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# UNRAVELING NOVEL GENES THAT ARE ESSENTIAL FOR ACUTE MYELOID LEUKEMIA AND NORMAL HEMATOPOIESIS

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# UNRAVELING NOVEL GENES THAT ARE ESSENTIAL FOR ACUTE MYELOID LEUKEMIA AND NORMAL HEMATOPOIESIS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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**Success is a science; if you have the conditions, you get the result.**

Oscar Wilde

**To every single person in the world…**

**ABSTRACT**

Acute myeloid leukemia (AML) is the most common hematological malignancy among adults. This disease results from an abnormal expansion of myeloid progenitors that are blocked in differentiation. Malignant transformation arises from various genetic and epigenetic changes in hematopoietic stem cells (HSCs) that accumulate with age. The overall aim of this thesis was to discover and characterize novel factors that are essential for AML and normal hematopoiesis.

In study I and study III, our aim was to discover and characterize novel genes that are selectively essential for AML. For this reason, we performed high throughput large-scale shRNA-based screens on human and mouse AML cell lines, and on non-transformed mouse hematopoietic Factor-Dependent Continuous Paterson Laboratories (FDCP mix) cell line as control cells. Based on the screening results, we have identified Chromatin Remodelling Factor 4 (CHD4) and General Transcription Factor II-I Repeat Domain-Containing Protein (GTF2IRD1) as the most promising hits, showing a highly significant selective importance in AML cell growth. Loss of function studies including both shRNA or CRISPR- Cas9 technology in normal mouse and human hematopoietic cells and various leukemia cell lines showed that either CHD4 or GTF2IRD1 were selectively required for AML cell growth, however their loss did not significantly affect normal hematopoietic cells. The importance of GTF2IRD1 or CHD4 was found to be conserved in primary AML cells, including leukemia-initiating cells, a using niche-like co-culture system. The importance of CHD4 in childhood AML was further supported by the fact that the shRNA-targeted childhood primary AML cells displayed a significantly lower level of engraftment when transplanted into a xenograft mouse model for AML. In addition, the downregulation of GTF2IRD1 in primary AML samples from both childhood and adult samples significantly prevented disease progression, compared to controls. The importance of CHD4 or GTF2IRD1 in AML was found to be associated with cell cycle progression via MYC and its target genes. Finally, using a heterologous reporter system we showed that GTF2IRD1 acts as a transcriptional repressor, us. In conclusion, we have shown that GTF2IRD1 is a transcriptional repressor required for AML maintenance both *in vitro* and *in vivo*, but not for normal hematopoietic cells. Our data demonstrated that CHD4 is a novel epigenetic factor required for the maintenance of childhood AML.

In study II, we aimed to determine the role of Euchromatic Histone Lysine Methyltransferase 1 (EHMT1) in AML and to investigate the link to its homolog, EHMT2. Our *in vitro* data showed that both EHMT1 and EHMT2 were required for growth of various leukemic cell lines. CRISPR-Cas9 based individual knock-out of EHMT1 and EHMT2 prevented AML cell growth

to a comparable degree, while double knock-out of EHMT1/2 did not show any additive or synergistic effect in growth of AML cell lines. Moreover, downregulation of EHMT1 using shRNA caused a remarkable reduction in engraftment of both childhood and adult primary AML cells in recipient mice, suggesting that downregulation of EHMT1 can prevent AML disease progression. Although downregulation of EHMT1 or EHMT2 caused a block in the G0 phase of the cell cycle, the apoptotic rate was not significantly increased. RNA-sequencing analysis showed that suppression of either EHMT1 or EHMT2 led to both overlapping and non-overlapping changes in gene expression linked to different biological processes.

In study IV, our goal was to identify and characterize novel factors in HSC function and normal hematopoiesis via bioinformatics analysis of various gene expression datasets of hematopoietic cells. From this analysis we identified an epigenetic factor, Nucleosome Assembly Protein 1 Like 3 (NAP1L3), that was consistently more highly expressed in HSCs compared to more mature cells. Loss of NAP1L3 function or overexpression of Nucleosome Assembly Protein 1 Like 3 (NAP1L3) caused a reduction in the number of myeloid progenitor cells and colony-forming cells *in vitro*. shRNA-mediated knock-down of NAP1L3 in umbilical cord blood (UCB) HSCs resulted in disruption of HSC proliferation and maintenance, both *in vitro* and *in vivo*. Moreover, loss of NAP1L3 function led to an impaired repopulation capacity of HSCs *in vivo*. Suppression of NAP1L3 in UCB HSCs results in block of cell cycle progression in the G0 phase and inhibits transcription of gene sets linked to cell cycle progression, including E2F and MYC. In addition, we observed upregulation of HOXA gene clusters such as HOXA3, HOXA5, HOXA6, and HOXA9 genes upon downregulation of NAP1L3 in UCB HSCs.

In summary; we determined functionally relevant genes (CHD4, EHMT1 and GTF2IRD1) that are specifically required for AML without significant effects on normal hematopoietic cells. We thus suggested that these target genes may serve as potential therapeutic targets in AML treatment. In addition, we demonstrated an important role of NAP1L3 in HSC homeostasis and hematopoietic differentiation**.**

**LIST OF SCIENTIFIC PAPERS**

I. **The chromatin-remodeling factor CHD4 is required for maintenance of childhood acute myeloid leukemia.**
Yaser Heshmati, **Gözde Türköz**, Aditya Harisankar, Shabnam Kharazi, Johan Boström, Esmat Kamali Dolatabadi, Aleksandra Krstic, David Chang, Robert Månsson, Mikael Altun, Hong Qian and Julian Walfridsson. *Haematologica, 2018, volume 103 (7), 1169-1181*.

II. **The histone methyltransferase EHMT1 plays both independent and cooperative regulatory role in the maintenance of Acute Myeloid Leukemia**
Yaser Heshmati*****, **Gözde Türköz***, Emma Wagner, Aditya Harisankar, Johan Boström, Mikael Altun, Hong Qian and Julian Walfridsson. *Manuscript*.
*Authors contributed equally to this study.

III. **Identification of GTF2IRD1 as a novel transcription factor essential for acute myeloid leukemia.**
Yaser Heshmati, **Gözde Türköz**, Marios Dimitriou, Aditya Harisankar, Johan Boström, Mikael Altun, Hong Qian, Nadir Kadri, Julian Walfridsson. *Manuscript*.

IV. **The histone chaperone NAP1L3 is required for hematopoietic stem cell maintenance and differentiation.**
Yaser Heshmati, Shabnam Kharazi, **Gözde Türköz**, David Chang, Esmat Kamali Dolatabadi, Johan Boström, Aleksandra Krstic, Theodora Boukoura, Emma Wagner, Nadir Kadri, Robert Månsson, Mikael Altun, Hong Qian, Julian Walfridsson. *Scientific reports, 2018, volume 8(1)11202*.

**CONTENTS**

**LIST OF ABBREVIATIONS**

| | |
|---|---|
| AML | Acute Myeloid Leukemia |
| BCL-2 | B-Cell Leukemia/Lymphoma-2 |
| BMI-1 | B Lymphoma Mo-MLV Insertion region 1 |
| CAR-T | Chimeric Antigen Receptor-Engineered T cell |
| CAS9 | CRISPR Associated Protein 9 |
| CD | Cluster of Differentiation |
| CFU | Colony Forming Unit |
| CFU-E/BFU-E | CFU-Erythrocytes |
| CFU-G/GM | CFU-Granulocytes/Macrophages |
| CFU-GEM | CFU-Granulocyte, Erythrocyte, Monocyte/macrophage |
| CFU-M | CFU-Macrophages |
| CHD4 | Chromodomain Helicase DNA Binding Protein 4 |
| CLP | Common Lymphoid Progenitor |
| CMP | Common Myeloid Progenitor |
| CRISPR | Clustered Regularly Interspaced Short Palindrome Repeats |
| CSC | Cancer Stem Cell |
| DEGs | Differential Expressed Genes |
| EHMT1/2 | Euchromatic Histone Lysine Methyltransferase ½ |
| DNMT | DNA Methyltransferase |
| DOT1L | Disruptor of Telomeric Silencing 1-Like |
| DSB | Double-Strand Break |
| ELN | European Leukemia Net |
| EMA | European Medicines Agency |
| EZH2 | Enhancer of Zeste Homolog 2 |
| FAB | French American British |
| FDA | Food and Drug Administration |
| FDCP | Factor-Dependent Continuous Paterson Laboratories |
| FLT3 | Fms-Like Tyrosine Kinase 3 |
| GM-CSF | Granulocyte-Macrophage Colony-Stimulating Factor |
| GMP | Granulocyte/Macrophage Progenitor |
| gRNA | Guide RNA |
| GSEA | Gene Set Enrichment Analysis |
| GTF2IRD1 | GTF2I Repeat Domain Containing 1 |

| | |
|---|---|
| GVHD | Graft Versus Host Disease |
| HAT | Histone Acetyl Transferase |
| HOX | Homeobox |
| HR | Homologous Recombination |
| HSC | Hematopoietic Stem Cell |
| HSPCs | Hemotopoietic Stem and Progenitor Cells |
| IDH | Isocitrate dehydrogenase |
| LT-HSC | Long-Term HSC |
| LSD1 | Lysine (K)-Specific Demethylase 1 |
| LIC | Leukemia Initiating Cell |
| MEP | Megakaryocyte/Erythroid Progenitor |
| MLL1 | Mixed Lineage Leukemia 1 |
| MLP | Multi-Lymphoid Progenitor |
| MPP | Multipotent progenitor |
| MRD | Minimal Residual Disease |
| MSC | Mesenchymal Stem Cells |
| MSigDB | Molecular Signature Data Base, |
| NAP1L3 | Nucleosome Assembly Protein 1 Like 3 |
| NGS | Next-Generation Sequencing |
| NHEJ | Non-Homologous End Joining |
| NK | Natural Killer Cell |
| NSG-SGM3 | NOD Scid IL2Rgnull-3-SCF/GM/IL3 |
| PAM | Proto-spacer Adjacent Motif |
| PcG | Polycomb Group |
| PLK1 | Polo-Like Kinase 1 |
| PRC | Polycomb Repressive Complex |
| SCF | Stem Cell Factor |
| shRNA | short hairpin RNA |
| ST-HSC | Short-Term HSC |
| TET2 | Tet methylcytosine dioxygenase 2 |
| UCB | Umbilical Cord Blood |
| WHO | World Health Organization |

# 1 INTRODUCTION

## 1.1 Normal Hematopoiesis and Hematopoietic Stem Cells (HSCs)

Hematopoiesis, the formation of blood cells that mainly occurs in the bone marrow, is a dynamic process that requires the coordination of various cell-fate choices throughout the life of an individual (Galloway and Zon 2003). It is a hierarchical process with hematopoietic stem cells (HSCs) at the apex of this hierarchy (Figure 1) (Orkin 2000). In the bone marrow, HSCs represent rare populations that possess multipotent and self-renewal properties (Attar and Scadden 2004; Lessard, Faubert et al. 2004). HSCs can undergo both symmetrical and asymmetrical divisions. The former generates two daughter HSCs leading to expansion of stem cells in the bone marrow. Asymmetrical division, conversely, results in one HSC which is identical to the parental HSC and another more mature progenitor which can differentiate into mature blood cells (Attar and Scadden 2004; Nakamura-Ishizu, Takizawa et al. 2014). During differentiation, HSCs gradually lose their proliferative potential and multi-lineage capacity while progressively gaining differentiated cell characteristics (Kondo, Wagers et al. 2003). HSC compartment can be divided into Short-Term HSCs (ST-HSCs) and Long-Term HSCs (LT-HSCs). LT-HSCs have been defined based on their reconstitution ability beyond twelve weeks of transplantation. ST-HSCs also exhibit the potential to self-renew and generate all hematopoietic lineages, though in a more limited capacity than LT-HSCs (Benveniste, Frelin et al. 2010).

