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Towards identification and targeting of Polycomb signaling pathways in leukemia

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TOWARDS IDENTIFICATION AND TARGETING OF POLYCOMB SIGNALING PATHWAYS IN LEUKEMIA

Henny Maat

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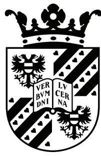

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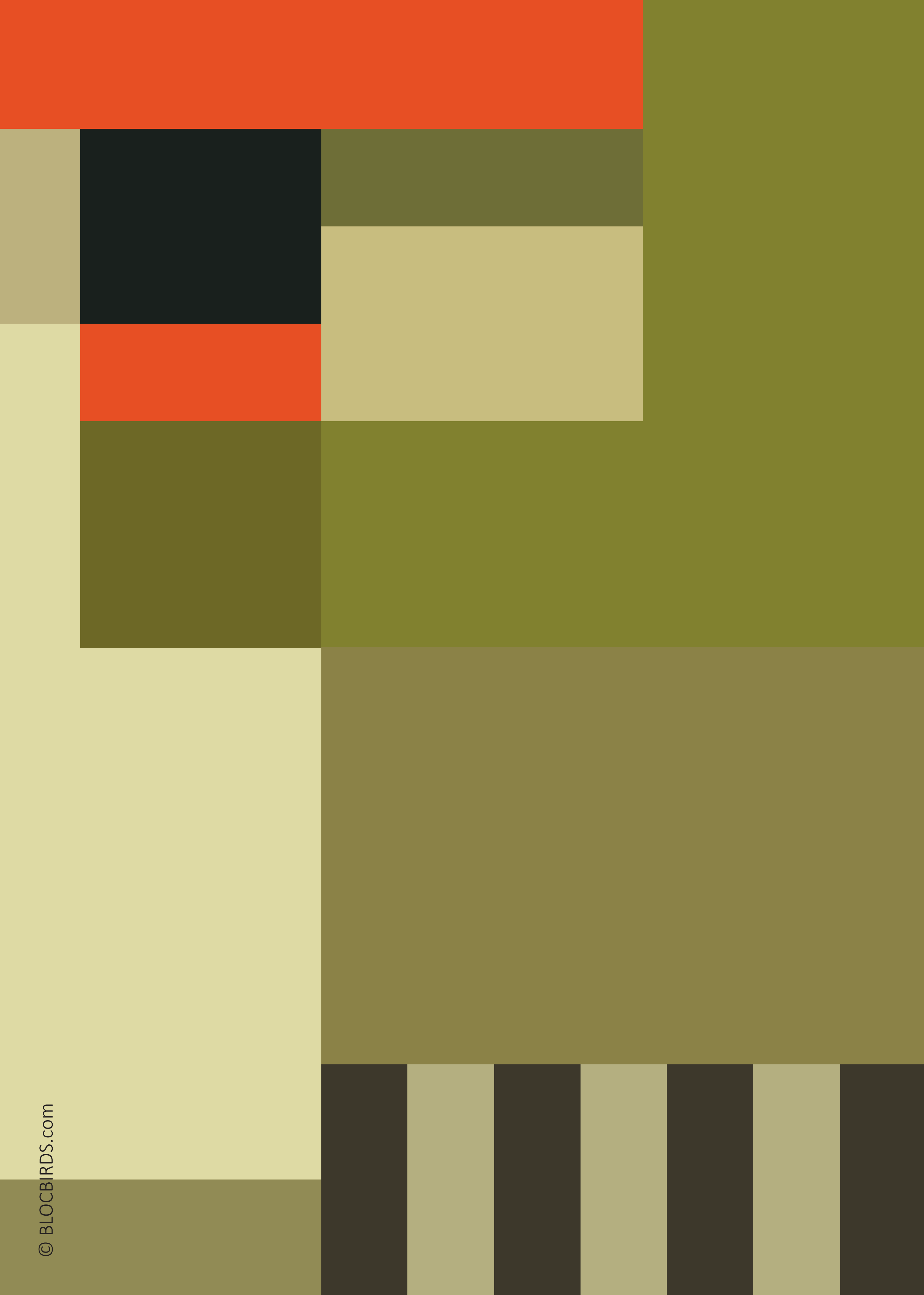
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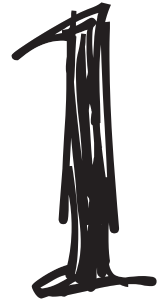
CHAPTER 

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

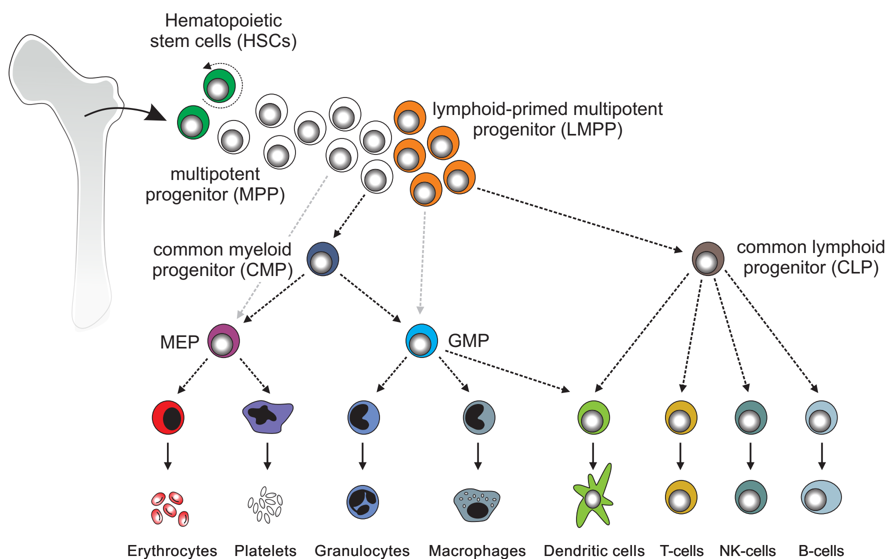


Figure 1. The hematopoietic system

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 transcriptional 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 $Lin^{-}CD34^{+}CD38^{-}CD45RA^{-}CD90^{+}Rho^{low}CD49f^{+}$ 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 Ca^{2+} levels and as a consequence enhanced mitochondrial activity can initiate cell division of HSCs (Umemoto 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 aKG 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.

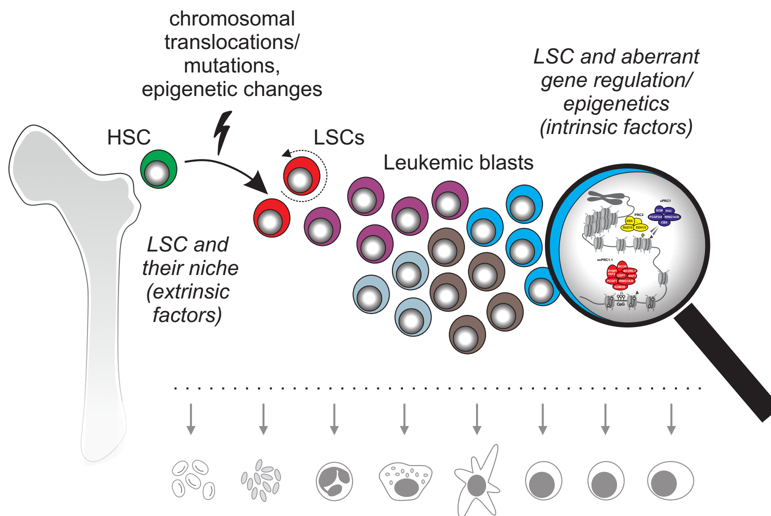


Figure 2. Schematic illustration of leukemia initiation and aberrant self-renewal

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 identify 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),

PHC1/2/3, CBX2/4/6/7/8, SCMH1/L1/L2 and RING1A/1B (See Table 1). 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).

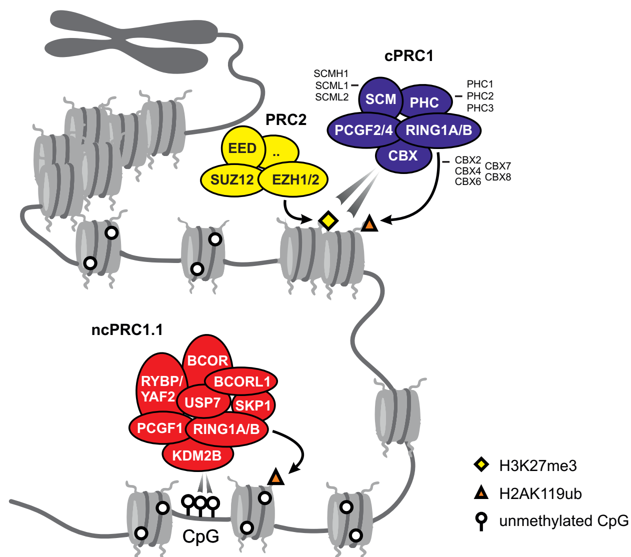


Figure 3. Polycomb proteins as chromatin-modifying complexes
Schematic illustration of canonical PRC1, PRC2 and non-canonical PRC1.1 (multi-protein) complexes targeted to the chromatin and associated histone modifications.

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

PRC2

Subunit	Function
EZH1/EZH2	Trimethylation of H3K27
SUZ12	Essential for enzymatic activity
EED	Essential for enzymatic activity
PCL1/2/3, JARID2	Accessory proteins, possibly recruitment

Canonical PRC1

Subunit	Function
CBX2/4/6/7/8	Chromodomain, binds H3K27me3
PCGF2/4	Essential for enzymatic activity
PHC1/2/3	Protein-protein interaction
SCMH1/L1/L2	Protein-protein interaction
RING1A/B	E3 ubiquitin ligase, monoubiquitylaiton of H2AK119

Non-canonical PRC1.1

Subunit	Function
RYBP/YAF2	DNA binding
PCGF1	Essential for enzymatic activity
KDM2B	CXXC domain, CpG binding, H3K36 demethylase
BCOR/L1	Protein-protein interaction
USP7	Ubiquitin specific protease
SKP1	F-box domain, protein-protein interaction
RING1A/B	E3 ubiquitin ligase, monoubiquitylaiton of H2AK119

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, Klauke 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.

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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CHAPTER 2

NON-CANONICAL PRC1.1 TARGETS ACTIVE GENES INDEPENDENT OF H3K27ME3 AND IS ESSENTIAL FOR LEUKEMOGENESIS

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ABSTRACT

Polycomb proteins are classical regulators of stem cell self-renewal and cell lineage commitment and are frequently deregulated in cancer. Here, we find that the non-canonical PRC1.1 complex, as identified by mass-spectrometry-based proteomics, is critically important for human leukemic stem cells. Downmodulation of PRC1.1 complex members, like the DNA-binding subunit KDM2B, strongly reduces cell proliferation *in vitro* and delays or even abrogates leukemogenesis *in vivo* in humanized xenograft models. PRC1.1 components are significantly overexpressed in primary AML CD34$^+$ cells. Besides a set of genes that is targeted by PRC1 and PRC2, ChIP-seq studies show that PRC1.1 also binds a distinct set of genes that are devoid of H3K27me3, suggesting a gene-regulatory role independent of PRC2. This set encompasses genes involved in metabolism, which have transcriptionally active chromatin profiles. These data indicate that PRC1.1 controls specific genes involved in unique cell biological processes required for leukemic cell viability.

INTRODUCTION

Stem cell self-renewal and lineage specification are tightly regulated processes that are of vital importance for proper embryonic development and maintenance of somatic stem cells in adults. The Polycomb group protein family of epigenetic modifiers is critically involved in the regulation of stem cell self-renewal and differentiation.

In general, Polycomb proteins reside in two complexes, the Polycomb repressive complex 1 (PRC1) and 2 (PRC2) (Simon and Kingston, 2013). The PRC2 complex, consisting of the core components EED, SUZ12 and EZH1 or EZH2, can trimethylate lysine 27 on histone H3 (H3K27me3) via EZH1 or EZH2 (Cao et al., 2002; Ezhkova et al., 2011; Kirmizis et al., 2004; Kuzmichev et al., 2002; Shen et al., 2008). The PRC1 complex has five subunits (PCGF, PHC, CBX, SCM and RING1) and displays RING1-mediated ubiquitination activity towards histone H2A at lysine 119 (H2AK119ub) (Buchwald et al., 2006; de Napoles et al., 2004; Levine et al., 2002; Wang et al., 2004). The human genome encodes for multiple paralogs for each of the PRC1 subunits: six PCGF members (PCGF1, PCGF2, PCGF3, PCGF4, PCGF5 and PCGF6), three PHC members (PHC1, PHC2 and PHC3), five CBX members (CBX2, CBX4, CBX6, CBX7 and CBX8), three SCM members (SCML1, SCML2 and SCMH1) and two RING1 members (RING1A and RING1B). Accumulating evidence suggests that PRC1 paralogs reside in the complex in a mutually exclusive manner allowing a so far poorly understood complexity of regulation by PRC1 (Gao et al., 2012; Maertens et al., 2009; Morey et al., 2012; van den Boom et al., 2013; Vandamme et al., 2011).

The classical view on Polycomb-mediated silencing is a consecutive model where PRC2 first trimethylates H3K27, followed by CBX-dependent binding of PRC1 to H3K27me3 and subsequent ubiquitination of H2AK119 (Cao et al., 2002; Bernstein et al., 2006; Kaustov et al., 2011). In line with this model, genome-wide chromatin binding studies showed frequent co-occupancy of PRC1 and PRC2 at Polycomb target genes in mammalian cells (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006). PRC1 complexes containing a CBX subunit and PCGF2 (MEL18) or PCGF4 (BMI1) are referred to as canonical PRC1 complexes (PRC1.2/PRC1.4) and often co-occupy target loci (Gao et al., 2012). However, recent work from various groups led to the identification of a class of non-canonical PRC1 complexes that contain RYBP but lack a CBX subunit and are targeted to chromatin independently of H3K27me3 (Morey et al., 2013; Tavares et al., 2012). In addition, other non-canonical PRC1 complexes were identified that are targeted to chromatin by KDM2B (PRC1.1) or L3MBTL2 (PRC1.6), the first being a DNA-binding protein that specifically targets non-methylated CpG islands via its CxxC domain (Gearhart et al., 2006; Farcas et al., 2012; He et al., 2013; Wu et al., 2013; van den Boom et al., 2013; Gao et al., 2012; Qin et al., 2012). Recent publications have shown that the H2AK119ub mark itself can also independently recruit the PRC2 complex (Cooper et al., 2014; Blackledge et al., 2014; Kalb et al., 2014). In this latter scenario, the ubiquitination of H2AK119 is dependent on non-canonical PRC1 complexes.

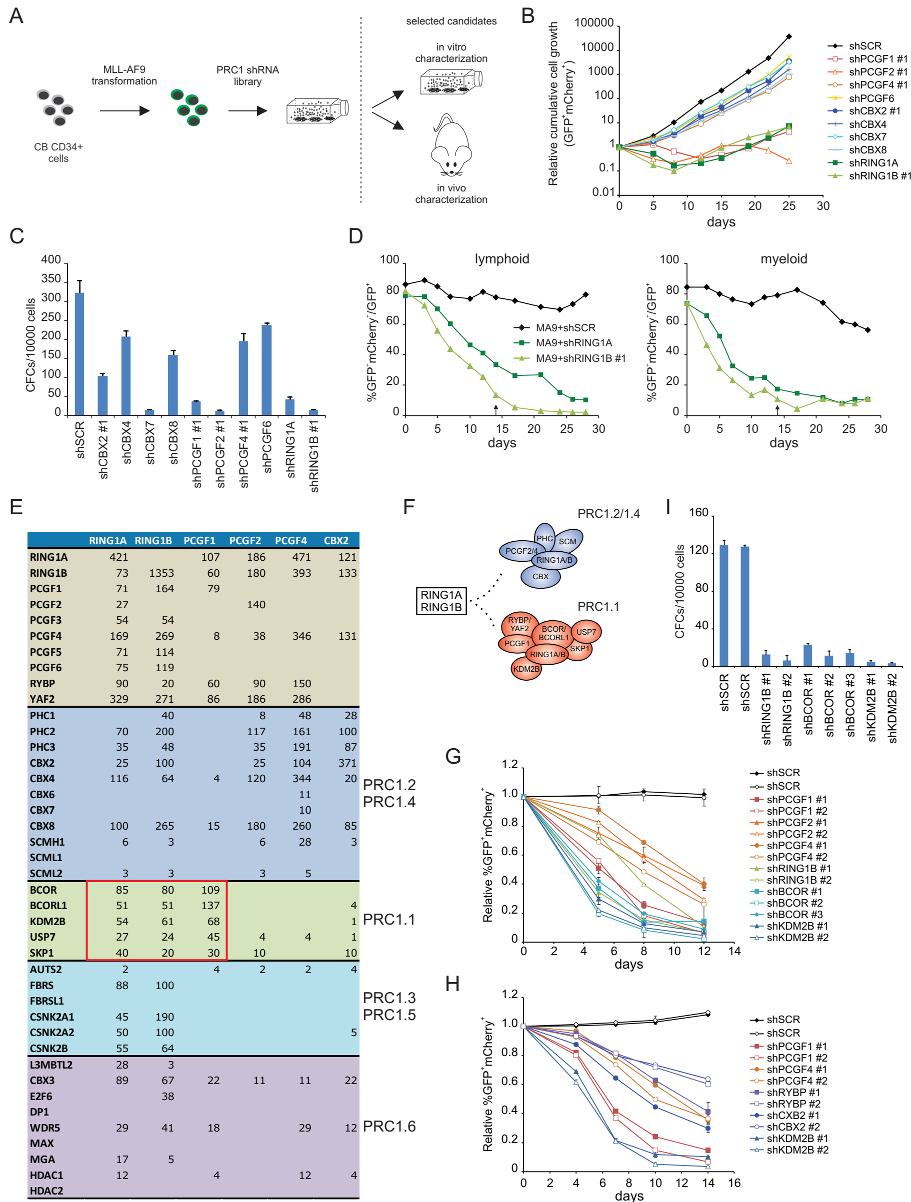
Self-renewal of hematopoietic stem cells (HSCs) critically depends on Polycomb protein function. Homozygous deletion of *Bmi1*, encoding PCGF4 (BMI1), resulted in reduced numbers of hematopoietic progenitors and more differentiated cells, eventually leading to hematopoietic failure (van der Lugt et al., 1994). Other studies showed that BMI1 has a central regulatory role in self-renewal of HSCs by inducing symmetric cell division(s) both in mouse and human model systems (Iwama et al., 2004; Lessard and Sauvageau, 2003; Park et al., 2003; Rizo et al., 2008; Rizo et al., 2009). Using an shRNA screen in human hematopoietic cells we recently showed that many PRC1 paralog family members lack functional redundancy suggesting that multiple PRC1 complexes exist that locate to specific target genes (van den Boom et al., 2013). In addition, murine hematopoietic cells display differentiation stage-specific expression of CBX paralogs and a leukemogenic role for CBX7 has been suggested (Klauke et al., 2013). Similarly, PRC1 complex composition changes upon differentiation of mouse embryonic stem (mES) cells. Whereas CBX7-PRC1 is present in self-renewing mES cells and important for pluripotency, CBX7 expression is lost upon mES cell differentiation. Instead, CBX2-, CBX4- and CBX8-containing PRC1 complexes appear to regulate lineage specification (Morey et al., 2012; O'Loghlen et al., 2012). Furthermore, Morey et al. showed that PCGF2-PRC1 is required for cardiac differentiation of mES cells and that exchange of subunits enables gene repressive and activating functions of the complex that are specific for the differentiation stage (Morey et al., 2015).

Here, we investigated PRC1 paralog dependency in human acute myeloid leukemia (AML). Using an shRNA strategy in a human lentiviral MLL-AF9 leukemia model and in primary AML patient cells combined with proteome analysis we identify the non-canonical PRC1.1 complex as an essential epigenetic regulator in leukemic cells *in vitro* and *in vivo*. Chromatin immunoprecipitation sequencing (ChIP-seq) analyses in K562 cells and primary CD34$^+$ AML patient cells show that PRC1.1 binds a unique set of active genes independent of PRC2. Gene Ontology (GO) analyses of these targets reveal enrichment for genes involved in metabolism and cell cycle regulation. Our data show that the non-canonical PRC1.1 complex is essential for leukemic stem cells and that inhibition of this complex may be beneficial for the treatment of AML.

RESULTS

Essential role for non-canonical PRC1.1 in leukemic cells

To characterize the requirement of PRC1 paralog family members for leukemic cell viability we performed an shRNA-mediated knockdown screen in our MLL-AF9 leukemic human model system (Horton et al., 2013). Cord blood (CB) CD34$^+$ cells were transduced with MLL-AF9 and subsequently allowed to transform along the myeloid lineage over the course of 3–4 weeks (Figure 1A). CB MLL-AF9 (MA9)-transformed cells were subsequently transduced with pLKO.1 shRNA vectors directed against various PRC1 paralog family members. Knockdown efficiencies of shRNAs are displayed in Figure S1A. Phenotypes were evaluated *in vitro* followed by more detailed *in vitro* and *in vivo* analyses of selected candidates (Figure 1A). Most PRC1 paralog knockdowns displayed a mild negative effect on cumulative cell growth in sorted myeloid liquid cultures in two independent experiments (Figures 1B and S1B). However, a strongly reduced proliferation was observed upon knockdown of PCGF1, PCGF2, RING1A, and RING1B, which was also reflected by colony-forming cell (CFC) analyses where a sharp decrease of progenitor frequencies was observed (Figure 1C). We noted that CBX7 knockdown resulted in moderate phenotypes in liquid cultures while strong phenotypes were observed in CFC assays, suggesting that CBX7 is relevant for cells capable of colony formation in methylcellulose but less so for cells that sustain long-term liquid cultures. Next, we focused on the two members of the RING1 paralog family, the E3 ubiquitin ligases RING1A and RING1B. Annexin V staining revealed that both RING1A and RING1B knockdown induced apoptosis in both CB MA9 cells and K562 leukemic cells (Figures S1C and S1D) as well as in several other leukemic cell lines (data not shown). Since MLL-AF9 can give rise to both myeloid leukemia (AML) and lymphoid leukemia (acute lymphoblastic leukemia (ALL)), in particular in pediatric patients, we tested whether CB MA9 cells grown under lymphoid-permissive conditions



	RING1A	RING1B	PCGF1	PCGF2	PCGF4	CBX2
RING1A	421		107	186	471	121
RING1B	73	1353	60	180	393	133
PCGF1	71	164	79			
PCGF2	27			140		
PCGF3	54	54				
PCGF4	169	269	8	38	346	131
PCGF5	71	114				
PCGF6	75	119				
RYBP	90	20	60	90	150	
YAF2	329	271	86	186	286	
PHC1		40		8	48	28
PHC2	70	200		117	161	100
PHC3	35	48		35	191	87
CBX2	25	100		25	104	371
CBX4	116	64	4	120	344	20
CBX6					11	
CBX7					10	
CBX8	100	265	15	180	260	85
SCMH1	6	3		6	28	3
SCML1						
SCML2	3	3		3	5	
BCOR	85	80	109			
BCORL1	51	51	137			4
KDM2B	54	61	68			1
USP7	27	24	45	4	4	1
SKP1	40	20	30	10		10
AUTS2	2		4	2	2	4
FBRS	88	100				
FBRSL1						
CSNK2A1	45	190				
CSNK2A2	50	100				5
CSNK2B	55	64				
L3MBTL2	28	3				
CBX3	89	67	22	11	11	22
E2F6		38				
DP1						
WDR5	29	41	18		29	12
MAX						
MGA	17	5				
HDAC1	12		4		12	4
HDAC2						

Figure 1. Primary MLL-AF9 leukemic cells critically depend on PRC1.1 (A) Schematic overview of Polycomb shRNA screen in primary MLL-AF9 (MA9)-transformed CB cells. (B) Cumulative cell growth of MA9 cells in a sorted liquid culture expressing indicated Polycomb shRNAs. (C) CFC analysis of Polycomb knockdown CB-MA9 cells. Error bars represent SD. (D) MS5 stromal co-cultures of CB-MA9 cells expressing SCR, RING1A or RING1B #1 shRNAs grown under lymphoid- and myeloid-permissive conditions. Arrows indicate time of replating. (E) Canonical and non-canonical PRC1 complex members identified by LC-MS/MS in Avi-RING1A, Avi-RING1B, Avi-PCGF1, Avi-PCGF2,

Avi-PCGF4 and CBX2-Avi pullouts from K562 cells. Total spectrum counts per protein corrected for expected peptides are shown. (F) Schematic model showing that RING1A and RING1B reside in both the canonical PRC1 complex and the non-canonical PRC1.1 complex. (G) Relative fraction of GFP$^+$mCh$^+$ cells in unsorted myeloid-permissive liquid cultures of CB MA9 cells expressing SCR, PCGF1, PCGF2, PCGF4, RING1B, BCOR and KDM2B shRNAs. Error bars represent SD. (H) Relative fraction of GFP$^+$mCh$^+$ CB MA9 cells as in panel G expressing SCR, PCGF1, PCGF4, RYBP, CBX2 and KDM2B shRNAs. Error bars represent SD. (I) CFC analysis of CB MA9 cells expressing SCR, RING1B, BCOR or KDM2B shRNAs. Error bars represent SD.

were also sensitive to RING1A or RING1B knockdown. CB CD34$^+$ cells were co-transduced with MLL-AF9 (GFP) and pLKO.1 mCherry (mCh) shRNA vectors, and unsorted MS5-driven bone marrow (BM) stromal cocultures under myeloid- or lymphoid-permissive conditions were initiated (Figure 1D). Next, the percentage of GFP$^+$mCh$^+$ cells within the total fraction of GFP$^+$ cells was measured over the course of the experiment. While in the MA9/shSCR control group the GFP$^+$mCh$^+$ fraction was relatively stable, it was rapidly reduced in the MA9/shRING1A and MA9/shRING1B groups, both under myeloid- and lymphoid-permissive conditions, suggesting that RING1A and RING1B are essential for transformation and maintenance of both myeloid and lymphoid MLL-AF9-driven leukemias.

To investigate the molecular background of RING1A and RING1B function, we identified the interactome of RING1A, RING1B, PCGF1, PCGF2, PCGF4, and CBX2. K562 cells were transduced with vectors expressing a bicistronic transcript encoding Avi-fusion proteins and the biotin ligase BirA fused to GFP and streptavidin-mediated pull outs were performed followed by LC-MS/MS analyses (Table S1) (van den Boom et al., 2013). Figure 1E shows a summary of the interactomes of these proteins, where we focused on known canonical and non-canonical PRC1 complexes. Since the total number of potentially identifiable peptides after trypsin digestion obviously differs between proteins, total spectra counts were corrected for expected peptides based on in silico protein digests. RING1A and RING1B both co-purified many proteins that reside in canonical PRC1 complexes (PRC1.2 and PRC1.4) such as PHC, CBX, and SCML proteins (Figure 1E). In RING1B pullouts, RING1A was not detected, and in RING1A pullouts, only little RING1B was identified, in line with earlier data from our lab and others showing that RING1A and RING1B are mutually exclusive in PRC1 complexes (Maertens et al., 2009; van den Boom et al., 2013). Interestingly, we found that the non-canonical PRC1.1 complex specifically co-purified with RING1A, RING1B, and PCGF1 (Figure 1E), but not with PCGF2, PCGF4, and CBX2. This led us to speculate that the phenotypic consequence of RING1A, RING1B, and PCGF1 knockdown in MLL-AF9 leukemic cells might be a consequence of compromised PRC1.1 complex activity (Figure 1F). To more specifically address the role of the PRC1.1 complex in leukemia, we generated shRNAs directed against the PRC1.1

subunits KDM2B and BCOR (knockdown efficiencies are shown in Figure S1A). CB MA9 cells were transduced with SCR, PCGF1, PCGF2, PCGF4, RING1B, BCOR, or KDM2B shRNAs, all with multiple independent shRNAs, and liquid cultures were initiated. Clearly, knockdown of KDM2B, BCOR, PCGF1, and RING1B induced a quick loss of the $GFP^{+}mCh^{+}$ fraction, whereas PCGF2 and PCGF4 knockdown showed a milder, though still negative phenotype (Figure 1G). Next, unsorted CB MA9 cultures were performed using two independent shRNAs directed against RYBP (a common component in various non-canonical PRC1 complexes;Gao et al., 2012; Garcia et al., 1999; Morey et al., 2013; Tavares et al., 2012), and we compared those with PCGF1, PCGF4, CBX2, and KDM2B knockdowns (Figure 1H). Interestingly, despite high knockdown efficiencies for both RYBP hairpins (Figure S1A), RYBP depletion resulted in a mild negative phenotype less severe than seen upon PCGF1 and KDM2B knockdowns. Finally, RING1B, BCOR, and KDM2B downmodulation also impaired the MLL-AF9 CFC frequency (Figure 1I). Taken together, these data show that the non-canonical PRC1.1 complex is pivotal for leukemic cell survival in vitro.

PRC1.1 is essential for MLL-AF9 induced leukemogenesis *in vivo*

Next, we investigated Polycomb-dependency of leukemic cells *in vivo*. CB $CD34^{+}$ cells were co-transduced with MLL-AF9 and SCR, RING1A, or RING1B shRNA vectors (Figure 2A). Next, $GFP^{+}mCh^{+}$ cells were sorted (Figure 2B), and 1×10^{5} cells were injected intravenously per mouse. Peripheral blood chimerism levels of $GFP^{+}mCh^{+}$ cells were monitored by regular blood sample analysis and mice were sacrificed when chimerism levels in the blood exceeded 30%. BM, spleen and liver analyses of sacrificed mice showed that all three organs displayed high levels of chimerism (>90%), indicative of a full-blown leukemia (Figure S2A). Leukemia development was first observed in the MA9/shSCR group. Downregulation of RING1A significantly delayed leukemia development, while knockdown of RING1B completely prevented MA9-induced leukemic transformation *in vivo* within the time frame of the experiment (Figures 2C and 2D). Spleen weights in MA9/shSCR leukemic mice were strongly increased compared to non-leukemic mice (Figure 2E). Despite the absence of leukemia development, MA9/shRING1B mice recurrently showed low but clearly detectable chimerism levels, which slowly increased over time (Figure 2C). Some mice transplanted with MA9/shRING1A cells did develop leukemia, but qRT-PCR analysis of BM cells from these leukemic mice showed that the reduction of RING1A mRNA expression levels was considerably less compared to knockdown efficiencies directly after transduction (Figure 2F). These data suggest that only clones with a relatively mild RING1A knockdown can persist, while clones with a strong RING1A knockdown do not expand or only slowly expand in vivo. In accordance with previous studies (Horton et al., 2013), leukemic mice mostly developed $CD19^{+}$ lymphoid leukemias (ALL), and small co-

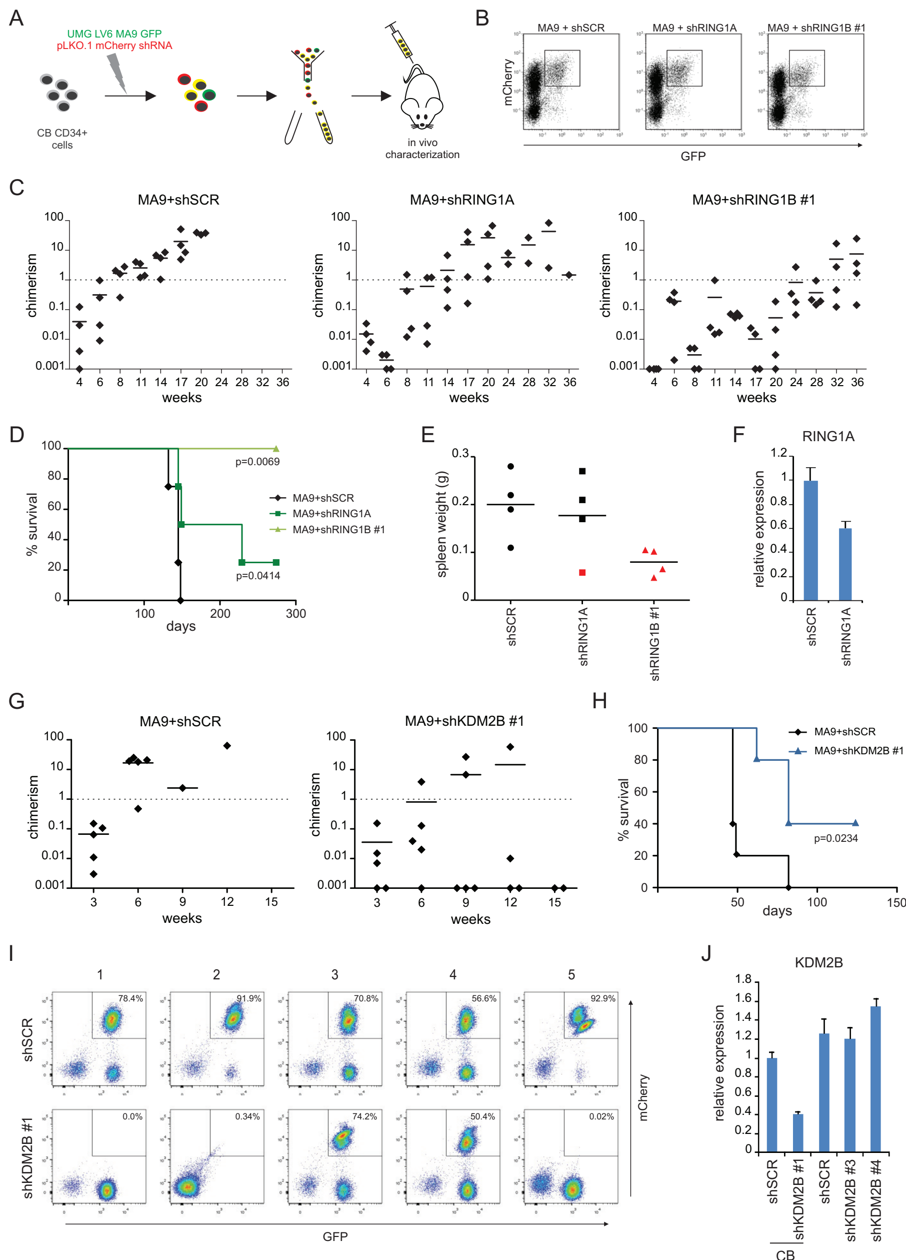


Figure 2. PRC1.1 depletion interferes with MLL-AF9 leukemogenesis *in vivo*. (A) Schematic overview of shRNA expression in a primary MLL-AF9 (MA9) xenograft model. (B) FACS sort of MA9 (GFP) and shRNA (mCh) expressing cells at the day of injection. (C) Peripheral blood chimerism of MA9/shSCR, MA9/shRING1A or MA9/shRING1B cells over the course of the experiment. (D) Kaplan-Meier survival plot of mice intravenously injected with MA9 shSCR, shRING1A or shRING1B expressing cells (n=4 per group). This survival plot is a representative example from two independent

experiments. Statistical analysis was performed using a log-rank test. (E) Spleen weights of leukemic mice (black symbols) at the day of sacrifice or non-leukemic mice (red symbols) at the end of the experiment. (F) Average knockdown efficiencies of RING1A in bone marrow of leukemic mice. Error bars represent SEM. (G) Peripheral blood chimerism of MA9/shSCR and MA9/shKDM2B cells over the course of the experiment. (H) Kaplan-Meier survival plot of mice intravenously injected with MA9/shSCR or MA9/shKDM2B expressing cells (n=5 per group). Statistical analysis was performed using a log-rank test. (I) FACS plots showing BM analyses at the day of sacrifice of MA9/shSCR and MA9/shKDM2B mice. (J) Average KDM2B knockdown efficiencies in bone marrow of SCR mice (n=5) and two individual KDM2B knockdown mice.

existing $CD33^+$/$CD19^-$ myeloid clones were observed only in some mice (Figure S2B).

Next, we selectively interfered with non-canonical PRC1.1 function by knocking down KDM2B. Here, an MLL-AF9 secondary transplantation model was used where leukemic cells were harvested from mice that developed a full-blown lymphoid leukemia after transplantation of MA9-transduced $CD34^+$ CB cells. Subsequently, these cells were transduced with SCR and KDM2B shRNA vectors, GFP^+mCh^+ cells were sorted and 5×10^5 cells were intravenously injected per mouse (n = 5). Peripheral blood analyses showed that MA9/shSCR mice quickly developed high chimerism levels, whereas MA9/shKDM2B mice displayed a slower increase in chimerism and sometimes lost chimerism at later stages of the experiment (Figure 2G). Survival analysis showed that MA9/shKDM2B mice have a significantly delayed onset of leukemia compared to MA9/shSCR controls (Figure 2H). Importantly, the MA9/shKDM2B mice that did develop leukemia either only showed chimerism of single GFP^+ MA9 cells (Figure 2I; likely due to sort impurities) or did not show knockdown of KDM2B (Figure 2J; KDM2B #3 and KDM2B #4).

