Towards identification and targeting of Polycomb signaling pathways in leukemia

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DOI:
10.33612/diss.101427699

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

Publication date:
2019

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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CHAPTER 1

GENERAL INTRODUCTION & SCOPE OF THIS THESIS
INTRODUCTION

1.1 Normal hematopoiesis

The hematopoietic system is a tightly controlled and regulated system that sustains the generation of all types of blood cells during the entire lifespan of an organism (Orkin and Zon, 2008). Every day one trillion ($10^{12}$) new blood cells are produced, a process called hematopoiesis. These include red blood cells (erythrocytes) necessary for oxygen transport throughout the body, white blood cells (leukocytes) including granulocytes, macrophages, dendritic cells, natural killer (NK) cells and T and B-lymphocytes for an active innate and adaptive immune system in response to infections and platelets (thrombocytes) for clot formation to stop bleeding (Doulatov et al., 2012). Under steady state conditions, red blood cells have a lifespan of approximately 120 days, while white blood cells have a lifespan that varies from hours to months and platelets 9-12 days. Because of the variety in turnover rates and the necessity to quickly respond to a bleeding or infection, hematopoiesis is a very active process in order to continuously replenish all mature blood cells to maintain normal blood homeostasis.

Identification of a hierarchy in the hematopoietic system

Hematopoiesis is organized as a hierarchy, in which all blood cell lineages are derived from a primitive cell, termed the hematopoietic stem cell (HSC). The first evidence for the existence of HSCs came in the 1960s from studies by Becker, McCulloch and Till which identified that transplantation of mouse bone marrow cells into an irradiated mouse recipient gave rise to mixed myeloerythroid lineage colonies in the spleen that were derived from a single cell (McCulloch and Till, 1960, Becker et al., 1963). Serial transplantation of these bone marrow cells into secondary recipient mice could still give rise to progeny of all blood lineages, indicating the self-renewal potential of these cells. Such serial transplantation assays are still being used today and are important to prove the existence of stem cells. The ability to self-renew and to differentiate into all mature blood cells are the two unique features of HSCs.

The development of in vitro assays, firstly the colony forming unit assay, followed by long-term initiating cell (LTC-IC) assays (Conneally et al., 1997, Bradley and Metcalf, 1966), as well as the identification of hematopoietic cell surface markers for flow cytometry have increased our understanding of the hematopoietic system and factors involved in regulating stem cell fate. HSCs reside within specialized regions of the bone marrow, the so-called stem cell niche (Morrison and Scadden, 2014). Studying stem and progenitor cell populations revealed that HSCs are extremely rare and very quiescent (Passegue et al., 2005). They can be divided into long-term (LT) or dormant HSCs that
divide minimally over time (Spangrude et al., 1988, Wilson et al., 2007) and capable of long-term self-renewal while short-term (ST) HSCs divide approximately once a month and provide short-term engraftment. To maintain the stem cell pool and the demand for mature blood cells, a controlled balance between symmetric and asymmetric cell divisions is critical (Fuchs et al., 2004). In an asymmetric cell division, one daughter cell will be an exact copy (self-renewal) and the other daughter cell will differentiate into a multipotent progenitor (MPP) with multi-lineage potential. In the classical model of hematopoiesis (Figure 1), MPPs will proliferate and can differentiate towards either the myeloid or lymphoid lineage, respectively a common myeloid progenitor (CMP) or common lymphoid progenitor (CLP) (Morrison et al., 1997, Akashi et al., 2000, Kondo et al., 1997). CLPs differentiate towards T- and B-cells, NK-cells or dendritic cells. The CMP can further differentiate into a granulocyte macrophage progenitor (GMP) that gives rise to granulocytes or macrophages (Akashi et al., 2000) or megakaryocyte erythrocyte progenitor (MEP) generating erythrocytes and platelets (Figure 1) (Majeti et al., 2007, Doulatov et al., 2012, Haas et al., 2018, Laurenti and Gottgens, 2018, Velten et al., 2017). Alternatively, a few years later it was proposed that MPPs can differentiate towards either a more lymphoid primed MPP (LMPP) or megakaryocyte/erythrocyte progenitor (Adolfsson et al., 2005). In that model, the LMPP can further differentiate into a CLP

Figure 1. The hematopoietic system
All blood cell lineages are derived from multipotent hematopoietic stem cells that reside in the bone marrow niche. Hematopoietic stem cells (HSCs); multipotent progenitor (MPP); lymphoid-primed multipotent progenitor (LMPP); common myeloid progenitor (CMP); common lymphoid progenitor (CLP); megakaryocyte erythroid progenitor (MEP); granulocyte macrophage progenitor (GMP).
or GMP to generate lymphoid and myeloid cells. The development of new techniques, including single cell genomic approaches have further challenged the accuracy of the classical model (Paul et al., 2015, Notta et al., 2016, Perie et al., 2015, Belluschi et al., 2018). Researchers found that individual cells cluster together and were transcriptionally primed suggesting already early commitment towards a certain lineage. No clusters were found that expressed both erythrocyte and megakaryocyte specific markers which argues against mixed-lineage progenitors (Paul et al., 2015). Perie and colleagues sorted different hematopoietic populations, labeled them and tracked them in vivo to follow their cell fate. Surprisingly, phenotypical CMPs that are thought to have multi-lineage potential were rather a mix of lineage-primed progenitors. This indicated that blood cells are pre-programmed and the stem/progenitor pool is a complex mix of cells with varying capability (Perie et al., 2015).