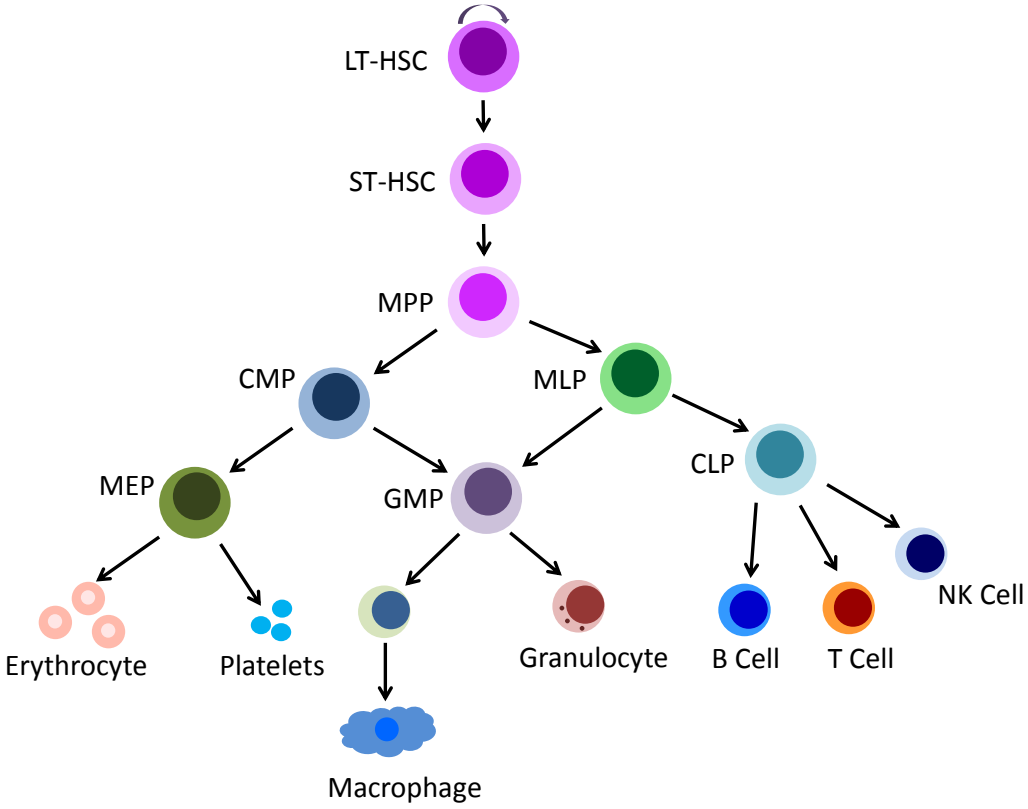


**Figure 1. Hematopoietic Hierarchy.** Hematopoietic stem cells are responsible for the formation of fully differentiated functional blood cells in a hierarchical manner. LT-HSC, Long term hematopoietic stem cell; ST-HSC, Short term hematopoietic stem cell; MPP, multipotent progenitor cell; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; MLP, multi-lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; NK, natural killer cell. Figure adapted with permission from publisher (Blank and Karlsson 2015).

### 1.1.1 Regulation of Normal Hematopoiesis

#### 1.1.1.1 Hematopoiesis is regulated by Intrinsic or Extrinsic Factors

HSCs are defined based on the self-renewal capacity and the ability to differentiate into all hematopoietic cell lineages (Orkin and Zon 2008). The balance between self-renewal and differentiation is tightly modulated by intrinsic and extrinsic factors in order to maintain a sufficient pool of primitive cells for sustaining hematopoiesis. Extrinsic factors are soluble molecules such as cytokines and growth factors that are supplied by the bone marrow microenvironment (Rizo, Vellenga et al. 2006). The bone marrow microenvironment provides a hypoxic and limited-nutrient milieu, supporting the maintenance of G0 phase and hence HSC quiescence. Moreover, the bone marrow niche contains various other cell types including mesenchymal stem cells (MSCs), osteoprogenitors, osteoblasts, osteocytes, and chondrocytes, which physically interact with HSCs and contribute to their maintenance (Kosan and Godmann

2016). Intrinsic factors include lineage-specific transcription factors and epigenetic factors. The regulation of lineage-specific gene expression is mediated by transcription factors in combination with epigenetic factors. This process is critical for regulating lineage commitment, HSC maintenance and differentiation during hematopoiesis (Rizo, Vellenga et al. 2006; Arinobu, Mizuno et al. 2007).

#### 1.1.1.2 Epigenetic Regulation of Normal Hematopoiesis

Epigenetic modifications are the inherited changes (e.g. DNA methylation, histone modifications, etc.) that do not comprise alterations in DNA sequences (Zhu and Reinberg 2011). These modifications modulate DNA accessibility through the reposition of nucleosome structure or the recruitment of proteins comprising chromatin-remodeling complexes (Bouazoune and Brehm 2006; Rippe, Schrader et al. 2007). Specific chromatin structures enable the transcription of certain lineage-related genes while repressing the unrelated ones during lineage specification of HSCs (Raghuwanshi, Dahariya et al. 2017).

Post-translational histone modifications are critical for the regulation of transcription, DNA replication, DNA repair, and DNA condensation (Kouzarides 2007). The most extensively studied histone modifications are the methylation and acetylation of histone proteins (Cullen, Mayle et al. 2014). Histone methylation is a reversible process that is governed by histone methyltransferases (Bannister and Kouzarides 2005). Methylation of histone H3 lysine 4 (H3K4), 36 (H3K36) and 79 (H3K79) are involved in transcriptional activation while di-/trimethylation of histone 3 Lysine 9 (H3K9) and 27 (H3K27) are involved in transcriptional repression (Goyama and Kitamura 2017).

Polycomb group (PcG) proteins are epigenetic modifiers that play a role in gene repression by mediating H3K27 methylation (Smith, Lee et al. 2008). PcG proteins form two major multiprotein chromatin-associated complexes known as Polycomb Repressive Complex (PRC) 1 and PRC2. PRC1 complex consists of RAE/MPH1, BMI1, M33/CBX, MEL18, and RING1/2 (Sharma and Gurudutta 2016). B lymphoma Mo-MLV insertion region 1 homolog (BMI1) is one of the best characterized proteins among PRC1 and its expression is known to be important in HSC function (Rizo, Olthof et al. 2009). Studies showed that BMI1 was also required for the self-renewal and long term repopulation capacity of HSC (Park, Qian et al. 2003; Nakauchi, Oguro et al. 2005; Rizo, Olthof et al. 2009). The PRC2 complex contains EZH2, EED, SUZ12 and RBAP46/48. Enhancer of Zeste Homolog 2 (EZH2) is the catalytic component of the PRC2 complex and involved in the di-/trimethylation of H3K27.

Overexpression of EZH2 caused an increase in HSC self-renewal (Kamminga, Bystrykh et al. 2006) and it is also shown to be important in T and B cell development (Su, Basavaraj et al. 2003; Su, Dobenecker et al. 2005).

The Mixed Lineage Leukemia 1 (MLL1) protein is a histone methyl transferase that introduces mono-/ di-/trimethylation to H3K4, leading to transcriptional activation (Nakamura, Mori et al. 2002). It was demonstrated that Mll1 plays a critical role in the maintenance of hemotopoietic stem and progenitor cells (HSPCs) by mediating the expression of Homeobox (Hox) genes (Jude, Climer et al. 2007; McMahon, Hiew et al. 2007).

The Disruptor of Telomeric Silencing 1-Like (DOT1L) protein is a methyltransferase that catalyzes the mono-/ di-/trimethylation of H3K79 (Min, Feng et al. 2003). H3K79 methylation was associated with transcriptional activation (Steger, Lefterova et al. 2008). Loss of Dot1l function caused a reduction in the number of HSCs and progenitor cells (Jo, Granowicz et al. 2011; Nguyen, He et al. 2011). It was also suggested that Dot1l has a critical role in erythropoiesis during early hematopoiesis by modulating transcriptional levels of the Gata2 and PU.1 transcription factors (Feng, Yang et al. 2010).

Another extensively studied histone modification is histone acetylation. This process involves the transfer of one acetyl group to lysine residues of histone proteins. Histone acetylation is governed by histone acetyl transferases (HATs) and is correlated with transcriptional activation (Sharma and Gurudutta 2016; Goyama and Kitamura 2017). HATs that are important in hematopoiesis include PCAF, Tip60, MOZ, and CBP/p300 (Sun XJ, Man N, Tan Y, Nimer SD, Wang L (Sun, Man et al. 2015). MOZ deficiency results in impairment in the number of HSPCs and B cell development in mice (Thomas, Corcoran et al. 2006; Perez-Campo, Borrow et al. 2009) while p300 was shown to be involved in hematopoietic differentiation (Oike, Takakura et al. 1999).

The other epigenetic modification that modulates gene expression in concert with histone modifications is DNA methylation. DNA methylation is a dynamic process that creates heritable epigenetic marks. DNA methyltransferases (DNMTs) including DNMT3a, DNMT3b and DNMT1 facilitate the addition of a methyl group to DNA (Kim and Costello 2017). DNA methylation regulates gene transcription by recruiting transcriptional activators or repressors which establish active or silent chromatin, respectively (Thomson, Skene et al. 2010; Fournier, Sasai et al. 2012). The genes important for sustaining undifferentiated state of

the HSCs or progenitors are highly methylated (Gereige and Mikkola 2009). However, as progenitors become primed for lineage differentiation, these genes lose their methylation pattern (Ji, Ehrlich et al. 2010; Hogart, Lichtenberg et al. 2012). Therefore, DNA methylation levels are tightly regulated during myeloid or lymphoid differentiation of HSCs and the DNA methylation pattern is uniquely present for specific cell types during hematopoietic differentiation (Ji, Ehrlich et al. 2010).

## 1.2 Malignant Hematopoiesis and Acute Myeloid Leukemia

### 1.2.1 Leukemogenesis

Leukemogenesis is a multistep process that includes both pre-leukemia and leukemia states (Jan, Snyder et al. 2012). Identification and characterization of a leukemia initiating cell (LIC) are major steps towards understanding the cellular and molecular mechanism of leukemogenesis (Somervaille and Cleary 2006). During the pre-leukemia phase, HSCs acquire primary and secondary mutations that provide a growth advantage. HSCs carrying pre-leukemic mutations maintain their normal differentiation and self-renewal capabilities (Corces-Zimmerman and Majeti 2014; Shlush and Mitchell 2015). Pre-leukemic HSCs transform into leukemia initiating cells (LICs) following the acquisition of additional mutations in the leukemic phase (Figure 2) (Uribesalgo and Di Croce 2011). A cohort study among Acute Myeloid Leukemia (AML) patients indicated that patients carrying pre-leukemic mutations with high allelic burden had a worse overall or relapse-free survival (Corces, Buenrostro et al. 2016). Accordingly, another study suggested that clones with pre-leukemic mutations were resistant to conventional chemotherapy and persistence of pre-leukemic clones might gain additional mutations, leading to relapse (Corces-Zimmerman, Hong et al. 2014). During the leukemic phase, pre-leukemic cells gain an indefinite self-renewal potential and generate blast cells that have severe differentiation abnormalities and high proliferative capacity. Subsequently, blast cells out-compete normal cells and take over the entire hematopoietic system, leading to bone marrow failure (Lapidot, Sirard et al. 1994; Bonnet and Dick 1997).