Altered expression of Polycomb proteins in AML

Given that the PRC1.1 complex was of vital importance for leukemic cells, we hypothesized that the expression of its components might be deregulated in primary leukemic patient samples. Previously, we performed transcriptome studies in AML $CD34^+$ cells (n = 60) and normal BM $CD34^+$ cells (n = 40) (de Jonge et al., 2011). Here, we investigated which PRC2, PRC1, or PRC1.1 complex partners were significantly differentially expressed between AML $CD34^+$ and normal BM $CD34^+$ cells. Among others, the PRC1.1 components BCOR, PCGF1, and RING1A were significantly upregulated in AML $CD34^+$ cells (Figure S2C; Table S2). Similarly, HemaExplorer datasets (http://servers.binf.ku.dk/hemaexplorer/) also showed that PRC1.1 members were significantly upregulated compared to normal HSC/progenitor fractions (Figure S2D; Table S2). In contrast, the expression of PRC2 complex members EZH2 and EED was significantly lower in AML $CD34^+$ cells, whereas EZH1 showed increased expression.

PRC1.1 is required for primary patient AML cell growth *in vitro* and *in vivo*

Given that the PRC1.1 complex was essential for MLL-AF9-transformed cells and its expression was increased in primary AML patient cells, we investigated the functional requirement of PRC1.1 in primary samples (patient details are provided in Table S2). Primary AML patient CD34$^+$ cells were transduced with SCR, RING1A, RING1B, or KDM2B shRNAs, and unsorted MS5 stromal co-cultures were initiated (Figures 3A and S3A). Knockdown of KDM2B led to a quick loss of mCh$^+$ cells over time compared to SCR control cultures, whereas shRING1B-expressing cells were lost as well but at lower rates (Figure 3B). Next, we performed co-cultures using CD34$^+$ AML cells (two patients) transduced with SCR, PCGF1, PCGF2, PCGF4, RING1A, RING1B, BCOR, or KDM2B shRNAs (Figures 3B and S3B). In both AMLs, mCh$^+$ cells were quickly lost upon knockdown of PRC1.1 components like KDM2B, BCOR, PCGF1, or RING1B. Slightly milder phenotypes were observed upon depletion of PCGF2, PCGF4, or RING1A. Together, these data suggest that although there is some heterogeneity between individual AML patients, the non-canonical PRC1.1 complex is critically important in AML.

Next, we tested the effect of RING1A, RING1B, or KDM2B knockdown on AML development *in vivo* using a humanized model that is based on subcutaneous implantation of human BM-like scaffolds as reported previously (Groen et al., 2012; Gutierrez et al., 2014; P. Sontakke and J.J.S., unpublished data). Shortly, four hybrid scaffolds consisting of three 2 to 3 mm biphasic calcium phosphate (BCP) particles loaded with human mesenchymal stromal cells (MSCs) were implanted subcutaneously into NSG mice, where they formed bone and differentiated into bone marrow stromal cells, together serving as a human niche for AML leukemic stem cells. Six weeks after implantation the scaffolds were well vascularized and scaffold 1 and 3 were injected with 200,000 mCh$^+$ AML cells (AML 8) expressing SCR, RING1A, RING1B, or KDM2B shRNAs (Figure 3C). Clearly, whereas all mice injected with shSCR cells developed leukemia after ~100-130 days, only one shRING1B mouse developed leukemia, but with severely delayed onset (day 188). The other shRING1B and shKDM2B mice did not develop tumors (Figure 3D). One shRING1A mouse developed leukemia, but also with longer latency compared to SCR control mice. At day 200 after intra-scaffold injection, all remaining mice were sacrificed and no signs of tumor initiation were observed (Figure 3E). Taken together, these data suggest that PRC1.1 is functionally relevant across a broad set of AML subtypes.

PRC1.1 targets active genes independent of H3K27me3

Next, we performed ChIP-seq studies to identify non-canonical PRC1.1 and canonical PRC1 target genes in leukemic cells. For this purpose, we expressed GFP fusions of RING1A, RING1B, PCGF1, PCGF2, PCGF4, and CBX2 or non-fused GFP in K562 leukemic cells and

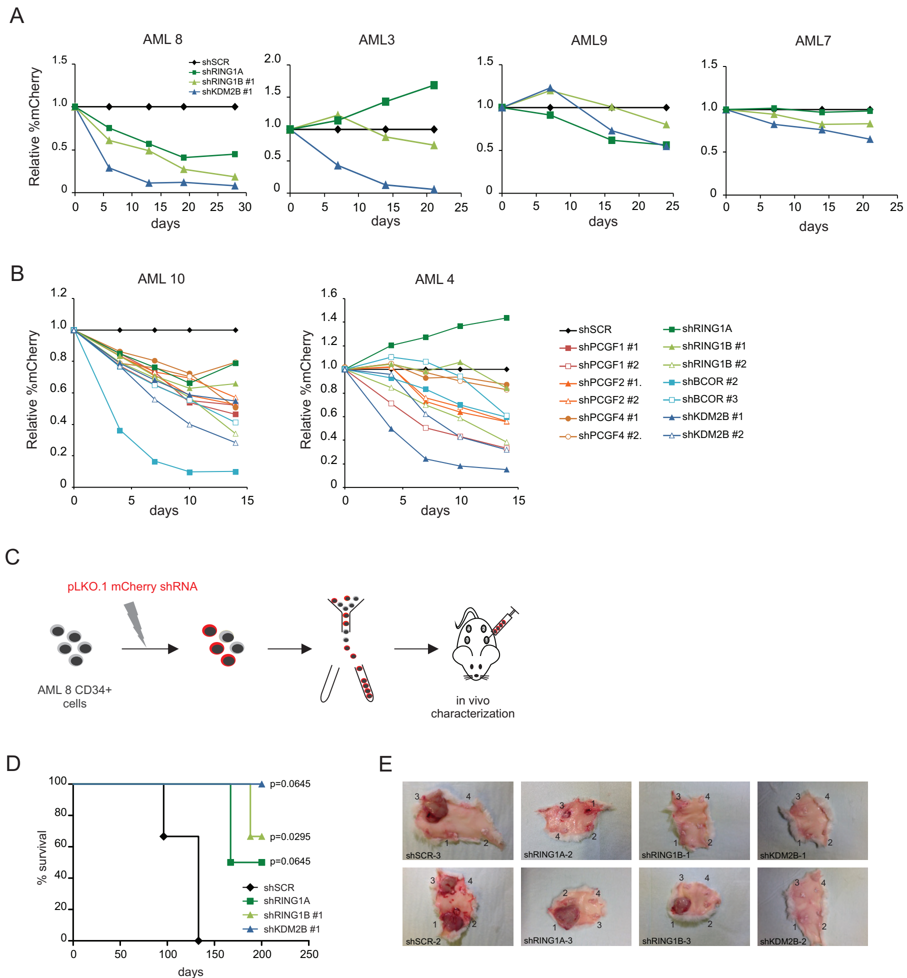


Figure 3. PRC1.1 is required for in vitro growth of primary AML patient cells and leukemogenesis *in vivo.* (A) Relative fraction mCh+ cells of primary AML patient cells from four independent patients transduced with SCR, RING1A, RING1B or KDM2B knockdown vectors grown on a stromal cell layer. (B) Relative fraction mCh+ cells as in (A) where primary AML patient cells from two independent patients were transduced with SCR and multiple PCGF1, PCGF2, PCGF4, RING1A, RING1B, BCOR and KDM2B shRNAs. (C) Experimental setup of our humanized niche scaffold xenograft model using primary AML patient cells transduced with pLKO.1 mCherry SCR, RING1A, RING1B or KDM2B shRNA vectors. (D) Kaplan-Meier survival plot of mice intra-scaffold injected with AML 8 CD34+ cells expressing SCR (n=3), RING1A (n=2), RING1B (n=3) or KDM2B (n=2) shRNAs. Statistical analysis was performed using a log-rank test. (E) Pictures from skin of sacrificed mice showing vascularized scaffolds and tumors in shSCR, shRING1A and shRING1B mice.

performed ChIP reactions using an α-GFP antibody. We carefully analyzed the expression levels of GFP-fusion proteins compared to endogenous protein expression. Fluorescence-activated cell sorting (FACS) analyses showed that all cell lines displayed comparable mean fluorescence intensities of GFP (Figure S4A), and western blot analyses showed that GFP-fusion proteins were expressed at levels comparable to their endogenous counterparts (Figure S4B). Furthermore, we compared our GFP-CBX2 and GFP-RING1B tracks with endogenous CBX2 and RING1B ChIP-seq datasets in K562 cells from ENCODE/Broad, which showed strong overlap in target genes, suggesting that the GFP moiety did not interfere with chromatin targeting of the proteins (Figures S4C and S4D).

In addition, we also generated H3K27me3 and H2AK119ub profiles in K562 cells. Peak calling was performed, and normalized read counts were calculated for each precipitated component at each called chromosomal position. Subsequently, we identified PRC1.1 binding sites (PCGF1^{+}, RING1B^{+}, and CBX2^{-}), PRC1 binding sites (RING1B^{+}, CBX2^{+}, and PCGF1^{-}), and genomic regions containing both PRC1.1 and PRC1 (PCGF1^{+}, RING1B^{+}, and CBX2^{+}; Figure 4A; Table S4). RING1A and RING1B binding sites showed a near to complete overlap (Figure 4B). Supervised clustering analysis was performed on PRC1.1 and/or PRC1 occupied loci, and heatmaps and density plots are shown in Figures 4C and S5A. Interestingly, and in contrast to PRC1, PRC1.1 binding sites were completely devoid of H3K27me3. H2AK119ub was enriched in all clusters, although distinct patterns could be observed. In addition, we performed ChIP-seq analyses using an antibody recognizing endogenous KDM2B (Figure 4C). Clearly, KDM2B was enriched at genomic loci assigned as PRC1.1 and "both" loci, but not PRC1 loci, supporting our annotation of PRC1.1 targets. Comparison of PCGF1, PCGF2, and PCGF4 showed that whereas PRC1 target genes were devoid of PCGF1, PRC1.1 target genes also showed some occupancy of PCGF2 and PCGF4 suggesting that PRC1.1 loci may, to some extent, also be co-occupied by canonical PRC1 (Figure S5B).

Genome-wide analysis of PRC1.1 and PRC1 peaks showed that PRC1.1 was mainly targeted to transcription start sites (TSSs) whereas the majority of PRC1 peaks were located in intergenic or intronic regions (Figures 4D and S5C; Table S4). Chromosomal regions harboring both PRC1 and PRC1.1 complexes generally located to TSSs or intergenic regions. In agreement with previously published data showing KDM2B-dependent PRC1.1 targeting to non-methylated CpG islands (CGIs) we observed preferential binding of PRC1.1 to CGIs (94.1%, Figure 4E) (Farcas et al., 2012; He et al., 2013; Wu et al., 2013). In contrast, PRC1 peaks did not enrich at CGIs (18.2%), and peaks targeted by both PRC1 and PRC1.1 showed intermediate enrichment for CGIs (68.9%). Genes were assigned as being regulated by PRC1.1, PRC1, or both when a peak was called within a −5 to +5 kb region relative to a TSS (GREAT; http://bejerano.stanford.edu/great/public/html/; Table S4 (McLean et al., 2010).

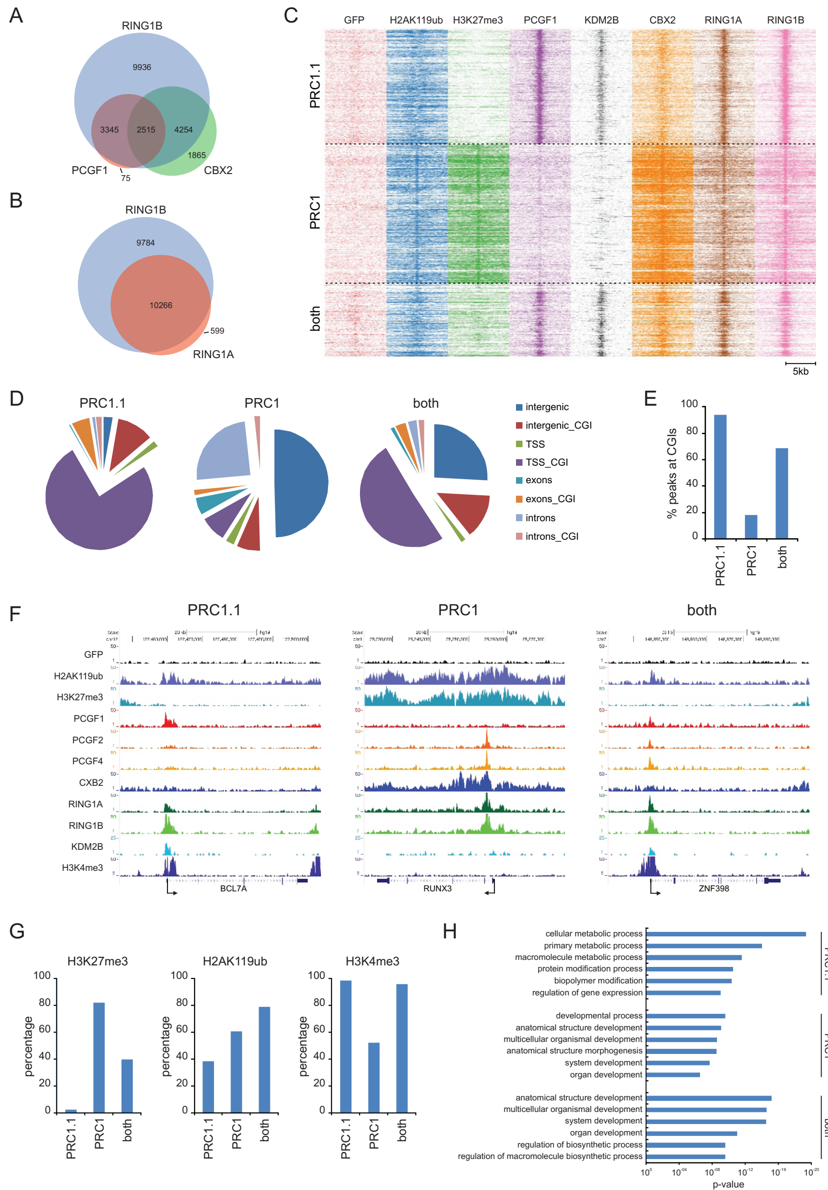


Figure 4. Non-canonical PRC1.1 and PRC1 target unique sets of genes involved in specific pathways. (A) Venn diagram showing overlap of RING1B, PCGF1 and CBX2 called peaks. (B) Venn diagram displaying overlap of RING1A and RING1B called peaks. (C) ChIP-seq heatmap of peaks and surrounding regions (-5 to +5 kb) targeted by PRC1.1 (n=3327), PRC1 (n=4016) or both (n=2122). (D) Localization analysis of identified PRC1.1, PRC1 and 'both' peaks across the genome. TSS, transcription stat site; CGI, CpG island. (E) Percentage of peaks targeted by PRC1.1, PRC1 or both that are localized to CGIs (F) Characteristic examples of genes targeted by PRC1.1, PRC1 or both complexes at the transcription start site (TSS). (G) Percentage of genes targeted by PRC1.1, PRC1 or 'both' based on

occupancy in a-5kb to +5kb window surrounding the TSS, which enrich for H3K27me3, H2AK119ub or H3K4me3. (H) Gene Ontology (GO) analysis of genes targeted by PRC1.1, PRC1 or both.

Thus, 2434 PRC1.1 target genes were identified, 386 genes targeted by PRC1 and 1029 genes bound by both complexes. Representative examples of ChIP-seq profiles of PRC1.1, PRC1, and both target genes are displayed in Figure 4F. Specific comparison of these genes with our H3K27me3 and H2AK119ub ChIP-seq tracks and ENCODE/Broad K562 H3K4me3 profiles showed strong enrichment for H2AK119ub and H3K4me3 but not H3K27me3 at PRC1.1 loci, whereas PRC1 target genes were enriched for H3K27me3, H2AK119ub, and, to a lesser extent, H3K4me3 (Figure 4G). Since PRC1.1 target genes were strongly enriched for the active chromatin mark H3K4me3 and devoid of H3K27me3, we hypothesized that PRC1.1 target genes may be actively transcribed in contrast to PRC1 target genes that are typically repressed. To investigate this, available K562 tracks (ENCODE/Broad) for H3K36me3, which enriches at actively transcribed genes throughout the gene body, and serine 5 phosphorylated active RNA polymerase II (RNAPII S5P) were analyzed. Both H3K36me3 and RNAPII S5P were strongly enriched at PRC1.1 target genes, whereas only weak enrichment was observed at PRC1 target genes (Figures S5D and S5E). GO analyses strikingly showed that PRC1.1 targeted genes involved in metabolism, whereas PRC1-bound genes were enriched for classical Polycomb-associated GO terms related to development and lineage specification (Figure 4H). Genes that were targeted by both PRC1 and PRC1.1 showed the strongest enrichment for developmental GO terms. Specific analyses for KEGG pathway-associated terms indicated that leukemia-associated pathways were enriched in the PRC1.1 as well as the “both” category of target genes.

Independent ChIP-qPCR experiments confirmed our ChIP-seq data and examples of ChIP-seq screenshots and ChIP-qPCRs are shown in Figures S6A and S6B. Strong binding of PCGF1, RING1A, and RING1B, but not PCGF2 or PCGF4, was observed around the TSSs of PRC1.1 targets LIMD2, GATA5, MYC, and PKM. These loci were also enriched for H2AK119ub and H3K4me3 marks but devoid of H3K27me3 marks. Interestingly, downmodulation of RING1A or RING1B resulted in a significant decrease in MYC expression, indicating that this locus is not repressed but likely activated by PRC1.1 (Figure S6C). In contrast, the CDKN1A locus was targeted by both canonical PRC1 and non-canonical PRC1.1 (Figures S6A and S6B) and knockdown of RING1A/B resulted in a significant increase in p21 expression, showing Polycomb repression of this locus (Figure S6C). Taken together, these data show that PRC1.1 regulates active genes involved in metabolism and cell cycle that are devoid of PRC2 activity.

Identification of non-canonical PRC1.1 targets in primary AML patient cells

Next, we identified PRC1.1 target genes in primary $CD34^+$ AML cells derived from six independent AML patients (patient details are provided in Table S3). ChIP-seq was performed using antibodies recognizing endogenous KDM2B, H2AK119ub, H3K27me3, and H3K4me3. Subsequently, three categories of target genes were defined: PRC1.1 ($KDM2B^+$, $H2AK119ub^+$, and $H3K27me3^-$), PRC1 ($KDM2B^-$, $H2AK119ub^+$, and $H3K27me3^+$), or genes targeted by both complexes that were positive for all three marks (Figure 5A; Table S4). Heatmaps of all annotated peak regions (−5 to +5 kb) in all AMLs are shown in Figure 5B. Similar to K562 cells, genome-wide peak localization analyses showed that PRC1.1 preferentially localized to CGI-containing TSSs, whereas the majority of PRC1 bound loci localized to intergenic regions (Figure 5C). Furthermore, PRC1.1 peaks were strongly enriched for H3K4me3 (~90%) across all AML samples, whereas PRC1-specific targets showed a much lower number of peaks with H3K4me3 (~30%; Figures 5B and 5D). Genomic regions targeted by both PRC1 and PRC1.1 were also highly enriched for H3K4me3 (~98%). Figure 5E shows examples of ChIP-seq profiles of PRC1.1, PRC1, or both target genes. Similar to K562 cells, PRC1.1 was found to target the MYC and PKM genes whereas CDKN1A was targeted by both PRC1.1 and PRC1. Next, we performed independent ChIP-qPCR experiments on AML2 and AML3 and analyzed H3K27me3, H2AK119ub, H3K4me3, KDM2B, and PCGF4 occupancy at PRC1.1, PRC1, and both loci (Figure 6A). Similar to our ChIP-seq data, we observed that PRC1.1 targets were enriched for H2AK119ub, H3K4me3, and KDM2B, but not H3K27me3. In contrast, PRC1 targets showed high levels of H3K27me3 and H2AK119ub but low levels of H3K4me3 and KDM2B. Genes targeted by both complexes were enriched for H3K27me3, H2AK119ub, H3K4me3, and KDM2B. PCGF4 showed the strongest enrichment at PRC1 target genes but was also observed at some PRC1.1 target genes. GO analyses showed that PRC1.1 target genes were enriched for metabolic processes, chromatin organization, and cell cycle, whereas the PRC1-specific and both targets were highly enriched for developmental GO terms (Figure 6B; Table S5). Finally, we also performed ChIP-seq analysis on $CD34^+$ cells derived from mobilized peripheral blood (PB $CD34^+$). PRC1.1, PRC1, and both target genes were annotated in this sample (Figure 6C), and we tested the overlap of PRC1.1 target genes between the AML samples and control PB (Figure 6D). Thus, common PRC1.1 targets were identified, as were targets that were specific for either AML $CD34^+$ cells or PB $CD34^+$ cells (Figure 6D; Table S6).

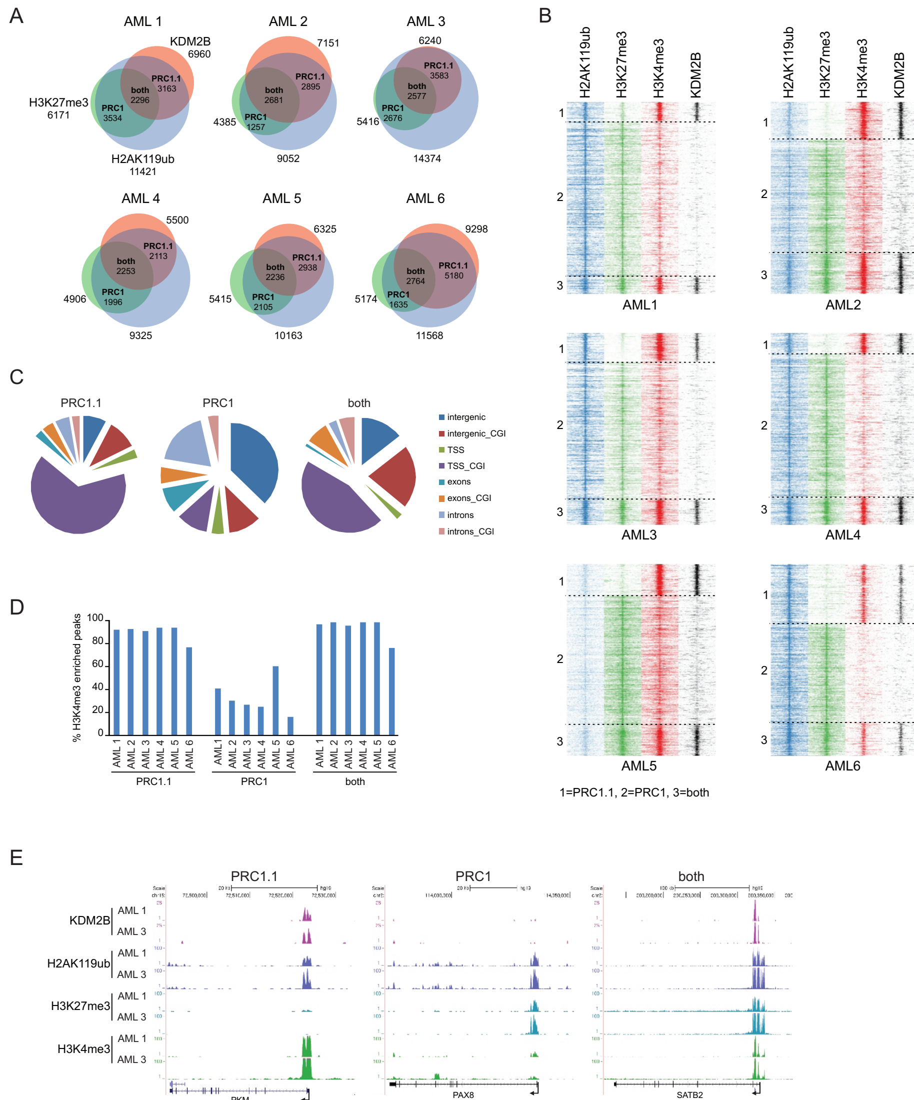


Figure 5. Distinct targeting of non-canonical PRC1.1 and PRC1 in primary CD34⁺ AML patient cells. (A) Venn diagrams showing overlap of genes targeted by KDM2B, H2AK119ub and H3K27me3 in six independent AML patient samples. (B) ChIP-seq heatmap of peaks (-5 to +5 kb) targeted by PRC1.1, PRC1 or both in all analyzed AML samples. (C) Chromosomal localization of peaks enriched for PRC1.1, PRC1 or both complexes. The average of all six AML samples is shown. (D) Percentage of H3K4me3-enriched peaks targeted by PRC1.1, PRC1 or both complexes in all measured primary AML patient samples. (E) Representative examples of genes targeted by PRC1.1 and/or PRC1 in two independent AMLs.

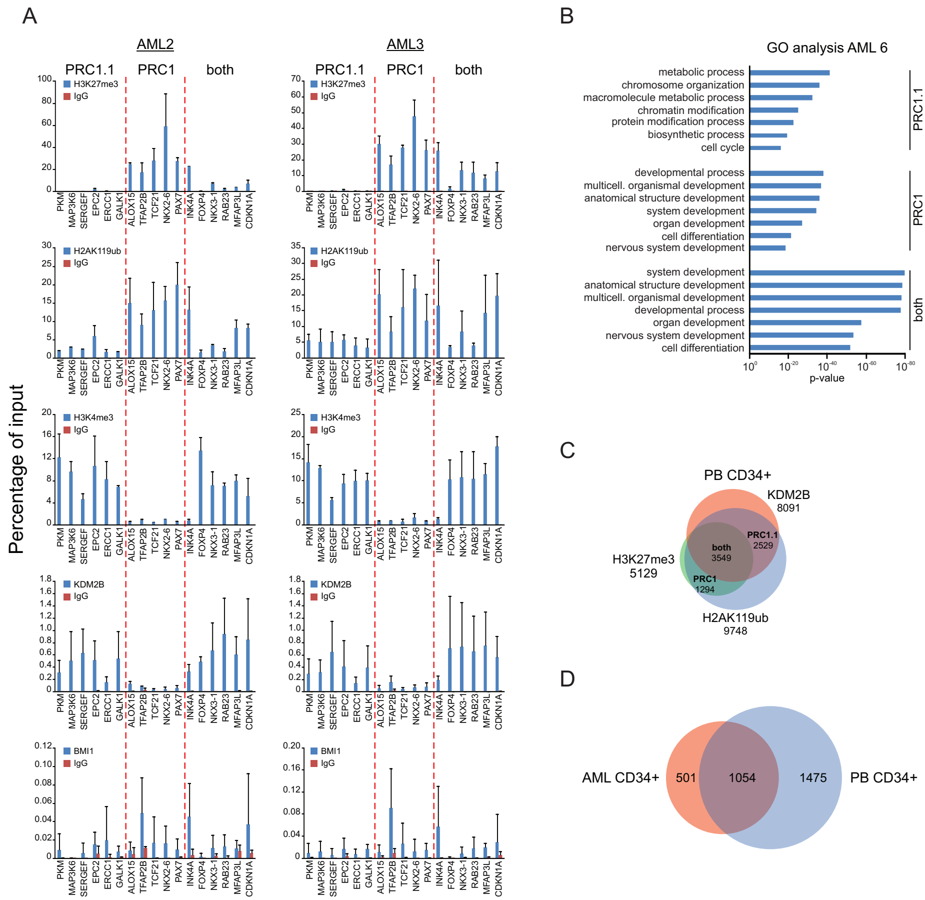


Figure 6. Specific PRC1.1 targeting in AML and normal PB CD34⁺ cells. (A) ChIP-qPCR on PRC1.1, PRC1 and 'both' target genes using antibodies directed against H3K27me3, H2AK119ub, H3K4me3, KDM2B and PCGF4. (B) GO analyses of gene sets targeted by PRC1.1, PRC1 or both complexes (AML6). (C) Venn diagram showing overlap of genes targeted by KDM2B, H2AK119ub and H3K27me3 in normal PB CD34⁺ cells. (D) Overlap in non-canonical PRC1.1 targeted genes in AML CD34⁺ cells (we considered a gene a PRC1.1 target gene if it was found in five out of six AMLs) compared to normal PB CD34⁺ cells.

DISCUSSION

Our data provided here demonstrate that leukemic cells from AML patients are critically dependent on a functional non-canonical PRC1.1 complex. Proteomics studies in leukemic cells revealed strong interactions between the RING1A/B ubiquitin ligases and non-canonical PRC1.1 proteins like KDM2B, PCGF1, and BCOR(L1). Knockdown of PRC1.1 subunits strongly impaired leukemic cell growth *in vitro*. PRC1.1 complex partners are frequently overexpressed in human AML patients, and using our *in vivo* MLL-AF9 and primary AML patient humanized niche xenograft models, we could demonstrate that leukemia initiation and maintenance both required the presence of a functional PRC1.1 complex. Finally, we observed that PRC1.1 targets a large set of active genes involved in metabolism and cell cycle in primary AML patient cells independent of H3K27me3.

A role for PRC1.1 in leukemic transformation and maintenance arose from our expression data showing upregulation of various members of the PRC1.1 complex in AML CD34$^+$ cells versus normal BM CD34$^+$ cells (de Jonge et al., 2011). Therefore, we hypothesize that increased PRC1.1 expression may act as an oncogenic hit in the process of leukemogenesis. In line with this idea, overexpression of murine KDM2B induces transformation of mouse BM cells and KDM2B knockdown conversely abrogates Hoxa9/Meis1-induced leukemogenesis (He et al., 2011). Interestingly, KDM2B was also overexpressed in human pancreatic ductal adenocarcinoma cells, and KDM2B collaborated with mutant KRAS to induce pancreatic tumors in mouse models (Tzatsos et al., 2013). Similarly, increased abundance of the PRC1.1 complex in human leukemic cells may act as a primary or secondary oncogenic hit.

The severe negative phenotype upon downregulation of PRC1.1 members in primary MLL-AF9 cells is in contrast with the milder phenotype observed upon knockdown of canonical PRC1 complex members like PCGF4 and CBX2. In contrast, normal CB CD34$^+$ cells critically depend on a functional PRC1 complex and display strong sensitivity to CBX2 knockdown (van den Boom et al., 2013). These data suggest that PRC1 paralog dependency in normal human hematopoietic stem/progenitor cells versus leukemic cells in AML is quite distinct. The mild phenotype of PCGF4 knockdown resembles the observation that MLL-AF9-induced leukemic transformation of mouse BM cells is independent of PCGF4/BMI1, and HOXA9 may replace PCGF4/BMI1 as a repressor of the CDKN2A locus (Smith et al., 2011). Previously, Tan and colleagues reported that MLL-AF9-induced leukemogenesis depends on CBX8 in a PRC1-independent manner and suggested a co-activating role for CBX8 on MLL-AF9 target genes (Tan et al., 2011). Similarly, we found that CBX8 knockdown reduced cell proliferation and colony formation in MLL-AF9 liquid cultures and CFC analyses. In contrast to our study, knockdown of RING1B did not affect MLL-AF9-dependent cell growth in their model system, at least

not in relatively short *in vitro* assays in which cells were analyzed for 5–10 days (Tan et al., 2011). It is currently not clear which mechanisms might underlie these different observations, but it is possible that PRC1 paralog dependency differs between human models and mouse models driven by leukemic granulocyte-macrophage progenitors.

Although knockdown of KDM2B, PCGF1, and BCOR strongly impaired MLL-AF9-induced leukemogenesis, we unexpectedly observed that downregulation of RYBP, an integral part of non-canonical PRC1 complexes, resulted in a rather mild negative phenotype in CB MLL-AF9 cells. An explanation for this phenotype could be that RYBP is replaced by its homolog YAF2, in line with previous data showing that YAF2 and RYBP can reside in variant PRC complexes in a mutually exclusive manner (Gao et al., 2012).

Using a ChIP-seq approach in K562 leukemic cells and primary $CD34^+$ AML patient cells, we identified genes that are targeted by PRC1.1 and/or PRC1. In line with previous studies in mouse embryonic stem cells, we find that PRC1.1 preferentially targets CGI-containing TSSs (Farcas et al., 2012; He et al., 2013; Wu et al., 2013). Where these studies showed that PRC1.1 often co-represses genes together with canonical PRC1 and PRC2 complexes, we observe a large fraction of genes that is preferentially targeted by PRC1.1 and enriched for H2AK119ub but devoid of H3K27me3 (Farcas et al., 2012; He et al., 2013; Wu et al., 2013). We do find some binding of PCGF2/4 at PRC1.1 sites suggesting that canonical PRC1 may also bind these loci, though with lower efficiency than PRC1.1. These data suggest that PRC1.1 can target chromatin independently of PRC2, in line with recent data that PRC1.1 can act as an initiating complex in Polycomb-mediated silencing (Cooper et al., 2014; Blackledge et al., 2014; Kalb et al., 2014). In contrast, PRC1 target genes are strongly enriched for H3K27me3, and a category of genes targeted by both PRC1 and PRC1.1 display an intermediate situation where H3K27me3 is found but to a lesser extent compared to exclusive PRC1 gene targets. Interestingly, Farcas and colleagues make note of low-magnitude RING1B binding sites in mES cells, which are lost upon either RING1B depletion or KDM2B knockdown and only infrequently coincide with PRC2 (Farcas et al., 2012). These sites may be similar to the PRC1.1 target genes that we identified, although we observed a strong enrichment for PRC1.1 complex members rather than weak binding. Farcas and colleagues suggested that these genes are targeted to make them susceptible to Polycomb-mediated silencing. We hypothesize that in leukemic cells, PRC1.1 might specifically regulate the activity of its target genes. In line with this idea, PRC1.1 bound genes displayed transcriptionally active chromatin profiles that were strongly enriched for H3K4me3, H3K36me3 and active RNA polymerase II. Furthermore, the expression of the PRC1.1 target gene MYC was increased upon RING1A/B knockdown, suggesting an activating role for the PRC1.1 complex. RING1B may play a role in recruitment of RNA polymerase II as recently suggested by Frangini and

colleagues, who showed that RING1B, together with Aurora B kinase, regulates active genes in resting B and T cells (Frangini et al., 2013). Interestingly, a recent study shows that KDM2B binding to non-methylated CGIs prevents CpG methylation at these sites (Boulard et al., 2015). Although not addressed in our current work, PRC1.1 may prevent CGI hypermethylation at target genes, thereby maintaining their transcriptional activity.

We compared non-canonical PRC1.1 target genes in AML CD34$^+$ samples with normal PB CD34$^+$ samples, and we observed that besides AML-specific and normal PB-specific loci, a considerable overlap exists, suggesting that these PRC1.1 genes are controlled by non-canonical signaling in both normal and leukemic cells. Future studies will be aimed at further unraveling similarities and differences between normal and leukemic cells, but what is clear now is that PRC1.1 mostly targeted genes involved in metabolism, whereas canonical PRC1/2 predominantly binds classical Polycomb target genes involved in developmental processes. Interestingly, the non-canonical RYBP-PRC1 complex was also found to target metabolic genes in mES cells (Morey et al., 2013). Here, RYBP-PRC1 targets were annotated by the presence of RING1B, RYBP, and H2AK119ub, but not CBX7. Since RING1B and RYBP are also PRC1.1 subunits, part of these enriched regions may in fact be PRC1.1 target genes. In addition, in human pancreatic ductal adenocarcinoma cells it was also found that KDM2B targets a large group of metabolic genes independent of EZH2 (Tzatsos et al., 2013). Furthermore, Brookes and colleagues previously identified a set of active PRC loci that were enriched for metabolic genes as well (Brookes et al., 2012). Taken together, we suggest that the non-canonical PRC1.1 complex targets a variety of active genes involved in metabolism independently of H3K27me3.

These metabolic PRC1.1 target genes include enzymes functioning in the glycolytic pathway like pyruvate kinase (PKM) and lactate dehydrogenase (LDHA). KDM2B recently was suggested to positively regulate the glycolytic pathway (Yu et al., 2015). Furthermore, the Scadden lab recently demonstrated that expression of both PKM (PKM2 splice variant) and LDHA are essential for leukemogenesis and that loss of either gene resulted in delayed leukemic onset of BCR-ABL and MLL-AF9 induced leukemias *in vivo* (Wang et al., 2014). We hypothesize that deregulated expression of these glycolytic genes upon PRC1.1 depletion contributes to the observed phenotypes in leukemogenesis. In addition, also other cancer-related genes, such as the cell-cycle regulatory gene MYC, were controlled by PRC1.1.

Taken together, we propose that the non-canonical PRC1.1 complex is essential for leukemic transformation and that its targeting might prove an excellent way to eradicate leukemic stem cells, with the ultimate aim to prevent relapse of the disease. It will be of great interest to investigate which PRC1.1-regulated cellular pathways are essential for leukemic stem cell function and whether pharmacological inhibition of either of these pathways, or PRC1.1 itself, may prove a rigid therapy in AML.

MATERIALS AND METHODS

Primary cell isolation

Cord blood (CB) was obtained from healthy full-term pregnancies after informed consent was obtained in accordance with the Declaration of Helsinki at the obstetrics departments at the Martini Hospital and University Medical Center Groningen. The study was approved by the UMCG Medical Ethical Committee. CB $CD34^+$ cells were isolated as described previously (Schuringa et al., 2004).