**Defining hematopoietic stem and progenitor cells using cell surface markers**

The identification of specific cell surface markers enabled to define and isolate hematopoietic stem and progenitor cells using fluorescence-activated cell sorting (FACS). Among several markers, CD34 was the first marker that was identified to be enriched on human hematopoietic stem and progenitor cells (Civin et al., 1984). LT-HSCs are enriched within the CD34⁺CD38⁻ fraction and several (serial) transplantation studies showed that they can successfully reconstitute and maintain hematopoiesis (Kondo et al., 2003, Bensinger et al., 1996, Civin et al., 1996). To further distinguish human stem and progenitor cells, the expression of CD90 and CD49f was found to further enrich for stem cells (Mayani et al., 1993, Notta et al., 2010, Notta et al., 2011, Baum et al., 1992). Single LinCD34⁺CD38⁻CD45RA⁻CD90⁺Rho⁻CD49f⁺ cells were capable of long-term multi-lineage engraftment in NSG mice (Notta et al., 2010). The loss of CD90 expression is characteristic for MPPs. The markers CD45RA, CD38, CD123 and CD10 can be used to isolate myeloid or lymphoid-restricted progenitors (Doulatov et al., 2012, Lansdorp et al., 1990, Bhatia et al., 1997).

**1.2 Factors involved in regulating stem cell fate**

Stem cell self-renewal and differentiation is a tightly controlled process since disturbance of regulatory mechanisms can ultimately lead to leukemic transformation. Both cell intrinsic signaling including epigenetic regulators that control gene expression and extrinsic signals provided by the niche like growth factors and cytokines that drive signal transduction are involved in regulating hematopoietic stem cell fate (Zon, 2008, Warr et al., 2011, Rizo et al., 2006).
Stem cell niche interactions

HSCs reside in the bone marrow niche and are surrounded by all kind of cells, including mesenchymal stromal cells, osteoblasts, chondrocytes, adipocytes, CAR cells, endothelial cells and neuronal cells (Adams and Scadden, 2006, Kiel and Morrison, 2008, Kumar and Geiger, 2017, Asada et al., 2017). The idea that cells in the bone marrow might create a ‘supportive’ niche for hematopoietic cells was suggested by one of the first studies that showed that osteoblasts produced granulocyte colony stimulating factor (G-CSF), that stimulated the growth of hematopoietic cells in culture (Taichman 1994). The continuous cross-talk between stem cells and the niche can be mediated via direct cell-cell contacts or via growth factor and cytokine-induced signaling that regulate maintenance, survival, proliferation and differentiation of hematopoietic cells (Kiel and Morrison, 2008, Kumar and Geiger, 2017). The niche is an important regulator of self-renewal and can influence whether symmetric or asymmetric cell division occurs (Takano et al., 2004, Fuchs et al., 2004). A study in *Drosophila* showed that male germline stem cells upon cell division orient their mitotic spindles perpendicular to the niche resulting in an asymmetric cell division whereby the cell close to the niche remains a stem cell while the cell furthest away differentiates (Yamashita et al., 2003). Moreover, several growth factors and cytokines secreted by cells in the niche can bind to their receptors on stem cells and regulate stem cell self-renewal and proliferation, like Stem Cell Factor (SCF), Thrombopoietin (TPO) and Flt3-ligand (Flt3-L) (Ramsfjell et al., 1996). Other important signaling molecules include WNT/β-Catenin signaling and the Notch pathway that influence self-renewal and expansion of HSCs (Butler et al., 2010, Reya et al., 2003). Optimal *in vitro* culture conditions to expand or differentiate hematopoietic cells requires the addition of cytokines and growth factors to culture medium. The presence of bone marrow stromal cells is essential for the maintenance of hematopoietic stem and progenitor cells (Itoh et al., 1989, Ding and Morrison, 2013, Ding et al., 2012, Calvi et al., 2003, Boulais and Frenette, 2015).