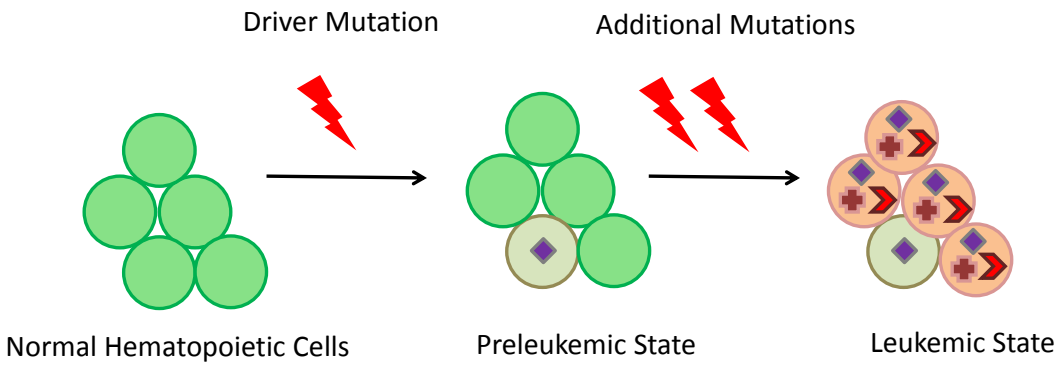


**Figure 2. Multistage Process of Leukemogenesis.**

A driver mutation in normal hematopoietic cells induces the pre-leukemic state and the further additional mutations lead to the leukemic state. Figure adapted with permission from publisher (Uribesalgo and Di Croce 2011).

### 1.2.2 Acute Myeloid Leukemia (AML)

AML is an aggressive hematological disease characterized by clonal expansion of immature myeloid cells (blasts) in the bone marrow and blood (Shlush and Minden 2015). Epidemiological risk factors for AML development include environmental factors such as smoking and benzene exposure, therapy-related factors such as chemotherapeutic agents or radiotherapy exposure, and familial/genetic factors such as Down syndrome or the presence of the Philadelphia chromosome (Zeeb and Blettner 1998). AML originates from HSC with a stepwise acquisition of genetic and epigenetic alterations. These accumulated mutations compromise normal HSC functions, blocking differentiation and increasing self-renewal capacity (Corces-Zimmerman and Majeti 2014). As a consequence, AML comprises heterogeneous populations of leukemic cells. The evolution of leukemic clones occurs during the leukemic phase and the heterogeneity of these clones depends on the length of this phase, environmental exposure, and mutation rate (Shlush and Mitchell 2015). Mutational acquisition is a dynamic process and different cell subpopulations harboring different mutations can be found within a leukemic patient (Cancer Genome Atlas Research, Ley et al. 2013). Distinct sub-clones with certain genetic changes can lead to relapse in AML patients (Shlush, Chapal-Ilani et al. 2012). These genetic changes include single nucleotide changes, deletions or insertions, translocations, copy number variations and loss of heterozygosity (Byrd, Mrozek et al. 2002; Cancer Genome Atlas Research, Ley et al. 2013). Each of these genetic changes has different mechanisms in the underlying pathogenesis of AML and the combination of these genetic changes within distinct sub-clones forms a complex leukemic profile (Paguirigan, Smith et al. 2015).

AML classification is important for understanding disease pathogenesis, the appropriate prognosis and therapy regimens (Cancer Genome Atlas Research, Ley et al. 2013). Previously, AML was classified using French American British (FAB) classification system. This classification system divided AML into eight subtypes, M0 to M7, based on the differentiation status of malignant cells (Table 1). The classification was established based on the morphology of malignant cells under the microscope and/or cytogenetic analysis (Bennett, Catovsky et al. 1976). In 2002, the FAB classification system was replaced with the World Health Organization (WHO) classification system, which was revised in 2008 and again in 2016 (Vardiman, Harris et al. 2002; Wandt, Haferlach et al. 2010; Arber, Orazi et al. 2016). The WHO classification system is based on morphology, immune-phenotype, genetic profile and clinical properties (Table 2). At least twenty percent of nucleated cells should be present in the bone marrow or blood for AML diagnosis, according to this system (Arber, Orazi et al. 2016). Moreover, an updated classification system was introduced to European Leukemia Net (ELN) risk stratification in 2017 based on patient genetics (Dohner, Estey et al. 2017). Today, whole genome sequencing has shown that AML is indeed an extremely complex disease, consisting of more than ten different subtypes based on molecular characteristics (Welch, Ley et al. 2012; Cancer Genome Atlas Research, Ley et al. 2013; Papaemmanuil, Gerstung et al. 2016).

**Table 1. French American British Classification System of AML.** Table reprinted with permission from publication (Kabel, Zamzami et al. 2017).

| **Subtype** | **Name** |
|---|---|
| M0 | Undifferentiated acute myeloblastic |
| M1 | Acute myeloblastic leukemia with minimal maturation |
| M2 | Acute myeloblastic leukemia with maturation |
| M3 | Acute promyelocytic leukemia |
| M4 | Acute myelomonocytic leukemia |
| M5 | Acute monocytic leukemia |
| M6 | Acute erythroid leukemia |
| M7 | Acute megakaryoblastic leukemia |

**Table 2. World Health Organization Classification System of AML (2016)** Table reprinted with permission from publisher (Arber, Orazi et al. 2016)

| **Acute myeloid leukemia and related neoplasms** |
|---|
| AML with recurrent genetic abnormalities |
| AML with t(8;21)(q22;q22.1); RUNX1-RUNX1T1<br>AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);CBFB-MYH11<br>APL with PML-RARA<br>AML with t(9;11)(p21.3;q23.3);MLLT3-KMT2A<br>AML with t(6;9)(p23;q34.1);DEK-NUP214<br>AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM<br>AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1<br>Provisional entity: AML with BCR-ABL1<br>AML with mutated NPM1<br>AML with biallelic mutations of CEBPA<br>Provisional entity: AML with mutated RUNX1 |
| AML with myelodysplasia-related changes |
| Therapy-related myeloid neoplasms |
| AML, not otherwise specified (NOS) |
| AML with minimal differentiation<br>AML without maturation<br>AML with maturation<br>Acute myelomonocytic leukemia<br>Acute monoblastic/monocytic leukemia<br>Pure erythroid leukemia<br>Acute megakaryoblastic leukemia<br>Acute basophilic leukemia<br>Acute panmyelosis with myelofibrosis |
| Myeloid sarcoma |
| Myeloid proliferations related to Down syndrome |
| Transient abnormal myelopoiesis (TAM)<br>Myeloid leukemia associated with Down syndrome |

**Table 3. ELN 2017 Risk Stratification by Genetics.** Table reprinted with permission from publisher (Dohner, Estey et al. 2017)

| Risk Category | Genetic Abnormality |
|---|---|
| Favorable | t(8;21)(q22;q22.1); RUNX1-RUNX1T1<br><br>inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11<br><br>Mutated NPM1 without FLT3-ITD or with FLT3-ITDlow[1]<br><br>Biallelic mutated CEBPA |
| Intermediate | Mutated NPM1 and FLT3-ITDhigh[1]<br>Wild-type NPM1 without FLT3-ITD or with FLT3-ITDlow[1] (without adverse-risk genetic lesions)<br>t(9;11)(p21.3;q23.3); MLLT3-KMT2A[2]<br>Cytogenetic abnormalities not classified as favorable or adverse |
| Adverse | t(6;9)(p23;q34.1); DEK-NUP214<br><br>t(v;11q23.3); KMT2A rearranged<br><br>t(9;22)(q34.1;q11.2); BCR-ABL1<br><br>inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2);GATA2,MECOM(EVI1)<br><br>25 or del(5q); 27; 217/abn(17p)<br><br>Complex karyotype[3], monosomal karyotype[4]<br><br>Wild-type NPM1 and FLT3-ITDhigh[1]<br><br>Mutated RUNX1[5]<br><br>Mutated ASXL1[5]<br><br>Mutated TP53[6] |

[1]Low, low allelic ratio (<0.5); high, high allelic ratio (≥0.5); semiquantitative assessment of FLT3-ITD allelic ratio (using DNA fragment analysis) is determined as ratio of the area under the curve "FLT3-ITD" divided by area under the curve "FLT3-wild type"; recent studies indicate that AML with NPM1 mutation and FLT3-ITD low allelic ratio may also have a more favorable prognosis and patients should not routinely be assigned to allogeneic hematopoietic cell transplantation.
[2]The presence of t(9;11)(p21.3;q23.3) takes precedence over rare, concurrent adverse-risk gene mutations.
[3]Three or more unrelated chromosome abnormalities in the absence of 1 of the WHO designated recurring translocations or inversions, that is, t(8;21), inv(16) or t(16;16), t(9;11), t(v;11)(v;q23.3), t(6;9), inv(3) or t(3;3);AML with BCR-ABL1.
[4]Defined by the presence of 1 single monosomy (excluding loss of X or Y) in association with at least 1 additional monosomy or structural chromosome abnormality (excluding core binding factor AML)
[5]These markers should not be used as an adverse prognostic marker if they co-occur with favorable-risk AML subtypes.
[6]TP53 mutations are significantly associated with AML with complex and monosomal karyotype.

#### 1.2.2.1 Leukemia Initiating Cells (LICs) in AML

The cancer stem cell (CSC) hypothesis suggests that a tumor arises from a rare population of tumor cells which possess stem cell characteristics and clonogenic growth potential. Evidence supporting the existence of the CSC was first introduced with a landmark study in AML by John Dick and colleagues. This study demonstrated that only a small population of leukemic cells, termed LICs, had the ability to initiate the disease in serial transplantation assay while the bulk of leukemic did not (Lapidot, Sirard et al. 1994).

LICs are highly tumorigenic and share similar functional properties, such as self-renewal and differentiation, with normal stem cells. This observation has led some to suggest LICs might be derived from normal stem cells (Huntly and Gilliland 2005). The regulatory pathways for self-renewal and differentiation are conserved between normal stem cells and LICs (e.g. Wnt/β catenin, Hedgehog, and Notch signaling pathways) (Kobune, Takimoto et al. 2009; Wang, Krivtsov et al. 2010; Liu, Zhang et al. 2013). These extrinsic signals are provided from the bone marrow microenvironment and affect the self-renewal and differentiation functions of HSC, consequently promoting LIC formation (Krause and Van Etten 2007). LICs might also arise from committed progenitors which have acquired the capacity for self-renewal, because LICs show phenotypic heterogeneity among AML patients (Krivtsov, Twomey et al. 2006; Kirstetter, Schuster et al. 2008). Two models have been proposed for committed progenitors as an origin of LICs. The first model proposes that an initiating mutation can occur in HSCs and additional mutations hit the downstream progenitors giving rise to the LIC (Reya, Morrison et al. 2001). Supporting this notion, a study using a C/EBPα mutant AML mouse model demonstrated that clonal expansion of pre-leukemic HSCs occurred and leukemia developed from a downstream progenitor (Kirstetter, Schuster et al. 2008). The second model suggests that downstream progenitors can be targets for the initial mutation and LICs can be generated directly from the committed progenitors. In line with this, retroviral overexpression of MLL-AF9 in committed progenitors was capable of transmitting AML into secondary recipients (Krivtsov, Twomey et al. 2006; Krivtsov, Figueroa et al. 2013).