Lentiviral transductions

For transduction CB $CD34^+$ cells were pre-stimulated and transduced as described previously (Horton et al., 2013; Schuringa et al., 2004; van den Boom et al., 2013). One round of transduction was performed and cells were harvested at day 2 after transduction. For MLL-AF9 transformation of CB $CD34^+$ cells either FEIGW MLL-AF9 IRES GFP (Figure 1B, 1C, S1B and S1C; (Horton et al., 2013)) or UMG LV6 MLL-AF9 (all other experiments, (Chiarella et al., 2014)) lentiviral vectors were used. For the *in vitro* PRC1 shRNA library screen MLL-AF9 transformed CB CD34+ cells, grown under myeloid-permissive conditions for 4 to 6 weeks, were transduced with pLKO.1 mCherry shRNA vectors. Short hairpin sequences used in this study are: shSCR:CAACAAGATGAAGAGCACCAA; shPCGF1(#1):CCACTCTAAAGCCCACTACTA;shPCGF1(#2):GCCACTGCTCAACCTCAAACT;shPCGF2(#1):GCTGAGCATCAGGTCTGACAA;shPCGF2(#2):GAGCCACTGAAGGAATACT;shPCGF4(#1):CGGAAAGTAAACAAAGACAAA;shPCGF4(#2):AGAAGGAATGGTCCACTT;shPCGF6:CCCATACATCTTGTGTTCCAT;shCXB2(#1):CGCCGAGTGCATCCTGAGCAA;shCXB2(#2):ACAGGAAGCATGCGTACAGTA;shCBX4:TGCCTACCTTTGCCCGTCGTT;shCBX7:CGGAAGGGTAAAGTCGAGTAT:shCBX8:CGTCACCATTAAGGAAAGTAA;shRING1A:AGACGAGGTATGTGAAGACAA;shRING1B(#1):CGAAGTCTACACAGTGAATTA;shRING1B(#2):GCTCATCAAGAGAGAGTATTA;shBCOR(#1):GGCACTTGGTGATATAACT;shBCOR(#2):GCTCTCCAATGGCAAGTATCC;shBCOR(#3):GCTTGTCTACGTAGACCTTCT;shKDM2B(#1):GGAAGTTGAGAGTCTGCTTTG;shKDM2B(#2):GCATGAGCTCTTGTACTTACA;shRYBP(#1):CACCGTCATTATCACAGACTT;shRYBP(#2):CCAAAGTCTGACATTCTGAAA.

Cell culture

CB MLL-AF9 liquid cultures and MS5 stromal cocultures were performed as described previously (Horton et al., 2013). For CB MLL-AF9 liquid cultures and MS5 cocultures under myeloid-permissive conditions cells were cultured in Gartner's medium supplemented with IL-3, SCF and Flt-3L (10 ng/ml each). For MS5 cocultures under lymphoid-permissive conditions the same conditions were used except that hydrocortisone, horse serum and

IL-3 were left out and ascorbic acid (50 g/ml, Sigma) and IL-7 (10 ng/ml) were added. MS5 stromal cells were cultured in alpha MEM supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin. The (human) erythromyeloblastoid leukemia cell line K562 was cultured in RPMI 1640 (containing L-glutamine) supplemented with 10% FCS and 1% penicillin/streptomycin (PAA Laboratories).

CFC assays

For colony-forming cell (CFC) assays of MLL-AF9 transformed cells 10.000 $GFP^+mCherry^+$ cells were plated in duplicate in 1 ml methylcellulose (H4230, Stem Cell Technologies) supplemented with 20 ng/ml IL-3, IL-6, SCF, G-CSF, Flt-3L, 10 ng/ml GM-CSF and 1U/ml Epo. Colonies were scored after 2 weeks.

Flow cytometry analysis and sorting procedures

Prior to staining, cells were blocked with anti-human FcR Block (Stem Cell Technologies) and murine cells were blocked with anti-Fcγ (BD Biosciences). Cells were stained with anti-CD14 APC-Cy7 (M5E2, Biolegend), anti-CD15 BV605 (W6D3, BD), anti-CD19 BV785 (HIB19, Biolegend), anti-CD20 (2H7, BV605), anti-CD33 APC (WM53, Biolegend), anti-CD34 APC (581, BD) and anti-CD45 BV421 (HI30, Biolegend). For AnnexinV stains, transduced cells were stained with APC-conjugated AnnexinV (IQ Products). Cell sorting was performed on a MoFlo-Astrios (Beckman Coulter). Analyses were done on a LSR-II (BD Biosciences). Data were analyzed using FlowJo 7.6.1 software (TreeStar, Ashland, OR).

Establishment of the humanized scaffold niche xenograft model and transplantations

The humanized scaffold niche xenograft models was applied as described previously (Groen et al., 2012; Gutierrez et al., 2014). For this purpose, four hybrid scaffolds containing three biphasic calcium phosphate particles (2–3mm) were loaded with human MSCs and subcutaneously implanted into NSG mice. NSG female mice were anesthetized by isoflurane, four subcutaneous pockets were made and 1 scaffold was implanted in each pocket. Subsequently. incisions were closed using Histoacryl and the mice were treated with temgesic buprenorphine (0.1mg/kg body weight) before surgery and housed in separate IVC cages. Seven weeks after scaffold implantation mice were sub-lethally irradiated using 1 Gy. One day after irradiation, 200.000 transduced CB cells were intra-scaffold injected. CD45 engraftment was analyzed by measuring peripheral blood chimerims (once in three weeks) and mice that had considerable engraftment and that showed signs of sickness were euthanized by cervical dislocation.

RNA isolation and qPCR

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's recommendations. For quantitative RT-PCR, RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad) and amplified using SsoAdvanced SYBR Green Supermix (Bio-Rad) on a MyIQ thermocycler (Bio-Rad). Data was quantified using MyIQ software (Bio-Rad). RPL27 was used as a housekeeping gene. Primer sequences are available on request.

GFP- and Avi-fusion constructs

Lentiviral pRRL SFFV GFP-fusion vectors for CBX2, PCGF2 and PCGF4 were generated as described previously (van den Boom et al., 2013). PCGF1 was amplified from pMSCV Avi-PCGF1 IRES GPF-BirA with flanking BamHI and AgeI sites and cloned into pJet1.2. Using BamHI and AgeI digestion PCGF4 was swapped with PCGF1 resulting in pRRL SFFV PCGF1-GFP. RING1A was amplified from pJet1.2 RING1A with flanking BsrGI sites and cloned into pJet1.2. Next, RING1A was cloned into pRRL SFFV GFP using BsrGI digestion resulting in pRRL SFFV GFP-RING1A. RING1B was cloned with flanking BsrGI sites into pJet1.2 and subsequently subcloned into pRRL SFFV mCherry using BsrGI. Subsequently, RING1B was cloned into the pRRL SFFV GFP vector using BsrGI digestion resulting in pRRL SFFV GFP-RING1B. For generation of lentiviral pRRL SFFV Avi-MCS IRES GFP-BirA and pRRL SFFV MCS-Avi IRES GFP-BirA vectors GFP-BirA was subcloned from pMSCV Avi-fusion IRES GFP-BirA vector that were described before and swapped with GFP in the pRRL SFFV IRES GFP vector (van den Boom et al., 2013). Linkers encoding Avi-MCS or MCS-Avi were cloned in front of the IRES. PCGF2 and RING1A were subcloned from pCR4 PCGF2 and pJet1.2 RING1A vectors and inserted into pRRL SFFV Avi-MCS IRES GFP-BirA using AgeI/MluI digestion. BMI1 was PCR-amplified with flanking AgeI and MluI sites from MiGR BMI1 and cloned into pJet1.2. Subsequently BMI1 was subcloned from pJet1.2 BMI1 into pRRL Avi-MCS IRES GFP-BirA using AgeI and MluI digestion. RING1B was cloned with flanking AgeI and MluI sites and cloned into pJet1.2. RING1B was subcloned from pJet1.2 RING1B into pRRL SFFV Avi-MCS IRES GFP-BirA by AgeI/MluI digestion resulting in pRRL SFFV Avi-RING1B IRES GFP-BirA. For PCGF1 pull outs we used the pMSCV Avi-PCGF1 IRES GPF-BirA vector.

Streptavidin-mediated pull-outs

Nuclear extracts were prepared from K562 cells stably co-expressing GFP-BirA and Avi-RING1A, Avi-RING1B, Avi-PCGF1, Avi-PCGF2, Avi-PCGF4 or CBX2-Avi. As a control we used K562 cells expressing GFP-BirA alone. Next, pull-outs were performed using magnetic Streptavidin M-280 Dynabeads (Invitrogen). Pull out material was separated on a 4-12% NuPAGE gel (Invitrogen), stained with Coomassie blue G250 and subsequently destained overnight. Gel lanes were cut into 24 slices for in-gel trypsin digestion. Details concerning

nuclear extract preparation, pull outs and LC-MS/MS analyses are described in van den Boom et al., 2013 (van den Boom et al., 2013). The MS data, obtained on an LTQ-Orbitrap XL™ (Thermo Scientific) were submitted to Mascot (Version 2.1, Matrix Science, London, UK) using the Proteome Discoverer 1.3 analysis platform (Thermo Scientific) and searched against the UniProtKB Human complete proteome. Since the number of potentially identified peptides after trypsin digestion obviously differs between proteins, PeptideMass (http://web.expasy.org/peptide_mass/) was used to obtain the number of expected trypsin digests with masses between 750 and 4000 Dalton, and the total spectra counts shown in Figure 1E were corrected for these numbers of expected peptides.

In vivo transplantations into NSG mice

8- to 10-week-old female NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice were purchased from the Centrale Dienst Proefdieren breeding facility within the University Medical Center Groningen. Mouse experiments were performed in accordance with national and institutional guidelines, and all experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen Prior to transplantations, mice were sublethally irradiated with a dose of 1.0 Gy (Rizo et al., 2010). Following irradiation, mice received neomycin (3.5 g/l in drinking water) and soft food daily for 2 weeks. Mice were injected intravenously with 1 3 105 sorted MA9/shSCR, MA9/shRING1A, or MA9/shRING1B CB CD34^{+} cells. Mice were sacrificed when chimerism levels in the PB exceeded 30% and/or when mice appeared lethargic.

ChIP

ChIP was essentially performed as described previously (Frank et al., 2001). Briefly, K562 cells were transduced with the lentiviral GFP-fusion vectors encoding GFP-CBX2, PCGF1-GPF, PCGF2-GFP, PCGF4-GFP, GFP-RING1A, or GFP-RING1B. K562 cells expressing GFP fusions at relatively low levels were sorted and expanded and subsequently crosslinked. ChIP reactions were performed using the following antibodies: anti-GFP (ab290, Abcam), anti-H3K27me3 (07-449, Millipore), anti-H3K4me3 (ab8580, Abcam), anti- H2AK119ub (D27C4, Cell Signaling Technology), anti-KDM2B (ab137547, Abcam), and anti-BMI1 (AF27). ChIP efficiencies were determined by qPCR. Additional materials and methods can be found in Supplemental Experimental Procedures.

ChIP-seq

Sequencing samples were prepared according to the manufacturer’s protocol (Illumina). End repair was performed using the precipitated DNA using Klenow and T4 PNK. A 3’ protruding A base was generated using Taq polymerase and adapters were ligated. The

DNA was loaded on gel and a band corresponding to ~300 bp (ChIP fragment + adapters) was excised. The DNA was isolated, amplified by PCR and used for cluster generation on the Illumina HiSeq 2000 genome analyzer. The 50 bp tags were mapped to the human genome HG19 using BWA (Li and Durbin, 2009). For processing and manipulation of SAM/BAM files SAMtools was used (Li et al., 2009). For each base pair in the genome the number of overlapping sequence reads was determined and averaged over a 10 bp window and visualized in the UCSC genome browser (http://genome.ucsc.edu).

Detection of enriched regions

Peak calling algorithm MACS was used to detect the binding sites at a q-value cut off for peak detection of 0.01. GFP-CBX2, PCGF1-GPF, PCGF2-GFP, PCGF4-GFP, GFP-RING1A or GFP-RING1B peaks were called relative to a control track (GFP) (Zhang et al., 2008).

Tag counting

Tags within a given region were counted and adjusted to represent the number of tags within a 1 kb region. Subsequently the percentage of these tags as a measure of the total number of sequenced tags of the sample was calculated.

Peak distribution analysis

To determine genomic locations of binding sites, the peak file was analyzed using genomic_distribution.py script that annotates binding sites according to all RefSeq genes. With this script every binding site is annotated either as promoter (-500 bp to the Transcription Start Site), exon, intron or intergenic (everything else).

Generation of profiles and heatmaps

All heatmaps and bandplot profiles were generated using fluff (http://simonvh.github.com/fluff).

Gene ontology analyses

For gene ontology (GO) analysis we used either DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/home.jsp) or BiNGO (Maere et al., 2005).

Western blotting

Western blot analysis was performed as published previously (van den Boom et al., 2013). The following antibodies were used: anti-PCGF1 (ab183499, Abcam), anti-MEL18 (H-115, Santa Cruz), anti-BMI1 (F6, Millipore), anti-CBX2 (N-20, Santa Cruz) and anti-RING1B (ab181140, Abcam), anti-STAT5 (C-16, Santa Cruz), anti-b-Actin (C4 and N-21, Santa Cruz).

Statistical analysis

For transcriptome studies previously published datasets were used (de Jonge et al., 2011; Metzeler et al., 2008; Valk et al., 2004). In the manuscript by De Jonge et al we described 46 AML CD34$^+$ and 31 NBM samples. Since then we have included more samples in our transcriptome studies and analyses described in Figure S2C includes 60 AML CD34$^+$ samples and 40 NBM CD34$^+$ samples. Statistically significant differences were determined with multiple testing correction (Benjamini-Hochberg, FDR $p<0.01$).

Accession numbers

The accession number for the ChIP-seq data reported in this paper is GEO: GSE54580.

Author contributions

V.v.d.B., H.M., and J.J.S. conceptualization and methodology. V.v.d.B., H.M., M.G., A.R.L., J.J., A.Z.B.-V., F.F., and J.J.S. performed experiments. V.v.d.B., H.M., E.V., and J.J.S. analyzed and interpreted data. V.v.d.B., H.M., A.M.S., H.G.S., J.H.A.M., and J.J.S. performed ChIP-seq experiments and interpreted data. R.W.J.G., H.Y., and A.C.M.M. helped to setup the scaffold *in vivo* mouse xenograft model. V.v.d.B., H.M., and J.J.S. wrote the manuscript.

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SUPPLEMENTARY FIGURES

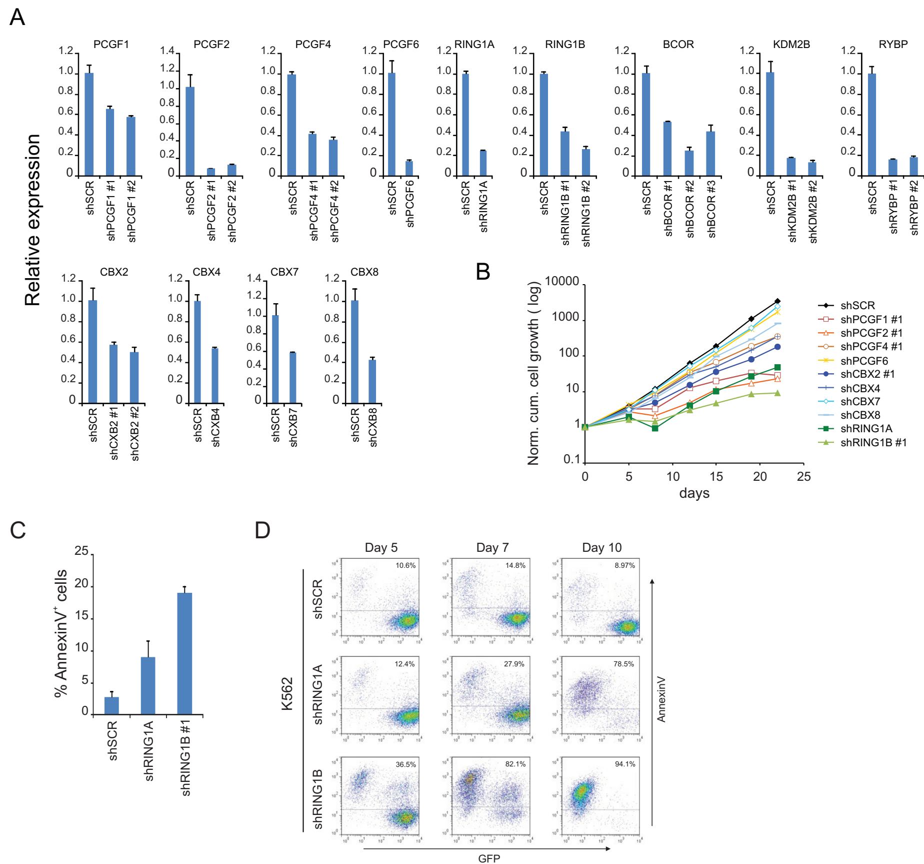

Figure S1. shRNA efficacy and effect on proliferation and apoptosis levels in CB MA9 cells. (A) Knockdown efficiencies of all used shRNA vectors CB MA9 cells 4 days after transduction as measured by qPCR. Error bars represent SEM of triplicate measurements. (B) Cumulative cell growth of CB MA9 cells in a sorted liquid culture under myeloid-permissive conditions, expressing indicated Polycomb shRNAs. (C) Apoptosis levels in CB MA9 cells transduced with SCR, RING1A or RING1B knockdown vectors as measured by AnnexinV stains. Graph shows average of two experiments and error bars represent standard deviations. (D) AnnexinV stains on K562 cells expressing SCR, RING1A or RING1B shRNAs.

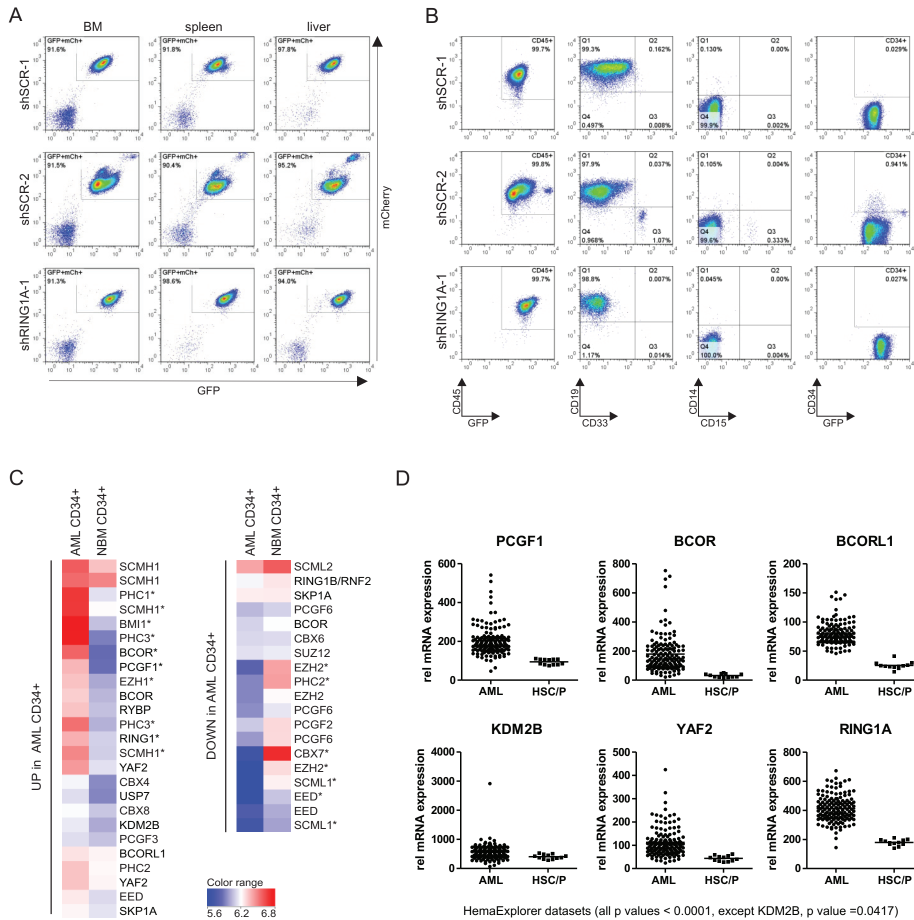

Figure S2. Analysis of leukemic mice and PcG expression in human AML patient cells. (A) FACS analysis of bone marrow (BM), spleen and liver of leukemic mice. (B) FACS analysis of differentiation markers of bone marrow cells from three independent leukemic mice. (C) Expression analysis of Polycomb proteins in AML CD34^{+} cells (n=60) compared to normal bone marrow (NBM) cells (n=40; based on (de Jonge et al, Leukemia 2011). Significant probesets (p<0.001) are indicated with asterisks. (D) HemaExplorer database shows increased expression of PRC1.1 components in primary AML samples compared to normal HSCs and progenitors.

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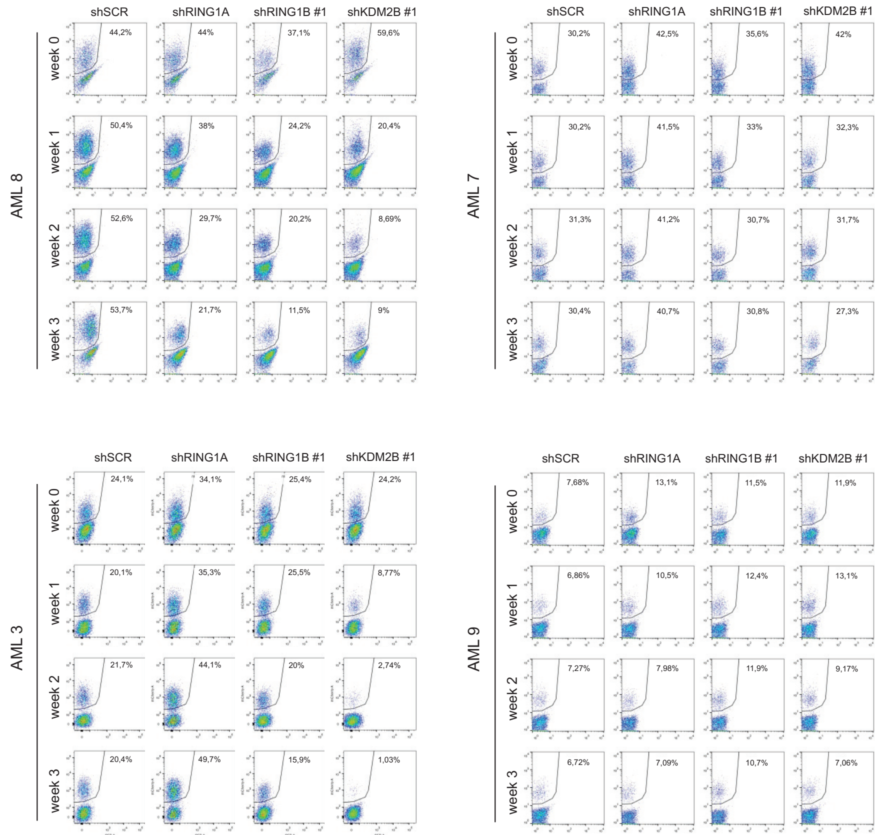

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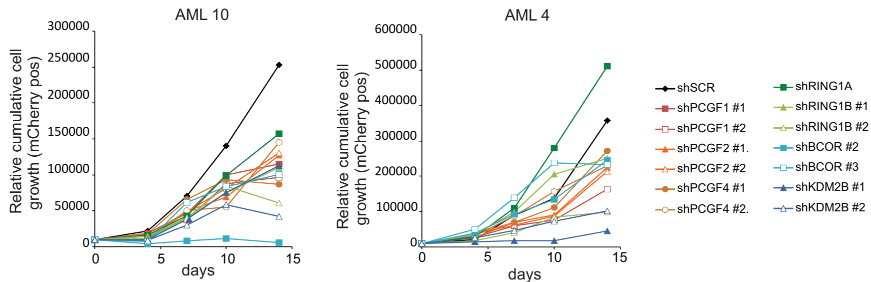

Figure S3. PRC1.1 knockdown affects proliferation of primary AML patient cells. (A) FACS analyses of mCh+ fraction in primary AML CD34^{+} cells transduced with various pLKO.1 mCherry shRNA vectors (shSCR,sh RING1A,sh RING1B or shKDM2B) and grown on a stroma cell layer. FACS analyses were performed at the indicated time points. (B) Relative cumulative cell growth of mCh^{+} cells in co-cultures of AML CD34^{+} cells, from two independent patients, transduced with various shRNA vectors.

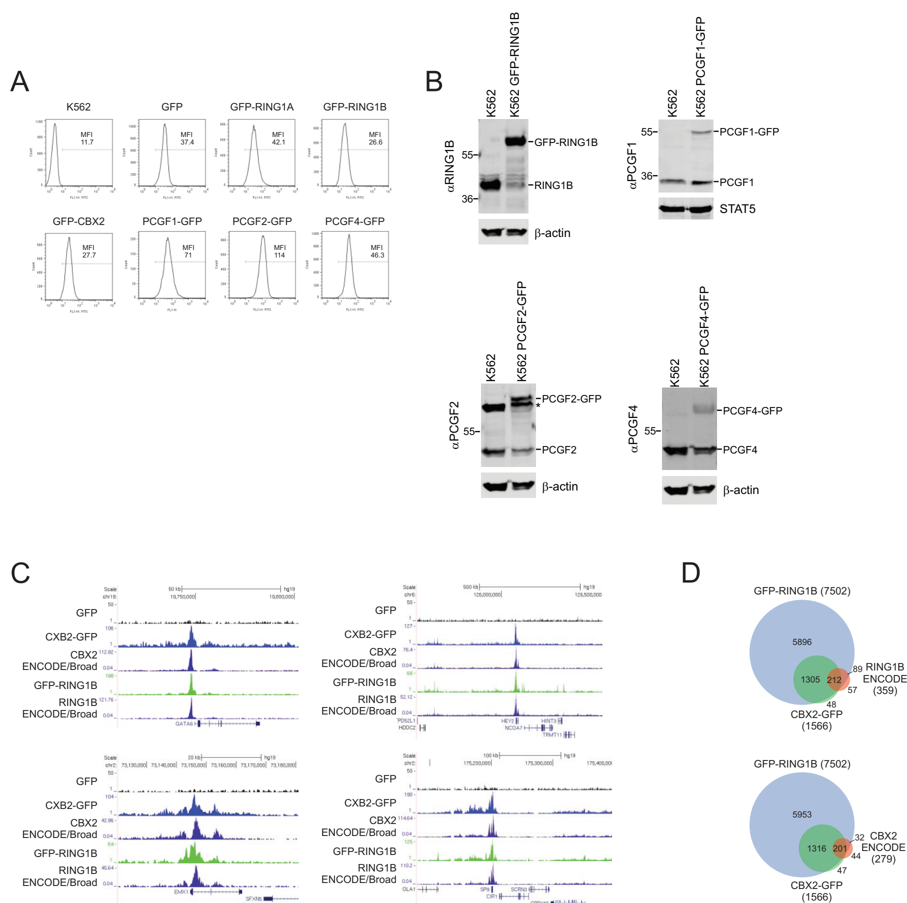

Figure S4. Analysis of GFP-fusion expression levels in K562 cells and comparison of GFP-fusion and ENCODE/Broad ChIP-seq tracks. (A) FACS analysis showing mean fluorescent intensity (MFI) of K562 cell lines stably expressing the indicated GFP-fusion proteins. (B) Western blot analysis with indicated antibodies showing expression levels of GFP-fusion proteins compared to their relative control in stable cell lines expressing GFP-fusions and untransduced K562 cells. (C) Comparison of ENCODE/Broad ChIP-Seq tracks for CBX2 and RING1B and K562 CBX2-GFP and GFP-RING1B tracks. All tracks show a strong overlap, indicating that the GFP-fusions are targeted similar to the endogenous proteins. (D) Venn diagrams showing overlap between identified target genes based on peak calling in K562 GFP-RING1B, CBX2-GFP and endogenous CBX2 and RING1B ENCODE/Broad tracks.

Figure S5. K562 ChIP-Seq data analyses. (A) Density plots of PRC1 paralogs, H3K27me3 and H2AK119ub at peak regions. (B) ChIP-seq heat map of K562 PCGF1-GFP, PCGF2-GFP and PCGF4-GFP tracks. (C) Positional information of annotated PRC1.1, PRC1 or 'both' peaks relative to the transcription start site of neighboring genes (http://bejerano.stanford.edu/great/public/html/). (D) Plots displaying average enrichment of H3K36me3 or RNAPII S5P (both ENCODE/Broad) across the gene body of PRC1.1, PRC1 or both target genes in K562 cells. Individual replicates from ENCODE/Broad were independently analyzed. (E) Heat map of H3K36me3 or RNAPII S5P enrichment across the gene bodies of indicated target genes. Heat map is based on a representative replicate from the ENCODE/Broad dataset.

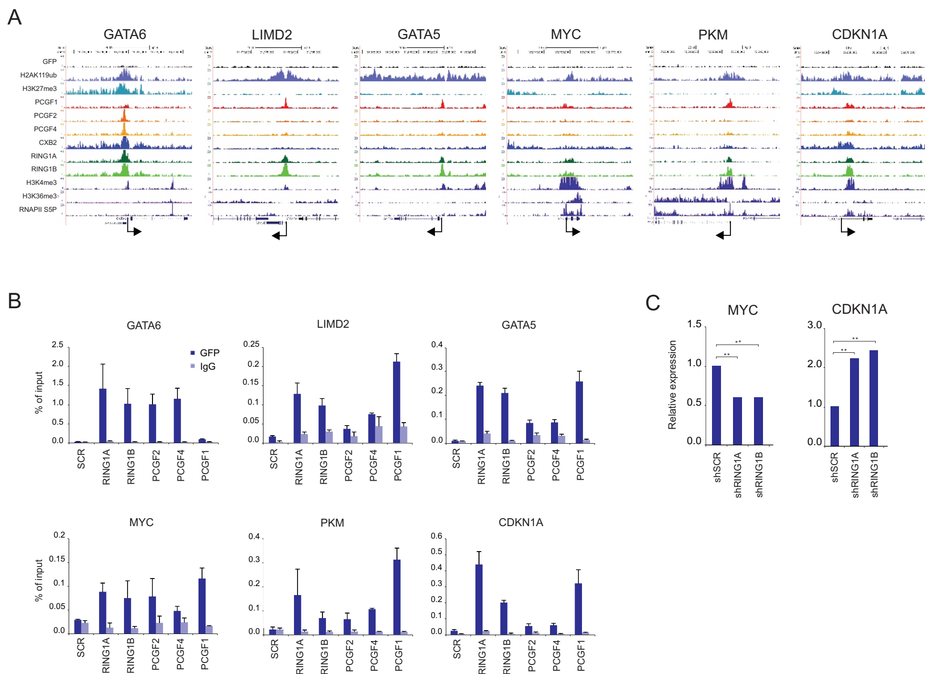

Figure S6. PRC1.1 complexes target genes with essential functions in metabolism and cell cycle regulation. (A) Examples of various identified PRC1 and/or PRC1.1 target genes. (B) Validation of ChIP-Seq data. Independent ChIP experiments were performed on K562 cells expressing GFP-fusions. For GATA6, MYC and PKM the average of two experiments is shown. For LIMD2, GATA5 and CDKN1A the average triplicate measurements of ChIP efficiency is shown. Error bars represent standard deviation. (C) Relative expression as measured by qPCR of MYC and CDKN1A in SCR, RING1A or RING1B knockdown cells. Statistical analysis was performed using Student t test. Error bars represent standard deviation, **p<0.005.

SUPPLEMENTARY INFORMATION

Supplementary Information, including Table S1-S6, can be found with this article online at https://doi.org/10.1016/j.celrep.2015.12.034

Table S1. Mass Spectrometry Data, Related to Figure 1
Table S2. Expression of Polycombs in Primary AML $CD34^+$ Cells, Related to Figure 3
Table S3. Patient Characteristics, Related to Figure 3
Table S4. Identification of Loci in K562 Cells Bound by PRC1, PRC1.1, or Both, Related to Figure 4
Table S5. KEGG Pathway Analysis of K562 PRC Complex Targets and GO Analysis of PRC Complex Targets in AML and PB $CD34^+$ cells, Related to Figures 4, 5, and 6
Table S6. Gene Names of Complex Targets for all AML $CD34^+$ Samples and PB $CD34^+$ Sample, Related to Figures 5 and 6

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CHAPTER 3

USP7 AS PART OF NON-CANONICAL PRC1.1 IS A DRUGGABLE TARGET IN LEUKEMIA

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ABSTRACT

Acute myeloid leukemia (AML) is a highly heterogeneous disease in which genetic and epigenetic changes disturb regulatory mechanisms controlling stem cell fate and maintenance. AML still remains difficult to treat, in particular in poor risk AML patients carrying TP53 mutations. Here, we identify the deubiquitinase USP7 as an integral member of non-canonical PRC1.1 and show that targeting of USP7 provides an alternative therapeutic approach for AML. USP7 inhibitors effectively induced apoptosis in (primary) AML cells, also independent of the USP7-MDM2-TP53 axis, whereby survival of both the cycling as well as quiescent populations was affected. MLL-AF9-induced leukemia was significantly delayed *in vivo* in human leukemia xenografts. We previously showed that non-canonical PRC1.1 is critically important for leukemic stem cell self-renewal, and that genetic knockdown of the PRC1.1 chromatin binding component KDM2B abrogated leukemia development *in vitro* and *in vivo* (van den Boom et al., 2016). Here, by performing KDM2B interactome studies in TP53mut cells we identify that USP7 is an essential component of PRC1.1 and is required for its stability and function. USP7 inhibition results in disassembly of the PRC1.1 complex and consequently loss of binding to its target loci. Loss of PRC1.1 binding coincided with reduced H2AK119ub and H3K27ac levels and diminished gene transcription, whereas H3K4me3 levels remained unaffected. Our studies highlight the diverse functions of USP7 and link it to Polycomb-mediated epigenetic control. USP7 inhibition provides an efficient therapeutic approach for AML, also in the most aggressive subtypes with mutations in TP53.

INTRODUCTION

Patients with AML often have a poor prognosis despite treatment with intensive chemotherapy and allogeneic stem cell transplantation. Dependent on risk category overall survival for adult AML patients varies between 10%-60% (Burnett et al., 2011). AML is a disease of the elderly accounting for 75% of the cases in patients >60 years of age which have a particularly poor outcome (Klepin, 2016; Rucker et al., 2012). These older patients usually have karyotypes associated with unfavorable risk and also TP53 mutations are more frequently seen in patients above 60, and this patient group generally does not respond well to standard chemotherapy (Bowen et al., 2009; Hou et al., 2015; Rucker et al., 2012). Therefore, alternative therapies need to be developed to achieve more effective treatment of AML patients.

A recurrent challenge in AML treatment is the notion that standard-of-care chemotherapeutic approaches do not effectively target quiescent leukemic stem cell

(LSC) populations, and as a consequence relapse of disease occurs frequently. A thorough understanding of how LSCs self-renew and maintain their quiescent state is therefore essential in order to be able to develop targeting approaches that also show efficacy in those cell populations. We and others have shown that Polycomb group (PcG) proteins are important regulators of hematopoietic stem cell fate, both in health and in leukemia (Iwama et al., 2004; Rizo et al., 2008; Rizo et al., 2010; Rizo et al., 2009; van den Boom et al., 2016; van den Boom et al., 2013; Yuan et al., 2011). PcG proteins are epigenetic regulators that are critically involved in controlling gene transcription by mediating post-translational modifications of histone proteins and chromatin remodeling (Kouzarides, 2007; Mas and Di, 2016; Simon and Kingston, 2013; Yuan et al., 2011). Genome-wide analyses of Polycomb target genes revealed the occupancy of PcG proteins at promoters of genes regulating cell fate, highlighting their importance for proper lineage specification (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006). Yet, how PcG proteins are recruited, recognize their target genes and regulate gene expression still remains poorly understood. Understanding these processes is important since deregulation of PcG proteins frequently contributes to cancer and hematopoietic malignancies, like leukemia (Bracken and Helin, 2009; Hu and Shilatifard, 2016; Piunti and Shilatifard, 2016).

PcG proteins form multi-protein chromatin modifying complexes of which Polycomb Repressive Complex 1 (PRC1) and 2 (PRC2) are best characterized (Cao et al., 2002; Gao et al., 2012; Simon and Kingston, 2009; Vandamme et al., 2011). We recently identified an essential role for non-canonical PRC1.1 proteins in human leukemias (van den Boom et al., 2016). PRC1.1 was first identified by the purification of the BCOR protein, which was found to interact with RING1A/B, RYBP, PCGF1, SKP1 and KDM2B (Gearhart et al., 2006; Sanchez et al., 2007). A potential oncogenic role of PRC1.1 is underlined by the fact that KDM2B is overexpressed in leukemias, breast and pancreatic cancers where it functions as an oncogene, conversely knockdown of KDM2B abrogated tumorigenicity (Andricovich et al., 2016; He et al., 2011; Kottakis et al., 2014; Ueda et al., 2015; van den Boom et al., 2016). The exact function of individual subunits in the PRC1 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 (de Napoles et al., 2004; Rose et al., 2016; Wang et al., 2004). For example, the H2AK119 E3 ligase activity is enhanced by the dimerization of RING1A/B with either PCGF2 or PCGF4 (Buchwald et al., 2006; Cao et al., 2005; Elderkin et al., 2007) or can be stimulated by the PCGF1-RYBP/YAF2 interaction in the case of non-canonical PRC1.1 (Gao et al., 2012; Rose et al., 2016). A shRNA approach for 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) and indeed

PRC1 complex composition changes upon lineage specification (Morey et al., 2015).

