokine. 2015;75(1):3-7.

363. Kiniwa T, Enomoto Y, Terazawa N, Omi A, Miyata N, Ishiwata K, et al. NK cells activated by Interleukin-4 in cooperation with Interleukin-15 exhibit distinctive characteristics. Proc Natl Acad Sci U S A. 2016;113(36):10139-44.

364. Milner JD, Orekov T, Ward JM, Cheng L, Torres-Velez F, Junttila I, et al. Sustained IL-4 exposure leads to a novel pathway for hemophagocytosis, inflammation, and tissue macrophage accumulation. Blood. 2010;116(14):2476-83.

365. Erb KJ, Ruger B, von Brevern M, Ryffel B, Schimpl A, Rivett K. Constitutive expression of interleukin (IL)-4 in vivo causes autoimmune-type disorders in mice. J Exp Med. 1997;185(2):329-39.

366. Jaiswal S, Chao MP, Majeti R, Weissman IL. Macrophages as mediators of tumor immunosurveillance. Trends Immunol. 2010;31(6):212-9.

367. Loke P, Nair MG, Parkinson J, Guiliano D, Blaxter M, Allen JE. IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype. BMC Immunol. 2002;3:7.

368. Jaiswal S, Jamieson CH, Pang WW, Park CY, Chao MP, Majeti R, et al. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. Cell. 2009;138(2):271-85.

369. Domanska UM, Kruizinga RC, Nagengast WB, Timmer-Bosscha H, Huls G, de Vries EG, et al. A review on CXCR4/CXCL12 axis in oncology: no place to hide. Eur J Cancer. 2013;49(1):219-30.

370. Bonci D, Musumeci M, Coppola V, Addario A, Conticello C, Hahne M, et al. Blocking the APRIL circuit enhances acute myeloid leukemia cell chemosensitivity. Haematologica. 2008;93(12):1899-902.

371. Mimitou EP, Cheng A, Montalbano A, Hao S, Stoeckius M, Legut M, et al. Multiplexed detection of proteins, transcriptomes, clonotypes and CRISPR perturbations in single cells. Nat Methods. 2019;16(5):409-12.