#### 1.2.2.2 Genetic Aberrations in AML

AML is a clonal disease characterized by cytogenetic and molecular genetic alterations. These alterations lead to a differentiation block, impaired apoptosis, enhanced self-renewal and proliferation of hematopoietic precursors (Zheng, Beissert et al. 2004). As a consequence, an accumulation of AML blast cells is observed in the bone marrow and peripheral blood during AML progression, out-competing the normal hematopoietic cell compartments (Boyd, Campbell et al. 2014).

Recurrent cytogenetic abnormalities have long been established as critical markers for AML diagnosis and prognosis (Schoch and Haferlach 2002). These cytogenetic changes, observed in 50-60% of AML patients, include translocations, deletions, insertions, inversions, aneuploidies (Chen, Cortes et al. 2011; Kumar 2011; Yang, Park et al. 2017). Some translocations or inversions result in oncogenic fusion proteins that disrupt the regulation of cell growth or differentiation (Grove and Vassiliou 2014; Meyer and Levine 2014; Liesveld and Lichtman 2018).

Around 45% of AML patients have a normal karyotype with somatic alterations and/or changes in gene expression (Bacher, Schnittger et al. 2010). The development of next generation sequencing allows us to identify recurrent mutated genes in AML (Lin, Li et al. 2017). A ‘double-hit’ model of leukemogenesis in AML proposes two classes of mutations which frequently occur in AML. Class I mutations are alterations in genes that are important to regulate cell proliferation and survival. Class II mutations includes genetic changes in transcription factors crucial for differentiation processes (Figure 3) (Kelly and Gilliland 2002; Shih, Abdel-Wahab et al. 2012). Although the combination of class I and class II mutations can give rise to the malignant transformation of hematopoietic stem cells, not every AML patient carries mutations that are categorized in these classes (Takahashi 2011).

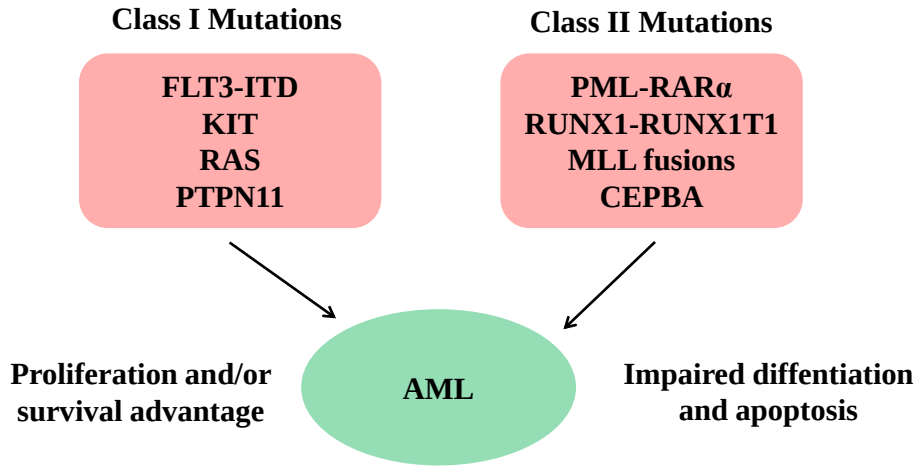


**Figure 3. Two hit process of leukemogenesis.**

Figure adapted with permission from publisher (Thapa 2013).

A genome-wide study identified genes that are commonly mutated in AML at a frequency higher than 5% including RUNX1, FLT3, NPM1, DNMT3A etc. These mutations were categorized based on their biological functions and potential roles in AML pathogenesis (Table 4) (Cancer Genome Atlas Research, Ley et al. 2013). The majority of mutations in cancer genomes might result in genomic instability and subsequent clonal evolution. However, genetic instability is not common in the AML genome, because the mutational status remains unchanged during disease progression (Welch, Ley et al. 2012; Tsai, Hou et al. 2017). In comparison to solid tumors, which might possess more than a hundred mutated genes, the AML genome has a small number of mutations with an average of only thirteen cancer-specific mutations per case (Cancer Genome Atlas Research, Ley et al. 2013; Xu, Gu et al. 2014).

**Table 4. Functional Category of Mutated Genes in AML.** Table reprinted with permission from publisher (Khwaja, Bjorkholm et al. 2016).

| Functional Category | Example of Mutations |
|---|---|
| Signaling pathways | FLT3, KRAS, NRAS, KIT, PTPN11, and NF1 |
| Epigenetic modifiers (DNA methylation and chromatin modification) | DNMT3A, IDH1, IDH2, TET2, ASXL1, EZH2, and MLL/KMT2A |
| Nucleophosmin | NPM1 |
| Transcription factors | CEBPA, RUNX1, and GATA2 |
| Tumor suppressors | TP53 |
| Spliceosome complex | SRSF2, U2AF1, SF3B1, and ZRSR2 |
| Cohesin complex | RAD21, STAG1, STAG2, SMC1A, and SMC3 |

### 1.2.2.3 Epigenetic Aberrations in AML

Epigenetic-regulator mutations have been shown to drive the development of various cancers, including AML. Mutations in epigenetic modifiers are known to be early events during AML development which are critical for AML initiation and/or disease progression (Jan, Snyder et al. 2012). Supporting this notion, studies have demonstrated that mutations in epigenetic regulators disrupt the self-renewal and/or differentiation functions of HSCs (Challen, Sun et al. 2011; Ko, Bandukwala et al. 2011). These mutations cause epigenetic alterations including DNA methylation (e.g. DNMT3A, TET2, IDH mutations etc.) and histone modifications (MLL fusions etc.) in the AML genome and trigger malignant transformation (Marcucci, Haferlach et al. 2011).

DNA methylation is an epigenetic process that regulates gene expression through the addition of a methyl group to DNA. CpG islands are DNA sequences containing a higher frequency of CpG dinucleotides. The methylation of CpG islands is mediated by DNMT1, DNMT3A and DNMT3B (Jones and Liang 2009). DNMT3A and DNMT3B contribute to the establishment of the methylated sites while DNMT1 facilitates their maintenance. CpG islands are enriched at promoter regions and their methylation is associated with heterochromatin and transcriptional repression. Alterations in DNA methylation are hallmark of cancer (Feinberg and Tycko 2004). Cancer genomes are notable by global hypomethylation at CpG islands of intragenic and intergenic regions (except for promoter regions) (Ehrlich 2009). These methylation signatures are similar to the ones in AML (Cancer Genome Atlas Research, Ley et al. 2013). The mechanism by which global hypomethylation contributes to cancer is not clear. However, hypomethylation was demonstrated to cause genomic instability and an increase in expression of cancer-associated genes (Shao, Lacey et al. 2009). In addition, hypermethylation at CpG islands of gene promoters of tumor suppressor genes or genes involved in cell cycle control, apoptosis and differentiation results in silencing of these genes. This is frequently observed in AML (Alvarez, Suela et al. 2010). Mutations in DNMT3A are well known to be frequent in AML patients having normal karyotype and were proposed to be initial events prior to AML development (Sun, Shen et al. 2016). In line with this notion, loss of DNMT3A function resulted in an increased self-renewal capacity and an impaired multi-lineage differentiation, serving as a pre-leukemic HSC (Ley, Ding et al. 2010). Moreover, it was shown that patients with DNMT3A mutations had global hypomethylation compared to DNMT3A wild type patients. Hypomethylated genomic regions were enriched for HOX genes, which have role in normal hematopoiesis and leukemogenesis (Qu, Lennartsson et al. 2014). Genome-wide methylation profiling showed the presence of unique DNA methylation signatures in AML as

compared to normal controls. These signatures were proposed to be used to identify the prognostically relevant AML subtypes. Moreover, methylation profiles revealed a gene subset specifically hypermethylated and inactivated in AML, suggesting a role of certain epigenetic pathways in AML transformation (Figueroa, Lugthart et al. 2010).

Isocitrate dehydrogenases (IDHs) are enzymes which convert isocitrate to $\alpha$-ketoglutarate. Mutations in IDH enzymes result in formation of the 2-hydroxyglutarate oncometabolite that inhibits the function of Tet Methylcytosine Dioxygenase 2 (TET2) (Marcucci, Maharry et al. 2010). The diminished TET2 function leads to the DNA hypermethlylation phenotype and dampens histone demethylation through histone demethylase inhibition. Impairment of the histone demethylation process led to the clonal expansion of HSCs and a block in myeloid differentiation (Figueroa, Abdel-Wahab et al. 2010). A large genetic and epigenetic cohort study demonstrated that AML patients with IDH mutations had global DNA hypermethylation (Figueroa, Lugthart et al. 2010). Similarly, another cohort study that included 398 AML patients, indicated that IDH1 or 2 mutations were correlated with abnormal hypermethylation at promoter regions of genes important for myeloid cell maturation and leukemic transformation processes (Fernandez, Sun et al. 2009).

TET2 accounts for DNA hydroxymethylation by converting 5-methylcytosine to 5-hydroxymethylcytosine (Metzeler, Maharry et al. 2011). Mutations in this enzyme result in the disruption of protein function and decreased 5-hydroxymethylcytosine levels (Liu, Zhang et al. 2013). In line of this evidence, it was shown that TET2 inactivation triggered the DNA hypermethylation at enhancer regions of tumor suppressor genes in AML, causing a reduction in H3K27Ac levels and their transcription. TET2 mutations have been suggested as pre-leukemic events in AML, because TET2 inactivation was observed in pre-leukemic stem cells (Metzeler, Maharry et al. 2011). Genetic TET2 inactivation in mice resulted in aberrant hematopoietic differentiation and an increased HSC pool (Li, Cai et al. 2011; Moran-Crusio, Reavie et al. 2011; Shide, Kameda et al. 2012), leading the leukemic development.

MLL is an epigenetic regulator protein that exhibits histone 3 lysine 4 (H3K4) methyltransferase activities. MLL has different fusion partners involved in leukemogenesis such as AF4, AF9 and ENL. MLL partners recruit multi-protein complexes involved in transcriptional elongation, histone acetylation or DNA/RNA binding in the nucleus (Scandura, Boccuni et al. 2002; Cosgrove and Patel 2010). MLL translocations disrupt its histone methyltransferase activity and are associated with AML aggressiveness. The most

frequent translocation found in AML patients is t(9;11) encoding the oncogenic MLL-AF9 fusion protein. MLL-AF9 is involved in the transcriptional activation of important genes such as HOX-A9 and MEIS-1 by catalyzing H3K4me2, resulting in a differentiation block and increased self-renewal of HSCs (Zhu, Chen et al. 2016).

#### 1.2.2.4 Standard Care of Treatment for AML

The treatment strategy for AML has remained unchanged over thirty years. Standard treatments include intense induction chemotherapy and consolidation therapy (Cheson, Bennett et al. 2003). Studies related to genetic lesions within AML clearly showed that AML possessed a complex genetic profile with clonal heterogeneity. The disease heterogeneity affects patients' outcomes in response to AML treatment (Shen, Zhu et al. 2011; Patel, Gonen et al. 2012; Cancer Genome Atlas Research, Ley et al. 2013). Moreover, chemotherapy also contributes to AML clonal diversity by generating new mutations, possibly resulting in therapy resistance and relapse (Ding, Ley et al. 2012).