Protein ubiquitination is an important post-translational modification that controls the stability of almost all cellular proteins. Mono-ubiquitination impacts on the activity of proteins or can promote or prevent protein-protein interactions, while poly-ubiquitinated proteins are typically targeted to and degraded by the proteasome (Hershko, 1983). USP7 is a ubiquitin-specific protease that displays a wide range of activities, making it an attractive candidate target for cancer treatment. USP7 inhibition destabilizes MDM2 resulting in increased levels of TP53, and recently a number of USP7-specific inhibitors were generated that effectively targeted various human cancer cells presumably in an TP53-dependent manner (Gavory et al., 2018; Kategaya et al., 2017; Turnbull et al., 2017). However, TP53-independent roles exist as well (Bhattacharya et al., 2018; Nicholson and Suresh Kumar, 2011). Here, using LC-MS/MS-based proteome studies we identify USP7 as an integral component of the KDM2B/PRC1.1 complex. USP7 inhibition results in PRC1.1 complex disassembly and reduced chromatin binding, with a concomitant reduction in gene expression of target loci. Our data show that USP7 is essential for leukemic cells and suggests that targeting of USP7 might provide an alternative therapeutic approach for leukemia, also for the most aggressive subtypes of AML which harbor mutations in TP53.

RESULTS

Targeting of quiescent and cycling primary AML cell populations upon USP7 inhibition

To study the functional consequences of inhibition of the ubiquitin-specific protease 7 (USP7), we tested P22077 (1-(5-((2,4-difluorophenyl)thio)-4-nitrothiophen-2-yl)ethanone) on a panel of AML cell lines and primary patient samples. USP7 inhibition severely impaired cell growth in all tested AML cell lines (Figure 1A). Since P22077 has been documented to also inhibit USP47 (Weinstock et al., 2012) we tested in addition the more specific USP7 inhibitor FT671 (Turnbull et al., 2017) which demonstrated similar reduced proliferation of MOLM-13, OCI-AML3, K562 and HL60 cells (Figure 1B). Cell lines that do not express functional TP53 (HL60 and K562, Supplementary Table 1) also showed sensitivity, indicating that at least in those cell lines the effect of P22077 and FT671 was independent of TP53. A strong dose-dependent reduction in cell viability and increase in apoptosis was observed as determined by Annexin V staining, while no significant changes in cell cycle distribution were noted (data not shown). Next, we assessed whether USP7 inhibition also affected the survival of primary AML cells grown in long-term stromal co-cultures. The long-term proliferation of primary AML CD34^{+} cells was clearly reduced in the presence of P22077 (Figure 1C). Patient characteristics are shown in Supplementary Table 1, and again

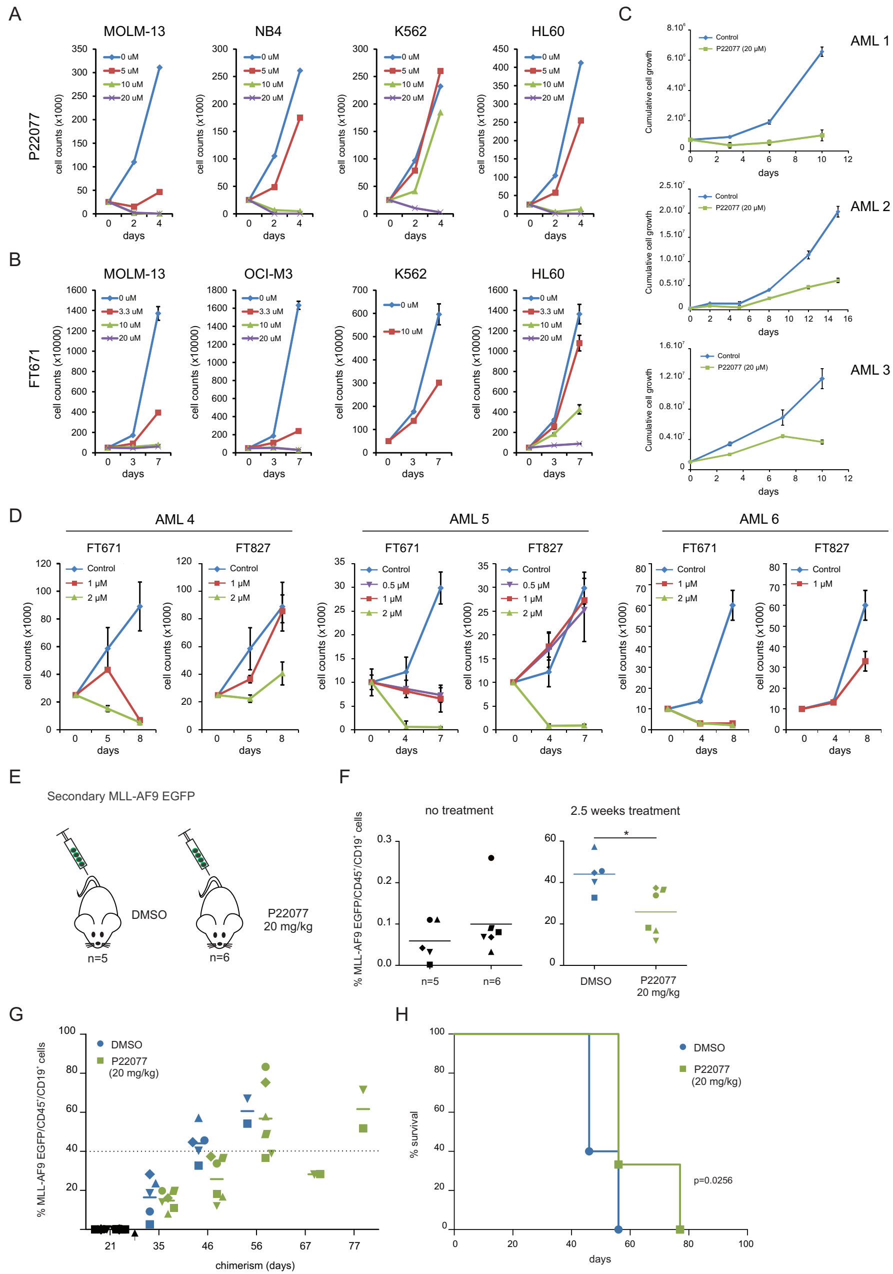

Figure 1: Sensitivity of leukemic cells towards USP7 inhibition. (A) Cumulative cell growth of various AML cell lines in control (DMSO) or P22077 treated conditions. (B) Cumulative cell growth of various AML cell lines in control (DMSO) or FT671 conditions performed in triplicate. (C) Cumulative cell growth of primary AML patient cells (n=3) grown on MS5 co-culture treated with DMSO also (control) or P22077 performed in duplicate. (D) Cumulative cell growth of primary AML patient

cells (n=3) grown on MS5 co-culture treated with DMSO, FT671 or FT827 performed in duplicate. (E) Experimental setup of our human CB MLL-AF9 xenograft mouse model. Here 5 x 10^4 MLL-AF9 EGFP cells from a primary leukemic mouse were IV injected into secondary recipients (n=11). (F) Peripheral blood analysis of MLL-AF9 EGFP/$CD45^+$/$CD19^+$ cells, three weeks after injection prior to treatment (left) and 2.5 weeks following treatment (right). Mice were treated daily with either DMSO as control (n=5) or 20 mg/kg P22077 (n=6). (G) Peripheral blood chimerism levels of control and P22077 (20 mg/kg) treated mice over the course of the experiment. Treatment was started at day 28 (4 weeks post-transplant) indicated with the arrow. (H) Kaplan-Meier curve of MLL-AF9 mice treated with DMSO or 20 mg/kg 22077. Statistical analysis was performed using a log-rank test.

a patient sample with a TP53(H179R) mutation showed strong sensitivity (Figure 1C, AML 1). The more USP7-specific inhibitor FT671 was also tested on primary AML samples together with the somewhat less potent FT827 inhibitor (Turnbull et al., 2017), and again we observed strong reductions in proliferation, in particular in response to FT671 (Figure 1D). With current therapies for leukemia patients the majority of the blast population is readily eradicated, while the rare quiescent population of leukemic stem cells is more difficult to target. To examine whether USP7 inhibition does target the quiescent (G0) leukemic cell population we performed Hoechst/PyroninY stainings (Suppl Fig 1). AML 2 was characterized by a large $CD34^+$/$CD38^+$ population that is high in cycling activity, while the $CD34^+$/$CD38^-$ population was predominantly in G_0 (Suppl Fig1A). In both populations, the G_0 fraction was equally or even more efficiently targeted by P22077 compared to the cycling fraction, indicating that cycling cells are not necessarily more sensitive than quiescent cells. AML3 was low in $CD34^+$ and therefore we gated on the whole blast population which also showed that both quiescent and cycling cells were sensitive for P22077 (Suppl Fig1B).

Next, we evaluated the effect of USP7 inhibition in our human CB MLL-AF9 xenograft mouse model (Figure 1E) (Horton et al., 2013; Sontakke et al., 2016). Importantly, these lymphoid MLL-AF9 cells when grown in co-culture on MS5 stroma *in vitro* were sensitive towards USP7 inhibition (data not shown). 5 x 10^4 MLL-AF9 leukemic cells, from a primary leukemic mouse, were intravenously (IV) injected into secondary recipients (n=11). Three weeks after injection 10/11 mice showed engraftment of MLL-AF9 $EGFP^+$/$CD45^+$/$CD19^+$ cells in peripheral blood and subsequently mice were divided into two groups that were treated either with DMSO (n=5) or 20 mg/kg P22077 (n=6) via intraperitoneal (IP) injections. Mice were treated daily starting four weeks post-transplant and peripheral blood chimerism levels were monitored by regular blood sample analysis and mice were sacrificed when chimerism levels in the blood exceeded 40%. Two-and-a-half weeks after the initiation of treatment chimerism levels were significantly lower in P22077-treated mice compared to DMSO (Figure 1F). The chimerism levels for DMSO treated mice rapidly increased to 44% (average) within 6 weeks after injection, whereas the chimerism levels of P22077

treated mice were around 25% on average. Notably, 3/6 mice gave a better response to USP7 inhibition with lower chimerism levels of 18.3, 16.8 and 12% respectively at day 46 (Figure G). Within 60 days all control mice exceeded 40% chimerism in the blood, indicative for a full blown leukemia and were sacrificed (Figure 6I). Bone marrow, spleen and liver analyses showed high levels (>90%) of chimerism (data not shown). Leukemia development was significantly delayed in USP7 inhibitor treated mice, and in particular in two mice a clear response to USP7 inhibition was observed and chimerism levels remained relatively stable between day 56-67 post-transplant, although ultimately those mice also did develop MLL-AF9-induced leukemia after day 77 (Figure 1H).

Identification of the deubiquitinase USP7 as a subunit of PRC1.1

One of the pathways downstream of USP7 via which its inhibition might contribute to reduced cell survival is the TP53 pathway (Fan et al., 2013). Yet, also in the absence of functional TP53, both in leukemic cell lines as well as in primary patient samples, we noted strong sensitivity towards USP7 inhibition. We recently identified KDM2B as a critically important factor for the survival of human leukemic stem cells (van den Boom et al., 2016). In our initial interactome screens we had already identified USP7 as a potential interaction partner within the non-canonical PRC1.1 complex. Therefore, we set out to further study the KDM2B interactome in detail in leukemic cells, specifically focussing on a potential role for USP7. Human K562 cells were transduced with KDM2B-EGFP followed by anti-EGFP pull outs and LC-MS/MS analysis to identify interacting proteins. Thus, 406 KDM2B-interacting proteins were identified involved in cellular processes like DNA/ chromatin binding, protein binding, RNA binding and RNA polymerase activity (Figure 2A and Supplementary Table 2). Gene Ontology analyses revealed that this list contained proteins that associated with rRNA processing, mRNA splicing, translation, mRNA processing, positive regulation of gene expression and DNA damage response (Figure 2B). The most abundant KDM2B interaction partners were non-canonical PRC1.1 proteins (Figure 2C and 2D), including USP7. Next, we questioned whether inhibition of the deubiquitinase activity of USP7 would impact on PRC1.1 function, which would provide further insight into possible mechanisms via which USP7 inhibitors would contribute to targeting of leukemic (stem) cell populations. To further validate the interaction of USP7 with PRC1.1 proteins, besides KDM2B, we also performed EGFP pull outs on nuclear extracts of stable K562 cell lines expressing PCGF1-EGFP and EGFP-RING1B followed by LC-MS/MS analysis (Supplementary Table 3). Figure 2D shows the spectral counts (MS/MS counts) corrected for expected peptides based on *in silico* protein digests. Clearly, USP7 was found to interact with KDM2B, PCGF1 and RING1B and all other PRC1.1 proteins were co-purified, indicating that USP7 is a bona fide subunit of PRC1.1. Importantly, USP47

was not found as interaction partner with any of the Polycomb proteins (Supplementary Table 2-3). In addition, Western blots of independent pull outs on canonical PRC1 (PCGF2, PCGF4, CBX2) and non-canonical PRC1.1 (PCGF1, RING1B) were performed using antibodies against USP7 and RING1B (Figure 2E). As expected, RING1B precipitated with both PRC1 and PRC1.1 proteins. The interaction of USP7 with RING1B and PCGF1 was confirmed by Western blot while less interaction was observed with the canonical PRC1 proteins PCGF2, PCGF4 or CBX2 (Figure 2E and Supplementary Table 4).

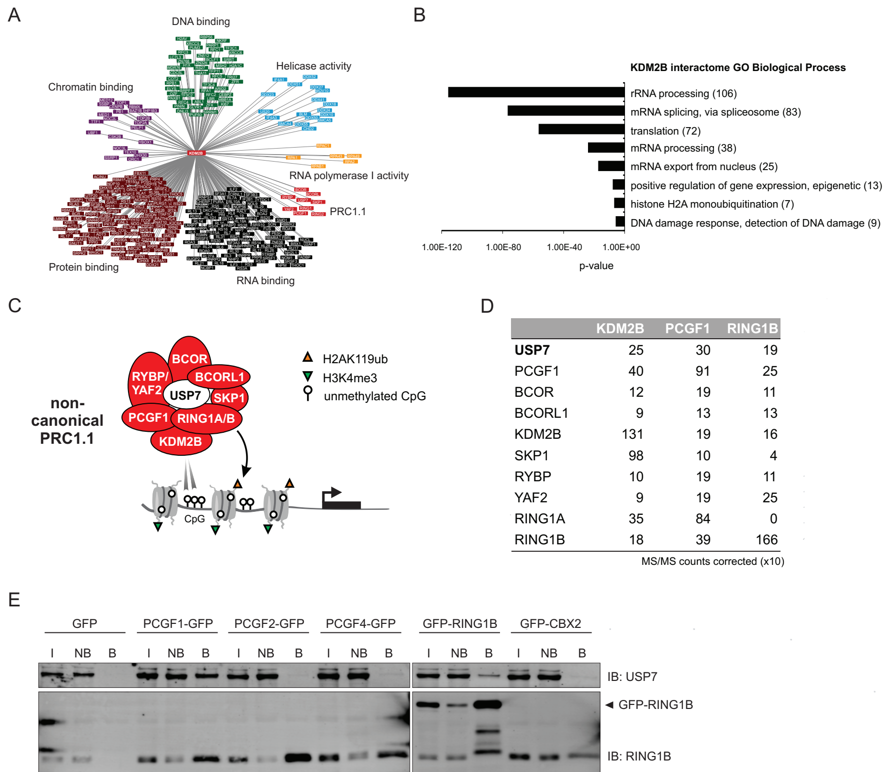

Figure 2. The deubiquitinase USP7 interacts with non-canonical PRC1.1 proteins. (A) Visualization of KDM2B interactome using Cytoscape, categorized by Molecular Function as annotated by DAVID. (B) Gene Ontology analysis of KDM2B interactome shown as Biological Process. (C) Schematic representation of the non-canonical PRC1.1 complex, including USP7. PRC1.1 preferentially binds to non-methylated CpG islands via the ZF-CxxC domain of KDM2B. The ubiquitination of histone H2A on lysine 119 is mediated by RING1A/B. PRC1.1 can be recruited to active genes in leukemic cells, indicated by H3K4me3. (D) Identification of USP7 and other PRC1.1 proteins by LC-MS/MS in KDM2B-EGFP, PCGF1-EGFP and EGFP-RING1B pull outs from nuclear extracts in K562 cells. The numbers indicate MS/MS counts for each interacting protein corrected for expected peptides based on *in silico* digests (x10). (E) Validation of USP7 and RING1B interactions as analysed by Western blot in canonical PRC1 (PCGF2/PCGF4/CBX2/RING1B) and non-canonical PRC1.1 (PCGF1/RING1B) pull outs. Input (I), non-bound (NB) and bound (B) fractions are shown.

USP7 inhibition results in disassembly of the PRC1.1 complex

Deubiquitinating enzymes (DUBs) exert a variety of important cellular functions, including the control over protein stabilization or degradation, protein localization, protein activity or by modulating protein-protein interactions (Leznicki and Kulathu, 2017). We therefore questioned whether inhibition of the deubiquitinase USP7 might affect PRC1.1 stability and function. In general, DUB inhibitors increase overall protein polyubiquitination of many target proteins (Altun et al., 2011). Similarly, we observed accumulation of polyubiquitinated proteins in our EGFP-RING1B and PCGF1-EGFP K562 cells treated for 24h with P22077 (Figure 3A). Next, we investigated the effect of USP7 inhibition on the stability of PRC1.1. EGFP pull outs were performed on nuclear extracts from K562 PCGF1-EGFP and EGFP-RING1B cells treated with DMSO or P22077 followed by LC-MS/MS analysis (Figure 3B and Supplementary Table 5). Volcano plots were generated using label-free quantification (LFQ) intensities of potential interactors of EGFP-RING1B (left) and PCGF1-EGFP (right) plotted as fold change difference (control/USP7i) against significance (t-test p-value). Interactions with several PRC1.1 proteins, highlighted in orange, were significantly reduced in both EGFP-RING1B and PCGF1-EGFP pull outs as a consequence of USP7 inhibition (Figure 3B). The ubiquitin protein, UBB, was enriched in both PCGF1-EGFP and EGFP-RING1B pull outs upon P22077 treatment, suggesting that these proteins or other Polycomb interaction proteins might be more ubiquitinated upon USP7 inhibition. In addition we analysed the intensity-based absolute quantification (iBAQ) values, as a measure for protein abundance, relative to either RING1B or PCGF1 and normalized to control pull outs (Figure 3C). Similarly, these data demonstrated a clear reduced interaction of PRC1.1 proteins with RING1B and PCGF1 after P22077 treatment. Independent EGFP pull outs performed on PCGF1-EGFP and EGFP-RING1B cell lines further confirmed that USP7 inhibition indeed resulted in reduced interaction of PCGF1-EGFP with endogenous RING1B and EGFP-RING1B with endogenous PCGF1 (Figure 3D). Importantly, input samples did not reveal reduced expression of EGFP-RING1B,PCGF1-EGFP or KDM2B-EGFP, which was further validated by FACS analysis (Figure 3E). Taken together these data indicate that USP7 is essential for PRC1.1 complex integrity.

PRC1.1 chromatin binding relies on functional USP7 deubiquitinase activity

To determine the functional consequences of disassembly of the PRC1.1 complex upon USP7 inhibition, we performed several ChIPs to investigate whether PRC1.1 chromatin targeting at target loci would be affected by USP7 inhibition. Previously, we identified PRC1 and PRC1.1 target loci by ChIP-seq for PCGF1/2/4, CBX2, RING1A/1B, KDM2B, H2AK119ub and H3K27me3 in the leukemic cell line K562 (van den Boom et al., 2016). Here, ChIP-qPCRs were performed for KDM2B, PCGF1 and RING1B, the core PRC1.1

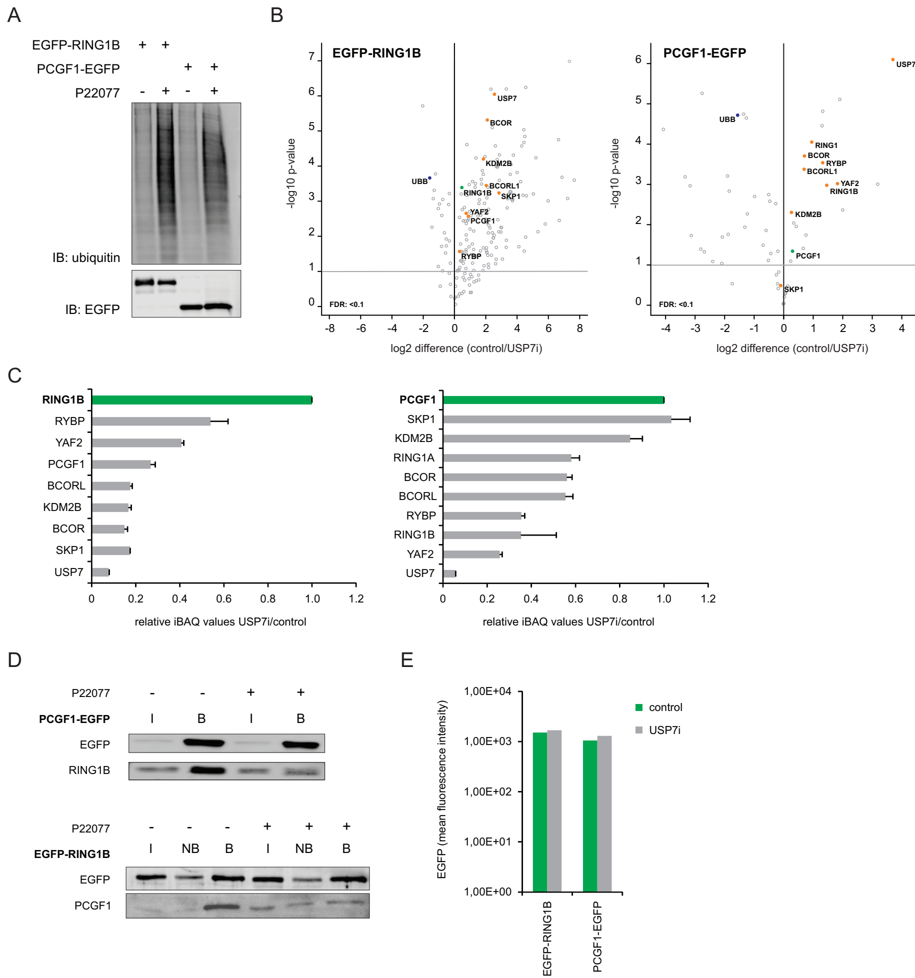

Figure 3. USP7 deubiquitinase activity is essential for PRC1.1 integrity. (A) Purification of his-tagged ubiquitinated proteins under denaturing conditions in EGFP-RING1B and PCGF1-EGFP cells treated with DMSO (-) or P22077 (+) for 24h followed by Western blot analysis. (B) Label-free quantification (LFQ) intensities of EGFP pull outs on EGFP-RING1B (left) and PCGF1-EGFP (right) interactions, performed in technical replicates in control (DMSO, 0.1%) and USP7i treated cells are illustrated as Volcano plot. The fold change difference (log2) of the EGFP pull out in control versus USP7i is plotted against the –log10 t-test p-value (y-axis). The bait protein (RING1B/PCGF1) is highlighted in green, PRC1.1 proteins in orange and UBB protein in purple. (C) Intensity based absolute quantification (iBAQ) values for several identified PRC1.1 proteins are shown in USP7i/control EGFP pull outs relative to RING1B (left) and PCGF1 (right). Data are shown as mean ± SD (n=2 or 3). (D) Western blot of EGFP pull outs on PCGF1-EGFP and EGFP-RING1B in the absence (-) or presence (+) of P22077 for 72h probed with antibodies for EGFP, RING1B and PCGF1. Input (I), non-bound (NB) and bound (B) fractions are shown. (E) Mean fluorescent intensity (MFI) analysis of EGFP-RING1B, PCGF1-EGFP AND KDM2B-EGFP K562 cells in control or USP7i treated cells at 72h.

subunits, on several loci in the absence or presence of P22077. Inhibition of USP7 resulted in a complete loss of KDM2B binding, with concomitant strong reductions in PCGF1 and RING1B chromatin binding (Figure 4A). Since RING1B mediates H2AK119 ubiquitination we then analysed the levels of H2AK119ub upon USP7 inhibition. Similarly, H2AK119ub was lost from PRC1.1 target loci (Figure 4B). These data indicate that USP7 inhibition severely impacts on PRC1.1 chromatin binding and as a consequence on H2AK119ub levels. Since non-canonical PRC1.1 can be recruited to active loci in leukemic cells, we also investigated the levels of H3K4me3, but no changes were observed on this histone mark upon USP7 inhibition, highlighting that not all posttranslational histone modifications are affected as a consequence of treatment (Figure 4C). To further investigate the kinetics of loss of H2AK119ub cells were cross-linked at 4h, 8h and 16h followed by a ChIP for H2AK119ub, KDM2B and RING1B (Figure 4D). The time-dependent loss of H2AK119ub coincided with reduced KDM2B and RING1B binding at the same target loci, suggesting that loss of *de novo* ubiquitination underlies these observations. Again, H3K4me3 levels remained unaffected, while H3K27ac levels were reduced upon USP7 inhibition indicative for reduced transcriptional activity at these loci (Figure 4E). Finally, to exclude a potential role of USP47 we also validated our findings using the more specific USP7 inhibitor FT671, and these data also clearly demonstrated a loss of KDM2B binding and reduced H2AK119ub levels at PRC1.1 loci upon inhibiting USP7 function (Figure 4F).

3

USP7 inhibition leads to downregulation of several PRC1.1 active target genes

Given that USP7 inhibition severely impaired PRC1.1 occupancy to several target loci we investigated whether this would also affect the expression of PRC1.1 target genes in leukemic cells. RNA-seq was performed to compare gene expression in DMSO or USP7 inhibitor treated K562 cells (P22077, 30 µM) for 4h, 8h, 16h and 24h. Since PRC1.1 can be associated with active genes in leukemic cells we focused on the genes that were downregulated upon USP7 inhibition. In total 297 genes were downregulated more than 2 fold after 24h of USP7 inhibition (Supplementary Table 6). Since USP7 has also PRC1.1 independent functions, for instance by controlling MDM2/TP53 (Li et al., 2004), we then analysed whether downregulated genes overlapped with previously identified PRC1.1 peaks by ChIP-seq (Figure 5A and Supplementary Table 6)(van den Boom et al., 2016). Twenty-four percent of these genes overlapped with PRC1.1 targets. Gene Ontology (GO) analysis revealed that this set was enriched for GO terms like transcription, regulation of gene expression and chromatin modification, while PRC1.1 independent downregulated genes were enriched for GO terms like mRNA splicing, protein folding and protein polyubiquitination (Figure 5A). ChIP-seq profiles of TOP2B, SIN3A, CHD1 and MYC are shown in Figure 5B as representative examples for PRC1.1 target genes. The tracks

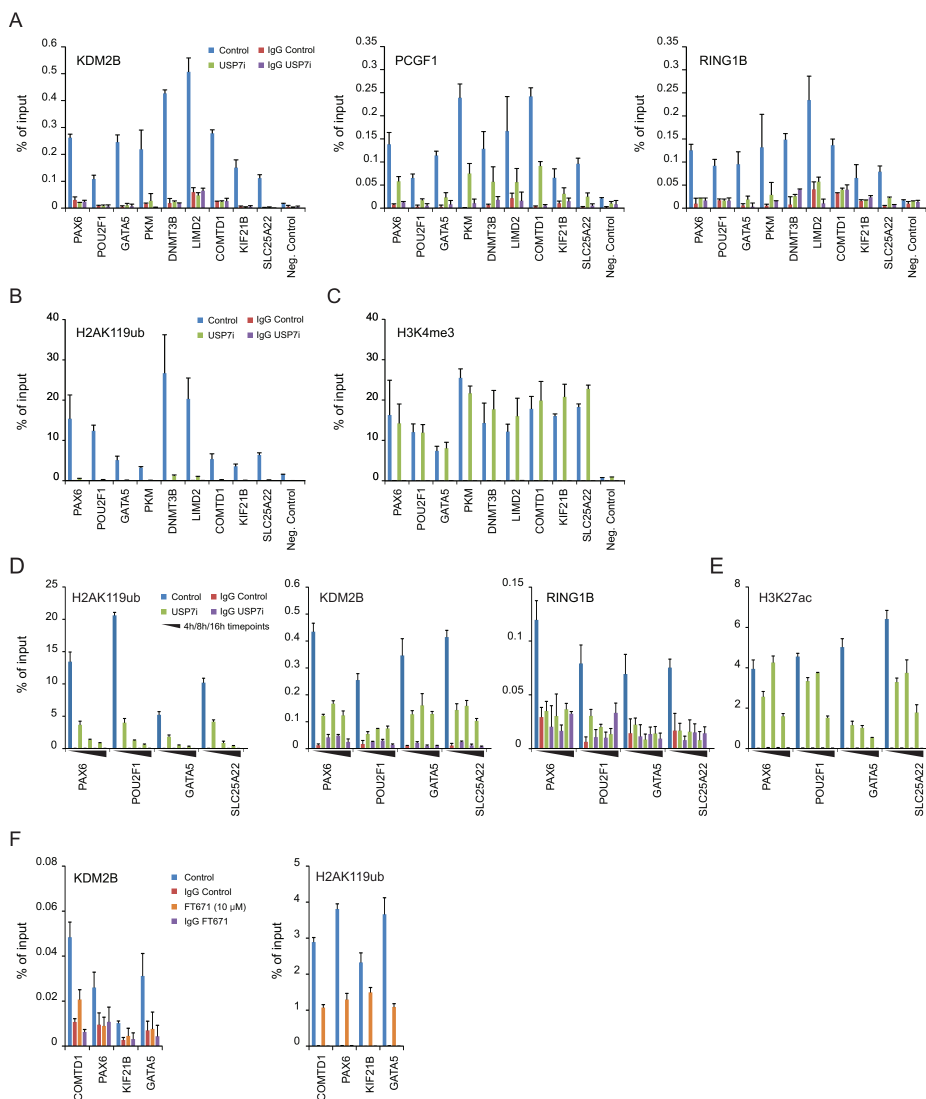

Figure 4. Loss of PRC1.1 occupancy and H2AK119ub at target loci upon USP7 inhibition. (A) ChIP-qPCRs on control or 72h P22077 (USP7i) treated K562 cells for endogenous KDM2B, PCGF1-EGFP, EGFP-RING1B (B) H2AK119ub and (C) H3K4me3 on several PRC1.1 loci previously identified by ChIP-seq. Error bars represent SD of technical qPCR replicates. (D) ChIP-qPCRs on control or USP7i treated K562 cells for 4h/8h/16h (◀) for H2AK119ub, KDM2B-EGFP, EGFP-RING1B and (E) H3K27ac on four PRC1.1 loci. Error bars represent SD of technical qPCR replicates. (F) ChIP-qPCRs on control or 24h FT671 (10 µM) treated K562 cells for endogenous KDM2B and H2AK119ub. Error bars represent SD of technical qPCR replicates.

show clear binding of PCGF1, RING1A/1B and KDM2B and little occupancy of canonical PRC1 proteins (PCGF2, PCGF4, CBX2). These loci were also enriched for H2AK119ub and active chromatin marks H3K4me3, H3K36me3, RNAPII and H3K27ac but devoid of the repressive mark H3K27me3. Loss of PRC1.1 binding was confirmed by ChIP-qPCRs for KDM2B, PCGF1 and RING1B on these 4 loci (Figure 5C). Again, a concomitant loss of H2AK119ub and H3K27ac marks was seen, without any alterations in H3K4me3 (Figure 5C). Subsequently, the observed downregulation based on RNA-seq data was validated by independent quantitative RT-PCRs (Figure 5D). Loss of PRC1.1 binding correlated with loss of gene expression. Taken together, these data suggest that the presence of PRC1.1 is required to maintain the transcriptional activity of several target genes.

DISCUSSION

In this study, we reveal that the deubiquitinase USP7 is essential for leukemic cells and suggests that targeting of USP7 might provide an alternative therapeutic approach for leukemia, also for the most aggressive subtypes of AML which harbor mutations in TP53. Mechanistically, using these TP53null AML cells as starting point, we uncover an important role for USP7 in controlling the stability and function of non-canonical PRC1.1 in leukemia. Our interactome proteomics studies identify USP7 as an integral component of PRC1.1, and inhibiting USP7 deubiquitinase activity resulted in disassembly of the PRC1.1 complex. Consequently, recruitment of the PRC1.1 complex to target loci was lost coinciding with the loss of its H2AK119ub catalytic activity. This resulted in repression of a subset of transcriptionally active PRC1.1 target genes and corresponded with a reduction in H3K27ac, highlighting an essential function for PRC1.1 in maintaining gene transcription. Our data indicates that USP7 stably interacts with PRC1.1 proteins, KDM2B, PCGF1 and RING1B (Figure 1). Sánchez and colleagues first identified the ubiquitin protease USP7 as an interactor of RING1B (Sanchez et al., 2007). Then, USP7 was also identified in proteomics analysis of PCGF1, RYBP, YAF2 and RING1A/B pullouts (Gao et al., 2012; Hein et al., 2015; van den Boom et al., 2016). Moreover, using quantitative proteomics and USP7 as bait specific interactions were identified in HeLa cells with PCGF1, BCOR, RING1A/B (Hein et al., 2015). Previously, USP7 has been shown to be associated with the canonical PRC1 protein BMI1 (PCGF4) and potentially also with MEL18 (PCGF2), although Lecona and colleagues suggested that USP7 interacts directly with SCML2 and thereby bridges the interaction with PRC1.4 (Lecona et al., 2015; Maertens et al., 2010). However, similar to Gao and colleagues (Gao et al., 2012), our PCGF2, PCGF4 and CBX2 proteome analyses showed little or no interaction with USP7 (van den Boom et al., 2016). We did notice that several canonical RING1B-Polycomb interactions were

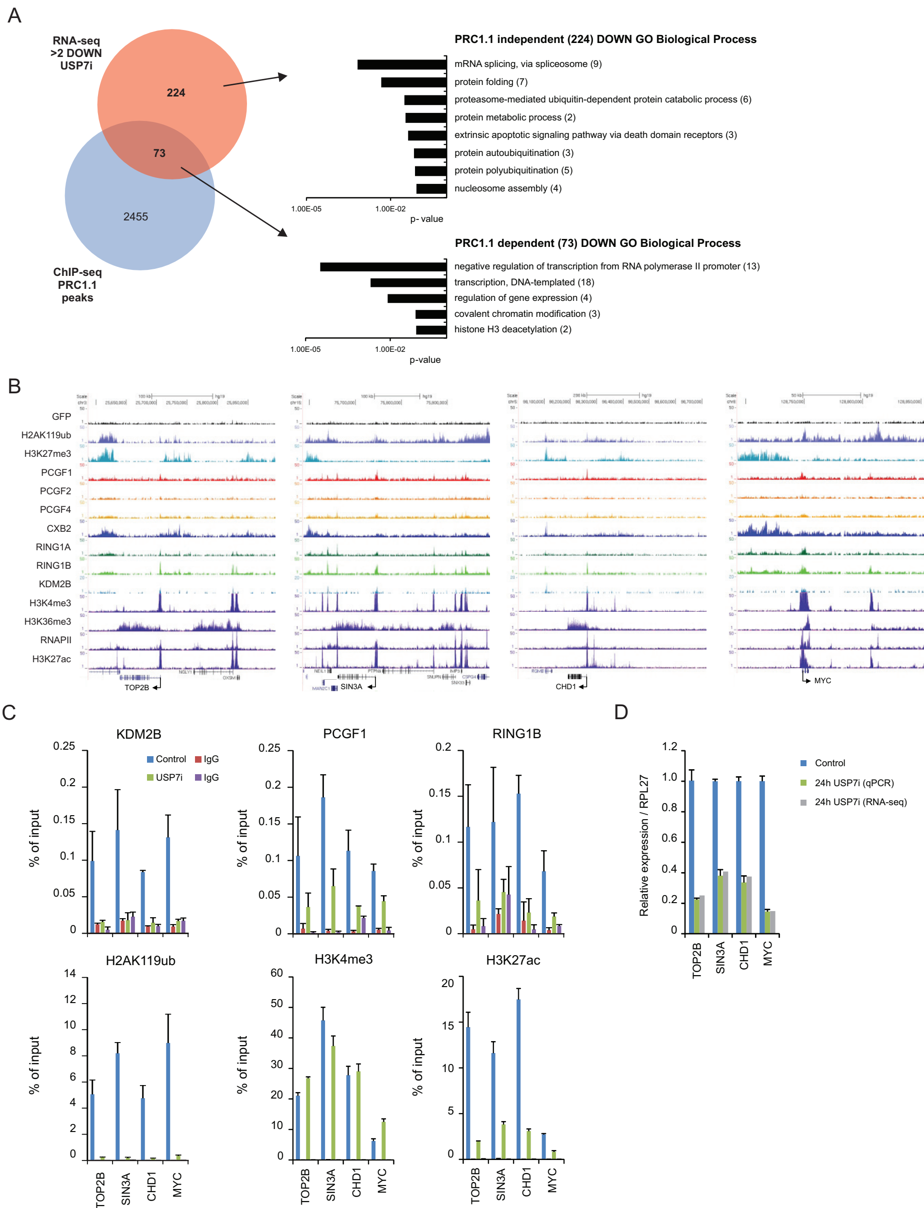

Figure 5. USP7 inhibition leads to downregulation of a subset of PRC1.1 active target genes. (A) Venn diagram showing overlap of genes downregulated by more than two fold after 24h of USP7 inhibition with previously identified PRC1.1 target genes. Gene Ontology analysis of downregulated genes identified as PRC1.1 targets (73) or Polycomb independent targets (224) shown as Biological Process. (B) ChIP-seq profiles of TOP2B, SIN3A, CHD1 and MYC as representative examples for PRC1.1 target genes (73). Our ChIP-seq tracks are shown for GFP (control), H2AK119ub, H3K27me3, PCGF1, PCGF2, PCGF4, CBX2, RING1A, RING1B and KDM2B. ChIP-seq tracks for H3K4me3, H3K36me3, RNAPII and

H3K27ac were downloaded from ENCODE/Broad. (C) ChIP-qPCRs in control or 72h USP7i for KDM2B, PCGF1-EGFP, EGFP-RING1B and H2AK119ub or 16h USP7i for H3K4me3 and H3K27ac. Error bars represent SD of technical qPCR replicates. (D) Validation of relative gene expression levels of PRC1.1 target genes by qRT-PCR in control and P22077 treated cells (24h). Data are represented as mean ± SD.

also reduced upon USP7 inhibition, including interactions with CBX2/4/8, SCML2 and SCMH1, though the interaction with PCGF2 and PCGF4 was not affected. This might also suggest that USP7 impacts on PRC1 protein stability, although further studies are required to clarify these issues. In line with that notion, a recent paper demonstrated that another deubiquitinase, USP26, controls the stability of CBX4 and CBX6 and thereby affects the complex composition of PRC1 during ESC reprogramming (Ning et al., 2017). Maertens and colleagues demonstrated that also USP11 interacts with PRC1 proteins and affects the stability of BMI1 (Maertens et al., 2010). Thus, DUBs clearly play an important role in controlling Polycomb stability and complex composition. Given that USP7 inhibition affects the complex composition of PRC1.1, its activity might control ubiquitin levels of Polycomb proteins, important for protein-protein interactions within PRC1.1. Upon USP7 inhibition an overall increase in protein ubiquitination was observed (Figure 2). Importantly, USP7 inhibition did not lead to degradation of PCGF1 or RING1B, allowing us to conclude that USP7 controls protein-protein interactions rather than proteasomal degradation. Overexpression of USP7 has been shown to deubiquitinate RING1B thereby stabilizing RING1B and preventing it from proteasomal degradation (de et al., 2010). Furthermore, RING1B self-ubiquitination is required for its ligase activity on histone H2A (Ben-Saadon et al., 2006). This is supported by the observation that H2AK119ub is lost upon USP7 inhibition, most likely as a consequence of loss of *de novo* ubiquitination mediated via RING1B. Similarly, knockout of USP7 reduced H2AK119ub levels coinciding with reduced levels of RING1B (Lecona et al., 2015). Interestingly, UbE2E1 was shown to be a essential for Polycomb-mediated H2AK119ub and regulated by USP7 (Sarkari et al., 2013; Wheaton et al., 2017). Other studies also show that USP7 is not likely a DUB for H2AK119 (Maertens et al., 2010; Sarkari et al., 2009; van der Knaap et al., 2005). Thus, regulating ubiquitin levels within the PRC1.1 complex itself is critically important and controlled by USP7 deubiquitinase activity. Inhibiting the catalytic core by removal of RING1A/B in ESCs does not lead to loss of KDM2B binding (Farcas et al., 2012). However, in leukemic cells we find that USP7 inhibition not only leads to reduced H2AK119ub but also induces loss of PRC1.1/KDM2B binding as a result of complex disassembly, suggesting that KDM2B-PCGF1-BCOR/L1/SKP1 interactions might be needed for targeting. This is in agreement with data that suggest that dimerization of PCGF1 and BCOR(L1) is required for binding to KDM2B and recruiting PRC1.1 to the chromatin (Wong et al., 2016).