Growth factor or cytokine-induced signaling

Growth factor and cytokine-induced signaling ultimately activates lineage-specific transcription factors (e.g. STATs, GATA1, C/EBPα, PU.1 and PAX5) to regulate gene transcription that instruct cell fate towards for example lymphoid or myeloid differentiation (Orkin, 1995, Rieger et al., 2009, Metcalf, 1998, Endele et al., 2014). The growth hormone Erythropoietin (EPO) is a critical factor in the development of erythrocytes, while the formation of platelets is stimulated by TPO (Klimchenko et al., 2009). Interleukin-7 (IL-7) stimulates the proliferation of cells in the lymphoid lineage, whereas IL-3 and colony stimulating factors (CSFs), like granulocyte/macrophage-CSF stimulate induction of myeloid lineage commitment (Kondo, 2010). For example, STAT5 is induced by several
cytokines like IL-3, G-CSF and GM-CSF (Han et al., 2009). Loss of STAT5A/B in knockout mice revealed an important role in erythropoiesis (Socolovsky et al., 2001, Bunting et al., 2002). Furthermore, adhesion molecules and chemokines are involved in the homing and retention of HSCs in the niche. For example, stromal derived factor 1 (SDF-1/CXCL12) attracts CXCR4-expressing stem cells to the bone marrow (Cashman et al., 2002). G-CSF treatment was shown to suppress the CXCL12/CXCR4 axis and thereby mobilizing hematopoietic stem and progenitor cells to the peripheral blood (Petit et al., 2002). Nowadays G-CSF-mobilized peripheral blood hematopoietic stem and progenitor cells are often used in the clinic for transplantation therapy (Harada et al., 1996).

**Metabolic pathways**

An important energy source for cells is glucose, which is catabolized to pyruvate in a multistep process called glycolysis. Usually, under normoxic conditions pyruvate enters the mitochondrial tricarboxylic acid (TCA) cycle and via oxidative phosphorylation (OXPHOS) is capable of generating a maximum of 36 molecules of ATP per glucose molecule. Under hypoxic conditions, pyruvate is fermented to lactate, generating only 2 molecules of ATP. The relatively quiescent HSC has been shown to rely on anaerobic glycolysis over mitochondrial OXPHOS for energy production (Simsek et al., 2010, Vannini et al., 2016). More committed progenitors rely on mitochondrial OXPHOS to meet the demands of proliferation and differentiation (Takubo et al., 2013, Yu et al., 2013). These dynamic changes in metabolism and the molecular mechanisms underlying the switch from a quiescent HSC towards actively proliferating and differentiating progenitors are still far from understood.

It has been proposed that oxygen levels play an essential role in influencing the metabolic program between HSCs that reside in a hypoxic niche versus more differentiated cells in normoxic conditions in peripheral blood. The hypoxic conditions in the bone marrow result in the stabilization and activation of HIF signaling, particular HIF1α and HIF2α. They have been shown to be critically involved in stem cell quiescence and maintenance by negatively regulating cell cycle genes and promoting several glycolytic enzymes, including pyruvate dehydrogenase kinases and glucose transporters (Wierenga et al., 2014, Takubo et al., 2010, Takubo et al., 2013, Rouault-Pierre et al., 2016, Suda et al., 2011). A deficiency in pyruvate kinase isoform M2 (PKM2) impaired progenitor function and lactate dehydrogenase A (LDHA) depletion resulted in both HSC exhaustion and impaired growth and expansion of progenitors (Wang et al., 2014). Furthermore, it has been shown that intracellular Ca²⁺ levels and as a consequence enhanced mitochondrial activity can initiate cell division of HSCs (Umamoto et al., 2018). Moreover, growth factors and amino acids that activate PI3K-AKT-mTOR pathways (Yu and Cui, 2016), glutamine metabolism (Oburoglu et al., 2014) and fatty acid metabolism (Ito et al., 2012) also have a
crucial role in regulating the balance between quiescence and proliferation of stem cells. PI3K/AKT signaling negatively regulates FOXO family of transcription factors, resulting in increased ROS and HSC exhaustion and differentiation (Bigarella et al., 2014). AKT/mTOR signaling can stimulate glycolysis and promotes cell cycling of HSCs (Ito and Suda, 2014). Taya et al reported that amino acids are important for HSC maintenance, depletion of valine heavily impaired the proliferation and survival of hematopoietic stem cells (Taya et al., 2016). Several metabolic pathways involved in regulating stem cell fate are linked with epigenetic regulation of gene expression. α-Ketoglutarate (αKG), an intermediate of the TCA cycle, is converted from isocitrate by isocitrate dehydrogenase 1 (IDH1) or IDH2. αKG is also a co-factor for ten-eleven translocation (TET) proteins involved in DNA demethylation. Mutations in IDH1/2 are commonly found in acute myeloid leukemia (AML) and results in 2-hydroxyglutarate, an inhibitory metabolite of TET2. Loss of TET2 results in hypermethylation and induces increased self-renewal and impaired myeloid differentiation (Figueroa et al., 2010, Scourzic et al., 2015). Recently, branched-chain amino acid transaminase 1 (BCAT1) was found to be overexpressed in AMLs, restricting αKG levels, and thereby creating a DNA hypermethylation status (Raffel et al., 2017). Thus, metabolic enzymes and metabolites are important for cell fate and can affect epigenetic processes involved in controlling gene expression essential for both normal as well as leukemic cells.