Induction therapy consists of an intensive Anthracycline and Cytarabine regimen. Administration of induction therapy continues for one week and the aim of this therapy is to reach complete remission, which is defined as having lower than 5% blasts in the bone marrow (Cheson, Bennett et al. 2003). Complete remission is achieved with the induction regimen in 60-85 % of patients who are 60 years old or younger. However, patients older than 60 years of age are not able to tolerate the intensive chemotherapy, therefore less intense therapy in combination with a palliative regimen including hypomethylating agents is utilized (Al-Ali, Jaekel et al. 2014).

Consolidation therapy, to avoid relapse and remove minimal residual disease (MRD) in bone marrow, includes two to four cycles of intermediate-dose Cytarabine and/or HSC transplantation (Byrd, Mrozek et al. 2002). Quantitative real time PCR is used to monitor MRD in patients given consolidation therapy (Grimwade and Freeman 2014). Consolidation therapy with allogeneic hematopoietic stem cell transplantation is known to be the most effective therapy for long-term survival of AML patients, with a success rate between 50% and 60% (Appelbaum 2003; Popat, de Lima et al. 2012).

Stem cell transplantation may be helpful for patients who fail to achieve first remission with induction therapy or for patients who achieve initial remission but develop relapse at some point after the treatment end (Koreth, Schlenk et al. 2009; Armand, Kim et al. 2014). Chemo-

radiotherapy conditioning is used to kill the leukemic cells that could be resistant to the chemotherapy. This conditioning is followed by transfusion of donor hematopoietic cells that mount immune responses to eradicate any residual leukemic cells, known as the graft-versus-leukemia effect (Gupta, Tallman et al. 2011). Although stem cell transplantation is the most effective AML therapy, it can induce severe complications including graft versus host disease (GVHD), that results in transplant-related morbidity and mortality (Dohner, Weisdorf et al. 2015).

#### 1.2.2.5 New Therapeutic Approaches in AML

AML is a heterogeneous disease due to the interplay between genetics and epigenetics (Cancer Genome Atlas Research, Ley et al. 2013; Papaemmanuil, Gerstung et al. 2016). Despite many efforts towards understanding the pathogenesis of AML and its heterogeneous molecular landscape, standard chemotherapy cannot eradicate the small population of quiescent LICs and the majority of patients have a high risk of relapse associated with remarkable mortality (Pollyea, Gutman et al. 2014). The success rate of AML treatment is 35-40% in patients who are 60 years old or younger and just 5-15% in patients older than 60 years of age (Dohner, Estey et al. 2010). For these reasons, researchers are studying to identify more effective therapeutic regimens for AML. New targeting therapies in AML against signaling pathways including tyrosine kinases, epigenetic processes and antigens that are specifically expressed in LIC were achieved by advances in genome wide studies (Dawson, Kouzarides et al. 2012; Abdel-Wahab and Levine 2013; Gasiorowski, Clark et al. 2014; Wander, Levis et al. 2014; DiNardo and Cortes 2015).

##### 1.2.2.5.1 Targeted Therapy for LICs

LICs, being critical in the initiation and progression of leukemia, are selectively resistant to standard treatments, resulting in treatment failure. Current treatments are not specific enough to eliminate LICs following disease recurrence and also have toxic effects on healthy tissues. Therefore, it is essential to develop novel therapeutic strategies specifically targeting LICs in to eradicate these cells and thus possibly cure the disease (Figure 4) (Jordan 2002; Somervaille and Cleary 2006).

Anti-apoptotic protein B-cell leukemia/lymphoma-2 (BCL-2) is overexpressed in AML LICs compared to normal compartments (Lagadinou, Sach et al. 2013). BCL-2 is crucial for chemo-resistant properties of LICs due to its role in supporting cell survival and maintenance. Clinical trials of Venetoclax, a BCL-2 inhibitor, are ongoing in combination

with Azacitidine/Decitabine and with low dose Cytarabine for the treatment of naive elderly patients who are not eligible for intensive chemotherapy (Lin, Strickland et al. 2016; Pollyea, Dinardo et al. 2016).

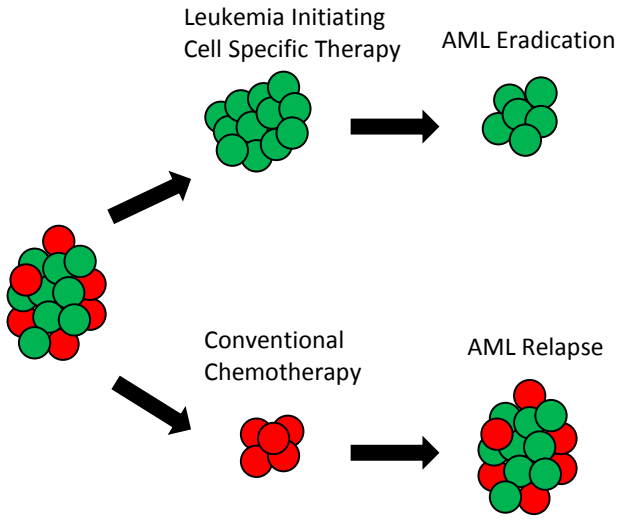


**Figure 4. LIC-specific therapy and conventional chemotherapy in AML.**
Conventional chemotherapy is effective for leukemic blasts; but it is not able to eliminate LICs and relapsed AML. However, a specific therapy for leukemia initiating cells can eradicate AML. Figure adapted with permission from publisher (Trendowski 2015). Red Circles: LICs, Green Circles: Leukemic blasts.

#### 1.2.2.5.2 Targeted Therapy for Tyrosine Kinases and the CD33 Antigen

Targeted therapies for AML have also been developed against tyrosine kinases and leukemic cell specific antigens (Walter, Raden et al. 2005; Gasiorowski, Clark et al. 2014; Wander, Levis et al. 2014). The Fms-Like Tyrosine Kinase 3 (FLT3) gene is frequently mutated in AML hence; pharmacological inhibitors against this protein have been developed for AML treatment. However, the first generation inhibitors of FLT3 were not successful because of their ineffectiveness and toxicity (Wander, Levis et al. 2014). Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved Midostaurin, a tyrosine kinase inhibitor with multiple targets including FLT3, for clinical use in AML patients (Stone, Manley et al. 2018). Midostaurin is used in combination with the conventional chemotherapy for AML treatment (Levis 2017). According to the RATIFY trial results, AML patients aged 18–59 years showed an increased median overall survival from 25.6 months to 74.7 months after the combination therapy (Stone, Mandrekar et al. 2017).

Polo-like kinase 1 (PLK1) is a critical signaling molecule for the $G_2$/M transition and the mitosis phase of the cell cycle. PLK1 has been showed to be overexpressed in AML patients

compared to normal progenitors (Santamaria, Neef et al. 2007; Renner, Dos Santos et al. 2009). Pharmacological inhibition of PLK1 using Volasertib induced cell-cycle arrest at M phase and thus apoptotic death, resulting in a block in primary AML-cell growth while sparing normal hematopoietic cells (Renner, Dos Santos et al. 2009; Yang and Wang 2018). An on-going phase III clinical trial of is testing the use of Volasertib in combination with low dose Cytarabine in elderly patients who are not eligible for intensive chemotherapy (NCT01721876) (DeAngelo, Sekeres et al. 2015).

CD33 is expressed on the majority of AML patient blasts (Dinndorf, Andrews et al. 1986). Gemtuzumab ozogamicin (GO) is the monoclonal antibody against CD33 which is conjugated with Calicheamicin (Ricart 2011). Binding of GO to the CD33 antigens on the surface of AML cells results in Calicheamicin release and causes AML cell death (van Der Velden, te Marvelde et al. 2001). A phase III randomized trial showed that older AML patients using low dose GO as a sole agent had increased overall survival (NCT00091234) (Amadori, Suciu et al. 2016). Moreover, Phase II studies also demonstrated that GO in combination with Azacitidine resulted in increased remission and survival rates in older patients (NCT00658814) (Nand, Othus et al. 2013).

#### 1.2.2.5.3 Targeted Therapy for Epigenetic Modulators

Epigenetic regulators include epigenetic writers, epigenetic erasers and epigenetic readers. Epigenetic writers catalyze the formation of epigenetic marks on DNA and histones while epigenetic erasers facilitate the reverse process to remove those marks. Epigenetic readers bind to epigenetic marks and modulate gene expression together with other epigenetic factors (Yang, Kim et al. 2016). Mutations in these epigenetic regulators are frequently found in AML. Epigenetic dysregulation promotes the transformation from a premalignant state to a malignant state in AML (Roboz 2014; Ahuja, Sharma et al. 2016; Sun, Chen et al. 2018). Unlike the genetic changes, epigenetic alterations are reversible. Thus, therapies targeting causative epigenetic regulators have become a focus in the development of AML treatments (Momparler, Cote et al. 2014).

DOT1L, an H3K79 histone methyltransferase, modulates the transcriptional elongation of genes critical for the maintenance of LICs in AML. DOT1L interacts with MLL fusion partners such as AF4, AF9 and AF10 (Okada, Feng et al. 2005; He, Chan et al. 2011). DOT1L loss of function, particularly loss of its H3K79 histone methyltransferase activity, slows disease progression in MLL-rearranged AML humanized mouse models. DOT1L has therefore been

recommended as a potential therapeutic target for AML with MLL rearrangement (Daigle, Olhava et al. 2011; Chen, Deshpande et al. 2013).

IDH mutations cause a block in hematopoietic differentiation and consequently promote AML development (Yang and Wang 2018). Enasidenib, a mutant IDH2 inhibitor, was approved by the FDA for use in relapsed/refractory AML patients with IDH2 mutations (Kim 2017). A clinical study with Enasidenib demonstrated that 41% of relapsed/refractory AML patients achieved an overall response (Stein, DiNardo et al. 2017).

In AML, DNA methylation has been found to be perturbed (Figueroa, Lugthart et al. 2010; Qu, Siggens et al. 2017). Consequently, therapies targeting DNMT enzymes have been used to restore the normal hematopoiesis via reversing abnormal DNA methylation (Navada, Steinmann et al. 2014; Deveau, Forrester et al. 2015). Hypomethylating agents including Azacitidine and Decitabine are DNMT inhibitors used for the treatment of older patients, and these agents are still under clinical investigations (Fenaux, Mufti et al. 2010; Nieto, Demolis et al. 2016; DiNardo, Pratz et al. 2018). Elderly patients who were ineligible for intensive chemotherapy exhibited clinical benefits upon treatment with Decitabine in a clinical trial. This study revealed a complete response rate of 47% among all patients (Blum, Garzon et al. 2010)

Lysine (K)-Specific Demethylase 1 (LSD1) is highly expressed in various blood diseases including AML (Amente, Lania et al. 2013; Wada, Koyama et al. 2015; Przespolewski and Wang 2016). LSD1 catalyzes the demethylation of mono- and di-methylated histone H3 lysine residues 4 and 9 or other non-histone proteins. Knock-down or pharmacological inhibition of LSD1 promotes myeloid differentiation of AML cells, which results in diminished AML cell growth and a prolonged AML latency period in AML mouse models (Harris, Huang et al. 2012; Maiques-Diaz, Spencer et al. 2018). Feng and colleagues (Feng, Yao et al. 2016) showed that small molecule inhibition of LSD1 in human MLL-rearranged AML cells led to an upregulation of pathways involved in cell apoptosis and differentiation. Taken together, these studies proposed that LSD1 might serve as an effective drug target for MLL-rearranged AML.