With the efficient loss of PRC1.1 binding and consequent loss of H2AK119ub from target loci upon USP7 inhibition we were able to study PRC1.1 function in relation to gene regulation in more detail. Previously we identified genes that are targeted by PRC1.1 independent of H3K27me3 and have a transcriptionally active profile suggested by H3K4me3, RNAPII and H3K27ac occupancy close to the transcription start site. Loss of PRC1.1 binding resulted in reduced gene expression coinciding with reduced levels of H3K27ac on several known PRC1.1 targets. We therefore hypothesize that PRC1.1 creates a transcriptionally permissive and open chromatin state which enables transcription factors to bind and initiate gene expression. Addressing a possible cross-talk between PRC1.1 proteins, CBP/p300-linked H3K27ac, recruitment of transcription factors and accessibility of chromatin is definitely a focus for future work. Since H3K4me3 levels remained unaffected following USP7 inhibition, this suggest that the H3K4 methyltransferase complex that is likely targeted to CpGs via CFP1 protein is not targeted in a PRC1.1-dependent manner (Lee and Skalnik, 2005).

Where we previously highlighted the importance of PRC1.1 for the survival of leukemic cells using genetic studies, small molecule inhibitors would be easier to implement in a therapeutic clinical setting (van den Boom et al., 2016). Inhibition of USP7 provided an efficient means to target PRC1.1. Of course it is evident that USP7 can function in several pathways, often through regulating protein stability of tumor suppressors or epigenetic regulators (Carra et al., 2017; Felle et al., 2011; van der Horst et al., 2006) and it is particularly the TP53 pathway that is strongly controlled by USP7 (Colland et al., 2009; Fan et al., 2013; Hu et al., 2006; Ye et al., 2015). Various USP7 inhibitors have been developed, and most recently selective USP7 inhibitors were generated that destabilize USP7 substrates including MDM2 and thereby increase TP53-mediated apoptosis of cancer cells (Kategaya et al., 2017a; Turnbull et al., 2017). While we cannot exclude the possibility that some of these pathways were also affected in some of our studies, we have analysed the TP53 status in our models and primary AML patient samples and see sensitivity even in the absence of a normal TP53 response. Furthermore, while USP7 knockout mice are embryonically lethal, deletion of p53 was not able to rescue this phenotype, further highlighting that p53-independent pathways downstream of USP7 exist as well (Agathanggelou et al., 2017; Kon et al., 2010). In conclusion, our data reveal an important role for USP7 deubiquitinase activity in the integrity of the PRC1.1 complex. We provide insight into the recruitment of PRC1.1 to target loci and function in gene regulation and show that PRC1.1 is a potential interesting therapeutic target in leukemia.

MATERIALS AND METHODS

GFP-mediated pull outs

Pull outs were performed on nuclear extracts from K562 cells stably expressing KDM2B-EGFP, PCGF1-EGFP and EGFP-RING1B. At least $80x10^6$ cells were collected for each pull out, nuclear extract preparation was done as described previously (van den Boom et al., 2013). Pre-clearing of cell lysates was done by adding 50 µl pre-equilibrated binding control magnetic agarose beads (Chromotek) and incubated for 30 min at 4°C on a rotating platform. Then pre-cleared lysate was incubated with 70 µl pre-equilibrated GFP-Trap magnetic agarose beads (Chromotek) overnight at 4°C on a rotating platform. Beads were separated using a magnetic rack and six times washed in wash buffer (TBS, 0.3% IGEPAL CA-630, 1x CLAP, 0.1 mM PMSF). Bound fractions were eluted from the beads by boiling for 10 min in 2x Laemmli sample buffer.

Chromatin immunoprecipitation

ChIP was essentially performed as described previously (Frank et al., 2001). K562 cells stably expressing low levels of EGFP-fusion vectors encoding, PCGF1-EGFP, EGFP-RING1B, KDM2B-EGFP or non-transduced K562 cells were treated with DMSO or P22077 for indicated timepoints and subsequently cross-linked. The following antibodies were used: anti-GFP (ab290, Abcam), anti-KDM2B (ab137547, Abcam), anti-H2AK119ub (D27C4, Cell Signaling Technology), anti-H3K4me3 (ab8580, Abcam), anti-H3K27ac (C15410196, Diagenode) and IgG (I8141, Sigma). ChIPs were analysed by qPCR as percentage of input, Supplementary Table 7 lists the qPCR primers used.

Cell culture

The AML cell lines K562, HL60 (ATCC: CCL-243,CCL-240), MOLM13, NB4 and OCI-AML3 (DSMZ: ACC-554, ACC-207, ACC-582) were cultured in RPMI 1640 (BioWhittaker, Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FCS, HyClone Laboratories, Logan, Utah, US) and 1% penicillin/streptomycin (p/s, PAA Laboratories). MS5 murine stromal cells (DSMZ: ACC-441) were cultured in alpha-MEM with 200 mM glutamine (BioWhittaker) supplemented with 10% FCS and 1% p/s. Primary AMLs were cultured in Gartner's medium as described before [44]. All cultures were kept at 37°C and 5% CO_2. For USP7 inhibition experiments, P22077 (1-(5-((2,4-difluorophenyl)thio)-4-nitrothiophen-2-yl)ethanone) was purchased from Merck Millipore (662142) (Billerica, MA, USA). FT671 and FT827 were generously provided by FORMA Therapeutics (Watertown, MA, USA (Turnbull et al., 2017)).

In gel trypsin digestion

Bound fractions of KDM2B-EGFP, PCGF1-EGFP (control/USP7i) and GFP-RING1B (control/USP7i) were loaded on a 4-12% pre-cast NuPAGE gel (Invitrogen) and were run briefly. Gels were stained with Coomassie dye R-250 (Thermo Scientific) and subsequently destained with ultrapure water overnight. Gel lanes were cut into one slice, further cut into small pieces and completely destained using 70% 50 mM NH_4HCO_3 and 30% acetonitrile (ACN). Reduction and alkylation of cysteines was performed by adding 10 mM DTT dissolved in 50 mM NH_4HCO_3 and incubated at 55°C for 30 min. Next, 55 mM iodoacetamide in 50 mM NH_4HCO_3 was added and incubated for 30 min, in dark, at room temperature. Remaining fluid was removed and 50 mM NH_4HCO_3 was added with 10 min. shaking. Then 100% ACN was added incubated for 30 min. while shaking. Fluid was removed and gel pieces were dried for 15 min. at 55°C. Proteins were digested with adding 10 ng/µl sequencing-grade modified trypsin (Promega) in 50 mM NH_4HCO_3 to the gel pieces and incubated overnight at 37°C. Next day, peptides were extracted using 5% formic acid followed by second elution with 5% formic acid in 75% acetonitrile. Samples were dried in a SpeedVac centrifuge and dissolved in 5% formic acid.

LC-MS/MS analysis

Online chromatography of the extracted tryptic peptides was performed with the Ultimate 3000 nano-HPLC system (Thermo Fisher Scientific) coupled online to a Q-Exactive-Plus mass spectrometer with a NanoFlex source (Thermo Fisher Scientific) equipped with a stainless steel emitter. Tryptic digests were loaded onto a 5 mm × 300 µm i.d. trapping micro column packed with PepMAP100 5 µm particles (Dionex) in 0.1% FA at the flow rate of 20 µL/min. After loading and washing for 3 minutes, peptides were forward-flush eluted onto a 50 cm × 75 µm i.d. nanocolumn, packed with Acclaim C18 PepMAP100 2 µm particles (Dionex). The following mobile phase gradient was delivered at the flow rate of 300 nL/min: 2–50% of solvent B in 90 min; 50–80% B in 1 min; 80% B during 9 min, and back to 2 % B in 1 min and held at 3% A for 19 minutes. Solvent A was 100:0 H_2O/acetonitrile (v/v) with 0.1% formic acid and solvent B was 0:100 H_2O/acetonitrile (v/v) with 0.1% formic acid. MS data were acquired using a data-dependent top-10 method dynamically choosing the most abundant not-yet-sequenced precursor ions from the survey scans (300–1650 Th) with a dynamic exclusion of 20 seconds. Sequencing was performed via higher energy collisional dissociation fragmentation with a target value of 2e5 ions determined with predictive automatic gain control. Isolation of precursors was performed with a window of 1.6. Survey scans were acquired at a resolution of 70,000 at m/z 200. Resolution for HCD spectra was set to 17,500 at m/z 200 with a maximum ion injection time of 110 ms. The normalized collision energy was set at 28. Furthermore,

the S-lens RF level was set at 60 and the capillary temperature was set at 250degr. C. Precursor ions with single, unassigned, or six and higher charge states were excluded from fragmentation selection.

Data analysis

Raw mass spectrometry data were analysed using MaxQuant version 1.5.2.8 (Cox and Mann, 2008), using default settings and LFQ/ iBAQ enabled, searched against the Human Uniprot/Swissprot database (downloaded June 26, 2016, 20197 entries). Further data processing was performed using Perseus software, version 1.5.6.0 (Tyanova et al., 2016). Network visualization of KDM2B interactome was performed using Cytoscape software, version 3.5.1. For Gene Ontology (GO) analysis we used DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/home.jsp). ChIP-seq tracks were visualized and analysed using UCSC genome browser (http://genome.ucsc.edu). Accession number for the Polycomb ChIP-seq data previously reported and used for analysis in this paper is GEO: GSE54580 (van den Boom et al., 2016). Previously published ChIP-seq used for analysis include H3K4me3, H3K36me3, H3K27ac and RNAPII/Pol2(b) from ENCODE/Broad Institute (GSE29611).

Patient samples

AML blasts from peripheral blood or bone marrow from untreated patients were studied after informed consent and the protocol was approved by the Medical Ethical Committee, in accordance with the Declaration of Helsinki. Mononuclear cells were isolated by density gradient centrifugation and $CD34^{+}$ cells were selected automatically by using autoMACS (Miltenyi Biotec).

Generation of lentiviral vectors and transductions

Lentiviral pRRL SFFV PCGF1-EGFP and EGFP-RING1B vectors were generated as described previously(van den Boom et al., 2016). pRRL SFFV KDM2B EGFP was generated as follows. KDM2B was PCR amplified from cDNA in two parts (from the ATG to the RsrII site [fragment 1] and from the RsrII site to end of KDM2B [excluding the stop codon, fragment 2]). Both fragments were independently subcloned into pJet1.2 resulting in the pJet1.2 KDM2B[1] and pJet1.2 KDM2B[2] plasmids and that were subsequenty verified by sequencing. Next, KDM2B fragment 2 was isolated from pJet1.2 KDM2B[2] using RsrII and XbaI digestion and ligated into pJet1.2 KDM2B[1] that was also digested with RsrII and XbaI, resulting in a pJet1.2 plasmid with the full length KDM2B ORF but excluding the stop codon. Finally, the KDM2B ORF was subcloned into the pRRL SFFV GFP vector using AgeI digestion, resulting in the pRRL SFFV KDM2B-GFP construct. A lentiviral pRRL SFFV-His-Ubiquitin-

mBlueberry2 vector was generated by PCR amplification of His-Ubiquitin from pCl His-Ubi (Addgene) plasmid using primers including BamHI sites. The PCR product was first ligated into pJet1.2 using Blunt-End cloning protocol. Subsequently His-Ubiquitin was isolated from pJet1.2 using BamHI digestion and ligated into pRRL SFFV-IRES-mBlueberry2 vector also using BamHI digestion and verified by sequencing. Generation of lentiviral viruses and transductions were performed as described previously (van Gosliga et al., 2007).

Flow cytometry analysis

Flow cytometry analyses were performed on the BD LSR II (Becton Dickinson (BD) Biosciences) and data were analysed using FlowJo (Tree Star Inc, Ashland, OR, USA). Cells were sorted on a MoFlo XDP or MoFLo Astrios (Beckman Coulter). For Hoechst/PyroninY staining, cells were resuspended in HPGM (Lonza, Leusden, The Netherlands) and stained with 5 ug/ml Hoechst 33342 (Invitrogen) at 37°C for 30-45 min. Then 1 ug/ml PyroninY (Sigma) was added and incubated for 30-45 min at 37°C. Upon FcR blocking (MACS miltenyi Biotec), cells were stained with CD34-APC (581, BD Biosciences) and CD38-AlexaFluor 700 (HIT2, Biolegend) at 4°C for 30 min. Cells were washed in medium containing Hoechst/PyroninY and analysed on the BD LSR II. *In vivo* engraftment levels were analysed in peripheral blood (PB), bone marrow, liver and spleen. Prior to staining, cells were blocked with anti-human FcR block (MACS miltenyi Biotec) and anti-mouse CD16/CD32 block (BD Biosciences) and stained with CD45-BV421 (HI30), CD19-BV785 (HIB19) and CD33-APC (WM53) all from Biolegend at 4°C for 30 min.

Western blotting

For detecting His-tagged ubiquitinated proteins cells were lysed in 1 ml denaturing lysis buffer (6M Guanidium-HCl, 100 mM Na_2HPO_4 x $2H_2O$, 10 mM Tris-HCl pH 8.0, 5 mM Imidazole, 10 mM β-mercaptoethanol) and sonicated on ice. Lysates were pre-cleared by centrifugation at 14000 rpm at 4°C and five volumes denaturing lysis buffer was added. His-tagged ubiquitinated proteins were purified by adding 75 µl pre-equilibrated Ni-NTA magnetic agarose beads (Jena Bioscience) and incubated for 4h on a rotating wheel. Beads were separated using a magnetic rack and washed 1x in denaturing lysis buffer without Imidazole, 1x in wash buffer pH 8.0 (8M Urea, 100 mM Na_2HPO_4 x $2H_2O$, 10 mM Tris-HCl pH 8.0 and 10 mM β-mercaptoethanol), 1x in wash buffer pH 6.3 (8M Urea, 100 mM Na_2HPO_4 x $2H_2O$, 10 mM Tris-HCl pH 6.3, 10 mM β-mercaptoethanol) plus 0.2% Triton X-100, and 1x in wash buffer pH 6.3 plus 0.1% Triton X-100. His-tagged ubiquitinated proteins were eluted from the beads by adding 75 µl elution buffer (200 mM Imidazole, 150 mM Tris-HCl pH 6.7, 30% glycerol, 5% SDS and 720 mM β-mercaptoethanol) and incubated for 20 min. on a rotating wheel. Elution samples were diluted 2x in Laemmli

sample buffer containing 10% β-mercaptoethanol and immediately boiled for 5 min. prior to Western blot analysis. GFP mediated pull out cell lysates, input, non-bound and bound fractions were boiled for 5-10 min. in Laemmli sample buffer prior to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to fluorescence polyvinylidene difluoride (PVDF FL, Millipore) membrane by semidry blotting. Membranes were blocked in Odyssey blocking buffer (Westburg). The following primary antibodies were used: anti-USP7 (A300-033A, Bethyl Laboratories), anti-RING1B (ab 181140, Abcam), anti-GFP (sc-9996, Santa Cruz), anti-Ubiquitin (FK2, Enzo Life Sciences), anti-PCGF1 (ab183499, Abcam). Fluorescent secondary antibodies either goat anti-mouse IRDye 800 or goat anti-rabbit IgG (H+L) Alexa Fluor 680 (Invitrogen) were used for detection. Membranes were scanned using the Odyssey CLx Imaging System (Li-Cor Biosciences).

RNA seq analysis and quantitative real-time PCR

RNA samples for sequencing were prepared for DMSO and P22077 (30 µM) treated K562 cells at 4h, 8h, 16h and 24h. Total RNA was isolated using the RNeasy Mini Kit from Qiagen (Venlo, The Netherlands) according to the manufacturer's recommendations. Initial quality check and RNA quantification of the samples was performed by capillary electrophoresis using the LabChip GX (Perkin Elmer). Sequence libraries were generated with 50 ng mRNA, using Lexogen Quantseq 3' prep kit (Lexogen GmbH) according to the manufacturer's recommendations. The obtained cDNA fragment libraries were sequenced on an Illumina NextSeq500 using default parameters (single read). Bioinformatics were performed on the Strand Avadis NGS (v3.0) software (Strand Life Sciences Pvt.Ltd). Sequence quality was checked for GC content, base quality and composition using FASTQC and StrandNGS. Quality trimmed reads were aligned to build Human Hg19 transcriptome. Ensembl Genes and transcripts (2014.01.02) was used as gene annotation database. Quantified reads were normalized using the DESeq package. Reads with failed vendor QC, quality score less than 24 (average), mapping quality score below 50 and length less than 20 were all filtered out. For quantitative RT-PCR, RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad) and amplified using SsoAdvanced SYBR Green Supermix (Bio-Rad) on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). RPL27 was used as housekeeping gene. Primer sequences are available on request.

USP7 inhibition *in vivo*

Eight to ten week old female NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice were purchased from the Centrale Dienst Proefdieren (CDP) breeding facility within the University Medical Center Groningen. Mouse experiments were performed in accordance with national and institutional guidelines, and all experiments were approved by the Institutional Animal

Care and Use Committee of the University of Groningen (IACUC-RuG). 24h prior to transplantations, mice were sub-lethally irradiated with a dose of 1.0 Gy (X-RAD 320 Unit, PXINC 2010). After irradiation mice received Neomycin (3.5 g/l) in their drinking water and soft food (RM Convalescence + BG SY (M); Special Diet Services; Witham, England) for two weeks. For secondary transplantations, 5 x 10^4 MLL-AF9 EGFP cells from primary leukemic mice (CB MLL-AF9 xenograft mouse model, (Horton et al., 2013; Sontakke et al., 2016)) were injected IV (lateral tail vein). Peripheral blood chimerism levels were monitored by regular blood sample analysis. Mice were randomly divided into two groups, weighted and treated with DMSO as control (n=5) or 20 mg/kg P22077 (n=6) via intraperitoneal (IP) injections daily starting four weeks post-transplant. Prior to injections, P22077 was dissolved in DMSO (or DMSO only as control) and directly mixed with Cremophor EL (1:1). This solution was then diluted 1:4 in saline, to get an end concentration of max. 10% DMSO. Mice were humanely terminated by cervical dislocation under isoflurane anesthesia when chimerism levels in the blood exceeded 40%. Peripheral blood, bone marrow, spleen and liver were analysed.

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Author contributions

HM designed and performed experiments, analyzed data, and wrote the manuscript; JJ, ARL, SMH, MPV, CG, AZBV, NM, VB performed experiments, analyzed data, and reviewed the manuscript; GH and EV provided patient samples, analyzed data and reviewed the manuscript; JJS designed the study, analyzed data and wrote the manuscript.

Conflict of interest disclosures

The authors declare no conflict of interests.

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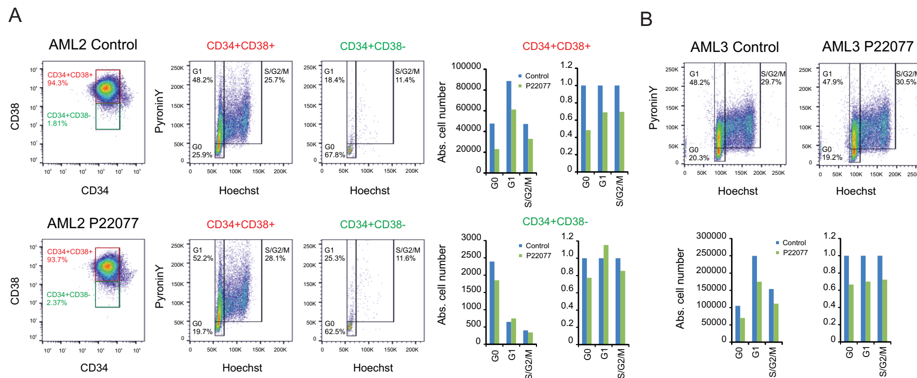
SUPPLEMENTARY FIGURES



(A) Analysis of both cycling and quiescent AML cells by Hoechst/PyroninY stainings within the CD34$^+$/CD38$^+$ and CD34$^+$/CD38$^-$ populations (B) or whole population (AML3). Cells were treated with DMSO (control) or P22077 (20 µM) for 3 days while AMLs were grown on MS5 co-culture. Absolute cell numbers were calculated in G0, G1 and S/G2/M fractions.

SUPPLEMENTARY INFORMATION

Supplementary Table 1 t/m 7 can be found with this article online at https://doi.org/10.1101/221093

Suppl. Table 1. Cell lines and patient characteristics

Suppl. Table 2. KDM2B EGFP interactome and Gene Ontology analysis

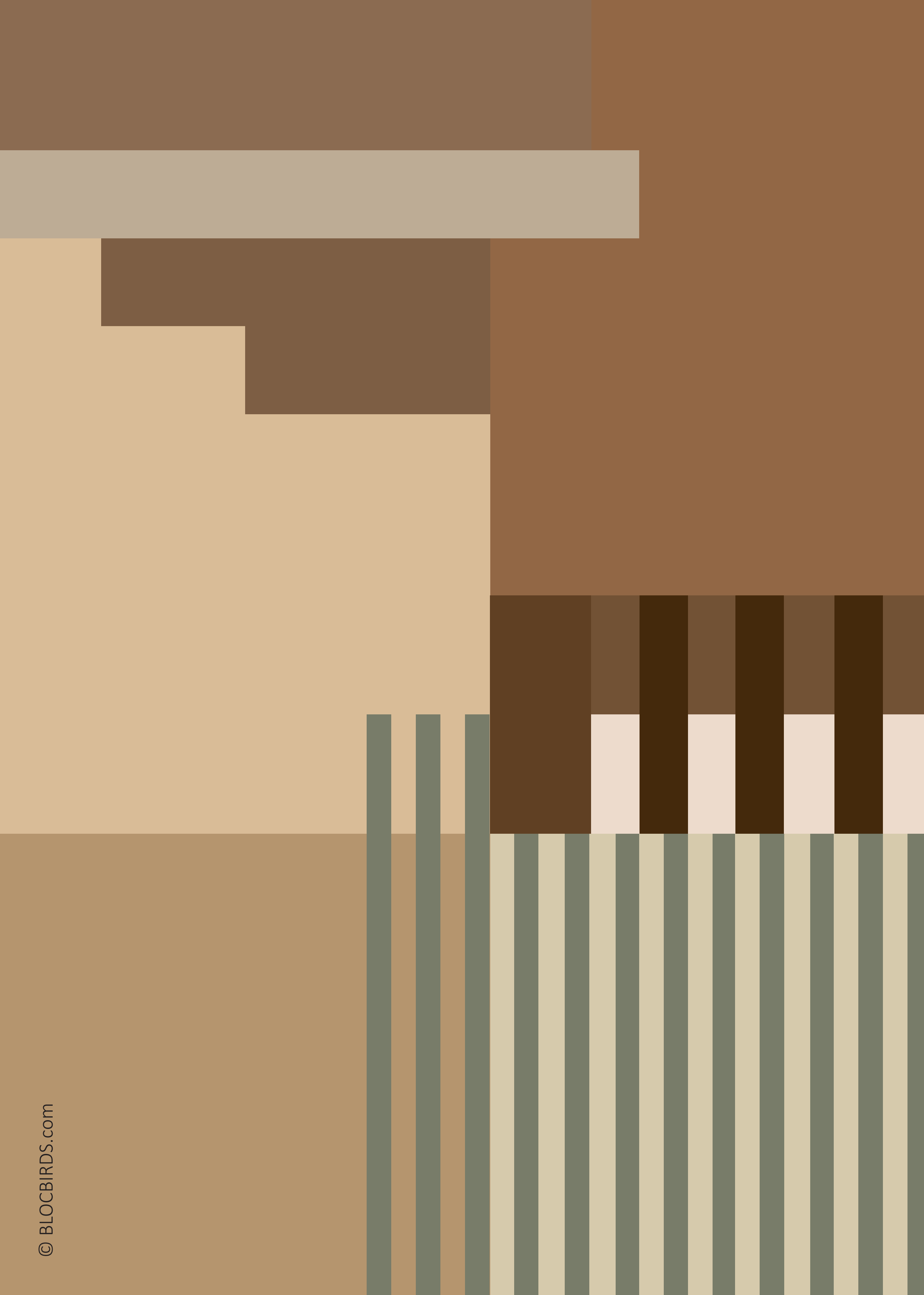
Suppl. Table 3. LC-MS/MS data K562 EGFP-RING1B, PCGF1-EGFP pull outs

Suppl. Table 4. MS data Avi-pull outs published in van den Boom et al (2016)

Suppl. Table 5. PCGF1-EGFP and EGFP-RING1B pull outs in control/USP7i

Suppl. Table 6. RNA seq data control/USP7i

Suppl. Table 7. Primer sequences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TRANSCRIPTIONAL CONTROL OF PRC1.1 TARGET GENES IN LEUKEMIC CELLS: ACTIVELY DRIVING TRANSCRIPTION OR MAINTAINING LOCI IN A 'TRANSCRIPTION PERMISSIVE' STATE?

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In progress

ABSTRACT

Polycomb complexes are essential epigenetic regulators of gene transcription and are critically involved in hematopoietic stem cell self-renewal and differentiation. Non-canonical PRC1.1 is essential for the survival of primary leukemic cells. Our previous work has shown that PRC1.1 targets a subset of loci independent of H3K27me3 and is associated with permissive or active chromatin, however, the molecular mechanism by which PRC1.1 affects transcriptional control is not well understood. When comparing ChIP-seq and DNA methylation data we find that PRC1.1 preferentially targets unmethylated CGI promoters associated with transcriptionally active chromatin. Inhibition of USP7, a core component of PRC1.1, resulted in disassembly of the complex and dislodgement of KDM2B from the chromatin, which coincided with slightly enhanced *de novo* DNA methylation on some PRC1.1 target loci. RNA-seq data revealed that several PRC1.1 target genes associated with the Gene Ontology (GO) terms 'transcription' and 'regulation of gene expression' were downregulated upon USP7 inhibition. A ChIP for H3K27ac on some of those loci showed reduced levels upon loss of PRC1.1 binding by treatment with the USP7 inhibitor, indicative for reduced transcriptional activity. While further research is needed to gain insight into the mechanism, we propose that PRC1.1 is important to maintain gene expression of several target genes critical for the survival of leukemic cells.

INTRODUCTION

The regulation of gene expression is mediated by growth factor or cytokine-induced signaling and controlled by several epigenetic processes that are critically involved in instructing hematopoietic stem cell fate. During development, hematopoietic stem cells with the same genetic information can either self-renew or differentiate. This is accompanied by dynamic changes in the chromatin state, allowing the activation of distinct gene expression programs (Chen et al., 2014; Cullen et al., 2014; Haas et al., 2018; Paul et al., 2015; Yu et al., 2016). It is well known that transcription factors play a central role in initiating and regulating gene expression, although the specific local chromatin architecture impacts on the transcriptional activity of the locus as well (Obier and Bonifer, 2016). Processes that are involved in the chromatin architecture include DNA modifications (e.g. methylation), post-translational modifications of histone proteins or RNA polymerase II, as well as nucleosome positioning which is influenced by chromatin remodeling complexes that increase or decrease the accessibility for transcription factors (Kouzarides, 2007). Although our understanding

of how the chromatin architecture and epigenetic state contribute to gene regulation is continuously increasing, several aspects such as the cross-talk between the epigenetic machinery and transcription factors, their recruitment to chromatin and their functional role on gene transcription are not fully understood (Henikoff and Shilatifard, 2011). Most CpG islands (CGIs) are sites of transcriptional regulation and recruit proteins that influence the chromatin architecture of CGIs. While the link with transcription is not entirely clear, unmethylated CGIs are mostly associated with transcriptional activity and when methylated are associated with transcriptional silencing (Deaton and Bird, 2011). DNA methylation is catalyzed by DNA methyltransferases (DNMTs) of which DNMT3A/B are involved in de novo DNA methylation and DNMT1 is a maintenance DNMT (Challen et al., 2014; Okano et al., 1999). Reversely, DNA demethylation is mediated by TET proteins, that catalyze the oxidation of methylcytosine (meC) into hydroxymethylcytosine (hmC). The observation that unmethylated CGIs serve as a substrate for several transcriptional regulators and recruit proteins with a ZF-CxxC domain that can alter local chromatin architecture was an important step forward in understanding CGI function (Blattler and Farnham, 2013; Jones, 2012; Long et al., 2013). CxxC finger protein 1 (CFP1) was the first one identified, after which a family of ZF-CxxC domain containing proteins was discovered including DNMT1, MLL1, MBD1, KDM2B and TET1 (Blackledge et al., 2013; Long et al., 2013; Voo et al., 2000). Several of them modulate specific histone lysine methylation marks. For example, CFP1 or MLL proteins exist in a SET1 containing methyltransferase complex that mediates H3K4 trimethylation and KDM2B is a H3K36 specific demethylase (Thomson et al., 2010; Wang et al., 2009; Wu et al., 2013). Notably, DNMT1 and TET1 proteins are implicated in DNA methylation and demethylation respectively. This raises the question of why they occupy unmethylated CGIs. Structural studies revealed that DNMT1 was catalytically inactive when bound to unmethylated CGIs (Song et al., 2011). Depletion of TET1 resulted in increased CGI methylation suggesting that ZF-CxxC proteins themselves might protect CGIs from methylation (Wu et al., 2011). Polycomb group (PcG) proteins are associated with CGIs and are important epigenetic regulators of gene transcription (Di Croce and Helin, 2013; Orlando et al., 2012; Schuettengruber et al., 2017; Tanay et al., 2007). They form multi-protein chromatin modifying complexes whose central functions include post-translational modifications of histones. Canonical Polycomb Repressive Complex 1 (PRC1) and PRC2 are well known as transcriptional repressors (Morey and Helin, 2010). PRC2 consists of the core components EED, SUZ12, and one of the two histone H3K27 methyltransferases EZH1 or EZH2 (Cao et al., 2002). Canonical Polycomb signaling is initiated by the PRC2 complex that catalyzes the trimethylation of histone H3 at lysine 27 (H3K27me3) and allow the recruitment of the PRC1 complex. The canonical PRC1 core complex is

composed of CBX2/4/6/7/8, PCGF2/4, PHC1/2/3, SCML1/L2/H1 and RING1A/B subunits which catalyzes the mono-ubiquitination of histone H2A on lysine 119 (H2AK119ub) (Simon and Kingston, 2013; Wang et al., 2004). Thus in addition to DNA methylation, CGI promoters can be silenced by PRC2/PRC1-mediated transcriptional repression. While PRC2/PRC1 are enriched at CGIs, the mechanism underlying their recruitment to chromatin is not fully understood and likely mediated via multiple interactions. It is suggested that DNA binding proteins like JARID2 and AEBP2, several transcription factors and noncoding RNAs might be involved (Schuettengruber et al., 2017). An alternative Polycomb complex, known as non-canonical PRC1.1 consists of the core proteins KDM2B, PCGF1, RING1A/B, BCOR(L1), RYBP/YAF2, USP7 and SKP1. Non-canonical PRC1.1 is targeted to unmethylated CGIs via the ZF-CxxC domain of KDM2B and catalyzes the ubiquitylation of H2AK119 mediated by the RING1A/B E3 ligases (Farcas et al., 2012; Wong et al., 2016; Wu et al., 2013) (Figure 1). PRC1.1 is critically important for leukemic stem cells, since genetic knockdown of PRC1.1 components impaired long-term self-renewal and leukemia progression *in vitro* and *in vivo* (van den Boom et al., 2016). Our ChIP-seq studies (van den Boom et al., 2016) revealed that besides non-canonical PRC1.1 targets a subset of loci co-occupied by canonical PRC2/PRC1, referred to as 'both' loci, it also targets a distinct set of loci that are devoid of the repressive PRC2/H3K27me3 mark. Instead, these loci are associated with transcriptionally permissive or active chromatin marked with SET1- or MLL-mediated H3K4me3, p300/CBP-mediated H3K27ac, active RNA polymerase II (RNAPII S5P) at the transcription start site (TSS) and SETD2-mediated H3K36me3 throughout the gene body (Figure 1). Thus, these data indicate that PRC1.1 controls gene expression via complex and diverse mechanisms.

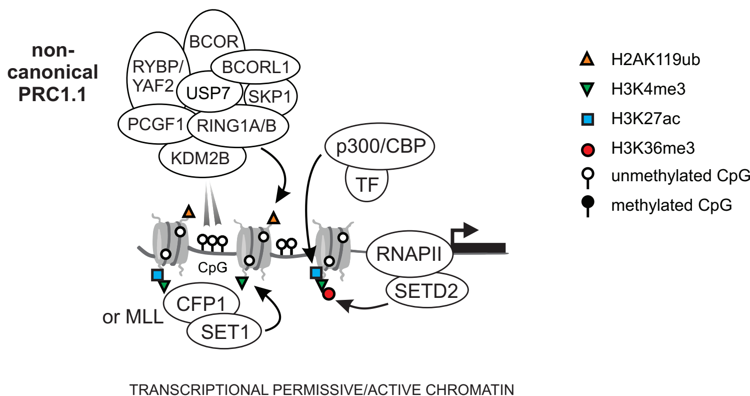

Figure 1. Schematic representation of transcriptionally permissive/active PRC1.1 targets in leukemic cells

In this study, we analyzed ChIP-seq and DNA methylation data to investigate how non-canonical PRC1.1 might exert transcriptional control in leukemic cells. We found that PRC1.1 preferentially targets unmethylated CGI promoters associated with transcriptionally active chromatin. Removal of the PRC1.1 complex, as a consequence of USP7 inhibition, resulted in slightly more de novo DNA methylation on loci that are typically unmethylated. Several PRC1.1 target genes were downregulated upon USP7 inhibition and some targets indeed revealed reduced transcriptional activity as shown by reduced H3K27ac levels. Lastly, our data indicate that PRC1.1 is important to maintain gene expression of several target genes in leukemic cells. Future studies are needed to shed light on the molecular mechanisms and cross-talk with chromatin regulators and transcriptional machinery.