**Epigenetic regulators**

Self-renewal and lineage-commitment is in part intrinsically regulated by epigenetic regulators including the Polycomb group protein family. Epigenetics involves control of gene expression by factors other than an individual’s DNA sequence. Stem cells, platelets, B-cells and granulocytes all have the same DNA, but all are distinct cell types with unique functions, which is strongly controlled by epigenetic regulators that affect local chromatin structure and DNA accessibility. Thereby they have a major influence on transcriptional activities of genes in regulating stem cell fate (Bernstein et al., 2007, Bracken et al., 2006). The nucleosome, involved in packaging the DNA, is the basic unit of chromatin consisting of DNA wrapped around a histone octamer containing two copies each of the core histones H2A, H2B, H3 and H4. Epigenetic modifications include DNA methylation and histone modifications that are the gatekeepers of gene expression during cell fate and control stem cell function or differentiation (Bannister and Kouzarides, 2011, Sashida and Iwama, 2012, Rice et al., 2007, Fuks, 2005).

Transcriptional repression via DNA methylation, is catalyzed by DNA methyltransferases (DNMTs) that transfer a methyl group to the fifth carbon of a cytosine residue and almost exclusively occurs at CpG islands. DNMT3A/B are involved in de novo DNA methylation
and DNMT1 in DNA maintenance following DNA replication (Challen et al., 2014). Loss of DNMT3A/B in double knockout (dKO) mice resulted in expansion of HSCs, coinciding with de-repression of self-renewal genes, and thereby a block in differentiation (Trowbridge and Orkin, 2011, Challen et al., 2014). Activated β-Catenin signaling contributed to the impaired differentiation of dKO HSCs (Scheller et al., 2006). Dnmt1 knockout in HSCs severely impaired engraftment levels and showed defects in differentiation (Trowbridge et al., 2009). Thus, a certain threshold of DNA methylation is required to maintain HSC self-renewal and differentiation. Mutations in DNMT3A/B and TET2 are thought to be an initiating event and thereby increase the risk for the development of hematological malignancies such as AML (Langemeijer et al., 2009). Insights into genome wide profiling of DNA methylation in hematopoietic stem cells revealed lineage-specific DNA methylation, associated with chromatin/DNA accessibility, between myeloid and lymphoid progenitors (Farlik et al., 2016). For example, in CD8 cells, regions with open chromatin were associated with reduced DNA methylation while being methylated in other cell lineages including B-cells, monocytes and neutrophils.

The histone tails of the core histone are subjected to posttranslational modifications, including methylation, ubiquitination, acetylation and phosphorylation that are also reversible (Bannister and Kouzarides, 2011, Bannister and Kouzarides, 2005). Acetylation of histone H3 at lysine 27 (H3K27ac), tri-methylation of histone H3 at lysine 4 (H3K4me3) and lysine 36 (H3K36me3) are linked to ‘open: accessible regions’ or euchromatin and associated with active transcription. Whereas tri-methylation of histone H3 at lysine 27 (H3K27me3) is linked to ‘condensed: packed regions’ or heterochromatin and associated with gene repression (Gillette and Hill, 2015, Henikoff and Shilatifard, 2011). Thus, dynamic changes in histone modifications help to maintain selective gene expression or silencing for regulating stem cell self-renewal and differentiation. Chromatin immunoprecipitation (ChIP) analysis in pluripotent embryonic stem cells (ESCs) revealed genomic loci that were co-occupied by H3K4me3 and H3K27me3, referred to as bivalent loci, allowing genes to be poised for activation during development (Bernstein et al., 2006). Chromatin-associated proteins, often part of multi-protein complexes, are implicated in chromatin remodeling, transcription and can act as histone ‘writers’, ‘erasers’ and ‘readers’ which add, remove or recognize histone modifications respectively (Gillette and Hill, 2015). The Polycomb group protein family is such a chromatin modifying complex which was identified in Drosophila melanogaster as regulators of body patterning by maintaining gene repression of Hox genes (Lewis, 1978). Polycomb proteins reside in multi-protein complexes of which Polycomb Repressive Complex 1 (PRC1) and 2 (PRC2) are best characterized (Simon and Kingston, 2013, Gao et al., 2012, Vandamme et al., 2011). Canonical PRC1 and PRC2 are involved in gene silencing by mediating post-translational modifications of histone proteins including
H3K27me3 and H2AK119ub (Morey and Helin, 2010, Wang et al., 2004, Cao et al., 2002). The Polycomb protein BMI1 (or PCGF4), part of the canonical PRC1 complex, has been shown to be a key regulator of self-renewal of both normal and leukemic stem cells (Rizo et al., 2008, Lessard and Sauvageau, 2003, Jacobs et al., 1999, Rizo et al., 2010). EZH2, the core component of PRC2, catalyzes H3K27me3 and is critically involved in the balance between self-renewal and differentiation (Bracken et al., 2006). Aberrant expression of EZH2 can contribute to different types of leukemia. EZH2 can act as oncogene but also as tumor suppressor suggesting complexity of EZH2-mediated regulation of gene expression (Gollner et al., 2016, Herrera-Merchan et al., 2012, Ernst et al., 2010, Safaei et al., 2018). A more detailed description about Polycomb signaling is provided below. The MLL/SET1 containing methyltransferase complex mediates H3K4me3, allowing transcriptional permissive/active chromatin. The SET domain is lost in MLL-rearrangements, like the MLL-AF9 fusion gene, and instead MLL interacts with the DOT1L methyltransferase mediating H3K79me resulting in aberrant gene expression of HOXA9, MEIS1 and BCL-2 (Krivtsov and Armstrong, 2007).