## 2 AIM OF THE THESIS

The aim of this thesis is to discover novel genes and relevant pathways that are required for AML and normal hematopoiesis.

### Study I

To discover and characterize novel genes that are selectively essential for AML and thus might represent potential drug targets against this disease.

### Study II

To determine the role of EHMT1 in AML and to investigate the link to its homolog, EHMT2. In addition, to investigate if EHMT1 can serve as a potential drug target.

### Study III

To identify and characterize novel factors that have a cancer-specific role in AML.

### Study IV

To investigate the role of NAP1L3 in normal hematopoiesis.

# 3 METHODOLOGICAL APPROACHES

## 3.1 Large Scale shRNA Screening

Genome wide shRNA screening is a powerful methodology that has been used to identify the genes that play roles in cancer maintenance or progression (Hoffman, Rahal et al. 2014; McDonald, de Weck et al. 2017). To identify and characterize novel factors/ pathways critical for AML cancer cell growth, we have performed high-throughput genome-wide screening using pooled lentiviral-based shRNA libraries (Cellecta Inc.). The shRNA libraries target 15,377 human and mouse protein coding genes in study I and III. We used two human AML cell lines (NOMO-1 and THP-1), a genetically defined AML mouse cell line and non-transformed mouse hematopoietic Factor-Dependent Continuous Paterson Laboratories (FDCP mix) cells as control for the screens. These shRNA libraries include a total of 82,500 hairpins and each gene is targeted by 5 shRNAs. Each shRNA in the library contains a unique barcode, allowing the identification of individual hairpins in individual cells using Next-Generation Sequencing (NGS). The rationale behind the screen is to infect a target cell population with the shRNA library as viral particles and under a suitable multiplicity of infection, majority of cells take up a single shRNA (Berns, Hijmans et al. 2004; Paddison, Silva et al. 2004). Large scale shRNA library screening allows measurement of the effects of numerous pooled hairpins at a time. This screening method results in silencing of target genes for long periods of time, facilitating result acquisition over weeks rather than days in cell culture after transduction. One of the limitations is the random integration of lentiviral vectors into the host genome, which can affect shRNA expression levels. Moreover, interpreting large scale shRNA screen data correctly is challenging because of the potential off-target effect of shRNAs, which leads to false positive outcomes (Echeverri, Beachy et al. 2006). Using multiple shRNAs against a target gene or enforcing its expression to rescue the phenotype is common ways to overcome off-target limitations (Unwalla, Li et al. 2006; Xu, Carlson et al. 2007).

## 3.2 CRISPR CAS-9 Genome Editing Technology

Class2 Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) and CRISPR associated protein 9 (Cas9) is a powerful genome editing technology capable of accurately recognizing target DNA (Cong and Zhang 2015). CRISPR was first found in bacteria as an adaptive immune system which responds to foreign genetic material (Barrangou 2015). CRISPR-Cas9 consists of two components including guide RNA (gRNA) and Cas9 (Sun, Lutz et al. 2016). gRNA, which is twenty nucleotides long, facilitates the formation of double-strand

breaks (DSBs) by bringing Cas9 to the target DNA region containing the Protospacer Adjacent Motif (PAM) sequence (Cong and Zhang 2015). When DSBs are generated, either homologous recombination (HR) or non-homologous end joining (NHEJ) is activated to repair DNA DSBs (Cong, Ran et al. 2013). The presence of homologous template induces the HR DNA repair system, allowing accurate repair (Chen, Lai et al. 2017). However, the NHEJ repair system is an error-prone pathway, leading to insertions or deletions within the genome (Cong, Ran et al. 2013). Thus, CRISPR-Cas9 technology is capable of inducing genetic changes in various organisms, including repression, activation, correction and disruption of target genes (Gilbert, Larson et al. 2013; Sander and Joung 2014; Ehrke-Schulz, Schiwon et al. 2017; Surun, Schwable et al. 2018). The cancer genome is genetically complex with multiple point mutations and chromosomal aberrations (Loeb and Loeb 2000). CRISPR Cas9 is an effective and multifaceted tool for generating *in vitro* or *in vivo* cancer models in order to investigate the molecular mechanisms of cancer initiation and progression. The knowledge gained using this system will facilitate the identification of new drug targets for cancer treatment (Chiou, Winters et al. 2015; Freedman, Brooks et al. 2015).

For knock-out studies, we took advantage of the CRISPR-Cas9 technology in study I, II, III and IV to reveal the functional importance of target genes in AML growth or in normal hematopoiesis. The rationale for using the CRISPR-Cas9 approach in addition to shRNA knock-down studies is to reduce the off-target effects of the shRNA approach. Since the performance of this technology depends on the gRNA design, in order to improve the specificity more than one gRNA were included for each target gene by targeting different exonic regions. Various human AML cell lines, a mouse AML cell line, and freshly sorted Lin-Sca+cKit+ mouse HSCs were used for the knock-out experiments. Knock-out efficiency was examined using western blot or flow cytometry approaches. The effect of knocking-out target genes on AML growth was monitored with flow cytometry. Single clones from Lin-Sca+cKit+ mouse HSCs following target-gene knock-out were selected for sequencing in order to determine any nucleotide changes. The loss of function effect of the target gene on normal hematopoiesis was monitored by counting the colonies from Colony Forming Unit (CFU) assays.

### 3.3 Colony Forming Unit Assay

The Colony forming unit (CFU) assay is a method to evaluate HSPC proliferation and differentiation into myeloid or lymphoid colonies in a semi-solid media (Bradley and Metcalf 1966). In study II and III, we investigated whether our target genes were involved in normal hematopoiesis with a CFU assay, in combination with shRNA approach. The CFU assay was performed according to manufacturer instructions. In study IV, due to the high expression of Nucleosome Assembly Protein 1 Like 3 (NAP1L3) in HSCs, we aimed to study NALP1L3 in normal hematopoiesis with CFU assays using shRNA, CRISPR-Cas9 and overexpression approaches. Total colonies were counted and myeloid colonies including mixed myelo-erythroid CFUs (CFU-GEM), a granulocyte/macrophage CFUs (CFU-G/GM), burst-forming unit erythroid cells (CFU-E/BFU-E,), macrophages (CFU-M) were identified based on their unique morphology.

This method has some drawbacks, making it time-consuming and error-prone. First, good skills are needed for the identification of distinct colonies. Additionally, the semi-solid media requires careful handling in order to be evenly mixed without bubbles being created before being manipulated into the plates.

### 3.4 Bone Marrow Transplantation Experiment

Bone marrow transplantation or HSC transplantation is a procedure to introduce donor derived multipotent HSCs, which are capable of reconstituting all blood cells, to the recipient animal (Duran-Struuck and Dysko 2009). The recipient animals are dosed with whole-body irradiation prior to prior to transplantation in order to eliminate recipient bone marrow cells and to create space for the engraftment of donor bone marrow cells (Bhattacharya, Ehrlich et al. 2008).

In study IV, we investigated the role of NAP1L3 in normal hematopoiesis for both mouse and human. Following knock-down, we transplanted mouse and human HSPCs into lethally irradiated congenic recipients or sub-lethally irradiated humanized NOD Scid IL2Rgnull-3-SCF/GM/IL3 (NSG-SMG3) mice, respectively. Ultimately, we determined the NAP1L3 knock-down effect on the engraftment, repopulation capacity and HSC maintenance with flow cytometric analysis. In study I, II, III, to determine the role of target genes in AML progression and maintenance, we performed AML transplantation experiments using both mouse MLL-AF9 AML cells and patient-derived primary AML samples. To determine whether loss of target genes leads to delay in AML progression, MLL-AF9 AML mouse cells upon knock-down of target genes were transplanted into non-irradiated congenic recipients. We did not irradiate the recipient mice because AML cells were able to out-compete normal

cells and, over time, take their space in the bone marrow. The advantage of using a congenic mouse model is that it does not lead to rejection of donor cells in the recipient mice, as donor and recipient mice are genetically identical (Duran-Struuck and Dysko 2009). However, using congenic mouse models for AML and normal hematopoiesis does not exactly mimic the human AML progression or human hematopoiesis, respectively, since this model has a different bone marrow microenvironment, cytokines and growth factors (Arber, Brenner et al. 2013). To investigate whether the role of target genes in normal hematopoiesis and AML disease progression is also conserved in human, we transplanted primary AML samples or normal hematopoietic cells following knock-down into sub-lethally irradiated NSG-SMG3 humanized mice. This mouse model expresses human IL3, GM-CSF and SCF cytokines, and allows the engraftment of diverse hematopoietic lineages and primary AML samples (Nicolini, Cashman et al. 2004).

### 3.5 A Niche-Like Culture System for Primary AML Samples and HSPCs

The determination of HSCs and LICs has been dependent on transplantation assays where these cells display their capability to self-renew and propagate themselves. However, transplantation experiments are time consuming, rendering it difficult for molecular characterization of HSCs and LICs and for drug discovery studies for LICs (Griessinger, Anjos-Afonso et al. 2014). M210B4/SlSl and MS5 stromal cell lines provide supportive environments to maintain the primitive characteristics and functions of HSCs and LICs, respectively, mimicking the bone marrow microenvironment *ex vivo* (Griessinger, Anjos-Afonso et al. 2014; Huang, Zhu et al. 2016).

To investigate target-gene roles in LICs maintenance and AML cell growth in study I, II, III, we performed a growth assay following downregulation of target genes using a co-culture system. This included primary AML cells on the top of the mouse MS5 stromal cells for 3-5 weeks. Flow cytometric analysis was performed to investigate the effect of target genes on primary AML cell growth of LICs and leukocytes. In study III, to determine whether General Transcription Factor II-I Repeat Domain-Containing Protein (GTF2IRD1) is required for primary human hematopoietic cells in the long term, we carried out a growth assay in which umbilical cord blood (UCB) cells were co-cultured with M210B4/SlSl stromal cells for 3 weeks. The effect of GTF2IRD1 downregulation on normal human hematopoietic cells was monitored by flow cytometry analysis. In study IV, we aimed to study the long-term effect of NAP1L3 downregulation in human UCB HSCs. M210B4/SlSl stromal cells were co-cultured with UCB HSCs for three weeks following shRNA-mediated knock-down of NAP1L3. The long-term role of NAP1L3 in human HSCs was assessed by flow cytometry.

### 3.6 RNA-Sequencing and Data Analysis

RNA-sequencing reveals the transcriptome in a biological sample using next generation sequencing. In comparison to an Affymetrix gene expression array, RNA-sequencing has various advantages including higher sensitivity, higher accuracy and better capability to capture novel transcripts, single nucleotide variants and gene fusion without prior knowledge (Wang, Gerstein et al. 2009; Kukurba and Montgomery 2015).