RESULTS AND DISCUSSION

PRC1.1 binds unmethylated CpG island promoters linked to transcriptionally permissive or active chromatin

To study the relationship between Polycomb complex binding to unmethylated or methylated CGIs, we compared previously performed ChIP-seq data in which we identified distinct non-canonical PRC1.1 and canonical PRC2/PRC1 target genes in leukemic cells (van den Boom et al., 2016) with differentially methylated regions (DMRs) from K562 cells obtained from ENCODE/Hudson Alpha (450k arrays and RRBS). Since approximately 70% of human gene promoters contain CGIs (Saxonov et al., 2006), we analyzed those Polycomb loci targeted to the TSS. This revealed that the majority (89%) of loci bound by non-canonical PRC1.1 at TSSs was enriched for unmethylated CGIs, while only 9% of PRC1.1-bound loci was suggested to be methylated. In contrast, 30% of all TSS loci bound by canonical PRC2/PRC1 was methylated, while for loci that were bound by both canonical PRC2/PRC1 and non-canonical PRC1.1 (referred to as ‘both’) this percentage was 20% (Figure 2A). According to literature, CGIs are usually unmethylated allowing a transcriptionally permissive chromatin state (Deaton and Bird, 2011; Long et al., 2016). Silencing of CGI promoters is either mediated by direct DNA methylation or by canonical PRC2/PRC1-mediated transcriptional repression and it is also suggested that the PRC2 subunit EZH2 can recruit DNMTs and thereby initiate DNA methylation (Deaton and Bird, 2011; Orlando et al., 2012; Vire et al., 2006). The finding that PRC1.1 is predominantly associated with unmethylated CGIs is supported by the fact that KDM2B recruits PRC1.1 to CGIs dependent on its ZF-CxxC domain, shown to specifically recognize unmethylated CGIs (Farcas et al., 2012; Long et al., 2013; Wu et al., 2013). Since PRC1.1 and canonical PRC2/PRC1 can target the same but also distinct loci it is still unclear what mechanism underlies the association with CGIs at specific chromatin sites. Why are some loci bound

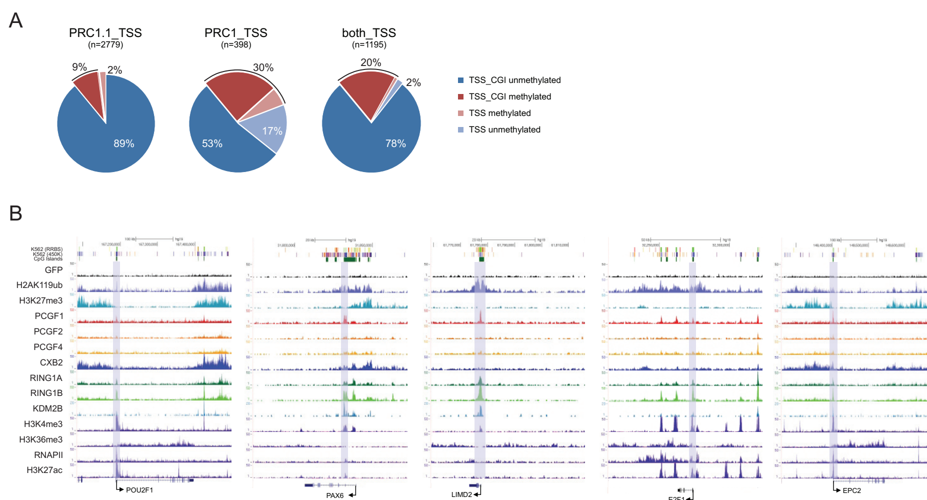

Figure 2. Analysis of Polycomb complex binding to TSSs (associated) with methylation status (A) Pie charts analyzing PRC1.1, PRC1 and 'both' target genes at the TSS or specifically TSS_CGI together with differentially methylated regions from K562 obtained from ENCODE/Hudson Alpha (450k arrays and RRBS). (B) Representative examples of genes targeted by PRC1.1 to TSS_CGI. K562 RRBS (green/yellow/red, green is 0% methylated) and 450k array tracks (bright blue/purple/orange, bright blue is unmethylated) are shown to indicate methylation status. K562 H3K4me3, H3K36me3, RNAPII and H3K27ac tracks were downloaded from ENCODE/Broad. Our Polycomb and H2AK119ub/H3K27me3 ChIP-seq tracks based on previously reported data are shown (van den Boom et al., 2016).

by both canonical PRC2/PRC1 and non-canonical PRC1.1, while others are exclusively bound by PRC1.1 and not by canonical Polycombs? Further studies are required to resolve these issues. Nevertheless, where canonical PRC2/PRC1 is associated with a repressed chromatin state, non-canonical PRC1.1 complexes can be targeted to chromatin independent of H3K27me3 (Morey et al., 2013; Tavares et al., 2012) associated with active chromatin (van den Boom et al., 2016). Figure 2B shows some representative examples of PRC1.1 loci at TSS_CGIs, illustrated by enrichment of PCGF1, KDM2B, RING1A/B (H2AK119ub) and low levels of canonical PRC1 proteins like CBX2. The RRBS and 450k array tracks indicated unmethylated regions and since this coincided with an enrichment of H3K4me3, RNAPII and H3K27ac, this indicates that PRC1.1 preferentially binds active CGI promoters. Transcriptionally active CGIs likely prevent PRC2 recruitment, as deleting transcription factor motifs at certain CGI promoters resulted in PRC2 recruitment and H3K27me3 (Ku et al., 2008; Mendenhall et al., 2010).

Evaluating the consequence of loss of PRC1.1 binding with a focus on DNA methylation

To examine whether KDM2B-mediated recruitment of PRC1.1 to unmethylated CGIs protect genes from hypermethylation, as suggested by Boulard et al (Boulard et al., 2015), we targeted PRC1.1 by inhibiting the deubiquitinase activity of USP7. We recently identified that the deubiquitinase USP7 is an essential component of PRC1.1 (Figure 3A) and is required to maintain its integrity and function (Maat et al, submitted). As a consequence of inhibiting USP7 deubiquitinase activity, the PRC1.1 complex disassembled which resulted in a complete loss of KDM2B binding with concomitant strong reductions in PCGF1 and RING1B binding to several PRC1.1 target loci as shown by ChIP-qPCRs (Figure 3B). To investigate DNA methylation, we used the methyl-Cap procedure as described by Brinkman et al (Brinkman et al., 2010). Methylated DNA was captured by incubation with a MBD domain fusion protein (MeCP2) and different methyl-CpG density fractions were eluted in a step-wise manner using increasing salt concentrations (NaCl). Unmethylated or low methylated CpGs are found in the low salt elutions steps, while high methylated CpGs were eluted at high salt concentrations. First we performed the methyl-Cap procedure on K562 cells treated with the hypomethylating agent decitabine (DAC) or DMSO as control (Figure 3C). qPCRs on three putative highly methylated loci showed reduced levels in fractions 4/5 or 5/6 as a consequence of DAC treatment and increased levels in fractions 2/3 (4). Therefore fractions 1-3 were interpreted as low methylated and 4-7 as high methylated. The data clearly indicated reduced methylation following DAC treatment, illustrated in black/white bar graphs. Next, to determine whether loss of PRC1.1 binding would affect changes in DNA methylation, we performed the methyl-Cap procedure on K562 cells treated with the USP7 inhibitor followed by qPCR on loci exclusively bound by PRC1.1 (Figure 3D). For all 5 investigated loci, a 5-10% increase in DNA methylation was observed upon USP7 inhibition. Since USP7 can function in several pathways, we cannot exclude the possibility that the observed effects were induced by PRC1.1 independent mechanisms (Kim and Sixma, 2017; Reyes-Turcu et al., 2009). For further conclusive evidence, it would be important to specifically interfere with PRC1.1 binding, for instance by introducing a mutation in the CxxC domain of KDM2B or by using an (inducible) KDM2B knockdown or knockout approach. Where USP7 has been implicated in the maintenance of DNA methylation by stabilizing UHRF1 and DNMT1, several methylated loci could be analyzed to control for possible effects of USP7 inhibition (Felle et al., 2011). Boulard and colleagues (Boulard et al., 2015) showed that only a subset of ES loci normally bound by KDM2B (also known as FBXL10) and PRC1/PRC2 became hypermethylated (>50%) in KDM2B depleted cells, indicating that KDM2B protects these loci from *de novo* methylation. It would be of interest to study

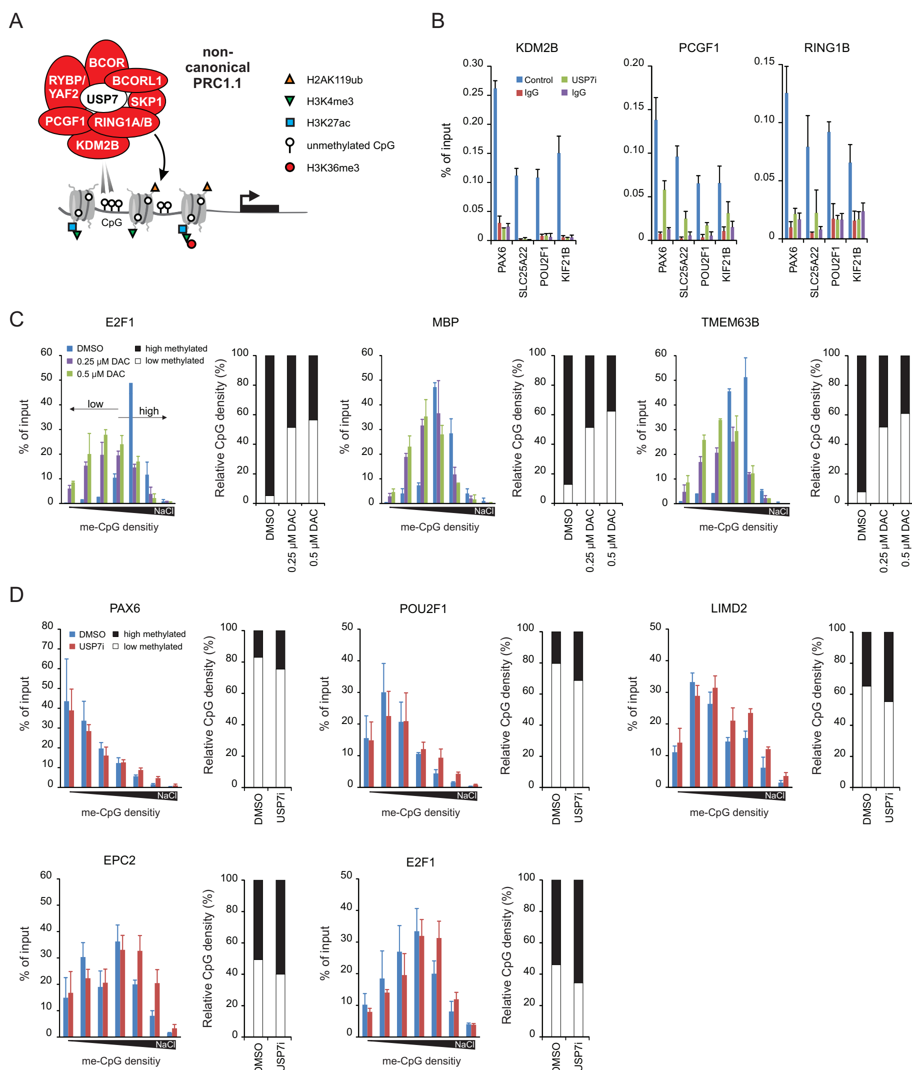

Figure 3. Analysis of differential DNA methylation on several PRC1.1 target loci upon USP7 inhibition
(A) Schematic representation of non-canonical PRC1.1. LC-MS/MS analysis revealed PRC1.1 complex members, KDM2B, PCGF1, RING1A/B, RYBP/YAF2, SKP1, BCOR(L1) and USP7. PRC1.1 is targeted to unmethylated CGIs on active promoters and catalyzes the ubiquitination of H2AK119. Here we targeted PRC1.1 by inhibiting the deubiquitinase activity of USP7, using P22077. (B) ChIP for KDM2B, PCGF1-GFP and GFP-RING1B in untreated/control K562s (blue bars) or following 72h of USP7 inhibition (green bars). PRC1.1 binding is lost upon USP7i at several target genes validated by ChIP-qPCR. (C) Methyl-Cap performed on K562 cells treated 72h with DMSO or decitabine (DAC), followed by qPCR on three highly methylated regions (n=2). Eluted fractions 1-3 are considered low methylated and 4-7 high methylated. Bar graphs represent normalized CpG densities as sum low

methylated fractions and sum high methylated fractions. (D) Methyl-Cap performed on K562 cells treated 72h with DMSO or USP7i, followed by qPCR on five PRC1.1 target genes (n=3).

if DNA methylation changes would be more prominent on the 'both' loci in leukemic cells as well. The underlying mechanism how DNMT3A/B would be recruited to these 'both' loci is still unclear. Our data suggests that PRC1.1 is likely not the key player in protecting CGIs from DNA methylation, but may act together with for instance other ZF-CxxC domain containing proteins like DNMT1 and TET1 that might protect CGIs from methylation (Song et al., 2011; Wu et al., 2011). Furthermore, transcription factors have been implicated in preventing DNA methylation (Brandeis et al., 1994; Gebhard et al., 2010) and studies suggested that H3K4me3 may have an inhibitory effect on DNA methyltansferase activity (Cheng, 2014; Li et al., 2011; Rose and Klose, 2014).

PRC1.1 is required to maintain gene expression of several target genes

In order to understand PRC1.1 function on unmethylated CGI promoters in relation to gene expression, we analyzed previously performed RNA-seq data of K562 cells treated for 24h with the USP7 inhibitor (30 µM P22077). Since PRC1.1 is associated with active CGI promoters, we expected that loss of PRC1.1 binding upon USP7 inhibition might reduce the expression of several PRC1.1 target genes. 355 PRC1.1 target genes were differentially expressed (fold change>1.5) of which the majority was downregulated (Figure 4A). GO analysis revealed that downregulated genes were enriched for processes like 'transcription', 'regulation of gene expression', including transcription factors and other transcriptional modulators (Figure 4B). Upregulated genes were enriched for 'cell-cell adhesion' processes (Figure 4B). Gene expression changes, upon USP7 inihbition, independent of PRC1.1 were enriched for GO terms like 'protein ubiquitination', 'cell division' and 'protein folding' (Figure 4C). The exact function of PRC1.1 is still not fully understood and also what mediates reduced or increased gene expression of several target genes is not clear. To study the effects of loss of PRC1.1 binding and if possible re-binding of PRC1.1 using ChIP-seq, RRBS and RNA-seq under the same conditions would provide a global view on PRC1.1 function and recruitment. USP7 inhibition resulted in loss of PRC1.1 binding and as a direct or indirect consequence this also affected the levels of several histone modifications (Figure 4D and E). The loss of H2AK119ub could be a consequence of loss of *de novo* ubiquitination mediated by RING1A/B E3 ligases or actively removed by a deubiquitinase with specificity for H2A. Since H2AK119ub marks are both present at repressed and active chromatin (de Napoles et al., 2004; Tavares et al., 2012; van den Boom et al., 2016; Wang et al., 2004), the mechanism by which H2AK119ub functions on gene transcription is unclear. In line with the notion that several

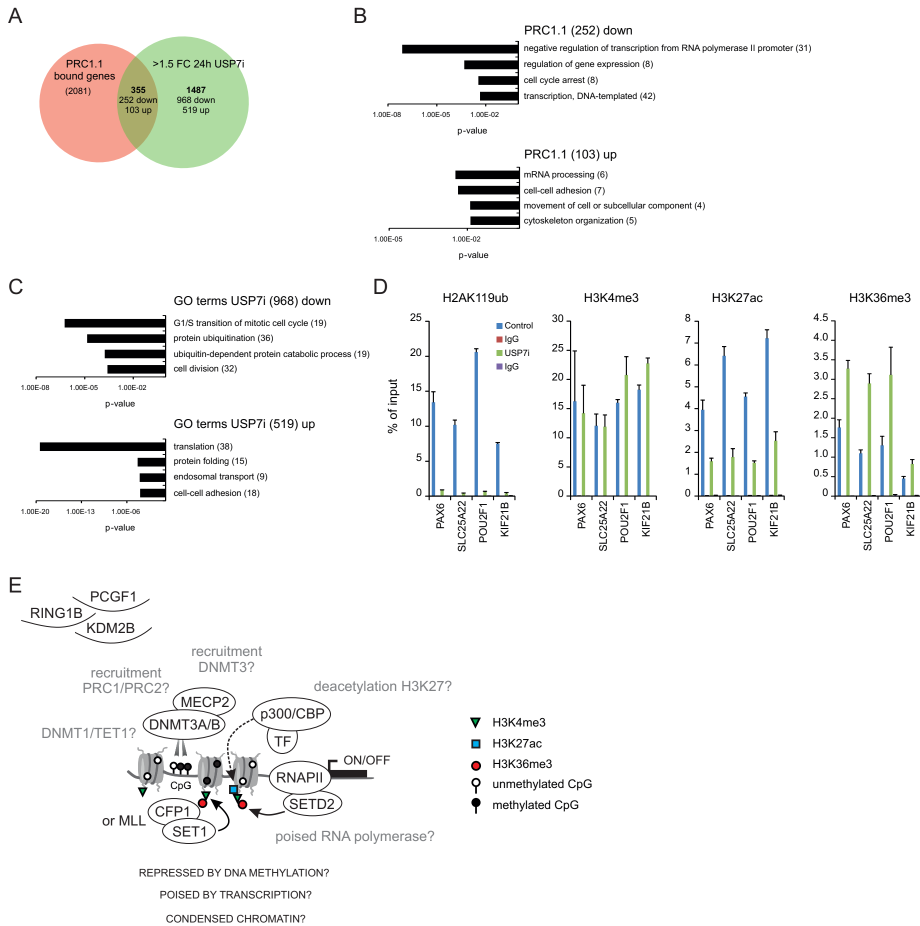

Figure 4. Comparison of PRC1.1 bound genes, gene expression and histone modification changes upon USP7i (A) Venn diagram comparing PRC1.1 bound genes in K562 (red) and gene expression changes as identified by RNA-seq (>1.5 FC) after 24h of USP7i in K562 cells (green). (B) Gene Ontology analysis of PRC1.1 bound genes, downregulated or upregulated upon USP7i. Biological Process terms are indicated. (C) Gene Ontology analysis of genes changing upon USP7i that are not directly regulated by PRC1.1 bound genes. Biological Process terms are indicated. (D) ChIP for H2AK119ub, H3K27ac and H3K36me3 in control or USP7i (16h) treated cells. H3K4me3 ChIP was performed following 72h of USP7i. (E) Schematic model of a transcriptionally permissive or active PRC1.1 locus following USP7 inhibition, resulting in disassembly of the PRC1.1 complex and consequently loss of binding to its target loci. This coincided with loss of H2AK119ub, likely as a consequence of loss of *de novo* ubiquitination. Furthermore, loss of PRC1.1 binding resulted in reduced levels of H3K27ac at several target loci, whereas H3K4me3 levels remained unaffected. Still many questions remain. Can loss of PRC1.1 result in *de novo* DNA methylation or do DNMT1/TET1 proteins or H3K4me3 still prevent DNA methylation? What is the status of RNA polymerase II?

Is there a link with PRC1.1 and accessibility of transcription factors to bind? What dictates gene expression changes?

target genes were downregulated, a reduction in H3K27ac was observed associated with reduced transcriptional activity (Creyghton et al., 2010). A potential mechanism could be that the accessibility of transcription factors is blocked and P300/CBP mediated H3K27ac is reduced or it is due to recruitment of histone deacetylases. A focus for future work is to also define RNA polymerase II occupancy and if it is in an inactive or poised state. H3K4me3 levels were not affected upon USP7 inhibition, suggesting that MLL or CFP1 proteins are still targeted to non-methylated CGIs via their ZF-CxxC domain (Lee and Skalnik, 2005; Long et al., 2013; Milne et al., 2005; Thomson et al., 2010). Lastly, we performed a ChIP for H3K36me3 since KDM2B contains the demethylase JmjC domain (He et al., 2008; He et al., 2011). While H3K36me3 is enriched throughout the gene body and can function as an elongation mark (Wagner and Carpenter, 2012), we analyzed some PRC1.1 target loci at the TSS. The loss of KDM2B coincided with increased levels of H3K36me3, but it is not known why KDM2B would demethylate H3K36 on the TSS. In addition, some studies suggest that there is a link between H3K36me3 and *de novo* DNA methylation in specific genomic contexts, but further work is needed to elucidate the underlying mechanism (Dhayalan et al., 2010; Lorincz et al., 2004; Rose and Klose, 2014).

CONCLUSIONS AND PERSPECTIVES

The current model that non-canonical PRC1.1 is preferentially targeted to unmethylated CGI promoters via the ZF-CxxC domain of KDM2B and associated with transcriptionally permissive or active chromatin in leukemic cells suggest that PRC1.1 is important to maintain or initiate gene expression of several target genes. Targeting the PRC1.1 complex resulted in downregulation of several target genes and reduced transcriptional activity. DNA methylation changes were not very pronounced at several PRC1.1 target genes, and it was even suggested that methylation on CGIs often takes place at genes marked by H3K27me3 (Boulard et al., 2015; Schlesinger et al., 2007). Thus, other chromatin regulators might be involved. It will be of interest in future work to perform DNaseI hypersensitive site mapping or assay for transposase accessible chromatin (ATAC)-seq to investigate sites that are accessible for transcription factor binding and interplay with chromatin remodeling complexes (Bonifer and Cockerill, 2017). A recent paper suggested that PRC1.1 could promote the recruitment of SS18-SSX1 containing SWI/SNF complexes to unmethylated CGIs in synovial sarcoma cells (Banito et al., 2018). SWI/SNF complexes facilitate transcription by nucleosome remodeling, allowing transcription factors to bind

(Clapier and Cairns, 2009). KDM2B depletion resulted in reduced SS18-SSX1 binding from chromatin and H3K27me3 levels increased at a sub-set of loci. Future studies are needed to understand the chromatin architecture at PRC1.1 target genes and its function on transcriptional control.

MATERIALS AND METHODS

Cell culture

K562 cells (ATCC:CCL-243) were maintained in RPMI 1640 (BioWhittaker, Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FCS, HyClone Laboratories, Logan, Utah, US) and 1% penicillin/streptomycin (p/s, PAA Laboratories). For USP7 inhibition experiments, P22077 (1-(5-((2,4-difluorophenyl)thio)-4-nitrothiophen-2-yl)ethanone) was purchased from Merck Millipore (662142) (Billerica, MA, USA) and used in a concentration of 30 µM. Stock solutions were dissolved in DMSO then added 1:1000 to the culture. Decitabine (DAC) experiments were performed for 72h and DAC was added freshly every day.

Chromatin immunoprecipitation (ChIP) and quantitative real-time PCR

ChIP was performed as described previously (Maat et al, submitted). K562 cells stably expressing low levels of EGFP-fusion vectors encoding, PCGF1-EGFP, EGFP-RING1B (van den Boom et al., 2016), KDM2B-EGFP (Maat et al, submitted) or non-transduced K562 cells were treated with DMSO or P22077 for indicated timepoints and subsequently cross-linked. Then ChIP reactions were performed using the following antibodies: anti-GFP (ab290, Abcam), anti-KDM2B (ab137547, Abcam), anti-H2AK119ub (D27C4, Cell Signaling Technology), anti-H3K4me3 (ab8580, Abcam), anti-H3K27ac (C15410196, Diagenode), H3K36me3 (C15410192, Diagenode) and IgG (I8141, Sigma). ChIPs were analyzed by qPCR on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) as percentage of input, primer sequences are available on request.

Me-Cap procedure

To study DNA methylation, we used the methyl-Cap procedure as described by Brinkman et al (Brinkman et al., 2010). Genomic DNA was isolated using the NucleoSpin Tissue kit according to manufacturer's protocol (Machery-Nagel). For DNA shearing, 3 ug of DNA was used in a concentration of 100 ng/ul and sonicated using the Bioruptor (Diagenode). Cycles of 15 sec ON/OFF were performed for a total time of 10 min. For binding, 1 ug of fragmented DNA was incubated with 2 ug of H6-GST-MBD fusion protein (C02020012, Diagenode) in Binding buffer (20 mM Tris-HCl pH8.5, 0.1% Triton X-100) with 200 mM

NaCl in a final volume of 200 ul, incubated for 2 hours on a rotating platform at 4C. Then 35 ul pre-cleared MagneGST-beads (Promega) were added and incubated for another hour at 4C. First the unmethylated fraction was collected using a magnetic rack, followed by step-wise elution of different methyl-CpG density fractions using increasing salt concentrations. Beads were washed/eluted with 200 ul Binding buffer with increasing concentrations of NaCl, 1x 300 mM, 2x 400 mM, 1x 500 mM, 1x 600 mM, 1x 800 mM and 1x 1M. After each step the fraction was collected and were all purified using the QIAquick PCR purification kit (Qiagen). Several loci were analyzed by qPCR as percentage of input, primer sequences are available on request.

Data analysis

Accession number for the Polycomb ChIP-seq data previously reported and used for analysis in this paper is GEO: GSE54580 (van den Boom et al., 2016). Previously published ChIP-seq used for analysis include H3K4me3, H3K36me3, H3K27ac and RNAPII/Pol2(b) from ENCODE/Broad Institute (GSE29611). DMRs from K562 cells were obtained from ENCODE/Hudson Alpha (450k arrays and RRBS). ChIP-seq tracks were visualized and analyzed using UCSC genome browser (http://genome.ucsc.edu). RNA-seq was previously performed as described in Maat et al, submitted. Gene expression changes were analyzed at timepoint 24h in DMSO and P22077 (30 µM) treated K562 cells. For Gene Ontology (GO) analysis we used DAVID Bioinformatics Resource (http://david.abcc.ncifcrf.gov/home.jsp).

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CHAPTER 5

USING A LENTIVIRAL TET-REGULATED miR-E shRNA DUAL COLOR VECTOR TO EVALUATE GENE FUNCTION IN HUMAN LEUKEMIC STEM CELLS *IN VIVO*

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ABSTRACT

RNA interference is a powerful tool to study loss-of-gene function in leukemic cells. Still, in order to identify effective novel treatment strategies to target and eradicate leukemic stem cells (LSCs), it is critically important to study gene function in a well-controlled and time-dependent manner. We implemented a lentiviral Tet-regulated miR-E shRNA dual color vector in our *in vitro* and *in vivo* human leukemia models. Thus, we were able to efficiently introduce doxycycline-inducible and reversible gene repression and trace and isolate transduced miR-E shRNA expressing cells over time. As proof of concept we focused on the non-canonical PRC1.1 Polycomb complex, which we previously identified to be essential for LSCs (van den Boom et al., 2016). Here, we show that inducible downmodulation of PCGF1 strongly impaired the growth of primary MLL-AF9 cells. Next, a Tet-regulated miR-E PCGF1 human xenograft MLL-AF9 leukemia mouse model was established, which revealed that early knockdown of PCGF1 at the onset of leukemia development significantly reduced peripheral blood chimerism levels and improved overall survival. In contrast, knockdown of PCGF1 when leukemia was already firmly established in the bone marrow proved insufficient to enhance overall survival. Despite these findings, FACS analysis of MLL-AF9/miR-E PCGF1/CD45^{+}/GFP^{+} populations suggested that particularly cells with inefficient PCGF1 knockdown contributed to leukemogenesis. In conclusion, by building *in vivo* xenograft leukemia inducible RNAi models, we show that timing of gene knockdown critically impacts on the efficacy of leukemia treatment and that clonal drift still plays a major role in the escape of LSCs.

INTRODUCTION

Leukemogenesis is a multistep process in which genetic and epigenetic changes affect normal growth and differentiation of hematopoietic stem and/or progenitor cells, which can ultimately lead to full leukemic transformation (Bonnet and Dick, 1997; Cancer Genome Atlas Research et al., 2013; Eriksson et al., 2015; Yang et al., 2017). With current therapies the majority of the blast population is readily eradicated, though the rare quiescent population of LSCs is more difficult to target and the major cause of relapse of the disease (Jan et al., 2012; Pandolfi et al., 2013; Rothenberg-Thurley et al., 2018). In order to eradicate these LSCs, a better understanding of the molecular mechanisms underlying human leukemia development is needed. Loss-of-function studies using RNA interference screens have shown to be very useful in identifying signaling pathways that are required for the proliferation and survival of leukemic cells (Karlsson et al., 2014; Miller et al., 2013; van den Boom et al., 2016; Zuber

et al., 2011). Lentiviral short hairpin RNA (shRNA) expression vectors enable sustained and sequence-specific downregulation of a target mRNA (Singer and Verma, 2008). The usual setup of these RNA interference screens involves the transduction of leukemic cells with shRNAs resulting in quick downregulation of designated targets, after which phenotypes are evaluated *in vitro* or *in vivo* upon transplantation in (xenograft) mouse models. This however does not reflect the situation in the clinic at which leukemia patients are diagnosed and treated at the full-blown stage. Therefore, in order to evaluate the role of target genes in the maintenance and propagation of leukemic (stem) cells and to truly evaluate the efficacy of novel treatment options, one needs to first establish a leukemia within the bone marrow microenvironment and then perform gene-function studies. The inducible Tet-regulated (Tet-On) shRNA expression system provides an attractive approach to study genes required for leukemic transformation (initiation) as well as for maintenance and survival (Centlivre et al., 2010; Shin et al., 2006; Zaiss et al., 2014; Zuber et al., 2011). It allows controlled gene regulation by doxycycline inducible and reversible gene repression to study direct consequences at any chosen time point during disease progression. In addition, essential genes can be studied where constitutive knockdown might be toxic. To introduce Tet-regulated shRNA expression vectors in *in vivo* xenograft leukemia models would be very promising as a predictive model for therapy in (leukemia) patients. Treatment strategies and potential relapse of disease can be further studied upon discontinuing doxycycline treatment. Identifying and prospectively isolating different AML subclones expressing Tet-regulated shRNAs using leukemia specific plasma membrane markers can be used to study clone-specific dynamics in response to shRNA-mediated knockdown (de Boer et al, in submission). In this study, we implemented a Tet-regulated miR-E shRNA expression vector, as reported by the Zuber lab, in our human *in vitro* and *in vivo* xenograft model systems to study gene function in the development and maintenance of leukemia (Fellmann et al., 2013). A single lentiviral dual-color vector allowed tracking of transduced miR-E shRNA expressing cells and demonstrated efficient inducible and reversible regulation of gene expression. Our data indicate that this doxycycline inducible knockdown approach is applicable to study the biological importance of a gene at any time in leukemia development *in vitro* and *in vivo*.

RESULTS

An efficient lentiviral Tet-regulated miR-E shRNA expression vector in stably transduced leukemic cells

In order to study the function of genes necessary for the maintenance and survival of

leukemic cells *in vitro* and *in vivo* in our human models, we took advantage of a Tet-regulated (inducible) lentiviral miR-E expression vector reported by the Zuber lab (Fellmann et al., 2013). We generated miR-E based shRNAs against PRC1.1 subunits RING1B, PCGF1 and KDM2B, plus a SCR control, that were cloned into the pRRL.TRE3G.GFP.miRE.PGK.mCherry.IRES.rtTA3 vector (LT3GECIR) (Fig 1A). These targets were selected based on our previous studies in which we identified non-canonical PRC1.1 as a critically important Polycomb complex in human leukemic cells (van den Boom et al., 2016). In K562 cells, the transduction efficiency reached ~40% and mCherry⁺ cells were sorted to establish stable cell lines. The tetracycline-derivate doxycycline (dox, 1 µg/ml) was used to induce GFP-coupled miR-E shRNA expression and therefore could be easily traced. Dox-induced shRNA expression was very efficient as demonstrated by >95% mCherry⁺/GFP⁺ cells (Fig 1A). To test the efficiency of the knockdown, qRT-PCR analysis

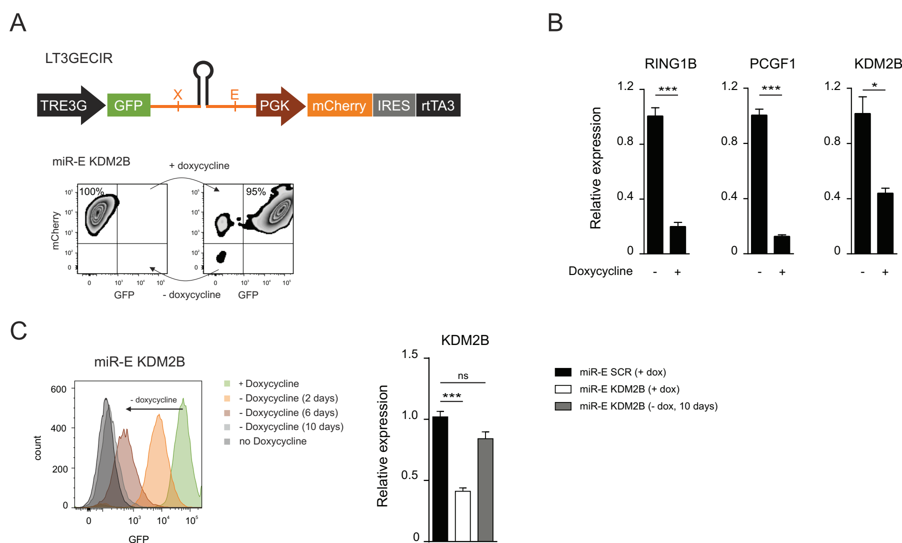

Fig 1. Inducible and reversible miR-E shRNA expression *in vitro*. (A) Schematic representation of the lentiviral Tet-regulated miR-E shRNA expression vector (illustration adapted from Fellmann et al., 2013) expressing fluorescence (mCherry/GFP) coupled miR-E shRNAs. As a representative example, doxycycline induced shRNA expression efficiency (*in vitro*) is shown as analyzed by FACS in miR-E KDM2B min/plus dox. (B) Knockdown efficiency of RING1B, PCGF1 and KDM2B in K562 miR-E expressing shRNAs respectively, with and without doxycycline for 4 days. Error bars represent SD. (C) Flow cytometry analysis of K562 miR-E KDM2B cells in the presence and/or withdrawal of doxycycline. The mean fluorescence intensity (MFI) for GFP, as a marker for doxycycline induced shRNA expression, is plotted for different time points. KDM2B expression was analyzed by qRT-PCR. Statistical analysis was performed using an unpaired t-test ($p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

was performed on K562 cells expressing miR-E RING1B, miR-E PCGF1 or miR-E KDM2B in the presence or absence of doxycycline for 4 days (Fig 1B). Next, we examined the effect of dox withdrawal to verify that the knockdown was reversible (Fig 1C). In the presence of dox, miR-E KDM2B cells became GFP-positive within two days, coinciding with a 60% knockdown in KDM2B expression (Fig 1C). In order to evaluate the reversibility of the system, cells were withdrawn from dox. Already after two days of dox withdrawal the GFP percentage started to drop, but it took 10 days before all GFP expression was completely gone. At that timepoint, KDM2B expression levels were also again up to normal levels (Fig 1C). Thus, these lentiviral vectors provide an efficient dox-inducible and reversible shRNA expression tool, which is useful to perform gene function studies in a time-dependent manner.

Primary human MLL-AF9 cells are sensitive to inducible downmodulation of PRC1.1 components

*N*ext, we examined the biological importance of downregulating PRC1.1 subunits RING1B, PCGF1 and KDM2B in human leukemic cells *in vitro*. First, AML cell lines NB4 (PML-RARA) and THP-1 (MLL-AF9) were transduced with the miR-E KDM2B shRNA vector. Upon dox treatment the majority of mCherry$^+$ cells became GFP$^+$, indicating efficient

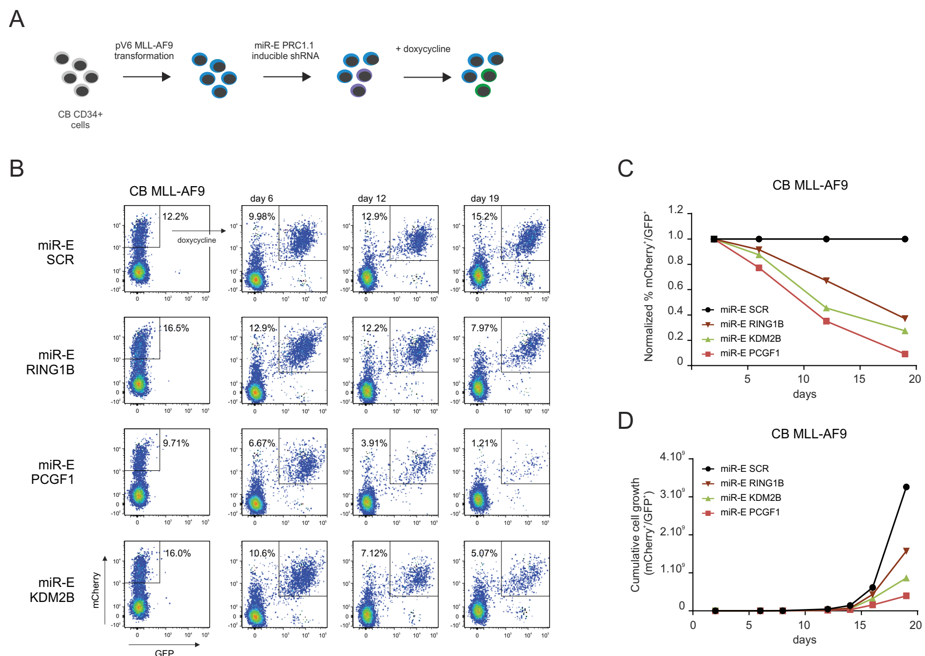

Fig. 2: Doxycycline induced miR-E RING1B, PCGF1 or KDM2B shRNA expression impaired cell growth of primary human MLL-AF9 cells. (A) Experimental setup of primary CB MLL-AF9 (mBlueberry)

miR-E shRNA (SCR, RING1B, PCGF1 and KDM2B) transductions. (B) Flow cytometry analysis of primary CB MLL-AF9 miR-E SCR, RING1B, PCGF1 and KDM2B cells in the presence of doxycycline over the course of 19 days. (C) Normalized percentage mCherry$^+$/GFP$^+$ and (D) cumulative cell growth (mCherry$^+$/GFP$^+$) of CB-MLL-AF9 cells expressing miR-E SCR, RING1B, PCGF1 or KDM2B.

miR-E induction (S1 Fig). Following three weeks, we observed a clear reduction in mCherry$^+$/GFP$^+$ cells in the miR-E KDM2B transduced group indicating that cells rely on KDM2B function (S1 Fig). Furthermore, we setup a primary cord blood (CB)-MLL-AF9 model expressing miR-E PRC1.1 inducible shRNAs (Fig 2A). CB derived CD34$^+$ cells were transduced with the lentiviral MLL-AF9 mBlueberry vector. Then, transformed MLL-AF9 cells were transduced with lentiviral constructs for miR-E SCR, miR-E RING1B, miR-E PCGF1 and miR-E KDM2B, reaching transduction efficiencies of 10-15% (Fig 2B). While dox-induced mCherry$^+$/GFP$^+$ percentages remained stable over time in the miR-E SCR transduced control group, downregulation of either RING1B, PCGF1 or KDM2B resulted in impaired growth of primary MLL-AF9 cells (Fig 2C and 2D).