1.3 Malignant hematopoiesis
Leukemogenesis is a multistep process in which genetic and epigenetic changes disturb regulatory mechanisms controlling stem cell fate and maintenance. Leukemia is characterized by an accumulation of immature blasts in the bone marrow and a block in differentiation, resulting in a deficit of fully mature and functional hematopoietic blood cells (Figure 2). This results in clinical symptoms of anemia, fatigue and impaired immune function. Leukemias can be classified into acute or chronic myeloid leukemia (AML or CML) and acute or chronic lymphoid leukemia (ALL or CLL). CML is characterized by the t(9;22) translocation resulting in BCR-ABL-induced activation of downstream signaling including PI3K, STAT and MAPK pathways (Shuai et al., 1996, Konig et al., 2008, Sawyers et al., 2002). The development and use of tyrosine kinase inhibitors, like imatinib or dasatinib, greatly improved prognosis and survival for CML patients. AML is a highly heterogeneous disease and characterized by subclonal expansion of immature blasts in the bone marrow (Hughes et al., 2014, Ding et al., 2012, Valent et al., 2012). Whole blood cell counts, cytogenetics, flow cytometry, immunohistochemistry tests and next generation or targeted sequencing are instrumental in the diagnosis and classification of AML subtypes and to subsequently design treatment strategies (Bennett et al., 1976, Hong and He, 2017). AML progresses rapidly and without treatment is fatal within months or even weeks.
Cytogenetic analysis and advances in sequencing technologies have revealed over 250 genetic abnormalities in AML, including chromosomal translocations, mutations, genetic deletions and insertions (Cancer Genome Atlas Research et al., 2013, Miller et al., 2013, Papaemmanuil et al., 2016). Mutations can be categorized in transcription-factor fusions, tumor-suppressor genes, DNA methylation related genes, signaling genes, myeloid transcription factor genes, chromatin-modifying genes, cohesion-complex genes, nucleophosmin mutation and spliceosome-complex genes (Cancer Genome Atlas Research et al., 2013). MLL-AF9 is a fusion gene caused by the t(9;11) translocation and is associated with aggressive leukemia. MLL target genes, including HOXA9, MEIS1 and BCL-2 are upregulated and regulate self-renewal and maintenance of leukemic cells (Zuber et al., 2011). Other frequent translocations in AML include t(15;17), t(8;21) and inv(16) encoding for PML-RARα, AML1/RUNX1-ETO and CBFβ/MYH11 respectively (Licht, 2001, Valk et al., 2004). The most frequently mutated genes across AML patients are FLT3, NPM1, DNMT3A, IDH1/2, TET2, RUNX1, TP53, N-RAS, C/EBPα and WT1 (Cancer Genome Atlas Research et al., 2013). AML is a very heterogeneous disease and although the complete lists of mutations found in leukemia patients is over 250, individual patients typically carry 5-15 mutations.