To reveal the molecular mechanism underlying the roles of target genes in AML disease progression or normal hematopoiesis, we carried out RNA-sequencing. Upon knock-down of target genes, we used mouse AML cells in study II and III, human AML cell line in study I and human UCB HSCs in study IV for RNA-sequencing. Extraction of total RNA from sorted cells was performed using the RNeasy Micro Kit. Strand specific and paired end cDNA libraries were prepared using Total script RNA-sequencing kit and were sequenced using the Illumina platform HiSeq2000 or Nextseq500. Using the STAR aligner, all raw sequence reads from RNA-sequencing were mapped to the Ensembl Homo sapiens GRCh38 reference genome. Feature Counts was used to assign the mapped reads to their corresponding genes. Normalization and differential expressed genes (DEGs) were assessed using the DESeq2 package. The differential expression analysis identified the transcripts with significantly different expression (FDR<0.05, -1 downregulated, 1 upregulated, 0 not significant). Gene Set Enrichment Analysis (GSEA) analysis was performed in order to identify biological states (Hallmark gene sets) or pathways (Biocarta or Reactome gene sets) enriched with differentially expressed genes. We used the log2 transformed expression values as the inputs and the Molecular Signature Data Base, (MSigDB), as the database of biological states or pathways (http://software.broadinstitute.org/gsea/msigdb/annotate.jsp).

## 4 RESULTS AND DISCUSSIONS

We aimed to identify and characterize novel genes and pathways essential for the maintenance of AML (study I, II, III) in order to better understand AML biology and to find potential drug targets against the disease. Furthermore, our goal was to investigate NAP1L3 roles in HSC activities and differentiation (study IV), to delineate the biology of normal hematopoiesis.

### 4.1 STUDY I

In study I, we used the lentiviral-based shRNA library screen targeting disease associated genes (Module 2, Cellecta Inc) to identify important genes for AML maintenance. We selected the Chromodomain Helicase DNA Binding Protein 4 (CHD4) for further investigation of its potential role in AML growth. For this reason, we carried out a cell growth assay with primary childhood AML cells using a stromal co-culture system. Specifically, we transduced various primary childhood AML samples with shRNA against CHD4 or mock then expanded positively transduced cells on stromal cells. Our data demonstrated that CHD4 knock-down caused a reduction in the number of AML bulk cells ($CD45^+$) and LICs ($CD45^+Lin^-CD34^+CD38^-$) of childhood AML samples with different genetic lesions *ex vivo*.

To delineate the role of CHD4 in normal hematopoietic cells, we performed a growth competition assay using UCB cells following CHD4 knock-down. Importantly, inhibition of CHD4 did not result in any strong effects on growth of human normal UCB cells *in vitro*. To investigate the role of the CHD4 in AML progression, we transplanted primary childhood AML samples upon CHD4 knock-down into humanized NSG-SMG3 mice. Flow cytometric analysis showed that shRNA-mediated downregulation of CHD4 resulted in a highly significant decrease in engraftment of childhood AML cells in the transplanted animals, which is consistent with previous data in adult AML (Sperlazza, Rahmani et al. 2015). Our data suggested that suppression of CHD4 can prevent AML growth and progression *in vivo*. Taken together, our findings demonstrate that CHD4 is essential for patient-derived AML cells and disease progression with less pronounced effects on normal hematopoietic cells.

To investigate the cellular mechanisms by which CHD4 affects AML growth and disease progression, we carried out cell cycle and apoptosis analysis. Suppression of CHD4 via shRNA also dramatically increased the percentage of AML cells in G0 phase, but diminished the corresponding percentages in G1, S and G2-M phases, suggesting that CHD4 is important in the regulation of cell cycle. Contrastingly, downregulation of CHD4 did not show a pronounced effect in an analysis of apoptosis. Although we could not exclude that cell cycle

arrest of AML cells will be followed by apoptosis, our data suggest that loss of CHD4 function may prevent AML cell proliferation and AML development by blocking cell cycle progression in G0 phase.

In order to identify the molecular mechanisms by which CHD4 was involved in AML cell growth and disease progression, we carried out RNA-sequencing of AML cells following downregulation of CHD4 using shRNA approach. RNA-sequencing data showed that 1011 genes were upregulated and 413 genes were downregulated following shRNA mediated knock-down of CHD4. To reveal the relevant biological pathways that are enriched upon CHD4 knock-down, we carried out GSEA analysis. Using the MSigDB hallmark gene sets, this analysis revealed that MYC targets and E2F transcription factor (E2F) targets, including MYC and other cell cycle associated genes, were enriched subsequent to CHD4 knock-down. Given the role of CHD4 in cell cycle progression (Polo, Kaidi et al. 2010; Sims and Wade 2011; D'Alesio, Punzi et al. 2016), Reactome data set analysis revealed that dysregulated genes following CHD4 knock-down were significantly enriched for cell cycle regulation such as S phase, synthesis of DNA and assembly of the pre-replicative complex. RNA-sequencing data revealed a reduction in the mRNA levels of MYC and MYC target genes known to be involved in cell cycle regulation, including Cyclin D1, D2, E1, E2F1 and E2F2 (Zörnig and Evan 1996; Zajac-Kaye 2001; Bretones, Delgado et al. 2015). In conclusion, the molecular mechanism of CHD4's role in childhood AML maintenance appears to be mediated in part by inducing expression of the MYC oncogene and its targets, which are in turn known to be important in cell cycle regulation.

### 4.2 STUDY II

In study II, we focused on investigating whether Euchromatin Histone Methyltransferase 1 (EHMT1), a histone 3 lysine 9 (H3K9) specific methyltransferase (Vedadi, Barsyte-Lovejoy et al. 2011), is required for adult and childhood AML. First, we determined that EHMT1 knock-down impaired the maintenance of primary adult or childhood AML cell growth, when co-cultured with stromal cells, resulting in a decrease in the frequency of CD45+ AML bulk cells and LinCD34+ CD38- LICs. Second, to delineate the role of EHMT1 in normal hematopoietic cell growth, we performed a growth assay using CD34+ bone marrow cells from healthy donors in combination with knock-down of EHMT1. EHMT1 knock-down resulted in only a modest reduction in growth of CD34+ bone marrow cells.

Next, to investigate if the essential role of EHMT1 in primary adult and childhood AML cells *ex vivo* was conserved *in vivo*, we transplanted primary childhood or adult AML samples in to

humanized NSG-SMG3 recipient mice following EHMT1 knock-down. Engraftment of transplanted cells was followed up to 16 weeks. Our data demonstrates that suppression of EHMT1 resulted in reduction of primary AML cell engraftment, both for childhood and adult samples. Overall, these results suggest that EHMT1 is required for both childhood and adult AML growth and disease progression, but EHMT1 loss has no dramatic effect on normal hematopoietic cell growth. EHMT1 is reported to form a heterodimer complex with EHMT2 (Tachibana, Ueda et al. 2005) and EHMT2 has recently shown to be involved in AML growth (Lehnertz, Pabst et al. 2014). To investigate whether these two methyltransferases were functioning cooperatively in AML, we used the CRISPR-Cas9 system to generate single or double knock-outs of EHMT1 and EHMT2 in two different AML cell lines. Single knock-out EHMT1 and EHMT2, or double knock-out EHMT1/2 all resulted in a similar reduction of AML growth in both AML cell lines *in vitro*, suggesting that loss of both EHMT1 and EHMT2 did not lead to an additional effect on AML cell growth. It should be pointed out that single knock-out of EHMT1 and EHMT2 caused a dramatic effect on AML growth in our assay, which might mask a putative synergistic effect of double knock-outs of EHMT1 and EHMT2 on AML cell growth. Therefore, further experiments are needed to answer this specific question.

In order to compare the common or specific cellular mechanisms by which EHMT1 and EHMT2 have a role in AML cell growth, the cell cycle and apoptosis assays were performed on mouse AML cell line following shRNA mediated knock-down of EHMT1 or EHMT2. Our data indicated that knock-down of either EHMT1 or EHMT2 led to AML cell growth arrest in G0 phase, but did not result in increased apoptosis.

To investigate the molecular mechanisms through which EHMT1 plays a role in AML cell growth/ disease maintenance, we performed RNA-sequencing with EHMT1 or EHMT2 downregulated mouse AML cell line. A comparison of differential gene expression from EHMT1 knocked-down versus EHMT2 knocked-down cells revealed a significant, though not complete, overlap in upregulated genes. Our next step was to identify the biological processes to which overlapping or uniquely dysregulated genes belong. For this reason, we performed GO-term analysis with overlapping upregulated genes. Our results showed that these genes were associated with various biological pathways including cytokine signaling, inflammatory response and cell differentiation. Moreover, the data revealed unique pathways such as DNA binding transcription activity and cell chemotaxis for EHMT1 and EHMT2, respectively. In conclusion, our data suggested that EHMT1 and EHMT2 predominantly share

a common biological function in AML cells, though it is likely that they have specific non-redundant roles as well.

### 4.3 STUDY III

In study III, we performed a screen for 5000 annotated genes playing a role in signaling pathways using a targeted shRNA library (Module 1, Cellecta Inc). From the results of this screen, GTF2IRD1 was chosen as a candidate target gene for follow-up to determine its role in adult and childhood AML growth and disease progression.

To assess the role of GTF2IRD1 in AML growth *in vitro*, a growth assay was carried out by co-culturing childhood and adult primary AML cells after shRNA-mediated knock-down of GTF2IRD1 on a stromal cell layer. Our data showed that GTF2IRD1 depletion diminished AML bulk cell ($CD45^+$) and leukemia initiating cell ($CD45^+Lin^-CD34^+CD38^-$) numbers, suggesting a potential role of GTF2IRD1 in childhood and adult AML cell growth *ex vivo*. As our long-term goal is to identify target genes that can potentially be used for AML therapy, we examined whether GTF2IRD1 had a role in normal hematopoiesis. For this purpose, we transplanted human UCB cells with and without knock-down of GTF2IRD1 in to humanized NSG-SMG3 recipient mice and investigated the effects of GTF2IRD1 in normal hematopoiesis and growth of the transplanted cells. Immunophenotypic analysis using flow cytometry showed that knock-down of GTF2IRD1 did not significantly affect normal hematopoiesis or expansion of the normal cells.

To investigate the importance of GTF2IRD1 in AML progression, we took advantage of a humanized NSG-SMG3 mouse model. GTF2IRD1 shRNA knock-down led to a remarkable reduction in engraftment of childhood and adult primary AML cells in recipient mice, suggesting involvement of GTF2IRD1 in AML progression. In conclusion, our data suggests that GTF2ITRD1 is essential for childhood and adult patient-derived AML cells and disease progression, without significant effects on normal cells and hematopoiesis.

To identify the cellular mechanisms by which GTF2IRD1 is involved in AML growth and disease progression, we performed cell cycle and apoptosis assays. Flow cytometric analysis indicated that downregulation of GTF2IRD1 caused an accumulation of cells in the G0 phase, and a reduction of cells in G1, S and G2-M cell cycle phases. Furthermore, flow cytometry analysis showed that shRNA-mediated knock-down of GTF2IRD1 led to an increase in the ratio of early apoptotic cells, but not in the ratio of late apoptotic cells. Together, these

findings suggest that the GTF2IRD1 is required for AML cell growth and disease progression by inducing cell cycle progression without causing apoptosis. In addition, we investigated the molecular mechanisms by which downregulation of GTF2IRD1 led to a block in AML cell growth and inhibition in disease progression. RNA-sequencing was utilized for this purpose, and the data obtained showed that 278 genes were upregulated and 378 genes were downregulated upon GTF2IRD1 downregulation. GSEA was also performed to determine the enriched pathways related to differently expressed genes following GTF2IRD1 downregulation. This showed that deregulated genes, upon GTF2IRD1 knock-down, were negatively associated with MYC targets and KRAS signaling based on MsigDB hallmark gene set analysis. In summary GTF2IRD1 is involved in various molecular processes in AML by modulating gene expression, including in known oncogenic pathways such as MYC targets and KRAS signaling.