Evaluation of Tet-regulated miR-E shRNA expression in a xenograft MLL-AF9 model

To examine loss-of-function in a time-dependent manner *in vivo*, we developed a xenograft MLL-AF9 Tet-regulated miR-E shRNA mouse model. Since PCGF1 knockdown *in vitro* strongly reduced the proliferation of MLL-AF9 cells, we studied the consequences of PCGF1 knockdown at several stages during the process of leukemia development *in vivo*. CB-derived CD34$^+$ cells were transduced with the lentiviral vector expressing miR-E PCGF1 (mCherry) and mCherry$^+$ cells (~17% efficiency) were sorted. Subsequently, cells were transduced with the lentiviral MLL-AF9 (mBlueberry) vector and were intravenously injected into sub-lethally irradiated NSG mice (Fig 3A). After 30 weeks, leukemia developed with high levels of engraftment of MLL-AF9/miR-E PCGF1/CD45$^+$/CD19$^+$ cells in BM, SP and LV (S2A Fig). Next, secondary transplantations were performed and to knockdown PCGF1, doxycycline was administered to the mice in food pellets (625 ppm) either at the start or at 10 weeks after secondary transplantation when leukemia was already established in the BM (Fig 3B). Control mice that did not receive any doxycycline, developed leukemia within 150 days (Fig 3C). Mice that were treated with doxycycline from the start showed significant prolonged survival until the end of the experiment (Fig 3C). Peripheral blood chimerism levels (MLL-AF9/miR-E PCGF1/CD45$^+$) remained low over time (Fig 3D). To investigate if we could treat mice at a later stage in the development of leukemia, we also started treatment of doxycycline 10 weeks after secondary transplantation when average blood chimerism levels were already 5%, a situation that would more faithfully mimic the

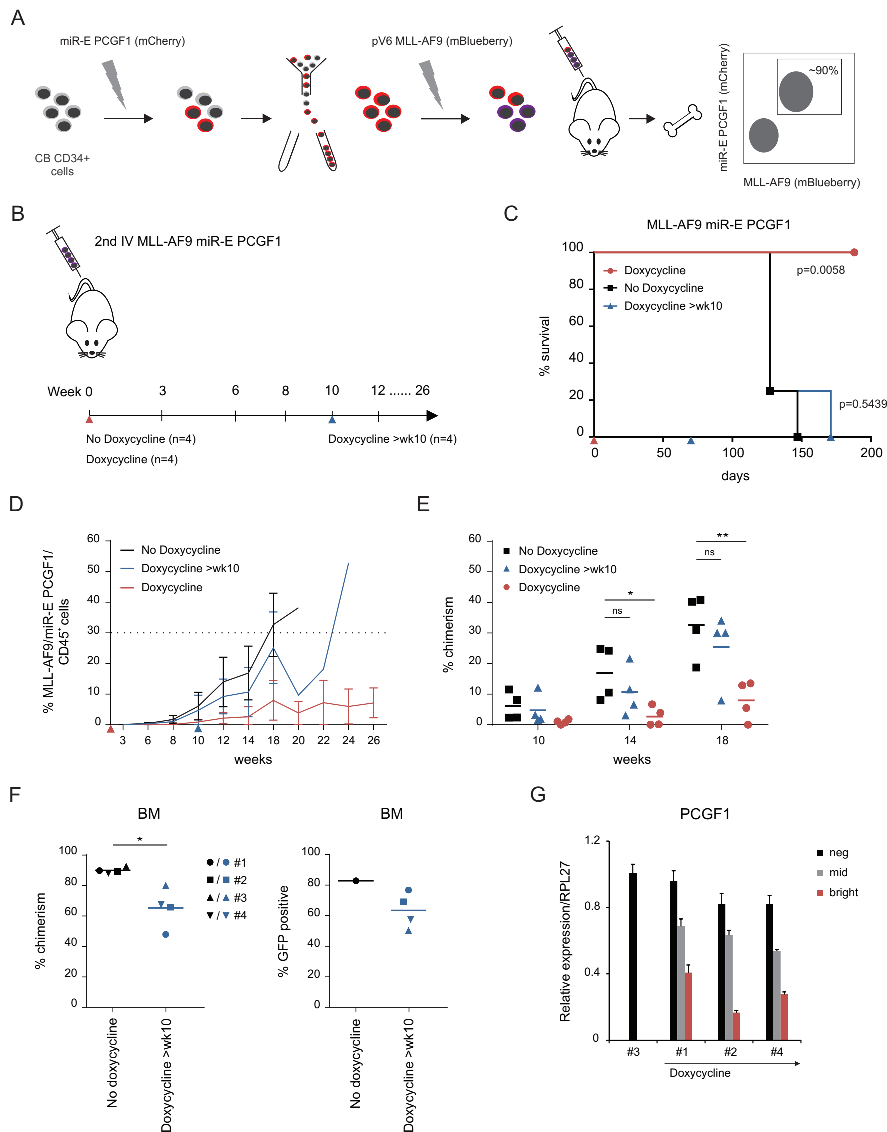

Fig 3. Evaluation of miR-E PCGF1 in xenograft CB-MLL-AF9 mouse model. (A) Schematic overview of developing an inducible miR-E shRNA against PCGF1 (mCherry) in a primary CB-MLL-AF9 (mBlueberry) *in vivo* model. FACS analysis of bone marrow cells showed ~90% engraftment of MLL-AF9 (mBlueberry) cells expressing miR-E PCGF1 (mCherry), which were used for secondary transplantations. (B) Experimental setup as illustrated where MLL-AF9 miR-E PCGF1 secondary transplantations were performed. Blood chimerism levels were monitored for indicated time points (weeks). Mice were divided into three groups, no doxycycline (n=4), doxycycline from the start (n=4) and doxycycline >wk10 (n=4). Doxycycline was administered in food pellets (625 ppm). (C) Kaplan-Meier curve of MLL-AF9 miR-E PCGF1 mice treated with no doxycycline, doxycycline from the start or

doxycycline >wk10. Statistical analysis was performed using a log-rank test. (D) Peripheral blood chimerism levels plotted over the course of the experiment between no doxycycline, doxycycline from the start (red triangle) and doxycycline >wk10 (blue triangle). Mice were sacrificed when chimerism levels exceeded 30%. (E) Peripheral blood chimerism levels of individual mice treated from the start or treated for 4-8 weeks. Statistical analysis was performed using unpaired t-test ($p \leq 0.05$ (*), $p \leq 0.01$ (**)). (F) Engraftment levels in BM shown as % chimerism of MLL-AF9 mBlueberry/ miR-E PCGF1 mCherry/ $CD45^+$ cells in no doxycycline and doxycycline>wk10 treated mice. Within the population of engrafted cells, GFP^+ cells are shown as a percentage. From the no doxycycline group, mouse #1 was administered doxycycline for 5 days before sacrifice to determine max GFP induction in the BM. (G) Expression of PCGF1 in BM samples of no doxycycline and doxycycline treated mice sorted on neg, mid and bright GFP populations, analyzed by qPCR.

patient situation (Fig 3E). While chimerism levels increased over time in untreated mice after week 10, this increase was clearly delayed in mice treated with doxycycline (Fig 3E). Nevertheless, leukemia did develop in mice treated from week 10 and mice were sacrificed when peripheral blood chimerism levels reached 30% and ultimately within this experiment no significant differences in overall survival were reached (Fig 3C, 3D and 3E). Even though substantial levels of ~65% chimerism (MLL-AF9/miR-E PCGF1/$CD45^+$) were observed in the BM of mice treated with doxycycline at week 10, these levels were still significantly lower compared to control mice (Fig 3F). Intriguingly, FACS analysis of MLL-AF9/miR-E PCGF1 $mCherry^+$/$CD45^+$ populations in doxycycline-treated mice revealed strong variation in GFP positivity (Fig 3F and S2B Fig). We questioned whether *in vivo* all BM cells would be equally sensitive to dox treatment as compared to *in vitro* conditions. To do so, a mouse (#1) that had not been treated with dox and had developed leukemia was administered with dox for 5 days before sacrifice. 82% of these cells were GFP positive and while this was somewhat lower compared to the 95% that was reached *in vitro*, this was still higher than percentages seen in mice that were treated with doxycycline for 8-14 weeks, which showed average GFP levels of 63% (Fig 3F). These data suggest that there was some selection for those clones that did not, or at low levels, express GFP. Indeed, the highest chimerism levels were seen in mice with the lowest percentage of GFP^+ cells. Moreover, we sorted the bright, mid and negative GFP populations and analyzed the efficiency of PCGF1 knockdown by qRT-PCR (Fig 3G). The GFP negative population showed similar PCGF1 expression levels as the no doxycycline control group, PCGF1 knockdown was more efficient in the GFP^{mid} population and the highest knockdown was achieved in the GFP^{bright} population (Fig 3G). Thus, these data indeed indicate that it were particularly those cells with inefficient or no PCGF1 knockdown that contributed to MLL-AF9-induced leukemogenesis. Re-analysis of the BM sample of the control mouse (#1) that was administered with dox for 5 days revealed that the highest miR-E PCGF1 $mCherry^+$ cells

(top) all expressed GFP, whereas the middle and bottom mCherry^{+} population represented GFP levels of 84% and 60% respectively (S2C Fig). These data suggest that the maximal dox-induced miR-E shRNA expression was technically limited to the high mCherry^{+} cells. Thus, in future studies to establish xenograft leukemia models expressing a dox-inducible miR-E shRNA vector, it is advisable to select the highest mCherry+ cells to reach maximal gene knockdown efficiencies.

DISCUSSION

Acute myeloid leukemia (AML) is characterised by (sub)clonal expansion of immature blasts in the bone marrow with a block in differentiation (Ding et al., 2012; Hughes et al., 2014). Whole blood cell counts, cytogenetics, flow cytometry and immunohistochemistry tests are instrumental in the diagnosis and classification of AML subtypes and subsequently treatment strategy (Bennett et al., 1976; Hong and He, 2017). Nevertheless, with currently available therapies the majority of patients will eventually still relapse. This is most likely due to the existence of therapy-resistant LSCs and many studies now focus on ways to identify, target and ultimately eradicate these LSCs in order to reach more curative therapies (Bonardi et al., 2013; Klco et al., 2014; Nieborowska-Skorska et al., 2017; Shlush et al., 2017; Valent et al., 2012). To understand the molecular mechanisms involved in initiation, maintenance and relapse of human leukemias, it is essential to study gene function in a time-dependent manner in *in vitro* and *in vivo* human leukemic models (Antonelli et al., 2016; Sontakke et al., 2014). Loss-of-gene function studies using constitutive RNAi expression result in a stable and quick downregulation of a designated target and while this is a powerful tool it will not reveal gene functions at specific stages of the disease. Therefore, an inducible RNAi system in a human leukemia model would allow for a controlled regulation of gene expression and be more promising as predictive model for therapy in patients. Where genetic knockout of essential genes using CRISPR-Cas9 might be too toxic for the cells, we reasoned that the best model would be an inducible knockdown system. While the application of CRISPR-Cas9 is very interesting, it remains a major challenge in primary AMLs (Morgens et al., 2017; Tzelepis et al., 2016).

We implemented an inducible lentiviral miR-E shRNA dual-color vector in our *in vitro* and *in vivo* human leukemia models that can be used to study the function of genes during any stage of leukemia development. This single lentiviral vector (LT3GECIR) based on an optimized miR-E backbone was shown to enhance shRNA levels and improve knockdown efficiency (Adams et al., 2017; Fellmann et al., 2013). The dual-color vector allows identification of mCherry^{+} transduced cells and in the presence of doxycycline, GFP-coupled miR-E shRNA is expressed. We showed that doxycycline-inducible miR-E

shRNA expression in leukemic cells was very efficient *in vitro*, resulting in effective gene repression and the effects were also reversible. Next, we demonstrated that the effects of a dox-inducible lentiviral miR-E shRNA on cell proliferation can be studied in AML cell lines and primary MLL-AF9 cells. Downregulation of Polycomb proteins RING1B, PCGF1 or KDM2B resulted in impaired growth of primary MLL-AF9 cells indicated by a strong reduction in mCherry$^+$/GFP$^+$ cells, which is in line with our previous constitutive shRNA reported data in which PRC1.1 was found to be essential for leukemic cells (van den Boom et al., 2016). Furthermore, we introduced the miR-E shRNA vector in a xenograft MLL-AF9 mouse model. Doxycycline was administered in food pellets because it is a reliable and effective method (Cawthorne et al., 2007; Redelsperger et al., 2016). In the BM, GFP-coupled miR-E shRNA levels reached maximum levels of 82%, indicating that the system is also nicely dox-inducible *in vivo*, albeit to a somewhat lesser extent compared to *in vitro* experiments where >95% GFP-positivity was reached. In mice carrying MLL-AF9 miR-E PCGF1 cells that were treated from the start with dox directly after transplantation, a significantly prolonged survival was observed. Chimerism levels in the blood remained low over time, indicating that the model can be used to study gene function *in vivo* and also underlining the importance of non-canonical PRC1.1 in leukemia development. In contrast, and somewhat unexpectedly, the survival of mice in which dox treatment was initiated at week 10 was not significantly prolonged. We decided to start the treatment of mice when peripheral blood chimerism levels had reached about 5%, which in our view would be a moment that would faithfully recapitulate the situation when patients would enter the clinic. These data strongly contrast our *in vitro* data in which we observed that PCGF1 knockdown strongly impaired MLL-AF9 cell growth, and also the effects seen *in vivo* when mice were treated directly after transplantation, which were very promising. However, once leukemia was established in the BM niche the effects were minor, which would have major implications for the efficacy of PCGF1 inhibition as a treatment modality. We also observed that doxycycline treated mice with low chimerism levels in the blood, still had substantial levels of engraftment in the BM, suggesting that the niche would provide protective factors that counteract loss of PCGF1. Clearly, the bone marrow microenvironment might play a protective role in the survival of leukemic cells (Carretta et al., 2017; Greim et al., 2014; Krause and Scadden, 2015). Indeed, we observed that MLL-AF9 cells cultured in the presence of murine MS5 stromal cells were less sensitive towards USP7 inhibition compared to cells grown in liquid culture conditions (see Discussion p.152). This even further emphasises that loss-of-gene function or inhibitor studies should be investigated in the context of a niche. Intriguingly, we noted strong variations in GFP positivity in FACS analysis of MLL-AF9/miR-E PCGF1 mCherry$^+$/CD45$^+$ populations in doxycycline-treated mice. Therefore, we questioned whether in this *in vivo* context all

BM cells would be equally sensitive to dox treatment compared to *in vitro* conditions. We indeed noted that, *in vivo*, the dox-inducibility was somewhat less compared to what was seen *in vitro*. Perhaps more importantly, we also noted that knockdown was most efficient in the brightest mCherry^{+}/GFP^{+} cells, and the lower 40% of the mCherry^{+} fraction did not express GFP at all and evaded shRNA expression. Also in the THP1 cell line there was a minor mCherry^{+} population that did not become GFP^{+} upon doxycycline treatment. This indicated that certain cells were insensitive for doxycycline potentially due to failure to undergo proper tetracycline-responsive element expression, as also experienced by Zuber and colleagues (Zuber et al., 2011). As a consequence, clonal selection had occurred in our mice treated with dox from week 10, whereby a shift towards GFP$^{mid/neg}$ cells was seen with considerable less efficient knockdown of PCGF1. Thus, in future studies to establish xenograft leukemia models expressing a dox-inducible miR-E shRNA vector, it is advisable to select the highest mCherry^{+} cells to reach maximal gene knockdown efficiencies. With this in mind, we conclude that the inducible miR-E shRNA dual-color vector can be used to study, trace and isolate shRNA expressing cells in a time-dependent manner both in *in vitro* and *in vivo* human leukemia models that most faithfully recapitulate human disease.

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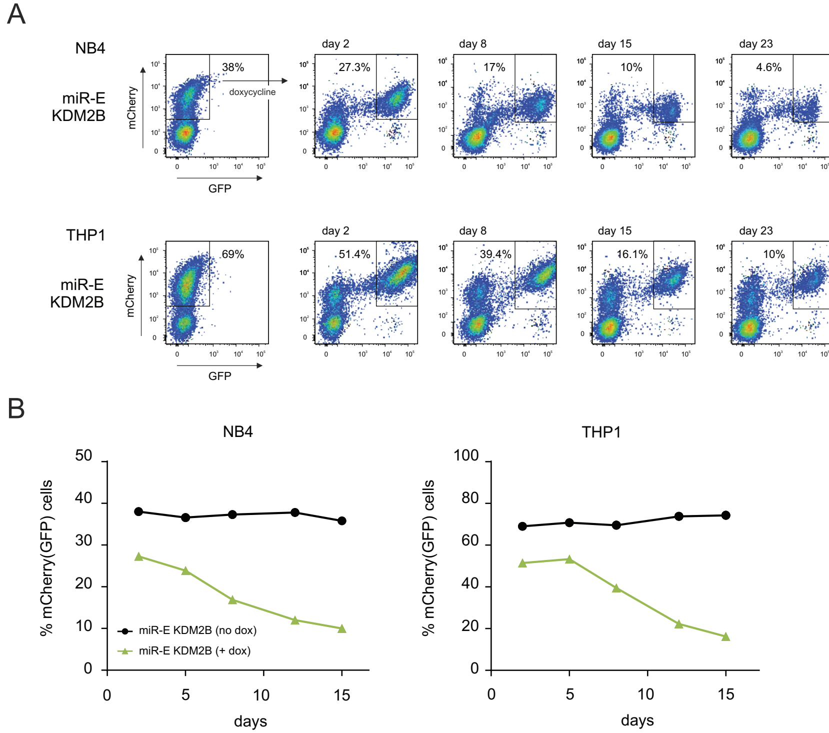
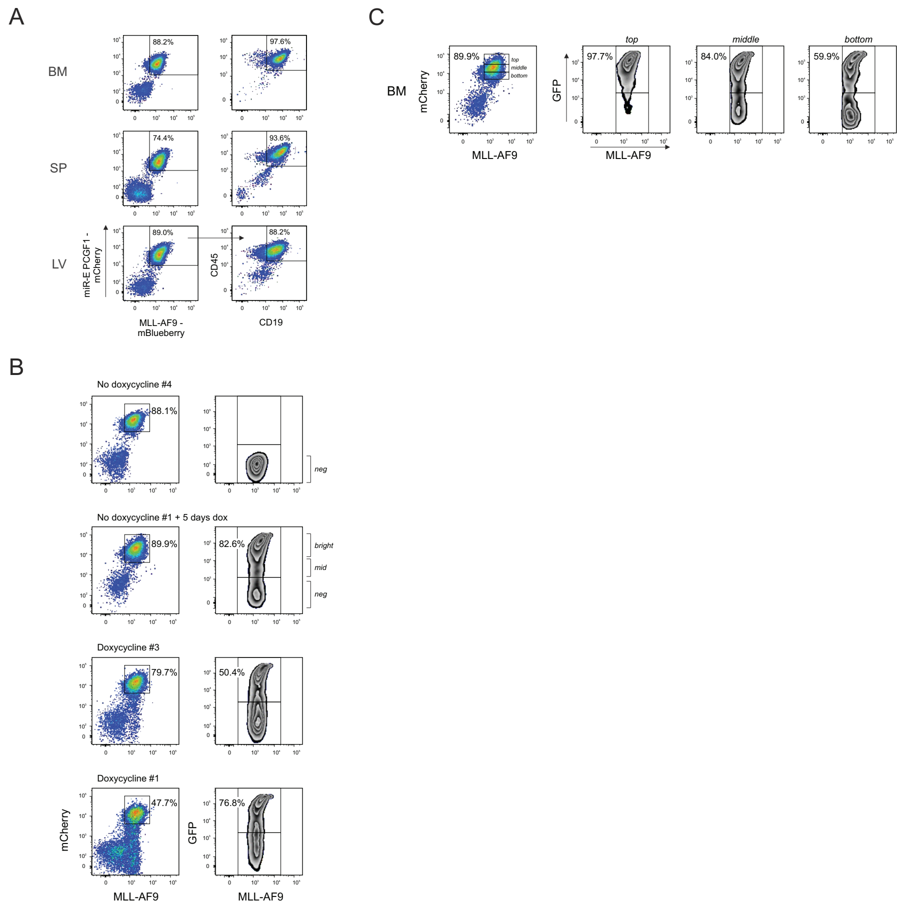
SUPPLEMENTARY FIGURES



S1 Fig. Inducible KDM2B knockdown impairs cell growth of leukemic cells. (A) Flow cytometry analysis of NB4 and THP1 miR-E KDM2B cells, unsorted, following doxycycline treatment for 3 weeks. (B) Analysis of miR-E KDM2B mCherry+ (no dox) or mCherry+/GFP+ NB4 or THP1 cells in time.



S2 Fig. FACS analysis of MLL-AF9 miR-E PCGF1 engraftment in BM, SP, LV and analysis of GFP induction. (A) FACS analysis of bone marrow (BM), spleen (SP) and liver (LV) of primary CB MLL-AF9 (mBlueberry) miR-E PCGF1 (mCherry) mouse. (B) FACS plots of engraftment levels in BM of MLL-AF9 mBlueberry/miR-E PCGF1 mCherry mice are illustrated and GFP levels were divided into negative (neg), mid and bright populations. Mouse #1 was administered doxycycline for 5 days before sacrifice to determine max GFP induction in the BM. (C) Example of chimerism level in BM (no doxycycline #1, 5 days dox), gated for top, middle and bottom mCherry+ population and further analysed for GFP percentage.

S1 Table. miR-E shRNAs, 97-mer oligonucleotides

Gene	mRNA target site	97-mer oligo
		5'basal stem - sense strand - loop - antisense strand - 3'basal stem
RING1B	TCGAAGTCTACACAGTGAATTA	TGCTGTTGACAGTGAGCGCCGAAGTCTACACAGTGAATTATAGTGAAGCCACAGATGTATAATTCACTGTGTAGACTTCGATGCCTACTGCCTCGGA
PCGF1	GCAGCTCCTTTTTGACAATGAA	TGCTGTTGACAGTGAGCGACAGCTCCTTTTTGACAATGAATAGTGAAGCCACAGATGTATTCATTGTCAAAAAGGAGCTGCTGCCTACTGCCTCGGA
KDM2B	TGCATGAGCTCTTGTACTTACA	TGCTGTTGACAGTGAGCGCGCATGAGCTCTTGTACTTACATAGTGAAGCCACAGATGTATGTAAGTACAAGAGCTCATGCTTGCCTACTGCCTCGGA
SCR/Luci	TAGGAATTATAATGCTTATCTA	TGCTGTTGACAGTGAGCGCAGGAATTATAATGCTTATCTATAGTGAAGCCACAGATGTATAGATAAGCATTATAATTCCTATGCCTACTGCCTCGGA

S2 Table. Primer sequences

Gene	Fwd/Rev	Sequence
RING1B	Fwd	5'-TAGTATTCAGGCCTCATCCCACA-3'
	Rev	5'-CTTGGATAAGTGATCAACAGTGGC-3'
PCGF1	Fwd	5'-CTGTTAGAGCTGAGGTACGCCA-3'
	Rev	5'-AAAGGAGCTGCACATGCTGAG-3'
KDM2B	Fwd	5'-GTCTTCAGCTACCTCAGCCACCAAG-3'
	Rev	5'-TGCAGTGGTTCAGGTCAATGCGG-3'
RPL27	Fwd	5'-TCCGGACGCAAAGCTGTCATCG-3'
	Rev	5'-TCTTGCCCATGGCAGCTGTCAC-3'

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CHAPTER 6

SUMMARY, DISCUSSION & FUTURE PERSPECTIVES

SUMMARY

The development of leukemia is a multistep process that can be caused by multiple genetic and epigenetic changes which affect normal growth and differentiation of hematopoietic stem and progenitor cells and ultimately lead to full leukemic transformation. Despite most leukemia patients initially achieving successful remission after intensive treatment, the persistence of a rare population of chemotherapy-resistant leukemic stem cells (LSCs) can result in relapse of disease in a relatively large cohort of patients. In order to identify effective novel treatment strategies to target and eradicate these LSCs, a better understanding of the molecular mechanisms involved in regulating stem cell fate, maintenance, survival and chemoresistance of leukemic cells is needed. Both extrinsic and intrinsic factors have been suggested to regulate stem cell fate and are essential for the survival of LSCs, including stem cell-niche interactions, growth factor or cytokine-induced signal transduction, metabolic signaling and epigenetic regulators including Polycomb proteins. The studies presented in this thesis were aimed to identify Polycomb signaling pathways in leukemia and whether they can be used to target and eradicate LSCs.

In **Chapter 2** we describe that the non-canonical Polycomb complex PRC1.1, containing KDM2B, RING1A/B, PCGF1, RYBP/YAF2, SKP1 and BCOR(L1), is essential for human leukemic cells. Using a lentiviral shRNA approach we showed that downregulation of PRC1.1 proteins strongly reduced cell proliferation of (primary) leukemic cells *in vitro* and delayed or even abrogated leukemogenesis *in vivo*. Transcriptome studies revealed that the PRC1.1 subunits BCOR, PCGF1 and RING1A were significantly upregulated in AML CD34$^+$ cells compared to normal BM CD34$^+$ cells. Since Polycomb proteins are epigenetic regulators of gene transcription, we performed ChIP-seq studies in K562 cells and primary AML CD34$^+$ cells to identify signaling pathways targeted by non-canonical PRC1.1 and/or canonical PRC2/PRC1. Whereas canonical PRC2 and PRC1 are well known as transcriptional repressors, we observed that non-canonical PRC1.1 targets a subset of loci devoid of H3K27me3 suggesting a role independent of PRC2. Notably these loci were associated with permissive or active chromatin, suggested by the occupancy of H3K4me3, RNAPII(S5P) and H3K27ac. Furthermore, Gene Ontology analysis of these targets revealed enrichment for genes involved in metabolism and cell cycle regulation. Together, our data suggest an essential role for non-canonical PRC1.1 in controlling distinct gene sets involved in unique cell biological processes required for the maintenance of leukemic cells.

In **Chapter 3** we identified the deubiquitinase USP7 as an integral member of non-canonical PRC1.1 and show that USP7 inhibitors provide an alternative approach in AML treatment. USP7 inhibition decreased the proliferation and survival of several leukemic cell lines and primary AMLs, also independent of the USP7-MDM2-TP53 axis. We identified that USP7 is part of non-canonical PRC1.1 and its enzymatic activity is critically important to maintain complex integrity. USP7 inhibition results in disassembly of the PRC1.1 complex and consequently loss of binding to its target loci. Furthermore, we observed that loss of PRC1.1 binding coincided with loss of H2AK119ub, reduced H3K27ac levels and reduced gene transcription on several target loci, whereas H3K4me3 levels remained unaffected. These data indicate that PRC1.1 is required to facilitate or maintain transcriptional activity of these genes. Our studies highlight the diverse functions of USP7 and link it to Polycomb-mediated epigenetic control which might aid further to ultimately eradicate LSCs.

In Chapter 2 we showed that non-canonical PRC1.1 targets a subset of loci independent of active PRC2 and instead is associated with transcriptionally permissive or active chromatin marks. This was further investigated in **Chapter 4** where we aimed to obtain insights into the mechanisms by which PRC1.1 might affect transcriptional control in leukemic cells. We observed that PRC1.1 preferentially targets unmethylated CpG island promoters. Therefore, we hypothesized that PRC1.1 could potentially prevent *de novo* DNA methylation on these loci. Treatment with an USP7 inhibitor resulted in loss of PRC1.1 binding and we observed only a slight increase in DNA methylation on several PRC1.1 target loci. Moreover, we observed that several PRC1.1 target genes were downregulated upon USP7 inhibition, coinciding with a loss of PRC1.1 chromatin binding, suggesting that PRC1.1 is required to maintain gene expression. Furthermore, ChIP analysis showed reduced levels of H3K27ac on some of those loci, indicative for reduced transcriptional activity. Nevertheless, several PRC1.1 target genes were also upregulated or no change in expression was observed, indicating that transcriptional control is a complicated multifactorial process and that beyond PRC1.1 other regulators play clearly important roles as well. Future work will focus on the underlying mechanisms and cross-talk with chromatin regulators and transcriptional machinery.

To identify signaling pathways underlying human leukemia development, it is critical to study gene function in a well-controlled and time-dependent manner. Therefore, in **Chapter 5** we established a doxycycline inducible (miR-E based) shRNA xenograft MLL-AF9 leukemia mouse model which allowed us to study timing of gene knockdown on the efficacy of leukemia treatment. We implemented a lentiviral Tet-regulated miR-E shRNA dual color vector that enabled tracing of mCherry^{+} transduced cells and expression of

GFP-coupled miR-E shRNAs in the presence of doxycycline. We have generated stably transduced leukemic cells expressing miR-E based shRNAs against non-canonical PRC1.1 subunits and showed efficient doxycycline inducible and reversible gene repression *in vitro*. Next, we showed that primary CB MLL-AF9 cells were sensitive to inducible downmodulation of PRC1.1 subunits, in particular PCGF1. We then developed a xenograft MLL-AF9 Tet-regulated miR-E PCGF1 mouse model and we observed that early treatment (knockdown of PCGF1) clearly reduced engraftment levels in the blood, but treatment at a later stage in the development of leukemia was not sufficient to enhance overall survival. Interestingly, we did observe that there was selection of clones that expressed low or even no GFP, with no or inefficient PCGF1 knockdown, and that these clones predominantly contributed to leukemogenesis. These findings suggest that building an efficient inducible RNAi xenograft mouse model is essential to study potential new targets for leukemia treatment.

DISCUSSION AND FUTURE PERSPECTIVES

The Polycomb group (PcG) protein family has emerged as a group of key epigenetic regulators of stem cell self-renewal and differentiation. Deregulation and dysfunction of PcG proteins is associated with cancer, including hematologic malignancies. PcG proteins reside in multi-protein chromatin modifying complexes, of which the function of individual subunits is only partially understood. Polycomb complexes are epigenetic regulators of gene transcription and act as 'gatekeepers' regulating cell fate during development. With the aid of mass spectrometry-based proteomic approaches the complex composition of distinct Polycomb complexes could be characterized, revealing proteins that form the stable core subunit, but also proteins that display sub stoichiometric interactions with Polycomb complexes. With the use of chromatin immunoprecipitation (ChIP) the identification of Polycomb target genes genome-wide has been made possible. The existence of multiple canonical and non-canonical Polycomb complexes, their dynamic protein composition during development and the dynamic occupancy of Polycomb complexes to different target loci adds to the complexity of how they are recruited and control gene expression. Quite possibly, the mechanisms by which Polycomb proteins maintain or regulate cell fate, might also have cell type or context-specific features. While many studies have indicated that Polycomb proteins are critically involved in regulating stem cell fate, their function in leukemogenesis in contrast is less well understood. In this thesis we therefore addressed if Polycomb proteins are essential for the self-renewal and maintenance of leukemic cells, which signaling pathways they control and how they might

contribute to gene regulation. Since aberrant Polycomb expression has been observed in cancer stem cells, a thorough understanding of Polycomb function might provide alternative possibilities to therapeutically target leukemic stem cells. These topics will be further discussed in the sections below.

6.1 PRC1 dependencies in leukemic cells

Initial insights about the role of Polycomb proteins in leukemic cells have mostly emerged from studies of the Polycomb group protein BMI1 (also known as PCGF4). BMI1 has been shown to have a key role in the self-renewal and maintenance of normal (hematopoietic) stem cells in part mediated by transcriptional repression of the cyclin-dependent kinase inhibitor 2A (CDKN2A) senescence pathway (Iwama et al., 2004; Lessard and Sauvageau, 2003) and was suggested to be involved in preventing inappropriate differentiation by suppression of several HOX genes (Biehs et al., 2013; Park et al., 2003). BMI1 is frequently found to be upregulated in various types of cancer, including CD34$^+$-enriched primary AML cells and is associated with poor prognosis in AML patients (Bhattacharya et al., 2015; de Jonge et al., 2011; Rizo et al., 2009). Aberrant expression of Polycomb proteins might therefore disturb the balance of downstream proto-oncogenic and tumor suppressor signaling pathways controlling self-renewal, proliferation, chemosensitivity and be implicated in cancer (Pardal et al., 2005). BMI1 has been shown to collaborate with BCR-ABL in the leukemic transformation of human CD34$^+$ cells, allowing long-term expansion of leukemic cells and serially transplantable lymphoid leukemia *in vivo* (Rizo et al., 2010; Sontakke et al., 2016). Introduction of the leukemia-associated fusion proteins AML1-ETO and PLZF-RARα into BMI1-deficient murine bone marrow cells revealed that BMI1 was indeed required for leukemic transformation driven by these oncogenes. In contrast, the MLL-AF9 fusion oncogene was able to induce leukemogenesis in BMI1-deficient cells, mainly driven by MLL-AF9-mediated HOXA9 expression that maintained the CDKN2A locus in a repressed state (Smith et al., 2011). Tan and colleagues observed that BMI1 was less efficiently recruited to HOXA9 and MEIS1 target genes in leukemic cells driven by the MLL-AF9 fusion oncogene resulting in their upregulated expression (Tan et al., 2016). Also in DNMT3A-mutated cells a reduced BMI1 chromatin binding was observed with consequently an upregulation of HOXA9 and MEIS1 expression (Tan et al., 2016). Since DNMT3A mutations are usually mutually exclusive with MLL-AF9 these data indicate that different upstream mechanisms can control the action of Polycomb proteins.

Several PCGF paralogs exist (PCGF1-PCGF6) and the detailed characterization of PRC1 complexes revealed that they can reside in different canonical or non-canonical PRC1 complexes (Gao et al., 2012). However, our understanding of the functional relevance of

these distinct PRC1 complexes is still limited. In our shRNA-mediated knockdown screen in cord blood (CB) CD34^{+} MLL-AF9 transformed cells we observed a strongly reduced proliferation upon knockdown of PCGF2, but a much milder phenotype upon knockdown of PCGF4. These observations are potentially in line with data described above where BMI1-deficient murine bone marrow cells could still be transformed with MLL-AF9 and upon transplantation in secondary recipients were able to induce leukemia in mice. Indicating that BMI1 is dispensable for the self-renewal of MLL-AF9 driven leukemic cells (Smith et al., 2011). Clearly, our data indicate that PCGF2 is playing a much more dominant role in these leukemias and it would be intriguing to further investigate the function of PCGF2 in MLL-AF9 cells. While transcription-independent functions of Polycomb proteins are less-well characterized, they might underlie the observed phenotypes as well. For example, knockdown of PCGF2 resulted in enhanced UBE2I-mediated sumoylation activity and subsequently increased PML-RARA degradation in NB4 cells, suggesting a novel role of PCGF2 as an anti-SUMO E3 protein (Jo et al., 2016). We observed that knockdown of PCGF1, BCOR and KDM2B severely impaired the growth of MLL-AF9 transformed cells, indicating that non-canonical PRC1.1 also plays an essential role in MLL-AF9 leukemogenesis. Furthermore, knockdown of either RING1A or RING1B, the catalytic core subunits of both canonical and non-canonical PRC1 complexes, also severely impaired the growth of leukemic cells indicating that the activity of PRC1 complexes is essential to maintain leukemic cells, which is in line with work from others (Rossi et al., 2016; Shima et al., 2018). The existence of multiple PRC1 complexes and their context-specific expression and recruitment to target loci suggests that they might regulate distinct biological processes, features that no doubt will be further investigated in detail in the future.