Figure 2. Schematic illustration of leukemia initiation and aberrant self-renewal
LSC self-renewal, maintenance, survival and chemoresistance is likely mediated by disturbed extrinsic and intrinsic factors involved in stem cell fate.
Current therapies for AML patients include intensive chemotherapy, in the first place to induce complete remission followed by consolidation/maintenance therapy to prevent recurrence of the disease. Based on karyotype and mutations, patients are categorized into good, intermediate and poor risk related to prognosis and treatment strategy, developed by the World Health Organization (Arber et al., 2016). After complete remission is achieved, good risk patients are mostly treated with autologous or allogeneic stem cell transplantation, while intermediate or poor risk patients are treated with allogeneic stem cell transplantation. In elderly patients (>65), due to increased toxicity or resistance to chemotherapy, other treatment strategies are being studied including hypomethylating agents like decitabine or azacitidine (van der Helm et al., 2013). AML patients with TET2, DNMT3A and TP53 mutations are associated with poor survival, but do sometimes benefit from treatment with hypomethylating agents (van der Helm et al., 2017, Traina et al., 2014, Welch et al., 2016). Even though the majority of patients do achieve complete remission, frequently relapse of the disease occurs within weeks or months after diagnosis with poor outcome. It is thought that AML is maintained by a small population of quiescent leukemic stem cells (LSCs) that is difficult to target and the major cause of relapse (Bonnet and Dick, 1997, Dick, 2008). AMLs consist of multiple subclones and some likely escape current treatment which leads to clonal selection and expansion (Shlush et al., 2014, Welch et al., 2012, de Boer et al., 2018). In the case of therapy-related myeloid neoplasms, gain of additional mutations or outgrowth of resistant subclones occur upon treatment (Berger et al., 2018, Godley and Larson, 2008). AML is thought to progress from a pre-leukemic state, which develops to full blown leukemia via accumulation of mutations (Valent et al., 2012, Klco et al., 2014). Some epigenetic mutations like DNMT3A and TET2 increase the risk, though additional mutations are required to induce leukemic transformation.

In order to understand human leukemia development and improve treatment strategies it is essential to establish in vivo xenograft models (Antonelli et al., 2016, Sontakke et al., 2016, Wunderlich et al., 2010, Barabe et al., 2007). Most knockout/knockdown models allow for a thorough analysis of gene function at the initiation of leukemia and novel treatment options can be evaluated in such model systems. Nevertheless, to be able to study the function of genes during the maintenance and propagation of leukemic cells, it is essential to first establish a leukemia within the bone marrow microenvironment of the mouse and then perform knockdown or knockout studies. For such studies inducible systems are essential, which we have contributed to in this thesis as well. Furthermore, in order to eradicate LSCs a better understanding of the molecular mechanisms underlying human leukemia development is needed. Therefore an important step forward is to find attractive targets, that are for instance involved in...
LSC self-renewal, maintenance/survival or chemoresistance, to ultimately be able to eradicate these LSCs (Shlush et al., 2017, Nieborowska-Skorska et al., 2017, Klco et al., 2014, de Boer et al., 2018, Valent et al., 2012, Bonardi et al., 2013). LSCs are the most primitive cells and capable of self-renewal, initiation and maintenance of leukemia upon transplantation in immune deficient mice (Lapidot et al., 1994). LSCs reside within the CD34+ fraction in the majority of cases and several new potential LSCs markers have been described, including CD123, TIM3, CD44, CD96, CD47, CD32, CD25 and CD99 (Bonardi et al., 2013, Majeti et al., 2009, Jordan et al., 2000, Jan et al., 2011, Chung et al., 2017). LSCs are relatively quiescent and give rise to leukemic blasts that are highly proliferative and characterized by a lack of differentiation capacity. LSCs share many similarities with normal stem cells and it is therefore challenging to identify targets for their identification.

In order to design better treatment strategies in leukemia, it is important to identify the molecular mechanisms that maintain LSCs. Since Polycomb proteins fulfill important functions in normal HSCs, we studied Polycomb signaling pathways in leukemia in detail in this thesis. In the next section an overview is provided of what have we learned so far and what we do not know yet.

1.4 Polycomb signaling

The Polycomb group (PcG) protein family of epigenetic regulators has been shown to be critically involved in regulating stem cell fate. In Drosophila, Polycomb mutants displayed a variety of developmental phenotypes (Jürgens, 1985) and the occupancy of Polycomb proteins at the promoters of key differentiation and developmental genes in embryonic cells suggested a critical role in regulating genes involved in cell identity and differentiation (Bracken et al., 2006, Sparmann and van Lohuizen, 2006, Lee et al., 2006, Valk-Lingbeek et al., 2004, Boyer et al., 2006, Bracken and Helin, 2009, Morey and Helin, 2010). Polycomb proteins are chromatin modifying factors and well known to function to maintain gene silencing via histone modifications and chromatin compaction or transcriptional inhibition (Muller and Verrijzer, 2009, Simon and Kingston, 2013).