Given the role of GTF2IRD1 as a transcription factor (Polly, Haddadi et al. 2003; Issa, Palmer et al. 2006), we next investigated whether GTF2IRD1 acts as a repressor or activator. To do so, we took an advantage of a heterologous reporter system in HEK293 cells with five different GAL4 DNA-binding sites and a luciferase reporter gene under control of the thymidine kinase promoter. GTF2IRD1 expression led to significant reduction of luciferase expression level. Our findings were consistent with other studies (O'Mahoney, Guven et al. 1998; Issa, Palmer et al. 2006) suggesting that GTF2IRD1 functions as a transcriptional repressor.

### 4.4 STUDY IV

In study IV, we studied the role of NAP1L3 in normal hematopoiesis. As recently reported (Riddell, Gazit et al. 2014; Bagger, Sasivarevic et al. 2016), we found that NAP1L3 is highly expressed in mouse HSCs, suggesting a potential role of NAP1L3 in HSC function.

To determine whether NAP1L3 was involved in HSC maintenance, we transduced sorted UCB HSCs with shRNA against NAP1L3 or mock, then expanded positively transduced cells on stromal cells for three weeks. Flow cytometry analysis indicated that NAP1L3 suppression caused a reduction in UCB HSC numbers *in vitro*. To further delineate the effect of loss of NAP1L3 function influences hematopoietic cell proliferation and differentiation, we cultured the NAP1L3 or mock shRNA-transduced CD34+ enriched UCB HSPCs on methylcellulose for 14 days. Our results show that suppression of NAP1L3 impaired the differentiation and proliferation of HSPCs. Consistent with the methylcellulose data; serial plating of UCB HSPCs

upon NAP1L3 knock-down also caused a reduction of total colony numbers after each plating process.

To investigate the role of NAP1L3 in HSC maintenance and differentiation *in vivo*, we transplanted NAP1L3 knocked-down UCB HSPCs into humanized NSG-SMG3 recipient mice. Downregulation of NAP1L3 significantly diminished the engraftment of human nucleated hematopoietic cells ($CD45^+$) and UCB HSCs in recipient mice after sixteen weeks of transplantation, demonstrating a potential role for NAP1L3 in HSC engraftment. In addition, we also observed a significant increase in the percentage of myeloid cells and a decrease in the percentage of lymphoid cells upon NAP1L3 suppression. Overall, our data suggests that NAP1L3 is essential for HSC maintenance and differentiation both *in vitro* and *in vivo*.

Aiming to understand the cellular and molecular mechanisms through which NAP1L3 is important in normal hematopoiesis, we performed cell cycle analysis and apoptosis assay and RNA-sequencing with NAP1L3-depleted UCB HSCs. Data from the cell cycle assay demonstrated that NAP1L3 suppression led to arrest of UCB HSC growth in the G0 phase of the cell cycle. The apoptosis assay showed that NAP1L3 knock-down led to an increased proportion of early, but not late, apoptotic cells. Taken together, these data suggest that the effect of loss of NAP1L3 function on HSC maintenance and differentiation both *in vitro* and *in vivo* can be explained by induction of G0 cell cycle arrest and apoptosis. To delineate the biological processes of dysregulated genes identified by RNA-sequencing, we carried out GSEA analysis. We observed that knock-down of NAP1L3 in UCB HSCs resulted in enrichment of deregulated genes for cell cycle regulation, including E2F and MYC targets. Moreover, depletion of NAP1L3 gave rise to upregulation of HOXA3 and HOXA5 genes in UCB HSCs.

## 4.5 DISCUSSION

Transcription factors and epigenetic modifiers are known to modulate the balance between self-renewal and differentiation of HSCs (Wilson, Laurenti et al. 2009). Disturbance of this balance can result in hematological malignancies, such as AML (Attar and Scadden 2004). Therefore, identification of the molecular mechanisms involved in normal hematopoiesis is of tremendous importance to understand the disease biology and for the development of new treatments. Conventional chemotherapy for AML cannot eliminate the LICs and the majority of patients relapse (Trendowski 2015), thus novel therapeutic strategies are needed to fight this disease.

Large-scale shRNA screening technology is a potent and versatile technique to identify genes and pathways that are important in disease development or progression, and to identify potential drug targets (Meacham, Ho et al. 2009). Using this technology allowed us to discover novel genes that play a role in AML. Loss of function of our candidate genes prevented both adult and childhood AML growth *in vitro* and disease progression *in vivo* through inhibiting the growth of LICs and AML bulk cells, without significant effects on normal hematopoietic cells. Moreover, further molecular and cellular characterization of these candidates provides a way to determine potential cellular processes and genes/pathways that are deregulated in AML. This deepens our knowledge of AML biology and will be of importance to understand the disease mechanisms in developing new therapeutic approaches. Since inhibition of our candidates resulted in elimination of both LICs and bulk cells in various primary AML samples with different genetic lesions, development of small molecule inhibitors against these candidates may serve as potential drug targets for different AML subtypes. In addition, they may also be suitable to treat relapse.

Our study related to normal hematopoiesis revealed that NAP1L3 has a role in HSC maintenance and differentiation, through regulation of the transcription of genes involved in cell cycle progression and differentiation. The HSC is the target of leukemic transformation, and regulatory pathways that play role in HSC differentiation, survival and proliferation are perturbed during the transformation process (Corces-Zimmerman and Majeti 2014; Shlush and Mitchell 2015). Identification of these pathways will give us a greater insight into malignancy, and can also lead to the development of novel therapeutic strategies for AML.

## 5 CONCLUDING REMARKS

AML is a heterogeneous disease at both molecular and clinical level. Genome wide studies allow us to examine the mutational complexity and diversity of AML (Walter, Shen et al. 2012; Welch, Ley et al. 2012; Cancer Genome Atlas Research, Ley et al. 2013; Shlush, Zandi et al. 2014). For instance, the Cancer Genome Atlas AML sub-study classified the commonly mutated genes in nine functional categories by profiling 200 de novo AML patients (Cancer Genome Atlas Research, Ley et al. 2013). Moreover, another study of more than 1500 AML patients revealed eleven different AML subgroups by analyzing 111 leukemia related genes (Papaemmanuil, Gerstung et al. 2016). Although advances in understanding the molecular mechanisms underlying AML development, they have not been translated into clinical practice. Over the past four decades, standard chemotherapy with little progress was utilized for all subgroups of patients and the majority of the patients relapse after treatment (Cheson, Bennett et al. 2003; Dohner, Estey et al. 2010). In addition, the current genomic classification systems have contributed to prediction of the disease prognosis (Cancer Genome Atlas Research, Ley et al. 2013; Papaemmanuil, Gerstung et al. 2016), but it is not clear that recurrent mutations have an initiating or cooperative role in AML development. Therefore, accurate pre-clinical models must be utilized in order to further assess the clinical implications of these mutated genes.

In this thesis, large scale shRNA-based approaches allowed us to identify AML-specific cell essential genes. Use of an *in vitro* co-culture system for the expansion of primary AML samples, and transplantation of primary AML samples into humanized AML mouse models revealed the functional importance of these genes in AML growth and disease progression. Our studies are also highly significant in terms of identification of molecular vulnerabilities both in adult and childhood AML.

As is known, relapse occurs as a result of LICs remaining after treatment (Dohner, Weisdorf et al. 2015). Conventional chemotherapy is not sufficient to eliminating LICs and is also toxic for normal cells (Pollyea, Gutman et al. 2014). Therefore, new treatment strategies that target LICs are needed in order to eradicate disease. It has been shown that BCL-2 is specifically expressed in LICs compared to the normal compartments (Lagadinou, Sach et al. 2013). A BCL-2 inhibitor, Venetoclax, is being studied in combination with low dose Cytarabine or with hypomethylating agents in elderly AML patients that are not eligible for conventional treatment (Lin, Strickland et al. 2016; DiNardo, Pratz et al. 2018). Clinical trial of Venetoclax in combination with Azacytidine/Cytarabine showed an encouraging response rate (CR/CRi) of

61%, suggesting a promising treatment strategy (DiNardo, Pratz et al. 2018). Our *in vitro* and *in vivo* studies also revealed that our target genes are required for LICs as well as for AML blasts, suggesting that they serve as potential therapeutic targets.

Another challenge in AML is that standard chemotherapy is not tolerated by elderly AML patients due to its cytotoxicity. This leads to high mortality rates among elderly patients (Walter and Estey 2015). Novel therapeutic regimens with low toxicity are needed for these patients. Hypomethylating agents including Azacytidine and Decitabine have been approved by the EMA for use in elderly patients (Derissen, Beijnen et al. 2013). Although these hypomethylating agents lead to clinical responses in elderly patients with low toxicity, their clinical activity is limited. Therefore, ongoing clinical trials are testing the use of Azacytidine in combination with Venetoclax or Gilteritinib in elderly patients who are ineligible for intensive chemotherapy (Cortes, Altman et al. 2017; Potluri, Xu et al. 2017).

Novel immunotherapies are also a promising approach to destroy leukemic cells in AML. These immunotherapies consist of targeted antibody based and chimeric antigen receptor-engineered T cell (CAR-T) therapies (Nagler, Xavier et al. 2017). It has been shown that around 90% of AML patients had CD33 antigen expression (Jilani, Estey et al. 2002). Gemtuzumab Ozogamicin, designed to target CD33-expressing leukemic cells, has been approved by the FDA for use in AML patients (Appelbaum and Bernstein 2017). Clinical trials are still ongoing for anti-CD33 monoclonal antibodies in combination with other therapies (e.g. ATRA or Daunorubicin+Clorofarabine) (NCT00893399), (UK NCRI AML16 trial) (Burnett, Russell et al. 2017). CAR-T cell therapy is a novel cellular immunotherapy using autologous or allogeneic T cells that are genetically modified with synthetic CARs to target leukemic cell antigens (Budde, Song et al. 2017). These synthetic CARs bind the antigens on the surface of leukemic cells and promote T cell activation, resulting in rapid tumor cell cytotoxicity (Bonifant, Jackson et al. 2016; Brudno and Kochenderfer 2016). The CD123 antigen has been suggested as an attractive therapeutic target in AML, because it is highly expressed in leukemia initiating cells compared to normal hematopoietic cells (Testa, Pelosi et al. 2014). A preclinical study indicated that CD123-directed CAR-T cells had an anti-leukemic effect which prolonged the survival of recipient animals (Petrov, Wada et al. 2018). Full clinical implications of immunotherapy are just beginning to manifest. Although immunotherapy holds much promise for AML treatment, therapeutic challenges remain. For example, AML has a complex clonal structure, as it arises from a malignant founding clone by acquiring additional mutations (Paguirigan, Smith et al. 2015). Some of these clones can survive after chemotherapy and lead to relapse (Jan and Majeti

2013). This clonal complexity may also cause heterogeneity in the cell surface antigens which could potentially be targeted by immunotherapy. Therefore, it will be helpful to understand the clonal dynamics and architecture of AML in order to implement this knowledge into clinical practice. On the other hand, genome-wide studies showed that inter-patient heterogeneity is the case for AML as well (Cancer Genome Atlas Research, Ley et al. 2013; Papaemmanuil, Gerstung et al. 2016). Individual heterogeneity among AML patients results in a discrepancy in treatment outcome. Thus, focusing on the development of individualized therapies targeting patient-specific mutations or dysregulated pathways might be a promising therapeutic approach to battle AML.

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