6.2 Biological function of non-canonical PRC1.1

In our studies we uncovered that the non-canonical PRC1.1 complex is critically important in human leukemias. Knockdown of PRC1.1 proteins, including KDM2B, PCGF1, RING1B and BCOR strongly reduced cell proliferation of (primary) leukemic cells *in vitro* and delayed or even abrogated MLL-AF9 induced leukemogenesis *in vivo*. Our ChIP-seq studies revealed that PRC1.1 complexes can associate with two distinct chromatin states. A subset of loci was co-occupied by PRC1.1 and canonical PRC2/PRC1 complexes, which we termed ‘both’ loci, enriched for both H3K4me3 and H3K27me3. But intriguingly, we also find that PRC1.1 targets a unique set of genes independent of the repressive PRC2/H3K27me3 mark, among them cell cycle and metabolic genes. In line with our observations that KDM2B/PRC1.1 is essential for leukemogenesis, overexpression of KDM2B (or FBXL10) in murine hematopoietic stem cells contributed to the development of myeloid or B-lymphoid leukemia in mice (Ueda et al., 2015). Interestingly, KDM2B

bound to actively transcribed metabolic genes and activated the expression of Nsg2 resulting in a block in differentiation. In addition, He et al showed that KDM2B is necessary for the development and maintenance of leukemia in a mouse AML model (He et al., 2011). KDM2B is overexpressed in various cancers and cooperated with K-RAS to promote pancreatic cancer in mouse models (Tzatsos et al., 2013). Co-binding with MYC and/or KDM5A positively regulated the transcription of metabolic genes. These data suggest that KDM2B might contribute to the metabolic requirements for the maintenance and proliferation of leukemic cells. Functional metabolic studies should further provide a link between KDM2B and cellular metabolism in human leukemic cells. Indeed, studies have already shown that KDM2B is a positive regulator of glycolysis in HeLa cells and in gastric cancer cells KDM2B has a regulatory role in autophagy via PI3K/Akt/mTOR signaling (Yu et al., 2015; Zhao et al., 2017).

Seemingly in contrast, BCOR/BCORL1 loss-of-function mutations have also been found in AML (3.8%) and myelodysplastic syndromes (4.2%), frequently associated with other mutations such as TET2, DNMT3A, FLT3-ITD and RUNX1 (Damm et al., 2013; Grossmann et al., 2011). BCOR mutations result in a truncated BCOR protein and loss-of-function. In normal mouse bone marrow cells, BCOR loss-of-function is associated with increased myeloid cell growth and differentiation coinciding with de-repression of myeloid genes including HOXA and CEPB (Cao et al., 2016; Tara et al., 2018). This is in line with another study that showed that PCGF1 was implicated in suppressing mouse myeloid progenitor cells by repressing HOXA genes (Ross et al., 2012). Loss of RUNX1 and PCGF1 resulted in a block in differentiation *in vitro*, but the authors could not observe leukemia development upon transplantation in mice, which warrants further investigation. Mice with a combined loss of BCOR and TET2 developed a lethal MDS suggesting that cooperative BCOR and TET2 mutations can drive myeloid transformation (Tara et al., 2018). In agreement, yet unpublished data, showed that loss of BCOR in human leukemic cells have reduced cell proliferation, whereas loss of both BCOR and TET2 resulted in enhanced proliferation (Schaefer et al., 2018). Moreover, knockdown of BCOR induced myeloid differentiation of NB4 and HL60 leukemic cells (Cao et al., 2016). Mice with a deletion of exon 4, lacking the BCL6 binding domain, developed T-cell lymphoblastic leukemia (T-ALL) suggesting a tumor suppressor role for the co-repressor function of BCOR (Tanaka et al., 2017). Several NOTCH1 target genes, co-bound by BCOR/PCGF1 were upregulated (in T-lymphocytes) and likely contributed to transformation. This indicates that BCOR might have a dual role: on the one hand it interacts and functions as a co-repressor of BCL6 and on the other hand it interacts with PCGF1/PRC1.1 allowing oncogenic activation of NOTCH1 target genes. Remarkably, mice lacking BCOR exons 9 and 10, expressing a truncated BCOR protein

that fails to interact with PRC1.1 proteins also developed T-ALL in a NOTCH1-dependent manner (Tara et al., 2018), but these mice showed activated oncogenic NOTCH1 signaling mediated by gain-of-function mutation or deletion of the NOTCH1 gene itself. Further studies are needed to determine what the BCOR(L1) dependencies are in human leukemias and if BCOR/PRC1.1 drives both oncogenic transcription and tumor suppression. Is it a cell type-specific context and/or are there differences between lymphoid versus myeloid dependencies? Further questions remain such as does the truncated BCOR protein have other interaction partners or functions contributing to transformation? Especially in the context of other mutations like TET2, what is the cooperative function? Understanding the underlying molecular mechanisms resulting in oncogenic or tumor suppressor function is necessary for potential therapeutic targeting of Polycomb signaling pathways.

6.3 Cross-talk between non-canonical PRC1.1, transcription factor signaling and epigenetic modifications

We and others observed that PRC1.1 can target a subset of genes independent of H3K27me3 and these loci are in a transcriptionally permissive or active chromatin state, suggested by the occupancy of H3K4me3, RNAPII (S5P) and H3K27ac. Among these loci are genes involved in controlling cell cycle and metabolism. Remarkably, we observed that loss of KDM2B-mediated recruitment of PRC1.1 resulted in the reduction of the expression of some target genes, but this was less clear for other target genes. Similar observations have been described for canonical PRC2/PRC1 target genes, where loss of PRC2 or PRC1 activity did not always result in de-repression of its target genes (Comet et al., 2016; Laugesen et al., 2019; Riising et al., 2014). These observations raise the question of whether Polycomb proteins are actively repressing or activating genes, or whether they are merely maintaining the chromatin in a transcriptionally repressed or transcriptionally permissive state, after which the recruitment of activators or repressors ultimately decide whether genes would or would not be expressed.

A conceptual overview is shown in Figure 1, where we distinguish 4 different chromatin states; (1) poised for transcription initiation, (2) active transcription, (3) initiation of repression and (4) active/maintenance of repression. In this hypothetical model, non-canonical PRC1.1 would not necessarily initiate transcription itself, but would maintain chromatin in an open conformation poised for transcription initiation. In response to extracellular growth factors or intracellular signal transduction activities, transcription factors would be the ultimate factors that actively drive gene expression. However, in the (transient) absence of transcription factor activity, loci would need to decide on whether to maintain an open conformation or whether they would adopt a repressed

conformation. The former might be the case for relevant genes controlling for instance cellular metabolism, loci that we indeed see heavily enriched for non-canonical PRC1.1. The latter might be the case for lineage specific genes that upon differentiation to a specific lineage are no longer required. This model would nicely align with the observations that interfering with Polycombs alone is not always sufficient to directly change gene expression of targets, but that this needs to be addressed in the context of transcription factor activities. Also transcription factor activities themselves can have a direct impact on the chromatin state, in part by impacting on chromatin binding of Polycomb proteins. For instance, Riising and colleagues have nicely shown that PRC2 is not required for the initiation of gene repression during differentiation but that first transcriptional inhibition occurs after which PRC2 binds to CpG islands (Riising et al., 2014). These data argue that only after removal of transcription factor activities, chromatin can be remodeled into a repressed state. Transcription factors have also been shown to be able to actively 'open up' chromatin (Choukrallah and Matthias, 2014). For instance, upon B-cell commitment, the transcription factor PAX5 can rapidly induce chromatin changes by recruiting chromatin-remodeling and histone-modifying enzymes (McManus et al., 2011).

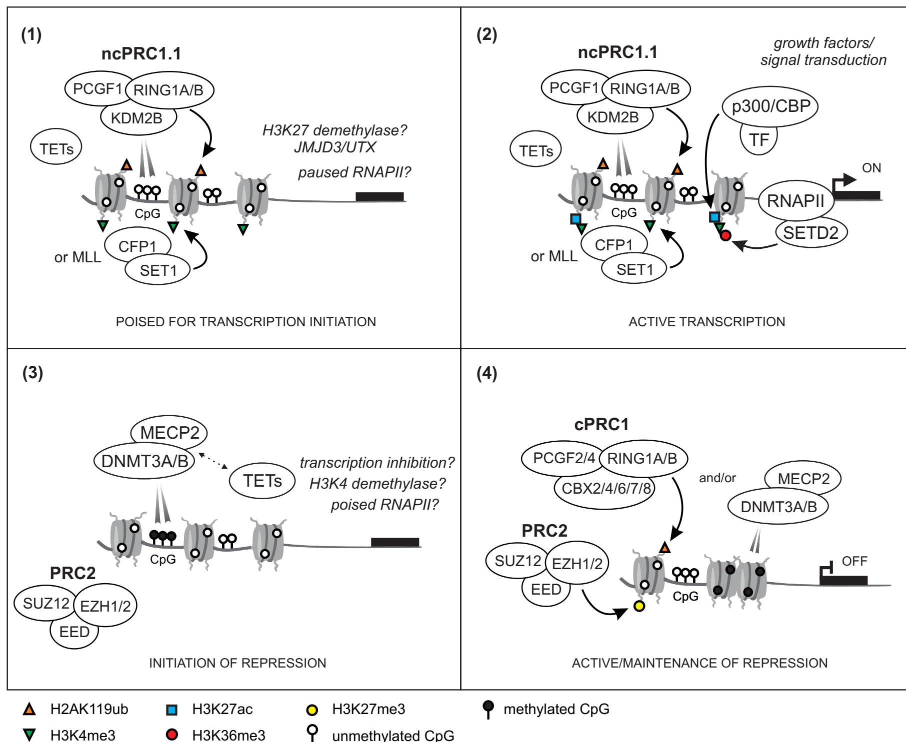

Fig 1. Conceptual overview of chromatin states linked to Polycomb-mediated transcription regulation.

In Chapter 4 of this thesis we have begun studies to address the hypotheses outlined in Figure 1. We and others have shown that PRC1.1 binds to unmethylated CpG island promoters, via the ZF-CxxC domain of KDM2B (Farcas et al., 2012; Wu et al., 2013). We therefore examined whether loss of PRC1.1 binding would be sufficient to induce DNA methylation of those loci resulting in reduced gene expression. Since we identified that USP7 is an essential component of PRC1.1 and its enzymatic activity is critically important for the stability and function of PRC1.1, we used USP7 inhibitors as a tool to induce loss of PRC1.1 occupancy at target loci. We indeed observed a slight increase in DNA methylation on some PRC1.1 target loci, maybe suggesting that the function of PRC1.1 would be to prevent CpG methylation at active promoters. While not addressed here, the recruitment of TET proteins also maintain an unmethylated status (Lei et al., 2018; Lei et al., 2017). Interestingly, we also noted that loss of PRC1.1 not only coincided with a loss of H2AK119ub marks, but also of H3K27ac marks, further indicating reduced transcriptional activity. Similarly, Farcas and colleagues showed that knockdown of KDM2B in ESCs causes a locus specific reduction in RING1B binding and reduced levels of H2AK119ub (Farcas et al., 2012). Some target genes were upregulated upon knockdown of KDM2B, coinciding with reduced levels of H2AK119ub, while for other target genes no effect on H2AK119ub levels and gene expression was observed. Thus, interfering with PRC1.1 signaling can affect gene expression on certain loci, but this is not always an all-or-nothing process. Transcriptional control occurs at several levels and it is still unclear when PRC1.1 is more critically important. Is it the affinity or quantity of Polycomb proteins with certain loci or their recruitment and cross-talk with other chromatin regulators controlling the epigenetic state? Another possibility is the activity of signaling pathways that might influence the epigenetic landscape. NOTCH1 has been shown to mediate the recruitment of JMJD3, resulting in the loss of H3K27me3 to oncogenic targets in T-ALL (Ntziachristos et al., 2014). Inhibiting JMJD3, by GSKJ4 treatment, greatly impaired the growth of (primary) T-ALL cells. While NOTCH1 signaling acts predominantly as a tumor suppressor in myeloid malignancies, it would be interesting to see if PRC1.1 permissive target genes in AML are co-bound by JMJD3 or another H3K27 demethylase UTX. If so, which signaling pathways are involved in its activation and underlie that a subset of PRC1.1 target genes are devoid of H3K27me3? One hypothesis is that other regulators of the chromatin landscape influence the function of PRC1.1 being more restrictive or permissive.

AML is a heterogeneous disease caused by a variety of mutations and chromosomal translocations resulting in aberrant transcriptional networks regulating cell fate. Unique gene regulatory networks underlie differences in the epigenome of RUNX1-ETO and RUNX1-EVI1 fusion proteins in AML (Loke et al., 2017). Recent work revealed that specific

AML subgroups carrying fusion proteins or mutations establish specific transcriptional networks and chromatin landscape as identified by DNase hypersensitive site mapping, which are distinct from normal cells (Assi et al., 2019). Furthermore, prospective isolation of genetically distinct subclones using leukemia-enriched plasma membrane proteins showed differential gene expression patterns that correlated with chromatin accessibility and differences in transcription factor occupancy (de Boer et al., 2018). Nevertheless, we know very little about how transcriptional networks impact on the epigenetic landscape. A study on t(8;21) AML showed that constitutive mitogen-activated protein (MAP) kinase signaling abrogated the binding of PRC1/PRC2 to the PAX5 promoter resulting in its activation (Ray et al., 2013). In normal myeloid cells PAX5 was repressed by PRC1/PRC2. Thus, aberrant transcriptional networks might affect the expression and or the recruitment/displacement of Polycomb proteins to sustain gene expression required for the growth and survival of leukemic cells (Bracken and Helin, 2009). From our ChIP-seq data we observed that differences exist between $CD34^+$ AMLs and also between $CD34^+$ AMLs and normal $CD34^+$ cells in the occupancy of KDM2B, H2AK119ub, H3K4me3 and H3K27me3 on a specific target gene. It would be important to understand what underlies these differences in binding and if it correlates with (aberrant) extrinsic or intrinsic signaling pathways. Future studies should aim at further dissecting the interplay between transcription factor networks and Polycomb-mediated transcriptional control. Although enriched for CD34, we identified PRC1.1, PRC1 and 'both' target genes within a heterogeneous population of primary AML cells which might underestimate the epigenetic landscape contributing to gene regulation of small subclones. Future work will focus on the epigenetic landscape of these subclones, their chromatin accessibility and differential gene expression and study their drug sensitivity. In order to understand aberrant gene expression in leukemia it is important to assess chromatin accessibility, transcription factor binding, epigenetic modifications including histone marks, Polycomb protein binding and complex composition, DNA methylation and other chromatin remodeling complexes together with gene expression analysis at the right time (Prange et al., 2014). The regulatory composition might be very complex, dynamic and cell type dependent or even controlled at single cell level. An interesting approach to study potential oncogenic targets is the isolation of locus-specific protein complexes using an *in vivo* biotinylated nuclease-deficient Cas9 protein and sequence specific guide RNAs (Liu et al., 2017). Transcriptional control is a complicated multifactorial process and with such an approach other regulators can be identified and further studied.

6.4 Targeting Polycomb signaling pathways: challenges and opportunities

Polycomb proteins are essential regulators of gene transcription and critically involved in regulating cell fate. The diversity of cell types during development and also the progression or transformation from normal to leukemic cells underlie differential gene expression patterns. Identifying aberrant Polycomb signaling pathways in leukemia and understanding underlying mechanisms and function in gene regulation might provide interesting alternative possibilities to target leukemic stem cells. Self-renewal is an essential feature for the maintenance of both normal and malignant stem cells. The molecular mechanisms that enable self-renewal are likely shared between normal stem cells and the rare population of chemotherapy-resistant leukemic stem cells. Therefore, both overlapping and unique functions of Polycomb signaling pathways in the self-renewal and maintenance of normal and leukemic cells are important for further studies.

The expression and activity of Polycomb proteins, their complex composition and affinity or recruitment to target loci can all influence their unique functions in regulating biological processes. In mouse ESCs, pluripotency and differentiation is controlled by dynamic assembly and disassembly of CBX associated PRC1 complexes (Morey et al., 2012). CBX7 was predominantly expressed in pluripotent ESCs and downregulated upon differentiation, while CBX2 and CBX4 protein expression was increased upon differentiation. Thus, what are the upstream regulators that dictate the switch between self-renewal and differentiation? Morey et al showed that CBX7 binds to the promoters of CBX2 and CBX4 and upon differentiation displacement of CBX7 resulted in its de-repression. Another study, published back-to-back, identified that the microRNAs miR-125 and miR-181 are expressed during differentiation and regulate CBX7 expression (O'Loghlen et al., 2012). MicroRNAs are small non-coding RNAs that target specific mRNAs for degradation or result in translational inhibition. Knockdown of Dicer, essential for the processing of microRNAs, revealed that several Polycomb proteins were upregulated (Cao et al., 2011). It is clear that deregulated expression of miRNAs is implicated in cancers, including hematological malignancies (Calin and Croce, 2006; Lechman et al., 2016; O'Connell et al., 2008), and in part this might also involve deregulation of Polycomb proteins. It is suggested that altered expression of Polycomb proteins could be one of the key events that allow leukemic stem cell self-renewal or maintenance (Martin-Perez et al., 2010). In our lab label-free quantification proteome analysis was performed on $CD34^+$ cells from AML patients (n=42) and $CD34^+$ peripheral blood cells from healthy controls (n=6) (de Boer et al., 2018). Here, we selected some Polycomb proteins and evaluated there expression levels. This revealed that several Polycomb proteins including KDM2B, RYBP and RING1B were most differentially expressed between AML $CD34^+$ cells and normal $CD34^+$ cells (Figure 2). The

expression of Polycomb proteins is highly variable between individual AML patients and it would be very important to understand these differences in the context of different subclones/subpopulations, disease progression and sensitivity for treatment. Polycomb proteins are regulated at the epigenetic, transcriptional and post-transcriptional level (Kottakis et al., 2014). Moreover, Polycomb proteins are subjected to post-translational modifications including ubiquitination and phosphorylation, that can influence their activity and function (Niessen et al., 2009). For example, self-ubiquitination of RING1B is important for its E3 ligase activity on histone H2A (de and Ciechanover, 2012). It is important to understand how the expression, activity and function of individual subunits in Polycomb protein complexes is regulated in order to interfere with their function. Recent advances in generating inducible CRISPR-Cas9 models, resulting in a complete knockout, could be used to further study Polycomb protein function in a time-dependent manner (Aubrey et al., 2015). Furthermore, various epigenetic inhibitors have been or are being developed against epigenetic proteins that add, remove or recognize histone modifications and are being tested for potential use in AML treatment (Lu and Wang, 2017).

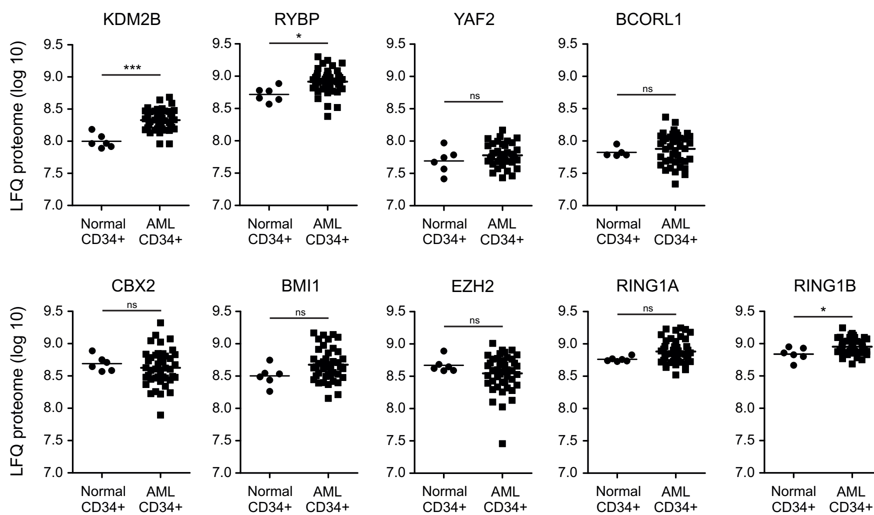

Fig 2. Label-free quantification (LFQ) proteome analysis was performed on 5.10^6 CD34$^+$ cells from AML patients (n=42) or CD34$^+$ cells from mobilized healthy/normal peripheral blood (n=6). The LFQ intensities are shown for several Polycomb proteins in normal and AML CD34$^+$ cells. Statistical differences were determined by Student's t-test (n.s. not significant, * or *** represents p<0.05 or p<0.001 respectively).

In our studies, we further aimed to target Polycomb signaling pathways. Therefore we introduced a Tet-regulated shRNA expression vector against PCGF1 in an *in vivo* xenograft MLL-AF9 leukemia model to study timing of gene knockdown on the efficacy of leukemia treatment. While chimerism levels in the blood remained low over time in MLL-AF9 miR-E PCGF1 mice treated with doxycyline at the onset of disease, mice that were treated at a later stage when leukemia was established in the bone marrow did reach high chimerism levels and developed leukemia. This suggested that the cells in the bone marrow can survive and counteract reduced levels of PCGF1. In another study we evaluated the effect of the USP7 inhibitor P22077 in the MLL-AF9 xenograft mouse model. While the efficacy of USP7 inhibition in several cell lines and primary AMLs was rather high, *in vivo* leukemia development was delayed but ultimatley all mice succumbed to leukemia. Several studies have suggested that the bone marrow niche can play a protective role in the survival of leukemic cells (Greim et al., 2014; Krause and Scadden, 2015; Schepers et al., 2013; Shafat et al., 2017). Therefore, we evaluated the efficacy of USP7 inhibition in a direct comparison of CB MLL-AF9 cells grown in liquid culture or in a co-culture setting on murine MS5 bone marrow stromal cells (Figure 3). In both settings P22077 impaired cell growth, but the efficacy was much higher in liquid culture conditions. Also the effects of the inhibitor on the binding of KDM2B to several target loci was more pronounced in liquid culture conditions. These studies do show the importance of studying loss-of-gene function on the effect of inhibitors in the context of a niche. Further studies are needed to see which niche factors protect the survival of the cells and/or dampen the response to therapeutic agents.

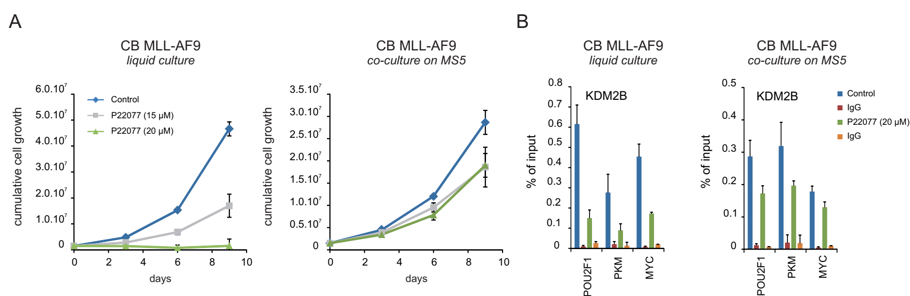

Fig 3. Evaluation of the efficacy of USP7 inhibition on CB MLL-AF9 cells grown in the absence or presence of bone marrow stromal cells. (A) Cumulative cell growth of CB MLL-AF9 cells cultured in Gartner's medium supplemented with IL-3, SCF and Flt-3L (10 ng/ml each) in liquid culture and co-culture on MS5, treated with DMSO (control) or P22077. (B) ChIP-qPCRs for KDM2B and IgG as control on a few loci in CB-MLL-AF9 cells treated with DMSO or P22077 (48h) under either liquid culture or MS5 co-culture conditions.

To conclude, our findings indicate that PRC1.1 signaling contributes to the maintenance or survival of leukemic cells. PRC1.1 is recruited to non-methylated CpGs associated with restrictive and permissive chromatin states in a H3K27me3 dependent or independent manner respectively. It is important to understand how the dynamic (open/closed) chromatin structure is regulated allowing gene transcription at the right place and right time for those genes critically involved in the maintenance of (chemotherapy-resistant) leukemic cells. Transcriptional networks initiated via intrinsic or extrinsic pathways are suggested to impact on the epigenetic landscape which might underlie differential gene expression and requires different targeted therapies. Understanding Polycomb signaling in leukemic cells and the cross-talk with the bone marrow niche is important to find new opportunities for therapeutically targeting leukemic stem cells.

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APPENDICES

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NEDERLANDSE SAMENVATTING

Leukemie is een verzamelnaam voor een aantal vormen van kanker die in het bloed voorkomen en kenmerkt zich door een woekering van afwijkende bloedcellen in het beenmerg met als gevolg een tekort aan functionele uitgerijpte bloedcellen. De aanmaak van onze bloedcellen vindt plaats in het beenmerg en wordt nauwkeurig gecontroleerd en aangestuurd. Het is een proces dat continu actief is, omdat bloedcellen maar een beperkte levensduur hebben. Wanneer er toch iets misgaat en de productie van functionele bloedcellen wordt verstoord heeft dat al snel consequenties. Door een tekort aan witte bloedcellen ben je meer vatbaar voor infecties, minder bloedplaatjes leiden tot een verminderde bloedstolling en een tekort aan rode bloedcellen resulteert in bloedarmoede. Bij acute leukemie kan er dan ook in korte tijd een levensbedreigende situatie ontstaan. Leukemie kent verschillende vormen, zo wordt er onderscheid gemaakt tussen acute of chronische myeloïde leukemie (AML en CML) waarbij er een tekort is aan myeloïde cellen, onder andere rode bloedcellen, bloedplaatjes en ook bepaalde witte bloedcellen. Bij acute of chronische lymfoïde leukemie (ALL en CLL) is er vooral een tekort aan lymfocyten, een soort witte bloedcellen.

Onze bloedcellen worden gevormd door zogenaamde hematopoietische stamcellen. Deze stamcellen hebben de bijzondere eigenschap dat na deling, één dochtercel een exacte kopie is van zichzelf (self-renewal) en vormt dus een nieuwe stamcel, terwijl de andere dochtercel zich kan ontwikkelen tot een voorlopercel en uiteindelijk kan uitrijpen (differentiëren) tot een functionele bloedcel. Deze voorlopercellen delen snel en voorzien de meeste tijd van het aantal bloedcellen dat nodig is. De stamcellen zelf delen maar heel weinig, ongeveer een keer per maand, maar zorgen dus levenslang voor de productie van onze bloedcellen. Het is erg belangrijk dat deze processen van self-renewal en differentiatie goed gecontroleerd worden, want veranderingen in factoren die dit aansturen of reguleren kunnen uiteindelijk leiden tot leukemie.

Leukemie wordt veroorzaakt door genetische veranderingen, zoals foutjes in het DNA, en ook door epigenetische veranderingen, waarbij andere factoren de expressie van genen kunnen beïnvloeden, waardoor processen als self-renewal (celgroei) en differentiatie verstoord raken. Dit kan uiteindelijk leiden tot een woekering van afwijkende niet functionele bloedcellen, ook wel leukemische blasten genoemd. Ondanks dat de meeste leukemie patiënten complete remissie bereiken na chemotherapie, komt de leukemie in veel gevallen toch weer terug. Eén van de gedachten is dat een aantal langzaam delende leukemische stamcellen overblijven en chemotherapie pakt vooral de snel delende leukemische blasten aan. Om betere behandelingsstrategieën te kunnen ontwikkelen om

ook deze leukemische stamcellen gericht aan te pakken moeten we dieper gaan kijken in een leukemiecel naar welke processen (of signaalroutes) ontregeld zijn en hoe dat komt. Het wel of niet tot expressie komen van bepaalde genen, de bouwstenen van ons DNA, en vervolgens de vertaling naar eiwitten is essentieel voor een cel om bepaalde signaalroutes aan of uit te zetten. Een leukemiecel kan hier gebruik van maken en daardoor sneller gaan delen of langer kan overleven of wellicht zelfs aan te passen waardoor ze ongevoelig worden voor chemotherapie. Die processen worden beïnvloed door zowel signalen van binnen als van buiten de cel. Factoren die hier een rol in spelen zijn bijvoorbeeld de interactie met cellen in de directe nabijheid (stamcel niche), groeifactoren en cytokines die bepaalde signaalroutes aansturen, metabole processen die zorgen voor de energiehuishouding in de cel en epigenetische factoren die de expressie van genen kunnen controleren. Het is belangrijk om inzicht te krijgen in dit soort processen om vervolgens nieuwe behandelmethoden te kunnen ontwikkelen. In dit proefschrift hebben we onderzoek gedaan naar epigenetische factoren en gefocust op het belang en functie van Polycomb eiwitten in leukemiecellen. Verder hebben we gekeken of het uitschakelen van Polycomb eiwitten de ontwikkeling van leukemie kan tegengaan.

Polycomb eiwitten zijn een grote familie en maken deel uit van verschillende eiwitcomplexen, die Polycomb Repressive Complex 1 en 2 (PRC1 en PRC2) worden genoemd. Dankzij eiwit-eiwit interactie studies weten we dat er verschillende PRC1 eiwitcomplexen zijn, onder andere het eiwitcomplex PRC1.1. De eiwitcomplexen PRC1 en PRC2 binden aan het DNA wat met andere eiwitten (histonen) opgevouwen zit in de celkern en onderdrukken (repressie) de expressie van genen. Dit wordt gemedieerd via markeringen die op histonen worden gezet, het PRC2 complex trimethyleert lysine 27 op histone H3 (H3K27me3) en het PRC1 complex ubiquitinyleert lysine 119 op histone H2A (H2AK119ub).

In **hoofdstuk 2** hebben we aangetoond dat het eiwitcomplex PRC1.1 essentieel is voor leukemiecellen. Eén voor één hebben we verschillende Polycomb eiwitten min of meer uitgeschakeld (een verlaagde expressie) in leukemiecellen. Met name door het uitschakelen van PRC1.1 eiwitten, waaronder KDM2B, RING1A/B, PCGF1 en BCOR was de celgroei sterk afgenomen. Ook de ontwikkeling van leukemie in een muizenmodel was sterk vertraagd door het uitschakelen van KDM2B. Omdat Polycomb eiwitten belangrijke factoren zijn bij het controleren van genexpressie hebben we in kaart gebracht waar die Polycomb eiwitcomplexen aan het DNA binden om daarmee inzicht te krijgen in welke processen (of signaalroutes) ze controleren in leukemiecellen. Daarnaast hebben we ook gekeken naar een aantal markeringen op histonen die geassocieerd zijn met repressie (H3K27me3, H2AK119ub) en activatie (H3K4me3, H3K27ac) van genen. Waar canonical PRC2 en PRC1 eiwitcomplexen geassocieerd zijn met de repressie van target genen,

A

ontdekten we dat het eiwitcomplex PRC1.1 een set van target genen controleert in de afwezigheid van H3K27me3 (dus onafhankelijk van PRC2). Opvallend was dat deze target genen geassocieerd waren met actieve markeringen zoals H3K4me3, H3K27ac en RNA Polymerase II betrokken bij transcriptie. Deze genen hebben invloed op processen als metabolisme (energiehuishouding van de cel) en celgroei. Deze data suggereert dat het eiwitcomplex PRC1.1 de expressie van een set genen controleert die essentieel zijn voor de overleving en groei van leukemiecellen.

In **hoofdstuk 3** laten de studies met USP7 inhibitors nieuwe inzichten zien in de biologie van het eiwitcomplex PRC1.1 en zijn mogelijk interessant als nieuw target voor de behandeling van AML. Door de activiteit van het eiwit USP7 te blokkeren nam de groei en overleving sterk af van leukemiecellen met verschillende genetische achtergronden. We ontdekten dat USP7 een belangrijke plaats kan innemen in het PRC1.1 eiwitcomplex en de deubiquitinase actviteit van USP7 belangrijk is voor de integriteit van het eiwitcomplex. USP7 inhibitie resulteerde in het uiteenvallen van het PRC1.1 complex en vervolgens ook het verlies van binding op target genen. Het verlies van PRC1.1 op een aantal target genen, leidde ook tot een verlies van H2AK119ub, verminderde H3K27ac en verminderde expressie, terwijl H3K4me3 niet veranderde. Hieruit blijkt dat het PRC1.1 complex belangrijk is voor dan wel het faciliteren dan wel het in stand houden van de expressie van deze genen. Dus, naast de vele functies van USP7 is er ook een belangrijke rol van USP7 activiteit weggelegd voor 'Polycomb-mediated epigenetic control' wat nieuwe aanknopingspunten biedt om leukemiecellen gericht aan te pakken.

De link tussen PRC1.1 en target genen die toegankelijk zijn voor transcriptiefactoren waardoor transcriptie kan plaatsvinden werd verder bestudeerd in **hoofdstuk 4**. We zijn begonnen met inzicht te krijgen in de functie van het eiwitcomplex PRC1.1 op transcriptie en hoe dit wordt gecontroleerd in leukemiecellen. Uit eerdere en ook onze studies bleek dat het eiwitcomplex PRC1.1 voornamelijk bindt aan niet-gemethyleerde CpG-eilanden, dat zijn stukjes DNA waar transcriptie van een gen kan plaatsvinden. Methylatie van CpG-eilanden daarentegen blokkeert transcriptie en leidt tot inactivatie van een gen. Eén van de ideeën was daarom dat PRC1.1 misschien DNA methylatie voorkomt en hiermee het chromatine toegankelijk houdt voor transcriptiefactoren. Dit leek toch niet heel waarschijnlijk, omdat het verlies van PRC1.1 op een aantal target genen maar tot een kleine toename in DNA methylatie leidde. Wel ging de expressie omlaag van een aantal target genen na verlies van PRC1.1 hetgeen toch suggereert dat PRC1.1 hier een belangrijke rol bij speelt. Bovendien zagen we een afname in H3K27ac wat past bij een verminderde transcriptionele activiteit. Toch waren er ook veel genen waarvan de

expressie niet veranderde na verlies van PRC1.1, wat er op wijst dat transcriptie een ingewikkeld complex proces is wat door meerdere factoren wordt gereguleerd. Verder onderzoek zal zich richten op onderliggende mechanismen en cross-talk met andere factoren die een belangrijke rol spelen bij transcriptie regulatie.

Om meer inzicht te krijgen in hoe leukemie zich ontwikkelt in de tijd en welke signaaltransductieroutes zijn ontregeld is het belangrijk om de functie van genen te bestuderen tijdens de progressie van leukemie. In **hoofdstuk 5** hebben we de ontwikkeling van leukemie in een muizenmodel gevolgd waarbij we een gen (hier PCGF1) konden uitschakelen op een bepaald moment tijdens de progressie van leukemie. Hierdoor hebben we kunnen bestuderen wanneer het uitschakelen van PCGF1 nog effect had om de ontwikkeling van leukemie tegen te gaan. Dit uitschakelen van een gen werd geïnduceerd in de aanwezigheid van doxycycline. De leukemiecellen konden we traceren (mCherry positief) en zodra we het gen uitschakelden werden ze daarbij ook GFP positief. Voordat we dit in muizen hebben onderzocht, hebben we eerst in leukemiecellen verschillende genen uitgeschakeld. Het toevoegen van doxycycline in het groeimedium van leukemiecellen resulteerde in een efficiënte inductie (95% van de leukemiecellen werd ook GFP positief) en het uitschakelen van het gen was ook omkeerbaar wanneer doxycyline niet meer aanwezig was. De groei van leukemiecellen was sterk afgenomen na het uitschakelen van PRC1.1 subunits *in vitro*, waaronder PCGF1. Door PCGF1 vroeg uit te schakelen in het beginstadium van leukemieontwikkeling kon het ziekteverloop sterk worden vertraagd, terwijl het later uitschakelen van PCGF1 niet voldoende was en de muizen toch leukemie ontwikkelden. Na analyse van de leukemiecellen uit het beenmerg, bleek dat een selectie had plaatsgevonden van leukemiecellen waarbij het uitschakelen van PCGF1 niet helemaal goed heeft gewerkt (lage of geen expressie van GFP) en het induceerbare systeem dus nog verdere optimalisatie nodig heeft. Een leukemie muizenmodel waarbij je efficiënt genen kunt uitschakelen tijdens de progressie van leukemie zal bijdragen aan het vinden van potentiële nieuwe targets voor de behandeling van leukemie.

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Henny

BIOGRAPHY

Henny Maat was born on December 12, 1988 in Luxwoude (Opsterland), The Netherlands. After completing her pre-university education in Heerenveen, she started in 2007 her undergraduate studies in Biology at the University of Groningen. With a major in Biomedical Sciences, she graduated with a Bachelor of Science (BSc) degree in 2010. In the same year she entered the Master's programme in Biomedical Sciences at the University of Groningen and obtained her Master of Science (MSc) degree in 2012. During this two-year programme she completed two research internships, the first of which was performed at the Department of Pathology and Medical Biology, University Medical Center Groningen. Under the supervision of dr. B.J. Kroesen she studied the function of microRNA-21 in primary naïve and memory T-cells in the T-cell activation process. Her second project was performed at the Department of Experimental Hematology, University Medical Center Groningen under the supervision of Prof. dr. J.J. Schuringa. There she focussed on identifying RAC1 and RAC2 specific interaction proteins to understand their function in human leukemic cells. In September 2012 she continued in the lab of Prof. dr. J.J. Schuringa starting a PhD position on the project entitled: "Towards identification and targeting of Polycomb signaling pathways in leukemia" funded by an ERC Starting Grant. She presented her work on several national and international conferences. The results of her studies are presented in this thesis.

LIST OF PUBLICATIONS

Maat H, Jaques J, Rodríguez López A, Hogeling SM, de Vries MP, Gravesteijn C, Brouwers-Vos AZ, van der Meer N, Huls G, Vellenga E, van den Boom V and Schuringa JJ. USP7 as part of non-canonical PRC1.1 is a druggable target in leukemia. Preprint bioRxiv 221093; https://doi.org/10.1101/221093

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