The complexity of PcG complexes

Polycomb proteins reside in multi-protein complexes, the best characterized of which are Polycomb Repressive Complex 1 (PRC1) and PRC2 (Figure 3). PRC1 can be (sub)divided into canonical and non-canonical PRC1 complexes, but share the core components RING1A or RING1B and one of the six PCGF proteins (PCGF1-PCGF6). Several proteomic studies have revealed their complex composition and existence of multiple paralogs (Gao et al., 2012, Gearhart et al., 2006, Vandamme et al., 2011, van den Boom et al., 2013, Sanchez et al., 2007)). The core canonical PRC1 subunits are PCGF2/4 (PRC1.2/1.4),
There is a huge diversity of Polycomb complexes which is suggested to vary dependent on cell identity and upon differentiation, although the complexity of regulation by PRC1 is still not fully understood (Kloet et al., 2016, Morey et al., 2013, Klauke et al., 2013, van den Boom et al., 2013). Non-canonical PRC1 contains RYBP or YAF2, PCGF1 (PRC1.1) or PCGF3/4/5/6 (PRC1.3/1.4/1.5/1.6) and several other specific interaction proteins. Additional core components of non-canonical PRC1.1 subunits include KDM2B, BCOR, BCORL1, SKP1 and USP7 (See Table 1). The PRC2 complex consists of the core proteins EZH1/2, EED and SUZ12 that can interact with accessory proteins that are involved in targeting and its enzymatic activity (Pasini et al., 2010, Beringer et al., 2016, Li et al., 2017). The exact function of individual subunits in the PRC1/2 complex is not fully understood, though it is suggested that they are involved in maintaining the integrity of the complex, in providing or controlling enzymatic activity or in targeting to chromatin (Rose et al., 2016, de Napoles et al., 2004, Kaustov et al., 2011, Wong et al., 2016). Knockdown of individual PRC1 subunits in hematopoietic stem cells revealed a lack of functional redundancy, suggesting unique functions of distinct PRC1 complexes (van den Boom et al., 2013).

Figure 3. Polycomb proteins as chromatin-modifying complexes
Schematic illustration of canonical PRC1, PRC2 and non-canonical PRC1.1 (multi-protein) complexes targeted to the chromatin and associated histone modifications.
Polycomb recruitment to chromatin and gene regulation

PRC1 and PRC2 usually co-occupy target loci which can be initiated by PRC2, that catalyzes EZH1/2 mediated H3K27me3. PRC1 can be recruited to chromatin via the binding of CBX with H3K27me3 and catalyzes RING1-mediated H2AK119ub, important for PcG mediated silencing (Endoh et al., 2012). H3K27me3 acts as a repressive mark, suggested by a study in *Drosophila* that showed that repression of PRC2 target genes is affected by a point mutation in H3K27 as well as in cells lacking the catalytic subunit of PRC2 (Pengelly et al., 2013). In the case of non-canonical PRC1.1, KDM2B recruits the complex to non-methylated CpG islands via its CXXC domain (Farcas et al., 2012, He et al., 2013, Wu et al., 2013, Gearhart et al., 2006). PRC1.1 also exerts E3 ligase activity towards H2AK119 via RING1A/B and can drive PRC2 recruitment to several target loci (Tavares et al., 2012, Rose et al., 2016, Blackledge

Table 1. PRC2, cPRC1 and ncPRC1.1 core complex subunits in human (leukemic) cells

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Function</th>
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<tbody>
<tr>
<td>EZH1/EZH2</td>
<td>Trimethylation of H3K27</td>
</tr>
<tr>
<td>SUZ12</td>
<td>Essential for enzymatic activity</td>
</tr>
<tr>
<td>EED</td>
<td>Essential for enzymatic activity</td>
</tr>
<tr>
<td>PCL1/2/3, JARID2</td>
<td>Accessory proteins, possibly recruitment</td>
</tr>
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 Canonical PRC1

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Function</th>
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<tr>
<td>CBX2/4/6/7/8</td>
<td>Chromodomain, binds H3K27me3</td>
</tr>
<tr>
<td>PCGF2/4</td>
<td>Essential for enzymatic activity</td>
</tr>
<tr>
<td>PHC1/2/3</td>
<td>Protein-protein interaction</td>
</tr>
<tr>
<td>SCMH1/L1/L2</td>
<td>Protein-protein interaction</td>
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<tr>
<td>RING1A/B</td>
<td>E3 ubiquitin ligase, monoubiquitylation of H2AK119</td>
</tr>
</tbody>
</table>

 Non-canonical PRC1.1

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>RYBP/YAF2</td>
<td>DNA binding</td>
</tr>
<tr>
<td>PCGF1</td>
<td>Essential for enzymatic activity</td>
</tr>
<tr>
<td>KDM2B</td>
<td>CXXC domain, CpG binding, H3K36 demethylase</td>
</tr>
<tr>
<td>BCOR/L1</td>
<td>Protein-protein interaction</td>
</tr>
<tr>
<td>USP7</td>
<td>Ubiquitin specific protease</td>
</tr>
<tr>
<td>SKP1</td>
<td>F-box domain, protein-protein interaction</td>
</tr>
<tr>
<td>RING1A/B</td>
<td>E3 ubiquitin ligase, monoubiquitylation of H2AK119</td>
</tr>
</tbody>
</table>
et al., 2014). Polycomb target loci can also be occupied by MLL/SET1-mediated H3K4me3, so called bivalent loci, kept in a silenced state but poised for transcriptional activation.

The role of PcG complexes in regulating self-renewal and differentiation have been most extensively studied in ESCs. PcG knockout studies in mice revealed key functions of several Polycomb proteins in embryonic development (Aloia et al., 2013). BMI1 is required for maintenance of self-renewal by repression of Ink4/Arf locus (Park et al., 2003, Schuringa and Vellenga, 2010). EZH2 conserves long-term self-renewal of mouse HSCs (Kamminga et al., 2006) and EZH1 complements EZH2 in maintaining stem cell identity (Mochizuki-Kashio et al., 2011). Moreover differentiation of ESCs or mouse HSCs is regulated by different CBX-associated PRC1 complexes (Morey et al., 2012, Klaue et al., 2013). The existence of multiple PcG complexes and there dynamic functions in different cell types and during development adds to the complex understanding on how they recognize or are recruited to their target genes and regulate gene expression.

Deregulated expression of Polycomb proteins is implicated in the development of cancer and maintenance of cancer stem cells (Piunti and Pasini, 2011, Martin-Perez et al., 2010, Sauvageau and Sauvageau, 2010, Feinberg et al., 2006). Moreover, transcriptome analysis of AML CD34+ cells versus normal BM CD34+ cells revealed aberrant expression of several Polycomb proteins (de Jonge et al., 2011). Therefore the aim of this thesis is to identify critical Polycomb signaling pathways in the maintenance and propagation of leukemic cells and understand underlying mechanisms and function in Polycomb-mediated gene regulation which might provide interesting alternative possibilities to target and eradicate LSCs.
CHAPTER 1

SCOPE OF THIS THESIS

Polycomb group (PcG) proteins are classical epigenetic regulators of gene transcription and critically involved in regulating stem cell fate. Deregulation of PcG protein expression and consequently altered downstream signaling could therefore attribute to leukemic stem cell (LSC) self-renewal and maintenance. It is essential to improve treatment strategies to eradicate LSCs in patients and targeting PcG proteins might provide an interesting approach, which was investigated in this thesis.

In Chapter 2, we set out to investigate the importance of several canonical and non-canonical PRC1 proteins for leukemic cell survival using an shRNA-mediated knockdown screen in a human lentiviral MLL-AF9 leukemic model system and in primary patient acute myeloid leukemia cells. We examined in more detail the interactome of Polycomb subunits RING1A, RING1B, PCGF1, PCGF2, PCGF4 and CBX2 by proteome studies in order to validate Polycomb complex composition in leukemic cells. Functional in vitro and in vivo human leukemia xenograft studies were performed in which we focused on PRC1.1 proteins since knockdown of these was most efficient in targeting LSCs. By performing extensive ChIP-seq studies in leukemic cells we identified signaling pathways targeted by non-canonical PRC1.1 and/or canonical PRC2/PRC1.

In Chapter 3 we studied the targetability of ubiquitin-specific peptidase 7 (USP7) as part of the non-canonical PRC1.1 as an alternative therapeutic approach for AML. We examined the efficacy of small molecule USP7 inhibitors, that block its deubiquitinase activity, on the survival of (primary) leukemic cells both in vitro and in vivo. Besides USP7 controls many downstream signaling pathways, including TP53, we identified USP7 as a potential interaction partner of non-canonical PRC1.1. Since TP53 mutant AMLs were also highly sensitive upon USP7 inhibition we investigated the effects of USP7 inhibition on the integrity of the PRC1.1 complex, its recruitment to chromatin and consequences on gene transcription.

The chromatin architecture and epigenetic state contribute to gene regulation, however the underlying mechanisms via which PRC1.1 is linked to transcriptionally permissive or active chromatin is far from understood. In Chapter 3 we find that USP7 as part of PRC1.1 is critically important to maintain its stability and function. With this insight, in Chapter 4 we have begun studies aimed at a better understanding of how PRC1.1 controls gene expression. We analyzed ChIP-seq and DNA methylation data in detail and evaluated whether loss of PRC1.1 from the chromatin had an impact on de novo DNA methylation linked
to genome wide gene expression changes. For several PRC1.1 target genes, we investigated the association between loss of PRC1.1 binding and changes in histone modification levels.

In order to identify effective novel treatment strategies to target and eradicate LSCs, it is important to study gene function at different stages during the development of leukemia. In Chapter 5 we implemented an inducible Tet-regulated shRNA expression system in an in vivo human MLL-AF9 leukemic model to study gene function in a well-controlled and time-dependent manner. After verifying efficient inducible and reversible regulation of gene expression in vitro, we established a human xenograft MLL-AF9 leukemia mouse model in which timing of PCGF1 knockdown was studied on the efficacy of leukemia treatment.

The results of the studies outlined above are summarized in Chapter 6 and future perspectives are discussed.
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